LDL Receptor Regulation in Human Liver Cells by Dietary Fatty acids and Antioxidants

A thesis presented for the Degree of Doctor of Philosophy at the University of Adelaide

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This Thesis is dedicated to my wonderful parents, Gourdas and Protima Pal, for whom I have the utmost love and respect.

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Declaration

This thesis has been composed by myself and has not been accepted in any previous application for a degree. The work, of which this is a record, has been done by myself and all sources of information have been acknowledged by means of references or quotations. I consent to this thesis, when deposited in the University of Adelaide library, being available for photocopying and loan.

SEBELY PAL 8 November 1996

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SUMMARY

The individual effects of saturated, monounsaturated and polyunsaturated fatty acids (PUFAs) on low density lipoprotein (LDL) receptor activity were determined in a rat liver cell line (H-35), a human liver cell line (HepG2) and two human monocytic cell lines (THP-1 and U937). Cellular LDL receptor activity was measured by calcium-dependent binding of colloidal gold-LDL to intact cells. The relative amounts of LDL receptor protein in HepG2 cells was quantified by densitometry of Western blots that used an anti-LDL receptor antibody and an enhanced chemiluminescence (ECL) detection system. The level of LDL receptor mRNA was measured in HepG2 cells by reverse transcribing cellular mRNA and amplifying the LDL receptor cDNA by the polymerase chain reaction (PCR) with incorporation of digoxygenin dUTP. The LDL receptor cDNA was then quantified against a positive control from Southern blots using an anti-digoxygenin antibody and an ECL detection system.

In both the human and rat liver cell lines, the LDL receptor activity was significantly suppressed by linoleic acid (ω-6 PUFA) and further suppressed with eicosapentaenoic acid (EPA, ω-3 PUFA) compared to cells incubated with a saturated (palmitic acid) and a monounsaturated fatty acid (oleic acid) whether cholesterol was present or absent in the media. There was an inverse relationship between the degree of fatty acid unsaturation and LDL receptor activity in both liver cell lines, demonstrating that human and rat liver cells respond to fatty acids in a like manner.

The inverse relationship between fatty acid unsaturation and LDL receptor activity was not, however, observed in the two human monocytic cell lines. There was no difference in the effect of palmitic acid, oleic acid, linoleic acid and EPA on LDL receptor activity in THP-1 cells, whereas LDL receptor activity was only suppressed by EPA in U937 cells. This indicated that there may be a tissue specific response to fatty acids within a species.

When the relative amount of LDL receptor protein was quantified, HepG2 cells incubated with the PUFAs had a lower level of LDL receptor protein than cells incubated with the monounsaturated and saturated fatty acids. The LDL receptor mRNA levels were also observed to parallel the level of LDL receptor protein and activity. Thus, the effect of the fatty acids on LDL receptor activity in HepG2 cells was attributable to their affect on gene transcription.

Vitamin E (α-tocopherol) was co-incubated with EPA and linoleic acid to test the possibility that fatty acid peroxides of these PUFAs were responsible for suppression of the LDL receptor. The decrease in the LDL receptor in cells incubated with EPA and linoleic acid did not appear to be due to fatty acid oxidation products as LDL receptor protein remained suppressed in cells incubated with these fatty acids in the presence of α-

tocopherol. Interestingly, these experiments also revealed the novel finding that α-tocopherol was, on its own, able to upregulate the LDL receptor.

The α-tocopherol had a bimodal effect on LDL receptor expression (activity, protein and mRNA levels). The LDL receptor expression increased with α-tocopherol concentrations up to 50 μM and then decreased at higher concentrations. The bimodal regulation of the HMG-CoA reductase gene was also observed with increasing concentrations of α-tocopherol. In contrast, δ- and γ-tocopherol analogues of vitamin E decreased LDL receptor expression (activity, mass and mRNA levels) at all concentrations examined. The effect of α-tocopherol on the LDL receptor was therefore due to a unique property of the analogue, such as its high antioxidant potential and its high biological activity compared to the other forms of tocopherols.

The effect of other antioxidants (vitamin A, vitamin C, ß-carotene, green tea extract and red wine) on the LDL receptor in human HepG2 cells was also examined. These compounds are highly diverse in their structure, metabolism, function and mechanism of action, but are similar in that they are all potent antioxidants. Vitamin A, vitamin C, ß-carotene, green tea extract and red wine all increased LDL receptor activity, mass and mRNA levels at the concentrations examined. The vitamin antioxidants were capable of increasing LDL receptor gene expression up to 11-fold above control (no vitamins). The LDL receptor mass and mRNA levels were increased 20-fold above control cells with increasing concentrations of green tea extract. The HMG-CoA reductase mRNA levels were also increased when cells were incubated with green tea extract. Thus, the upregulation of the LDL receptor may be a "general antioxidant phenomenon" which is mediated through a common pathway.

As PUFAs were observed to downregulate the LDL receptor and antioxidants were shown to upregulate the LDL receptor, the interrelationship between antioxidants and fatty acids on the LDL receptor was investigated by incubating cells with both agents simultaneously. The LDL receptor activity, mass and mRNA levels were observed to be independently controlled by vitamin E (α-tocopherol) and EPA. Independent effects were also seen with vitamin A, vitamin C and ß-carotene, in the presence and absence of the PUFAs (EPA and linoleic acid), and *vice versa*.

In summary, the work in this thesis demonstrates that fatty acids and antioxidants can regulate the LDL receptor at the level of gene transcription in cultured liver cells. PUFAs were specifically shown to downregulate the LDL receptor compared to saturated and monounsaturated fatty acids in the presence or absence of cholesterol. Furthermore, these experiments led to the unique and important discovery that antioxidants can upregulate the LDL receptor in HepG2 cells. When cells were incubated with both antioxidants and PUFAs simultaneously, the final level of LDL receptor expression was a result of the combined effects of both. Furthermore, the opposing effects of the PUFAs and the antioxidants appeared to be independent of each other.

Chapter 1

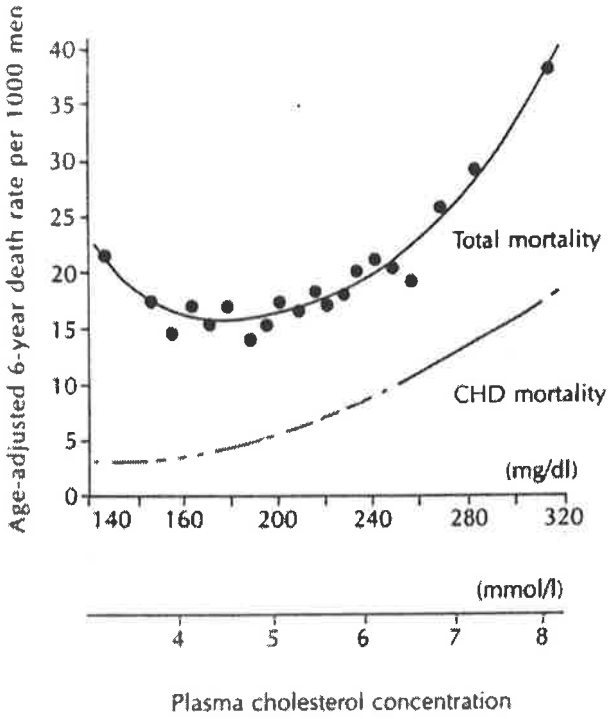
1.0 INTRODUCTION

1.1 Introduction

Cholesterol was first identified almost two centuries ago and was characterised as "the waxy, white component of gallstones" (Stallone, 1980). In 1816, Chevreul, a French scientist, gave cholesterol its original name "cholesterine", from the Greek words chole (bile) and steros (solid) (Bloch, 1965). Later, this compound was found to have a reactive hydroxyl group and the name changed to "cholesterol" (Bloch, 1965). In the early 1900s, extensive efforts focused on the structural studies of the sterol molecule but, remarkable findings about the role of cholesterol in atherosclerosis were also being made. In 1906, Aschoff first drew attention to the high content of cholesterol in atherosclerotic aortas and in 1910, Windaus showed that cholesterol deposited in atherosclerotic arteries was present chiefly as cholesterol esters (Goodman, 1989).

It is the role of cholesterol in the development of coronary artery disease that has spurred the greatest interest in cholesterol metabolism. Coronary artery disease has been called the "black plague" of the 20th century since it is the leading cause of premature death in western countries today (Havel, 1988). It has clearly been established that high serum cholesterol is the major risk factor for coronary heart disease (CHD) (Martin, 1986) (Figure 1.1). The demonstration that elevated blood cholesterol levels have a positive causal relationship in the development of atherosclerosis has firmly established the significance of this small molecule in the study of human metabolism. Nevertheless, several other risk factors have been identified, the two most relevant risk factors being cigarette smoking and hypertension. The presence of hypertension more than doubles the risk for CHD at any given level of serum cholesterol and a similar but smaller increment is attributable to cigarette smoking. The coexistence of all three factors results in a multiplicative rather than an additive increase in risk. Physical exercise, obesity, age, and sex are also associated with CHD but are considered to be only minor risk factors (Wilson, 1987).

Figure 1.1: Relationship between total plasma cholesterol levels and coronary heart disease. (From Martin, 1986).



1.2 Cholesterol Metabolism.

1.2.1 Cholesterol Synthesis.

Cholesterol is required by all cells of the body and is acquired from two sources. It is either absorbed from dietary sources across the gastrointestinal mucosa or it is synthesised *de novo* from acetyl coenzyme A (CoA) (Figure 1.2).

The liver is the major organ for cholesterol synthesis in the body. It is responsible for at least 50% of total body synthesis of cholesterol (Rudney and Sankhavaram, 1993). The pathway of cholesterol biosynthesis involves three distinct groups of reactions and requires 20 steps (Frantz and Schoepfer, 1967). The formation of cholesterol begins with the metabolic conversion of acetyl CoA to 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). Since the intermediates at this stage are not committed to cholesterol biosynthesis, they can be utilised in a variety of other metabolic processes. HMG-CoA is reduced to mevalonate in a reaction catalysed by HMG-CoA reductase, a microsomal enzyme considered to catalyse the rate limiting step in the pathway of cholesterol synthesis.

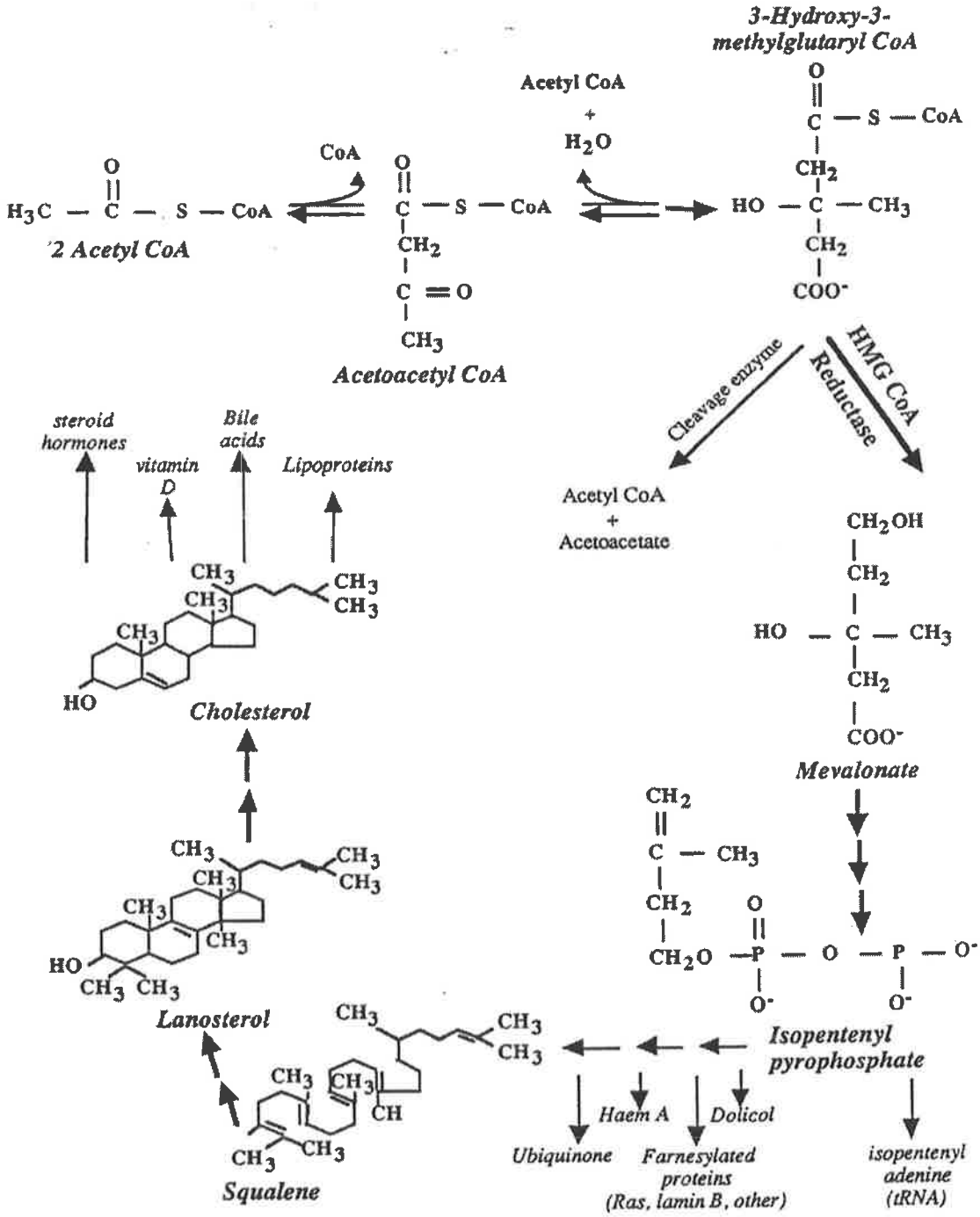
In the second phase, mevalonate undergoes a series of condensation reactions to form the long chain hydrocarbon squalene. Mevalonate is phosphorylated by ATP to form several active phosphorylated intermediates. The active isoprenoid unit then forms isopentenylpyrophosphate (IPP) by decarboxylation. The condensation of 3 molecules of IPP forms farnesyl pyrophosphate (FPP). Two molecules of FPP condense at the pyrophosphate to form pre-squalene pyrophosphate, which is then followed by a reduction reaction with NADPH to form squalene. In the liver, most of the mevalonate is ultimately converted into squalene.

The third phase of cholesterol biosynthesis involves the conversion of squalene to cholesterol. Specialised cytosolic proteins (sterol carrier proteins) are required for the last steps of cholesterol biosynthesis since squalene and the subsequent sterol precursors are poorly soluble in water. Squalene is cyclised into a four member ring structure with the introduction of a hydroxyl group. The resultant compound, lanosterol, differs from cholesterol by having three extra methyl groups and two double bonds. Subsequent reactions remove the methyl groups, shift one double bond to the five position, and saturate the other double bond. The three-dimensional structure of the final sterol product, cholesterol, allows it to intercalate among the phospholipids of the membrane and act as a moderator molecule producing intermediate states of membrane fluidity.

There appears to be a common mechanism of regulation of the sterol synthesis genes, specifically HMG CoA synthase, HMG CoA reductase, mevalonate kinase, and farnesyl diphosphate synthase (FPS). This common mechanism involves the interaction of one or more *trans*-acting factors with the SRE-1 sequences that are present in the promoter region of each of these genes (Smith et al., 1988). The expression of HMG-CoA synthase, HMG-CoA reductase, and FPS are all controlled at the level of gene transcription (Rudney and Sankhavaram, 1993). However, reductase expression appears to be the only protein in the pathway that exhibits control not only at the level of transcription, but at the level of both protein translation and protein stability as well. The reductase activity is also modulated by phosphorylation, making it one of the most highly regulated enzymes.

Any factor that ultimately affects the amount of free cholesterol in the cell will alter the rate of cholesterogenesis (Havel, 1988). The rate of cholesterol formation by the liver is highly responsive to cholesterol absorbed from dietary sources; the synthesis of HMG-CoA reductase in the liver is suppressed and existing enzyme molecules are inactivated. Dietary cholesterol may suppress hepatic cholesterol biosynthesis as a result of increased cholesterol entry into the liver via the chylomicron remnant pathway. Likewise, diversion of alimentary cholesterol from the liver by the creation of a lymph fistula reduces hepatic cholesterol content and increases the rate of biosynthesis (Rudney and Sankhavaram, 1993).

Figure 1.2: The pathway of cholesterol biosynthesis. (Adapted from Rudney and Sankhavaram, 1993).



1.2.2 Cholesterol esterification.

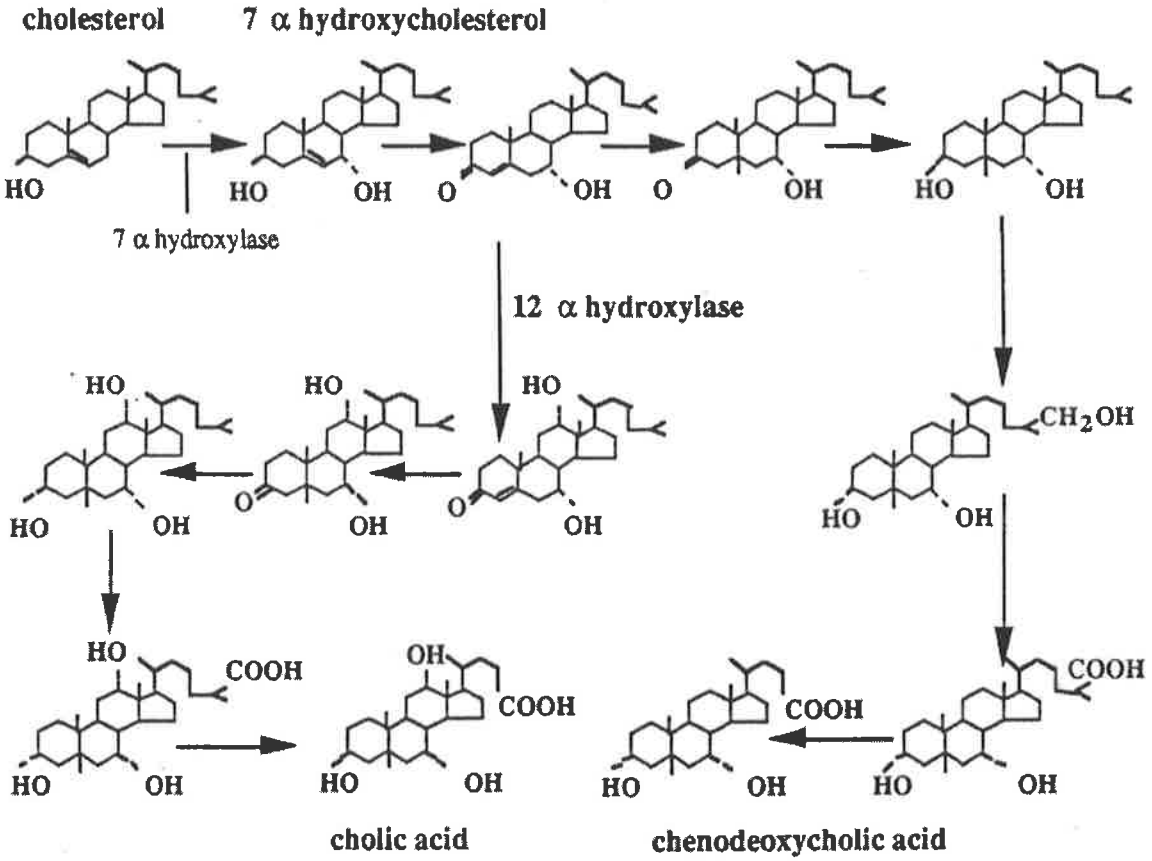
Excess intracellular cholesterol that is not needed for membrane synthesis or for the formation of bile salts, activates the endoplasmic reticulum ACAT (Suckling and Stange, 1985). The enzyme catalyses the intracellular formation of cholesterol esters from cholesterol and coenzyme A activated fatty acids. The composition of the cholesteryl esters formed *in vivo* is determined by the enzyme's preference for certain fatty acids. The order of preference in the rat is oleate < palmitate < stearate < linoleate. This process leads to the storage of cholesterol by the formation of cytoplasmic cholesteryl ester droplets and thereby ACAT plays an important role in cellular cholesterol metabolism. Esterification of cholesterol regulates the amount of free or unesterified cholesterol present in the cells. Defects in ACAT can overload the cell with cholesterol, shut down cholesterol synthesis within the cell, and decrease LDL receptor synthesis. The transformation of unesterified cholesterol, which is potentially cytotoxic, into metabolically inert cholesterol esters protects the cell and prevents deterioration of membranes. Under conditions where cellular cholesterol levels decline, cholesterol is released from storage by the hydrolysis of cytoplasmic cholesterol ester droplets to free cholesterol and fatty acids by neutral cholesterol ester hydrolase.

1.2.3 Cholesterol degradation.

More cholesterol usually enters the body pool through absorption of dietary cholesterol and *de novo* synthesis than is used during normal metabolic turnover. Thus, the excess must be metabolised (into cholesterol esters) and/or excreted to prevent a potential accumulation of cholesterol. Mammalian tissues do not possess enzymes capable of extensive degradation of the sterol nucleus. Instead, certain enzymes substitute groups on the hydrocarbon tail or on the ring structure of the sterol molecule to produce other sterol products such as bile acids and steroid hormones (Stravitz et al., 1993) (Figure 1.3).

The liver is the only organ that can excrete significant amounts of cholesterol from the body, predominantly after being converted into bile acids (Liscum et al., 1994). The conversion of cholesterol to bile acids accounts for approximately 70% of the cholesterol disposed daily (Siperstein et al., 1952). Direct secretion into the bile is the other qualitatively important route of cholesterol elimination from the body. The rate of conversion of cholesterol into bile acids is one of the major factors controlling the concentration of cholesterol in the liver cell. This is an enzymatic pathway governed by the rate-limiting enzyme cholesterol 7 α-hydroxylase.

Figure 1.3: Bile Acid Biosynthetic Pathway. The enzyme 7 α-hydroxylase catalyses the inital and rate limiting step in bile acid formation. The 12 α-hydroxylase determines whether cholic acid or chenodeoxycholic acid will be formed (adapted from Cooper, 1990).



1.3 Cholesterol, Lipoproteins and Coronary Artery Disease.

Cholesterol, like most lipids, is transported in body fluids by macromolecular complexes called lipoproteins. Considerable interest in lipoprotein metabolism has been generated in the past few decades because of the apparent correlation between the levels of certain serum lipoproteins and atherosclerosis (Brown and Goldstein., 1984). The low density lipoprotein (LDL) is the major lipoprotein responsible for the delivery of cholesterol to peripheral tissues including those of the vasculature. The cholesterol of atherosclerotic plaques is derived from the LDL particles that circulate in the blood stream and there is a strong positive relationship between the level of LDL in the plasma and the risk of coronary artery disease (McNamara, 1987; Nordoy and Goodnight, 1990). The fact that cholesterol in the arterial wall is derived from LDL has led to this lipoprotein being referred to as "bad cholesterol" (Brown and Goldstein, 1986; Steinberg, 1988).

Plasma high density lipoproteins (HDL) are also cholesterol rich particles. Evidence suggests that HDL plays an important role in the movement of cholesterol from non-hepatic tissues to the liver for catabolism and excretion. This process is known as "reverse cholesterol transport" (Glomset, 1968). High levels of HDL appear to be antiatherogenic since it acts as a scavenger of surplus free cholesterol from other lipoproteins and also from cells of the arterial wall where atherogenic plaques form (Gordon et al., 1977; Frick et al., 1989; Nordoy and Goodnight, 1990). Therefore, there is a strong negative relationship between the level of HDL in the plasma and the risk of CHD. This relationship is independent of plasma LDL, other lipoproteins such as very low density lipoprotein (VLDL) and other risk factors such as obesity, smoking and blood pressure. So HDL is often referred to as "good cholesterol".

The maintenance of cellular cholesterol homeostasis is undoubtedly the result of two opposing processes: the ability of LDL to deliver cholesterol to arteries and the ability of HDL to remove excess cholesterol from them. Therefore, it is the ratio of plasma LDL/HDL and not just plasma LDL levels alone which is the best predictor for the development of coronary artery disease (Nordoy and Goodnight, 1990).

1.4 Theories of Atherosclerosis.

A striking feature of atherosclerosis is the massive accumulation of esterified cholesterol that forms the "heart" of the atheromatous plaque. The name "atheroma" itself stems from the Greek word meaning gruel. It was first used by pathologists in the 18th century to describe a thickened area in the wall of a major artery which exuded a yellow grumous lipid upon sectioning. This lipid is now known to be nearly pure cholesterol ester (Goldstein and Brown, 1977; Brown and Goldstein, 1986).

Two schools of thought have emerged about the biochemical and physiological basis underlying the early stages in atherogenesis. These two hypotheses have generally been termed the "endothelial injury hypothesis" (Figure 1.4) and the "lipid infiltration hypothesis" (Figure 1.5) (Steinberg, 1988). It is not clear which process occurs first and the relationship between the two.

The endothelial injury hypothesis suggests that damage to the monolayer of the endothelial cells lining the artery may initiate plaque formation (Ross and Glomset, 1976). This initial damage could stem from various causes, such as smoking or elevated blood pressure, or cholesterol. Loss of the structural integrity of the endothelium may cause the release of platelet derived growth factors (PDGF) which could stimulate smooth muscle cell proliferation and secretion of other growth factors. Thus, repeated episodes of endothelial damage and smooth muscle cell proliferation can lead to the development of lesions (Ross and Glomset, 1976; Ross, 1981).

According to the classical lipid infiltration hypothesis, lipoproteins, especially LDL, infiltrate the arterial intima prior to endothelial cell damage (Small, 1977; Steinberg, 1988).

The major initiator of atherogenesis in this case is the uptake of lipid which does not involve cell damage of the arterial wall. Endothelial cells, smooth muscle cells and monocyte macrophages in the intima appear to accumulate lipids, particularly cholesterol, from these LDL particles and possibly from other lipoproteins such as chylomicron remnants, very low density lipoproteins (VLDL) and ß-VLDL (see section 1.5) (Steinberg, 1988). This implies that circulating cholesterol, principally carried in LDL, is central to the atherogenic process, and hence atherogenesis does not progress at lower plasma levels (Steinberg, 1988).

The lipid infiltration hypothesis has been further extended as recent evidence has emerged which states that the oxidative modification of LDL (or other lipoproteins) is important and possibly obligatory in the pathogenesis of the atherosclerotic lesion (Figure 1.5). It appears that macrophages can only accumulate LDL that has been modified by oxidation (Ox-LDL). The oxidation process is presumed to start with the polyunsaturated fatty acids of phospholipids on the surface of LDL and then propagates to the cholesterol moiety and to the apolipoprotein B. It has been shown that macrophages and other cells in the arterial wall such as smooth muscle and endothelial cells are capable of oxidatively modifying LDL.

The antioxidant content (vitamin E, ß-carotene etc.) of the LDL particle is also critical for its protection and, theoretically, if sufficient lipophilic antioxidant compounds are present then the LDL particle is protected from oxidant challenge. Whether or not LDL becomes oxidised *in vivo*, is therefore a result of a balance between the extent of the pro-oxidant challenge and the capacity of the antioxidant defences (Witztum, 1994).

Atherosclerosis is now believed to be a complicated interaction of endothelial cell damage, lipoproteins and their interactions with the cells and matrix proteins of the arterial wall, thus atherogenesis is a multifactorial disease (Witztum, 1994). The result is the unification of both hypothesis which couples the lipid infiltration hypothesis with the endothelial injury hypothesis to account for transition from fatty acid streak to an advanced lesion (Steinberg, 1988) (Figure 1.6). In fact, it is recognised that endothelial injury may not be the initial event, but rather a secondary event that makes an important contribution to the development and progression of fatty streaks within the arterial wall (Steinberg, 1988; Witztum, 1991). Nevertheless, LDL infiltration into the intima subsequently leading to the development of fatty streaks, can not alone account for advanced lesion formation without the series of events following endothelial injury. It has been suggested that the earliest event in atherogenesis is the focal accumulation of LDL within the intima of an apparently normal artery where they are susceptible to oxidation by arterial cells (Schwenke and Carew, 1989) (Figure 1.6). This seems to occur in response to elevated plasma LDL and this accumulation of LDL appears to be the first identifiable event in lesion-prone sites of the aorta. Monocyte macrophages then adhere to the luminal surface of the overlying endothelium at the sites of LDL accumulation. Subsequent to endothelium binding, monocyte macrophages migrate into the subintimal space under an intact endothelium. Here they take on the classical

appearance of foam cells. This term arises from the foam-like appearance of these cells in electron micrographs as a result from enrichment of the macrophages with cholesterol esters (Steinberg, 1988). As more monocytes enter the intima, more foam cells are generated. Increases in the size and number of these macrophages eventually results in the rupture of the endothelium. At this stage, the macrophages are exposed to the circulating blood elements, allowing platelet adherence and aggregation to occur. Release of factors such as PDGF serve both to recruit smooth muscle cells into the intimal layer and to stimulate their proliferation (Steinberg, 1988; Witztum, 1991). The accumulated cholesterol, cells and debris constitutes an atherosclerotic plaque which, in time, can narrow the lumen of the artery and lead to occlusion (Brown and Goldstein, 1986). Whatever the relative contributions of lipid infiltration and endothelial damage to the development of arterial lesions, the accumulation of LDL cholesterol ester in the arterial intima is a crucial event in atherogenesis.

Figure 1.4: The response-to-injury hypothesis of atherosclerosis. (From Bender, 1994).

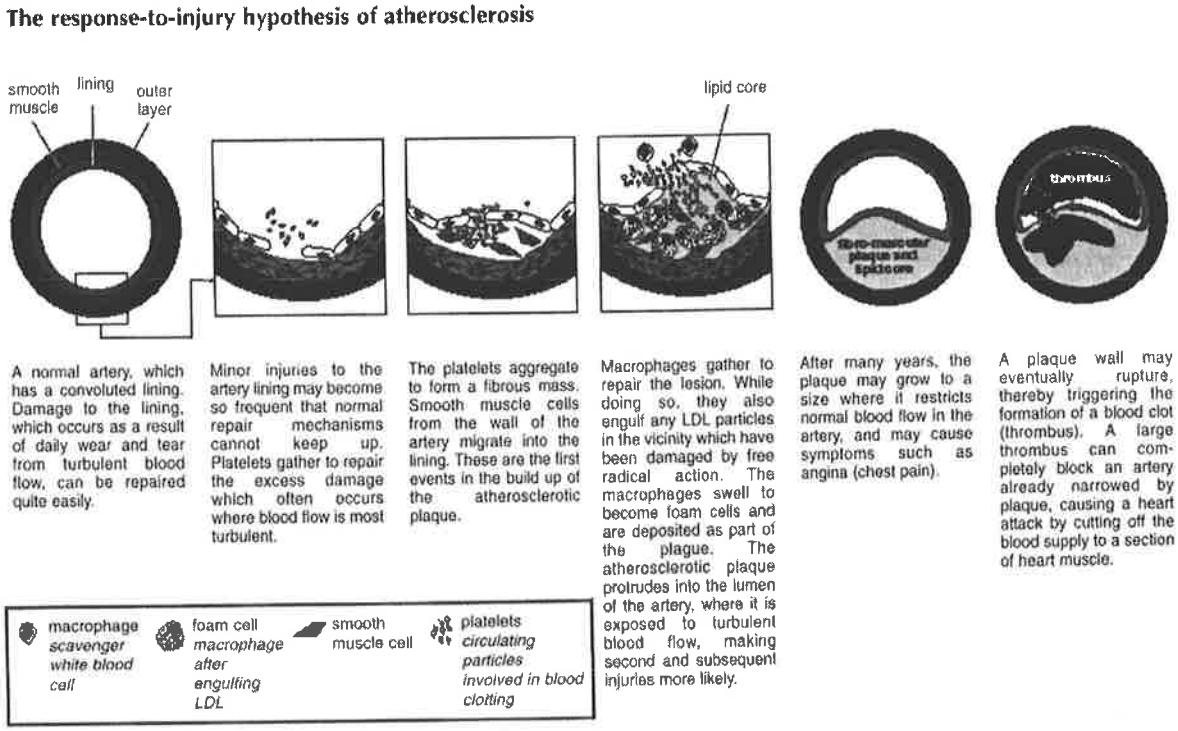


Figure 1.5: The lipid infiltration hypothesis. Postulated sequence of events by which LDL, after oxidative modification, could in itself lead to foam cell formation and the fatty streak lesion (from Steinberg, 1988).

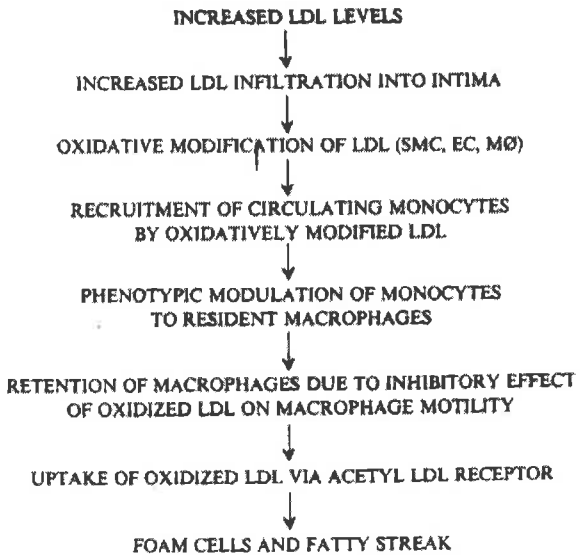


Figure 1.6: Unified Hypothesis. A unified hypothesis that couples the lipid infiltration hypothesis with the endothelial injury hypothesis to account for transition from fatty streak to advanced lesion (from Steinberg, 1988).

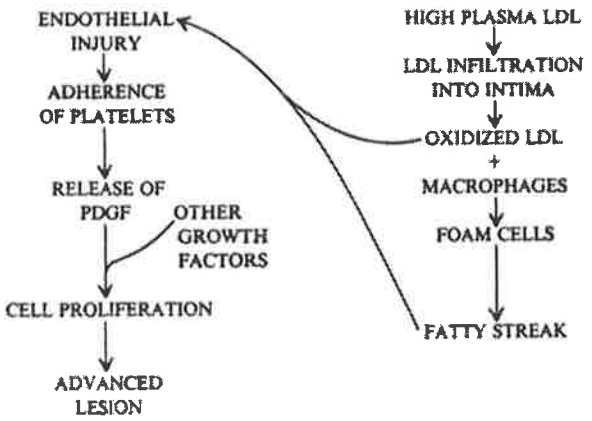


FIG. 4. A unified hypothesis that couples the lipid infiltration hypothesis with the endothelial injury hypothesis to account for transition from fatty streak to advanced lesion.

1.5 Lipoproteins.

Lipoproteins consist of a variety of different heterogenous groups of particles which are divided into 4 classes due to differences in density, size, chemical composition and apoprotein content (Table 1.1).

Table 1.1: Composition and characteristics of human plasma lipoproteins. (Taken from Thompson, 1989).

Characteristics	**Chylomicrons**	**VLDL**	**LDL**	**HDL**
Protein (% particle mass)	2	7	20	50
Triacylglycerols (% particle mass)	83	50	10	8
Cholesterol (% particle mass) (free + esterified)	8	22	48	20
Phospholipids (% particle mass)	7	20	22	22
Particle mass (x 10^6 Daltons)	0.4-30	10-100	2-3.5	0.175-0.36
Density range (g/ml)	<0.95	0.95-1.006	1.019-1.063	1.063-1.210
Diameter (nm)	>70	30-90	18-22	5-12
Major apolipoproteins	A_1, B_{48}, C_1, C_2, C_3	B_{100}, C	B_{100}	A_1, A_2, C
Trace apolipoproteins	E	A, E	A, C, E	E, D
Site of synthesis	gut	gut, liver	capillaries of peripheral tissues, liver	gut, liver
Major function(s)	transport of dietary fat	transport of endogenous fat	transport of cholesterol to peripheral tissues	reverse transport of cholesterol to liver

1.5.1 Chylomicrons.

Chylomicrons are the largest and least dense of the lipoproteins because they contain a high proportion of lipid relative to protein. Their function is to transport lipids of dietary origin (Davis, 1991). If blood plasma obtained from a patient soon after a meal containing fat is allowed to stand in a test tube overnight, a creamy layer forms at the top. In contrast, a plasma sample taken from a fasting person will not produce the creamy layer. The creamy layer is composed of chylomicrons, which are the major lipid secretory products of the intestine.

Dietary intake of fat in Western civilisation ranges from 50-100g/day and most of it is in the form of triacylglycerol and a small fraction consists of cholesterol esters and phospholipids. The triacylglycerols contain fatty acids generally with chain lengths greater than 14 carbon atoms which can be saturated (no double bond such as palmitic and stearic) as well as unsaturated (one or more double bonds such as oleic and linoleic). Emulsification of dietary lipid begins in the stomach, but it is in the intestinal lumen where the triacylglycerols and phospholipids are hydrolysed to free fatty acid and monoglycerols. They are then absorbed by the intestinal enterocytes, re-esterified into triacylglycerol and packaged into triacylglycerol-rich chylomicron particles. The fat soluble vitamins such as A, D, E and K are also packed with the triacylglycerols. They are all incorporated with cholesterol esters into the nonpolar core of neutral lipid that is surrounded by a coat of phospholipid, apoproteins, and unesterified cholesterol (Patsch, 1987).

The lipid composition of the chylomicron is a reflection of the composition of the diet. For example, a high cholesterol meal will result in relatively small, cholesterol ester rich chylomicrons, while a triacylglycerol-rich meal will result in large, triacylglycerol-rich chylomicron particles. The major apoprotein (apo) of chylomicrons is apo-B48, which is a large, insoluble apoprotein with a molecular weight of 264,000 Dalton's. Chylomicrons contain other apoproteins such as apo A-I, apo A-IV, apo E and apo C. Their size depends on factors such as the rate of lipid absorption and the type of dietary fatty acids that predominates in the diet. Thus, larger chylomicrons are produced after the consumption of large amounts of fat at the peak of absorption or when apoprotein synthesis is limiting. When fatty acids are largely unsaturated, the chylomicrons tend to be larger than when saturated fatty acids are the major fat in the diet .

Chylomicrons are secreted into the intestinal lymph and enter the systemic circulation. As they pass into the peripheral circulation they come into contact with an enzyme, lipoprotein lipase, which is located on the surface of the capillary endothelial cells and hydrolyses the triglycerides. This results in a smaller chylomicron particle called a remnant which is released into the circulation (Figure 1.7).

The liver is the site of removal of almost all chylomicron remnants which occurs via a number of uptake processes. The initial removal of chylomicron remnants by the liver

appears to involve interaction with several macromolecules on the cells surface, including, heparin-sulfate-bound hepatic lipase, apoE, as well as the LDL receptor, which binds the chylomicron remnant with high affinity. Interaction of remnants with the LDL receptor related protein (LRP), which recognises apoE as the ligand, or another endocytic receptor with similar properties, occurs slowly. This process requires the modification of surface-bound chylomicron remnants, and serves as a backup mechanism that is utilised primarily when LDL receptors are deficient or down-regulated. This backup mechanism is sufficient to prevent an appreciable accumulation of chylomicron remnants in humans and other mammals lacking functional LDL receptors (Havel, 1995)

1.5.2 VLDL.

The liver, like the gut, synthesises triacylglycerol rich lipoproteins and these are called very low density lipoprotein (VLDL). The main differences between chylomicrons and VLDL are their site of synthesis and the source of the triglyceride being transported. VLDL are similar to chylomicrons but are smaller, ranging in size from 25 to 100 nm, and contain less triglyceride but more cholesterol, phospholipid and protein. The protein consists of a mixture of apo C, apo E and apo B_{100} (referred to as apo B). The triacylglycerol and the apoproteins of these lipoproteins are synthesised in the ER and they emerge together with phospholipids and unesterified cholesterol to form a VLDL particle. Once synthesised, VLDL is responsible for delivering endogenously synthesised fat (in the form of triacylglycerols) from the liver to extra hepatic cells. VLDL particles deliver lipid to other tissues in a similar manner as chylomicrons. VLDL particles show a considerable variation in size, smaller particles having a lower ratio of apo C : apo B than larger ones. These particles can undergo lipolysis and the subsequent smaller particles are referred to as VLDL remants or IDL (see section 1.5.3) (Figure 1.7).

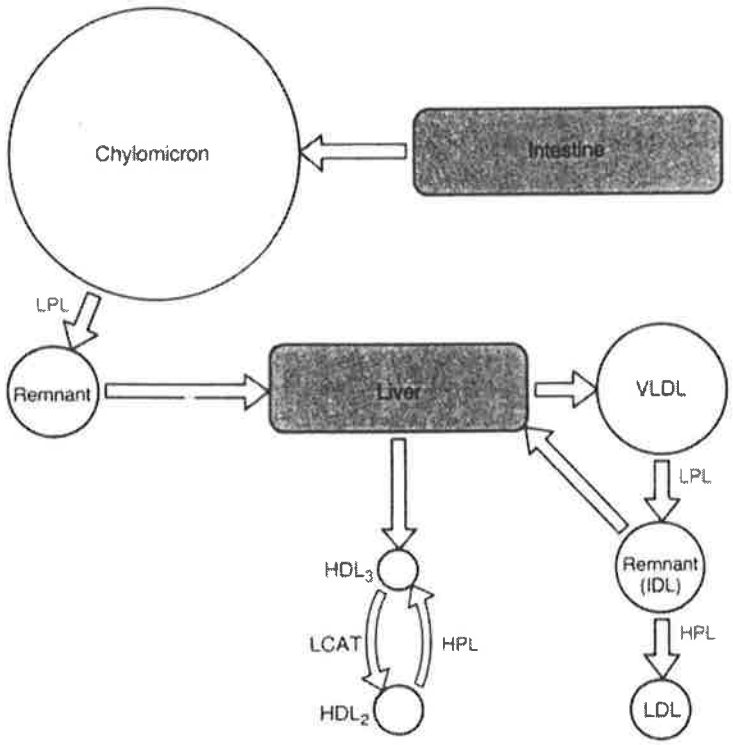
Apolipoprotein B formed in the liver is the major protein in VLDL and it is essential for the assembly and secretion of VLDL. The production of apo B does not appear to be regulated. The mRNA synthesis, as well as protein production has been shown to be constant under a number of different experimental conditions (Lusis et al., 1987; Adeli, 1994). However, most of the apo B_{100} formed in the liver is destroyed in the endoplasmic reticulum, but its secretion into VLDL is influenced by lipid metabolism in the liver.

The signal for producing VLDL is triggered by the availability of triacylglycerol for secretion in the lumen of the endoplasmic reticulum. The lipids probably pull trapped apo B out of the reticulum membrane for making VLDL. The fatty acid composition of the triacylglycerol formed in the liver has been found not only to effect VLDL assembly and/or secretion by the liver cells, but it may have some influence on the size of the VLDL particle secreted.

The rate of VLDL secretion varies directly with the rate of apo B secretion. In addition to apo B, nascent hepatic VLDL contains apo E and a small amount of apo C. As

nascent VLDL enters the plasma it acquires apo C as well as cholesterol esters from HDL in exchange for TG as catalysed by the cholesterol ester transfer protein (CETP). The VLDL particle undergoes lipolysis by lipoprotein lipase at the surface of endothelial cells and hydrolysis of the TG's results in the formation of a VLDL remnant. About 50-60% of these particles are directly cleared by the liver, probably by the chylomicron remnant removal systems (Grundy, 1986).

Figure 1.7: Lipoprotein metabolism. A simplified scheme of lipoprotein metabolism demonstrating the roles of lipoprotein lipase (LPL) and hepatic lipase (HPL) in the conversion of triglyceride-rich chylomicrons and VLDL into cholesterol-rich HDL and LDL (from Thompson, 1989).



1.5.3 LDL.

The role of low density lipoprotein is to transport cholesterol to tissues where the cholesterol may be required for membrane structure or conversion into various metabolites such as steroid hormones. LDL is the major carrier of plasma cholesterol in man, although this is not so in all other mammals. In some animal species such as ruminants and some rodents, most of the cholesterol is transported in high density lipoprotein (Davis, 1991).

LDL is characterised as a large spherical particle whose fluid core is composed of neutral lipids, mostly cholesterol ester (Brown and Goldstein, 1986). This core of cholesterol ester is surrounded by a monolayer of phospholipid and unesterified cholesterol molecules (Figure 1.8). The phospholipids are arrayed so that their hydrophilic heads are on

the outside surface allowing the LDL to be soluble in the blood or interstitial fluid. Embedded in this ampiphatic coat is the large protein molecule designated apoprotein B_{100} (refer to as apo B) (Brown and Goldstein, 1986). It is apo B which is recognised and bound by the LDL receptor on the surface of the cell. LDL particles are normally isolated from plasma by ultracentrifugation at salt densities between 1.019-1.063 g/ml. Each lipoprotein particle contains the same mass of apo B, but each can differ with respect to the amount of bound lipid.

In most mammals, the protein and most of the lipid component of LDL is derived almost exclusively from hepatic VLDL, one particle of VLDL generates one particle of LDL. LDL differs from its precursor VLDL as it contains a much lower amount of triglycerides, higher content of cholesterol and retains only one of the various apoproteins found in VLDL, apo B. The synthesis of LDL begins when the liver secretes VLDL into the bloodstream. LDL is then derived from the breakdown of VLDL by lipoprotein lipase (Figure 1.7). Lipolysis of VLDL produces a cascade of VLDL intermediates which contain a progressively lower proportion of triacylglycerols and correspondingly richer proportion of cholesterol and phospholipids. These particles are collectively termed intermediate density lipoprotein (IDL) and specifically refer to the intermediate particles formed during the conversion of VLDL to LDL. During the transformation, the apo B component remains with these particles and the apo C and apo E components are progressively lost. The IDL may be cleared from the circulation by the liver or they may be further processed by hydrolysis of the surface and core lipids whereby hepatic lipase progressively removes the rest of the triacylglycerols and LDL is formed. The major component of LDL is cholesterol esters, which is located in the neutral lipid core of the particle.

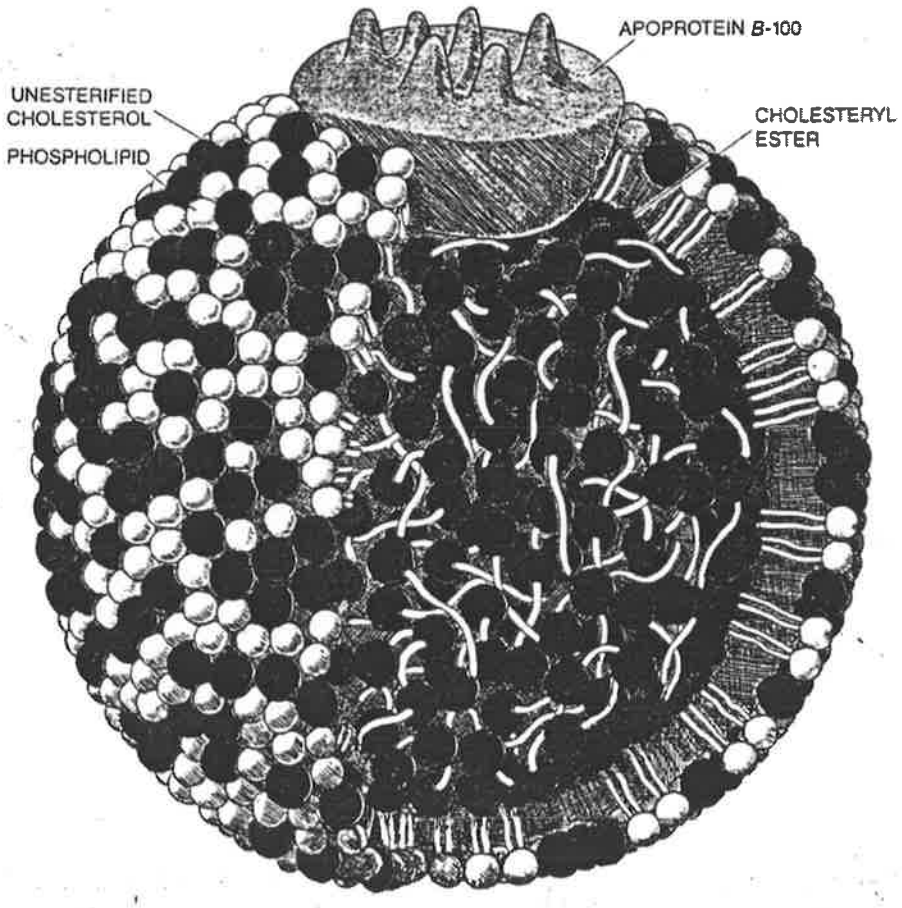
LDL plasma concentration is determined by the difference between the rate at which it is synthesised and removed from the body (Grundy, 1991). Turnover studies suggest that the rate of turnover of VLDL-apo B can account for all the LDL apo B synthesised (Soutar et al., 1977). Apart from the synthetic rate, the other major determinant of LDL concentration in plasma is the rate at which it is catabolised (Grundy, 1991). Normally, 30-40% of the intravascular pool of LDL/day is catabolised (this is equivalent to a fractional catabolic rate of LDL in the region of 0.3-0.4), most of which is mediated by the LDL receptor (see LDL receptor pathway, section 1.6.1). The liver accounts for the uptake and degradation of 60% of LDL that is turned over in the humans (Dietschy et al., 1993). LDL metabolism (synthesis and catabolism) is influenced by both environmental and genetic factors, such as the type of fat eaten (see section 1.8), the mutations in apo B and the mutations of the LDL receptor gene (also see section 1.7.4).

A mathematical model is used to analyse the disappearance of plasma radiolabelled LDL *in vivo* and to estimate LDL receptor activity. The absolute rate of catabolism of LDL is estimated from fractional catabolic rate (FCR) of a tracer dose of injected radio iodinated LDL and the LDL plasma concentration. Measurement of the rate of disappearance of

labelled native LDL from circulation provide the basis for the estimates of total rate of catabolism of LDL by all routes in the whole body, including LDL receptor dependent and independent pathways (Dietschy et al., 1993).

The LDL particle is also thought to be modified by oxidation, and these oxidised LDL particles can then be removed rapidly from the plasma by scavenger receptors residing in cells of the arterial wall. If dietary antioxidants are insufficient, then this may promote enhanced uptake of oxidised LDL (Steinberg, 1988) and may decrease the plasma concentration of LDL (see section 1.4 and 1.6.2).

Figure 1.8: **The structure of the LDL particle.** The major cholesterol carrier in the blood stream which is a spherical particle which consists of about 1500 cholesterol ester, surrounded by about 800 phospholipids and 500 molecules of unesterified cholesterol. The lipoprotein contains one large apolipoprotein B100 (from Brown and Goldstein, 1984).



1.5.4 HDL.

High density lipoprotein (HDL) particles have a spherical shape and possess a core of neutral lipids consisting of mostly cholesterol esters and variable amounts of triacylglycerols, depending on the plasma triacylglycerol levels. High density lipoproteins are initially synthesised in hepatocytes and intestinal cells as disc shaped particles rather than spherical shaped particles like other lipoproteins because they are devoid core lipids. They consist of a bilayer composed mainly of phosphtidylcholine with apo A and apo E at the margins of the disc. On entering plasma, they are rapidly converted to spherical HDL by acquiring cholesterol. The cholesterol is esterified by the plasma enzyme lecithin:cholesterol acyltransferase (LCAT) and is driven away from the surface of the HDL and into the core (space that is occupied by fatty acid acyl chains of the phospholipids). Additional lipid such as phospholipids and apoproteins are also acquired by transfer from chylomicrons and VLDL (Patsch and Gotto, 1987) (Figure 1.7).

HDL is generally divided into two subclasses, HDL_2 and HDL_3, because centrifugation of HDL gives rise to a bimodal distribution in the HDL region. The two subclasses also have different metabolic significance; HDL_2 appears to have a stronger inverse relationship with coronary artery disease than HDL_3. The major apoproteins of HDL are apo A-I and apo A-II but it also contains apo C, apo E and a protein which is unique to HDL, apo D (Miller, 1987) (Figure 1.7).

HDL carries cholesterol from peripheral cells to the liver either for excretion from the body or for recycling, a process called "reverse cholesterol transport". This process is believed to be antiatherogenic because HDL is able to promote efflux of cholesterol from the artery wall and more importantly, it is the only means by which cholesterol can be eliminated from cells in most tissues of the body.

1.6 LDL metabolism.

1.6.1 Uptake by the LDL receptor pathway.

The mechanism of cellular of uptake of LDL from the circulation by the LDL receptor pathway was established by Brown and Goldstein for which they were awarded the 1985 Nobel prize in medicine (Brown and Goldstein, 1986). The discovery of the LDL receptor has had a profound impact on cell biology, clinical investigations, therapeutics, and on the study of atherosclerosis in general (Brown and Goldstein, 1986: Steinberg, 1988).

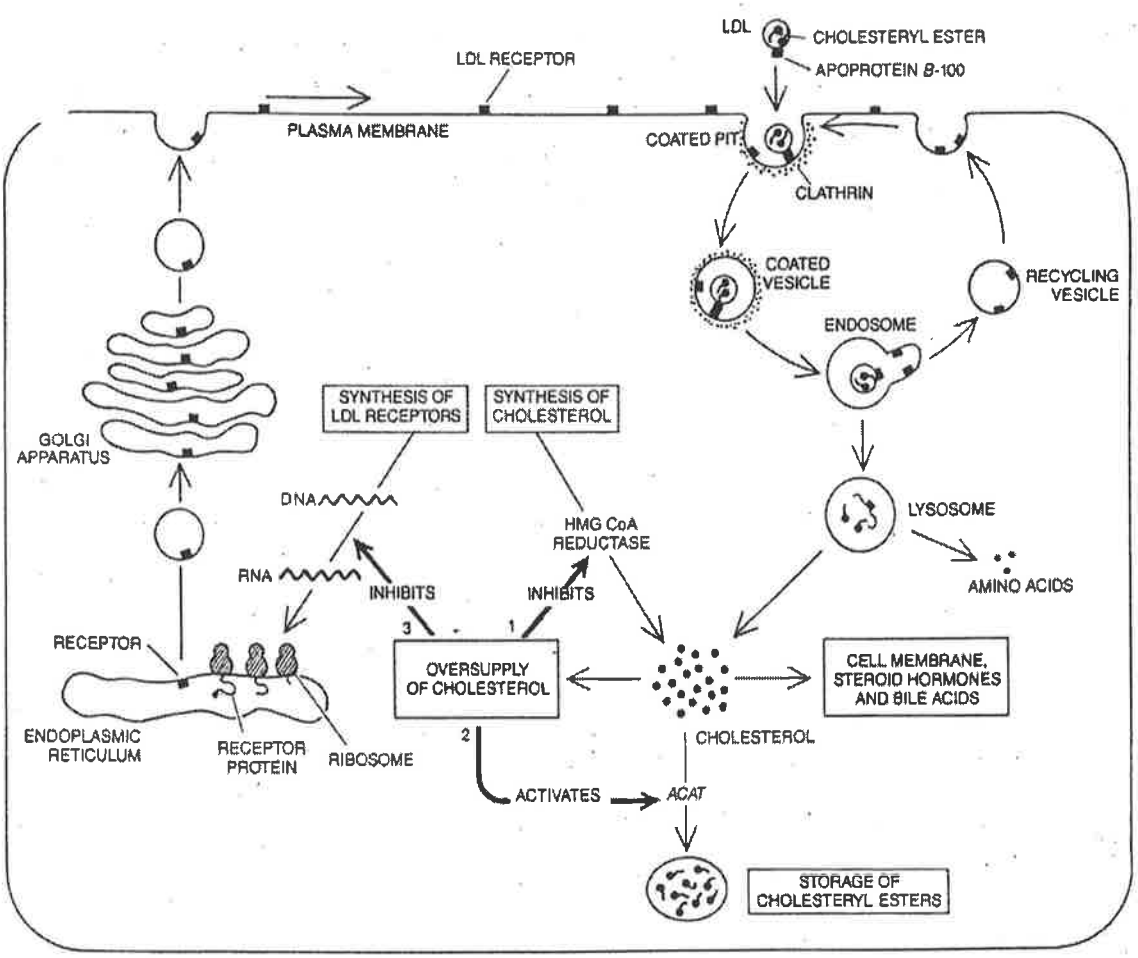
LDL receptors are located on the surface of the cell and are concentrated in the regions of the plasma membrane that contain indentations called coated pits (Figure 1.9). These pits are covered by a protein, clathrin, which imparts a fuzzy appearance to the pit. The receptor spans the membrane and carries a binding site that protrudes from the cell surface. When LDL binds to these receptors, the resulting lipoprotein and receptor complexes are internalised by invagination of the coated pit to form endocytotic vesicles or endosomes (Figure 1.4). These vesicles enter the cytoplasm where their acidification is believed to promote separation of LDL from its receptor. The receptor then recycles back to the plasma membrane where it can bind to another particle of LDL and initiate another round of internalisation. Each receptor migrates into and out of the cell approximately once every 10 minutes, carrying one LDL particle each time.

After its release from its receptor, the LDL is segregated into vesicles which fuse with lysosomes (Figure 1.9). In the lysosomes, acid lipases hydrolyse the LDL and its components, liberating unesterified cholesterol which enters the cellular cholesterol pool. The cholesterol liberated from LDL mediates a complex series of feedback control mechanisms that protects the cell from an over accumulation of cholesterol. First, it reduces the cell's ability to make its own cholesterol by inhibiting the synthesis of an enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyses the rate limiting step in the cholesterol biosynthetic pathway. Second, the LDL derived cholesterol promotes the storage of cholesterol in the cell by activating acyl CoA:cholesterol acyl transferase (ACAT). This enzyme esterifies a fatty acid to the ß-OH of cholesterol and the resulting cholesterol esters are deposited as cytoplasmic droplets. Third, the accumulation of cholesterol within the cell drives a feedback mechanism that suppresses the transcription of the LDL receptor gene, preventing the synthesis of new LDL receptors and thereby reducing the number of LDL receptors present on the cell membrane.

The LDL receptor-mediated regulatory system allows the cell to coordinate the utilisation of intra- and extracellular sources of cholesterol. Human fibroblasts and other mammalian cells in culture are able to survive in the absence of a large amount of lipoproteins because they can synthesise cholesterol from acetyl-CoA. When cholesterol is available, however, the cells primarily use the LDL receptor to import LDL and keep their

own synthetic activity suppressed. Thus, a constant level of cholesterol can be maintained within the cell even when the external supply in the form of lipoproteins undergoes large fluctuations. *In vivo*, the main task of LDL receptors is to supply the cells with cholesterol, thereby mediating the removal of cholesterol rich lipoproteins particles from the bloodstream. Having such precise feedback suppression of endogenous cholesterol synthesis as a primary metabolic response and, also, the inhibition of LDL receptor synthesis allows individuals to maintain a relatively constant plasma cholesterol level and avoid massive accumulation of excess cholesterol in body tissues and cells of the arterial wall.

Figure 1.9: The LDL receptor pathway. Circulating LDL is taken up into the cell by receptor mediated endocytosis. LDL is bound by a receptor in a coated pit, which invaginates and pinches off to form a coated vesicle. Several vesicles give rise to an endosome where the acidic environment causes the LDL receptor to dissociate from the LDL and recycles back to the cell surface. The LDL is delivered to the lysosome where apolipoprotein B100 is broken down into amino acids and cholesterol ester is hydrolysed to free cholesterol, which is released into the cell. This released cholesterol is self-regulating, and has three metabolic effects (see section 1.6.1) (from Brown and Goldstein, 1984).



1.6.2 Uptake by the scavenger receptor.

The cells of the arterial wall, such as monocyte macrophages and smooth muscle cells, can control the amount of cholesterol entering the cell via the LDL receptor pathway. This highly regulated LDL receptor pathway in these cells does not, therefore, account for the accumulation and deposition of large quantities of LDL-cholesterol in cells that are associated with atheroma (Brown and Goldstein, 1986). Several lines of evidence suggest that the accumulation of native LDL by macrophages in the arterial wall does not occur to any significant extent (Steinberg, 1988; Witztum, 1991). It has been shown in cell culture that macrophages cannot be transformed into foam cells simply by exposure to high levels of native LDL (Witztum, 1991). Furthermore, LDL receptor deficient humans and rabbits develop severe and rapid atherosclerosis with marked foam cell formation despite a total absence of functional LDL receptors. This suggests that there may be another pathway involved in the uptake of LDL by cells (Witztum, 1991). So, how does LDL cholesterol get into cells, such as macrophages and smooth muscle cells, and cause foam cell formation ?

Goldstein et al. (1979) have reported that LDL modified by acetylation (acetyl LDL), is taken up by monocyte-macrophages by a pathway that is not downregulated. Acetyl LDL is taken up rapidly by macrophages in culture, transforming these cells into lipid laden cells that resemble the foam cells observed *in vivo*. The acetyl LDL is taken up by a saturable, specific process, implying the presence of an acetyl LDL receptor that has now been designated "the scavenger receptor" (Steinberg, 1988; Witztum, 1991). Other chemically modified forms of LDL have also been shown to compete with acetyl LDL for the scavenger receptor. These include methylated LDL (Gajdusek et al., 1980) and acetoacetylated LDL (Fogelman et al., 1980). These chemically modified forms of LDL all have the lysine amino groups of apo B_{100} blocked. *In vitro*, LDL can bind copper which can promote rapid lipid peroxidation (Witztum, 1994).

Endothelial cells, arterial smooth muscle cells and macrophages can also modify LDL by an oxidative mechanism to a form that becomes recognisable to the scavenger receptor (see section 1.4). These cells can initiate the oxidation of polyunsaturated fatty acids of the LDL-surface phospholipids which results in oxidative modification of the cholesterol moiety and the modification of the apolipoprotein B. It is postulated that the oxidative modification of LDL occurs in the arterial intima if sufficient antioxidants are not present (Witztum, 1994).

It appears to be the scavenger receptor pathway and not the LDL receptor pathway, that is responsible for lipid accumulation within the arterial wall. Unlike the LDL receptor, the scavenger receptor pathway does not appear to be regulated by the cholesterol content of the cells, thereby enabling cells to accumulate massive amounts of cholesterol. Deposition of uncontrolled levels of lipid can transform these cells of the arterial wall into foam cells and eventually lead to the formation of atherosclerotic lesions.

1.7 The LDL receptor.

1.7.1 Introduction

The scavenger receptor is thought to be directly responsible for arterial plaque formation by allowing the uncontrolled influx of lipid into cells. In contrast, the accumulation of cellular cholesterol is tightly regulated by the LDL receptor. Therefore, it is thought that the LDL receptor is not likely to be directly involved in the atherosclerotic process. The preventive role of the LDL receptor in atherosclerosis was first appreciated when it was shown that its genetic absence is responsible for the severe atherosclerotic disease, familial hypercholesterolemia (FH). In 1939, Muller identified the disease as an inborn error of metabolism causing high cholesterol levels and heart attacks in young people (Brown and Goldstein, 1984). He showed that it was transmitted as a single dominant trait determined by a single gene. Khachadurian in the 1970's demonstrated that there are two forms of FH, a heterozygous form and a more severe homozygous form (Khachadurian, 1971).

Approximately 1 in 500 individuals has one mutant gene for the LDL receptor (Brown and Goldstein, 1986). They produce on average one half of the normal number of LDL receptors since they only have one normal gene. Their plasma LDL level can be twice the normal level and they start to experience heart attacks in their early 40's.

Approximately 1 in 1,000,000 people is a FH homozygote. Their plasma LDL levels can be up to 6 times higher than normal and they typically develop heart disease before the age of 20, often before age 10 (Brown and Goldstein, 1986). Studies with radioactively labelled LDL show that in FH homozygotes, the LDL particles remain in the blood stream about two and half times longer than they do in people with normal functioning LDL receptors (Langer et al., 1972). In addition, homozygotes actually produce twice as much LDL per day as normal people. This is because the precursors of LDL, VLDL and IDL also remain in the plasma longer since they are not removed from plasma through the LDL receptor. Therefore, elevated levels of these precursor lipoproteins cause an increase in the production of LDL. This synergistically acts with the decreased removal of LDL to increase the level of LDL in plasma. Children with FH have none of the risk factors for atherosclerosis other than elevated LDL levels. They have normal blood pressure, do not smoke and do not have high blood glucose levels. This condition demonstrates unequivocally the causal relationship between an elevated circulating LDL level and atherosclerosis. In addition, it demonstrates that the LDL receptor has a protective effect against atherosclerosis because the absence of LDL receptors causes the disease.

1.7.2 LDL receptor synthesis, recycling and structure.

The LDL receptors are synthesised in the rough endoplasmic reticulum (RER) and initially have an apparent molecular weight (M_r) of 120,000 Dalton's. They are transported to the Golgi apparatus where they are converted to a mature form with an apparent M_r of 160,000 Dalton's by the addition of carbohydrate (Gianturco et al., 1987). The receptor proteins can then move to the cell surface and migrate to the coated pits where they cluster by virtue of their cytoplasmic segment. When this segment is absent, the receptor can bind on the surface, but it can not slide laterally into the coated pits and take LDL into the cell. (Goldstein and Brown, 1977; Brown and Goldstein, 1986). About 80% of the LDL receptors are concentrated in these coated pits which cover about 2% of the cell surface.

The LDL receptors on the cell surface internalise once every 10 minutes. Since there is no reservoir of LDL receptors in the cell, the LDL receptor can recycle up to 100 times from the cell surface to the cell interior and back again every 20 hours. The half life of the receptor is 20-25 hours when the synthesis of new receptor is inhibited by cyclohexamide (Goldstein et al., 1979).

The structure of the LDL receptor has been extensively studied in the laboratories of Goldstein and Brown (Figure 1.10). The LDL receptor protein contains 839 amino acids and has an apparent molecular mass of 160,000 Dalton's. The receptor has five structural domains: 1) a ligand binding domain, 2) a domain having homology to epidermal growth factor (EGF) 3) a o-linked sugars region 4) a membrane spanning region, and 5) a cytoplasmic tail (Innerarity, 1991).

The ligand domain of the LDL receptor is located on the external surface of the plasma membrane (Figure 1.8) and consists of the amino-terminal 292 amino acids. This domain contains a 40 amino acid sequence which is repeated with some variation seven times (Goldstein et al., 1985; Sudhof et al., 1985; Schneider et al., 1983). Each of the seven 40 amino acid repeats contains six cysteine residues, which are all disulfide-bonded. The disulfide-bonding allows the region to exist in a tightly cross-linked structure and gives the binding domain of the receptor its stability.

Evidence also suggests that interactions between the LDL receptor and the LDL particle is an ionic attraction between the clusters of acidic residues of the LDL receptor binding repeats and the cluster of basic amino acid residues of its ligands, apo B (Figure 1.11) (Innerarity, 1991). The LDL receptor binding region of apo B consists of two regions containing a high proportion of basic amino acid residues (3147-3157 and 3359-3367). These two regions are brought together by a proline-rich segment with potential for forming amphipathic ß-sheets. There is also a disulfide bridge between the cysteines at positions 3167 and 3297 linking the two regions (Figure 1.11). It is within these regions that apo B binds to the LDL receptor, as monoclonal antibodies whose epitopes have been mapped to this region inhibit the binding of the LDL receptor to apo B. Also, selective

chemical modification of some of the basic residues of apo B abolish its ability to bind to the LDL receptor (Corsini et al., 1992).

The ligand binding domain of the LDL receptor contains a binding site for divalent cations such as calcium in its repeat 1 (Figure 1.10). It is thought that calcium may modulate a conformation change of the repeats in the binding domain when bound to repeat 1 (Innearity, 1990). However, repeat 1 is not necessary for lipoprotein binding. Lipoprotein binding to mutant receptors not containing repeat 1 was found to require the presence of a divalent cation, such as calcium, and is probably related to the LDL receptor interaction with its ligand, apo B. This interaction is abolished by the chelating agent EDTA.

The EGF-like domain of the LDL receptor consists of approximately 400 amino acids and is 35% homologous to a portion of the extracellular domain of the precursor for EGF (Yamamoto et al., 1984; Russell et al., 1984; Sudhof et al., 1985). This region is required for dissociation of the receptor from the ligand in the endosomes and recycling of the receptor to the plasma membrane (Figure 1.10).

The third domain of the LDL receptor lies immediately external to the membrane spanning domain and is encoded within a single exon (Russell et al., 1984) (Figure 1.10). It consists of a stretch of 58 amino acids containing 18 serine and threonine residues, and a cluster of o-linked sugar chains. This arrangement is similar to the interleukin-2 receptor on T-lymphocytes which has been shown to have o-linked sugars and a cluster of serine and threonine residues immediately external to the membrane spanning region (Goldstein et al., 1985). The functional significance of this region is not known as deletion of this domain does not affect the binding, internalisation, recycling or the stability of the LDL receptor in cultured CHO cells (Davis et al., 1986).

The fourth domain consists of a stretch of 22 hydrophobic amino acids which spans the plasma membrane (Yamamoto et al., 1984; Russell et al., 1984). This region is important in anchoring the LDL receptor to the plasma membrane (Figure 1.10).

The fifth domain is the COOH- terminal segment of 50 amino acids which projects into the cytoplasm (Yamamoto et al., 1984; Russell et al., 1984) (Figure 1.10). This cytoplasmic tail appears to play an important role in clustering in coated pits, either through an interaction with clathrin or the cytoplasmic side of the membrane (Goldstein et al., 1979; Brown et al., 1983). Using oligonucleotide-directed mutagenesis, it has been shown that the first 22 amino acids of the cytoplasmic domain are sufficient for internalisation. In particular, the amino acid at position 807 is critical for clustering in coated pits. Replacement of aromatic residues at that position with charged or uncharged aliphatic residues resulted in non effective clustering and internalisation (Davis et al., 1986).

Figure 1.10: The LDL receptor with 5 structural domains. A model showing the arrangement of elements of the ligand-binding domain of the human LDL receptor. The hexagonal structure is composed of repeats 2, 3 and 4 joined to repeats 5, 6 and 7 by a linker sequence of eight amino acids. Repeat 1 has no role in ligand binding (from Esser et al., 1988).

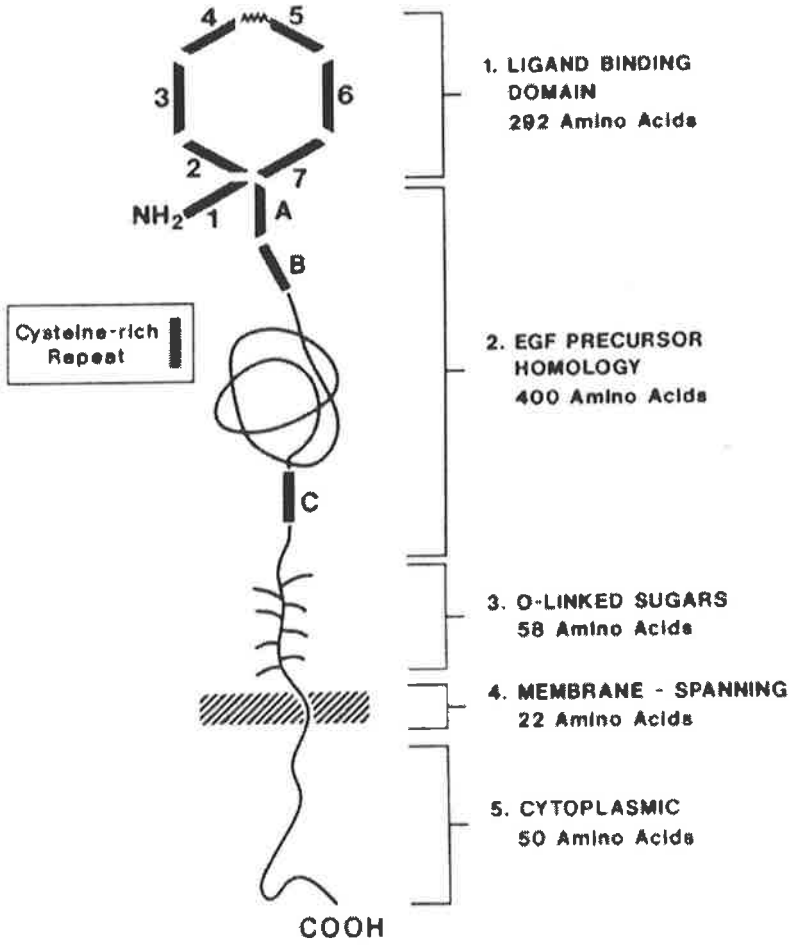
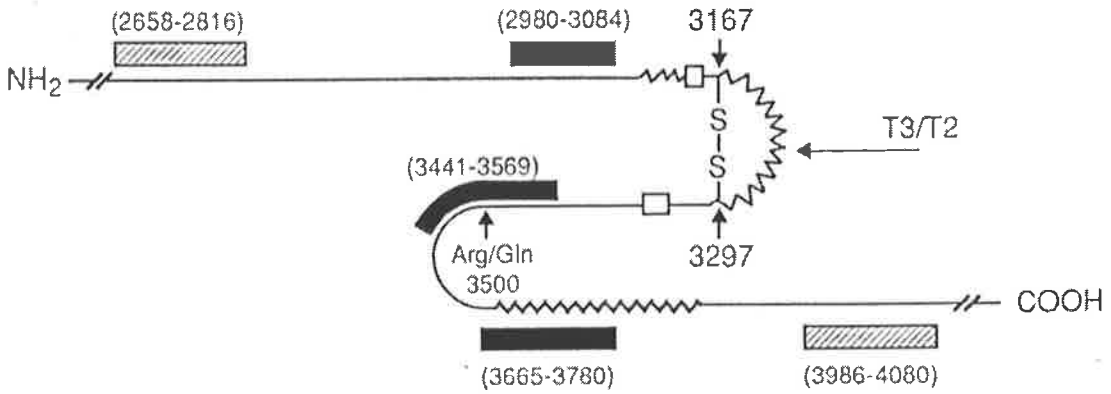


Figure 1.11: Interaction between the Ligand binding domain of the LDL receptor and apoB100. (From Scott, 1989).



1.7.3 LDL receptor Gene.

The gene for the LDL receptor is approximately 50 kb and has been mapped to bands p13.1-13.3 of the distal short arm of human chromosome 19. The gene includes 18 exons separated by 17 introns. The 5.3 kb mRNA transcribed from the gene consists of approximately 2.5 kb of coding sequence. An unusual feature of the human LDL receptor mRNA is that the 3' untranslated sequence harbours three copies of the *Alu* SINE repeat sequence. This is where some of the mutations in the receptor occur (Russell, 1985).

Structurally the gene appears to be a mosaic of exons that have been shuffled to and from other genes throughout evolution (Sudhof et al., 1985). The repeat unit that makes up the ligand binding domain of the receptor is also found in the complement component C9. Other regions in the LDL receptor sequence have been found to be homologous with sequences found in the EGF precursor, blood clotting factors IX and X, and protein C genes. The correlation between sequences shared by these proteins and exons strongly suggests that the LDL receptor gene evolved by exon shuffling. The mechanism by which these exons were shuffled throughout evolutionary time may have involved either RNA intermediates or recombination events between DNA strands.

1.7.4 Mutations of the LDL receptor.

More than 150 mutations including insertions, deletions, nonsense and missense mutations, have been described in the LDL receptor gene. These affect either the synthesis, post-transcriptional processing, ligand binding activity or internalisation of the LDL receptor (Lombardi et al., 1995).

Different mutations in the LDL receptor gene produce different phenotypic effects upon the receptor protein. Approximately two-thirds of the mutations are single base changes or small structural rearrangements and about one third of the mutations consist of major structural rearrangements detectable by Southern blotting. In most individuals with these mutations, the amount of mRNA is markedly reduced, although in some a defective mRNA is present. (Hobbs et al., 1988). Thus, in most of these individuals with this mutations, the LDL receptor is absent.

In the second class of mutations, the receptor is synthesised, but it folds improperly and therefore cannot be transported from its site of synthesis in the endoplasmic reticulum to the cell surface (Leitersdorf et al., 1989). The receptor is, however, found intracellularly. The Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal model for FH, appears to have a mutation of this class (Yamamoto et al., 1986).

In the third class of mutations, the receptors fold sufficiently well so as to move to the surface but can not bind LDL or bind LDL abnormally (sometimes less than 15% of normal binding). It is thought that the glycosylation of this receptor is normal and that the abnormal LDL binding is due to alterations within the receptor-binding domain (Hobbs et

al., 1989). A fourth mutant type occurs when the receptor is made, processed normally and binds LDL, but does not cluster in the coated pits.

Although these mutations produce different phenotypes at the protein level, they produce a uniform clinical syndrome. All of them impair the removal of LDL from blood, causing plasma LDL to accumulate and lead to atherosclerosis (Brown and Goldstein, 1986).

1.7.5 The regulation of the LDL receptor gene.

The expression of the LDL receptor is tightly regulated and geared to maintain an optimum cellular content of cholesterol. Consequently, synthesis of LDL receptor can be upregulated or downregulated (Brown and Goldstein, 1986) exclusively at the level of transcription. Cellular sterol balance is efficiently maintained by a feedback mechanism. When cellular cholesterol increases, receptor synthesis is suppressed; conversely when cellular cholesterol declines, synthesis increases. Available evidence suggests that only a metabolically active pool of unesterified cholesterol within the cell is regulatory. The location of this pool, however, is unknown (Grundy, 1991) (see below). Cholesterol balance is also maintained in the cell by sterol mediated feedback repression of several other genes including HMG-CoA reductase and synthase which are involved the synthetic pathway of cholesterol. These genes are coordinately regulated by the sterol status of the cell in parallel with the LDL receptor (Rudling, 1992). However, the expression of the LDL receptor is mainly regulated at the transcriptional level whereas, HMG-CoA reductase has been found to be regulated at the transcriptional, translational and the protein level (Rudling, 1992).

Transcription of the LDL receptor gene is efficiently regulated by its promoter. The promoter sequence is in the 5'-flanking region of the gene and acts as a switch which is turned off by cholesterol (Figure 1.12). The promoter consists of three related sequence elements of 16 base pairs each, referred to as repeats 1, 2 and 3, which are located upstream of a TATA box-like element (Figure 1.12). Similar *cis* -acting sequences have also been localised in the promoter region of the HMG-CoA reductase gene (Vallett et al., 1996). Repeats 1 and 3 bind the universal transcription factor Sp1, a general positive transcription factor (binds to sequences containing the core hexanucleotide CCGCCC plus four surrounding nucleotides) which is involved in the activation of transcription of a wide variety of promoters (Figure 1.12) (Kadonaga and Tjian, 1986). It is repeat 2 that harbours a special element called SRE-1 that is directly responsible for regulation by sterols. The sterol regulatory element 1 (SRE-1) within repeat 2 was identified by mutational studies as a 10-base pair contiguous sequence that binds a family of basic helix-loop-helix zipper (bHLH-Zip) proteins called the sterol regulatory element binding protein 1 (SREBP-1). The SRE-1 functions as a conditionally positive element, activating expression only when sterol levels are low. It acts in a synergistic manner with repeats 1 and 3 which are also positive

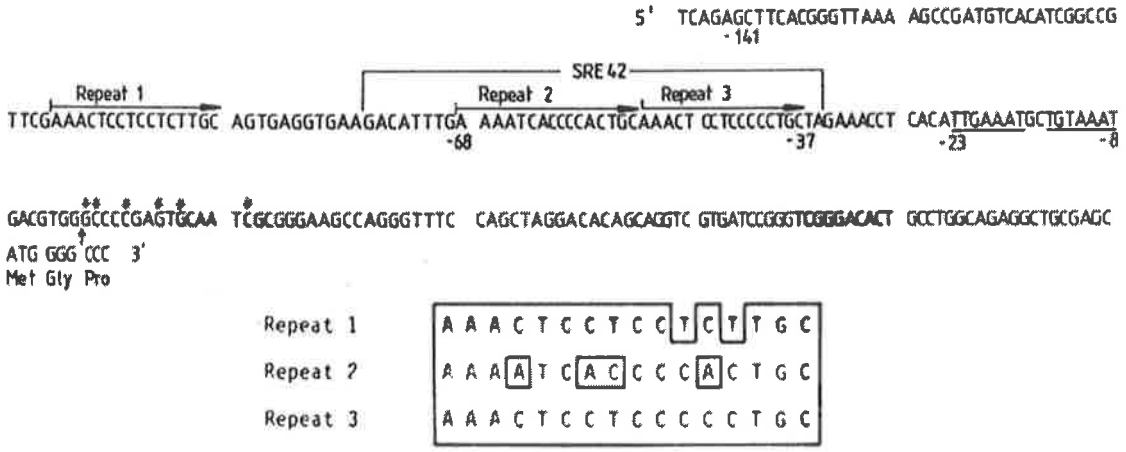
transcriptional elements. *In vivo*, the SRE-1 and the two Sp1 sequences are all necessary for high transcription in the absence of sterols (Sudhof et al., 1987(b); Yieh et al., 1995; Wang et al., 1994). The SRE-1 is also present in the promoters of other genes involved in cholesterol metabolism such as human HMG-CoA reductase, HMG-CoA synthase, mouse apoA-IV and rat farnesyl pyrophosphate synthase (Smith et al., 1988).

SREBP-1 is activated by a novel proteolytic mechanism that controls the concentration of the protein and the transcription rate of the LDL receptor. SREBP-1 is synthesised as a 120 kD precursor protein that is embedded in the membranes of the endoplasmic reticulum and nuclear envelope. The amino-terminal segment of the protein contains an acidic transcriptional activation domain and the bHLH-Zip region that mediates protein dimerisation and DNA binding (see below).

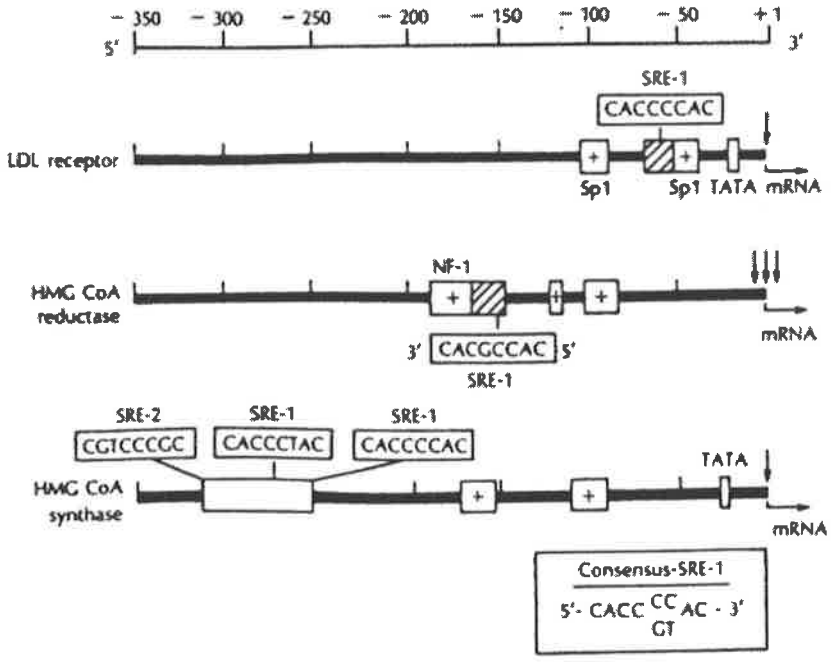
Sanchez et al. (1995) have suggested a model for how SREBP-1 and Sp1 function together to provide tight regulated expression of the LDL receptor. When cellular sterol levels fall, SREBP-1 is processed from its 120 kD membrane-bound form to a soluble 65 kD mature form (amino terminal fragment) which is translocated to the nucleus. The nuclear SREBP-1 then binds to repeat 2 and increases binding of Sp1 to the adjacent repeat 3 sequence. After Sp1 binds to this site, transcription is stimulated. With Sp1 bound adjacently, the SREBP-1-DNA interaction would be relatively unstable. However, under conditions of chronic low cellular sterol levels, SREBP-1 would accumulate in the nucleus and a continuous high level of LDL receptor expression would be sustained. When cellular sterol levels rise again due to high LDL receptor activity and lipoprotein-cholesterol uptake, the flux of SREBP-1 into the nucleus would be curtailed due to inhibition of the cleavage of membrane-bound SREBP-1 to its soluble form. This would result in dissociation of DNA-bound SREBP-1 at the LDL receptor promoter following a rapid decline in its concentration due to degradation of free SREBP-1. The net result would be a significant decline in LDL receptor mRNA synthesis (Sanchez et al., 1995). As the existence of other post-transcriptional mechanisms have not yet been found to effect the processing of the LDL receptor protein, the regulation of the LDL receptor gene transcription by binding of SREBP-1 to the promoter region (dictated by the availability of cholesterol) is the most important determinant for LDL receptor expression in the cell.

Figure 1.12: The LDL receptor gene. (A) The nucleotide sequence of the 5' end and the 5'-flanking region of the human LDL receptor gene, showing the position of repeats 1, 2, and 3, the TATA-like boxes (underlined). Transcription initiation sites are denoted by asterisks (from Sudhof et al., 1987) (B) The organization of transcriptional elements in the sterol regulatory promoter of the LDL receptor gene (from Smith et al., 1988).

(A)



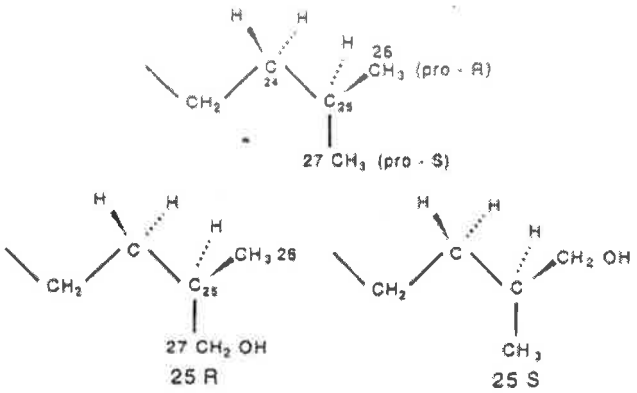
(B)



The availability of cholesterol to the cells has been established to be the only factor that can switch on/off the events which control LDL receptor expression. When cellular cholesterol increases, receptor synthesis is suppressed, conversely, when cellular cholesterol declines, synthesis increases. However, it appears that only a "metabolically active" pool of free (unesterified) cholesterol within the cell is regulatory, although the precise location of this pool is not known (see section 1.7.6, and Figure 1.14). It seems that cholesterol itself is not the regulatory sterol, but instead, the regulator appears to be an oxygenated derivative of cholesterol (Grundy, 1991). Therefore, the regulatory cellular cholesterol pool is actually represented by the concentration of oxysterols, which repress the transcription of the LDL receptor by inhibiting the cleavage of SREBP-1 (Sanchez et al., 1995; Wang et al., 1994). Oxysterols are now known to be much more potent inhibitors than cholesterol itself and are responsible for the regulation of HMG-CoA reductase and the LDL receptor (Figure 1.13) (Kandutsh et al., 1973, 1974; Kreiger et al., 1978).

Oxysterols are sterols containing an extra hydroxy or ketone group at positions 7, 20, 25, and 27 (also referred to as 26) (Smith, 1996) (Figure 1.13). Oxysterols are probably metabolised from the intermediates of cholesterol synthesis (during the conversion of lanosterol into cholesterol) or from cholesterol itself derived either directly from intracellular synthesis or released from LDL by lysosomal cholesterol esterase.

Figure 1.13: Oxysterols. Structures of 27-hydroxycholesterol (also referred to as 26-hydroxycholesterol) and 25-hydroxycholesterol (from Pinkerton et al., 1993).



Although, 25-hydroxycholesterol has been shown *in vitro* to be a potent oxysterols, it has been proposed that oxysterols such as 25-hydroxycholesterol may not achieve sufficient concentration within the cell *in vivo* to be physiologically relevant (Smith, 1996). The formation of 27-hydroxycholesterol and 7ß-hydroxycholesterol from cholesterol is probably more relevant *in vivo*. Studies have demonstrated that 27-hydroxycholesterol and

7ß-hydroxycholesterol can be found in surprisingly high amounts in atherosclerotic human arteries (Bjorkhem et al., 1994; Carpenter et al., 1995). Axelson and Larsson (1995), recently demonstrated that 27-hydroxycholesterol was necessary in order to mediate downregulation of the LDL receptor in cultured human fibroblasts. After the addition of LDL-cholesterol to the incubation medium, normal cells were found to convert the LDL cholesterol to 27-hydroxycholesterol within 5-8 h . During this time, the activity of HMG-CoA reductase decreased 73% and the formation of other biologically active oxysterols such as 7α-hydroxycholesterol, 24-hydroxycholesterol, and 25-hydroxycholesterol, were not observed. When 27-hydroxycholesterol formation was prevented by treating normal cells with cyclosporin (an immunosuppressant which has been shown to inhibit the 27-hydroxylation of sterol) or by using fibroblasts genetically lacking 27-hydroxylase, the suppressive effect of LDL on HMG-CoA reductase was reduced by a factor of 10. In the absence of LDL or when the fibroblasts lacked LDL receptors, the cells did not produce detectable amounts of 27-hydroxycholesterol and HMG-CoA was upregulated. These results indicate that 27-hydroxycholesterol is an important intracellular mediator between LDL and the suppression of HMG-CoA reductase in fibroblasts, and may also be linked to the suppression of the LDL receptor as these genes are coordinately regulated.

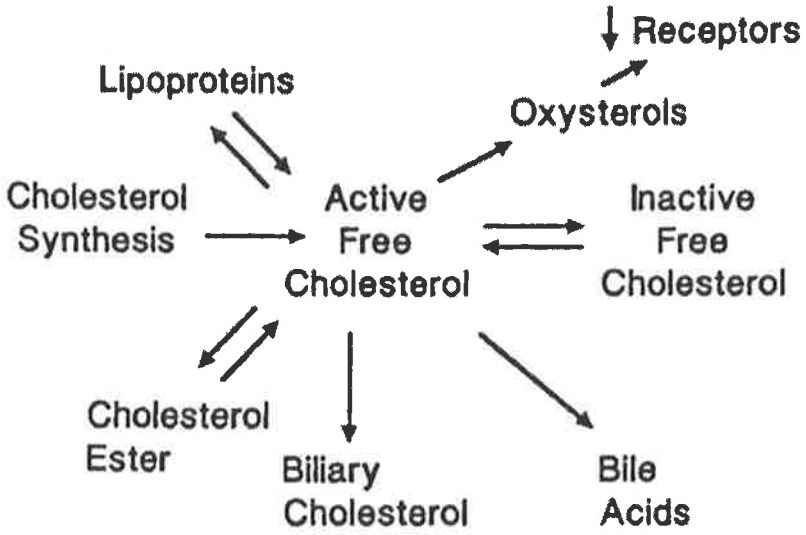
1.7.6 Regulation of Hepatic LDL receptor.

The risk of developing atherosclerotic disease is determined by the steady state concentration of cholesterol in the plasma that is carried in LDL. The organ most closely linked to the development of atherosclerosis is the liver. The liver is the major site of production and removal of the lipoproteins involved in atherogenesis and, therefore, it plays a key role in regulating whole body cholesterol balance and cholesterol trafficking (Figure 1.14). The liver is the key organ in control of this balance not only because it harbours the majority of the LDL receptors in the body, but also because it is responsible for a large part of the cholesterol synthesised within the body (Havel, 1988).

The concentration of cholesterol in hepatocytes seems to be a key factor in influencing the production of LDL receptors, and these receptors are tightly regulated to maintain an optimum cellular content of cholesterol (Grundy, 1991). As mentioned in section 1.7.5, the location of the "metabolically active" pool of unesterified cholesterol which regulates LDL receptor expression is unknown, but the factors influencing this active pool of unesterified cholesterol will determine LDL receptor synthesis (Liscum and Faust, 1994) in liver cells (Figure 1.14). The various factors that influence the amount of "metabolically active" cholesterol in the liver cells by adding to the pool of hepatic cholesterol include newly synthesised cholesterol, lipoprotein derived cholesterol (chylomicron remnants, VLDL and VLDL remants, LDL, and HDL), and hydrolysis of cholesterol ester. At the same time, active free cholesterol may be depleted by the

incorporation into lipoproteins, esterification, transfer to the inactive pool, or by excretion through its conversion into bile acids or direct secretion into bile. The net result of these various inputs and outputs determines the quantity of active free cholesterol which, in turn, regulates the activity of the LDL receptors.

Figure 1.14: Hepatic cholesterol metabolism. Diagram of factors regulating the intrahepatic concentration of active free (unesterified) cholesterol (from Grundy, 1991).



1.7.7 Inhibitors of cholesterol synthesis.

In 1976, Endo and his coworkers isolated several natural products from *Penicillium citrinum* that were powerful competitive inhibitors of HMG-CoA reductase. They were structurally similar to the natural substrate of the enzyme and so were able to exert their actions when present at low concentrations (Endo, 1992). Other fungal metabolites, lovastatin or simvastatin and mevastatin are from *Aspergillus terrus* . These drugs exist as inactive forms until they are enzymatically hydrolysed *in vivo*. Their open hydroxyl acid forms are potent competitive inhibitors of the cholesterol biosynthetic pathway. The affinity of HMG-CoA reductase for these fungal inhibitors is approximately 1000-fold greater than for its substrate. Treatment with these inhibitors results in a large increase in the mRNA and protein levels of HMG-CoA reductase, HMG-CoA synthase, FFP and the LDL receptor. *In vivo,* these fungal compounds show a marked degree of tissue specificity. They are concentrated in the liver where they are metabolically converted to their active form, inhibiting mevalonic acid production and thus cholesterol synthesis. This depletes the supply of cholesterol in the liver that is required for bile acid production and assembly of VLDL. The deficit in liver cholesterol is compensated by an enhanced removal of LDL from the circulation by the liver, thereby effectively lowering the plasma cholesterol.

1.8 Nutritional Effects on Cholesterol Metabolism.

1.8.1 Introduction

A relationship between dietary fat and coronary artery disease was suggested as early as 1916 (Goodman, 1989). A Dutch medical practitioner suggested that the Javanese people had minimal atherosclerosis because their traditional diet contained little fat and cholesterol. When these people were subsequently fed European diets rich in animal fat and cholesterol, their blood cholesterol level increased substantially (Nordoy and Goodnight, 1989). Subsequently, a series of epidemiological studies which included diverse populations from all over the world, showed a significant correlation between the daily intake of fat and cholesterol and mortality from coronary heart disease (Nordoy and Goodnight, 1989). However, it is now known that not only the amount of dietary fat and cholesterol is correlated to hyperlipidemia and atherosclerosis but, also the type of dietary fat has a direct relationship. Various epidemiological, animal and dietary intervention studies have reported that a reduction in total fat consumption and the replacement of saturated fats with polyunsaturated fats can inhibit or even reverse pre-existing vascular lesions (Nordoy and Goodnight, 1989; McNamara, 1987)

1.8.2 Dietary Cholesterol.

In 1906, Anitschkow and Chalatow showed that feeding cholesterol to rabbits caused atherosclerosis (Grundy and Denke, 1990). Numerous studies have since revealed that a high intake of dietary cholesterol causes severe hypercholesterolemia and atherosclerosis in many animal species. In primates, the major effect of dietary cholesterol is to raise LDL-cholesterol levels. Not only does a high intake of cholesterol increase the number of circulating LDL particles but it can also change the size and composition of these particles (Rudel et al., 1985). The rise in serum cholesterol levels in animals fed high cholesterol diets may also contribute to an enrichment of cholesterol ester in newly secreted lipoproteins at the expense of triacylglycerol. Initial studies in tissue culture have clearly demonstrated that increasing the cholesterol content of cells downregulates the synthesis of LDL receptors (Goldstein and Brown, 1975). A down regulation of LDL receptors is certainly seen in rabbits and hamsters fed cholesterol (Spady et al., 1985; Kovanen et al., 1981). Therefore, this may be the principal mechanism whereby plasma LDL increases with dietary cholesterol.

It can be speculated that a high dietary intake of cholesterol enhances cholesterol transport of chylomicrons from the intestine to the liver (Miettinen and Kasaniemi, 1986). The resultant overloading of hepatocytes with cholesterol leads to several alterations in cholesterol metabolism: **(a)** bile acid synthesis increases, **(b)** cholesterol secretion into bile increases, **(c)** cholesterol synthesis is reduced, **(d)** ACAT activation enhances the

esterification and storage of cholesterol esters in the liver, (**e**) LDL receptor is downregulated, and (**f**) the release of cholesterol in VLDL increases.

It has been suggested that in most species the first response by the liver to cholesterol feeding is downregulation of cholesterol synthesis. The downregulation of the LDL receptor only occurs if the synthesis response is not adequate enough to compensate for the increase in dietary cholesterol. In the rabbit (Kovanen et al., 1981) and hamsters (Spady et al., 1985), dietary cholesterol causes marked hypercholesterolemia, as factors (**a**) and (**d**) do not operate sufficiently well, and factor (**f**) occurs. Plasma cholesterol in these animals can increase by up to 10-fold and it appears to be mainly a result of downregulation of LDL receptor expression by 60-70%. Hence, the decrease in LDL receptor expression in these cholesterol fed rabbits is the causative factor in their hypercholesterolemia (Kovanen et al., 1981). Rats, however, are remarkably resistant to hypercholesterolemia when fed cholesterol as factors (**a**) through (**d**) appear to operate effectively. The LDL receptor expression is also not downregulated as in rabbits (Roach et al., 1993). Rats have an efficient capacity to reduce cholesterol synthesis and increase cholesterol secretion as bile acids to compensate for the incoming cholesterol in order to maintain cellular cholesterol homeostasis. There is also a marked increase in the secretion of cholesterol-enriched VLDL.

The response to dietary cholesterol in humans is more similar to the response observed in rats than rabbits, in that plasma cholesterol levels are not increased to a large extent with cholesterol administration. There appears to be adequate compensatory changes in various metabolic pathways which allow the maintenance of cholesterol homeostasis in man. The primary response is the suppression of cholesterol synthesis, but there is also a decrease in fractional absorption of cholesterol from the intestine and an increase in biliary excretion (McNamara, 1987). Nevertheless, there is still an effect when dietary cholesterol is consumed. There appears to be, however, a variable effect on serum cholesterol concentrations among individuals with the same intake of dietary cholesterol (Grundy and Denke, 1990). This may be related to the individual's ability to process dietary cholesterol (Miettinen and Kasaniemi, 1986; Clifton et al., 1990) resulting in some people being responsive and others being non responsive to dietary cholesterol. Nestel and Poyser (1976) demonstrated that in a group of individuals with moderate intakes of cholesterol, only 10% had a significant increase in their plasma cholesterol. These individuals failed to downregulate cholesterol synthesis and tended to have a higher fractional cholesterol absorption.

1.8.3 Dietary Fats.

1.8.3.1 Introduction

In the 1950's and 1960's the studies of Keys et al. (1957) and Hegsted et al. (1965) provided the first quantitative estimates of the effects of different fatty acids on blood cholesterol. They studied the responses to consuming diets rich in saturated, monounsaturated or polyunsaturated fatty acids compared to a low-fat, high carbohydrate intake. Monounsaturated fats had no effect on plasma cholesterol levels and they were therefore called neutral fatty acids. Keys reported that compared to carbohydrates, the potency of saturated fat to increase plasma cholesterol was twice that of polyunsaturated fat, which lowered plasma cholesterol. Hegsted et al. (1965) found similar responses, but reported that saturated fatty acids raised cholesterol levels less in their subjects than in Keys subjects and that polyunsaturated fatty acids lowered cholesterol levels somewhat more. Based on these studies, Keys and Hegsted developed similar equations to predict the changes in blood cholesterol with changes in dietary fat (Table 1.2). However, only saturated and polyunsaturated fatty acids were used in these equations. Carbohydrates and monounsaturates were left out because of their neutral effect on plasma cholesterol levels.

Through the years, the equations have been continuously tested and modified as experiments with different saturated and polyunsaturated fats have indicated that the relationship was not as simple as first thought. Investigators assumed that the diet-induced changes in total cholesterol levels was essentially due to alterations in LDL-cholesterol. This is not necessarily true as it is now apparent that the original studies did not differentiate between the effects of dietary fat on LDL-cholesterol and HDL-cholesterol when measuring total cholesterol levels. This distinction is required when measuring total plasma cholesterol levels. For example, a specific fatty acid may cause a decrease in plasma LDL levels and a rise in HDL levels with an overall effect that shows a rise in plasma cholesterol levels.

Table 1.2: Equations for predicting effects of diet on total serum cholesterol.

Keys equation (1957):

Δ plasma Cholesterol (mg/dl) = $2.7\Delta S - 1.35\Delta P + 1.5\Delta C^{1/2}$ mg/1000 cal-day

Hegsted equation (1965):

Δ plasma Cholesterol (mg/dl) = $2.16\Delta S - 1.65\Delta P + 0.068\Delta C$ (mg/day)

S = saturated fatty acids (% of total calories)
P = polyunsaturated fatty acids (% of total calories)
C = dietary cholesterol
Δ = change in

1.8.3.2 Saturated fatty acids.

The rise in blood cholesterol in humans associated with dietary saturated fats appears to be limited to lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids (Figure 1.15). Saturated fats with a chain length of less than 12 and more than 16 appear to have little effect on blood cholesterol. Palmitic acid constitutes approximately 60% of the total saturated acid intake in the Western diet and the evidence is very strong that palmitic raises total and LDL-cholesterol (Hegsted et al., 1965; Keys et al., 1965; Grundy and Vega, 1985; Denke and Grundy, 1991, Schwab et al., 1993). However, Hayes and Khosla (1992) have suggested that palmitic acid may have a neutral effect on total and LDL-cholesterol when dietary cholesterol is low or in people with normal or low blood cholesterol levels.

Myristic acid appears to be the most potent elevator of total and LDL-cholesterol compared to all other fatty acids (Grundy and Denke, 1990; Katan et al., 1994). The Hegsted equation assigned myristic to be four times more potent in raising cholesterol levels than palmitic acid. However, studies to investigate the cholesterol raising capacity of myristic acid are difficult to design as myristic and lauric acid tend to appear together in natural fats and differentiating their individual effects is difficult. Mensink and Katan (1992) studied the effects of lauric acid and found that it has a cholesterol-raising potential almost equal to palmitic acid.

Stearic acid (C18:0) is a long chain saturated fatty acid which makes up about 7-10% of the total fat in the Western diet (Grundy, 1994) (Figure 1.15). It is the predominant fatty acid in cocoa butter and it can constitute 5-25% of the total fatty acids in meats. The majority of studies indicate the effects of stearic acid on blood cholesterol is close to neutral when substituted for carbohydrate (Denke, 1994 ; Katan et al., 1994; Grundy, 1994). A number of possible reasons have been suggested for this effect. Over 90% of the available stearic acid is absorbed and is converted to oleic acid (C18:1). Oleic acid is thought to have a neutral effect on plasma cholesterol levels (see section 1.8.3.3).

How do saturated fatty acids increase total plasma cholesterol and LDL-cholesterol levels ? The increase is not due to an enhanced production of LDL particles since these fatty acids do not stimulate the synthesis of VLDL (the precursor of LDL synthesis). This has been supported by the observation that saturated fatty acids do not increase triacylglycerol concentration nor the synthesis of apo B_{100} as measured by hepatic mRNA apo B_{100} abundance (Sorci-Thomas et al., 1989; Spady and Dietschy, 1989). There is a strong possibility that they interfere with LDL receptor mediated clearance. Clearance studies of radiolabelled LDL B_{100} indicate that dietary saturated fatty acids impair the removal of LDL from the circulation in humans (Shepard et al., 1990). Similar results have been found in laboratory animals (eg. cebus monkeys and hamsters) where saturated fatty acids also

suppress LDL receptor-mediated clearance of LDL particles (Nicolosi et al., 1990; Spady and Dietschy, 1985).

It is possible that the delayed clearance of LDL from the circulation is as a result of an impairment in the LDL receptor pathway. This may include all or any one of the following processes: decreased synthesis of the LDL receptor at the level of transcription and/or protein translation, decreased binding of LDL to its receptor or decreased recycling of the LDL receptor-LDL complex. There is some experimental evidence that saturated fatty acids may effect gene transcription of the LDL receptor. Grundy (1991) reported that dietary saturated fatty acids decreased LDL receptor gene expression in baboons as measured by mRNA abundance. Decreased levels of LDL receptor mRNA could result in the reduction of the number of LDL receptors expressed at the cell surface. This could explain why LDL-cholesterol levels may be elevated when dietary saturated fatty acids are consumed.

The molecular mechanism whereby these fatty acids could be downregulating gene transcription of the LDL receptor is unknown. They may be directly binding to the DNA and effecting gene transcription or they may be indirectly suppressing LDL receptor expression. For example, the fatty acids could inhibit cholesterol esterification by ACAT. Inhibition of cholesterol esterification would result in an increased concentration of free cholesterol within the liver cell which in turn would lead to the suppression of LDL receptor gene transcription. Alternatively, saturated fatty acids may be mediating their suppressing effect on the LDL receptor gene by binding to a binding protein required for gene transcription and/or by enhancing oxysterol production in the cell.

1.8.3.3 Monounsaturated fatty acids.

Monounsaturated fatty acids are those fatty acids that contain one double bond in their hydrocarbon chain. The major monounsaturated fatty acid in the diet is oleic acid (18 carbons, one double bond 9 carbons from the terminal methyl end; C18:1 n-9). The richest sources of oleic acid are olive oil and rapeseed oil (Figure 1.15). It is not an essential fatty acid and can be synthesised *de novo* from carbohydrate or other fatty acids. Until recently, oleic acid was considered to be "neutral" as it appeared to affect LDL-cholesterol levels similarly to carbohydrate. This neutrality appeared to extend to all of the lipoprotein fractions: VLDL, LDL and HDL (Grundy and Denke, 1990; Hegsted et al., 1993). It is now recognised that when oleic acid is substituted for palmitic and other cholesterol elevating saturates, the LDL-cholesterol levels fall (Mensink and Katan, 1989). This response is similar to the major dietary polyunsaturated fatty acid, linoleic acid (see section 1.8.3.4). Therefore, in this sense, oleic acid is considered cholesterol lowering.

Monounsaturated fatty acids exist as *cis* and *trans* isomers and unlike the *cis* isomer, oleic acid, the *trans* monounsaturated fatty acids are not neutral fatty acids (Figure 1.16). The most prevalent *trans* fatty acid is elaidic acid (C18:1, n-9), a *trans* isomer of oleic acid. Some *trans* fatty acids are naturally found in milk and meat of sheep and cattle.

However, they are also synthesised when liquid vegetable or marine oils are chemically hydrogenated to produce margarine and other hardened fat products. The *cis* double bond makes oleic acid a flexible molecule and accounts for the liquid state of high oleic acid containing oils, such as olive oil. The *trans* monounsaturated fatty acids are more rigid molecules and therefore oils high in *trans* fatty acids tend to be solids (Grundy, 1994; Katan et al., 1994). In Australia, such *trans* isomers now constitute about 1.0-2.5% of the total energy intake with half coming from margarines (Noakes and Nestel, 1994).

In contrast to oleic acid, dietary *trans* monounsaturated fatty acids appear to raise the plasma LDL-cholesterol concentration and are therefore considered equivalent to saturated fatty acids (Zock and Katan, 1992; Mensink and Katan, 1990; Nestel et al., 1992). However, in some of these studies where relatively high doses of *trans* fatty acids were used, an HDL-cholesterol lowering effect was also observed. There are many conflicting reports on the consumption of *trans* fatty acids and the development of coronary artery disease. Until more evidence becomes available, it is premature to consider *trans* fatty acids as a risk factor for atherosclerosis.

1.8.3.4 ω-6 Polyunsaturated fatty acids.

Fatty acids with more than one double bond in their hydrocarbon chain are known as polyunsaturated fatty acids (PUFAs). Two types occur in the diet. They are classed as either ω-6 and ω-3 polyunsaturated fatty acids based on the position of the first double bond in the fatty acid chain (Figure 1.15). The ω-6 PUFAs have the first double bond inserted six carbons from the terminal methyl group. The predominant ω-6 fatty acid is linoleic acid. Linoleic acid cannot be synthesised by animals and hence is an essential fatty acid that must be consumed. This fatty acid is found in vegetables and vegetable oils, which are the major sources of PUFAs in most Western countries (Harris, 1985; Ziboh and Miller, 1990). Animal fats may also contain small quantities of linoleic acid, which originated from plant sources. For many years, linoleic acid was considered a cholesterol lowering fatty acid as studies which showed that vegetable oils rich in linoleic acid lowered serum cholesterol levels when substituted for dietary saturated fatty acids (Kinsell et al., 1955; Ahrens et al., 1954). The early work of Keys and Hegsted also indicated that linoleic acid was the only fatty acid to appreciably lower total blood cholesterol when substituted for oleic acid or carbohydrate in the diet (Keys et al., 1957; Hegsted et al., 1965). In general, oleic acid is used as a baseline, and the studies by Keys and Hegsted found that linoleic acid lowers cholesterol about half as much as saturated fatty acids increase it. The relationship, however, between the intake of dietary linoleic acid and cholesterol lowering is not linear since there is a desirable maximum level of PUFAs in human diets at which point they are no longer effective in cholesterol lowering. Mensink and Katan (1992) and Hegsted et al. (1993) have devised simple linear models for predicting blood lipid levels and they have found that the blood cholesterol response to linoleic acid is linear when intake is within 0-

12% of total energy. Current average intake of ω-6 PUFAs in Australia is approximately 6% of energy intake and this is considered to be adequate and safe.

How do PUFAs, such as linoleic acid, lower plasma cholesterol and LDL-cholesterol levels? Some studies have suggested that individuals consuming linoleic acid have enhanced fecal excretion of cholesterol compared to those individuals consuming saturated fats (Grundy, 1983). Vega et al. (1982) and Kuksis et al. (1982) have found that linoleic acid reduced the number of LDL particles in the circulation. The fall in LDL-apo B concentrations suggest that the production of LDL may be affected and this is related to VLDL production. Linoleic acid appears to cause a reduction in VLDL levels (and ultimately LDL concentration) by not only affecting VLDL synthesis but by also affecting its secretion from the liver cell and its rate of conversion from VLDL to LDL (Turner et al., 1981; Chait et al., 1974; Wong and Nestel, 1987, Wong et al., 1989; Lang and Davis, 1990). A decreased synthesis of any one of the two major components of VLDL, triacylglycerol and apo B_{100}, will result in decreased VLDL levels. Chait et al. (1974) reported that a high intake of PUFAs decreased serum triacylglycerol concentrations and Cortese et al. (1983) have reported a definite decrease in hepatic apo B_{100} production. These findings, however, are highly variable in individuals since these changes occur in some individuals but in not others. This indicates that there must be some other explanation as to why PUFAs consistently lower plasma LDL-cholesterol.

There is mounting evidence to suggest that PUFAs may lower plasma LDL cholesterol levels by affecting the hepatic expression and/or metabolism of the LDL receptor. This has been demonstrated in human and animal models through LDL clearance studies and measuring LDL receptor binding activity. Guinea pigs fed corn oil (58% linoleic acid) had increased hepatic LDL activity compared with animals fed lard (Lin et al., 1994). Recently, studies have indicated that dietary fat and cholesterol have independent effects that determine not only plasma LDL-cholesterol levels but also LDL receptor activity. Spady and Dietschy (1988) have reported that LDL receptor mediated LDL clearance was increased in hamsters fed polyunsaturated diets compared to animals fed saturated or monounsaturated fat diets. They also demonstrated that the downregulation of the LDL receptor by dietary cholesterol can be affected by the type of dietary fat. They found that the unsaturated fatty acids were able to prevent the cholesterol induced LDL receptor suppression relative to saturated fatty acids. *In vivo* turnover studies in cebus monkeys support these observations as the LDL receptor activity is higher in animals fed corn oil compared with animals fed saturated coconut oil diets. This was also seen when 0.1% cholesterol was added to the diet (Nicolosi et al., 1990). However, these studies do not elucidate the mode of action of these fatty acids. The question then arises, where in the LDL receptor pathway do these fatty acids affect the metabolism of the receptor such that the LDL receptor activity on the cell surface is increased ? There was no increase in mRNA abundance for the LDL receptor measured in African green monkeys fed large quantities of linoleic acid. This suggested that the LDL

receptor activity is not regulated at the level of transcription (Sorci-Thomas et al., 1989). This study did not further to elucidate at which level these fatty acids were affecting LDL receptor metabolism. However, another study found that hepatic mRNA levels increased in baboons when they were fed dietary corn oil compared with animals fed lard (Kushwaha et al., 1991).

Even though there is evidence indicating that the increase in LDL receptor function is a primary factor responsible for the decrease of plasma LDL-cholesterol, the number of studies directed toward elucidating the mechanisms by which these fatty acids are upregulating the receptor function are few. It is not clear whether they directly or indirectly act at the level of transcription and/or translation of the LDL receptor gene. The possibility does exist that they may effect receptor recycling or they may interact directly with the receptor to increase binding with the LDL particle. Changes in one or even all of these states would be the same. That is, there would be an increase in LDL receptor function at the cell surface which will increase the cells' ability to remove LDL-cholesterol from the plasma.

1.8.3.5 ω-3 Polyunsaturated fatty acids.

It now appears that different types of PUFAs may differ in their effectiveness in preventing coronary arterial lesions. As early as 1952, Nelson observed that feeding coronary disease patients large amounts of fatty fish significantly improved the 20-year survival rates from 8% to 35% (Nelson, 1972). Bang and Dyerberg in 1970 reported that the incidence of coronary heart disease among the Eskimo population of Northern Greenland was less than 10% of that in Eskimos living in Denmark (Bang and Dyerberg, 1972). They suggested that the absence of atherosclerosis in the Greenland Eskimos might be attributable to the high content of ω-3 PUFAs in the Eskimos' marine based diet. The two major ω-3 fatty acids common in fish are eicosapentaenoic acid (EPA) (C20:5) and docosapentaenoic acid (DHA) (C22:6). They both have the first double bond located three carbons from the terminal methyl group, and hence are called ω-3 fatty acids (Figure 1.15). Together they constitute about 26% of fish oil fatty acids.

The ω-3 series also includes the essential fatty acid, α-linolenic acid (18:3) which is found in some vegetable oils such as canola oil. There does not appear to be conclusive evidence on the effect of α-linolenic acid on plasma lipids. Chan et al. (1991) and Singer at al. (1986) found a small lowering effect in humans. However, this was contradicted by the results of Kestin et al. (1990). It is difficult to analyse the effect of α-linolenic acid because of the low level intake of this fatty acid in humans.

The ability of diets rich in fish oil ω-3 PUFAs to protect against atherosclerosis has been confirmed in several controlled animal studies, including those on pigs and rhesus monkeys (Davis et al., 1987; Wiener et al., 1986; Thiery and Siedel, 1987). In the pig study, the intima of the coronary arteries was damaged by a balloon catheter at the same time that the animals were fed cholesterol and fat (Wiener et al., 1986). Considerably less

atherosclerosis was found in pigs fed cod liver oil despite a rise of plasma lipid levels. These results suggest that fish oil may have an effect on atherosclerosis unrelated to plasma lipid concentrations.

Dietary ω-3 fatty acids have a variety of effects on plasma lipid and lipoproteins in humans (Nestel, 1990). The results of ω-3 PUFAs on HDL levels in human studies have been inconsistent, but it would appear that HDL levels in plasma are not altered by fish oil supplemented diets when compared with vegetable oil diets. Some of the discrepancies relate to dosage, source of the fish oil and hence the nature of the ω-3 fatty acid, the type of the hyperlipidemia treated, and the matching of the control and test diets. As fish oil is a heterogenous mixture of ω-3 PUFAs, monounsaturated fatty acids and saturated fatty acids, it is difficult to dissect out the effects of the individual fatty acids present in the oil. It has not been established in humans or in animals models whether the effect on lipoprotein levels are due to eicosapentaenoic, docosahexaenoic acid or a synergistic interaction of the two.

On the other hand, ω-3 polyunsaturates, especially EPA and DHA, have been shown to consistently lower plasma triglyceride levels in virtually all human and animal experiments (Harris, 1989; Land, 1986; Nestel, 1990). Therefore, these fatty acids are especially useful when fed to individuals with hypertriglyceridemia. The mechanism of triglyceride lowering appears to be an inhibition of secretion of VLDL triglycerides as the fatty acids interfere with the hepatic triglyceride synthesis (Wong and Nestel, 1987, Wong et al., 1989; Lang et al., 1990) but not apo B synthesis (Wong et al., 1989). This has been shown in perfused rat liver and isolated rat hepatocytes. Even though fish oil fatty acids do not effect apo B mRNA abundance, they do however increase the intracellular degradation of apo B protein, which leads to a decrease the secretion of newly synthesised apo B (Wang et al., 1993). Fish oil feeding has been also shown to diminish lipogenesis, increase ketogenesis and fatty acid oxidation, which can also contribute to the overall reduction in triglyceride secretion by the liver and lower plasma VLDL.

The effects of ω-3 polyunsaturates in humans on circulating levels of LDL have been shown to be variable. Plasma total and LDL cholesterol do fall when saturated fat is replaced by fish oils (Harris, 1989). However, compared to ω-6 PUFAs, it would appear that total plasma cholesterol and LDL cholesterol levels are higher with ω-3 PUFAs in humans and animals (Harris et al., 1983, 1988; Nestel, 1986; Illingworth et al., 1984). It is unlikely that the increase in LDL-cholesterol concentrations is related to an increase in the number of VLDL particles, as it has been established that dietary fish oils decrease plasma VLDL levels (precursor for LDL). However, Huff and Telford (1989) measured the rate of conversion of VLDL to LDL in miniature pigs fed ω-3 fish oil and found that the conversion rate was slightly increased. Even with this increased conversion to LDL, it did not contribute to an overall increase in total plasma LDL and thus, does not explain the rise in plasma LDL levels with fish oil feeding observed by others.

The concentration of LDL in plasma is determined by the rate at which LDL is produced relative to the rate at which it is cleared from the plasma by various tissues of the body. The major organ responsible for this clearance is the liver (see section 1.7.6). As the studies examining the effect of dietary ω-3 fatty acids on the production rate of LDL are unclear, research has been directed towards examining the clearance of the LDL particle by the hepatic LDL receptor. Studies indicate that increases in LDL levels caused by dietary fish oils may be due to the down-regulation of the hepatic LDL receptor.

There have been a number of animal and tissue culture studies examining the effect of EPA on hepatic LDL receptor activity. A study by Wong and Nestel (1986) found that human liver HepG2 cells enriched with EPA inhibited LDL binding to its receptor compared with cells which had been enriched in oleic acid. Decreased LDL receptor activity has also been observed in rat hepatic membranes isolated from rats fed 8% fish oils compared with rats fed safflower and olive oil diets (Roach et al., 1987). Therefore, it is possible that the observed increase in plasma LDL levels with the intake of dietary fish oils could be a direct result of suppression of LDL receptor activity.

However, the mechanism of action of these fatty acids remains elusive. It is possible that they may be downregulating the LDL receptor at the level of gene transcription or translation. Lindsey et al. (1992) observed that the mRNA for the LDL receptor was depressed in HepG2 cells which were incubated with LDL derived from individuals before and after they had consumed dietary fish oils. However, these observations were inconsistent when different LDL concentrations were used. As LDL receptor and HMG-CoA reductase are coordinately regulated at the level of gene transcription (Rudling, 1992), in this study the changes in LDL receptor mRNA levels did not correlate with the changes in HMG-CoA reductase mRNA levels. Moreover, contradictory to most other studies, they observed an increase of apo B mRNA levels in cells incubated with fish oil enriched LDL (Lindsey et al., 1992).

It remains to be shown whether fish oil fatty acids, EPA and/or DHA, reduce LDL receptor activity by suppressing gene transcription. The downregulation of the LDL receptor by ω-3 fatty acids at the level of gene transcription could be explained by indirect effects on cholesterol and lipoprotein metabolism. As these fatty acids inhibit VLDL synthesis, the build up of unincorporated endogenous cholesterol into the VLDL could contribute to the overall mass accumulation of free cholesterol in the cell and lead to the suppression of LDL receptor expression. Gene transcription is the most likely point of regulation whereby fish oil fatty acids may exert their effects as there is no evidence that the LDL receptor is regulated post-transcriptionally. It is unlikely that these fatty acids are inhibiting at any other level, such as LDL receptor protein processing, synthesis, or receptor recycling. However, it can not be dismissed that ω-3 fatty acids could still potentially affect LDL receptor activity by interfering with the interaction of the receptor with the LDL particle. As the exact molecular mechanism for the reduction of LDL receptor activity by fish oils remains

unknown, further studies to elucidate the exact molecular mechanism whereby fish oil fatty acids such as EPA regulate the LDL receptor expression in the liver are required.

Figure 1.15: Structure of fatty acids and their nomenclature. (From Bender, 1994).

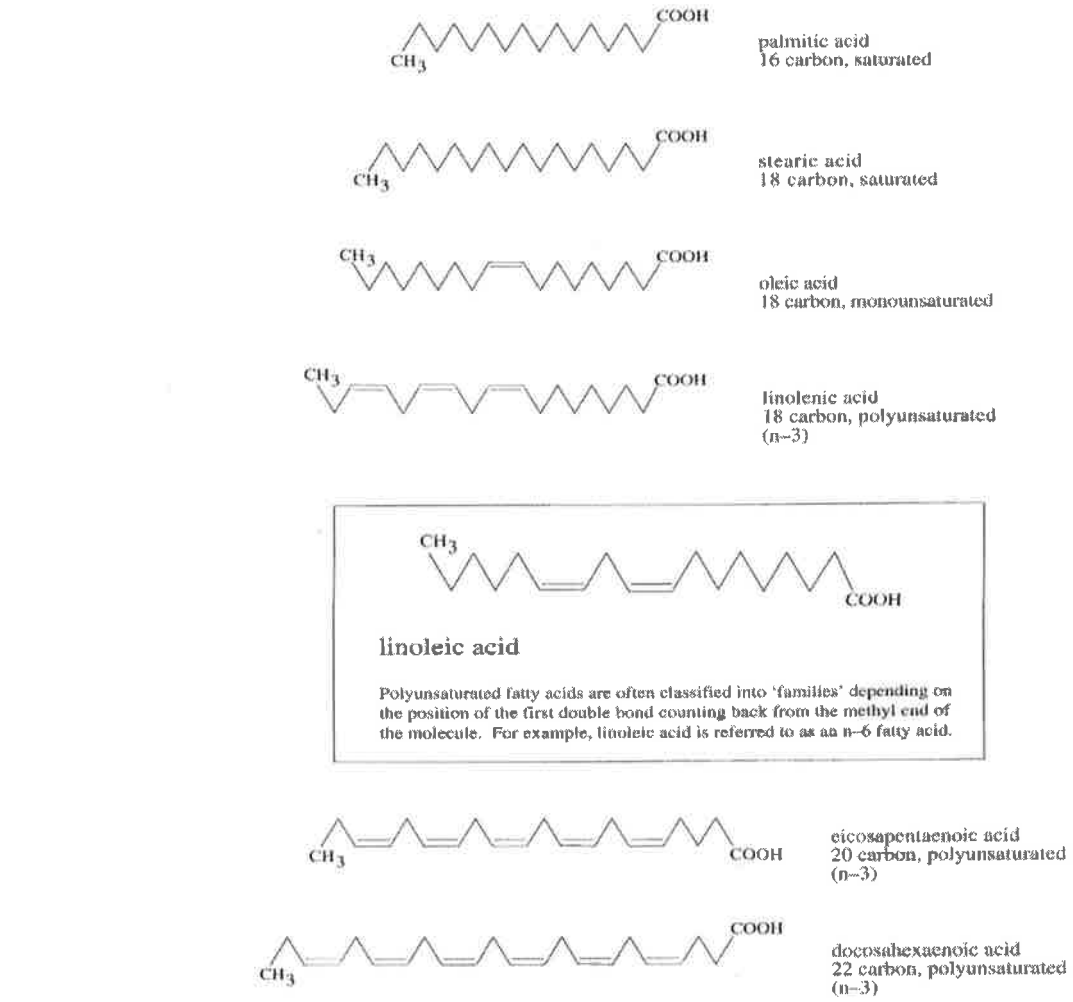
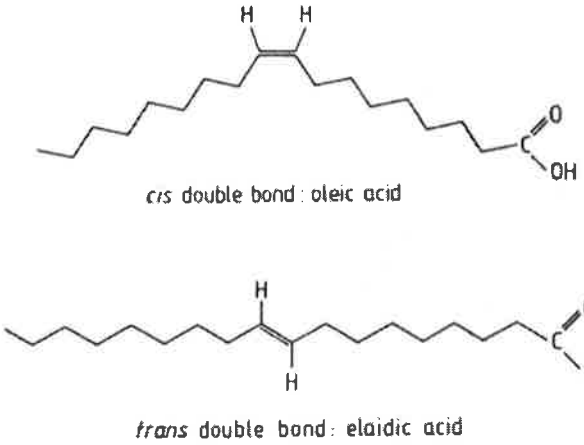


Figure 1.16: Structure of *cis* and *trans* 18:1 n-9. Partially hydrogenated fats contain a mixture of positional isomers of which elaidic acid is one (from Katan et al., 1994).



1.8.4 Polyunsaturated fatty acids and the need for dietary antioxidants.

High intakes of PUFAs in animals and humans can result in many positive effects. However, some PUFAs, especially ω-3 fatty acids, contain highly unsaturated and potentially unstable double bonds making them highly susceptible to peroxidation. Lipid peroxidation has been shown to cause cytotoxic effects in cell culture and leads to disruption of membrane structure and function. Oxidation of polyunsaturates occurs quite rapidly when free radicals, such as nitric oxide, superoxide or hydroxyl radicals, attack the fatty acid (Halliwell, 1994, 1995). These reactive radicals (R·) attack the fatty acid side chains in membranes and lipoproteins (L-H) and abstract an hydrogen atom, leaving a carbon centred radical (L·) and initiating the process of lipid peroxidation.

$$L\text{-}H + R\cdot \text{ --------> } L\cdot + RH$$

These L· radicals generated from PUFA side chains undergo molecular rearrangements to a conjugated diene structure. This is followed by a reaction with oxygen (oxygen is concentrated in the hydrophobic regions of membranes) to give a conjugated diene peroxyl radical ($LO_2\cdot$). These lipid peroxyl radicals can then move from the hydrophobic region to the membrane surface.

$$L\cdot + O_2 \text{ --------> } LO_2\cdot$$

The $LO_2\cdot$ radicals can damage proteins and membranes, deplete antioxidants and react with adjacent PUFA side chains to propagate lipid peroxidation. Lipid peroxides (LO_2H) are generated from the $LO_2\cdot$ radicals.

$$LO_2\cdot + L\text{-}H \text{ --------> } LO_2H + L\cdot$$

Once initiated, peroxidation of lipids accelerates in an autocatalytic manner until the substrate (PUFAs and /or oxygen) is exhausted or if the chain is broken by antioxidants. Chain breaking antioxidants (AH) prevent the autocatalytic phase of lipid peroxidation by competing with PUFAs for propagating radicals. The resulting antioxidant radical (A·) does have some capacity to abstract another hydrogen for PUFAs but they are far less reactive than are $LO_2\cdot$ radicals. In addition, A· can be recycled to AH by other antioxidants.

$$LO_2\cdot + A\text{-}H \text{ --------> } A\cdot + LO_2H$$

Vitamin E (or α-tocopherol) is the major lipid soluble, chain breaking antioxidant in biological systems and has been shown to protect membranes from PUFA peroxidation (Muggli, 1994; Van Acker et al., 1993). Other antioxidants, such as ascorbic acid (vitamin C) are water soluble and function to scavenge reactive oxygen species in the hydrophilic

environment (Njus and Kelley, 1991). The requirement for antioxidants is dependent on the amount and the degree of unsaturation of PUFA in the diet. It is quite common, therefore, that natural antioxidants are added to products, such as margarines, that are high in PUFAs. In recent years, the consumption of saturated fatty acids has been more favourably replaced by PUFAs. Consequently, more antioxidants are consumed. However, the exact amount of antioxidants to compensate for an increased intake of PUFAs has not been investigated in man and must be extrapolated from animal data. Due to the increased intake of dietary antioxidants, there is a growing interest in examining the effects of antioxidants on cell function and other metabolic pathways. Their functions in the cell may be dependent or independent of their antioxidant activities.

Epidemiological evidence is accumulating that antioxidants, such as tocopherols decrease the risk of coronary artery disease (Gaziano et al., 1990). However, there is no clear evidence as yet as to what mechanisms are operative in humans. Whether these antioxidants exert an effect on lipid and lipoprotein metabolism has not been examined, although some studies suggest that they do affect hepatic cholesterol metabolism. As antioxidants have been used to protect against the oxidation of PUFAs in most experiments, it has become quite apparent that some of the effects of dietary PUFAs that have been observed may be related to the antioxidant consumption or an interaction of both. To ensure that only the fatty acid effects are measured it will be necessary to control for antioxidants in any fatty acid experiments and/or examine these antioxidants in isolation.

1.8.5 Antioxidants and Coronary Artery Disease.

1.8.5.1 Introduction

An area of emerging interest is the protective effect of antioxidant nutrients in the early stages of heart disease (Gaziano et al., 1990). Studies suggest that the oxidation of LDL may be a key step in the initiation of atherosclerosis. Antioxidants may play an antiatherogenic role by protecting the LDL macromolecule from oxidation, and hence preventing uptake of this oxidised LDL by the cells in the arterial wall. Certain antioxidants have been shown to modulate endothelium relaxation, arterial cell proliferation and exhibit hypocholesterolemic properties. Recently, epidemiological studies have demonstrated that there is an inverse association between some serum antioxidants, such as vitamin E (α-tocopherol), vitamin C (ascorbic acid) and β-carotene, and the risk of developing coronary heart disease (Gaziano et al., 1990; Gey et al., 1991; Manson et al., 1993; Jaques, 1992).

Vitamin C, vitamin E, and β-carotene are three important naturally occurring antioxidants. Vitamin C is a water soluble dietary antioxidant found in many fruits and vegetables. Vitamin E is found in liver, egg yolks, milk fat, cereal grains, nuts and several vegetable oils. β-carotene is found in carrots, green leafy vegetables, squash, melons and

tomatoes. Vitamin E and β-carotene are fat soluble vitamins that reside in circulating lipoproteins and lipid membranes.

1.8.5.2 Vitamin E.

There are naturally occurring homologues of vitamin E: four tocopherols (α, β, δ and γ) and four tocotrienols. The tocopherols differ from each other in the number and position of the methyl groups around the ring structure (Figure 1.17). They are all absorbed equally by the intestine and transported to the liver by chylomicrons (Kayden and Traber, 1993). Only α-tocopherol is usually secreted from the liver into the circulation in nascent VLDL. (George Wolf, 1993). Unlike the other tocopherols, α-tocopherol is retained within the body since it selectively binds to the 30 kD hepatic α-tocopherol binding protein (TBP) in the liver (Figure 1.18). The TBP in the liver is thought to be responsible for intracellular distribution, transport of α-tocopherol and for specific incorporation of α-tocopherol into VLDL. Therefore, the liver discriminates against the β, δ and γ tocopherols which are secreted into bile with any excess α-tocopherol.

Since α-tocopherol re-enters the circulation from the liver via VLDL, it eventually becomes an ubiquitous constituent of all lipoproteins. The majority of α-tocopherol ends up in the LDL fraction (George Wolf, 1993). The LDL receptor in tissues is probably responsible for the uptake of most α-tocopherol from lipoproteins, thereby allowing α-tocopherol to become an ubiquitous constituent of all cell membranes. It is, therefore, α-tocopherol which is the most abundant and the most biologically active of the tocopherols *in vivo*. Thus, α-tocopherol is recognised as a highly effective antioxidant. However, a small proportion of the other tocopherols also reach the various tissues when they are associated with circulating chylomicrons (before entering the liver).

Vitamin E is generally considered to be of low toxicity. The recommended dietary intake (RDI) for vitamin E is 7-10 mg/day which is normally attainable through a balanced daily diet. With such a diet, the average plasma concentration of vitamin E in humans is approximately 12 mM. However, epidemiological studies indicate that when vitamin E supplementation of 100-10,000 IU/day (70-700 mg/day) have been taken for long periods of time, plasma vitamin E levels can reach 20-55 mM (Morrissey et al., 1993; Bendich et al., 1988).

Vitamin E is the principle, if not the sole, chain breaking antioxidant in tissues and plasma because it can readily donate the hydrogen atom from the hydroxyl group on its ring structure to a free radical. Vitamin E appears to prevent the modification of LDL into its atherogenic oxidised form, and hence it has been implicated in playing a preventative role in the pathogenesis of coronary artery disease.

Epidemiological studies have found compelling evidence that there is an inverse relationship between mortality from coronary heart disease and dietary intake of vitamin E

(Rimm et al., 1993; Stampher et al., 1993) or plasma vitamin E levels (Gey et al., 1991; Manson et al., 1993). Intervention studies in animals have also indicated that α-tocopherol is antiatherogenic (Chupukcharoen et al., 1985; Westrope et al., 1982). For example, studies indicate that vitamin E can influence the response of cells that contribute to the initiation and/or progression of spontaneous atherosclerosis (Janero, 1991). Vitamin E has also been shown to inhibit endothelial injury under oxidative stress and help maintain homeostasis by regulating important endothelium metabolic and enzymatic processes (Keaney et al., 1993; Hennig et al., 1988). Furthermore, in cell culture studies, vitamin E has been shown to inhibit monocyte-macrophage cytotoxicity, smooth muscle cell proliferation, and reduce the cholesterol ester content in these cells (Orekhov et al., 1986; Miller et al., 1979).

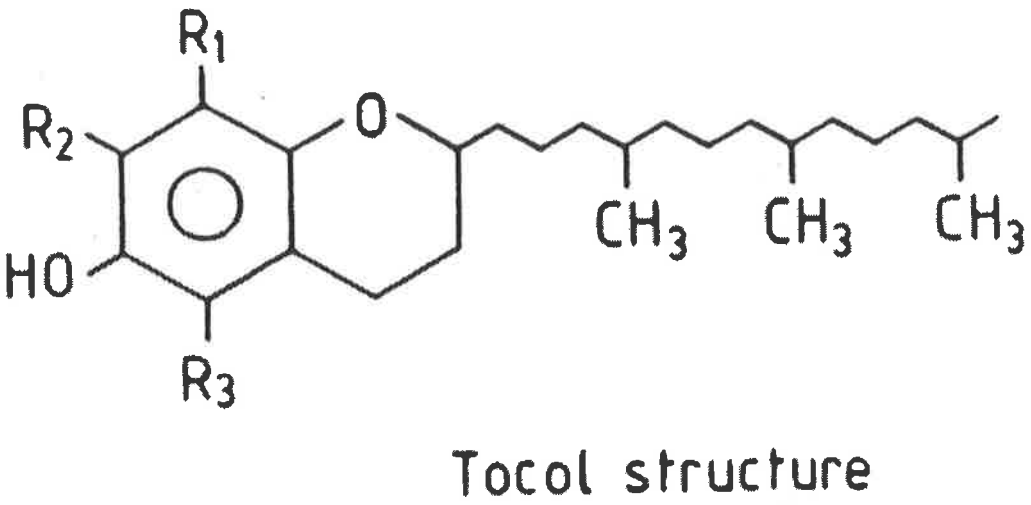
Vitamin E deficiency has been observed to cause hyperlipidemia in experimental animals. When rabbits were fed a vitamin E-free basal diet for 6 weeks, the total plasma cholesterol levels where higher than that of control rabbits (Chupukcharoen et al., 1985). The increase in plasma cholesterol was found in LDL, VLDL and chylomicron fractions but not in the HDL fraction of plasma lipoproteins. Elevated plasma cholesterol levels have also been reported in vitamin E deficient rats (Shirai et al., 1980), and guinea pigs (Chupukchaoen et al., 1985). Mechanisms responsible for the elevation of plasma LDL, VLDL and cholesterol levels are not yet known, but could be related to increased cholesterol synthesis, rearrangement of cholesterol transport by lipoproteins, or interference with normal cholesterol catabolism from the plasma.

The presence of vitamin E may contribute to the prevention of hypercholesterolemia and hence the development of atherosclerosis. Experiments on rabbits (Wilson et al., 1978; Westrope et al., 1982), guinea pigs (Qiao et al., 1993), and birds (Donaldson, 1982) have shown that vitamin E supplementation can ameliorate the hyperlipidemia that is induced by cholesterol and/or lard feeding. When rabbits were fed an atherogenic diet containing coconut oil supplemented with or without 1% vitamin E for 10 weeks, total plasma cholesterol and the level of plasma LDL significantly decreased in animals supplemented with vitamin E. Aortic and coronary atherosclerosis were also less frequent in these animals, suggesting that vitamin E can inhibit atherogenesis by preventing hypercholesterolemia. A recent study in guinea pigs reported that vitamin E not only prevented the hypercholesterolemia when these animals were fed a 2.5% cholesterol diet, but vitamin E was also able to preserve the morphological and functional integrity of the vascular wall, and hence inhibit atherogenesis (Qiao et al., 1993).

However, Fruebis et al. (1994) and Kleinveld et al. (1994) have recently demonstrated that this hyperlipidemia can not be ameliorated with vitamin E supplementation in WHHL rabbits which are deficient in LDL receptors. Vitamin E treatment had no effect on total plasma cholesterol, lipoprotein profile or the extent of atherosclerosis compared to the control group. These studies, therefore, seem to indicate that the role of vitamin E in

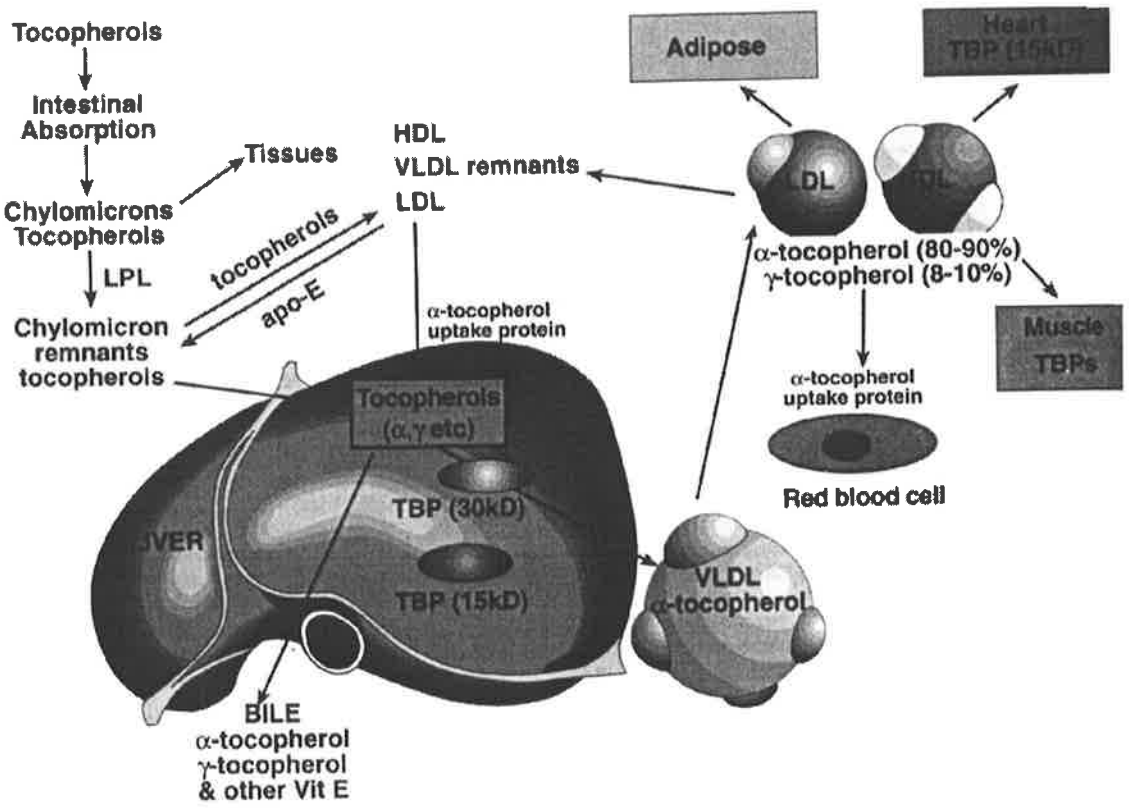
preventing hypercholesterolemia may be due to vitamin E upregulating the LDL receptor. An understanding of the process by which vitamin E affects LDL receptor metabolism clearly remains incomplete. Upregulation of LDL receptor expression in the liver is fundamental to clinically regulating the progression of atherosclerosis. If vitamin E affects the metabolism of the hepatic LDL receptor, then it may play an important role in the pathogenesis of coronary artery disease.

Figure 1.17: Structure of tocopherol. (From Bjorneboe et al., 1990).



Compound	R¹	R²	R³
α-Tocopherol	Me	Me	Me
β-Tocopherol	Me	H	Me
γ-Tocopherol	H	Me	Me
δ-Tocopherol	H	H	Me

Figure 1.18: Pathway of vitamin E transport. Tocopherols are absorbed from the diet in chylomicrons and then transported to the liver where α-tocopherol is usually secreted in nascent VLDL. The other vitamin E homologues, as well as excess α-tocopherol, are secreted in the bile. The 30 kD TBP (α-tocopherol binding protein) is thought to be responsible for specific incorporation of α-tocopherol into VLDL. The LDL receptor in tissues is probably responsible for the uptake of α-tocopherol from lipoproteins.



1.8.5.3 Vitamin C.

Vitamin C (ascorbic acid) appears to be the most effective water soluble antioxidant in plasma (Jialial and Grundy, 1992; Jialial and Scaccini, 1992). It can trap lipid peroxyl radicals before they can initiate lipid peroxidation and helps to recycle vitamin E *in vivo* (Halliwell , 1994). Although vitamin C is hydrophilic, synergistic activity with vitamin E in protecting LDL from oxidation has been demonstrated *in vitro*. Vitamin C preserves α–tocopherol levels in LDL during oxidative stress by converting the α-tocopherol radical back to the reduced state so that it may function again as an antioxidant (Esterbauer et al., 1992). However, in the presence of transition metal ions (iron and copper), vitamin C can become a pro-oxidant, acting as a reducing agent and generating oxygen, hydroxyl and peroxyl radicals (Ingold et al., 1993; Gorbunov et al., 1996). Therefore, vitamin C can be toxic if given to iron overloaded patients.

In an epidemiological study, Gey et al. (1993) have shown a significant inverse correlation between plasma ascorbate and coronary artery disease. Several lines of evidence indirectly suggest that low vitamin C levels increase the development of atherosclerosis. It has been observed that vitamin C levels are significantly lower in the aorta of patients with atherosclerotic vascular disease compared with controls, suggesting that vitamin C is needed to protect the vasculature. Diabetics, smokers and patients with coronary artery disease generally have lower levels of vitamin C (Stankova et al., 1984; Mezzeti et al., 1995). A study by Jaques (1992) reported a positive relationship between plasma ascorbic levels, higher levels of HDL cholesterol, and lower levels of plasma LDL cholesterol. Vitamin C deficiency has been shown to cause atherosclerotic lesions in guinea pigs. These lesions regress after administration of this vitamin (Willis, 1957). Altman et al. (1980) reported delayed progression, and in some cases, regression of atherosclerotic lesions among hypercholesterolemic rats supplemented with vitamin C. Little is known about how vitamin C protects against the development of atherosclerosis. It may be required for maintaining the endothelium in the arterial wall or it may be involved in regulating cholesterol or lipoprotein metabolism. Since it can affect LDL levels, vitamin C may be also able to increase the clearance of these atherogenic particles by upregulating hepatic LDL receptor activity.

1.8.5.4 Vitamin A.

The term vitamin A is used as a generic descriptor for all derivatives having the same (ß-ionine) ring structure and the same biological activity as retinol, excluding provitamin carotenoids. Retinol is a low molecular weight (286 g/mole), fat soluble compound that can partition into membranes. If present in excessive amounts, retinol can disrupt normal membrane structure and function. Therefore, to be transported through the aqueous environment and to limit its level in membranes, retinol is normally either bound to proteins both extracellularly and intracellularly, is esterified to long chain fatty acids for transport in lipoproteins, or is stored in cytoplasmic lipid droplets (Blomhoff et al., 1991). Vitamin A

occurs naturally only in animals. The best sources are liver, dairy products, kidney and eggs (Pitt, 1985) but the richest sources of vitamin A are fish oils and fish liver oils. The recommended daily allowance of vitamin A is approximately 5,000 IU. Hypervitaminosis A in humans is becoming a clinical problem of increasing frequency due to self-medication and over prescription. However, hypervitaminosis A in humans varies considerably depending on the age of individual and the duration of the excessive intake. Children are especially susceptible with toxicity occurring at therapeutic doses prescribed for various skin problems. Toxicity can occur if consumption reaches 100,000 IU a day for periods ranging from weeks to months. Symptoms include headache, dermatological changes, skin rash and hydrocephalus in children. Arctic and Antarctic explorers have suffered from acute toxicity after eating polar bear, seal or dog liver. It has been calculated that consumption of 500 g of polar bear liver (which contains 13,000-18,000 IU/g), of vitamin A would result in a toxic dose within 2-4 h (Bauernfeind, 1972).

Dietary retinol within the recommended safe range does not increase serum retinol levels. Absorbed retinol is esterified with long-chain fatty acids (mainly palmitic), incorporated into chylomicrons, and removed from the blood stream by the liver. The liver normally contains about 90% of total body vitamin A (Haghpassand and Moberly, 1995; Nagasaki et al., 1994). The retinyl esters in the liver that serve as the body's reserve of vitamin A. The esters are used to maintain a steady state concentration of vitamin A in plasma, where in the post-absorptive state it is almost all in the form of retinol. Retinyl esters have to be hydrolysed to retinol for their delivery into the blood. In plasma, the retinol is attached to a specific carrier protein called plasma retinol-binding protein (RBP). Serum retinol levels are homeostatically maintained within a narrow range by RBP, which delivers it to target tissues. Surface receptors for RBP have been found on many cells, including intestinal cells, epithelial testicular cells and interstitial cells. Specific binding proteins known as cellular retinol binding protein (CRBP) have been detected in these tissues and seem to be responsible for binding retinol once it has entered the cell (Haghpassand and Moberly, 1995; Nagasaki et al., 1994)..

Vitamin A or retinol can function as a lipoperoxyl radical scavenger. It has potential to behave as an antioxidant in a range of model natural membrane systems and has been shown to contribute to the antioxidant defence of LDL (Livrea et al., 1995). However, Livrea et al. (1995) observed that retinol and retinyl esters differ in their contributions to the antioxidant protection of LDL. It appears that retinol is just as effective as α–tocopherol in delaying the onset of lipid peroxidation, while retinyl palmitate is only used in the reaction when all the other antioxidants are depleted. Therefore, it has been suggested that the antioxidant activity of vitamin A could be of potential pathophysiological significance in delaying or preventing the atherosclerotic changes in LDL. Vitamin A has been shown to be antiatherogenic, as epidemiological investigations have shown that vitamin A is inversely correlated with coronary artery disease (Gey and Puska, 1989). The mechanism of this

action is unknown. Vitamin A may modulate lipid and/or lipoprotein metabolism as Nagasaki et al. (1994) have observed. Administration of vitamin A to normal and vitamin A deficient rats caused an increase in apo AI and CIII mRNA levels in the intestine. These apoproteins are the major proteins of HDL and VLDL. This effect of vitamin A on apoprotein gene expression would suggest that it may modulate lipid transport.

1.8.5.5 ß-carotene.

Approximately 600 naturally occurring carotenoids have been characterised, five of which are found in blood. ß-carotene represents about 15-30% of total serum carotenoids in humans and is concentrated mainly in the liver, adrenal gland, kidney, ovary and adipose tissue (Davison et al., 1993). It is abundant in vegetables and fruits and is a precursor for vitamin A. The ß-carotene can be found in many isomeric forms but all-*trans* isomers are the more stable geometric form and hence more biologically active than the *cis* forms (Bauernfeind, 1972). In humans, a significant fraction of ß-carotene is absorbed from the intestine and is converted to its metabolites in the liver and peripheral tissues. In contrast to humans, the intestinal epithelium in most other species rapidly cleaves ß-carotene to retinoic acid, retinal and other products, and very little ß-carotene is absorbed intact. Retinal is then converted to retinol for transport in plasma and esterified to retinyl palmitate by tissues (Palozza and Krinsky, 1992; Blomhoff et al., 1990).

Early epidemiological studies indicated that the incidence of several types of cancer (stomach, colon, and lung) is slightly lower among individuals with an above average intake of ß-carotene (Peto et al., 1981). However, recent evidence indicates that dietary supplementation of ß-carotene might not be protective and might even increase the risk of lung cancer (Heinonen and Demetrius, 1994). The National Cancer Institute in America has just announced that two major clinical trials that it had sponsored have yielded negative results and therefore the studies have been stopped 21 months early. The studies comprised of 18,314 participants, who were smokers and people exposed to asbestos, on a daily dose of ß-carotene, vitamin A or a placebo for about 8 years. It was reported that those taking ß-carotene had 28% more lung cancers and 17% more deaths than the group taking the placebo. These outcomes may be due to the nature of the group participating in the study and the effect of ß-carotene may not pertain to other types of cancers.

However, recent evidence indicates that ß-carotene may directly protect against cardiovascular disease. Heart attack patients supplemented with 50 mg/day experienced 50% fewer subsequent events than those receiving placebo (Gaziano et al , 1990). A study of 1,299 elderly Massachusetts residents demonstrated that individuals with large amounts of ß-carotene in their diet had the lowest risk for heart disease mortality (Gaziano et al., 1992). In another major study, ß-carotene supplementation was associated with a significant reduction in cardiovascular events, including heart attack, stroke, and death from heart

disease among a subgroup of physicians with chronic angina who had been taking 50 mg of ß-carotene every day (Gaziano et al., 1990).

Several mechanisms for the protective action of ß-carotene have been suggested. ß-carotene deactivates reactive chemical species, such as singlet oxygen and free radicals, that would otherwise initiate harmful reactions such as lipid peroxidation. The process of lipid peroxidation is thought to cause cancer and atherosclerosis. As LDL is the major carrier of plasma ß-carotene, the lipid soluble antioxidant may protect against the development of coronary artery disease by protecting the LDL particle from oxidation. Although ß-carotene has been shown to scavenge reactive oxygen intermediates *in vitro* (Ozhogina and Kasaikina, 1995), its ability to do the same *in vivo* is controversial. At higher oxygen pressures, ß-carotene can lose its antioxidant acivity and show an autocatalytic, pro-oxidant effect, particularly at relatively high concentrations (Burton and Ingold, 1984). Therefore, many people have suggested alternative explanations as to why ß-carotene may prevent the development of atherosclerosis. Shaish et al. (1995) showed that ß-carotene had very little effect on LDL oxidation, in contrast with vitamin E, but all *trans* ß-carotenes inhibited arterial lesion formation in hypercholesterolemic rabbits to the same degree as vitamin E. Therefore, the effect of *trans* ß-carotene on atherosclerosis can be dissociated from the protection of LDL from oxidation, indicating that other mechanisms may account for the ability of this compound to prevent vascular disease. These mechanisms may involve the modulation of lipid and/or lipoprotein metabolism, specifically, the enhanced clearance of atherogenic LDL particles. However, there is yet no evidence linking ß-carotene to these actions.

1.9 The experimental rationale, aims, and preview of this thesis.

The experimental evidence implying that certain fatty acids may be altering lipoprotein levels by affecting their clearance through the hepatic LDL receptor in animals has been discussed. However, whether this clearance is mediated through the LDL receptor or through other mechanisms has not been clearly defined. Furthermore, from currently available data, it is difficult to determine whether the fatty acids can exert their effects at the level of LDL receptor gene transcription. The immediate aim of this study, therefore, was to ascertain in an *in vitro* cell culture system if fatty acids can influence the LDL receptor binding activity, protein (translation) and mRNA (transcription) levels.

Based on the findings from many other studies, it was apparent that animal models were not going to be appropriate for this project due to the presence of extrinsic factors which could interfere with the direct effect of the fatty acids at the cellular level. Tissue culture was considered to be the ideal model system to show the effect of the fatty acids in isolation without the confounding effects of *in vivo* factors. Liver cell lines were chosen due to the importance of hepatic LDL receptor function in relation to plasma cholesterol levels and coronary artery disease. A human hepatic cell line (HepG2) and a rat hepatic cell line

(H-35) were chosen to determine if any intra-species variation at the hepatic cellular level exists. In addition, two human monocytic cell lines, U937 and THP-1, were chosen to allow a comparison between different tissues. These latter two cell lines provide a basis to compare whether LDL receptor binding activity in cells from nonhepatic tissues is modulated by fatty acids in a similar manner to hepatic cells.

The effect of saturated fatty acids (palmitic), monounsaturated fatty acids (oleic), ω-3 PUFAs (eicosapentaenoic) and ω-6 PUFAs (linoleic) on LDL receptor activity was investigated in the four cell lines (Chapter 4). Based on other studies, it was hypothesised that LDL receptor binding activity in the rat and human liver cells would respond to PUFAs similarly and display decreased LDL receptor activities compared to cells incubated with saturated fatty acids. On the other hand, the monocytic cells were expected to have increased LDL receptor activity with PUFAs compared to saturated fatty acids.

Further experiments were performed only on human hepatic HepG2 cells to define what influence fatty acids were having on mRNA translation and gene transcription, as well as LDL receptor binding activity (Chapter 5). The level of LDL receptor protein and mRNA were measured in the HepG2 cells incubated with the various fatty acids to determine whether they were acting at the level of protein translation and/or gene transcription. The amount of LDL receptor protein was quantified using the enhanced chemiluminescence method and the level of mRNA for the LDL receptor was quantified by the reverse transcription polymerase chain reaction.

However, there was concern that the observed fatty acid effects on LDL receptor expression may have been the result of fatty acid oxidation products formed from lipid peroxidation during the lengthy incubations. To ensure that fatty acid oxidation products were not responsible for the effects on the LDL receptor, an antioxidant, vitamin E (α-tocopherol), was added to cells simultaneously with the various fatty acids. Even in the presence of α-tocopherol, the fatty acids regulated LDL receptor expression in the same manner as previously observed. However, it was discovered that α-tocopherol was having an effect on LDL receptor activity independent to the effect observed of the fatty acids (Chapter 5).

To further examine the effects of vitamin E, α, δ and γ-tocopherols were investigated to determine if they were able to affect LDL receptor binding activity, mRNA translation and gene transcription (Chapter 6). In contrast to α-tocopherol, δ and γ-tocopherol had an opposite effect on the LDL receptor. This may have been a result of their lower antioxidant capacities.

Consequently, other well known antioxidants, including ß-carotene, vitamin A, and vitamin C, were also investigated to examine if they could have the same effect as α-tocopherol on LDL receptor expression (Chapter 7). Red wine and green tea were also chosen (Chapter 7) because the natural antioxidants occurring in red wine and green tea are though to be antiatherogenic. In addition, green tea has been shown to be an

hypocholesterolemic agent. Therefore, if green tea upregulates the LDL receptor in cells, it would provide a mechanism by which green tea lowers plasma cholesterol.

Separately, fatty acids and antioxidants had an inverse effect on the LDL receptor. The interactive effects of both agents on the LDL receptor were examined to determine whether one could overpower the effect of the other or whether their effects were independent (Chapter 8).

Chapter 2

2.0 METHODS

2.1 Cell Culture.

2.1.1 Maintaining culture.

Cells (HepG2, H-35, U937, THP-1 cell lines from American Type Culture Collection, Rockville, MD, USA) were grown in continuous cell culture at 37°C under 5% CO_2, in Dulbecco's Modified Eagles Medium (DMEM), supplemented with 12 µg/ml penicillin, 16 µg/ml gentamycin, 20 mM HEPES, 10 mM NaOH, 2 mM L-glutamine, and 10% (v/v) fetal calf serum (FCS) (CSL, Australia). The FCS, antibiotics and glutamine were added to the media just before use. Cells were seeded at 5 x 10^5 cells in 25 cm^3 flasks or 2 x 10^6 cells in 175 cm^3 flasks. The media was routinely replaced every 2-3 days, unless a visible colour change of the media had occurred from red/orange to yellow, indicating a drop in media pH. All experiments were performed with cells maintained for 2-15 passages from receipt.

2.1.2 Cell subculturing.

HepG2 and H-35 cells

When adherent HepG2 and H-35 liver cells reached confluence, they were removed from the flask by 0.1% trypsin-EDTA treatment. The culture medium was removed and the cells were washed with calcium and magnesium-free phosphate buffered saline (PBS) (136 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4, pH 7.4) to remove all traces of free salts from the cells. Approximately 5 ml of the 0.1% trypsin-EDTA solution was added directly to a 175 cm^3 flask of cells and the flask was incubated at 37°C for 5 min or until the cells had detached. The time required to remove the cells from the culture surface was dependent on the cell type, population density, serum concentration, potency of the trypsin and time since last subculture. When trypsinisation was complete, 5 ml DMEM containing 10% FCS was immediately added to the cell suspension to inhibit further trypsin activity which could cause cell damage. The cells were resuspended by gently pipetting the cell suspension several times. This was critical in order to ensure that cell clumping did not occur and that the cells grew as a monolayer. Cells were diluted with 100 ml of culture medium and the cell suspension was evenly distributed into three 175 cm^3 flasks. Cells were then grown (section 2.1.1) without disturbance for at least 24 h in order to allow attachment.

U937 and THP-1 cells

U937 cells and THP-1 cells grow in suspension without adhering to the flask and so were subcultured by diluting the cells with culture media to achieve the required cell density. To allow continuous cell growth, every week a 10 ml aliquot from the cell culture flask was transferred into a new culture flask. These cells were then diluted with 100 ml of fresh media and allowed to grow (section 2.1.1).

2.2 Fatty Acid Enrichment of Cells.

2.2.1 Preparation of lipoprotein deficient serum (LPDS) and LPDS culture media.

Lipoprotein deficient serum (LPDS) was prepared from either fetal calf serum or human plasma (obtained from the Australian Red Cross, Adelaide, SA). Plasma lipoproteins were removed by ultracentrifugation (Goldstein et al., 1983). Fetal calf serum (initial density 1.006 g/ml) was adjusted to a final density of 1.215 g/ml with potassium bromide and centrifuged for 40 h at 10°C at 38,000 rpm in a 50 Ti Beckman rotor. The bottom fraction, containing the lipoprotein deficient fraction of plasma, was collected and dialysed at 4°C against 150 mM NaCl (5 changes of 2 litres each) for 48-72 h. After filter dialysis, the serum was heat treated at 56°C for 30 min. The resulting clot was removed by centrifugation at 400 x *g* for 10 min. The lipoprotein-deficient serum was sterilised by passage through a 0.45μ Millipore filter, and then allotted into 50 ml fractions and kept frozen at -20°C until required.

LPDS (10%) culture media was prepared by the addition of 10 ml of LPDS to 100 ml of DMEM media containing 12 μg/ml penicillin, 16 μg/ml gentamycin, 20 mM HEPES, 10 mM NaOH, and 2 mM L-glutamine. This media was stored at 4°C and was used within 2 weeks.

2.2.2 Testing cells for normal LDL receptor modulation before fatty acid enrichment.

The LDL receptor in HepG2 cells is regulated by the amount of cholesterol in the media. Cells exposed to cholesterol will downregulate their receptors in order to inhibit any more cholesterol from entering the cell, and conversely cells will upregulate the LDL receptor when it is absent from the media (Brown and Goldstein, 1986). Therefore, this criterion was used as a test in order to verify normal cellular LDL receptor activity before proceeding to subsequent experiments.

Cells were incubated in media containing cholesterol (DMEM supplemented with 10% FCS) or in media lacking cholesterol (DMEM supplemented with 10% LPDS) before they were enriched with the fatty acids of interest. Cells were grown in 175 cm^3 flasks containing DMEM supplemented with 10% FCS until they were confluent. They were then subcultured at 5 x 10^5 cells into ten 25 cm^3 flasks containing the same media. After 48 h, the media of 5 flasks was replaced with 10 ml of fresh DMEM containing 10% FCS. The other 5 flasks received 10 ml of DMEM containing 10% LPDS. Cells were incubated for 24 h, after which the media was replaced with fresh media (corresponding to the previous incubation media of each flask) containing different fatty acids of interest (section 2.2.3). Fatty acid enrichment experiments were initiated when cells were ≈80% confluent or ≈1.5-2 x 10^5 cells/cm^2. At this point of cell growth, fatty acids of interest were added to cells until they were 95-100% confluent.

2.2.3 Preparation of fatty acids and enrichment of cells.

Sodium (Na^+) salts of various fatty acids (Sigma, Australia) were dissolved in either ethanol + PBS or ethanol alone, depending on the solubility of the fatty acid salt (see Table 2.1). As fatty acids are poorly soluble in water, they are bound to albumin before they are added to cells for enrichment. While vortexing, an appropriate concentration of fatty acid was added to a solution of bovine serum albumin (BSA) (0.2 g/ml in PBS) (fraction V, fatty acid free, Sigma). This solution was vortexed for a further 2 min. A 5:1 ratio of fatty acid to albumin was used. This ratio is important to maintain adequate fatty acid solubility in solution, prevent critical miscelle formation between the free fatty acids and prevent the detergent-like effects of the fatty acids that might disrupt cell membranes (Potter et al., 1989).

Fatty acid enrichment of cells was accomplished using a modified procedure of Spector et al. (1981). The fatty acid-albumin mixture was added to cells incubated in DMEM culture medium which either contained 10% FCS or 10% LPDS. A range of fatty acid concentrations (0-500 μM) was used for various experiments by adjusting the amount albumin added to the fatty acid-sodium salt so that a 5:1 fatty acid-albumin ratio was always maintained. The cells were incubated with the various fatty acids for approximately 24 h and then harvested (see section 2.2.4).

Table 2.1: The solubility of various fatty acids in ethanol and PBS.

Fatty acid (Na^+ salt)	Ratio of Ethanol to PBS
palmitic	50:50
oleic	50:50
linoleic	100:0
EPA	100:0

2.2.4 Cell harvesting.

Following a 24 h incubation with various fatty acids, culture media was decanted, and cells were washed 3 times in cold PBS buffer. This ensures that all traces of culture media are removed from the culture flasks. Cells were resuspended in 5 ml cold PBS by scraping with a rubber scraper. This suspension was transferred to a 10 ml polypropylene centrifuge tube and the cells pelleted at 400 x *g* for 10 min at 4°C. The supernatant was removed, the cells resuspended in 250 μl of PBS and transferred to a 1.5 ml eppendorf tube.

2.2.5 Fatty Acid analysis of cells after fatty acid enrichment.

Cellular lipids were extracted using a modification of the procedure reported by Bligh and Dyer (1959) to determine the degree of fatty acid enrichment. Fatty acid analysis was conducted on cells incubated with various fatty acids. Cells were harvested (section 2.2.4), and 750 μl of chloroform:methanol (1:2) was added to the cell suspension. Cellular proteins

were pelleted at 12,000 x *g* for 5 min at 4°C. The protein pellet was discarded and the supernatant was transferred to a fresh 1.5 ml eppendorf tube. 500 µl of chloroform:water (1:1) was added and the mix was centrifuged at 12,000 x *g* for 5 minutes at 4°C. The top aqueous layer, including the interface which contained some residual proteins, was discarded by carefully removing with a Pasteur pipette. The lower fraction, containing all the cellular lipids, was retained and 25 µg of the internal standard (heptadecanoic acid) was added to this fraction. This solution was transferred to a 10 ml glass tube. The chloroform was evaporated under N_2, and the remaining lipids were resuspended in 1 ml of 1% H_2SO_4 in dry methanol.

The lipids were transmethylated for 1 h at 100°C using a modified method of Keough and Davis (1979). Lipids were extracted with 5 ml of petroleum ether and 2 ml of water. The top layer was transferred to a fresh 10 ml glass tube and dried under nitrogen. Lipids were then resuspended in 5 ml of petroleum ether. The fatty acid esters were selectively isolated using a DMSC-treated quartz wool column (Alltech, USA). The column was prepared by filling a Pasteur pipette with DMSC-treated quartz wool. The column was washed with hexane, the sample loaded onto the column and allowed to pass through the pipette. The fatty acid esters were collected from the column by eluting with 10% ether hexane, dried under N_2, and resuspended in 50 µl of isooctane.

Separations of methyl esters were carried out on an H/P 5890 gas chromatograph fitted with a Supelcowax 10 fused capillary column (30 m, 0.53 mm ID and 1.0 mm film thickness) using helium as the carrier gas (flow rate 15 ml/min) and operated isothermally at a temperature of 190°C. The fatty acids were quantified relative to the internal standard.

2.3 Antioxidant enrichment of cells.

2.3.1 Vitamin E.

Vitamin E stock solutions (20, 40, and 60 mg/ml) were prepared by dissolving vitamin E in ethanol. This solution was stored in the dark at -20°C under nitrogen gas. The solution was used within 3 weeks as it is highly susceptible to breakdown.

Vitamin E enrichment of HepG2 cells : Cells were grown under the same conditions as described previously (section 2.1.1). They were then subcultured from 175 cm^3 flasks into 25 cm^3 flasks, and allowed to grow without disruption for 48 h in a background medium of DMEM supplemented with 10% FCS. The media was then replaced with fresh, pre-warmed (37°C) DMEM containing 10% FCS. Various amounts of vitamin E (0-100 µM) were then added directly to the media in the cell culture flasks. The vitamin E was dissolved into the cell culture media by inverting the flask upside down so that the media could be shaken without disrupting the cells. Cells were incubated with vitamin E medium for 24 h. After this incubation period, the media was removed and stored at -20°C for future vitamin E analysis (section 2.3.3). The cells were washed several times in cold PBS to ensure the removal of any residual media. Cells were then harvested in 1 ml of PBS

solution by scraping. They were pelleted by centrifugation at 400 x *g* for 10 min at 4°C, and resuspended gently either in 1.5 ml of PBS for LDL receptor binding analysis (section 2.6.3) or in 300 µl PBS for receptor protein analysis (section 2.7). Cells were harvested for mRNA analysis as described in section 2.8.

2.3.2 Vitamin A, vitamin C, ß-carotene, green tea and red wine.

Vitamin A stock solution (14.3 mg/ml) was prepared in ethanol, Vitamin C stock solution (8.8 mg/ml) was prepared in PBS, and ß-carotene stock solution (27.8 mg/ml) was dissolved in chloroform. Enrichment of cells with various concentrations of these antioxidants was carried out as for vitamin E (section 2.3.1).

Green tea extracts (5% w/v) were prepared by Ian Record (CSIRO Division of Human Nutrition, Adelaide) according to the method of Wang et al. (1992). This extract contained all the catechins normally present in green tea. The amount of green tea extract used to enrich the cells was based on the concentration of the major catechin, (-)-epigallocatechin gallate (EGCg) (1.96 mg/ml in 5% green tea extract). Enrichment assays with various concentrations of green tea extract were performed as described for vitamin E (section 2.3.1).

Red wine was obtained from Mavis Abbey (CSIRO Division of Human Nutrition, Adelaide) and its concentration was calculated based on the quercertin (1.508 mg/ml) content. Enrichment assays with various concentrations of red wine were performed as described for vitamin E (section 2.3.1).

2.3.3 Antioxidant analysis.

Preparation of samples

Vitamin E (α-tocopherol), ß-carotene (*trans* ß-carotene) and vitamin A (retinol) content of cells and expended growth media was measured by high performance liquid chromatography (HPLC) by the method of Yang and Lee (1987). Cells were grown in the presence of various antioxidants, and subsequently harvested in the manner described in section 2.2.4. A small aliquot of the cell suspension was analysed to determine cell protein content by the method of Lowry et al. (1951). The expended media was also analysed for antioxidant content.

In a 1.5 ml dark coloured eppendorf tube (or a foil wrapped eppendorf tube), a 200 µl sample of media or cell suspension containing 0.1 mg of cell protein (in PBS), and 25 µl of the internal standard (α-tocopherol acetate at 0.5 mg/ml in ethanol), was extracted with 175 µl of ethanol and 400 µl hexane. The sample was vortexed, and then centrifuged for 10 min at 10,000 x *g* at 4°C . 300 µl of the top hexane phase was transferred to a fresh 1.5 ml eppendorf tube, dried under N_2, and the precipitate redissolved in 200 µl of the mobile phase (methanol : acetonitrile : dichloromethane : hexane {22:55:11.5:11.5}). The sample was then analysed by reverse-phase HPLC using a LC-18-DB 25 cm x 4 mm column (Supelcosil).

Preparation of external standards

External standards of the vitamins of interest (α-tocopherol, *trans* ß-carotene and retinol) were prepared by combining 12.5 µg retinol, 220 µg α-tocopherol and 10 µg *trans* ß-carotene and reconstituting in 10 ml of the mobile phase. These standards were then diluted with the mobile phase to obtain specific concentrations (Table 2.2) which were used to produce a standard curve. In a separate tube, 25 µl of 0.5 mg/ml α-tocopherol acetate, the internal standard, was added to 5 empty 1.5 ml eppendorf tubes and evaporated under N_2. To each of these tubes, 200µl of the external dilutions were added and mixed. These samples were subjected to HPLC analysis to obtain a standard curve.

Table 2.2: Dilution of external standards for standard curve.

Retinol Final conc (µg/ml)	α-tocopherol Final conc (µg/ml)	trans ß-carotene Final conc (µg/ml)	Volume of standard (µl)	Volume of mobile phase (µl)
0.125	2.2	0.1	100	900
0.250	4.4	0.2	200	800
0.375	6.6	0.3	300	700
0.500	8.8	0.4	400	600
0.625	11.0	0.5	500	500

Sample analysis

The α-tocopherol, *trans* ß-carotene and retinol content of the cells and media was quantified by extrapolating from standard curves. The internal standard was used to account for sample losses only, and not for concentration determinations. The peak area of the internal standard should be the same in both the external and the cell or media samples. Therefore, any losses in the sample can be corrected for by using the multiplication factor : Actual area of internal standard/Area of internal standard in cell or media sample.

2.4 Lipid analysis.

Cholesterol and triaclgylcerol in FCS and LPDS were assayed using enzyme kits (Boehringer Mannheim, Mannheim,Germany).

2.5 Measurement of total protein synthesis in cells.

Total cellular protein synthesis was estimated to ensure that vitamin enrichment did not perturb cellular processes as a result of vitamin toxicity. Protein synthesis was measured by examining the incorporation of radiolabelled serine into cells. Cells were split from a 175 cm^3 flask into 5 x 25 cm^3 flasks and left undisturbed for 48 h. At day 2, cells were incubated with different amounts of vitamin E (ranging from 0-100 µM) in a background of 10 ml of DMEM containing 10% FCS. On day 3, the media in each flask was replaced by

10 ml media containing [^{14}C]-serine (0.1 μCi/ml) and 0.2 mM serine. The appropriate amount of vitamin E was again added to this media. After a 24 h incubation, cells were washed four times with PBS, pelleted at 400 x *g* and resuspended in 300 μl of solubilisation buffer (section 2.7.1). An 100 μl aliquot of the solubilised sample was transferred into a 10 ml tube and 100 μl of water, 5 μl of 10% BSA and 2 ml of 10% TCA was added. The tube was incubated on ice for 10 min, boiled for 15 min and again placed on ice for another 15 min. The sample was vacuum filtered (0.25μ filter) and the filter was washed under vacuum with 5 ml of 10% TCA, followed by 5 ml of 100% ethanol. [^{14}C]-serine incorporation was determined by scintillation counting (Beckman LS3801 Scintillation counter) of the filter and the radioactivity was measured in counts per min (cpm).

2.6 Measurement of LDL receptor binding activity.

2.6.1 Low Density Lipoprotein (LDL) preparation.

Human plasma was obtained from the Australian Red Cross, Adelaide, SA. Human LDL (1.025>d>1.063 g/ml) was isolated from 2-4 day old blood using sequential ultracentrifugation (Havel, 1988; Havel et al., 1955). Lipoproteins were dialysed against 150 mM NaCl, 50 mM Tris, 5 mM EDTA (pH 7.4), for 24 h before use. The method of Lowry et al. (1951) was used to determine the amount of protein per ml of the LDL fraction.

2.6.2 Preparation of colloidal gold-LDL.

Colloidal gold: A 400 ml solution of 0.01% (w/v) chloroauric acid was brought to boiling under reflux in a clean siliconised round bottom flask. While boiling, 11.2 ml of 1% (w/v) trisodium citrate solution was added. Boiling continued until the solution became red and stabilised in this characteristic colour of colloidal gold. The solution was stored at 4°C, and used within 2 weeks, although the colloidal gold is stable indefinitely if stored under sterile conditions.

LDL conjugation to colloidal gold: The conjugation of LDL to colloidal gold was by the method of Frens (1973). LDL (prepared as in section 2.6.1) was diluted to 1 mg/ml and dialysed overnight against 50 mM EDTA (pH 8.0). 150 μg (protein) of LDL was diluted to 0.5 ml with deionised water in each 10 ml glass round bottom tube used. Colloidal gold solution (5 ml per tube) was added rapidly while vortexing. The conjugates were pelleted at 20,000 x *g* for 20 min at 10°C. Tubes were left to stand until no more conjugates came off the sides of the tubes. The supernatant was removed by aspiration. The conjugates collected from the bottom of the tubes were combined and deionised water was added up to 1/50th of the original starting volume (eg, if started with 20 tubes of 5 ml each, then the final volume was made up to 2 ml). Sucrose was added to the colloidal gold-LDL to a concentration of 20% (w/v). The protein concentration of the colloidal gold-LDL was measured by the method of Lowry et al. (1951). The conjugates were allotted (0.5 ml) in

1.5 ml eppendorf tubes and stored at -20°C. When required, the colloidal gold-LDL was thawed in lukewarm water for 5 min. The LDL-gold stored in 20% sucrose at -20°C was stable for at least 2 months, however, this stability increased to 6 months when stored at -80°C. Enough colloidal gold-LDL was made for all samples for a comparative set of experiments to reduce the variability in binding activity between batches of LDL. Colloidal gold-LDL standards (12.5, 25, 50, 100, 200, and 400 ng in 120 µl of 4% (w/v) gum arabic) were prepared by serial dilution of the highest concentration and stored in 120 µl aliquots at -80°C. Blanks of gum arabic were also included as a control for each experiment. Gum arabic (4% w/v), calcium and EDTA solutions needed for the assay were made in bulk. These bulk solutions were prepared at the same time as colloidal gold-LDL conjugates were made in order to use the same batch of solutions for each batch of colloidal gold-LDL. These solutions were stored in aliquots at -80°C until required. Using the same buffer solutions for each batch of colloidal gold-LDL also reduces variability in the binding assay.

2.6.3 LDL receptor binding activity.

To determine cellular LDL receptor binding activity, cells were harvested, pelleted and resuspended in PBS (section 2.2.4). The cellular protein concentration was measured by the method of Lowry et al. (1951). 100 µg of cellular protein was incubated in a total assay volume of 300 µl for one hour at room temperature with human colloidal gold-LDL (20 µg protein/ml final concentration), 75 µl of buffer 1 (60 mM Tris-HCl (pH 8.0), 20 mg/ml BSA), and either with 60 µl of 2 mM $Ca(NO_3)_2$ to measure total binding or with 60 µl of 20 mM EDTA to measure nonspecific binding. The assay was done in triplicate in 1.5 ml eppendorf tubes. After 1 h, the cells were pelleted at 400 x *g* for 10 min at 4°C. The cell pellets were resuspended and washed in 300 µl of 2 mM $Ca(NO_3)_2$ for total binding or in 300 µl of 20 mM EDTA (pH 8.0) for nonspecific binding. Cells were pelleted at 400 x *g* for 10 min at 4°C. They were then resuspended in 120 µl of 4% (w/v) gum arabic and transferred to 200 µl Cobas tubes (specific tubes required for the Cobas Bio Autoanalyser).

LDL receptor activity was quantified with silver enhancement solution (IntenSE BL kit, Amersham, UK) in the Cobas Bio Autoanalyser (Roche Diagnostica, Nutley, NJ). The autoanalyser was programmed to mix 50 µl of sample, 20 µl of water and 200 µl of silver enhancement solution for 20 min in the dark at 37°C. After this time, the autoanalyser was programmed to measure the absorbance of the solution at 500 nm. Colloidal gold-LDL standards (section 2.6.2) were used to construct a standard curve. The amount of colloidal gold-LDL bound to the cells was expressed as ng LDL protein bound per mg cell protein (ng LDL/mg cell protein). LDL receptor specific binding activity was calculated by taking the difference between the total and nonspecific binding. There are approximately 10 LDL particles per colloidal gold particle in a conjugate but only one LDL at a time can be bound by a receptor. Therefore, the value obtained was divided by 10 to obtain an estimate of the LDL receptor activity.

2.7 Measurement of LDL receptor protein.

2.7.1 Solubilisation of cellular protein.

To determine LDL receptor protein, cells were harvested using cold PBS and pelleted at 400 x *g* for 10 min at 4°C (section 2.2.4). Cells could be solubilised immediately by resuspending in a 300 µl solution of cold solubilising buffer (1.5% Triton X-100, 50 mM Tris-maleate, 2 mM $CaCl_2$, 1 mM phenylmethyl-sulphonyl fluoride (PMSF) and 10 mM n-ethylmalemide (pH 6)). Cells were resuspended vigorously in solubilising solution by pipetting to reduce cell clumping and improve solubilisation of the protein. The cells were then left to solubilise in the dark for 12 h at 4°C in a rotating tube mixer. The samples were then centrifuged at 10,000 x *g* for 20 min at 4°C, and the supernatant carefully removed and transferred to another tube. The protein content of the supernatant was determined using the method of Lowry et al. (1951).

Solubilisation of cellular protein appeared to be greatly improved when cells were frozen at -20°C in PBS for at least 24 h immediately after harvesting, pelleted again at 400 x *g* for 10 mins at 4°C, and resuspended in 300 µl cold solubilisation buffer. The procedure was then continued as described above. The latter procedure was used if the amount of cells was limiting.

2.7.2 Separation of cellular protein.

Solubilised cellular protein was electrophoresed within 24 h after extraction to reduce LDL receptor breakdown. Solubilised cell protein (100-200 µg) samples were first adjusted to 100 µl by the addition of solubilisation buffer, 10 µl of glycerol, 7.5 µl of 20% SDS and 1 µl of tracking dye (0.5% bromophenol blue, 40% sucrose). RAINBOW molecular weight-markers (Pharmacia LKB, Uppsala, Sweden) were also prepared for separation by adding 1.6 µl ß-mercaptoethanol, 3 µl 20% SDS, and 12 µl sample buffer (0.1 M sodium phosphate (pH 6.8), 3% SDS, 1% ß-mercaptoethanol) to 16 µl of the marker. The marker solution was then heated to 100°C and boiled for 2 min. The cell protein and markers were then separated by electrophoresis on a 2-15% SDS polyacrylamide gradient gel at 30 mA for 5 h in running buffer (3.0 g/L Tris base, 14.4 g/L glycine, 1.0 g/L SDS, pH 8.3). Electrophoresis was stopped when the dye front was one inch away from the bottom of the gel. The gel was incubated in transfer buffer (25 mM Tris base, 192 mM glycine, 20% (v/v) methanol) for 30 min whilst rocking gently. The nitrocellulose membrane (0.45µ) (Schleicher and Schuell, Dassel, Germany) was soaked in transfer buffer for 30 min. Separated proteins were electro-transferred to nitrocellulose membranes (45V, 12 h, 4°C) and detected by immunoblotting using an enhanced chemiluminescence (ECL) method (section 2.7.3).

2.7.3 Detection of LDL receptor protein.

The nitrocellulose membrane was blocked for one hour at room temperature in 10 mM Tris-HCl (pH 7.4), 154 mM NaCl containing 10% skim milk powder. The membrane was equilibrated in 1% skim milk powder in 10 mM Tris-HCl (pH 7.4), 154 mM NaCl by washing it twice for 5 min. It was then incubated for 30 min with an anti-LDL receptor antibody (3.7 μg protein/ml in 1% skim milk powder). This is a polyclonal antibody that was raised in rabbits against a purified LDL receptor from bovine adrenal cortex (Roach et al., 1993). The membrane was incubated for a further 30 min with anti-rabbit IgG linked to horse radish peroxidase (Amersham, UK) that was diluted 1:5000 in 1% skim milk powder in 10 mM Tris-HCl (pH 7.4), 154 mM NaCl. After this incubation period, the membrane was washed twice in 10 mM Tris-HCl (pH 7.4), 154 mM NaCl containing 2 mM $CaCl_2$. Detection of the antibody conjugate was performed as per the ECL kit (Amersham, UK). Bands were detected after exposure for 4-6 min on hyper-film ECL (Amersham, UK).

2.7.4 Quantification of LDL receptor protein.

LDL receptor protein was quantified on autoradiographs using an LKB Ultrascan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, North Ryde, NSW, Australia). Results were expressed as relative absorbances against the background absorbance of the film and results were calculated as the % change compared to control cells.

2.8 Measurement of cellular LDL receptor mRNA and HMG-CoA reductase mRNA levels.

The level of cellular LDL receptor mRNA was quantified using a modified method of Powell and Kroon (1992). Total RNA from cells was reverse transcribed (RT) along with a synthetic internal control (Perkin Elmer Cetus, USA) containing sequences corresponding to the LDL receptor. The cDNAs were amplified using the polymerase chain reaction (PCR) incorporating digoxygenin (DIG) labelled dUTP (Boehringer Mannheim, Germany). cDNA was electrophoresed on agarose gels and then transferred to a nylon membrane. DIG labelled cDNAs were detected by the ECL method (section 2.7.3) using an antiDIG antibody. Comparison of cellular cDNA with the internal control by densitometry allowed quantification of LDL receptor mRNA levels.

Similarly, HMG-CoA reductase mRNA levels in HepG2 cells were quantified using the same synthetic internal control which also contains sequences corresponding to the HMG-CoA reductase coding sequence. The cDNAs were amplified with specific HMG-CoA reductase primers (section 2.8.4) by PCR and incorporating digoxygenin (DIG) labelled dUTP.

2.8.1 Total RNA isolation.

To avoid contamination with ribonuclease (RNase) and consequent degradation of RNA, all glassware was baked at 180°C overnight. Plastic ware which was not purchased sterile was autoclaved before use. Contamination with RNase was minimised by wearing gloves at all times. 1.5 ml eppendorf tubes were siliconised by placing the tubes in 5% dichloro-dimethylsilane (v/v) in chloroform. Tubes were removed and the chloroform allowed to evaporate. The tubes were washed several times with DEPC treated water (Sambrook et al., 1989) and autoclaved (15 min at 121°C). Solutions were autoclaved and prepared in baked glassware and autoclaved DEPC treated distilled water. Bottles and plastic lids were washed with 0.2% DEPC and autoclaved to inactivate DEPC (Sambrook et al., 1989).

Cellular RNA was isolated using a modified procedure of Chomocznski and Sacchi (1987). HepG2 cells were washed 3 times in PBS in order to remove all traces of culture medium. The cells were denatured by adding 500 µl pre-chilled denaturing solution (4.2 M guanidinium isothiocyanate, 42 mM sodium citrate (pH 7.0), 0.83 % N-lauryl-sarcosine and 0.2 mM 2-mercaptoethanol) directly into the culture flask. The denatured cells were transferred from the culture flasks to a 1.5 ml eppendorf tube. 40 µl of 2 M sodium acetate and 400 µl of phenol:chloroform:isoamyl alcohol (25:24:1) were added. The samples were chilled on ice for 15 min, and then centrifuged at 10,000 x *g* for 20 min at 4°C. The top aqueous phase was transferred to a clean siliconised 1.5 ml eppendorf tube. An equal volume of isopropanol was added and the sample incubated at -20°C for 30 min. The RNA was pelleted by centrifugation at 10,000 x *g* for 20 min at 4°C, and the resulting pellet was resuspended in 166 µl of denaturing solution. An equal volume of isopropanol (166µl) was added to the sample which was then incubated for 30 min at -20°C. RNA was pelleted by centrifugation at 10,000 x *g* for 10 min at 4°C. The pellet was twice washed with ice-cold 75% ethanol and then dried under vacuum for 2 min. The pellet was resuspended in 200 µl RNase-free deionised water. Samples were stored at -20°C.

Total cellular RNA was also prepared from liver cells by the procedure of Chirgwin et al. (1979). Cells were homogenised in 1.2 ml denaturing solution. This was layered over 1 ml of a 5.7 M caesium chloride cushion and spun in a TLS-55 Rotor at 33,000 rpm in a TL-100 centrifuge (Beckman) for 15-18 h at 4°C. The RNA pellet was resuspended in 90 µl TES Buffer (10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% SDS), then precipitated twice with 0.1 volume 3M sodium acetate and 2.5 volumes ethanol. The cellular RNA pellets were resuspended in 200 µl TES buffer, extracted once with phenol:choroform:isoamyl alcohol (10:9.5:0.5), and once with chloroform:isoamyl alcohol (9.5:0.5), and finally resuspended in 10 µl of sterile RNase free deionised water.

2.8.2 Measurement of RNA quality and quantity.

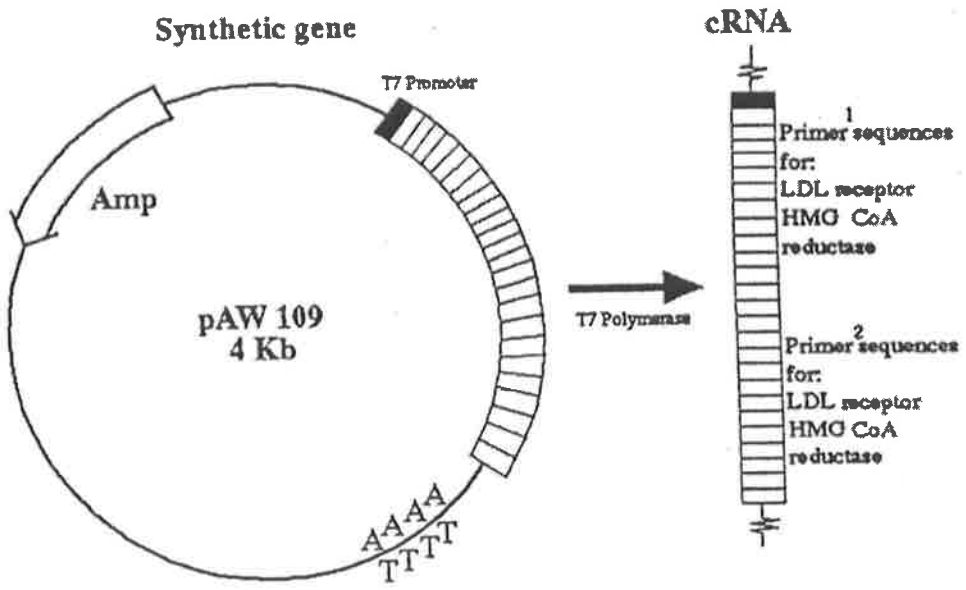
RNA was resuspended in 200 µl of sterile RNase free water. A 2 µl aliquot of resuspended RNA was further diluted to 400 µl in a separate tube with sterile deionised water. Absorbance of the 400 µl sample was measured at 260 (A_{260}) and 280 (A_{280}) nm in a spectrophotometer using 500 µl quartz cuvettes with a 1 cm light path against sterile deionised water. The amount of RNA was calculated using the optical density at A_{260}, knowing that an A_{260} of 1.0 = 37 µg/ml single-stranded RNA. The A_{260}/A_{280} ratio was used as an indicator of nucleic acid purity. Only samples with ratios greater than 1.8 were used for RT/PCR experiments (section 2.8.3).

2.8.3 Reverse transcription (RT).

2.8.3.1 Internal standard.

Cellular RNA was reverse transcribed into cDNA in the presence of a synthetic piece of RNA, AW109 cRNA (Perkin-Elmer Cetus Instruments, Norwalk, CT). This synthetic cRNA was used as an internal standard since it contains primer sites corresponding to the LDL receptor. AW109 cRNA is synthesised as a sense strand from the plasmid pAW109 by T7 RNA polymerase. The structure of the plasmid pAW109 is shown in Figure 2.1. It contains an insert of a synthetic linear array of primer sequences for multiple target genes including the LDL receptor and HMG-CoA reductase (Figure 2.1). The PCR product from each set is 300-308 base pairs (bp) depending on the primer set used and is designed not to overlap in size with the PCR products (240-260 bp) from the target mRNA (Table 2.3). The size difference between the PCR products permits easy separation of the cRNA product from the target mRNA product by gel electrophoresis

Figure 2.1: Plasmid pAW109



2.8.3.2 Reverse transcription (RT) protocol.

The 11.93 µl reverse transcription reaction mixture contained 1 µl of total cellular RNA (120 ng/µl), 1 µl of AW109 cRNA (4 x 10^4 copies/µl), 1 µl of 10 x PCR buffer (100 mM Tris HCl, 500 mM KCl, pH 8.3), 2 µl of 25 mM $MgCl_2$, 0.5 µl of RNasin (20 U/µl) (Perkin Elmer Cetus), 0.5 µl of random hexanucleotide primers (50 µM) (Perkin Elmer Cetus), 1.5 µl each of dGTP (10 mM), dATP (10 mM), dCTP (10 mM), 0.937 µl of mM dTTP (10 mM) (Perkin Elmer Cetus), and 0.5 µl of Moloney Murine Leukemia Virus reverse transcriptase (50 U/µl) (Perkin Elmer Cetus). The sample was incubated for 10 min at room temperature (23°C) to allow annealing and extension of the primers to begin before the reverse transcription at 45°C for 15 min in a Perkin Elmer Thermal cycler. The reaction was heated to 95°C for 5 min (to denature RNA-cDNA hybrids and to inactivate the reverse transcriptase) and then quick chilled on ice.

2.8.4 Polymerase chain reaction (PCR) protocol.

Amplification of the LDL receptor cDNA was performed by the polymerase chain reaction. The reaction mixture was prepared by including 5 µl of the reverse transcription reaction, 0.5 µl of digoxygenin (DIG)-11-dUTP (1 mM), 2 µl of 10 x PCR buffer, 0.25 µl of AmpliTaq DNA Polymerase (5 U/µl) (Cetus), 11.68 µl deionised H_2O, 0.60 µl (15 pmol) each of the LDL receptor downstream primer, AW125 (25 µM), and upstream primer, AW126 (25 µM) (Perkin Elmer Cetus, Norwalk, CT) (see Table 2.3), to give a total reaction volume of 20 µl. The same reaction mixture was used to amplify HMG-CoA reductase cDNA, except amplification was done using 0.30 µl each of the HMG-CoA reductase specific primers, downstream primer AW102 (25 µM) and upstream primer AW104 (25µM) (Perkin Elmer Cetus, Norwalk, CT) (see Table 2.3).

The mixture was overlaid with mineral oil and amplification carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) using the following conditions: denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min, for 27 cycles, and then a further extension at the end for 10 min at 72°C. The DIG-dUTP was used as a non radioactive label for DNA. DIG-dUTP partially replaces dTTP in the PCR reaction, so that the PCR products are labelled with DIG. Therefore, the correct ratio of DIG-UTP to dTTP plays a significant role in the determination of the optimal PCR products.

Table 2.3: Primer sequences for amplification of target and synthetic RNA.

mRNA species	5' primers (name/sequence)	3' primers (name/sequence)	Size of PCR product	
			mRNA	cRNA
	5'- -3'	5'- -3'		
LDL-R	AW125 CAATGTCTCACCAAGCTCTG	AW126 TCTGTCTCGAGGGGTAGCTG	258 bp	301 bp
HMG-CoA	AW102 TACCATGTCAGGGGTACGTC	AW104 CAAGCCTAGAGAGACATAATCAT	246 bp	303 bp

2.8.5 Detection of PCR products by chemiluminescence.

10 µl of each PCR reaction mixture was size fractionated by electrophoresis on a 3.0% agarose gel in TAE buffer (0.8 mM Tris acetate, 0.04 mM EDTA, pH 8.5) for 90 min at 90 V. It was necessary to run the gel at low voltage for a longer period of time in order to adequately separate the target cDNA from the internal control since there is only a 50 bp difference in size between the products. The DNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) by blotting for 4 h in 10 x SSC (1.5 M NaCl, 0.15 M Na_3 Citrate, pH 7.6). The blot membrane was baked for 1 hour at 100°C and rinsed in 2 x SSC. The membrane was then washed in Buffer A (0.1 M Tris-HCl, 0.1 M NaCl) for 5 min and blocked for 30 min in blocking buffer (10% skim milk powder). This was followed by a 30 min incubation with an anti-digoxygenin-IgG conjugated to alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in a blocking solution of 1% skim milk powder. The blot was washed 3 x 20 min in Buffer A and incubated with buffer B (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 min. Chemiluminescence detection was performed by incubating the membrane for 5 min in a solution of CSPD (Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2-(5-chloro) tricyclo[3.3.1.1]decan}-4-y)phenyl phosphate) (Boehringer Mannheim) at a concentration of 100 mg/ml in Buffer B. This diluted substrate solution can be stored at 4°C and reused at least 6 times. The membrane was dried by lightly blotting on filter paper, sealed in plastic and incubated at 37°C for 20 min. Exposure to X-ray film was performed in an X-ray cassette at room temperature for 5-30 min. The length of time for exposure was dependent on the potency of the substrate and the amount of sample present. Two sharp distinct bands corresponding to the cellular LDL receptor mRNA at 258 bp and synthetic cRNA at 301 bp could be detected when the blot was exposed to X-ray film. Similarly, distinct bands corresponding to cellular HMG-CoA reductase mRNA at 246 bp and the synthetic internal control at 303 bp could be detected. Typically, several different exposures were made for the same blot to ensure that band intensities were within an appropriate range for densitometry analysis.

2.8.6 mRNA quantification.

Each pair of signals corresponding to the target mRNA and the internal standard detected on autoradiograph film was scanned by laser densitometry. Quantification of the target mRNA was performed by a comparison with the cRNA internal standard and expressed per µg of total cellular RNA. Analysis was generally limited to exposures which gave absorbance readings of less than 1. The concentration of LDL receptor or HMG-CoA reductase mRNA was calculated from the relative sample and control peak areas and the known number of molecules of cRNA added to the PCR reaction using the equation :
mRNA copies/µg RNA = R x (copies of cRNA added)/(µg of cellular RNA added); where R = sample peak area/control peak area.

The reverse transcription and the co-amplification of the synthetic and cellular RNA in the same tube overcomes any variation that may have arisen due to sample preparation, RT or PCR conditions. Since the same primers were used in the PCR amplification of both templates, there are no differences in primer efficiency for cDNA derived from either cellular or synthetic RNA. The direct incorporation of DIG-labeled dUTP makes it possible to compare the relative intensities of both bands.

2.9 Statistical analysis.

Genstat (Numerical Algorithms Group, Oford, UK) analysis of variance (ANOVA) was conducted on the data described in Chapters 4 and 5. The analyses on the experiments were done assuming a 5 x 2 factorial experiment in a randomised complete block design. For each experiment 10 flasks of cells were used corresponding to 5 levels of fatty acids and 2 levels of media.

Data are shown in most cases as means ± SEM. When measurements were made in only two treatment groups, statistical evaluation was done using unpaired Student's *t*-test. When measurements were made in more than 2 treatment groups, statistical evaluation was done by ANOVA using Genstat and the pooled variance was then used to determine the least significant difference when comparing individual groups. Significance was defined as $P< 0.01$ (Chapters 4-8).

Chapter 3

3.0 OPTIMISATION OF METHODS

Chapter 3 describes the establishment of conditions for accurate and reproducible results. These conditions were used during the experiments discussed in Chapters 4-8, in which LDL receptor activity, protein and mRNA levels were quantified in cultured cells.

3.1 Optimisation of Cell culture.

Optimal conditions were established for maintaining the continous line of HepG2 cells to ensure proper growth and function (section 2.1). HepG2 cells have a tendency to grow over each other as clumps, rather than as a monolayer. This type of cell growth appeared to impair LDL receptor function as results for LDL receptor binding activity were inconsistent. Aggregation of the cells may have been due to a number of conditions, such as cell subculturing and improper growth media composition.

Growth media

Cells were initially grown and maintained in DMEM supplemented with 5% FCS. However, the growth rate of the cells was impaired and cell aggregation was increased in 5% FCS. Cells maintained in DMEM supplemented with 10% FCS reduced aggregation. Therefore, cells were maintained in DMEM supplemented with 10% FCS, and cells were cultured under these conditions prior to the experiments.

Cell subculturing

The trypsin treatment procedure (section 2.1.2) for subculturing was an important factor in alleviating cell aggregation. Trypsin concentrations could not exceed 0.1% and the incubation time with trypsin was no greater than 5 min, as damage to cell attachment mechanisms caused serious aggregation. After the 5 min incubation with trypsin solution, and cell detachment from the flasks was visible, the trypsin solution was diluted immediately with DMEM. Cells were resuspended by vigorous pipetting to destroy aggregates, and only then were cells further diluted with DMEM. These modifications to the cell culture methods were vital for monolayer cell growth and hence, accurate repeatability in the experiments on LDL receptor binding activity, protein and mRNA measurements.

3.2 Tests for normal LDL receptor function.

A number of tests were undertaken in order to ensure that LDL receptor function in HepG2 cells was as expected. Cells were examined periodically during the study under specific test conditions.

In the beginning of this project, when cells were tested to ensure that LDL receptor activity responded to the presence or absence of cholesterol, the liver cells (HepG2 and H-35) failed to show normal cholesterol response. These tests were carried out on cells grown in DMEM supplemented with 10% FCS (bovine) and DMEM supplemented with 10% LPDS (human). There was no significant difference in LDL receptor protein levels between the

cells grown in these different media. This implied that there were problems with the cells and/or the culture media. The problem was resolved as described below.

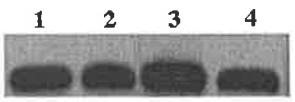
3.2.1 LDL receptor response in the presence of Pravastin.

First, to test whether cellular LDL receptor function was impaired due to a malfunction in cellular cholesterol metabolism, an HMG-CoA reductase inhibitor, Pravastatin, was used. Pravastatin is also known to upregulate the LDL receptor.

Cells were subcultured (section 2.1.2) into 25 cm^3 flasks and left undisturbed for 24 h. Cells were then incubated for another 24 h with media containing 10% human LPDS or 10% bovine FCS. Separate flasks of cells were set aside and incubated with either 10% human LPDS containing Pravastatin (10 µg/ml of media) or 10% bovine FCS with cholesterol (100 µM final concentration) for a final 24 h. Cells were harvested and LDL receptor protein was quantified using the ECL method (section 2.7).

When cholesterol was added to DMEM supplemented with 10% FCS, there was no change in cellular LDL receptor protein levels. This showed that the amount of cholesterol in the FCS media was optimal to downregulate the LDL receptor. However, Pravastatin was able to further increase the amount of LDL receptor protein in LPDS grown cells (Figure 3.1). This suggested that the LPDS media was not optimal to upregulate the LDL receptor and it may be contaminated with cholesterol. A the lack of difference in LDL receptor protein levels between FCS and LPDS grown cells could be attributable to the human LPDS used in these initial experiments, other sources of serum for LPDS production were explored.

Figure 3.1: LDL receptor protein levels in cells incubated in DMEM supplemented with either 10% human LPDS ± Pravastatin or 10% FCS ± 100 µM cholesterol. (Lanes : 1 = LPDS; 2 = FCS; 3 = LPDS + Pravastatin; 4 = FCS + cholesterol).



3.2.2 Cholesterol content of LPDS and FCS containing media.

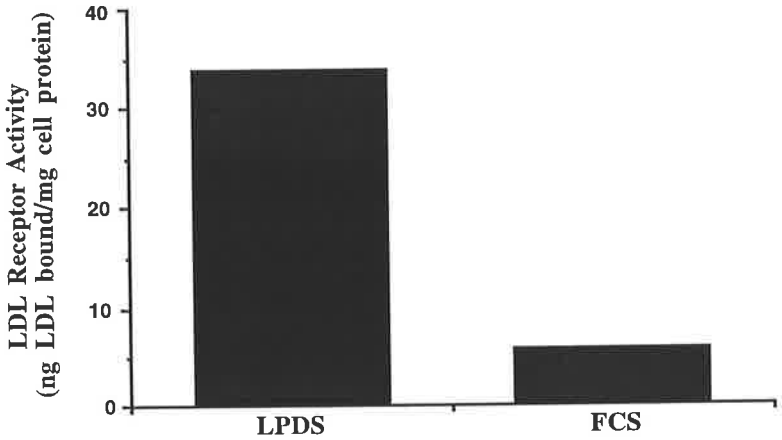
Given that the problems could be due to the human LPDS solution, the cholesterol content of all the different media preparations was measured to ensure that there was sufficient cholesterol in the FCS media to downregulate the LDL receptor and that there was no cholesterol in the human LPDS media. The cholesterol content of the media was determined in the Cobas Bio Autoanalyser (Roche Diagnostica, Nutley, NJ) (section 2.4). The cholesterol content of the human LPDS media preparation was found to be 17.4 µg/ml and media containing 10% bovine FCS contained 45.2 µg/ml. Since LPDS media should contain less than 10% of cholesterol found in FCS media, new LPDS was prepared from bovine FCS instead of human plasma (section 2.2.1).

3.2.3 Human vs Bovine LPDS.

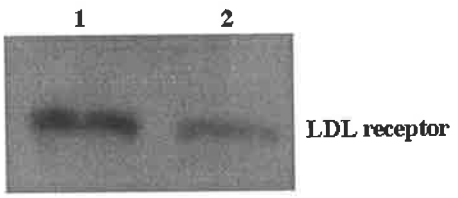
New media was prepared containing 10% bovine LPDS and cells were incubated with this media for 24 h. The level of LDL receptor activity (section 2.6) and protein (section 2.7) were measured in these cells and compared to levels in cells that had been incubated with DMEM supplemented with 10% FCS. Cells upregulated their LDL receptor activity (Figure 3.2a) and receptor protein (Figure 3.2b) when they were incubated with media containing 10% bovine LPDS compared to cells incubated with 10% bovine FCS. Therefore, throughout the remainder of the project, LPDS was derived from bovine FCS.

Figure 3.2: LDL receptor binding activity and protein of cells incubated in media containing bovine LPDS or bovine FCS.

(a) LDL receptor activity in cells incubated in the presence of 10% bovine LPDS compared to cells incubated with 10% bovine FCS.



(b) LDL receptor protein levels in cells incubated in the presence of 10% bovine LPDS (lane 1) compared to cells incubated with 10% bovine FCS (lane 2).



3.3 Optimisation of LDL receptor protein determination.

Polyclonal (Beisiegel et al., 1981(a)) and monoclonal (Beisiegel et al., 1981(b)) anti-LDL receptor antibodies have been used to detect the presence of LDL receptor protein in isolated intact cells. Immunoblot analysis with monoclonal antibodies has also been used to detect LDL receptor protein in tissue (Roach et al., 1987). Quantification of LDL receptor protein in liver cell lines utilised polyclonal antibodies (section 2.7). The optimal solubilisation buffer, blocking solutions, and antibody concentrations were determined for accurate quantification of cellular LDL receptor protein (section 2.7).

3.3.1 Solubilisation buffer.

Cell protein needed to be effectively solubilised in order to extract sufficient protein from the cells for LDL receptor protein determinations. The procedure also had to ensure that the LDL receptor protein could be extracted with the minimal degradation. A series of solubilisation buffers (Table 3.1) were compared to determine the most suitable for cell solubilisation.

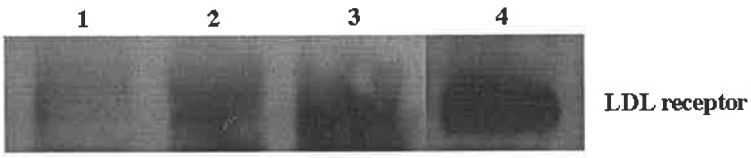
Table 3.1: Composition of various solubilisation buffers tested.

	Buffer 1	Buffer 2	Buffer 3	Buffer 4
Tris Maleate	50 mM	50 mM	50 mM	50 mM
$CaCl_2$	2 mM	2 mM	2 mM	2 mM
N-ethylmalimide	10 mM	10 mM	10 mM	10 mM
Triton X-100	1.0%	1.0%	1.0%	1.5%
PMSF *	0.1 mM	1.0 mM	1.0 mM	1.0 mM
Urea		5 M		
Iodoacetemide			0.1 mM	
Leupeptin			0.1 mM	
pH	6.0	6.0	6.0	6.0

*PMSF (phenylmethyl-sulfonyl fluoride) is unstable, binds reversibly to proteases and dissociates at high salt concentrations. This solution was prepared fresh when needed.

Insufficient solubilisation of cell protein led to smears and undefined bands in the protein blots (Figure 3.3). Clear resolution of the LDL receptor bands was necessary in order to accurately quantify the amount of LDL receptor protein by densitometry. Buffers 1, 2 and 3 produced indefinable bands (similar to those shown in Figure 3.3). Buffer 4 was the most effective solubilisation buffer as it produced clear sharp bands on protein gels and Western blots of the LDL receptor. This buffer was therefore used to solubilise cells for all subsequent experimental determination of LDL receptor protein levels (section 2.7).

Figure 3.3: Recovery of cellular protein with various solubilisation buffers.
(See Table 3.1 for buffer compositions. Lanes : 1 = buffer 1 ; 2 = buffer 2; 3 = buffer 3; 4 = buffer 4).



3.3.2 Antibody concentrations for LDL receptor protein detection.

Detection of the LDL receptor protein on the nitrocellulose membranes involved a number of steps including primary and secondary antibody treatments, washings, and treatment with a chemiluminscent substrate. After electro-elution of proteins from polyacrylamide gels (section 2.7.2) onto nitrocellulose membranes, the membranes were probed with the primary antibody (anti-LDL receptor antibody at 3.7 mg protein/ml), and a subsequent incubation with a secondary antibody (anti-rabbit IgG linked to horse radish peroxidase) (Amersham, UK) (Roach et al., 1993 (a)). Protein detection was by the ECL method (section 2.7.3).

It is important to optimise the detection method, especially for quantitative procedures in which optimal performance is a prerequisite. Distorted bands and poor resolution of the LDL receptor protein band on the X-ray film were due to problems associated with incorrect antibody concentrations, incubation times and blocking buffer composition. The optimisation of antibody concentrations was a critical step for an adequate signal from the LDL receptor protein. Rat liver homogenates were used to optimise antibody concentrations (Table 3.2). The conditions used in all subsequent experiments on LDL receptor protein was as for sample 6 (Table 3.2 and Figure 3.4).

Table 3.2: Primary and secondary antibody concentrations for LDL receptor protein detection. (where RLH = rat liver homogenates).

Sample	Primary Antibody (# µg in 20 ml buffer)	Secondary Antibody (Dilution)	Lane in Figure 3.4
1 RLH	148 µg	1/150	1
2 RLH	148 µg	1/300	2
3 RLH	148 µg	1/5000	3
4 RLH	74 µg	1/150	4
5 RLH	74 µg	1/300	5
6 RLH	**74 µg**	**1/5000**	**6**

Figure 3.4: Effects of various antibody concentrations on LDL receptor protein detection. (See Table 3.2 for sample treatment. Lanes: 1 = sample 1; 2 = sample 2; 3 = sample 3; 4 = sample 4; 5 = sample 5; 6 = sample 6).

3.4 Optimisation of LDL receptor mRNA determination.

3.4.1 RNA quality.

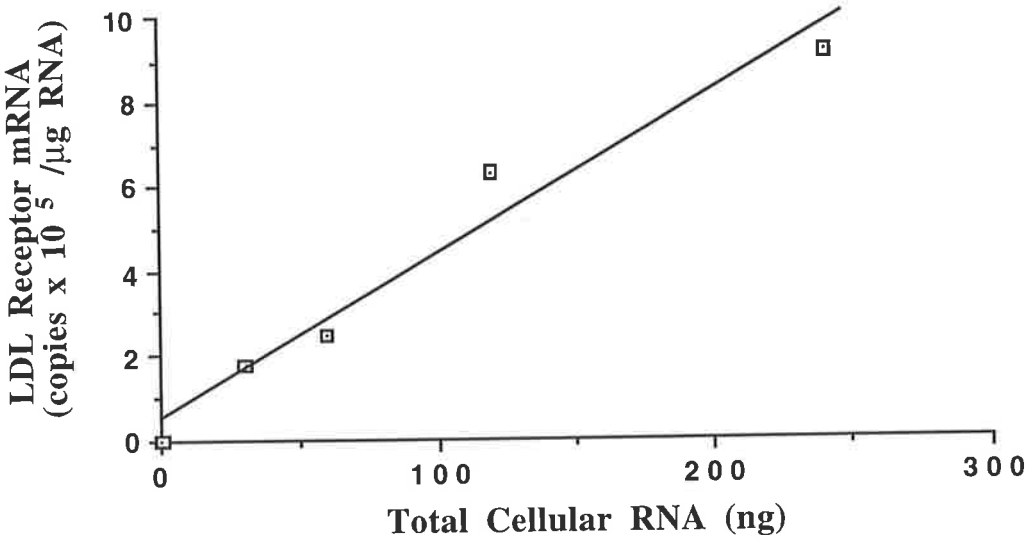
RNA samples that had an A260:A280 < 1.8 (section 2.8) were re-extracted with 50:50 phenol:chloroform and ethanol precipitated with sodium acetate (section 2.8). Only RNA with an A260:A280 > 1.8 was used in experiments. To ensure that the RNA was not degraded, samples were run on 1.2% denaturing (formaldehyde) agarose gels (Sambrook et al., 1989). If 28S and 18S ribosomal RNA's exhibited a 2:1 ratio of ethidium bromide staining, no gross degradation had occurred. Ratios less than this indicate that the 28S ribosomal RNA had been degraded to the 18S like species, and hence, degradation of total RNA isolated had occurred.

3.4.2 Linearity of amplification with increasing amounts of cell RNA.

The cDNA from the cRNA internal standard and cellular RNA must amplify and incorporate labelled bases uniformly, otherwise quantifying the amount of cellular RNA in a sample was inaccurate (McPherson et al., 1995). To ensure that the amplification with DIG-dUTP (section 2.8) showed linearity to the amount of RNA, a number of experiments were performed to demonstrate that the intensity of the chemiluminescent band was directly proportional to the amount of RNA that was used in the reverse transcription reaction. Experiments were performed with 4 x 10^4 copies of AW109 cRNA in combination with a range of different concentrations (0 to 240 ng/reaction) of total cellular RNA isolated from HepG2 cells (section 2.8). Following reverse transcription, PCR amplification was performed with LDL receptor specific primers (sections 2.8.3 and 2.8.4) for 27 cycles and the products analysed by chemiluminescence (section 2.8.5) and densitometry (section 2.8.6).

The intensity of the band was found to be directly proportional to the amount of total cellular RNA used in the reverse transcription reaction (Figure 3.5). Therefore, differences in the band intensity relative to the control cRNA represent direct variations in the amount of LDL receptor mRNA present in the sample. The final concentration of cellular RNA subsequently used in all experiments was 120 ng/reaction.

Figure 3.5: Linearity of amplification with increasing amount of cellular RNA.



3.4.3 Optimising the number of PCR cycles.

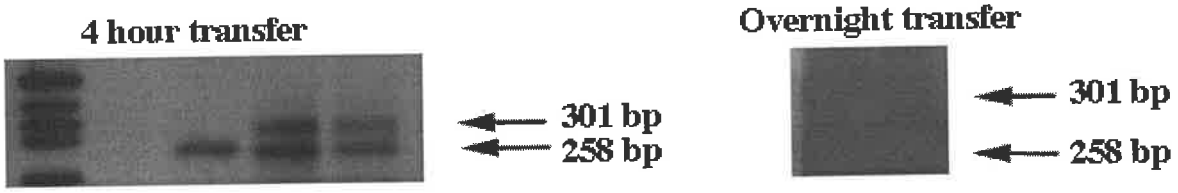
The number of PCR cycles is crucial as the linearity of incorporation of DIG-dUTP decreases at high cycle numbers (McPherson et al., 1995), but it is also necessary to amplify sufficient cDNA such that detection by ECL is possible. To ensure that the number of PCR cycles did not affect the efficiency of the formation of the PCR product, cellular RNA and synthetic cRNA were amplified using 20-40 cycles. Cellular RNA (120 ng) and 4 x 10^4 copies of synthetic cRNA was reverse transcribed (section 2.8.3) and co-amplified using the LDL receptor specific primers (section 2.8.4). The intensity of the two bands was measured using densitometry (section 2.8.5 and 2.8.6).

The efficiency of the product formation decreased after 30 cycles, but there was not much variation between 24-28 cycles. Therefore, a 27 cycle protocol was used in all subsequent experiments on LDL receptor mRNA determination (section 2.8).

3.4.4 Blotting.

The transfer of cDNA to nitrocellulose membranes by capillary blotting overnight led to diffuse bands on X-ray film after detection (Figure 3.6). The optimum transfer time which allowed sufficient transfer of DNA to the membranes, yet maintained sharp banding, was 4 h (Figure 3.6). The 4 h transfer time was used in all subsequent experiments on LDL receptor mRNA levels (section 2.8).

Figure 3.6: Overnight transfer of DNA in Southern analysis. Samples were transferred from agarose gels by capillary blotting onto nitrocellulose membranes, for 4 hours or overnight. Detection was by ECL.



3.4.5 Chemiluminscent detection of LDL receptor mRNA.

Chemiluminscence is the conversion of chemical energy into light. The energy required for the emission of light can be generated by the oxidation of a specific substrate, luminol in this case. This reaction is quite complex and occurs when hydrogen peroxide and the enzyme horseradish peroxidase catalyses the oxidation of cyclic diacylhydrazines (luminol) to form luminol radicals. These radicals then decompose through an endoperoxide intermediate to form the excited state 3-aminothalate dianion. Immediately following oxidation, these molecules decay from the excited state to the ground state emitting light. The light output has a maximum emission at 428 nm at which the chemiluminscence exhibits flash-type kinetics with an initial maximum light intensity peak after 1-5 min, and then decaying rapidly within 10 min, and then slowly over an hour. The light emissions can be detected on X-ray film.

During the course of this project two new chemiluminscent substrates, called AMPPD and CSPD, were available. The AMPPD molecule is stable, emits energy at the wavelength of visible light and has a source of energy within its peroxyl bond. Alkaline phosphatase dephoshorylates AMPPD to form a dioxetane anion, which fragments into adamantanone and the excited state of methly metaoxybenzoate anion, the light emitter. Light is emitted at 477 nm at a constant rate as a "glow". The high intensity of the emitted blue light ensures that the response of the film is linear to incoming light (Luskey, 1990). Because the non-enzymatic decomposition of AMPPD produces fragments which do not exhibit chemiluminscence there is no background signal. No background was observed during the brief X-ray exposures (10 min) required to produce strong signals.

Another major problem with chemiluminescent substrates is background luminescence due to intramolecular aggregation effects. This causes a decrease in the sensitivity of the detection reaction due to a lowered signal-to-noise ratio. CSPD has significantly lower background chemiluminescence due to hyper-conjugation effects which cause the light emission kinetics of CSPD to be twice as fast as AMPPD, enabling a rapid rise to maximum light emission. This enables the production of background-free autoradiographs that exhibited strong, sharp bands.

CSPD is also enzymatically dephosphoraylated by alkaline phosphatase leading to the metastable phenolate anion which decomposes and emits light at a maximum wavelength of 477 nm. The chemiluminescent signal from CSPD can persist for days on nylon membranes, in contrast to the 24 h signal produced from AMPPD. CSPD was used for the experimental work carried out in the last year of the project as Boehringer Mannheim replaced all AMPPD substrates by CSPD.

3.5 Quantification of mRNA using fluorescent assays.

A fluorescent assay was developed to examine the accuracy of the DIG method (section 2.8) in quantifying LDL receptor mRNA levels. Due to the exorbitant cost of the fluorescent method, it was employed only occassionally to verify the results from the DIG applications. The RT/PCR reactions were carried out similarly to the DIG method, however, the detection of the RT/PCR product was done directly from the electrophoretic gel. The samples were run on an Applied Biosystems Inc. (ABI) GENE SCANNER, which is also routinely used for DNA sequencing. This is a rapid, convenient and precise method of cellular mRNA quantification.

Initial experiments were performed by incorporating fluorescent nucleotide FluoroGreen (fluorescein-11-dUTP) into the PCR amplification reaction, similar to the DIG method. However, after many failed attempts to adequately incorporate the FluoroGreen, this method was abandoned and the fluorescent method was tackled using dye labelled primers (section 5.1 and 5.2).

3.5.1 Primer labelling.

The AW125 primer was linked to 6-FAM on the 5' end. Only one labelled primer is required to have a fluorescent tag. The fluorescent primer was prepared in the Applied Biosystems DNA/RNA synthesiser by linking a fluorescent dye, 6-carboxyfluorescein phosphoamidite (6-FAM Amidite), onto the 5' oligonucleotide during synthesis. The dye and its linkage to the oligonucleotide are stable during DNA synthesis. The 6-FAM-labelled oligonucleotide appears greenish-yellow at an absorbance maxima of 500 nm.

3.5.2 Reverse transcription (RT)-polymerase chain reaction (PCR).

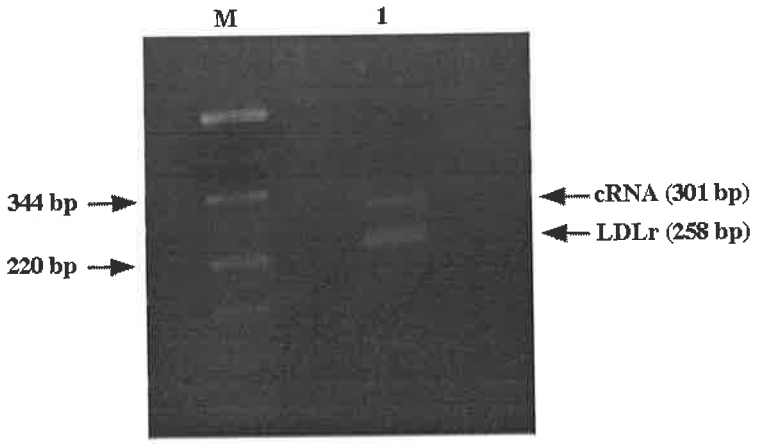
Total RNA was prepared (section 2.8.1) similarly to that of the DIG method. However, the RT and PCR reactions were carried out using different conditions to those used with the DIG method.

Cellular RNA was reversed transcribed into cDNA in the presence of AW109 cRNA. The 10 µl reverse transcription reaction mixture contained 0.5 µl of total cellular RNA (120 ng/µl), 1µl of AW109 cRNA ($4x10^4$ copies/µl) , 1µl of a 10 x PCR buffer (100 mM Tris HCl, 500 mM KCL, pH 8.3), 2 µl of 25 mM MgCl, 0.5 µl of RNasin (20 U/µl) (Perkin Elmer Cetus), 0.5 µl of random hexanucleotide primers (50 µM) (Perkin Elmer Cetus), 1.0

µl each of dGTP (10 mM), dATP (10 mM), dCTP (10 mM) and dTTP (10 mM) (Perkin Elmer Cetus), and 0.5 µl of Moloney Murine Leukemia Virus reverse transcriptase (50 U/µl) (Perkin Elmer Cetus). The sample was incubated for 10 min at room temperature (23°C) to allow annealing and extension of the primers to begin before completion of the reverse transcription at 45°C for 15 min in a Perkin - Elmer Thermal cycler. The reaction was heated at 95°C for 5 min and then quickly chilled on ice.

A 40 µl reaction mixture was prepared by including 10 µl of the reverse transcription reaction, 4 µl of 10 x PCR buffer (100 mM Tris HCl, 500 mM KCL, pH 8.3), 0.5 µl of AmpliTaq DNA Polymerase (5 U/µl), 24.15 µl deionised H_2O and 0.753 µl of the dye labeled downstream primer AW125 (19.9 µM) and 0.6 µl upstream primer AW126 (25 µM) (Perkin Elmer Cetus, Norwalk, CT) to give 7.5 pmoles of each primer in the reaction mixture. The mixture was overlaid with mineral oil and amplified in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) using the following conditions: denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min for 30 cycles, and then a further extension for 10 min at 72°C. To ensure that amplification with these primers and conditions was adequate, parallel samples were amplified for 35 cycles, and subsequently viewed on ethidium bromide stained 1.5% agarose gels (Figure 3.7) (Sambrook et al., 1989).

Figure 3.7 Amplification of cellular and internal cDNA with flouroprimer before detection. (Ethidium bromide stained agarose gel. Lanes: M = DNA size marker; 1 = RT/PCR of AW109 cRNA and cellular RNA for 35 cycles with fluoroprimer. LDLr = LDL receptor).



The PCR amplification products were combined with a red labeled size standard, GENESCAN-1000 6-carboxy-X-rhodamine (ROX), loaded on a 5.5% native copolymer gel, and placed in the 373A DNA sequencer for automated electrophoresis, direct detection, and data analysis. Electrophoresis was carried out for 1 h at 620 V. The PCR products corresponding to cellular LDL receptor mRNA and synthetic LDL receptor cRNA amplified with 6-FAM appear blue and are clearly separated from each other. The results could be

displayed as a gel view, electrophoretogram, and quantified tabular data. The quantified data show time of peak elution, dye color, fragment size, peak height (fluorescence intensity) and peak area.

Results indicated that there was a linear relationship between the number of copies of synthetic cRNA and fluorescence intensity (peak height) which was linear over 3 orders of magnitude (Table 3.3a). The number of copies of LDL receptor mRNA/μg RNA in cells incubated with DMEM supplemented with 10% LPDS was considerably more than in cells incubated in DMEM supplemented with 10% FCS, verifying that the method is reliable (Table 3.3b). Quantification of cellular RNA by this method was used to confirm the results obtained with the DIG method in Chapter 5 (Figure 5.4). Since the effect on LDL receptor mRNA levels was similar to that observed with the results obtained using the DIG method, only the DIG method was used for further LDL receptor mRNA quantification work.

Table 3.3 : Fluorometric analysis of LDL receptor mRNA levels.

a)

copies of AW109 cRNA	Fluorescence intensity (peak height)
0	0
4×10^3	94
4×10^4	564
4×10^5	6700

b)

Incubation media	Copies of AW109	Copies of cellular LDL receptor mRNA/μg RNA
10% LPDS	4×10^4	7.9×10^4
10% FCS	4×10^4	2.2×10^4

3.6 Conclusions.

The work herein established accurate and reproducible conditions for the experiments described in Chapters 4-8, which demonstrate the effects of fatty acids and antioxidants on LDL receptor binding activity, protein and mRNA levels.

Chapter 4

4.0 THE EFFECT OF FATTY ACIDS ON LDL RECEPTOR ACTIVITY IN HUMAN AND RAT LIVER CELL LINES AND HUMAN MONOCYTIC CELL LINES

4.1 Introduction

Many studies have examined which dietary fatty acids modulate plasma cholesterol levels in humans and the mechanism whereby these fatty acids induce these changes. Dietary saturated fatty acids have been shown to raise plasma cholesterol and LDL cholesterol levels compared to carbohydrates (or compared to polyunsaturated fatty acids) in humans. Such effects have been limited to lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids (Hegsted et al., 1965; Keys et al., 1965; Grundy and Vega, 1985; Denke and Grundy, 1991). Monounsaturated fatty acids, such as oleic acid, normally do not affect plasma cholesterol levels when compared to carbohydrates. However, some studies have shown that cholesterol levels do fall when oleic acid is substituted for palmitic acid and other cholesterol elevating saturated fatty acids (Grundy and Denke, 1990; Hegsted et al., 1990).

The ω-6 polyunsaturated fatty acids (PUFAs), such as linoleic acid, have been found to lower plasma cholesterol and LDL-cholesterol levels in humans when substituted for dietary saturated fatty acids (Kinsell and Micheal, 1955; Ahrens et al., 1954). Similarly, when dietary long chain ω-3 PUFAs of marine origin replace saturated fats, total plasma and LDL cholesterol can fall in humans (Harris et al., 1983; Harris, 1989; Nestel, 1986; Illingworth et al., 1984). These ω-3 PUFAs are also highly effective in lowering plasma triacylglycerol levels in man (Harris, 1989, Land, 1986; Nestel, 1990) and some animal species such as the rat (Roach et al., 1987) and the swine (Wiener et al., 1986). However, as discussed earlier (see section 1.8.3.5), the effects of ω-3 PUFAs on circulating levels of LDL when compared to ω-6 PUFAs, have been shown to be variable in humans (Harris et al., 1989; Land, 1986; Nestel, 1990). Nevertheless, the majority of studies in humans have shown a general rise in total plasma cholesterol and LDL cholesterol levels when ω-3 PUFAs are comsumed compared with ω-6 PUFAs (Harris et al 1983, 1988; Nestel, 1986; Illingworth et al., 1984).

In the past 10 years, a spectrum of studies have been undertaken to unravel how dietary fatty acids modulate lipid and lipoprotein levels in humans (Vega et al., 1982; Kuksis et al., 1982; Lang et al., 1990; Spady and Dietschy, 1988). Such studies, however, have not yet elucidated the mechanisms involved. For example, the decreased levels of plasma LDL cholesterol observed with dietary ω-6 PUFAs can not be entirely explained by enhanced faecal excretion of cholesterol or decreased LDL production (Grundy, 1983) (section 1.8.3.4). These changes only occur in some individuals, while in others ω-6

PUFAs appear to have no effect, despite significant decreases in plasma cholesterol (Grundy, 1983). Evidence also indicates that the rise in plasma LDL with ω-3 PUFAs is unlikely to be related to an increase in LDL-cholesterol production since it has been established that dietary fish oils decrease plasma VLDL levels, the precursor for LDL (Huff and Telford, 1989) (see section 1.8.3.5 for more detail).

Since none of the above proposed mechanisms were able to adequately explain how PUFAs modulate plasma LDL levels, attention then focused on the effects of PUFAs on the hepatic LDL receptor, the major mechanism by which LDL is cleared from plasma (Brown and Goldstein, 1986).

Animal Studies

Researchers have investigated whether PUFAs modulate the hepatic LDL receptor activity using animal models with the assumption that the effect of fatty acids on the LDL receptor in animal species is mimicked in humans. However, there are inconsistencies in these whole animal studies which make it difficult to reach a consensus. For example, Fernandez et al. (1992) reported that the fractional catabolic rate (FCR) of radiolabelled LDL was significantly higher in guinea pigs fed a commercial diet supplemented with corn oil (ω-6 PUFA) compared to animals supplemented with lard (saturated fat). They inferred that increased clearance of LDL was attributable to increased hepatic LDL receptor mediated uptake. In a later study, LDL receptor binding activity was measured in hepatic membranes in which they found increased LDL receptor activity in hepatocytes isolated from guinea pigs fed corn oil diets (58% linoleic acid) compared to animals fed lard diets (24% palmitic/14% stearic acid) (Lin et al., 1994). Similarly, Spady and Dietschy (1988) reported in a number of studies (Woolett et al., 1988; Spady and Dietschy, 1985) that LDL receptor mediated LDL clearance was increased in Syrian hamsters fed triacylglycerols rich in ω-6 PUFAs compared to animals fed saturated or monounsaturated triaclglycerols.

Roach et al. (1987) quantified LDL receptor activity in solubilised liver proteins which were isolated from rats fed either a ration diet or a diet supplemented with marine fish oil or safflower oil. In contrast to guinea pigs, LDL receptor activity was depressed (but not significantly) in liver cells isolated from rats supplemented with safflower oil compared to those on chow diets. However, rats supplemented with marine oil exhibited lower LDL receptor activity than those consuming safflower oil diets and significantly lower than animals fed chow diets. On the other hand, Spady et al. (1995) reported that rats fed a diet rich in fish oil had enhanced hepatic uptake of radiolabelled LDL than animals fed corn oil or normal chow diets. Although they implied that increased hepatic LDL receptor activity was responsible for the observed increased clearance in fish oil fed animals, when they quantified hepatic LDL receptor protein and mRNA levels in these animals, they found these levels unchanged. In contrast to the rat findings, Spady et al. (1995) reported in the same study that clearance of radiolabelled LDL was significantly lower in hamsters fed dietary fish oils

compared to animals fed corn oil, implying that LDL receptor activity had decreased due to fish oil feeding. They observed that hepatic LDL receptor mRNA levels in hamsters had significantly decreased with fish oil supplementation, unlike their results with rats.

The inconsistencies of the studies described above may be explained by a number of reasons. First, most animal studies investigating the effect of fatty acids on LDL clearance usually feed the animals diets supplemented with oils instead of the specific fatty acids of interest. For example, fish oil is fed to animals when one investigates the effect of ω-3 fatty acids. However, oils could contain other constituents such as vitamin E, phytochemicals, squalene and other compounds which could have their own effects on LDL clearance and LDL receptor activity. Second, the variability of the background chow diets associated with commercially purchased chow can often make a significant contribution to the animals' response to the fatty acids consumed. For example, Topping et al. (1990) showed that feeding rice bran to rats increases the hepatic LDL receptor activity relative to wheat bran and prevents the downregulation of LDL receptor by fish oils. Similarly, Roach et al. (1992) found that LDL receptor activity was significantly decreased when rats were fed wheat bran plus fish oils, but LDL receptor activity was unaffected when rats were fed oat bran plus fish oil compared to oat bran or wheat bran alone. Thus, interpretation of the effects of fish oils on LDL receptor activity in animal feeding trials can be misleading if other constituents are present in the diet. Background diet, for instance, may explain the disparity between the rat studies of Roach et al. (1987) and Spady (1995) (see above). It is, therefore, important to consider the background diets when evaluating studies with dietary fatty acids. Moreover, these examples stress the importance in developing a culture cell system to study fatty acid effects in isolation as this eliminates many of these confounding factors present in many animal studies.

It is important to note that the animal clearance studies may not actually represent the effect of PUFAs on hepatic LDL receptor binding activity. One can not assume that measuring the rate of LDL clearance from plasma reflects LDL receptor binding activity. It is certainly possible that the plasma clearance of LDL may occur through a number of different receptor and non-receptor dependent pathways which are not accountable. This may also vary between different species and may contribute to the inconsistencies observed between the animal studies.

Although there are inconsistencies between the guinea pig, hamsters and rat studies, these studies suggest that PUFAs could affect hepatic LDL receptor activity. However, it is difficult to draw any analogies from these animal experiments to humans on the grounds that the fatty acid effects may be species specific. For example, in the rat, lipid levels are unaffected by ω-6 polyunsaturated fatty acids, but on the other hand, triaclyglycerol levels and LDL cholesterol is lowered by fish oil fatty acids (Spady, 1993). Even though the animal clearance studies may imply that PUFAs affect hepatic LDL receptor activity, the fatty

acids may be actually having a very different effect at the cellular level and thus, may account for some of the inconsistencies.

Certainly, there are differences in lipoprotein metabolism between animal species and man. The effect of dietary fatty acids at the cellular level of LDL receptor activity in animals may, thus, be further complicated by differences in whole animal lipoprotein metabolism. For example, humans are unique with respect to their high LDL to HDL ratio. In most species such as rats, mice, hamster, and dogs, it is HDL, not LDL, that is the major transporter of cholesterol. This may explain why fatty acids have very different effects on lipoprotein metabolism in different species. On the other hand, in the guinea pig, LDL is the major lipoprotein and both dietary fat and cholesterol can modulate plasma LDL levels similarly to humans (Lin et al., 1994). However, HDL in guinea pigs is usually undetectable and in addition, the effect of cholesterol is usually stronger in guinea pigs than in man, implying the effect of PUFAs on LDL receptor activity in guinea pigs may not resemble that in humans (Lin et al., 1994). Thus, it is difficult to determine whether these observation in animals would truly reflect those in humans consuming similar oils.

It could be argued on the premise of gene conservation that cellular LDL receptor activity in similar tissues derived from different mammalian species should be affected in a like manner. The question is, therefore, whether PUFAs would affect hepatic LDL receptor activity in humans in a manner identical to other species. There is some direct evidence from a study by Wong and Nestel (1987) which suggests PUFAs added to cell culture media do affect LDL receptor binding activity in cultured human liver HepG2 cells the same way dietary PUFAs affect the LDL receptor activity in rat liver. They observed that LDL receptor activity was suppressed when human HepG2 cells were enriched with linoleic acid and was further suppressed when cells were incubated with eicosapentaenoic acid (EPA) compared to those cells preincubated with no fatty acids or oleic acid. If linoleic acid and EPA does affect LDL receptor binding activity in human liver cells *in vitro*, then the same also occur *in vivo*.

Human Cell Culture Studies

Nevertheless, studies examining the effects of PUFAs on LDL receptor activity in established human cell lines have also shown extreme variability, suggesting that there may be a distinct effect of fatty acids on different tissues even within one species. For example, Kuo et al. (1990) observed that human monocytic U937 cells enriched in both oleic and linoleic acid exhibited increased LDL receptor binding activity compared to cells enriched with stearic or no fatty acids. Furthermore, the linoleic acid enriched cells exhibited 50% greater LDL receptor binding activity compared to oleic acid. In contrast, a study by Nestel and May (1979) showed that the uptake and degradation of radiolabelled LDL by human fibroblasts isolated from human subjects before and after a linoleate rich diet were similar. In the same study, fibroblasts enriched with oleic and linoleic acid also displayed similar LDL receptor binding activity.

The differences in LDL receptor binding activity observed with PUFAs in the various studies may actually reflect the distinct manner in which different tissues handle fatty acids. Thus, in humans, the effect of fatty acids on peripheral cells, such as monocyte macrophages and fibroblasts, may be very different from fatty acid effects on LDL receptor activity in hepatic cells such as HepG2 (see above).

Establishing Cell Culture Studies

For this project, choosing a suitable model system to examine the effect of various fatty acids on the LDL receptor was the first priority. Based on the findings from many studies, many extrinsic factors could potentially interfere with the original effect of the fatty acids at the cellular level. Hence, it was apparent that animal models were not appropriate for this work. Therefore, to examine the response of fatty acids in isolation in one cell type, cell culture was utilised. Cell culture has the advantage in that fatty acid composition and cholesterol concentrations in the cell can be easily manipulated. These changes are achievable within a short period of time and their effects are easily monitored. For example, under the microscope, detrimental perturbations can be detected if they stunt cell growth or cause cell death.

Cells of interest can be either cultured from primary cells or immortalised cells. Primary cell culture can have its disadvantages as well, depending on the tissue of interest. For instance, primary cell cultures of liver are difficult to prepare and maintain as survival ranges from a few weeks to only a few days, making long term experiments very difficult. Therefore, immortalised liver cell lines were considered the best experimental model as fresh tissue samples from human livers are impractical to obtain.

To elucidate the effects of PUFAs on LDL receptor activity, immortalised cultured cells derived from different species and tissues were used in the initial work. Two different immortalised liver cell lines, human HepG2 and rat H-35 cells, were chosen for species comparisons to investigate the effect of fatty acids on hepatic LDL receptor activity. Liver cells were used since this organ is the major contributor to LDL metabolism in the body. It is the major site of LDL catabolism and has high LDL receptor activity. In addition, two immortalised human monocytic cell lines, U937 and THP-1 cells, were chosen as these cell types are peripheral cells and unlike liver cells, they can not synthesise lipoproteins or bile acids. These cells represent non-hepatic LDL receptor metabolism.

i) Liver cell lines: human HepG2 cells and rat H-35 cells

HepG2 is an immortalised human hepatoma cell line and is widely used as an *in vitro* model to study human hepatic lipid and lipoprotein metabolism (Gibbons, 1994; Gerbhardt, 1993; Javitt, 1990). HepG2 cells have proven to be of particular value in the direct investigation of factors which are associated with the risk of coronary artery disease (Wong and Nestel, 1987; Rumsey et al., 1995). They are fairly differentiated and still possess

several of the characteristics of human hepatocytes including lipoprotein synthesis, secretion, and cholesterol synthesis. Most importantly, HepG2 cells display essentially normal hepatic LDL receptor metabolism (Gibbons, 1994; Gerhart, 1993; Javitt, 1990). The rate of fatty acid synthesis in HepG2 cells is also similar to that of freshly prepared hepatocytes (Gibbons, 1994).

The H-35 cells are derived from rat hepatic carcinoma cells. This is a well differentiated rat hepatoma cell line which retains several differentiated functions of hepatocytes (Massague et al., 1982; Tamai et al., 1983). This cell line appears to possess three functionally distinct receptor activities that appear to mediate the specific binding and degradation of rat chylomicron remnants, LDL and non-apolipoprotein E HDL (Tamai et al., 1983, 1988). LDL binds to specific receptors on the cells' surface with high affinity, and binding at this site can be inhibited with suramin and EDTA (Tamai et al., 1983, 1988). Here, rat hepatocytes (H-35 cells) were selected to compare LDL receptor activity with the human liver cell line, HepG2, in the presence of different dietary fatty acids and exogenous cholesterol. This will clarify whether the differences in the plasma cholesterol response to fatty acids between rats and humans is related to differences in the effects of fatty acids on the LDL receptor.

ii) Monocytic cell lines: human THP-1 cells and U937 cells

The THP-1 cells are an immortalised leukemia cell line. They have been used widely as a model for mononuclear cells to study lipid and lipoprotein metabolism (Galella et al., 1993). They express and regulate LDL receptor activity similar to HepG2 cells, but they do not synthesise lipoproteins and bile acids. THP-1 cells were primarily chosen as they represent peripheral LDL receptor metabolism. The interesting question is whether the effect of fatty acids on the LDL receptor in these cells is similar to the effect of the same fatty acids in human or rat liver cells. If the LDL receptor in THP-1 cells responds similarly to dietary fatty acids, then it may be a potential *in vivo* model system which can be used for the study of non-hepatic LDL receptor metabolism.

The human tumour cell line, U937, is derived from a lymphoma with monoblastic characteristics. The U937 cells were chosen since they can not synthesise their own cholesterol (metabolic block in the cholesterol synthetic pathway), and therefore, require cholesterol from the growth media in the form of LDL. This uptake is mediated by binding of LDL to the LDL receptor (Frostegrad et al., 1990). These cells are useful for investigating the response of LDL receptors to fatty acids since their effects on the receptor would be independent of their influence on the endogenous cholesterol biosynthetic pathway.

Monocytic cells can transform into macrophages and can migrate into the arterial wall where they have the potential to differentiate into the atherogenic foam cells which are responsible for the formation of arterial plaque. The metamorphosis of these monocytic cells

into macrophages is associated with their expression of the scavenger receptor. As the expression of the scavenger receptor in these cells is inversely proportional to the expression of the LDL receptor, then increased expression of the LDL receptor delays transformation of the monocytes (Hara et al., 1987). Understanding the regulation of LDL receptor by fatty acids in monocytic cells is relevant to foam cell formation in arterial wall.

Objective

The work discussed in this chapter describes the establishment of 4 immortalised cell line cultures (HepG2, H-35, U937, THP-1cells), and effects of different dietary fatty acids on LDL receptor activity of these cell lines, in the presence or absence of cholesterol. Palmitic, oleic, linoleic and eicosapentaenoic acid were the fatty acids chosen to compare the differences between the four fatty acid groups (saturated, monounsaturated, ω-6 polyunsaturated and ω-3 polyunsaturated fatty acids, respectively).

One can hypothesise based on previous experiments by Wong and Nestel (1987), that linoleic acid and EPA would suppress LDL receptor activity in human liver HepG2 cells with EPA having a greater effect. Likewise, it would be expected that rat liver H-35 cells would respond in the same manner to these fatty acids, as suggested by the feeding studies in rats (Roach et al., 1987).

In contrast, based on the observations of Kuo et al. (1990) in U937 cells, one would expect to observe an increase in LDL receptor activity with the PUFAs in this cell line. Hence, PUFAs would probably have the same effect in human THP-1 cells as in human U937 cells on the basis that monocytic cells from the same species (human) would respond to PUFAs in a like manner.

Using identical experimental conditions, a comparative analysis was made in these cells to test these hypotheses, and thereby determine whether LDL receptor activity in different cell types is affected by the various fatty acids, and furthermore, whether the response was different in the peripheral cell lines versus hepatic cell lines, and similar in the rat hepatic cell line versus the human hepatic cell line.

4.2 METHODS

Cell cultures were established as described in section 2.2. Once cell lines were established, the next task was to incorporate the specific fatty acids into the cells and determine the extent of this incorporation. The LDL receptor activity assay, using the colloidal gold method had to be standardised in all these cells, as this procedure had not been used previously in these cell lines. One way to determine whether these cells were displaying normal LDL receptor activity (ie: downregulation of LDL receptor activity in the presence of cholesterol section 1.4.3.2.1), was to incubate cells in either media that contained cholesterol (DMEM containing 10% FCS) or in media that was deficient in cholesterol (DMEM containing 10% LPDS). Once suitable conditions were determined and experiments were standardised, cells were incubated with either no fatty acid, palmitic, oleic, linoleic acid or EPA, either in the presence or absence of cholesterol containing media. The effects of various fatty acids on LDL receptor activity in liver cells and monocytic cells were then examined. A comparative analysis of LDL receptor binding activity was made.

4.2.1 LDL Receptor Activity with Increasing Cell Protein.

To investigate the effect of increasing protein concentration on LDL receptor activity, HepG2, H-35, THP-1 and U937 cells were seeded at 2 x 10^6 cells in 175 cm^3 flasks in DMEM containing 10% FCS. After 2 days, the media was replaced with DMEM containing 5% LPDS, and the cells were then incubated in this media for 2 days. Cells were harvested as described in section 2.2.4, resuspended in 500 µl of PBS and total protein was analysed by the method of Lowry et al. (1951). The LDL-gold receptor binding assay was performed (as described in section 2.6.3) with an increasing amount of cell protein up to 200 µg.

4.2.2 Downregulation of the LDL receptor with increasing LDL.

Downregulation of the LDL receptor was achieved by increasing the concentration of LDL cholesterol in the incubation media. The LDL was prepared from human plasma according to the method described in section 2.6.1. HepG2 and H-35 cells usually attach to the flask surface, and therefore, were grown in 175 cm^3 flasks containing DMEM supplemented with 10% FCS until they were confluent. They were then subcultured at 5 x 10^5 cells in eight small 25 cm^3 flasks containing the same media.

U937 cells and THP-1 cells do not attach to the surface of the flasks. These cells were also subcultured at 5 x 10^5 cells (from large stock flasks) in eight small 25 cm^3 flasks containing DMEM supplemented with 10% FCS. After 48 h, the media of 5 flasks was replaced with 10ml of DMEM containing 5% LPDS (see section 2.2.1), and cells were left in this media for another 48 h. The media was then replaced by fresh DMEM containing 5% LPDS and increasing concentrations of LDL-protein ranging from 0 to 200 µg/ml. After 48

h, cells were harvested and LDL receptor activity was measured according to the LDL-gold method described in section 2.6.3.

4.2.3 Fatty Acid Enrichment of Cells.

Fatty acid enrichment of cells was accomplished using the method described in section 2.2.3 and 2.2.4 with a few modifications. HepG2 and H-35 cells were grown in 175 cm^3 flasks containing DMEM supplemented with 10% FCS until they were confluent. The cells were then subcultured at 5 x 10^5 cells in ten small 25 cm^3 flasks containing the same media. U937 cells and THP-1 cells were also subcultured at 5 x 10^5 cells in ten small 25 cm^3 flasks (from large stock flasks) containing DMEM supplemented with 10% FCS. After 48 h, the media of 5 flasks was replaced with 10 ml of DMEM containing 10% FCS. The other 5 flasks received 10 ml of DMEM containing 5% LPDS (see section 2.2.1). Following a 48 h incubation in these 2 media, new media (either LPDS or FCS media depending on the previous incubation) containing 500 µM of the different fatty acids of interest was added to the cells. Fatty acids were prepared as described in section 2.2.3. After another 48 h incubation in the fatty acid enriched media, cells were harvested (section 2.2.4) and LDL receptor activity was assayed using the LDL-gold method described in section 2.6.3.

4.2.4 Statistical Analysis.

Analysis of variance (ANOVA) was conducted using Genstat program (Numerical Algorithms Group, Oford, UK) describing LDL receptor activity in HepG2, H-35, THP-1 and U937 cells, in the presence of different fatty acids ± cholesterol. Experiments were analysed as 5 x 2 factorial experiments in a randomised complete block design. For each experiment, 10 flasks of cells were used corresponding to 5 levels of fatty acids and 2 levels of media (± cholesterol treatment). Data are shown as the mean ± SEM. The pooled variance was used to determine the least significant difference when comparing individual groups. Significance was defined at $P< 0.01$.

4.3 RESULTS

4.3.1 LDL Receptor Activity with Increasing Cell Protein Concentration.

The LDL receptor activity was measured in all cell lines with increasing concentrations of cell protein to maximise binding. In all cells, LDL receptor activity increased with increasing amounts of cell protein (Figures 4.1, 4.2, 4.3, 4.4). LDL receptor activity reached a plateau around 50-100 μg of cell protein from H-35, THP-1 and U937 cells. This presumably occurs because the LDL-gold becomes a limiting factor with more cells (Figures 4.2, 4.3, 4.4). LDL receptor activity in HepG2 cells also increased with higher amounts of cell protein. However, LDL receptor activity did not reach a maximum until 100-150 μg of cell protein (Figure 4.1). In all subsequent measurements of LDL receptor activity, 100 μg of cell protein was used per assay.

Figure 4.1: Human HepG2 liver cells.

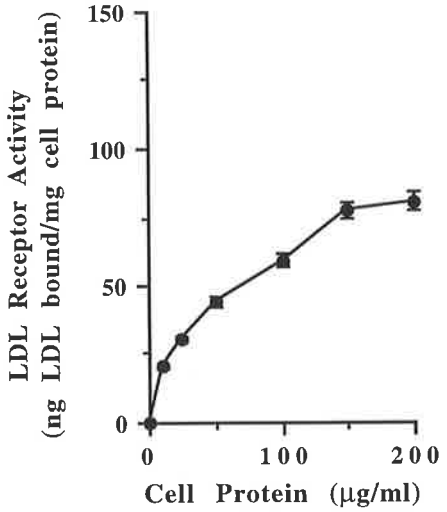


Figure 4.2: Rat H-35 liver cells.

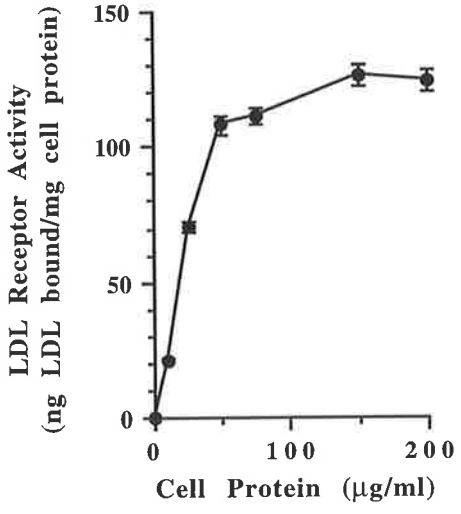


Figure 4.3: Human monocytic THP-1 cells.

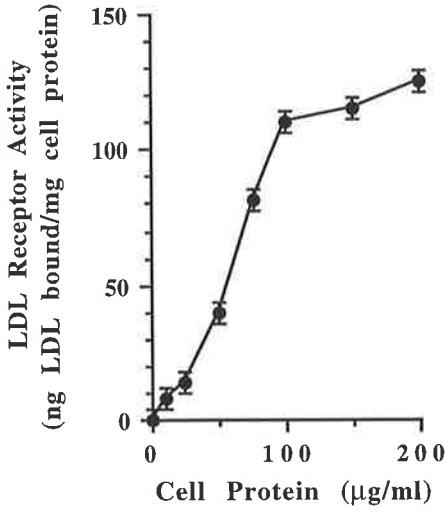
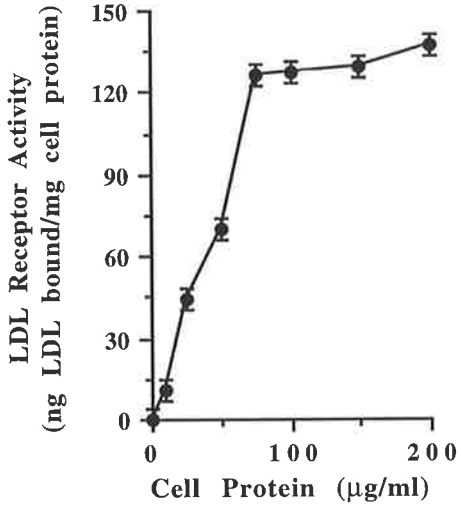


Figure 4.4: Human monocytic U937 cells.



Figures 4.1-4.4: LDL receptor activity in cells with increasing concentrations of cellular protein. LDL receptor activity was measured using LDL-gold conjugates (20 µg protein/ml) and various amounts of cell protein. Cells were incubated for 24 h with increasing concentrations of human LDL, in a background medium of 10% LPDS. Cells were harvested as described in section 4.2.1. Gold conjugates were incubated with 100 µg of cellular protein for 1 h at room temperature, either in the presence of $Ca(NO_3)_2$ to measure total binding or in the presence of EDTA to measure nonspecific binding. Specific binding = (total binding - nonspecific binding). Experiments were performed in triplicate and the results represent the average specific LDL receptor activity (ng LDL bound/mg cell protein). The data is expressed as the mean ± SEM of 3 experiments.

4.3.2 Downregulation of the LDL receptor with increasing concentrations of LDL.

Liver and monocytic cells were incubated with increasing concentrations of human LDL for 48 h. Subsequently, cells were harvested and LDL receptor activity measured using the LDL receptor binding assay. The LDL receptor activity was found to be downregulated in all 4 cell lines with increasing concentrations of human LDL in the incubation media.

The LDL receptor binding activity rapidly declined in HepG2 cells in response to cholesterol (in the form of human LDL) in the media (Figure 4.5). The LDL receptor activity decreased 70% with 100 μg protein/ml LDL (~200 μg cholesterol/ml). Downregulation of LDL receptor in rat liver H-35 cells was also achieved with increasing amounts of LDL protein and was suppressed by 60% in these cells in the presence of 100 μg protein/ml LDL (Figure 4.6).

Unlike in the liver cell lines, LDL receptor activity in the monocytic THP-1 cells only decreased gradually with increasing LDL-protein concentration in the media (Figure 4.7). LDL receptor activity was decreased only 20 % with 100 μg protein/ml LDL and 45 % with 200 μg protein/ml LDL. THP-1 cells appeared to be the most resistant to downregulation by human LDL. On the other hand, the monocytic cell line, U937, showed a rapid decline of 50 % in LDL receptor activity with only 25 μg protein/ml LDL in the media. However, with higher concentrations of human LDL, LDL receptor activity did not decline any further.

Regardless of the distinct manner in which these cells respond to LDL cholesterol, these findings demonstrate that all 4 cell lines behave in a classical "Brown and Goldstein" manner in which LDL receptor activity is suppressed in the presence of increasing concentrations of LDL cholesterol.

Figure 4.5: Human HepG2 liver cells.

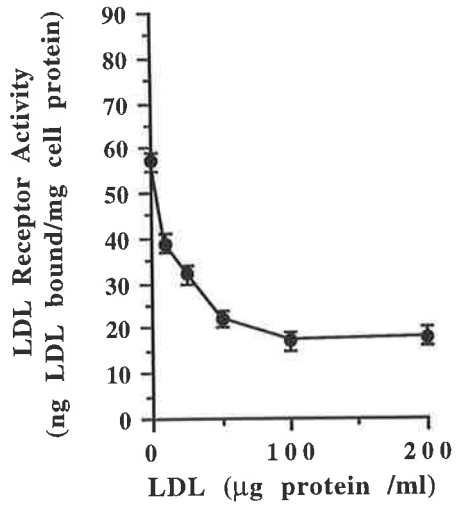


Figure 4.6: Rat H-35 liver cells.

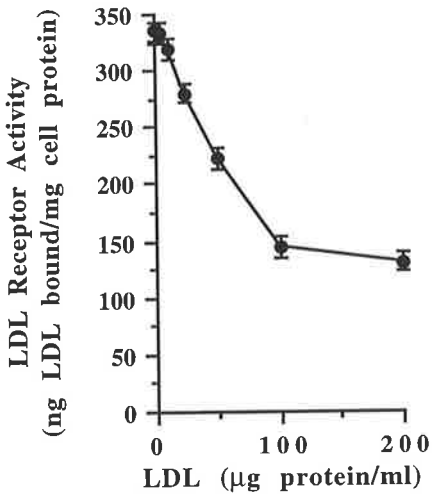


Figure 4.7: Human monocytic THP-1 cells.

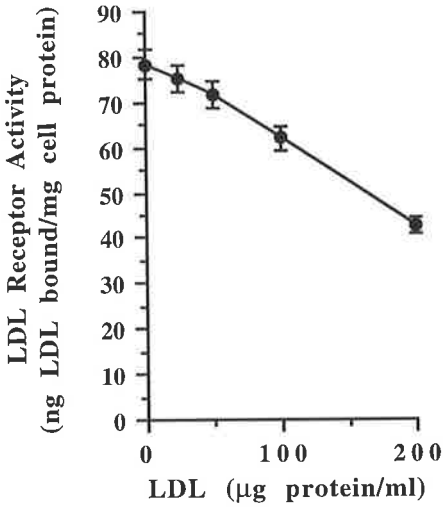
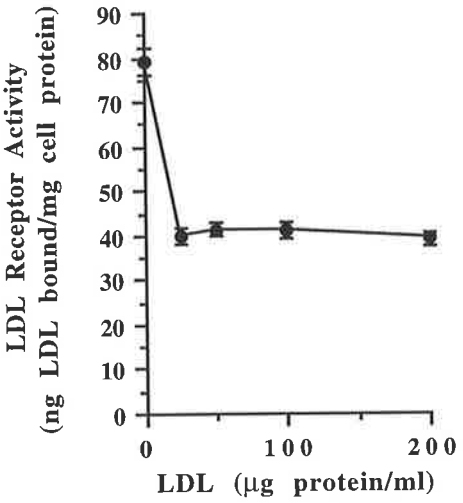


Figure 4.8: Human monocytic U937 cells.



Figures 4.5-4.8 : LDL receptor activity in cells with increasing concentrations of LDL. Cells were incubated for 24 h with increasing concentrations of human LDL, in a background medium of 10% LPDS (-cholesterol). LDL receptor binding activity was measured in triplicate using the colloidal gold-LDL method (section 4.2.2). Gold conjugates (20 µg/ml) were incubated with 100 µg of cellular protein for 1 h at room temperature, either in the presence of $Ca(NO_3)_2$ to measure total binding or in the presence of EDTA to measure nonspecific binding (section 4.2.2). Specific binding = (total binding - nonspecific binding). Experiments were performed in triplicate and the results represent the average specific LDL receptor activity (ng LDL bound/mg cell protein) ± SEM from 3 experiments.

4.3.3 Fatty Acid Enrichment of Cells.

HepG2

The effect of various fatty acids on LDL receptor activity was measured either in the presence or absence of cholesterol in the media. In the absence of fatty acids, the LDL receptor was significantly downregulated by 65% in cells incubated with DMEM containing 10% FCS for 48 h compared to cells incubated for 48 h with DMEM containing 5% LPDS (Figure 4.9). In this case, LDL receptor activity was inhibited by cholesterol present in the FCS. This indicated that cells were responding to cholesterol in the expected manner.

LDL receptor activity was unaffected by the presence of palmitic or oleic acid compared to the control cells incubated with DMEM supplemented with 5% LPDS without fatty acids. In LPDS media, however, LDL receptor activity was significantly decreased by 21% in cells incubated with linoleic acid and EPA, compared to cells incubated with no fatty acids, palmitic or oleic acid (Figure 4.9). In this case, LDL receptor activity in HepG2 cells appeared only to be sensitive to polyunsaturated fatty acids (Figure 4.9).

In contrast, the LDL receptor activity was significantly upregulated in cells when palmitic, oleic or linoleic acid were added to cells in DMEM containing cholesterol (in the form of 10% FCS) compared to control cells incubated with same background media without fatty acids. In essence, palmitic, oleic and linoleic acid were able to mask the suppression of LDL receptor activity by cholesterol (FCS, no fatty acid control). However, compared to the control cells supplemented with DMEM containing 10% FCS, cellular LDL receptor activity was unaffected by the addition of EPA into DMEM containing cholesterol. Thus, EPA could not override the suppression of LDL receptor activity by cholesterol. This dominant effect of cholesterol on LDL receptor activity was only observed with EPA, whereas, the other fatty acids exerted their own effects on LDL receptor activity independent of the suppressive effects of cholesterol.

Analysis of variance showed that there was a significant difference in LDL receptor activity in cells incubated with palmitic, oleic, linoleic acid and EPA. Compared to palmitic and oleic acid treatments, LDL receptor activity in cells was significantly lower in cells incubated with linoleic acid and EPA. Moreover, LDL receptor activity was significantly lower in cells incubated with EPA than cells incubated with linoleic acid (in a background media containing 10% FCS). Essentially, cellular LDL receptor activity was lower in the presence of polyunsaturated fatty acids compared to saturated or monounsaturated fatty acids.

On the other hand, the interaction between fatty acids and cholesterol was not consistent and was dependent on the type of fatty acid. Cellular LDL receptor activity in the presence of palmitic acid was unaffected by the absence or presence of cholesterol in the

cellular growth media. Similarly, LDL receptor activity in cells incubated with oleic acid and linoleic acid was not affected by the absence or presence of cholesterol. In contrast, LDL receptor activity in HepG2 cells was significantly suppressed by EPA when it was added to DMEM containing cholesterol (10% FCS) compared to when it was added to cells incubated with DMEM without cholesterol (5% LPDS) (Figure 4.9). In this case, the effect of EPA was modified by the presence of cholesterol in the background media due to an interaction effect between fatty acid and cholesterol.

H-35 Rat Liver cells

When there were no fatty acids in the media, there was a small but significant downregulation of 18% in LDL receptor activity in H-35 cells incubated in DMEM supplemented with 10% FCS compared to cells incubated in media supplemented with 5% LPDS (Figure 4.10).

In the LPDS (cholesterol free) background media, palmitic and oleic acid had no significant effect on LDL receptor activity compared to the no fatty acid control. However, when cells were incubated with linoleic acid or with EPA, LDL receptor activity was significantly decreased by 50% and 60% respectively, compared to cells incubated with DMEM containing no fatty acids, palmitic or oleic acid (Figure 4.10). In addition, LDL receptor activity in cells incubated with EPA was also significantly lower than in cells incubated with linoleic acid (Figure 4.10).

When palmitic acid was added to cells incubated in DMEM supplemented with 10% FCS, LDL receptor activity was unaffected compared to the control cells without fatty acids. In contrast, however, oleic, linoleic acid and EPA all downregulated LDL receptor activity (present in the FCS media) and their effects were greater than that of cholesterol in the absence of fatty acids (Figure 4.10). Cellular LDL receptor activity was significantly decreased by approximately 35%, 42% and 60% by oleic acid, linoleic acid and EPA respectively, compared to cells incubated in DMEM supplemented with 10% FCS without fatty acids (Figure 4.10). LDL receptor activity was also observed to decrease in cells incubated with linoleic acid than cells incubated with oleic acid (Figure 4.10). Moreover, cells incubated with EPA had a significantly lower LDL receptor activity than those cells incubated with linoleic acid (Figure 4.10).

Analysis of variance revealed that LDL receptor activity in H-35 cells was dependent on the type of fatty acid treatment (significant at the 1% level). LDL receptor activity in rat H-35 cells was lower when cells were incubated with polyunsaturated fatty acids, linoleic acid and EPA compared to cells incubated with saturated or monounsaturated fatty acids, palmitic acid and oleic acid. This occurred irrespective of whether or not cholesterol was supplemented in the growth media. Furthermore, LDL receptor activity in cells incubated with EPA was significantly lower compared to cells incubated with linoleic acid, regardless of cholesterol treatment.

Interestingly, analysis of variance also revealed that the cholesterol treatment did influence the effect of fatty acid on LDL receptor activity, but this was restricted to only one out of the four fatty acids examined. LDL receptor activity was unaffected by the absence or the presence of cholesterol when cells were incubated with palmitic acid. Similar results were found when cells were incubated with linoleic acid and EPA ± cholesterol. However, LDL receptor activity was higher in cells incubated without cholesterol and oleic acid than cells incubated with cholesterol and oleic acid. Even though oleic acid on its own has an independent effect on LDL receptor activity, the extent to which oleic acid reduces LDL receptor activity in H-35 cells is somewhat dependent on the amount of cholesterol in the growth media (Figure 4.10).

THP-1 Human Monocytic cells

The LDL receptor activity was significantly decreased by 60% in THP-1 cells incubated in cholesterol containing media (DMEM supplemented with 10% FCS) compared with cells incubated in cholesterol-free media (5% LPDS, no fatty acids) (Figure 4.11).

Compared to control cells incubated in DMEM and 5% LPDS without fatty acids, LDL receptor activity in THP-1 cells appeared to be sensitive to all of the test fatty acids (Figure 4.11). LDL receptor activity in cells incubated in DMEM containing 5% LPDS was suppressed significantly by approximately 60% in the presence of either palmitic, oleic, linoleic acid or EPA compared to control cells incubated without fatty acids (Figure 4.11).

In contrast, when cells were incubated in DMEM containing cholesterol (10% FCS), there was no significant difference ($p < 0.01$) in LDL receptor activity between any of the cells incubated with the test fatty acids and control cells incubated without fatty acids (Figure 4.11). In this case, it is difficult to distinguish whether the fatty acid effect was independent of cholesterol or whether cholesterol was masking the fatty acid effect.

There was an indiscriminate suppression of the LDL receptor activity in cells by palmitic, oleic, linoleic and EPA, regardless of whether or not the growth media contained cholesterol. Analysis of variance indicated that there was no significant difference in LDL receptor activity between cells treated with palmitic, oleic, linoleic acid and EPA in the absence or presence of cholesterol in the growth media. In addition, there was no significant interaction effect of fatty acids and cholesterol on LDL receptor activity. The effect of the fatty acids in monocytic THP-1 cells was quite different to those observed in the hepatic cell lines (HepG2 and H-35 cells) and appears to be a real effect as the results were confirmed by repeating the experiment 15 times.

4.3.2 U937 Human Monocytic cells

The LDL receptor activity decreased significantly by 20% when U937 cells were incubated with cholesterol (DMEM supplemented with 10% FCS) compared to cells in a cholesterol free media (DMEM supplemented with 5% LPDS).

Compared to control cells grown in DMEM in the absence of cholesterol (5% LPDS) and no fatty acids, LDL receptor activity was not significantly affected by the addition of palmitic, oleic or linoleic acid to the growth media. However, LDL receptor activity was significantly downregulated by 50% when EPA was added to the culture media compared to control cells without fatty acids or compared to cells incubated with palmitic, oleic and linoleic acid (Figure 4.12).

When the DMEM growth media contained 10% FCS, LDL receptor activity was not affected by the addition of palmitic, oleic or linoleic acid compared to the control cells incubated without fatty acids. However, cellular LDL receptor activity was significantly downregulated in the presence of EPA compared to control cells without fatty acids (DMEM supplemented with 10% FCS) or compared to cells incubated with palmitic, oleic and linoleic acid in growth media containing 10% FCS (Figure 4.12).

Analysis of variance conducted on the effects of the fatty acid treatments on LDL receptor activity with the 2 levels of media (± cholesterol) showed that LDL receptor activity was influenced by the various fatty acid treatments, but there was no interactions between these individual fatty acids and cholesterol. Results showed that LDL receptor activity in U937 cells was only affected by the EPA treatment but not palmitic, oleic or linoleic acid treatments, irrespective of whether or not the background media contained cholesterol.

Figure 4.9: Human HepG2 liver cells.

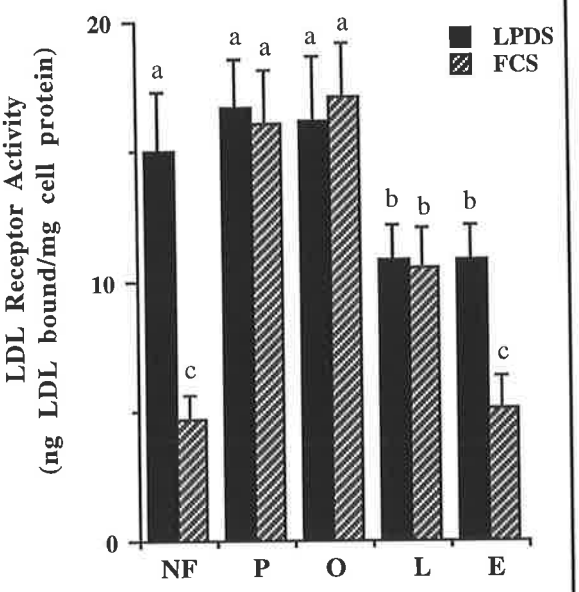


Figure 4.10: Rat H-35 liver cells.

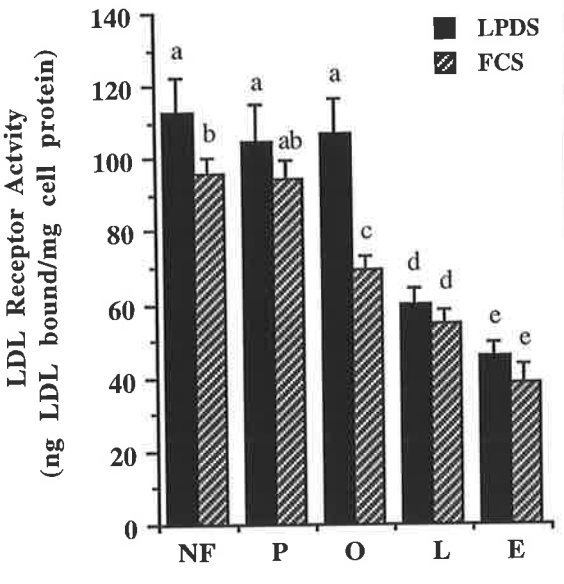


Figure 4.11: Human monocytic THP-1 cells.

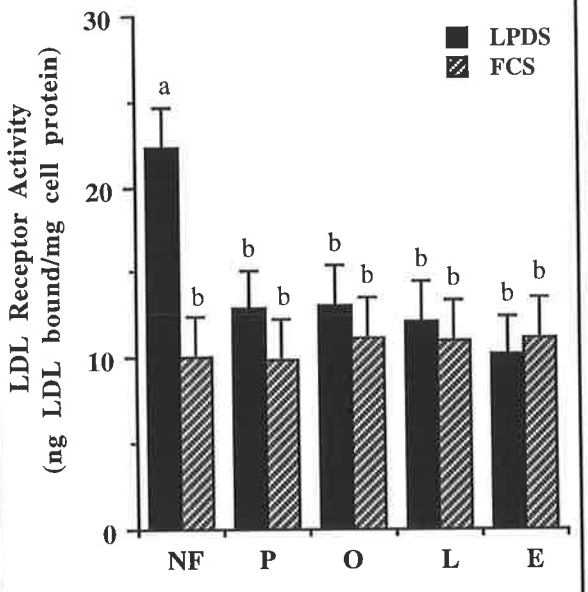
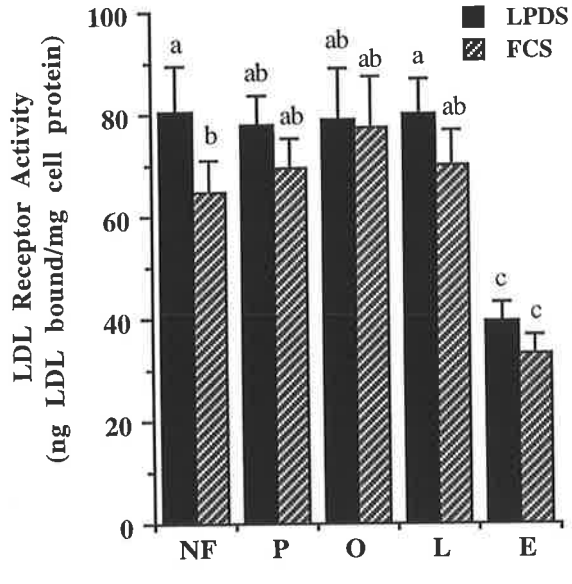


Figure 4.12: Human monocytic U937 cells.



Figures 4.9-12 : Fatty acid effects on LDL receptor activity in cells, -cholesterol (LPDS=lipoprotein deficient serum) or +cholesterol (FCS=fetal calf serum). Cells were preincubated for 48 h in media containing LPDS or FCS and then incubated for another 48 h in either LPDS or FCS media containing either no fatty acid (NF) or 500 μM palmitic acid = P (C16:0), oleic acid = O (C18:1), linoleic acid = L (C18:2), eicosapentaenoic acid = E (C20:5). LDL receptor activity was measured in triplicate using the colloidal-LDL gold method (section 4.2.3). Results represent specific LDL cell binding activity (ng of LDL/mg cell protein) and data is the mean LDL receptor activity (± SEM) of 4 experiments for HepG2, H-35, U937 cells and 15 experiments for THP-1 cells. Means with the same letters above them are not significantly different at $P < 0.01$.

4.4 DISCUSSION

LDL receptor activity measurements in various cells incubated with increasing amounts of cell protein.

Preliminary experiments involved the optimisation of the assay conditions for measuring cellular LDL receptor binding activity. The LDL receptor binding activity was measured in each cell line with increasing amounts of cell protein in order to determine a suitable cell concentration for subsequent experiments. The LDL receptor activity increased similarly in all four cell lines with increasing amounts of cell protein, and reached a plateau with 50-100 μg cell protein of H-35, THP-1 and U937 cells. HepG2 cells, on the other hand, reached maximum LDL receptor activity with 100-150 μg of cell protein. A plateau was presumably reached because the LDL-gold became a limiting factor (Figure 4.1, 4.2, 4.3, and 4.4).

Downregulation of LDL receptor activity with increasing LDL concentrations.

The metabolic downregulation of the LDL receptor in all four cell lines was achieved with increasing concentrations of human LDL, although there appeared to be a difference in the degree of inhibition between the various cell types (Figures 4.5, 4.6, 4.7, and 4.8).

The LDL receptor activity in the two liver cell lines, HepG2 and H-35 cells, responded similarly to increasing concentrations of human LDL, with a maximum inhibition of receptor activity of 60-70% with 100 μg LDL protein/ml (Figure 4.5 and 4.6). LDL receptor activity could not be completely inhibited in these two liver cell lines probably because they require a large amount of cholesterol for bile acid and lipoprotein synthesis. LDL receptor activity in rat liver cells was observed to be slightly more resistant to LDL cholesterol downregulation compared to human HepG2 liver cells (Figure 4.6). One can speculate that rat liver cells may require more cholesterol for its metabolic needs, or that the pathway involved in the regulation of LDL receptor activity is not as sensitive to cholesterol compared to that in humans. Roach et al. (1993) have observed that rats fed dietary cholesterol were extremely resistant to hypercholesterolemia compared to rabbits, and attributed this observation to the upregulation of the LDL receptor. It was interesting to note that the rat H-35 cells also had a much higher LDL receptor activity (Figure 4.6) compared to the human HepG2 cells (Figure 4.5). Thus, rats may withstand the rise in plasma cholesterol upon cholesterol feeding not just by having higher receptor activity, but also because their LDL receptors may be more resistant to downregulation by LDL (Figure 4.6).

The downregulation of the LDL receptor was most rapid in U937 cells, compared to the other cells lines, with maximum inhibition of 50% occurring at 25 μg LDL protein/ml (Figure 4.8). These cells are totally dependent on extracellular cholesterol for survival, and they would be expected to be the most sensitive to LDL cholesterol in the media. Unlike

hepatic cells, but similar to other peripheral cells, U937 cells are not capable of synthesising lipoproteins. Thus, surplus cholesterol can not be excreted through the packaging of lipoproteins. The U937 cells may, therefore, be able to respond quickly to intracellular cholesterol concentrations and rapidly shut down the activity of LDL receptor to accumulation of excess cholesterol (Figure 4.7).

LDL receptor activity was inhibited to a greater extent in the liver cell lines, with 70% suppression in HepG2 cells and 60 % in H-35 cells in the presence of 100 μg protein /ml of human LDL. By comparison, LDL receptor activity in the monocytic cell lines (THP-1 and U937) could only be inhibited by 20% and 45%, respectively. This would imply that hepatic cells are more sensitive to the influx of LDL cholesterol, and subsequently respond in this manner to try to maintain cellular cholesterol balance.

It is interesting to note that effective downregulation of LDL receptor activity was observed in HepG2 and THP-1 cells incubated with media supplemented with 10% FCS (Figures 4.9 and 4.11), whereas in H-35 cells and U937 cells, downregulation of the LDL receptor was far greater with human LDL (Figure 4.6 and Figure 4.8) than with 10% FCS (Figures 4.10 and 4.12). This may be attributable to the fact that the concentration of cholesterol in FCS is low, and probably explains why LDL receptor activity was not significantly downregulated with 10% FCS when used as a cholesterol source in the fatty acid experiments. Media supplemented with 10% FCS only contained 4.5 μg/ml of cholesterol, which is equivalent to 2.25 μg/ml protein of human LDL. Extrapolating from the graphs in Figures 4.5, 4.6, 4.7, and 4.8, the LDL receptor was not downregulated to any significant degree at this concentration of human LDL.

In the case of HepG2 cells and THP-1 cells, LDL receptor regulation may be sensitive to an active constituent present in FCS and, thus the LDL receptor is downregulated when incubated in media supplemented with FCS. The inability of FCS-cholesterol to efficiently downregulate LDL receptor in H-35 and U937 cell lines may be further compounded by the very low concentration of the LDL in FCS and, most importantly by the fact that nearly all of the cholesterol is carried in the HDL fraction of FCS. Thus, the cholesterol contained in FCS may not be as readily accessible *via* the LDL receptor to H-35 cells and U937 cells. This may explain why a far greater suppression of the LDL receptor was accomplished in these two cell lines when human LDL was used as a cholesterol source as opposed to FCS.

The effect of fatty acids on LDL receptor activity

The most interesting findings pertain to the effects of the fatty acids on LDL receptor activity of the four cell lines. An observation consistent in all four cell lines was the effect of EPA on LDL receptor activity when the background growth media was cholesterol-free. Cellular LDL receptor activity was significantly suppressed when cells were incubated with EPA compared to cells incubated in the absence of fatty acid and cholesterol (5% LPDS, no

fatty acid control). This effect may be related to the distinct characteristics of this dietary fatty acid.

Analysis of variance revealed that the fatty acid and cholesterol effects were not consistent between all the cell lines, but were dependent on the type of cell line. With the exception of HepG2 cells incubated with EPA and H-35 cells incubated with oleic acid, cholesterol and fatty acid interactions were not observed in the four cell lines. In general, the fatty acid effect on LDL receptor activity was independent of the suppressive actions of cholesterol. In both liver cell lines, LDL receptor activity was significantly decreased by incubation of cells with linoleic and EPA compared to cells incubated with palmitic or oleic acid. In addition, HepG2, H-35 and U937 cell lines incubated with EPA had significantly lower LDL receptor activity than cells incubated with linoleic acid (in background media supplemented with 10% FCS). Also interesting to note, there appeared to be no significant difference in LDL receptor activity in the four cell lines incubated with either palmitic or oleic acid in cholesterol free media.

i) Liver Cell Lines: Human HepG2 and Rat H-35 Cells

The two liver cell lines of human and rat origin responded similarly in the presence of the various fatty acids as predicted, and thus, supported the hypothesis that PUFAs effect LDL receptor activity in the two different hepatic cell lines in a like manner (Figure 4.9 and 4.10). In both cell lines, HepG2 cells and H-35 cells, linoleic acid and EPA decreased LDL receptor activity relative to their saturated (palmitic acid) and monounsaturated (oleic acid) counterparts. The decrease in LDL receptor activity by these two PUFAs compared to cells incubated with palmitic or oleic acid is consistent with the observations of Wong and Nestel (1987) in HepG2 cells, Roach et al. (1987) in rat liver cells and Rumsey et al. (1995) in J774 macrophages. Additionally, in both cell lines LDL receptor activity was further suppressed when cells were incubated with EPA compared to cells incubated with linoleic acid. This finding is supported by the results of Rumsey et al. (1995).

These results suggest that a relationship exists between the fatty acid chain length, double bond number and LDL receptor activity. It appears, based on the observations in the 2 liver cell lines, that as the polyunsaturation and chain length of the fatty acid increases, the LDL receptor activity decreases. The relationship between fatty acid chain length, saturation and LDL receptor activity is still observed in the presence of cholesterol (Figure 4.9 and 4.10). LDL receptor activity in both HepG2 and H-35 cell lines was significantly decreased when cells were incubated with linoleic acid and EPA compared to cells incubated with palmitic and oleic acid, even when cells were grown in media containing FCS.

It is interesting to note that the independent effect of fatty acids on LDL receptor activity in the two liver cell lines did not apply to HepG2 cells incubated with EPA and H-35 cells incubated with oleic acid in the presence of cholesterol. In both of these cases, it appears that the presence of cholesterol had a significant effect on LDL receptor activity.

LDL receptor activity was further suppressed in these two liver cell lines when they were incubated with the fatty acids plus cholesterol compared to the fatty acids alone (Figure 4.9 and Figure 4.10). As shown by these results, the effect of the fatty acids on LDL receptor activity can be dependent on external variables, such as cholesterol. However, this external influence of cholesterol also appears to be species specific.

It may be inappropriate to compare the fatty acid effects in the two liver cell lines to the control cells because the presence of cholesterol (DMEM containing 10% FCS) did not downregulate HepG2 cells and H-35 cells to the same extent (Figure 4.9 and 4.10). However, an interesting observation emerges if one does undertake this comparison. In both of these liver cell lines, the fatty acids appeared to exert an independent effect on LDL receptor activity, in that they overrode the effect of cholesterol in an opposing manner. For example, in HepG2 cells, palmitic, oleic and linoleic acid were able to "knockout" or mask the suppression of cholesterol (10% FCS, no fatty acid control) by augmenting LDL receptor activity from this suppressed level. The cholesterol effect, however, was dominant in cells incubated with EPA (Figure 4.9). In contrast, compared to control cells incubated with DMEM containing 10% FCS without fatty acids, oleic, linoleic acid and EPA had a greater effect on LDL receptor activity than cholesterol in H-35 cells, by further depressing LDL receptor activity (Figure 4.10). In summary, these results indicate that fatty acids (except for EPA in HepG2 cells) are exerting their own distinct effects in both cell types, irrespective of the presence of cholesterol. Most importantly, these results also demonstrate that at the cellular level, the rat cells are very similar to the human cells in the way they respond to fatty acids.

The intracellular cholesterol content has been shown to be a major factor in regulating the LDL receptor (Brown and Goldstein, 1986). The mechanisms by which the fatty acids may be affecting LDL receptor activity of these liver cell lines could be related to the their effects on intracellular cholesterol levels (discussed further in Chapters 5 and 9). For example, the perturbation of fatty acids on the VLDL secretion in liver cells may affect the cellular cholesterol content, indirectly leading to the regulation of LDL receptor (Wong and Nestel, 1987;Cortese et al., 1983; Wang et al., 1983). Alternatively, modulation of LDL receptor activity by the various fatty acids may also be linked to the affect of the fatty acids on cholesterol biosynthesis, esterification and/or secretion (Schaefer and Kattermann, 1992; Pal and Davis, 1991; Rustan et al., 1988; Murthy et al., 1988).

Although human HepG2 and rats H-35 liver cells responded similarly to fatty acids at the cellular level, whole animal studies in humans and rats show that they differ markedly in the way they respond to fatty acids (Harris, 1989; Land, 1986; Nestel, 1990; Roach et al., 1987; Spady et al., 1993). LDL cholesterol levels are usually elevated in humans in response to dietary saturated fatty acids and ω-3 PUFAs, whereas polyunsaturated fatty acids of the ω-6 series usually lower plasma LDL cholesterol (Keys et al., 1957; Hegsted et al., 1965; Nestel, 1990; Harris, 1989). In contrast, ω-6 PUFAs have very little effect on

plasma lipid levels in rats, and ω-3 PUFAs generally lower total plasma cholesterol and triacylglycerol concentrations (Roach et al., 1987; Harris, 1996; Spady et al, 1993). The variations in response to fatty acids between rats and humans *in vivo* may attributable to the differences in lipoprotein metabolism between these two species. For example, LDL cholesterol is not generally measured in animal studies (such as rats, mice, rabbits, hamsters) because HDL is the major carrier of cholesterol (Surrette et al., 1992; Roach et al., 1987; Riener et al., 1993; Rich et al., 1989).

At the cellular level, one would expect most species to respond in a similar manner to fatty acids due to gene conservation seen across species. The results herein support that this may the case as rat liver cells and human liver cells responded similarly to the various fatty acids at the level of LDL receptor activity (Figure 4.9 and 4.10). These findings are also consistent with the study by Roach et al. (1987), in which LDL receptor activity was depressed in rat liver cells isolated from rats supplemented with safflower oil and marine fish oils compared to those on chow diets. It is interesting to note that the findings *in vivo* suggest that rats are not even receptive to most dietary fatty acids (as lipid levels are generally unaffected). Since the sensitivity of LDL receptor activity to fatty acids in rats H-35 cells (Figure 4.10) are observed *in vitro*, it would suggest that at the cellular level rats do respond to dietary fatty acids.

In humans, a rise in plasma LDL cholesterol is associated with saturated fatty acids Grundy, 1994; Denke, 1994; Katan et al., 1994). Therefore, one would predict that saturated fatty acids cause hepatic LDL receptor activity to be decreased. In contrast, the findings herein show that LDL receptor activity was considerably in higher in human liver cells enriched in palmitic acid than cells enriched in linoleic acid or EPA. Although this is not definitive for what may occur *in vivo,* it does suggest that fatty acids may be have an entirely different effect at the cellular level. The implication is that the response of hepatic LDL receptor activity to saturated fatty acid intake does not necessarily correspond to the changes observed in LDL cholesterol in humans. Thus, the findings herein actually oppose the expected results when examining plasma LDL levels in response to saturated fatty acid intake.

Epidemiological dietary studies have shown that ω-6 PUFAs, such as linoleic acid, lower serum cholesterol in humans compared to saturated fatty acids like palmitate (Keys et al., 1957; Hegsted et al., 1965; Kinsell et al., 1955; Ahrens et al., 1954). The results show that human HepG2 cells incubated with linoleic acid have a lower LDL receptor activity compared to cells incubated with palmitic and oleic acid (Figure 4.9). How can decreased LDL receptor activity in HepG2 cells with linoleic acid explain the decrease in plasma LDL levels observed when humans are supplemented with dietary ω-6 PUFA? The decrease in LDL receptor activity would presumably cause a subsequent increase in plasma LDL cholesterol levels. Therefore, the results herein do not account for the *in vivo* observations and thus, do not support the general hypothesis. It does, however, suggest that the effect of

fatty acids on lipoprotein levels *in vivo* occur by mechanisms other than their effects on LDL receptor activity. Fatty acids may have greater effects elsewhere on cholesterol metabolism or indirect effects *in vivo* on the LDL receptor (eg. via hormones or cytokines {Endres and von Schacky, 1996}). For this reason, one can assume that the decrease in LDL receptor activity observed in H-35 cells incubated with EPA compared to cells enriched with linoleic acid (Figure 4.10) does not correspond to the decline in cholesterol levels in rats fed fish oil. Thus, humans and rats are probably alike with respect to fatty acid metabolism, where a fatty acid effect at one level may be entirely independent to its effects at another level.

On the other hand, the decrease in LDL receptor activity in human HepG2 cells by EPA (Figure 4.9) can adequately explain the elevation in LDL cholesterol observed in humans. Whether the effect of EPA on LDL receptor activity in human cells is associated with the observed increase in plasma LDL levels *in vivo* is, however, difficult to evaluate. It does imply, though, that the relationship between certain fatty acids, LDL receptor activity and lipid levels may be species specific.

ii) Monocytic cell lines: human U937 and THP-1 cells

The assumption that LDL receptor activity in both monocytic cell lines would be affected similarly by the various fatty acids was not supported by the results. Although the monocytic cells lines, THP-1 and U937, are both human in origin, the LDL receptor activity in these cell lines was observed to be modulated differently by the various fatty acids tested. The major finding in both the monocytic cell lines was the observation that LDL receptor activity was significantly lower when incubated with EPA compared to cells incubated without fatty acids in a cholesterol free media (5% LPDS). Only under these conditions did the monocytic cells respond in a manner similar to that observed in the liver cells (Figure 4.9 and 4.10).

Contrary to the expected results based on the study by Kuo et al. (1990) in U937 cells, LDL receptor activity was not upregulated by linoleic acid and EPA compared to palmitic acid in either U937 or THP-1 cells. LDL receptor activity in U937 cells appeared to be unaffected by the various fatty acids either in the presence or absence of cholesterol, except when cells were incubated with EPA. In contrast, THP-1 cells were sensitive to all the fatty acids examined either in the absence or presence of cholesterol. LDL receptor activity was significantly suppressed by 54% in THP-1 cells incubated with all of the fatty acids tested (without cholesterol in the background media, 5% LPDS). This indiscriminate suppression of the receptor appears to be independent of the different characteristics of the various fatty acids.

Although the LDL receptor was suppressed with the fatty acids in the presence of cholesterol, it is extremely difficult to ascertain whether any of the suppression of the LDL receptor activity was attributable to cholesterol (10% FCS) or entirely a separate effect of the fatty acids. The exogenous cholesterol may have the potential to mask the effect of the fatty

acids, but this effect may be tissue and species specific. This is an important observation as it demonstrates the necessity of examining the fatty acids in isolation to exclude confounding extrinsic factors (such as cholesterol) which may interfere or mask the fatty acid effect. Extrinsic factors may explain why many studies using the same animal species have produced conflicting results.

The effect of fatty acids on LDL receptor in human monocytic cells suggests that the fatty acid effect on LDL receptor binding activity can be tissue or cell specific within a species. For example, the lack of any effect in LDL receptor activity by linoleic acid compared to palmitic acid in human U937 and THP-1 monocytic cells contrasts with findings in the human HepG2 liver cells (Figure 4.9). This clearly implies that the effect of fatty acids on LDL receptor activity is tissue specific. Furthermore, if the response of LDL receptor activity to external factors is tissue specific, it demonstrates the complexity that is involved in fatty acid effects, and makes comparisons between studies extremely difficult to assess if different species and tissues are used for analysis. One can speculate that the monocytic cell lines are insensitive to fatty acids (as seen in U937 cell line) or indiscriminately responsive to fatty acids (as seen in the THP-1 cell line) due to the fact that these cell lines do not synthesise or secrete VLDL. Presumably, monocytic cells will be insensitive to the perturbations of fatty acids on VLDL secretion which is observed in liver cells (Cortese et al., 1983; Wang et al., 1983). This may explain the differences seen between the liver and monocytic cell lines in LDL receptor activity when exposed to various fatty acids.

The mechanism whereby fatty acids influence the LDL receptor in THP-1, but not in U937 cells may be associated with the individual cells' ability to synthesise cholesterol. The effect of all fatty acids on LDL receptor activity may be through a common mechanism in which the fatty acids act on enzymes and/or substrates which are involved in cholesterol synthesis (Nestel, 1990). Thus, fatty acids may directly affect cellular cholesterol synthesis and thereby, alter the total cholesterol concentrations within the cells. By changing the total cholesterol concentration within the cell, fatty acids could indirectly regulate LDL receptor activity. One can take this a step further and speculate that only the "metabolically active cholesterol pool" (Grundy, 1991) can modulate LDL receptor activity. Although the active pool of cholesterol is composed of the cholesterol that is derived from endogenous synthesis and exogenous LDL, the regulatory pool of cholesterol may be more sensitive to the concentration of cholesterol arriving from endogenous synthesis. If this is the case, then the fatty acids which influence the endogenous synthesis of cholesterol will have a greater influence in regulating LDL receptor activity. If these are the mechanisms by which fatty acids modulate the LDL receptor, then LDL receptor activity will be sensitive to fatty acids in cells such as THP-1, but not in cells such as U937, which lack the cholesterol synthetic pathway.

The effect of the fatty acids on LDL receptor activity in U937 cells (and not THP-1 cells) may more closely resemble how other peripheral cells respond to dietary fatty acids. THP-1 cells appear to be different from monocytic cells in the manner in which they incorporate fatty acids into their phospholipids. Galella et al. (1993) have shown that arachidonic acid and EPA are incorporated into phospholipids in THP-1 by a separate process that is different from other monocytes. Therefore, the THP-1 cells may respond differently to the various fatty acids compared to the other cell lines due to the unique processing of the fatty acids. If a unique processing of EPA also occurs in U937 cells, it may explain why these cells were susceptible to downregulation by only this fatty acid.

The U937 and THP-1 cells represent monocytic cells which can further differentiate into macrophages and have the potential to develop into foam cells in the arterial wall. Thus, the downregulation of the LDL receptor by linoleic acid (THP-1) and EPA (THP-1 and U937) compared to saturated fatty acids (Figure 4.11 and 4.12) could protect against the accumulation of cholesterol and CHD, if the same event occurs in macrophages and smooth muscle cells of the arterial wall in the presence of these fatty acids. Thus, a downregulation of LDL receptors by these fatty acids in cells of the arterial wall would be highly beneficial in inhibiting foam cell formation, and could explain why these fatty acids have been observed to be antiatherogenic. Nevertheless, it can be argued that it is the scavenger receptors present within macrophages which allow the unregulated accumulation of cholesterol. It has been shown that the presence of scavenger receptors is negatively correlated with the presence of the LDL receptor in these cells (Hara et al., 1987). If the modulation of scavenger receptors is associated with LDL receptor activity, these findings can not explain how fatty acids such as linoleic acid and EPA protect against CHD, unless the scavenger receptor activity can also be downregulated by these fatty acids. Although the scavenger receptor does not appear to be regulated by the accumulation of cholesterol, the activity of the scavenger receptor has been shown to be subject to regulation by phorbol esters and mezerein (a nonphorbal activator of protein kinase C) (Pitas et al., 1992). However, at this point, it is not known if fatty acids can exert such effects on scavenger receptor activity in monocyte macrophages.

Summary

The results herein demonstrated that the cultured hepatoma and monocytic cells used in this study possess high affinity LDL receptors and that these receptors respond to metabolic perturbations, such as cholesterol, in a manner which parallels that previously shown in human fibroblasts (Brown and Goldstein, 1986). LDL receptor activity in all of these cells was stimulated when the cells were grown in cholesterol depleted media (LPDS), and was repressed by incubation with human LDL. However, the suppressive effects of LDL cholesterol on LDL receptor activity in H-35 cells and U937 cells was far greater than that observed when these two cell lines were incubated with media supplemented with 10% FCS.

An important finding was the observation that in both rat H-35 and human HepG2 liver cells lines, linoleic acid and EPA suppressed LDL receptor activity compared to palmitic and oleic acid. As expected, these two liver cell lines responded to PUFAs similarly and thus proved the hypothesis that PUFAs suppress LDL receptor activity in liver cells. It was concluded from the results that the fatty acid effects on cellular LDL receptor activity in humans and rats are most likely independent from their influence on lipoprotein levels. Thus, the difference in lipoprotein levels between humans and rats in response to various fatty acids may be due to differences in lipoprotein metabolism in these two species (see Chapter 9, section 9.1).

In contrast to liver cells, the fatty acids exerted a different effect on LDL receptor activity in the monocytic cell lines, THP-1 and U937 cells. These cell lines appeared either nonresponsive to most of the test fatty acids (as observed in the U937 cell line) or indiscriminately responsive to the fatty acids (as observed in the THP-1 cell line). Linoleic acid and EPA did not upregulate LDL receptor activity in these two monocytic cell lines as previously anticipated from the studies of Kuo et al. (1990). In fact, linoleic acid had no effect on LDL receptor activity compared to palmitic acid in either of these cell lines. Other than the effect of EPA, there appeared to be no similarity in the effects of the various fatty acids between human liver cells and monocytic cells. Therefore, the findings suggest that rats and humans do not differ in the way they respond to fatty acids with regards to hepatic LDL receptor activity. However, in humans, fatty acids may affect LDL receptor activity in a tissue specific manner.

One can hypothesise that the distinct fatty acid effects on LDL receptor activity in liver cells may be due to the effect of the fatty acids at the level of transcription, post-transcription or both depending on the cell type. Unravelling the mechanisms responsible for the distinct effect of different fatty acids on LDL receptor activity in each of the cell lines is an involved and complicated process. Therefore, for further investigation, only one cell line (HepG2) was chosen as a model system in order to elucidate the mode of action by which various fatty acids regulate LDL receptor activity.

Chapter 5

5.0: THE EFFECT OF DIETARY FATTY ACIDS ON LDL RECEPTOR EXPRESSION (ACTIVITY, PROTEIN AND mRNA) IN HEPG2 CELLS

5.1 Introduction

The work described in chapter 4 clearly demonstrated that ω-6 and ω-3 PUFAs suppressed hepatic LDL receptor binding activity compared to saturated and monounsaturated fatty acids in human HepG2 liver cells as well as in rat H-35 liver cells. The question which now needs to be addressed is, by what mechanism(s) do these fatty acids modulate LDL receptor activity ? It may be that ω-6 and ω-3 PUFAs exert their effects by inhibiting cellular activities, such as RNA translation, receptor recycling, receptor degradation or the interaction of the receptor with the LDL. Effects at one or all of these levels would result in one thing, that is, a suppression in LDL receptor function at the cell surface. However, these mechanisms seem highly unlikely because LDL receptor metabolism appears to be regulated at the level of gene transcription (Goldstein and Brown, 1977; Brown and Goldstein, 1986; Rudling, 1992; Sanchez et al., 1995).

ω-6 PUFAs

There has been very little research on the effects of ω-6 PUFAs at the level of LDL receptor gene transcription. Some animal studies do imply that LDL receptor mRNA levels may vary with dietary ω-6 PUFAs. Most studies quantify cellular LDL receptor mRNA levels as a measure of LDL receptor gene transcription. A report by Kushwaha et al. (1991) found that hepatic LDL receptor mRNA levels increased in baboons when they were fed dietary corn oil compared with animals fed lard. Another study by Sorci-Thomas et al. (1989), indicated that mRNA abundance for the LDL receptor did not increase in African green monkeys fed large quantities of linoleic acid. Sorci-Thomas et al. (1989), therefore, suggested that the LDL receptor activity was probably not regulated at the level of transcription. As they did not measure LDL receptor binding activity, this assumption can not be validated. In this case, fatty acid treatment may have failed to have an effect even at the LDL receptor binding level. Moreover, their assumption that LDL receptor activity is not regulated at the level of gene transcription contradicts all existing evidence on the regulation of LDL receptor (Brown and Goldstein, 1986; Rudling, 1992; Sanchez et al., 1995). The inconsistencies in results between these two studies make it difficult to decipher whether these fatty acids are acting at the level of hepatic LDL receptor gene transcription. Furthermore, it is not known whether the effect of fatty acids on LDL receptor gene transcription in these animal species parallels those in humans.

ω-3 PUFAs

Does the effect of ω-3 PUFAs on LDL receptor activity originate from its effects on LDL receptor gene transcription ? Spady et al. (1995) reported that LDL receptor protein and mRNA levels were unchanged in rats fed a diet rich in fish oil compared to animals fed corn oil or normal chow diets. Although they did not measure LDL receptor binding activity, they did observe that rats fed a diet rich in fish oil had enhanced hepatic uptake of radiolabelled LDL than animals fed corn oil or normal chow diets. In contrast, hamsters in the same study had hepatic LDL receptor mRNA levels significantly decreased with fish oil supplementation compared to animals fed corn oil. Moreover, clearance of radiolabelled LDL was significantly lower in the hamsters fed dietary fish oils compared to animals fed corn oil, implying LDL receptor activity had decreased due to fish oil feeding.

Intake of dietary PUFAs may induce the secretion of growth factors or hormones which intervene at the level of transcription or translation and illicit their own effects, thus masking the original effect of the fatty acid. For example, the synthesis and secretion of cytokines (TNF-α or IL-α) is suppressed in mononuclear cells of individuals consuming dietary ω-6 and ω-3 PUFAs (Endes and von Schacky, 1996). As TNF-α or IL-α have also been shown to affect LDL receptor activity (Hamaka et al., 1992), the final expression of the LDL receptor *in vivo* may thus be determined indirectly by extracellular factors. This may explain why there are discrepancies in the above mentioned studies, in assessing the effects of particular fatty acids on the expression of the LDL receptor gene. It is important to study the isolated and independent effects of all fatty acids at the molecular level using a single cell system in order to eliminate any confounding factors that may be present *in vivo*.

To date, there has only been one *in vitro* study which describes the effect of ω-3 PUFAs on LDL receptor mRNA levels. A study done by Linsey et al. (1992) would suggest that these fish oil fatty acids do affect LDL receptor gene transcription. They observed that the mRNA for the LDL receptor was slightly depressed in HepG2 cells which had been incubated with LDL derived from individuals after they had consumed dietary fish oils. Contradictory to other studies (Wong and Nestel, 1987; Wong et al., 1989; Wang et al., 1993; Lang and Davis, 1990), however, they also observed an increase in the level of apolipoprotein B mRNA in cells incubated with fish oil enriched-LDL (refer to section 1.8.3.5). As the HepG2 cells themselves were not enriched in these fatty acids to any great extent, it was hard to distinguish whether the fish oil effects on LDL receptor gene expression was actually due to the small alteration in cell membrane composition, a result of the increased concentration of ω-3 fatty acids in the LDL phospholipids, or whether the fatty acids themselves were having a direct effect on LDL receptor gene transcription. Hence, it is difficult to conclude from just one study whether the ω-3 fatty acids are exerting a direct effect at the level of LDL receptor gene transcription.

Aims of this Chapter:

The experiments described in this Chapter were designed to investigate the molecular mechanism whereby different PUFAs affect LDL receptor metabolism using a cell culture system. The previous Chapter described the effects of the various fatty acids on LDL receptor activity in the 4 different cell lines originating from different tissues and species. It was apparent from these results that LDL receptor activity in humans and rat liver cells respond similarly to saturated, monounsaturated and polyunsaturated fatty acids (Figure 4.9 and 4.10). To further investigate the molecular mechanism whereby fatty acids regulate hepatic LDL receptor binding activity in humans, it was decided that HepG2 cells would be used as the model system.

Based on the observations of Linsey et al. (1992) and previous work on LDL receptor gene regulation (Goldstein and Brown, 1977; Brown and Goldstein, 1986; Rudling, 1992; Sanchez et al., 1995), one can hypothesise that the changes induced by PUFAs on LDL receptor binding activity in HepG2 cells (observed in Chapter 4) were the result of corresponding changes occurring at the level of LDL receptor protein translation and gene transcription. The objective of the work in this Chapter was, therefore, to verify the hypothesis that fatty acids affect the LDL receptor in HepG2 cells at the level of gene transcription, which in turn influences protein synthesis, and subsequently LDL receptor binding activity.

5.2: METHODS

5.2.1 Optimisation of methods for a new batch of HepG2 cells.

The experiments in Chapter 4 examining the effects of various fatty acids on HepG2 cells were conducted on a stock of cells that had been obtained and established in 1992 (Ming Chan, IMVS, Adelaide). However, by passaging the HepG2 cells over many generations, the cells started to grow abnormally in clumps instead of in a linear array. This may have resulted from a loss of surface attachment proteins and/or genetic mutations leading to abnormal growth. The cells did not attain confluence within the expected time frame. They also did not respond as normal hepatocytes, in that LDL receptor activity was not regulated in the presence or absence of cholesterol. Due to these irregularities in cell growth, it was not appropriate to use this HepG2 cell batch for further investigations. Thus, a new HepG2 cell culture was established from a different source of frozen cells (Mark Thomas, IMVS, Adelaide).

The new batch of HepG2 cells was tested for standard growth and normal LDL receptor activity (see section 2.6.3) to ensure that they possessed the normal characteristics of human hepatocytes. The rate of growth of this new batch was observed to be normal, and they reached confluence within 4-5 days of subculturing. Cells were incubated in the absence or presence of cholesterol in order to examine whether LDL receptor activity could be regulated in the classical "Brown and Goldstein" manner. The new cells responded by downregulating their LDL receptor activity when grown for 24 h in the presence of DMEM supplemented with 10% FCS (cholesterol containing media) compared to cells incubated for 24 h with DMEM which contained no cholesterol, 10% LPDS (Figure 5.1).

The experimental conditions in Chapter 4 were designed so that they were appropriate for comparing the effect of fatty acids on LDL receptor activity in all 4 cell lines. However, in the work described here, the experiments to measure LDL receptor activity with various fatty acids were optimised for only HepG2 cells. The major differences in the experimental conditions for the new HepG2 cell batches included LPDS concentration, fatty acid concentration, and length of incubation (Table 5.1).

Table 5.1 Experimental conditions of the two different HepG2 cell culture batches.

Conditions	HepG2 batch-1992	New HepG2 batch-1994
% LPDS	5%	10%
# hours in background media before fatty acid enrichment	48	24
Concentration of fatty acids	500 µM	100 µM
# hours incubation with fatty acids	48	24
Incubation temperature of LDL receptor binding assay	4°C	Room Temperature (21-25°C)
% FCS	10%	10%
protein concentration	100 µg/ml	100 µg/ml

5.2.2 Measuring LDL receptor activity, protein and mRNA levels in human HepG2 cells.

Cells were subcultured from one large flask into 12 small flasks and maintained on DMEM supplemented with 10% FCS (Figure 5.2). On day 2, the media was replaced in 6 flasks with DMEM containing 10% LPDS, and in the other 6 flasks the cells received DMEM containing 10% FCS. After 24 h, this media was decanted, and cells were incubated for another 24 h with 100 µM fatty acid in DMEM supplemented with either 10% LPDS or 10% FCS. LDL receptor activity was measured using the LDL-gold method (section 2.6.3). LDL receptor protein and mRNA levels were measured according to the methods described previously (section 2.7 and 2.8, respectively).

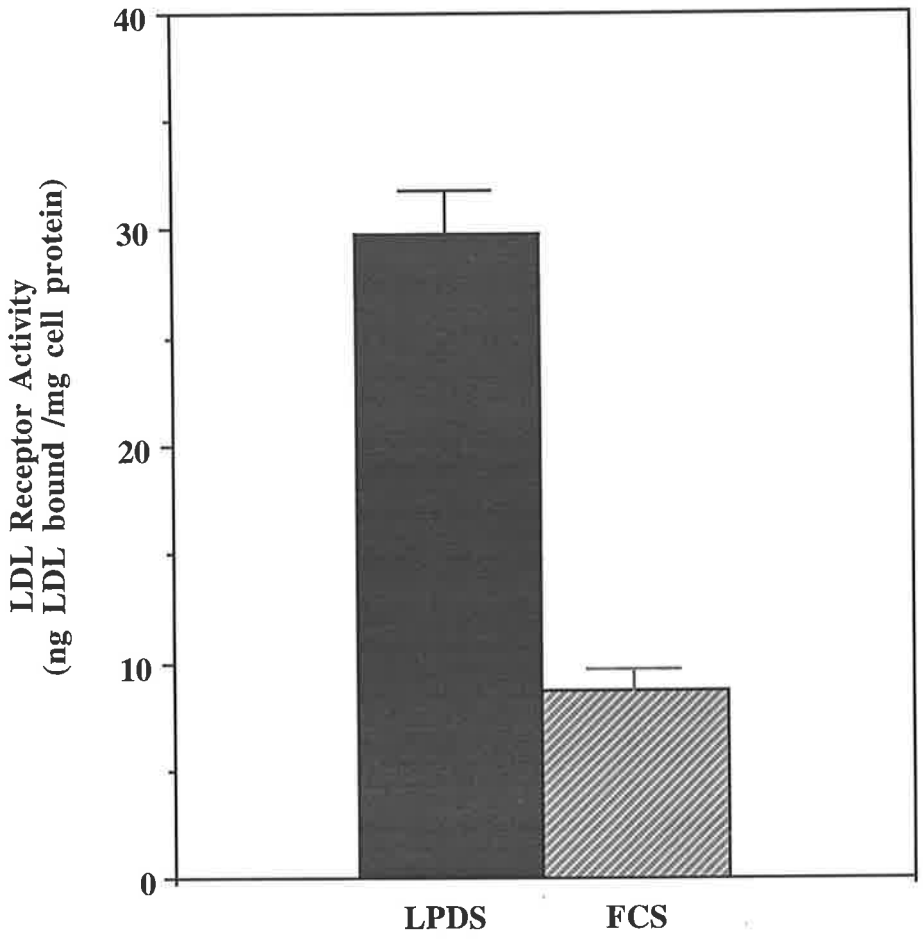


Figure 5.1: LDL receptor activity in the new stock of Hep G2 cells grown in media without cholesterol (LPDS = lipoprotein deficient serum) or with cholesterol (FCS = foetal calf serum). LDL receptor binding activity was measured using the colloidal LDL gold method as described in section 2.6.3. Results represent specific binding (ng of LDL/mg of cell protein) which was calculated by taking the difference between total binding (binding measured in the presence of $Ca(NO_3)_2$) and nonspecific binding (binding measured in the presence of EDTA). LDL receptor binding activity was measured in triplicate using the colloidal gold-LDL method described in section 2.6.3. Results represent specific LDL receptor binding activity (ng of LDL bound/mg cell protein) which expressed as the mean ± SEM of 3 experiments.

5.3 RESULTS

5.3.1 The effect of different fatty acids on cell fatty acid composition.

HepG2 cells were incubated with 100 μM concentration of various fatty acids for 24 h. Cells were then harvested, their lipids extracted (see section 2.4), and analysed for their fatty acid content by gas liquid chromatography. When cells were incubated without fatty acids, their lipids predominantly contained the saturated fatty acids, palmitic (16:0) and stearic (18:0) acid (Table 5.2). These contributed approximately 30% of the total cellular fatty acid. In essence, this fatty acid profile reflects the fatty acid content of the media (DMEM supplemented with 10% FCS). Cells incubated with media supplemented with palmitic acid were enriched predominantly with this saturated fatty acid (34% of total fatty acids) compared to cells incubated in the absence of any fatty acid (NF) (19%) (Table 5.2). The palmitic acid content appeared to dominate the relative proportion of total fatty acid. Cells incubated with oleic acid were enriched by this fatty acid from 27% in control cells (NF) to 43%. This was almost two times higher than cells incubated without fatty acids (NF). The increase in the relative amount of oleic acid in these cells was followed by a compensatory decrease in the relative palmitic acid content (compared to the no fatty acid control). Similarly, cells incubated with linoleic acid were enriched with this fatty acid (Table 5.2). The relative proportion of linoleic acid (18:2) increased from 4% in cells incubated without any fatty acids (NF) up to 20% in cells incubated with media supplemented with this fatty acid (which was 5 times the linoleic acid content of the control cells) (Table 5.2). Interestingly, the cells incubated with linoleic acid also had an increased relative proportion of 20:3, eicosatrienoic acid (11.3%) compared to the 20:3 fatty acid content in control cells (NF) (Table 5.2). The 20:3 fatty acid can be formed from linoleic acid when linoleic acid is desaturated to 18:3 (ω-6) by Δ6 desaturase, followed by chain elongation. Cells incubated with the ω-3 fatty acid, eicosapentaenoic acid (20:5), were not only enriched with this specific fatty acid to 13% (compared to 0.9% in control cells), but the relative content of docosapentaenoic acid (22:5) also increased to 4.1% (compared to 0.76% in control cells) (Table 5.2). In contrast, the relative content of docosahexanaenoic acid (22:6) decreased from 3.71% in control cells to 1.64% in cells incubated with EPA (Table 5.2).

Table 5.2: Fatty acid composition (relative % of total fatty acid) in HepG2 cells incubated with various fatty acids for 24 h. NF = no fatty acid, Palmitic = palmitic acid, Oleic = oleic acid, Linoleic = linoleic acid, EPA = eicosapentaenoic acid.

Fatty Acids	NF	Palmitic (16:0)	Oleic (18:1)	Linoleic (18:2)	EPA (20:5)
14:0	2.0 ± 0.03	1.6 ± 0.09	1.54 ± 0.90	1.5 ± 0.80	2.20 ± 0.50
14:1	0.3 ± 0.01	0.3 ± 0.08	0.30 ± 0.10	0.2 ± 0.10	0.16 ± 0.06
16:0	19.0 ± 1.20	33.5 ± 2.30	14.37 ± 1.70	14.98 ± 2.10	20.84 ± 2.30
16:1	4.4 ± 0.08	6.5 ± 1.10	2.77 ± 0.88	2.7 ± 0.67	2.90 ± 0.45
18:0	11.3 ± 1.10	9.4 ± 0.80	9.7 ± 1.30	8.4 ± 1.90	10.10 ± 1.40
18:1	27.0 ± 1.80	21.0 ± 1.80	43.1 ± 3.20	20.0 ± 2.20	24.00 ± 3.10
18:2	4.0 ± 0.50	4.0 ± 0.50	3.1 ± 0.50	20.7 ± 2.40	2.90 ± 1.10
20:3	1.5 ± 0.80	1.4 ± 0.06	1.05 ± 0.20	11.34 ± 0.20	0.60 ± 0.50
20:4	13.7 ± 1.70	10.3 ± 0.90	10.30 ± 1.30	6.2 ± 2.10	5.34 ± 1.50
20:5	0.90 ± 0.10	0.82 ± 0.10	0.4 ± 0.09	0.7 ± 0.20	13.60 ± 0.90
22:5	0.76 ± 0.10	0.62 ± 0.20	0.3 ± 0.10	0.8 ± 0.20	4.10 ± 0.70
22:6	3.71 ± 0.30	2.39 ± 0.67	3.1 ± 0.40	3.72 ± 0.80	1.64 ± 0.67

5.3.2 The effect of various fatty acids on LDL receptor binding activity.

Cells incubated with palmitic and oleic acid, in a background media of 10% LPDS, showed no significant difference in LDL receptor activity compared to the no fatty acid control (Figure 5.2). However, LDL receptor activity in cells incubated with DMEM containing 10 % LPDS was suppressed by 35% and 68% by the addition of linoleic acid and EPA, respectively, to the media compared to cells incubated without fatty acids, palmitic or oleic acid. LDL receptor activity appeared to decrease as the polyunsaturation of the fatty acid increased (Figure 5.2).

When cholesterol was supplemented in the media in the form of 10% FCS, LDL receptor activity was significantly downregulated compared to cells incubated in media that lacked this sterol. Under conditions in which cholesterol was present in the background media, palmitic, oleic, and linoleic acid, all increased LDL receptor activity compared to cells incubated with no fatty acid (Figure 5.2). In contrast, when cells were enriched with EPA, LDL receptor activity remained relatively unchanged compared to the control cells (no fatty acid + cholesterol) (Figure 5.2). Even in the presence of cholesterol, LDL receptor activity was lower in cells incubated with PUFAs compared to cells incubated with saturated and monounsaturated fatty acids.

In essence, it appeared that regardless of whether or not cholesterol was supplemented in the DMEM, there was an obvious trend between the type of fatty acid enrichment and cellular LDL receptor activity. The LDL receptor activity decreased in HepG2 cells as the degree of the fatty acid polyunsaturation increased (Figure 5.2). These results were as those observed previously in HepG2 cells (Chapter 4, Figure 4.9).

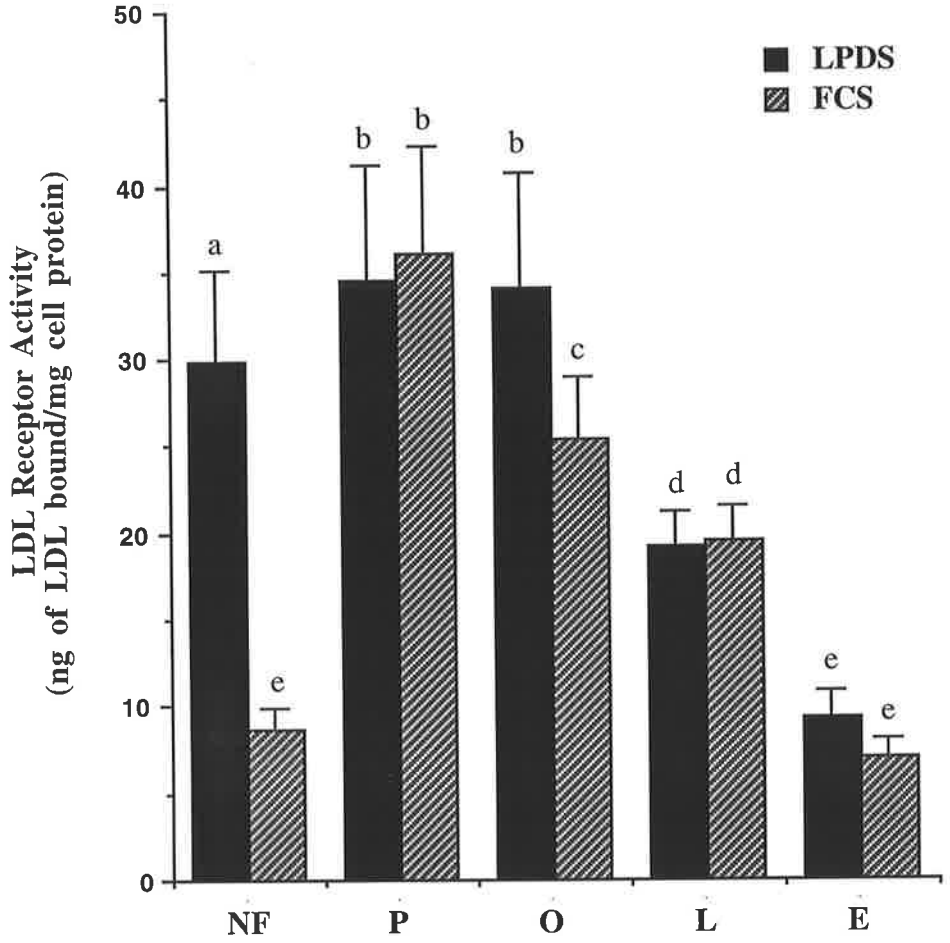


Figure 5.2: The effect of different fatty acids on LDL receptor activity in HepG2 cells. Cells were incubated for 24 h with various fatty acids, either minus cholesterol (LPDS = lipoprotein deficient serum) or plus cholesterol (FCS = foetal calf serum). NF = no fatty acids, P = palmitic acid (C16:0), O = oleic acid (C18:1), L = linoleic acid (C18:2), E = eicosapentaenoic acid (C20:5). LDL receptor binding activity was measured in triplicate using the colloidal gold-LDL method described in section 2.6.3. Results represent specific LDL receptor binding activity (ng of LDL bound/mg cell protein) which are expressed as a mean ± SEM of 4 experiments. Means with the same letters above them are not significantly different at $P < 0.01$.

5.3.3 The effect of various fatty acids on relative amounts of LDL receptor protein.

The LDL receptor protein in the cells was measured in order to ascertain whether the effect of the fatty acids on LDL receptor activity was due to a change in the number of LDL receptors synthesised in the cells.

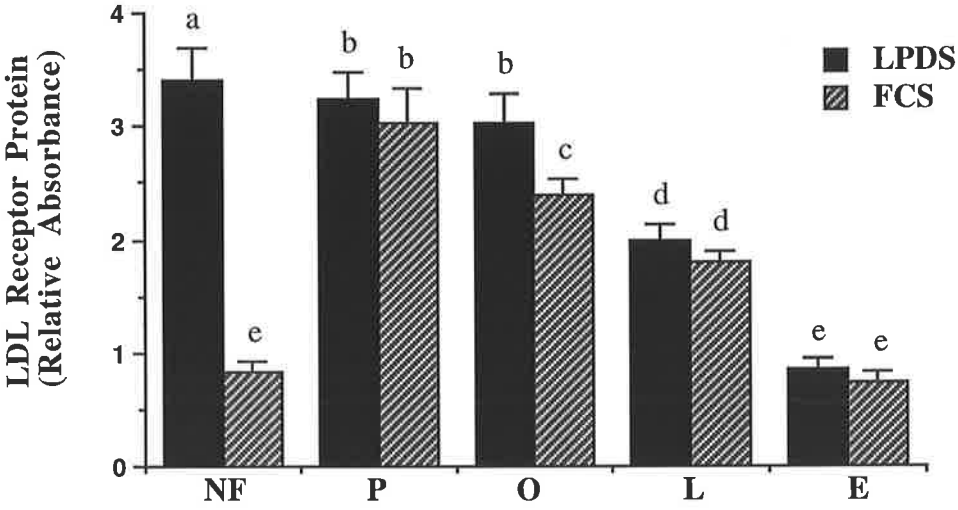
The relative amount of LDL receptor protein in HepG2 cells was significantly decreased when cells were incubated in media that contained cholesterol (DMEM supplemented with 10% FCS) compared to cells incubated in media that was cholesterol free (DMEM supplemented with 10% LPDS) (Figure 5.3). Thus, the reduction in the amount of LDL receptor protein in the presence of cholesterol most likely explains why the LDL receptor activity was also observed to be decreased under the same conditions (see Figure 5.2).

There was no difference between the relative LDL receptor protein in cells enriched in either palmitic or oleic acid compared to cells which were incubated without fatty acids in DMEM supplemented with 10% LPDS (Figure 5.3). In contrast, under the same growth conditions, the relative amount of protein was reduced by approximately 58% and 75% when cells were incubated with linoleic acid and EPA, respectively, compared to cells either incubated with no fatty acid, palmitic or oleic acid.

When cholesterol was supplemented in the DMEM growth media in the form of 10% FCS, the amount of LDL receptor protein appeared to be significantly increased in cells in the presence of palmitic, oleic and linoleic acid (Figure 5.3) compared to the no fatty acid control cells. However, the relative amount of LDL receptor protein remained unchanged in cells incubated with EPA compared to the control cells incubated with cholesterol and no fatty acids.

PUFA enriched cells (linoleic acid and EPA enriched cells) had significantly less LDL receptor protein compared to cells enriched with the saturated and monounsaturated fatty acids (palmitic and oleic acid enriched cells), regardless of whether the background media contained cholesterol or not. This is consistent with the trend observed in receptor activity (Figure 5.2), in that the relative protein of the LDL receptor decreased as the degree of fatty acid polyunsaturation increased (Figure 5.3). Compared to ω-6 enriched cells, LDL receptor protein was even further depressed in ω-3 PUFA enriched cells (Figure 5.3). The changes in the amount of total LDL receptor protein after incubation with the different fatty acids was correlated with the changes in LDL receptor binding activity (see Figure 5.2).

A)



B)

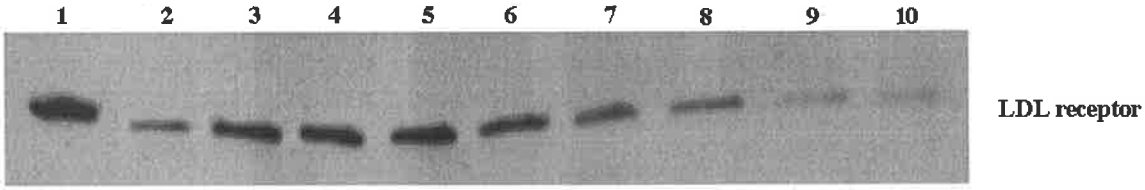


Figure 5.3: The effect of various fatty acids on LDL receptor protein in HepG2 cells. A) Cells were isolated after 24 h incubation with various fatty acids, either minus cholesterol (LPDS = lipoprotein deficient serum) or plus cholesterol (FCS = foetal calf serum). NF = no fatty acids, P = palmitic acid (C16:0), O = oleic acid (C18:1), L = linoleic acid (C18:2), E = eicosapentaenoic acid (C20:5). Cellular proteins were solubilised with Triton-X 100 detergent, electrophoresed, transferred to nitrocellulose and quantified by the ECL method as described in section 2.7. Experiments were performed in duplicate and the relative densities of the receptor band on the autoradiograph were quantified using laser densitometry. **A)** Results are expressed as relative absorbance and data represents the mean ± SEM of 4 experiments. Means with the same letters above them are not significantly different at $P< 0.01$. **B)** Autoradiograph of LDL receptor protein : lanes (1) LPDS + NF, (2) FCS + NF, (3) LPDS + P, (4) FCS + P, (5) LPDS + O, (6) FCS + O, (7) LPDS + L, (8) FCS + L, (9) LPDS + E, (10) FCS + E.

5.3.4 The effect of various fatty acids on LDL receptor mRNA levels.

The effect of the fatty acids on the relative LDL receptor protein was hypothesised to be a consequence of an effect at the level of LDL receptor gene transcription. Therefore, the amount of LDL receptor mRNA was quantified in HepG2 cells (see section 2.8) enriched with the fatty acids of interest in order to elucidate whether the fatty acids were influencing gene transcription.

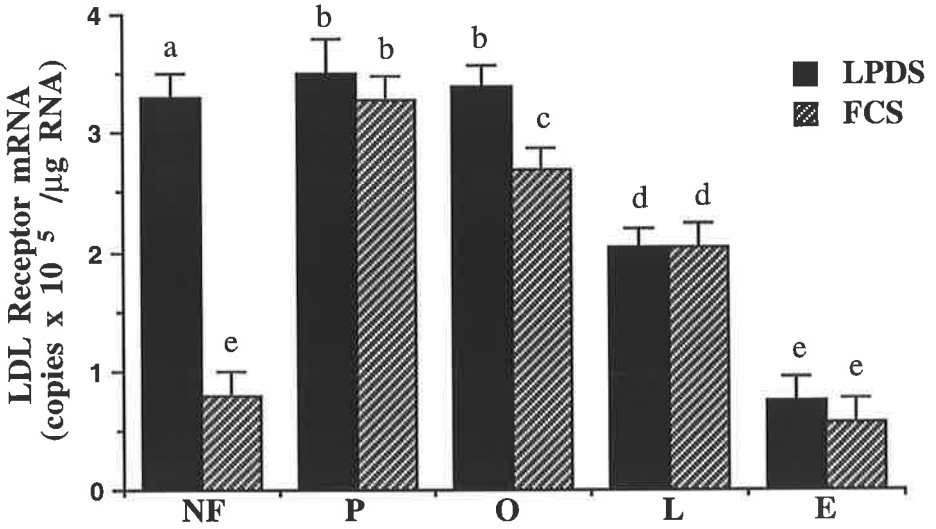
In the absence of fatty acids, the LDL receptor mRNA levels were reduced when cells were incubated in DMEM containing 10% FCS for 24 h compared to cells incubated in a cholesterol deficient media (DMEM supplemented with 10% LPDS) (Figure 5.4). In the absence of cholesterol (DMEM supplemented with 10% LPDS), the LDL receptor mRNA levels in cells incubated with palmitic or oleic acid enrichment did not differ from cells incubated without fatty acid (control). However, the amount of LDL receptor mRNA was decreased by 38% and 77% with linoleic acid and EPA, respectively (Figure 5.4).

When DMEM was supplemented with 10% FCS, LDL receptor mRNA levels were increased by palmitic, oleic and linoleic acid, but not EPA compared to cells incubated without fatty acid. Under these conditions, EPA enrichment of HepG2 cells had no effect on LDL receptor gene transcription compared to control cells.

Regression analysis of the data was conducted for LDL receptor activity and the amount of LDL receptor protein ($r= 0.946$, $P< 0.01$), LDL receptor activity and the level of LDL receptor mRNA ($r= 0.975$, $P< 0.01$), and the amount of LDL receptor protein and the level of LDL receptor mRNA levels ($r = 0.982$, $P< 0.01$). Regression analysis of the data indicate that there is a strong correlation between the LDL receptor activity, protein and mRNA levels.

In summary, the LDL receptor mRNA abundance within the cells decreased when the cells were incubated with PUFAs compared to saturated and monounsaturated enriched cells. This trend corresponded to that observed in LDL receptor protein and activity in the presence of the same fatty acids. This implies that the regulation of the LDL receptor binding activity by fatty acids is due to their effects at the level of LDL receptor gene transcription.

A)



B)

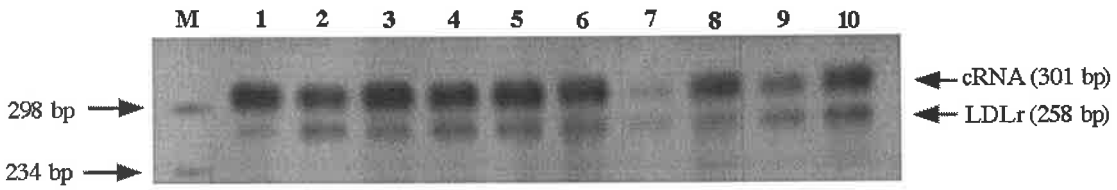


Figure 5.4: The effect of different fatty acids on cellular mRNA levels. Cells were incubated with various fatty acids for 24 h, either minus cholesterol (LPDS = lipoprotein deficient serum) or plus cholesterol (FCS = foetal calf serum). NF = no fatty acids, P = palmitic acid (C16:0), O = oleic acid (C18:1), L = linoleic acid (C18:2), E = eicosapentaenoic acid (C20:5). Total cellular mRNA was isolated, reverse transcribed and amplified using PCR with a synthetic piece of RNA used as an internal control (section 2.8). The amplified products were electrophoresed, transferred to nitrocellulose and quantified by the ECL method as described in section 2.8. **A)** Cellular mRNA levels were measured relative to the internal control and expressed as the mean ± SEM of 4 experiments. Means with the same letters above them are not significantly different at $P< 0.01$. **B)** Autoradiograph of LDL receptor mRNA: lanes (1) FCS + NF, (2) LPDS + NF, (3) LPDS + P, (4) FCS + P, (5) LPDS + O, (6) FCS + O, (7) FCS + E, (8) FCS + L, (9) LPDS + E, (10) LPDS + L.

5.3.5 The effect of increasing concentrations of EPA on LDL receptor activity and protein levels.

To further investigate the influence of EPA on LDL receptor activity, HepG2 cells were incubated for 24 h with increasing concentrations of EPA. Since there was no effect of EPA on the LDL receptor in the presence of cholesterol (Figure 5.2-5.4), cells were only supplemented with increasing concentrations of EPA in DMEM containing 10% LPDS.

The effect of EPA on LDL receptor was found to be dose dependent (Figure 5.5). LDL receptor activity was suppressed in a curvilinear manner as the concentration of EPA was increased to 500 μM (Figure 5.5). Concomitantly, the amount of LDL receptor protein decreased with increasing concentrations up to 250 μM EPA in a background media containing 10% LPDS (Figure 5.6). Therefore, the decrease in LDL receptor binding activity (see Figure 5.5) with increasing concentrations of EPA may be explained by the decrease in the amount of LDL receptor protein synthesised in HepG2 cells.

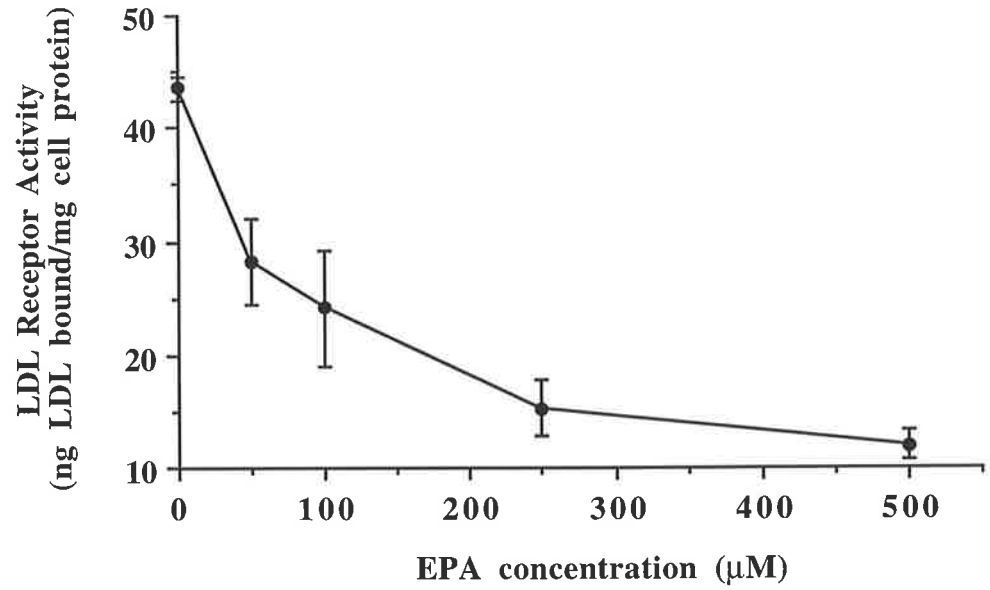


Figure 5.5: The dose dependent effect of EPA on LDL receptor activity. Cells were incubated with increasing concentrations (0-500 μM) of EPA (eicosapentaenoic acid), without cholesterol (LPDS = lipoprotein deficient serum). LDL receptor binding activity was measured in triplicate using the LDL colloidal gold method described in section 2.6.3. Results represent specific LDL receptor binding activity (ng of LDL bound/mg cell protein) which is expressed as the mean ± SEM of 2 experiments.

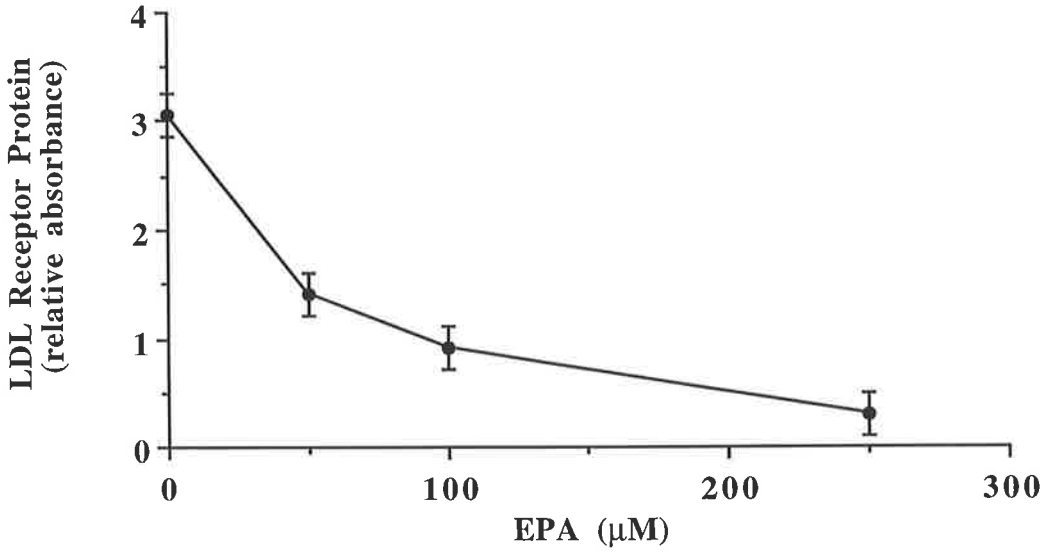


Figure 5.6: The effect of increasing concentrations of EPA on LDL receptor protein in HepG2 cells. Cells were isolated after 24 h incubation with increasing concentrations (0-500 μM) of EPA (eicosapentaenoic acid), without cholesterol (LPDS = lipoprotein deficient serum). Cellular proteins were solubilised with Triton-X 100 detergent, electrophoresed, transferred to nitrocellulose and quantified by the ECL method as described in section 2.7. The experiment was performed in duplicate and relative densities of the receptor band were quantified using laser densitometry. Results are expressed as relative absorbance and data represent the mean ± SEM of 2 experiment.

5.3.6 The effect of EPA on LDL receptor protein in the presence of vitamin E.

Long chain polyunsaturated fatty acids, specifically ω-3 fatty acids, are highly susceptible to oxidation due to the content of a high number of double bonds (see section 1.8.4). Therefore, in order to the eliminate the possibility that the effects of the EPA on the LDL receptor was not a consequence of EPA oxidation products which may have formed in the media while cells were incubated with this fatty acid, an antioxidant, vitamin E (α-tocopherol) was added to the EPA enrichment media. The amount of LDL receptor protein was measured in cells in the absence of vitamin E or EPA (control), EPA alone, in presence of vitamin E alone, and in the presence of 250 μM EPA + 50 μM vitamin E.

It was observed that the amount of LDL receptor protein decreased in cells with 250 μM EPA even in the presence of 50 μM vitamin E (Figure 5.7). Similarly, LDL receptor mass in cells incubated with linoleic acid + 50 μM vitamin E was also depressed regardless of the presence of the vitamin (Figure 5.8). This demonstrates that the suppression of the LDL receptor by EPA or linoleic acid was not a result of fatty acid oxidation products.

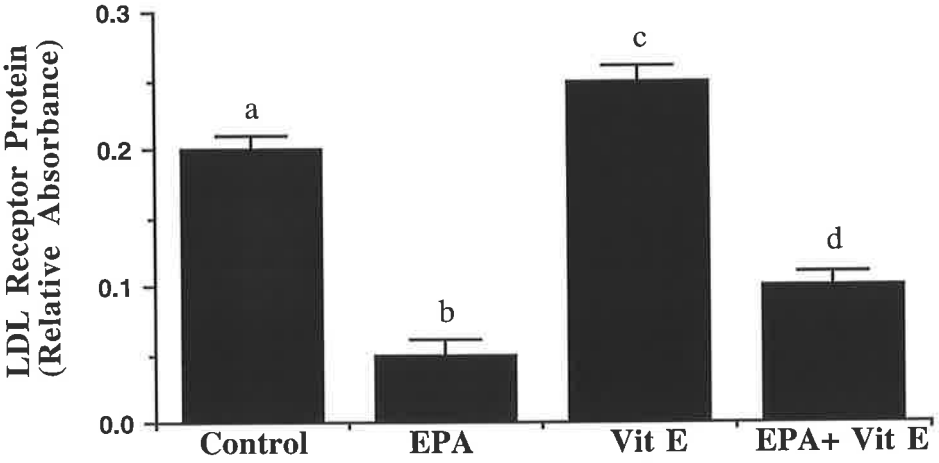


Figure 5.7: The effect of EPA and vitamin E on LDL receptor protein. Cells were isolated after 24 h incubation with either 250 μM EPA, 50 μM vitamin E, or 50 μM vitamin E + 250 μM EPA. Cellular proteins were solubilised with Triton-X 100 detergent, electrophoresed, transferred to nitrocellulose and quantified by the ECL method as described in section 2.7. Experiments were performed in duplicate and the relative densities of the receptor band were quantified using laser densitometry. Results are expressed as relative absorbance and data represents the mean ± SEM of 2 experiments. Means with different letters above are significantly different at $P< 0.01$.

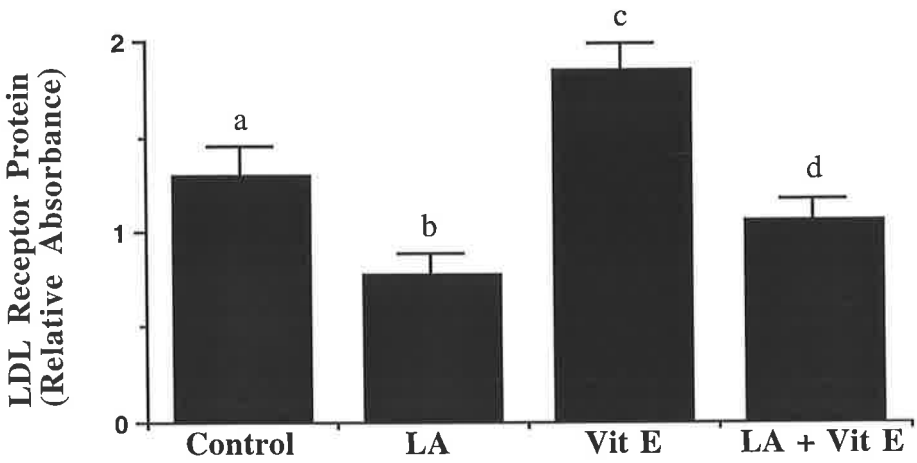


Figure 5.8: The effect of linoleic acid (LA) and vitamin E on LDL receptor protein. Cells were isolated after 24 h incubation with either 250 μM LA, 50 μM vitamin E, or 50 μM vitamin E + 250 μM LA. Cellular proteins were solubilised with Triton-X 100 detergent, electrophoresed, transferred to nitrocellulose and quantified by the ECL method as described in section 2.7. Experiments were performed in duplicate and the relative densities of the receptor band were quantified using laser densitometry. Results are expressed as relative absorbance and data represents the mean ± SEM of 2 experiments. Means with different letters above are significantly different at $P< 0.01$.

5.4. DISCUSSION

As anticipated, results showed that linoleic acid and EPA significantly suppressed LDL receptor activity in human HepG2 cells compared to palmitic and oleic acid. The PUFAs depressed LDL receptor protein in a similar manner. This is likely to be due to these fatty acids having an effect on LDL receptor gene transcription as receptor mRNA levels (Figure 5.4) corresponded to the changes in LDL receptor protein and activity (Figure 5.2). These observations are consistent with the current theory that the LDL receptor is only regulated at the level of gene transcription (Goldstein and Brown, 1977; Brown and Goldstein, 1986; Rudling, 1992; Sanchez et al., 1995). The results herein support the hypothesis that the changes in hepatic LDL receptor binding activity with PUFAs were due to their effects on LDL receptor gene transcription.

The effect of different fatty acids on cell fatty acid composition

When HepG2 cells were incubated with either saturated, monounsaturated, ω-6 or ω-3 polyunsaturated fatty acids for 24 h, GLC analysis revealed that cells were enriched with the fatty acids of interest, namely palmitic, oleic, linoleic and EPA (Table 5.2). Previous findings have shown that cells are able to take up these specific fatty acids from the culture media and incorporate them into the phospholipid component of their membranes (Pal and Davis, 1991). It was also observed that a greater percentage of EPA was preferentially incorporated into the phosphotidylethanolamine (PE) fraction of the phospholipid bilayer compared to the phosphtidylcholine (PC) fraction, whereas linoleic acid appears to be equally distributed in both PE and PC fractions (Pal, 1990).

Interestingly, cells incubated with EPA were enriched not only in this fatty acid, but compared to control cells (NF), there was also a marked increase in docosapentaenoic acid (DPA), an immediate elongation product of EPA (Table 5.2). This suggests that HepG2 cells can effectively convert EPA to DPA. In contrast, no significant increase in docosahexanaenoic acid (22:6, ω-3) was observed (Table 5.2). This indicates that this cell line, like human skin fibroblasts, lacks the Δ4 desaturase activity which is required for the conversion of DPA to docosahexanaenoic acid and thus, explains why the cells are not enriched in this fatty acid (Aeberhard et al., 1978).

Relationship between fatty acid unsaturation and LDL receptor expression (activity, protein, mRNA)

The work described herein also demonstrated that LDL receptor activity, protein and mRNA levels all decreased as the degree of unsaturation of the incubated fatty acid increased (Figure 5.2, 5.3, 5.3). In addition, this inverse association between the LDL receptor and the unsaturated properties of the fatty acids held whether or not cholesterol was included in the DMEM growth media.

Palmitic acid (16 carbons), a saturated fatty acid and oleic acid (18 carbons), which contains one double bond, induced the highest LDL receptor expression (activity, protein and mRNA levels) in HepG2 cells compared to the PUFAs, linoleic acid (18 carbons with 2 double bonds) and EPA (20 carbons and 5 double bonds). EPA enriched cells had the lowest LDL receptor activity, protein and mRNA levels. LDL receptor activity in cells incubated with linoleic acid and EPA decreased approximately 35% and 68%, respectively, compared to cells incubated with palmitic acid under conditions in which cholesterol was absent in the background media (DMEM supplemented with 10% LPDS) (Figure 5.2). Similarly, compared to cells incubated with palmitic acid (DMEM supplemented with 10% LPDS), the amount of LDL receptor protein was decreased by 58% and 75% in cells incubated with linoleic acid and EPA, respectively (Figure 5.3). Concomitantly, LDL receptor mRNA was decreased by 38% with linoleic acid and 68% with EPA compared to cells incubated with palmitic acid (Figure 5.4). In addition, the suppression of LDL receptor activity and protein by EPA was shown to be dose dependent in HepG2 cells incubated in a cholesterol free media (DMEM supplemented with 10% LPDS) (Figure 5.5 and 5.6).

A question that one can ask is whether it is the number of double bonds in the fatty acid chain or the fatty acid chain length itself which determines the effect of the fatty acid on the LDL receptor. For instance, does EPA have a greater effect on the LDL receptor than linoleic because it contains 5 double bonds or because of its 20 carbon chain length? Although the experiments herein were not designed to directly address this issue, one can extrapolate, based on the findings, that the LDL receptor was affected more by the fatty acid unsaturation rather than fatty acid chain length. For example, there is no difference in the LDL receptor expression (activity, protein and mRNA levels) between palmitic and oleic acid even though there is a difference of two carbons in their fatty acid chain length and one double bond. In contrast, oleic and linoleic acids have the same number of carbons atoms, but they differ in the number of double bonds they possess. In this case, it was observed that there was a greater suppression on the LDL receptor when cells were incubated with linoleic acid (18:2) than with oleic acid (18:1).

From the two examples given above, it appears that the degree of fatty acid unsaturation had a greater effect on LDL receptor expression than the fatty acid chain length. Therefore, one can speculate based on the above argument that EPA had a greater effect on the LDL receptor than linoleic acid because it contains 2 more double bonds, and not due to the fact that it contains 2 more carbons atoms in its fatty acid chain. Nevertheless, an experiment that could be done to unravel this further would be to examine the effect of arachidonic acid (C20:4, ω-6) and docosapentaenoic acid (C22:5, ω-3) versus EPA on the LDL receptor. Arachidonic acid would be appropriate as it contains the same number of carbon atoms as EPA, but has one less double bond in its carbon chain. As docosapentaenoic acid has the same degree of fatty acid unsaturation as EPA, these two fatty

acids could be examined together to determine whether there is correlation between chain length and LDL receptor expression.

While one can not completely rule out the influence of the fatty acids' chain length as a factor, based on the results reported herein, there appears to be stronger evidence favouring fatty acid unsaturation having a greater influence on the LDL receptor.

The effect of fatty acids at the transcriptional vs translational level.

Although there appears to be powerful evidence to demonstrate that LDL receptor activity is regulated at the level of gene transcription (Goldstein and Brown, 1977; Brown and Goldstein, 1986; Rudling, 1992; Sanchez et al., 1995), one can still question whether the effect of fatty acids on LDL receptor activity is as a consequence of a true transcriptional event or an outcome of a translational or post-translational event.

Regression analysis revealed that a strong positive correlation exists between LDL receptor activity and the amount of LDL receptor protein ($r= 0.946$, $P< 0.01$). A strong positive relationship was also observed between LDL receptor activity and the level of LDL receptor mRNA ($r= 0.975$, $P< 0.01$). Concomitantly, a highly significant positive correlation was found with the amount of LDL receptor protein and the level of LDL receptor mRNA levels ($r = 0.982$, $P< 0.01$). Therefore, regression analysis of the data indicates that there is a strong association between the LDL receptor activity, protein and mRNA levels. Thus, the effect of the fatty acids on LDL receptor activity is most likely due to their effects on gene transcription.

Other studies have also suggested that fatty acids affect LDL receptor activity at the level of gene transcription. A study by Lindsey et al. (1990) has shown that when liver samples were excised and examined from hamsters fed various fatty acid diets, LDL receptor mRNA levels did not vary between animals fed a diet rich in palmitic acid (90% palm oil) compared to animals fed a mixed diet (which had a lower content of palmitic acid). However, hepatic LDL receptor mRNA levels were significantly lower in animals fed a diet containing a high content of linoleic acid than a diet containing a higher content of palmitic acid. Even though LDL receptor activity was not measured in this study, these results corresponded to changes in LDL receptor mRNA levels in HepG2 cells incubated with similar fatty acids (Figure 5.4). Kushwaha et al. (1991) reported that hepatic LDL receptor mRNA levels increased in baboons when they were fed dietary corn oil compared with animals fed lard diets. In another study by Lindsey et al. (1992), LDL receptor mRNA levels were slightly depressed by 20% in HepG2 cells which had been incubated with LDL derived from individuals after they had consumed dietary fish oils, with a corresponding depression in LDL receptor binding activity. Although LDL receptor protein was not measured in this case, the changes in LDL receptor mRNA levels in HepG2 cells in Lindsey's study agree with the results observed herein. Thus, the studies described above,

along with the observations described in this Chapter, strongly support the hypothesis that fatty acids modulate LDL receptor activity by their effect on LDL receptor gene transcription.

The association between LDL receptor activity, protein and mRNA levels has, however, been questioned by some investigators examining the effects of fatty acids on LDL receptor gene transcription. Srivastava et al. (1995) reported a slight 25% decrease in LDL receptor activity in HepG2 cells incubated with palmitate, but found that LDL receptor mass and mRNA levels were not affected compared to BSA control cells. Srivastava et al. (1995) suggested that the suppressive effects of palmitate on LDL receptor activity is most likely to be post-translational. They based these conclusions on their own findings and from other *in vivo* studies which have shown that LDL receptor-mediated clearance of LDL from plasma decreases and plasma LDL levels increase as a consequence of feeding hamsters saturated fatty acids (Woollet et al., 1988, 1992a, 1992b). A solid argument can be made against these *in vivo* studies since the clearance of plasma LDL does not necessarily confirm that the uptake of plasma LDL was mediated by the hepatic LDL receptor. As a number of cells are capable of taking up LDL from plasma through other mechanisms, one has to consider that the results of such *in vivo* studies may be ambiguous.

Similar arguments can be made when analysing other studies investigating the effects of PUFAs on LDL receptor gene expression. Spady et al. (1995) observed that rats fed a diet rich in fish oil had enhanced hepatic uptake of radiolabelled LDL compared to animals fed corn oil or normal chow diets. Although they did not measure LDL receptor binding activity, they reported LDL receptor protein and mRNA levels were unchanged in rats fed a diet rich in fish oil compared to animals fed corn oil or normal chow diets. Even though they implied that the increased clearance of LDL in fish oil fed rats was attributable to increased LDL receptor activity, they suggested that this was not due to changes in LDL receptor mass and mRNA levels, and thus had to be post-translational. It is interesting that in the same study, they found that the hepatic LDL receptor mRNA levels had significantly decreased in hamsters with fish oil supplementation compared to animals fed corn oil. Again, even though they did not directly measure LDL receptor activity, they did find in this case that clearance of radiolabelled LDL was significantly lower in hamsters fed dietary fish oils compared to animals fed corn oil, implying LDL receptor activity had decreased due to fish oil feeding. Therefore, it is unclear from these *in vivo* studies whether LDL clearance can be used as a true measurement of hepatic LDL receptor activity. *In vivo* studies reporting changes in LDL clearance in the presence of specific fatty acids without observing the corresponding changes in LDL receptor protein levels and mRNA levels may be misleading.

One may propose that the association between LDL receptor activity, mass, and mRNA levels may be species specific. For example, the findings described in this Chapter show that ω-6 PUFAs suppress LDL receptor activity, mass and mRNA levels in human liver cells compared to saturated fatty acids (Figure 5.4). In contrast, Kushwaha et al. (1991) reported that hepatic LDL receptor mRNA levels increased in baboons when they

were fed dietary corn oil compared with animals fed lard diets. However, since they did not measure hepatic LDL receptor activity, it was difficult to decipher whether the changes in LDL receptor mRNA levels corresponded to LDL changes in receptor activity. Kushwaha's observations were not validated by Sorci-Thomas et al. (1989), who observed an increase in cholesterol plasma clearance but no increase in LDL receptor mRNA abundance in African green monkeys fed large quantities of linoleic acid. Even though Sorci-Thomas et al. (1989) did not measure LDL receptor binding activity, they suggested that the LDL receptor activity was not regulated at the level of transcription. Thus, it is possible that within a particular animal species, certain exogenous factors may be induced by fatty acid feeding which may play a significant role in altering and/or masking the true effect of the fatty acids at the level of hepatic LDL receptor gene transcription. The absence of any effect of fatty acids *in vivo* may be a consequence of exogenous factors other than the fatty acid themselves. Such arguments could explain the discrepancies between studies using different animal models to investigate the effect of fatty acids on LDL receptor gene transcription. It is especially difficult to discriminate against events in which exogenous factors may interfere with the fatty acid effect particularly as many *in vivo* studies do not directly measure LDL receptor binding activity, but measure plasma LDL clearance.

Independent effetcts of extracellular cholesterol and fatty acids

The results show that LDL receptor activity in HepG2 cells respond in a classical Brown and Goldstein manner in the presence of cholesterol. The LDL receptor activity was downregulated when 10% FCS (containing cholesterol in the form of LDL) was added to DMEM compared to cells without cholesterol supplementation (DMEM plus 10% LPDS) (Figure 5.1 and 5.2). However, results showed that this suppression by extracellular cholesterol was overridden by the addition of either palmitic, oleic, or linoleic acid in the growth media at the level of LDL receptor activity, protein and mRNA (Figure 5.2-5.4). The LDL receptor was upregulated when cells were incubated with palmitic, oleic and linoleic acid compared to control cells (no added fatty acids) incubated in DMEM containing cholesterol (10% FCS). As cellular LDL receptor binding was increased by these fatty acids despite the potential increase in intracellular cholesterol in the presence of 10% FCS, it emphasises that the distribution of cholesterol within the cells is probably more influential and hence, supports the idea that the media cholesterol concentration is unimportant when these fatty acids are present.

The ability of fatty acids to knockout the suppressive effects of external cholesterol appears to be fatty acid specific. Palmitic and oleic acid were better able to ameliorate the suppression of LDL receptor activity by cholesterol than linoleic acid. Furthermore, EPA did not appear to have the similar overriding effects compared to other fatty acids at the level of LDL receptor activity, protein or mRNA (Figure 5.2-5.4). Nevertheless, the effects with palmitic, linoleic and EPA appear to be independent of the extracellular cholesterol

concentration as similar effects were observed in the absence of cholesterol in the background media. However, that is not to say that extracellular cholesterol content is completely irrelevant, as LDL receptor gene expression was significantly lower in cells incubated with oleic acid and cholesterol containing media (DMEM supplemented with 10% FCS) than cells incubated with oleic acid without cholesterol (DMEM supplemented with 10% LPDS) (Figure 5.2-5.4). Despite the interaction of cholesterol with oleic acid, oleic acid still appeared to have a separate effect on cellular LDL receptor activity even in the presence of cholesterol (Figure 5.2-5.4). This was shown from the findings that LDL receptor activity was significantly higher in cells incubated with oleic acid plus cholesterol in the background media (DMEM supplemented with 10% FCS) compared to cells incubated without fatty acids in the media (DMEM supplemented with 10% FCS). In this case, oleic acid was still able to upregulate the LDL receptor in the presence of cholesterol, or in other words, "knockout" the suppressive effects of cholesterol to a certain extent (Figure 5.2-5.4).

Similar to the findings in this Chapter, Rumsey et al. (1995) demonstrated that LDL receptor binding activity was increased in fibroblasts incubated with oleic acid in a background media containing 10% FCS compared to cells incubated in the absence of oleic acid. In this case, oleic acid was able to upregulate the LDL receptor, despite the downregulation induced by 10% foetal calf serum. They attributed their results to changes in cellular cholesterol distribution caused by oleic acid supplementation. They implied that the increase in LDL receptor binding activity was due to a decrease in cellular free cholesterol, in which oleic acid increased the cellular cholesterol protein and the cholesterol ester to free cholesterol ratio. In contrast to this study, Srivastava et al. (1995) reported no difference in LDL receptor activity in HepG2 cells when incubated with oleic acid compared to oleic acid plus 25-hydroxycholesterol. Although the addition of 25-hydroxycholesterol did not further suppress LDL receptor activity when cells were incubated with oleic acid, the LDL receptor activity may have already been affected by the cholesterol contained in the growth media (from FCS).

Other studies have also observed that dietary fatty acids can have an overpowering effect on LDL receptor activity even in the presence of cholesterol. Spady and Dietschy (1988) demonstrated that the downregulation of the LDL receptor activity by dietary cholesterol can be affected by the type of dietary fat. They found that the unsaturated fatty acids were able to alleviate the cholesterol induced LDL receptor suppression relative to saturated fatty acids. Mott et al. (1993) found that the hepatic mRNA concentration for the LDL receptor was increased in infant baboons that were given breast milk compared to those infants that were weaned on formula milk. Since breast milk contains a higher percentage of saturated fat compared to formula, it can be speculated that increased LDL receptor gene expression was due to its high content of saturated fatty acids. They suggested that it may be a result of increased ACAT activity by saturated fatty acids. Interestingly, the breast milk also contained a higher amount of cholesterol than formula, thus demonstrating that the

cholesterol content of the milk had little bearing of final outcome on LDL receptor gene transciption.

One can conclude from these findings described above that, generally, fatty acids have an independent effect on the LDL receptor, regardless of the potential suppressive actions of extracellular cholesterol. Therefore, if the effects of the fatty acids on the LDL receptor are associated to changes in cholesterol then it is more likely a consequence of their effects on intracellular cholesterol metabolism (ACAT, cholesterol biosynthesis, cholesterol excretion, etc). The work herein indicate that the extracellular cholesterol concentration may not be as important compared to the fatty acid effects in determining the final effect on the LDL receptor.

Possible mechanisms

There are a number of possible mechanisms by which PUFAs could influence LDL receptor expression in liver cells. The influence of fatty acids on the receptor may be due to their effects on other cellular processes (eg. cholesterol metabolism), involvement of nuclear transcription factors or their direct effects on LDL receptor gene transcription (also see chapter 9). Some of these are explored below.

1) Fatty acid oxidation

Although the consumption of PUFAs can have many positive effects in preventing CHD in humans, long chain PUFAs, especially ω-3 fatty acids, possess several double bonds which make them potentially unstable in the membrane. These multiple double bonds make them highly susceptible to peroxidation by free radicals present either in the intracellular or extracellular environment of the cell. Consequently, oxidised PUFAs can be cytotoxic to the cell, having many deleterious effects, such as the disruption of cell membrane function and structure. As greater suppression of LDL receptor expression (activity, protein and mRNA levels) was observed by fatty acids with a higher number of double bonds, then it opens up the possibility that fatty acid oxidation products formed from these PUFA species cause these suppressive effects.

It is necessary, therefore, to protect fatty acid double bonds from highly reactive radical species, such as nitric oxide, superoxide or hydroxyl radical, by having sufficient concentrations of antioxidants present (Halliwell, 1994, 1995). Since the effect of EPA or linoleic acid on LDL receptor expression could have been attributed to the formation of fatty acid oxidation products due to the lack of sufficient antioxidants in the system, 50 μM vitamin E was added to the media with EPA or linoleic acid (Figure 5.7 and Figure 5.8). Results showed that even in the presence of the antioxidant, EPA was still able to inhibit LDL receptor protein (Figure 5.7). Similarly, LDL receptor protein was inhibited by linoleic acid with or without the presence of vitamin E in the background media (Figure 5.8). Hence, the effect of PUFAs on LDL receptor appears to be a true effect and not a consequence of anomalies arising from any oxidative products of EPA or linoleic acid which

may have formed as a result of an inadequate amount of antioxidant in the system. Thus, the inhibition of the LDL receptor by EPA is unlikely to be attributable to fatty acid peroxides, but is presumably due to the nature and particular characteristics of this fatty acid.

2) Fluidity effects

Fatty acids may also have their effects on LDL receptor expression (activity, protein and mRNA levels) through their influence on membrane fluidity. Dietary modification of fatty acid composition of biological membranes has been postulated to alter a number of membrane properties, including the degree of exposure of surface proteins and the activity of membrane bound enzymes by modulating the biophysical characteristics of membranes (Spector et al., 1979; Sandermann, 1978). Since dietary PUFAs are readily incorporated into the phospholipid fraction of cellular membranes, the physical properties of these membranes may be dramatically affected by changes in dietary fat (Spector et al., 1979). Evidence suggests that increasing the membrane PUFA content increases the fluidity of the membrane (Borell, personal communication; Kuo et al., 1990). Conversely, increasing the saturated fatty acid content, decreases membrane fluidity (Stryer, 1988). These changes in the microviscosity of the membrane lipids appear to affect the activity of certain membrane bound enzymes. Of special interest is the HMG-CoA reductase activity, which has been shown to be depressed when fibroblast microsomes are enriched with saturated PC, but are then reactivated by unsaturated PC (Davis and Poznansky, 1987; George et al., 1990). Thus, an increase in HMG-CoA reductase activity by the incorporation of membrane PUFAs may ultimately lead to the increase in cholesterol biosynthesis. Consequently, the total amount of cellular cholesterol would be increased, thereby causing a feedback suppression of LDL receptor gene transcription.

However, the idea that downregulation of the LDL receptor by ω-6 and ω-3 PUFAs is linked to their fluidity effects on HMG-CoA reductase activity is contradicted by the fact that the expression of these two genes has been shown to be coordinately regulated (Powell and Kroon, 1994; Rudling, 1992; Rudling and Collins, 1996). It appears that under most conditions tested, these genes are regulated at the mRNA level, suggesting a common mechanism may be responsible for controlling gene transcription of these two genes (Powell and Kroon, 1994; Rudling, 1992; Rudling and Collins, 1996). This implies that if fatty acids induce changes in gene transcription of HMG-CoA reductase, then the same change will also occur on LDL receptor gene transcription because they are coordinately regulated. As LDL receptor gene transcription is decreased with PUFAs, then one can assume that HMG-CoA reductase gene transcription would also be decreased.

The relationship between LDL receptor and HMG-CoA reductase at gene transcription does not appear to hold beyond this level, as HMG-CoA reductase has been shown to be regulated post-transcriptionally and post-translationally (Goldstein and Brown, 1990; Brown and Goldstein, 1984), unlike the LDL receptor (Goldstein and Brown, 1977;

Brown and Goldstein, 1984, 1986; Rudling, 1992; Sanchez et al., 1995). Therefore, one could argue that although transcription of these two genes is coordinately regulated, PUFAs may still increase HMG-CoA-reductase activity by influencing fluidity. However, many other studies have reported that HMG-CoA-reductase activity or cholesterol biosynthesis is inhibited by PUFAs (Field et al., 1987; Schafer and Katterman, 1992; Murthy et al., 1988). HMG-CoA reductase activity has been shown to be inhibited in rabbits fed fish oils (Field et al., 1987). Hepatic cholesterol biosynthesis was reduced by more than 50% in rats supplemented with 8% dietary fish oil compared with animals either supplemented with 8% safflower oil or control chow diet. Animals that had been supplemented with 8% safflower oil had a reduction of approximately 30% in cholesterol synthesis compared to the control animals (Field et al., 1987). Schafer and Katterman (1992) have also reported that cholesterol biosynthesis was decreased in HepG2 cells incubated with EPA, presumably due to a decrease in HMG-CoA reductase activity. Thus, in this case, fluidity effects by PUFAs seem an unlikely explanation.

The relationship between fluidity and LDL receptor binding activity has been also examined by Hannah et al. (1995), using a Chinese hamster ovary cell (CHO) line that constitutively expresses the human LDL receptor. They observed that LDL receptor activity was greater in cells enriched with palmitic and oleic acid than cells enriched in linoleic acid. They found that the correlation between LDL receptor binding activity and membrane fluidity (assessed by measuring diphenylhexatriene (DPH) fluorescence anisotropy) was weak ($r=0.24$, $P=0.27$) and did appear to explain the effect of fatty acid modification on LDL receptor binding.

The time frame whereby these PUFAs seem to act on the LDL receptor further supports the idea that fatty acids are not likely to be acting through mechanisms involving alterations in membrane fluidity. Results herein show that PUFAs had a swift effect on LDL receptor activity, translation and transcription in HepG2 cells (Figure 5.3 and 5.4). Others, such as Wong and Nestel (1987), have observed that linoleic acid and EPA suppressed LDL receptor activity as early as 6 h. Rumsey et al. (1995) have reported changes in ACAT activity by fatty acids within 2 hours. Incorporation of fatty acids into the phospholipid membrane of the cells, fluidity changes and the effect on enzyme activities appears to be a time consuming process which does not correspond to the rapid effects of these fatty acids one observes in LDL receptor gene transcription, HMG-CoA reductase activity and ACAT activity. Thus, there does not appear to be adequate proof to support the theory that the downregulation of the LDL receptor by ω-6 and ω-3 PUFAs is a consequence of their fluidity effects on HMG-CoA reductase activity.

3) The LDL receptor gene

If time is factor which is important in the process by which fatty acids modulate gene transcription, then one can hypothesise that fatty acids may directly act on both the LDL receptor and HMG-CoA reductase genes. This would imply that their effects on these genes may be independent of their actions on other cellular components, such as fluidity and cholesterol metabolism (eg. cholesterol biosynthesis, cholesterol esterification etc). This idea is supported by the findings of Murthy et al. (1988). They reported that both ACAT activity and HMG-CoA reductase activity were lower in intestinal CaCo-2 cells enriched with 100 μM EPA compared with oleic acid enriched cells. ACAT activity has also been shown to be inhibited in various cell types enriched with ω-3 fatty acids (Pal and Davis, 1991; Rustan et al., 1988; Murthy et al., 1988). The findings of Murthy et al. (1988) were unexpected. The consensus from other studies is that the regulation of these two enzymes is usually reciprocal under different test conditions and in cell culture systems where cells are actively processing cholesterol (Field et al., 1987; Brown et al., 1980; Goldstein et al., 1974). In theory, if ω-3 fatty acid enrichment of cells was responsible for the expansion of the intracellular cholesterol content, then this would subsequently cause a downregulation in both HMG-CoA reductase activity and upregulation of ACAT activity. Cells respond in this manner in order to maintain intracellular cholesterol homeostasis. Therefore, Murthy's results and others' results can be interpreted to mean that the concurrent decrease in both HMG-CoA reductase and ACAT activity in the presence of ω-3 PUFAs is not related to changes in cholesterol metabolism, but due to a direct effect elicited by these fatty acids on the gene expression of the LDL receptor, HMG-CoA reductase and ACAT and/or their activites.

The mechanism of feedback regulation of mRNA production of the LDL receptor by sterols has been shown to be modulated by a sterol regulatory element binding protein (SREBP-1) that recognises the sterol regulatory element (SRE-1) (Sudhof et al., 1987b; Wang et al., 1994; Sanchez et al., 1995) (see section 1.7.5). In the presence of sterols, transcription of the LDL receptor gene is silenced by preventing the cleavage of cytosolic SREBP-1 to a 68 kd protein and thereby inhibiting its migration to the nucleus to induce LDL receptor gene transcription. The cell inhibits the overaccumulation of cholesterol by suppressing the LDL receptor gene transcription through this mechanism. It is possible that, like cholesterol, PUFAs may also directly inhibit the proteolytic enzymes and not allow cleavage of SREBP-1. If this is the case, SREBP-1 would not be allowed to translocate to the nucleus and activate LDL receptor gene transcription. Thus, this describes how fatty acids do not have to act directly on a specific region of the gene, but how they can indirectly effect LDL receptor gene transcription through the sterol binding proteins. Non-sterol regulation of LDL receptor gene transcription is not a unique phenomenon as it has been also demonstrated by growth hormone (Pak et al., 1996). Regulation of the LDL receptor gene

transcription by these compounds is thought to occur not by altering sterol balance within the cell, but by directly acting upon the SRE-1 and SREBP-1 system.

Fatty acids have been observed to affect the transcription of other genes involved in lipid metabolism. Recent studies have shown that dietary PUFAs suppress the hepatic level of S14 protein, glucose-6-phosphate dehydrogenase and fatty acid synthase by inhibiting the transcription of the genes encoding these proteins (Clarke et al., 1990; Jump et al., 1993, 1994; Tomlinson et al., 1988). Montalto and Bensadoun (1993) demonstrated that adipose cell lipoprotein lipase (LPL) mRNA levels were depressed when cells were enriched with linoleic acid and EPA relative to oleic acid enriched cells, and they suggested that the fatty acids themselves maybe regulating the expression of this gene. The transcription rate and mRNA levels of fatty acid synthase, S14 protein and pyruvate kinase in rats fed a high glucose diet containing 10% triolein did not differ from rats fed a high carbohydrate, fat free diet (Jump et al., 1994). However, when the triolein was replaced with 10% menhaden oil for a single meal, the transcription rate of these genes decreased by 60% within 3 h. Conversely, when the menhaden oil was removed from the diet gene transcription was rapidly induced. In the case of the S14 gene, PUFA-regulated *cis* -acting elements have been localised to the proximal promoter of this gene (Jump et al., 1993). When PUFAs, triolinolein, trilinolenin, and triarachidonin were fed to mice, they were shown to decrease the level of hepatic stearoyl CoA desaturase-1 (SCD-1) mRNA, whereas saturated and monounsaturated fatty acids had very little effect on expression of this gene (Ntambi, 1995). These observations all suggest that PUFAs regulate the expression of several genes involved in lipid metabolism and that these genes may share common *cis* regulatory elements and/or *trans* factors that mediate PUFA repression of gene transcription. It is, therefore, possible that there may be regions present within the LDL receptor gene that also may be responsive to the binding of PUFAs.

Although modulation of the LDL receptor gene transcription by the direct binding of PUFAs to regulatory regions within the gene suggests a possible mechanism whereby PUFAs downregulate gene transcription of the LDL receptor in HepG2 cells, there does not, however, appear to be any direct evidence thus far to support the arguments stated herein. Thus, alternate mechanisms must be explored.

4) Extracellular factors

An alternate theory is that fatty acids, such as PUFAs, can act directly or indirectly through other factors to regulate the LDL receptor. This may, or may not be independent of fatty acid effects on intracellular sterol levels. For example, the plasma levels of certain factors, such as insulin (Wade et al., 1988) or tumour necrosis factor-α (TNF-α), have been shown to be altered by PUFA intake (Hamaka et al., 1992). Interestingly, LDL receptor activity has also been shown to be affected by these factors. This introduces the possibility that the effect of PUFAs on the LDL receptor may represent the effect of extracellular factors

which have been induced as a consequence of PUFA intake. However, it is exceedingly difficult to distinguish between the effect of the fatty acid and the effect of these extracellular factors on the LDL receptor. The problem becomes even more staggering when one examines the number of potential factors that can have an affect on LDL receptor gene transciption.

Many serum factors and hormones have been reported to regulate the LDL receptor in liver, such as interleukin-1ß (IL-ß) (Hamaka et al., 1992) oncostatin-M (Grove et al., 1991a), epidermal growth factor (EGF) (Grove et al., 1991b), insulin (Wade et al., 1988), estrogen (Semenkovich and Ostlund, 1988), and growth hormone (Pak et al., 1996). The mechanism(s) by which such a diverse group of factors would regulate the LDL receptor is unclear. The best studied example of LDL receptor gene regulation to date is the sterol-mediated feedback repression of transcription. If this were a common event, induction of LDL receptor by growth factors, cytokines, and hormones would be initiated by a change in a critical regulatory pool of sterol that could then alter the amount or activities of a nuclear protein, such as SREBP, to activate transcription of the LDL receptor promoter. However, could these hormones also directly affect the SREBP/SRE system independent of their effects on intracellular sterol?

Evidence supporting that nonsterol regulation of the LDL receptor occurs in cells is increasing. In addition, it opens an extremely interesting possibility that fatty acids may also operate through the SREBP mechanism. The questions, that can be raised from this hypothesis would be (1) why would cells need or allow the existence of such control mechanisms by fatty acids and (2) would fatty acid regulation override or occur simultaneously with sterol regulation?

5) Equilibrium of cell fatty acids

The results herein have strongly suggested that fatty acids can override the downregulation of the LDL receptor by exogenous cholesterol in HepG2 cells. The implication is that sterols may not be the sole regulator of LDL receptor gene transcription as many think, but that fatty acids may be just as important. This hypothesis is based on the idea that it is the cell's requirement for various fatty acids, and not cholesterol, may dictate the induction or repression of the LDL receptor. In addition, this type of regulation can be independent of the cell's requirement for cholesterol. If the cells can maintain a constant level of cholesterol despite the modulation of the LDL receptor for fatty acid balance, then this type of regulation is possible.

As evidence suggests that cholesterol is regulated at multiple levels including biosynthesis, esterification, and receptor mediated endocytosis, (Grundy, 1991; Goldstein and Brown, 1977; Brown and Goldstein, 1986), then cholesterol entering the cell may not have major detrimental consequences to cell metabolism. For example, when cholesterol comes into the cell, the cell can either store it as cholesterol esters, excrete it out of the body

as bile acids, inhibit the endogenous synthesis of cholesterol, or package the cholesterol as VLDL and release VLDL into the plasma (Grundy, 1991; Goldstein and Brown, 1977; Brown and Goldstein, 1986) (see section 1.7.6 for detail). Therefore, the control of cholesterol coming into the liver cell can be accommodated in many ways, and strict control of the LDL receptor is not required to maintain cellular cholesterol homeostasis or meet cholesterol needs.

It can be speculated that the role of the LDL receptor may be more important in maintaining fatty acid balance in the liver cell. Although the liver is responsible for the synthesis of fatty acids and triacylglycerols (TG), it can not store large quantities of these fatty acids in the form of triacylglycerols, and therefore, exports them to the adipose tissue for storage (Sweester et al., 1987; Thompson, 1989). In contrast to cholesterol, the processing of hepatic TGs is limited. Fatty acids from TGs can be hydrolysed via ß-oxidation, or they can be packaged up in VLDL particles and sent to other tissues in the body for storage (Thompson, 1989). Cells must be able to store or metabolise the fatty acids so that over-accumulation does not cause cell membrane damage (Potter et al., 1989). If the pathways mentioned above are inadequate for handling the influx of fatty acids coming into the cell, then the main control may lie in repressing the LDL receptor pathway to inhibit the uptake of these fatty acids. This type of control can become especially important with long chain, potentially cytotoxic fatty acids, such as linoleic acid and EPA, which enter the cell via LDL. Therefore, the regulation of hepatic LDL receptor by fatty acids rather than by cholesterol would be more important in order to maintain cellular fatty acid balance rather than cholesterol homeostasis.

If the regulation of LDL receptor expression (activity, protein and mRNA levels) is more important and more powerful by fatty acids than by cholesterol, then it would follow that the fatty acid effects should be dominant. In other words, the fatty acids should be able to overpower and negate the suppressive effects of cholesterol at the level of LDL receptor gene transcription. This hypothesis is supported by the findings herein (Figure 5.4) in which palmitic, oleic and linoleic acid were all able to overpower the suppression of cholesterol at the level of gene transcription, although they differed in their ability to overcome this suppression.

LDL receptor was suppressed by EPA even in the absence of cholesterol (Figure 5.2-5.4). Therefore, while it appears as if EPA was not able to completely overpower the suppressive effects of cholesterol, it can be argued that the suppression of the LDL receptor was due the independent effect of EPA. It has to be taken into consideration that LDL receptor activity with EPA in the absence of cholesterol in the background media was identical to the effect of EPA in the presence of cholesterol. It may be coincindental that the effect of EPA is exactly the same as control cells with or without cholesterol. Thus, one can argue that EPA, like the other test fatty acids, could have an independent effect from the actions of cholesterol. The cells may be more susceptible to downregulation of the LDL

receptor by linoleic acid and EPA, as these long chain fatty acids are potentially more cytotoxic than saturated and monounsaturated fatty acids at the same concentration (Potter et al., 1989). Therefore, it may necessary for the cells to have more stringent regulation over these fatty acids entering into the cell via the LDL receptor.

It seems that the LDL receptor may have dual regulation by both fatty acids and cholesterol rather than sole control by sterols. Dual control of the LDL receptor expression by these compounds may occur through similar mechanisms. However, control may involve fatty acid specific transcription factors or distinct elements within the control region of LDL receptor gene which can recognise fatty acids such that LDL receptor gene transcription can be regulated to satisfy the cells fatty acid requirements as discussed above.

6) Cholesterol and lipoprotein metabolism

An alternative theory is based the idea that LDL receptor expression (activity, protein and mRNA levels) is associated with only endogenous cholesterol metabolism. There appears to be more documented evidence in the literature demonstrating fatty acid effects on cholesterol esterification, cholesterol biosynthesis, and lipoprotein metabolism. Perturbations in the intracellular cholesterol concentration or the putative "metabolic pool of cholesterol" by fatty acids through one of the processes decribed below may lead to changes in LDL receptor gene transcription.

6a) Biliary cholesterol excretion

One can speculate that if fatty acids interfere with the hepatic biliary excretion of cholesterol, then they may indirectly contribute to an increase in the concentration of metabolically active cholesterol within liver cells. According to the study by Berr et al. (1993), hamsters fed 9% (w/w) ω-3 PUFA rich diet had a very low excretion of cholesterol, compared to animals fed a diet supplemented with 9% (w/w) saturated coconut fat. Inhibition of cholesterol excretion by ω-3 fatty acids could also lead to an increase in the concentration of cellular cholesterol in liver cells. In order to maintain cholesterol balance, liver cells probably have to compensate by suppressing both endogenous cholesterol synthesis and the LDL receptor.

6b) VLDL synthesis and secretion

The interference in VLDL synthesis and secretion by PUFAs may also ultimately lead to the expansion of the free cholesterol pool, thereby causing a decrease in the LDL receptor. Studies have demonstrated that linoleic acid appears to cause a reduction in VLDL levels by affecting VLDL synthesis and by decreasing its secretion from the liver (Chait et al., 1974; Cortesse et al., 1983). It is even better documented that the intake of marine fish oils rich in ω-3 PUFAs can significantly reduce plasma VLDL triacylglycerol concentrations. The effect with ω-3 PUFAs, however, is far greater than the effect exhibited with equal amounts of ω-6

rich vegetable oils (Nestel et al., 1984; Harris et al., 1983). Nestel and others have shown both *in vivo* and *in vitro* that the reduction in plasma VLDL concentration that is associated with fish oil consumption is due to reduced synthesis and secretion of VLDL triacylglycerol and apoprotein B (Nestel et al., 1984; Wong and Nestel, 1987; Wong et al., 1989). The mechanism of triacylglycerol lowering appears related to the inhibition of VLDL triacylglyceride secretion due to a decrease in hepatic triacylglycerol synthesis (Wong and Nestel, 1987; Wong et al., 1989; Lang et al., 1990) but not apo B synthesis (Wong et al., 1989). This has been shown in perfused rat liver and isolated rat hepatocytes. Even though fish oil fatty acids do not affect apo B mRNA abundance, they do, however, stimulate the intracellular degradation of newly synthesised apo B protein, leading to a decrease in the secretion of apo B (Wang et al., 1993; Wong et al., 1989). Furthermore, fish oil feeding has been shown to diminish lipogenesis, and to increase ketogenesis and fatty acid oxidation (Illingworth et al., 1984; Wong et al., 1983). These can all contribute to the overall reduction in triacylglyceride secretion by the liver and lower plasma VLDL. The decreased synthesis and secretion of triacylglycerol, in addition to decreased concentration of apo B, with EPA enrichment contributes to the diminished synthesis and secretion of the VLDL particles out of the cell. As cholesterol is also a constituent of VLDL, the decreased synthesis and secretion of VLDL could also, in theory, result in the accumulation of unpackaged endogenous cholesterol within the cell. Consequently, the increase of unincorporated cholesterol with ω-3 fatty acid intake can lead to an overall increase in hepatic free cholesterol leading to the decline of the LDL receptor.

6c) Cholesterol biosynthesis and secretion

Further evidence from Schafer and Kattermann (1992) indicates that cholesterol biosynthesis and secretion in HepG2 cells is affected to various degrees when they are incubated with palmitic, oleic, linoleic acids and EPA. They observed that cholesterol biosynthesis and secretion in HepG2 cells incubated with 200 μM palmitic acid increased 60% and 40%, respectively, compared to control cells incubated without fatty acids. In contrast, when cells were incubated with 200 μM linoleic acid, cholesterol biosynthesis and secretion decreased by 65% and 65% compared to cells incubated without fatty acids. Similarly, cholesterol biosynthesis and secretion decreased by 75% and 50% in cells incubated with 200 μM EPA compared to cells incubated without fatty acids. Thus, the decrease in cholesterol secretion would imply that these fatty acids may indirectly lead to the build up of cholesterol in the cell and thereby, cause a decrease in LDL receptor production. Concomitant with the effect of fatty acids on cholesterol biosynthesis and secretion, the fatty acids influenced cholesterol esterification such that palmitic acid increased cholesterol esterification, while linoleic acid and EPA decreased cholesterol esterification compared to control cells (see below). Thus, the effect of the fatty acids on the LDL receptor seen in the work herein maybe a reflection of the fatty acid effects on cholesterol secretion.

6d) Cholesterol esterification

If fatty acids affect cholesterol esterification by influencing ACAT activity then, indirectly, they will change the intracellular cholesterol content. Numerous studies have documented that human fibroblasts, rat liver cells and CaCo-2 (human colon carcinoma cell line which differentiates in cell culture) enriched with EPA exhibit 50-60% lower ACAT activity compared to cells enriched with saturated, monounsaturated and ω-6 PUFAs (Pal and Davis, 1991; Rustan et al., 1988; Murthy et al., 1988). Furthermore, fibroblasts incubated with linoleic acid have decreased ACAT activity compared to cells incubated with saturated fatty acids (Mathur et al., 1983; Pal, 1990). Decreased cholesterol esterification by EPA or linoleic acid may result in an expansion of the intracellular "metabolic cholesterol pool", thereby forcing cholesterol to induce a negative feedback effect on LDL receptor gene transcription. Inhibition of the LDL receptor in HepG2 cells by EPA or linoleic acid enrichment, therefore, may be a consequence of its primary effect on cholesterol esterification.

Conversely, Rumsey et al. (1995) reported that more cholesterol esters were formed in human J774 macrophages incubated with palmitic acid than cells incubated with linoleic acid. Thus, saturated fatty acids may efficiently activate ACAT activity, thereby causing a reduction in the regulatory pool of unesterified cholesterol and an increase in the LDL receptor gene expression.

Interestingly, Schafer and Katterman (1992) also investigated the effects of palmitic, oleic, linoleic acid and EPA on cholesterol esterification in HepG2 cells. Compared to cells incubated with 100 μM palmitic acid or oleic acid, which had similar effects, cholesterol ester biosynthesis was reduced by linoleic acid and EPA. The findings from this paper and others discussed above (Pal and Davis, 1991; Rustan et al., 1988; Murthy et al., 1988) parallels the effect of the fatty acids on LDL receptor gene expression observed herein.

6e) Conclusions: Effects of PUFAs on cholesterol metabolism

There appears to be strong support from numerous studies (see above) to show that PUFAs can increase the intracellular cholesterol content by affecting many pathways of cholesterol metabolism, such as, cholesterol esterification, secretion, biliary excretion, VLDL synthesis and secretion. Thus, the increase in intracellular cholesterol content by the action of PUFAs on these metabolic parameters may be the primary mechanism whereby they modulate LDL receptor gene transcription.

SUMMARY

In summary, LDL receptor expression (activity, protein and mRNA levels) in human liver HepG2 cells decreased as the degree of unsaturation of the fatty acid increased. This inverse association was observed either in the presence or absence of cholesterol in the

growth media. The response of LDL receptor binding activity to dietary fatty acids was directly correlated to the changes that had occurred in LDL receptor protein and mRNA levels. This implies that the changes in LDL receptor activity by fatty acids were due to perturbations at the level of LDL receptor gene transcription. Furthermore, the effect of the fatty acids observed on LDL receptor mRNA levels was reproducible using a fluorescent assay to quantify cellular LDL receptor mRNA (data not shown, see section 3.5 for details).

Evidence advocating that the LDL receptor is affected by fatty acids through their influence on intracellular cholesterol levels is compelling compared to the theory that they may affect the LDL receptor directly by binding to an element within the LDL receptor gene promoter or through their effect on membrane fluidity. Using a cell culture system ensured that extracellular factors were not responsible for the observed effects on the LDL receptor, but rather the fatty acids were directly associated with the response of the cell.

Chapter 6

6.0 THE EFFECT OF α-TOCOPHEROL ON LDL RECEPTOR EXPRESSION (ACTIVITY, PROTEIN, mRNA)

6.1 Introduction

The findings in Chapter 5 indicated that different fatty acids can affect the LDL receptor expression (activity, protein, mRNA) in a distinct manner in HepG2 cells. There was an inverse relationship between the number of double bonds in the fatty acid carbon chain and the LDL receptor. In other words, expression of the LDL receptor decreased as the polyunsaturation of the fatty acid increased (Figure 5.4). Furthermore, the effect of EPA on the LDL receptor was observed even in the presence of the antioxidant, vitamin E (α-tocopherol form), suggesting that the modulation of the LDL receptor by EPA was unlikely to be as a result of its oxidation (Figure 5.7). Similarly, LDL receptor protein levels were also decreased when cells were incubated with linoleic acid ± vitamin E (see Chapter 5). Interestingly, it was also observed during these experiments that vitamin E alone also had an effect on LDL receptor activity in HepG2 cells (Figure 5.7). This effect of vitamin E was independent of the effect exhibited by the PUFAs, EPA and linoleic acid (vitamin E herein is referred to as α-tocopherol analogue of vitamin E, unless otherwise specified).

Evidence suggests that there is a strong inverse relationship between the consumption of vitamin E and the risk of coronary heart disease (CHD) (Rimm et al., 1993; Stampher et al., 1993; Gey et al., 1991). Intervention studies in animals have also indicated that vitamin E is antiatherogenic (Chupukcharoen et al., 1985; Westrope et al., 1982). There are many postulated mechanisms whereby this nutrient could prevent CHD. Many studies have suggested that vitamin E inhibits the oxidation of LDL (Muggli, 1994; Van Acker et al., 1993) (see section 1.4 and 1.6.2 for details) and may protect arteries from atherogenic effects of oxidised LDL. The oxidation of LDL is believed to be a key step in promoting atherogenesis and it is thought to be prevented by antioxidants (Witztum, 1994).

LDL oxidation may be vital in the pathogenesis of CHD, however, some studies have shown that a decrease in the oxidative susceptibility of the LDL particle by vitamin E (α-tocopherol) was not sufficient to repress atherogenesis when cholesterol levels were markedly elevated and also when the LDL receptor was not functioning (Kleinveld et al., 1994; Fruebis et al., 1994). This was demonstrated in WHHL rabbits (LDL receptor deficient rabbits) with diets supplemented with vitamin E (Kleinveld et al., 1994; Fruebis et al., 1994). Vitamin E treatment had no significant effect on body weight, lipoprotein profiles or cholesterol levels. The protection of plasma LDL against oxidation was determined *in vivo* by measuring the lag time in the formation of conjugated dienes in a standard Cu^{2+}-containing system. This lag time was increased four-fold in the vitamin E treated group over that in controls, indicating a greater degree of antioxidant protection of

LDL. Vitamin E, however, failed to impede atherosclerosis in these vitamin E supplemented WHHL rabbits, suggesting that the prevention of LDL oxidative modification by antioxidants had no relevance to protection against atherosclerosis. Another study by Suarna et al., (1995) suggested that there was no connection between the presence vitamin E in the arterial wall and the formation of oxidised lipids in plaque. They reported that the amount of vitamin E was similar in normal and atherosclerotic arteries, and that human atherosclerotic plaque co-existed with large amounts of oxidised lipids and vitamin E. Thus, the protection afforded by vitamin E against atherosclerosis, when seen, may have been due to other effects of vitamin E.

In studies with animals and cultured cells, vitamin E (α-tocopherol) has been observed to inhibit abnormal cell processes and interactions which can contribute to the initiation and progression of atherosclerosis (Janero, 1991; Shiina, et al., 1993; Slim et al., 1996). For example, studies indicate that vitamin E can influence the response of cells that contribute to the initiation and/or progression of spontaneous atherosclerosis (Janero, 1991). Vitamin E has been shown to inhibit endothelial injury under oxidative stress and help maintain homeostasis by regulating important endothelium metabolic and enzymatic processes (Keaney et al., 1993; Hennig et al., 1988). Furthermore, in cell culture studies, vitamin E has been shown to inhibit monocyte-macrophage cytotoxicity, smooth muscle cell proliferation, and reduce the cholesterol ester content in these types of cells (Orekhov et al., 1986; Miller et al., 1979).

Given that vitamin E (α-tocopherol) can modulate such cellular processes, it is conceivable that this essential nutrient can regulate the LDL receptor pathway as the preliminary observations reported in Chapter 5 indicated. The vitamin E regulation of this pathway could be a major contributing factor in the prevention against CHD. As a fat soluble vitamin, α-tocopherol is generally carried in the plasma by lipoproteins, mainly LDL, (George Wolf, 1993; Kayden and Traber, 1993) (see introduction, section 1.8.5.2) and taken up by the cells through the LDL receptor. It is interesting, then, that this vitamin may affect LDL receptor activity and possibly its gene expression because the cellular uptake of this vitamin occurs through the LDL receptor pathway, similar to the mechanism of cholesterol homeostasis. It may, therefore, follow that the cellular requirement of α-tocopherol, like cholesterol, may regulate the expression of the LDL receptor. Nevertheless, this is the first evidence that α-tocopherol can regulate the LDL receptor expression.

The observation that vitamin E (α-tocopherol) affected LDL receptor activity independent of fatty acids (Figure 5.7), therefore led to a series of experiments investigating the effect of α-tocopherol on LDL receptor expression (activity, protein and mRNA). As mentioned earlier (section 1.8.5.2), there are 8 naturally occurring homologues of vitamin E; four tocopherols (alpha (α), beta (β), delta (δ) and gamma (γ)) and four tocotrienols (George Wolf, 1993; Kayden and Traber, 1993). The tocopherols differ from each other in the number and position of the methyl groups around the ring structure which gives them

different biological potencies (George Wolf, 1993; Kayden and Traber, 1993; Van Acker et al., 1993). Thus, other tocopherol species were also examined to further investigate whether the upregulation of the LDL receptor expression was uniquely associated with α-tocopherol or whether other tocopherol species would have similar or different effects depending on their biological and/or antioxidant properties. The δ- and γ-tocopherols were chosen since these species have lower biological (10 % and 3 %, respectively) and lower antioxidant capacities (50% and 10%, respectively) compared to α-tocopherol (Van Acker et al, 1993). The major form of vitamin E is the α-tocopherol species as it is preferentially retained in the liver by the hepatic α-tocopherol binding protein (see introduction section 1.8.5.2).

The aim of the present study was, therefore, to ascertain whether α-tocopherol can regulate the LDL receptor activity. LDL receptor protein and mRNA levels were measured in an attempt to determine whether the regulation was transcriptional, translational or post-translational. To determine whether the regulation by α-tocopherol was specifically due to its high antioxidant capacity or its high biological activity, other tocopherols (δ- and γ-tocopherol) possessing lower antioxidant and/or biological properties were also examined.

6.2 METHODS

HepG2 cells were grown at 37^{o}C in DMEM supplemented with 10 % fetal calf serum (FCS). Cells were subcultured from large flask 175 cm^3 into small 25 cm^3 flasks, and allowed to grow without disruption for 48 hr in a background medium of DMEM supplemented with 10% FCS. Cells were enriched with various concentrations of vitamin E by directly adding this vitamin to DMEM, containing 10% FCS. Cells were incubated with this medium for 24 hours, harvested and analysed for vitamin E content (section 2.3), LDL receptor binding activity (section 2.6), LDL receptor protein (section 2.7) and LDL receptor mRNA levels (section 2.7).

6.3 RESULTS

6.3.1 Enrichment of HepG2 cells with α-tocopherol.

After incubation for 24 h in media containing from 0-100 μM α-tocopherol, cultured HepG2 cells and their media were immediately analysed by HPLC for their vitamin E content to ensure minimal breakdown of this vitamin (section 2.3). The analysis revealed that the α-tocopherol content of the cells and the media increased linearly relative to the concentration present at the start of the 24 h incubation (Figure 6.1). HepG2 cells were, therefore, able to effectively take up α-tocopherol at all concentrations.

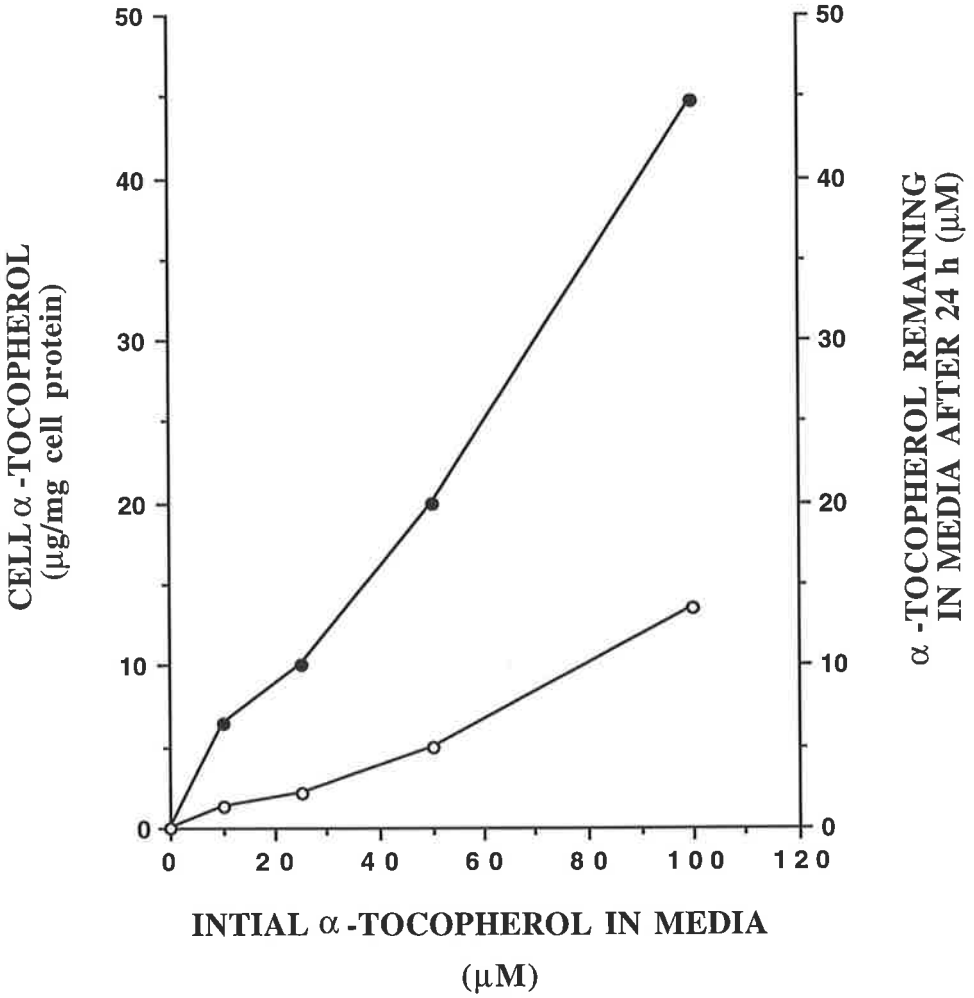


Figure 6.1: Enrichment of cells with α-tocopherol. HepG2 cells were incubated for 24 h at 37°C in media containing varying initial concentrations of α-tocopherol. After 24 h, the vitamin content of the cells (μg/mg cell protein) and the concentration of α-tocopherol remaining in the media (open circles) (μM) was measured by HPLC as described in section 2.3.

6.3.2 The effect of α-tocopherol on LDL receptor binding activity.

The LDL receptor activity of HepG2 cells incubated for 24 h in DMEM supplemented with 10% FCS and containing 0-100 μM α-tocopherol was measured by calcium-dependent binding of colloidal gold-LDL (section 2.6). The effect of α-tocopherol on the LDL receptor activity in HepG2 cells was found to be biphasic (Figure 6.2). The receptor binding activity progressively increased to 120% of control with increasing α-tocopherol concentrations up to 50 μM (Figure 6.2), but then decreased from this level with higher concentrations of the vitamin.

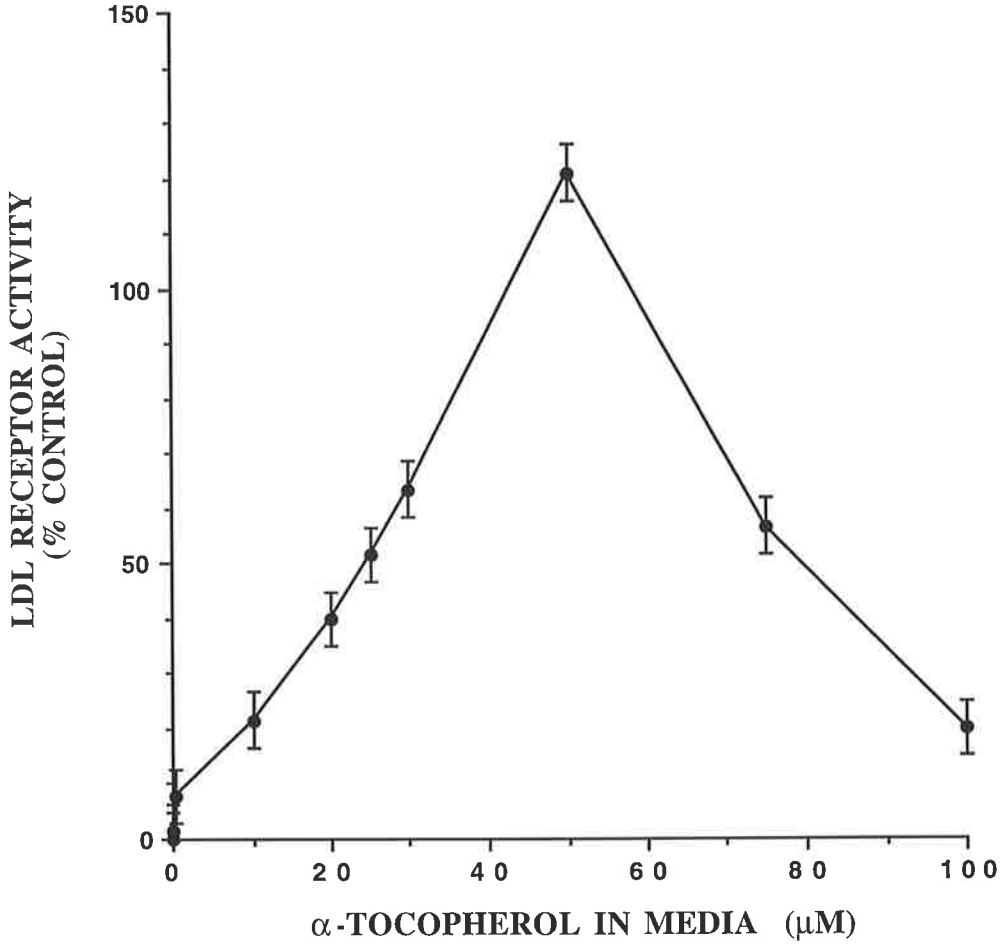
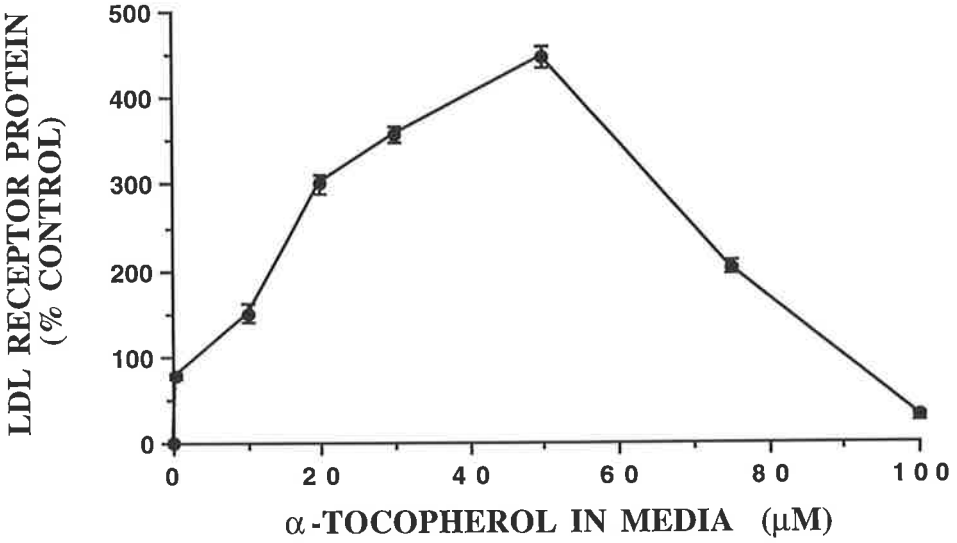


Figure 6.2: The dose dependent effect of α-tocopherol on LDL receptor binding in HepG2 cells. Cells were incubated for 24 h with increasing concentrations of α-tocopherol up to 100 μM in a background medium of DMEM containing 10% FCS. LDL receptor binding activity was measured in triplicate using the colloidal gold-LDL method as described section 2.6.3. Gold conjugates were incubated with 100 μg of cellular protein for 1 h at room temperature, either in the presence of $Ca(NO_3)_2$ to measure total binding or in the presence of EDTA to measure nonspecific binding. Specific binding was then calculated by taking the difference (section 2.6.3). Results represent the calcium-dependent (specific) LDL binding activity (ng of LDL/mg of cell protein) of the cells and data is expressed as a percentage (mean ± SEM of 3 experiments) of LDL receptor activity found in control cells which were not incubated with α-tocopherol. The absolute activity in control cells was 30 ± 4.4 ng LDL bound/mg cell protein.

6.3.3 LDL receptor protein in HepG2 cells with increasing concentrations of α-tocopherol.

Since changes in cellular LDL receptor binding activity usually reflect changes in receptor number (Brown and Goldstein, 1988, 1986; Rudling, 1992; Sanchez et al., 1995; Innerarity, 1991), the amount of LDL receptor protein was measured in HepG2 cells incubated for 24 h in media (supplemented with 10% FCS) containing 0-100 μM α-tocopherol. Using a polyclonal antibody against the LDL receptor, a single band at 130 Kd was visualised corresponding to a protein with the same mass as the LDL receptor. The effect of α-tocopherol on the amount of cellular LDL receptor protein was also biphasic. The intensity of the LDL receptor band progressively increased 4-fold above control with increasing concentrations of α-tocopherol up to 50 μM, but then decreased from this level with higher concentrations of the vitamin in the media (Figure 6.3). The biphasic changes observed in the LDL receptor activity in the HepG2 cells, are therefore, attributable to biphasic changes in the amount of LDL receptors in the cells.

A)



B)

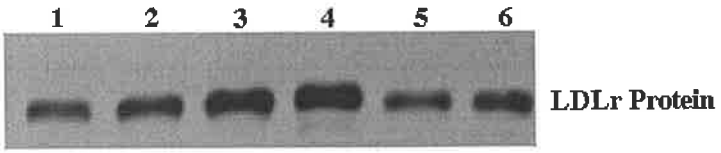


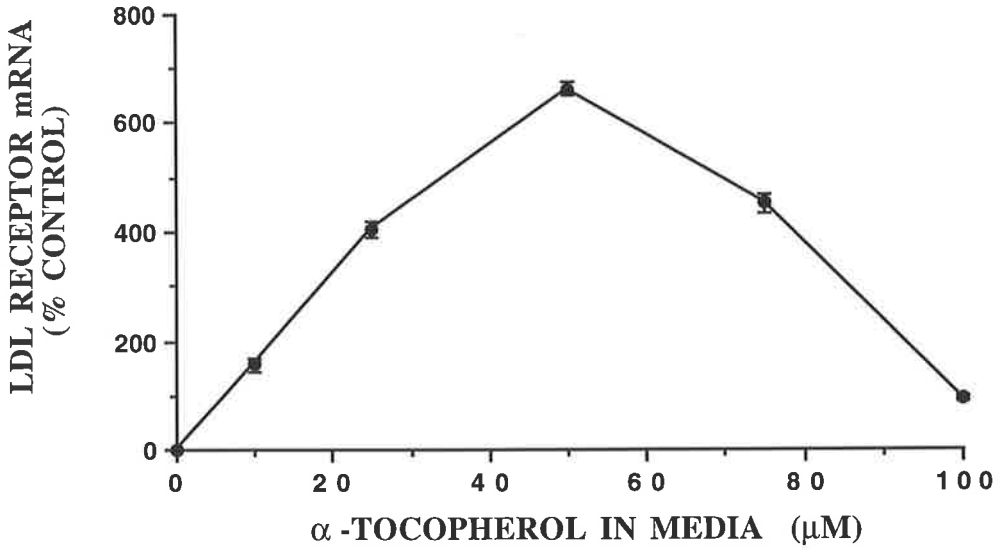
Figure 6.3: Dose dependent effect of α-tocopherol supplementation on LDL receptor protein. HepG2 cells were isolated after 24 h incubation with increasing concentrations of α-tocopherol. Cellular protein were solubilised with Triton-X 100 detergent, electrophoresed, transferred to nitrocellulose and quantified by the ECL method as described in section in 2.7. Experiments were performed in triplicate, and the relative densities of the receptor band were quantified using laser densitometry. **(A)** Data was expressed as a percentage (mean ± SEM of 3 experiments) of the LDL receptor protein found in cells not incubated with α-tocopherol. The absolute amount of LDL receptor protein present in control cells was 0.2 ± 0.09 (relative absorbance).**(B)** Autoradiograph of LDL receptor protein : different lanes represent various α-tocopherol concentrations (1) 0 μM, (2) 10 μM, (3) 25 μM, (4) 50μM (5) 100 μM.

6.3.4 The effect of α-tocopherol on the level of LDL receptor mRNA.

The LDL receptor was regulated by fatty acids at the level of gene transcription (Chapter 5). This regulation is usually reflected by changes in the amount of receptor mRNA. The effect of α-tocopherol on LDL receptor mRNA, was therefore determined using reverse transcription of cellular mRNA followed by PCR to amplify a specific cDNA sequence corresponding to the LDL receptor gene. Digoxygenin labelled dUTP was incorporated into the cDNA as a label in order for detection using an antibody (section 2.8).

The effect of α-tocopherol on LDL receptor mRNA was found to be biphasic (Figure 6.4). The amount of receptor mRNA progressively increased up to 7-fold above control levels with increasing concentrations of the α-tocopherol up to 50 μM. The amount of LDL receptor mRNA, however, then decreased from this level at higher concentrations of the vitamin (Figure 6.4). The biphasic changes observed in LDL receptor binding activity and protein in HepG2 cells are consequently attributable to biphasic changes in the amount of LDL receptor mRNA. This suggests a biphasic effect of α-tocopherol on the transcription of the LDL receptor gene.

A)



B)

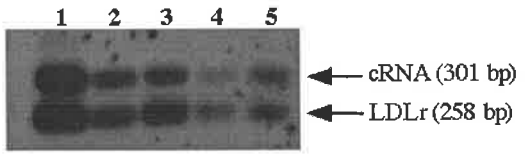


Figure 6.4: The dose-dependent effect of α-tocopherol on cellular mRNA levels. Cells were incubated with increasing concentrations of α-tocopherol for 24 h. Once total cellular RNA was isolated, it was reverse transcribed and amplified using PCR, along with a synthetic segment of RNA used as an quantitative internal control (section 2.8). Experiments were performed in triplicate, and the relative densities of the receptor band were quantified using laser densitometry.**(A)** Cellular mRNA levels were measured relative to the internal control and expressed as a percentage (mean ± SEM of 3 experiments) of the mRNA in control cells not incubated with α-tocopherol. The absolute value of the LDL receptor mRNA levels found in control cells was 4 ± 0.54 x 10^5 copies/μg RNA. **(B)** Autoradiograph of LDL receptor protein : where different lanes represent various α-tocopherol concentrations (1) 0 μM, (2) 10 μM, (3) 25 μM, (4) 50μM (5) 100 μM.

6.3.5: The effect of α-tocopherol on LDL receptor activity in the absence of cholesterol.

The previous experiments examined the effect of α-tocopherol on LDL receptor binding in HepG2 cells in the presence of normal growth media containing cholesterol added in the form of 10% FCS. To investigate whether α-tocopherol would induce the upregulation of the LDL receptor in the absence of cholesterol, HepG2 cells were incubated with 10% LPDS media for 24 h, and then subsequently for another 24 h with 50 μM α-tocopherol (Figure 6.5). Similar to the earlier results (Figure 6.2), α-tocopherol upregulated the LDL receptor binding activity by 128% at 50 μM in the absence of cholesterol. These results indicate that the upregulation of the LDL receptor by α-tocopherol is independent of cholesterol concentration.

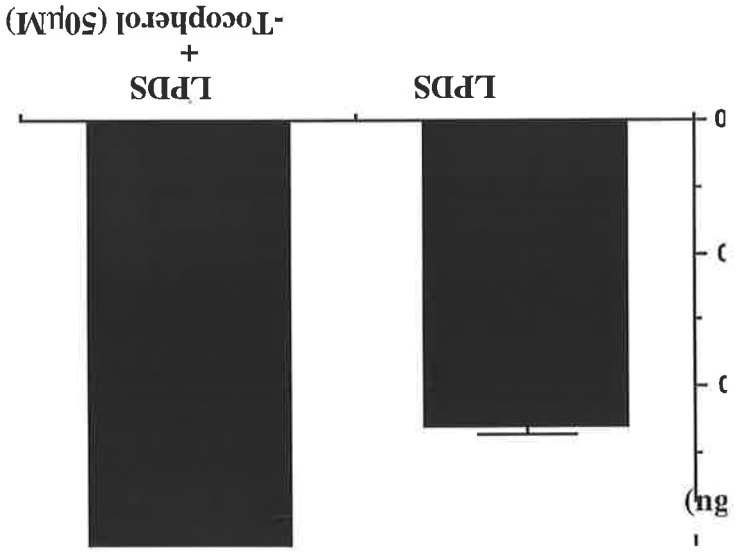


Figure 6.5: The effect of α-tocopherol on LDL receptor binding in HepG2 cells incubated in culture medium without cholesterol. Cells were incubated for 24 h with 50 μM α-tocopherol in a background medium of 10% LPDS. LDL receptor binding activity was measured in triplicate using the colloidal gold-LDL method as described section 2.6.3. Gold conjugates were incubated with 100 μg of cellular protein for 1 h at room temperature, either in the presence of $Ca(NO_3)_2$ to measure total binding or in the presence of EDTA to measure nonspecific binding. Specific binding was then calculated by taking the difference. Results represent specific LDL cell binding activity (ng of LDL/mg of cell protein) which is the mean ± SEM of 2 experiments.

6.3.6: The effect of α-tocopherol on the level of HMG-CoA reductase mRNA in HepG2 cells.

The LDL receptor and HMG-CoA reductase gene transcription has been shown to be coordinately regulated (Rudling, 1991). Thus, the level of HMG-CoA reductase mRNA was measured in cells incubated with increasing concentrations from 0-100 μM of α-tocopherol, to investigate whether α-tocopherol would also have a biphasic effect on HMG-CoA reductase gene transcription.

The level of HMG-CoA reductase mRNA increased up to 50 μM of α-tocopherol, but declined at higher concentrations (Figure 6.6). Thus, the transcription of HMG-CoA reductase gene was also found to be biphasic with increasing concentrations of the vitamin, similar to the mode of expression of the LDL receptor. This suggests that α-tocopherol may be coordinately regulating the gene transcription of both the LDL receptor and HMG-CoA reductase.

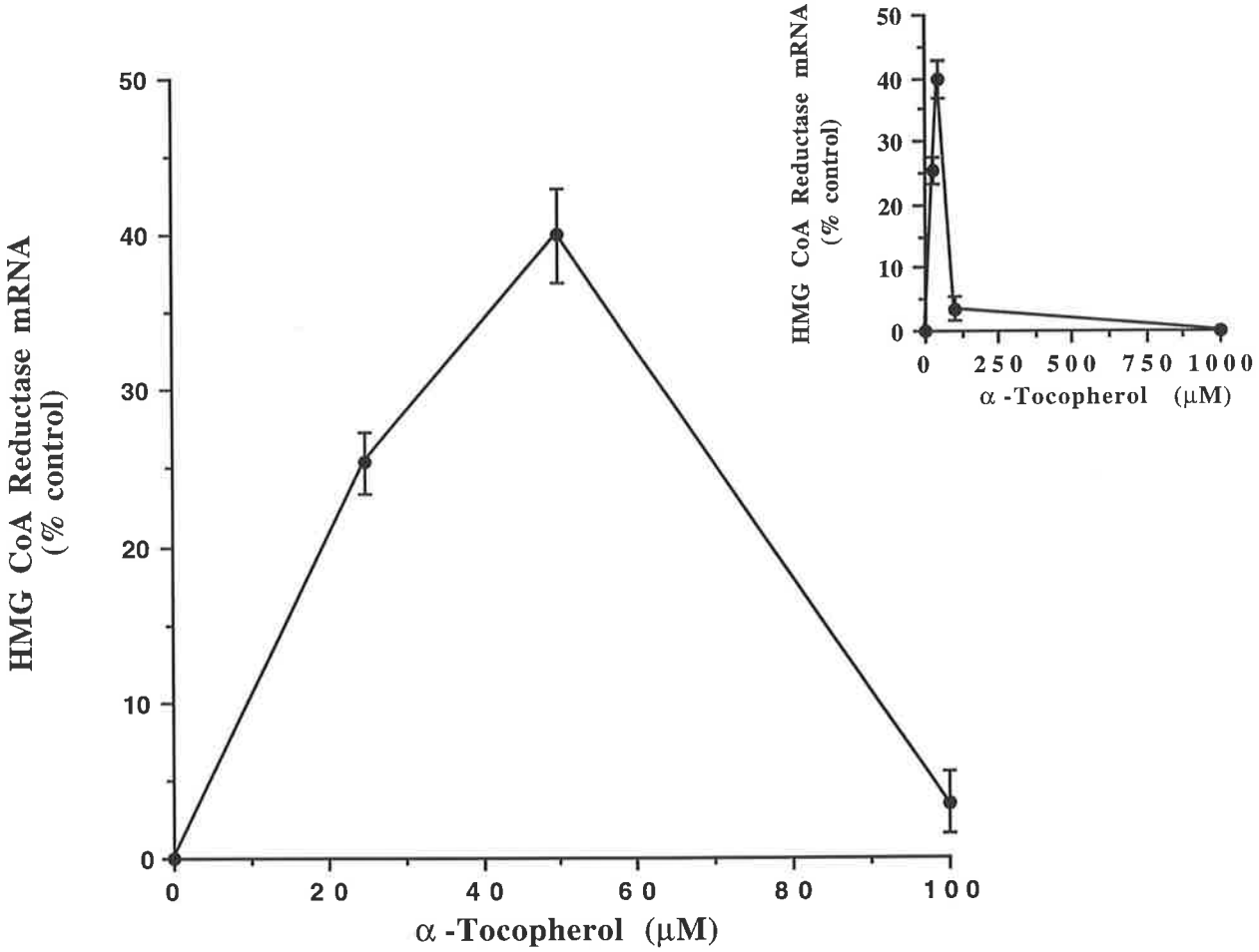


Figure 6.6: The dose dependent effect of α-tocopherol on cellular HMG-CoA reductase mRNA levels. Cells were incubated with increasing concentrations of α-tocopherol for 24 h. Once total cellular RNA was isolated, it was reverse transcribed and amplified using PCR, along with a synthetic segment of RNA used as a quantitative internal control (section 2.8). Experiments were done using triplicate measurements. Cellular HMG-CoA reductase mRNA levels were measured relative to the internal control and expressed as a percentage (mean ± SEM) of the reductase mRNA levels in control cells not incubated with α-tocopherol of 2 experiments. The absolute value of HMG-CoA reductase mRNA levels in control cells is 4.2 ± 0.8 x 10^5 copies/μg RNA.

6.3.7: The enrichment of HepG2 cells with different tocopherols.

The LDL receptor expression (activity, protein and mRNA) was measured in the presence of other tocopherol species to investigate whether the biphasic expression of the LDL receptor was uniquely associated with α-tocopherol or whether other tocopherol species could induce a similar effect. Compared to α-tocopherol, δ and γ tocopherol have lower biological activities and lower antioxidant capacities (Van Acker et al., 1993).

HepG2 cells were incubated for 24 h in media containing 0 to 100 μM α-, δ- and γ-tocopherols. Analysis of the cellular content using HPLC revealed that all three tocopherols were taken up equally well (Figure 6.7). The cellular content of each tocopherol increased linearly relative to the concentration of the vitamins present at the start of the 24 h incubation (Figure 6.7).

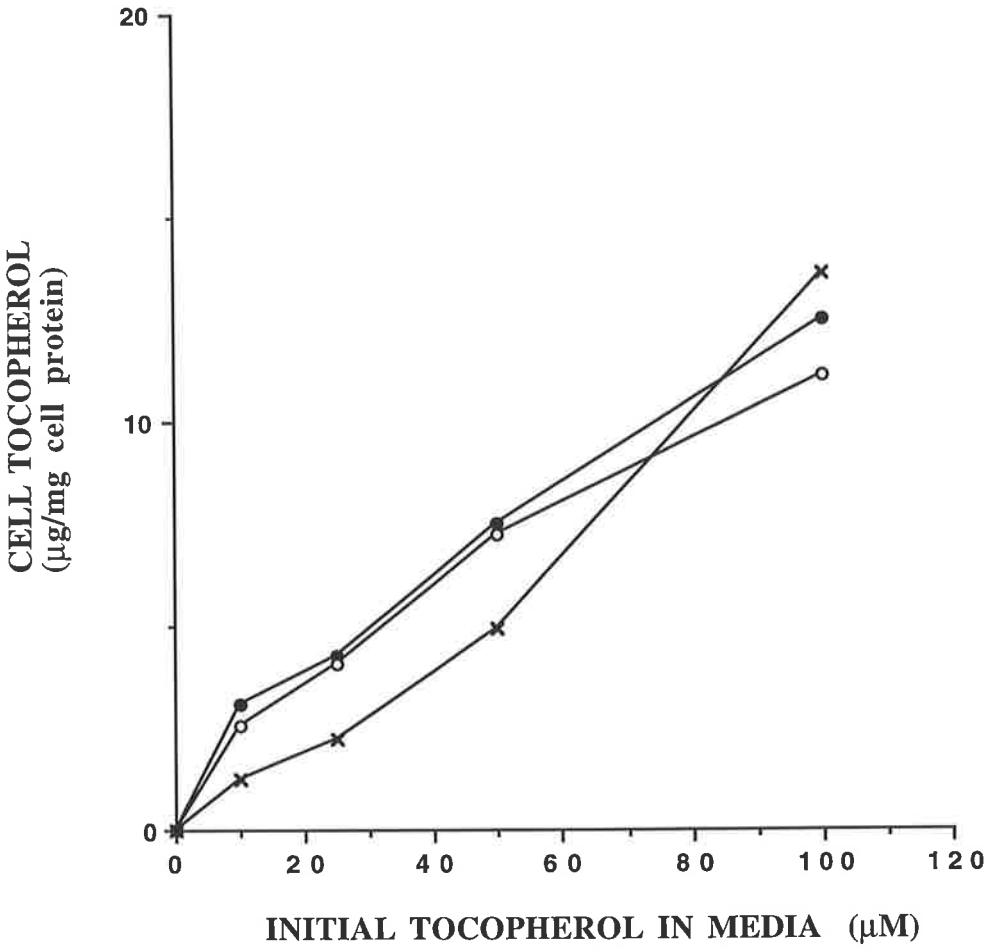


Figure 6.7: The enrichment of HepG2 cells with different tocopherols. Cells were incubated for 24 h in the presence of increasing concentrations of either α- (closed circles), δ- (open circles) or γ- (crosses) tocopherols. Cells were isolated and the tocopherol content of the cells was quantified using HPLC and expressed as μg of tocopherol/mg of cell protein (section 2.3).

6.3.8 The effect of different Tocopherols on LDL receptor binding activity, protein and mRNA levels in HepG2 cells.

The effect of δ- and γ-tocopherols on the LDL receptor was investigated in relation to α-tocopherol. The biphasic regulation with increasing α-tocopherol concentrations was again observed for LDL receptor binding activity (Figure 6.8), protein mass (Figure 6.9) and mRNA (Figure 6.10). However, in contrast to the biphasic effect associated with α-tocopherol, both δ- and γ-tocopherols suppressed LDL receptor activity (Figure 6.8). This decrease in LDL receptor activity was observed at all δ- and γ-tocopherol concentrations examined. Concomitantly, LDL receptor protein and mRNA levels were suppressed when cells were incubated with increasing concentrations of δ- and γ-tocopherol (Figure 6.9 and Figure 6.10). There was no evidence that the LDL receptor expression was upregulated at any concentration of δ- and γ-tocopherol tested. The decrease in LDL receptor activity in cells incubated with δ- and γ-tocopherols was, therefore a result of their effects on gene transcription. Thus, unlike α-tocopherol, these two tocopherol species suppressed LDL receptor expression.

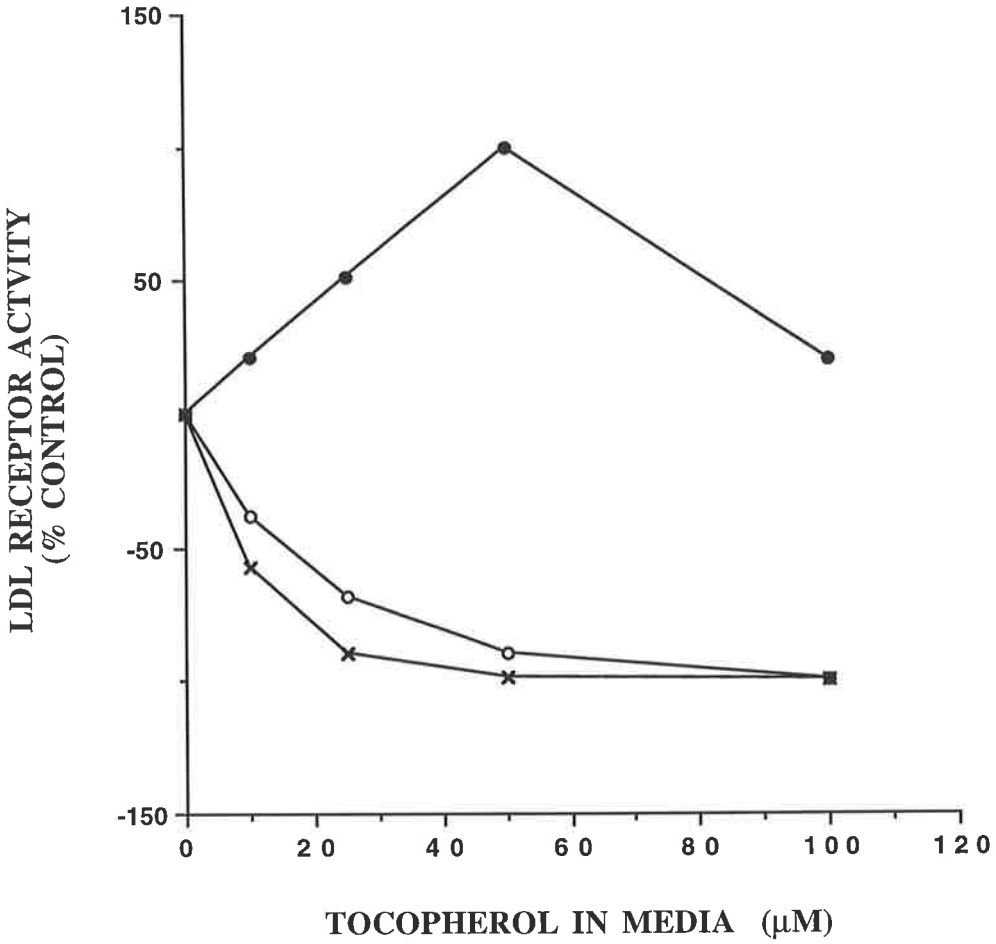


Figure 6.8: Comparison of different tocopherols on LDL receptor binding activity. HepG2 cells were incubated for 24 h with media containing increasing concentrations of either α- (closed circles), δ- (open circles) or γ- (crosses) tocopherols. LDL receptor activity was measured using the colloidal gold-LDL method (see section 2.6.3). Results represent the calcium-dependent (specific) LDL binding activity (ng of LDL/mg of cell protein) of the cells and data is expressed as a percentage of LDL receptor activity found in control cells (40 ng of LDL/mg of cell protein) which were not incubated with tocopherol.

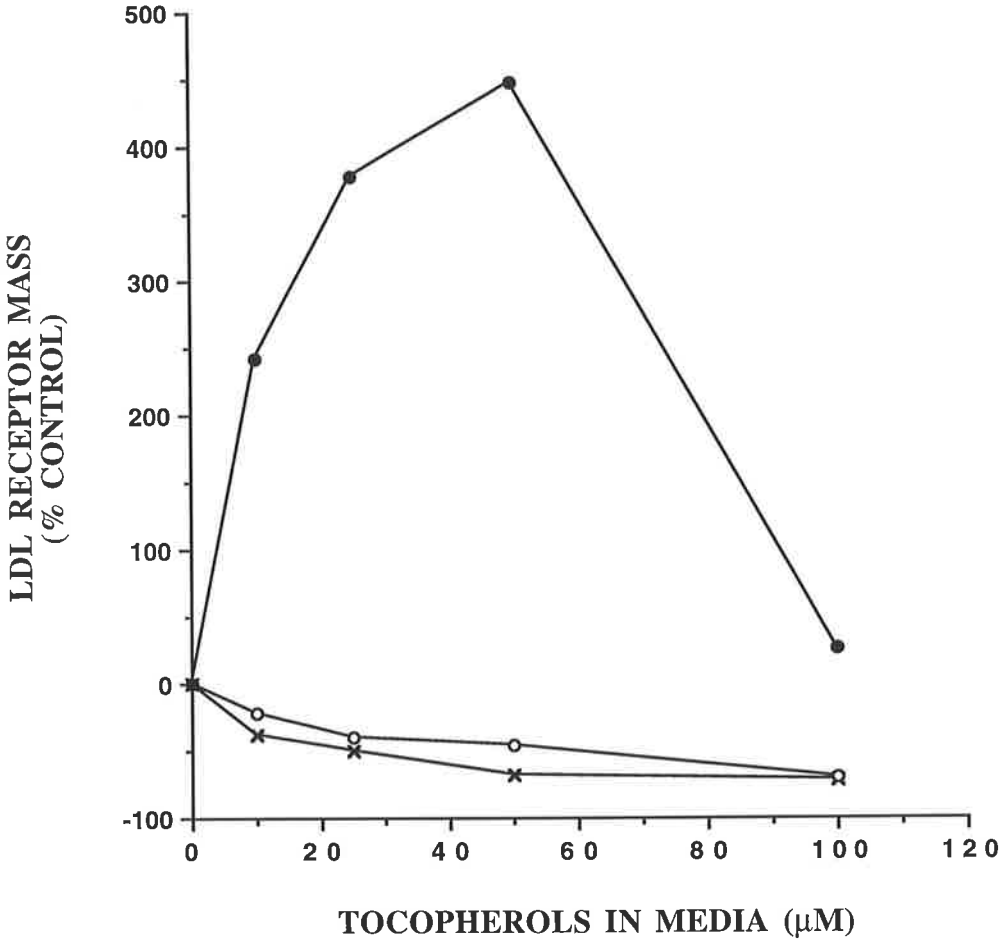


Figure 6.9: Comparison of LDL receptor protein in cells incubated with different tocopherols. LDL receptor protein was measured in cells incubated for 24 h with media containing increasing concentrations of either α- (closed circles), δ- (open circles) or γ- (crosses) tocopherols. The receptor protein in cells was measured using the ECL method as described in section 2.7. Relative densities of the LDL receptor protein band were quantified by densitometry and were expressed as a percentage of the control cells (0.85 relative absorbance) not incubated with any tocopherol.

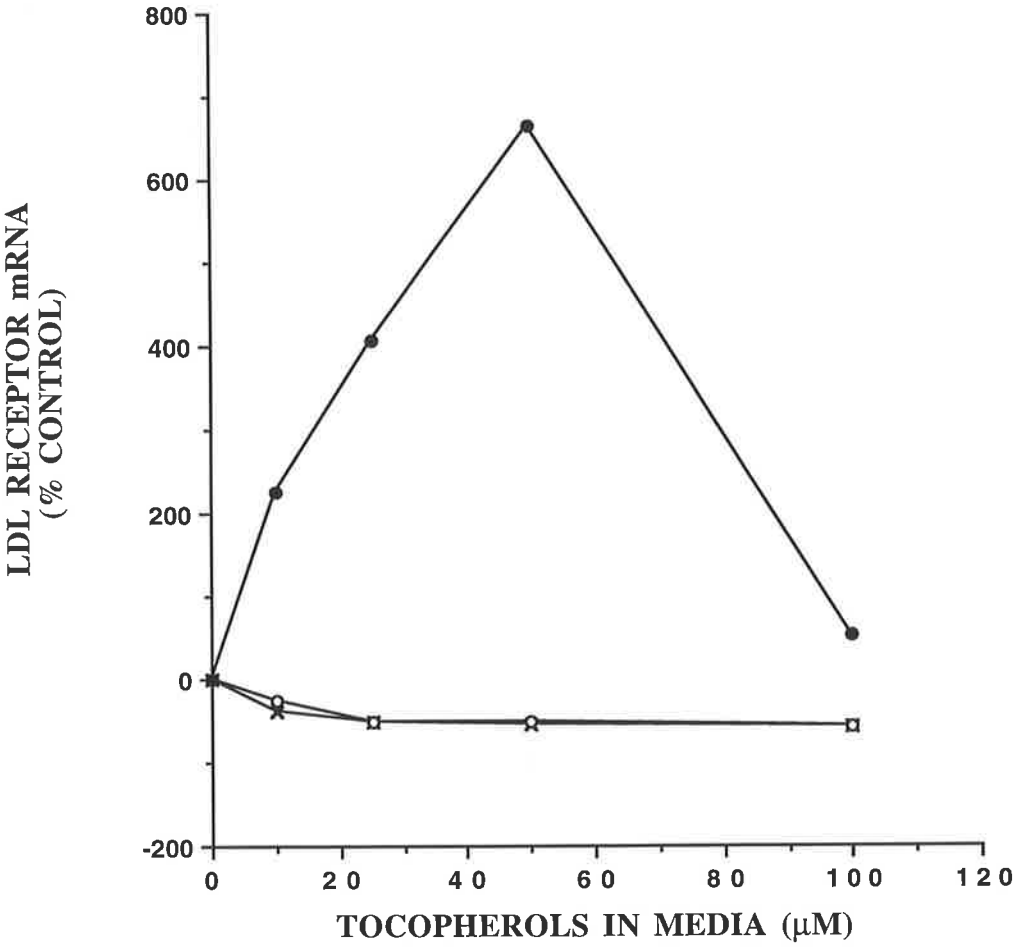


Figure 6.10: Comparison of LDL receptor mRNA levels in cells incubated with different tocopherols. The mRNA levels were quantified in cells incubated for 24 h in media containing either α- (closed circles) or δ- (open circles) or γ- (crosses) tocopherols. Total cell RNA was isolated, reversed transcribed, and amplified using PCR together with the internal control, pAW109 (as described in section 2.8). LDL receptor mRNA was quantified using the internal control and the data is expressed as a percentage of the control cells (4 x10 ^{5} copies/μg RNA) not incubated with any tocopherol.

6.4: DISCUSSION

The vitamin E homologue, α-tocopherol was found to regulate the LDL receptor in HepG2 cells in a biphasic manner. The LDL receptor was progressively upregulated with increasing concentrations of α-tocopherol up to a maximum of 50 μM. The expression of the receptor then decreased from this peak level at higher vitamin concentrations. This biphasic regulation was observed with LDL receptor binding activity, protein and mRNA, suggesting that the modulation of LDL receptor binding activity at the cell surface was a direct consequence of α-tocopherol inducing changes at the level of gene transcription. Similarly, a biphasic regulation of HMG-CoA reductase mRNA was observed with increasing concentrations of α-tocopherol, with a maximum level of mRNA attained at 50 μM. In contrast to α-tocopherol, δ- and γ-tocopherols suppressed the expression (activity, protein and mRNA) of the LDL receptor. These two vitamin E homologues progressively decreased LDL receptor mRNA, protein and binding activity with increasing concentrations, indicating that the upregulation of the LDL receptor was specific for α-tocopherol.

Changes in LDL receptor binding activity usually reflects the number of LDL receptor protein molecules present in the cell (Brown and Goldstein, 1986). Consistent with this mode of regulation, LDL receptor protein (Figure 6.3) was enhanced with increasing concentrations up to 50 μM α-tocopherol, concomitant with the changes in LDL receptor binding activity. There appeared to be, however, a small discrepancy in the magnitude of change between that observed in LDL binding activity (2-fold at 50 μM α-tocopherol, Figure 6.2) and LDL receptor protein (5-fold at 50 μM α-tocopherol, Figure 6.3). This can be accounted for by the fact that only a relatively small proportion of the LDL receptors are actually on the cell surface at any given point in time. The majority (approximately 80%) of the LDL receptors are localised within the intracellular recycling endosomes because the number of receptors that can be accommodated in the cell surface coated pits at one time is limited (Brown and Goldstein, 1986; Goldstein and Brown, 1977).

The amount of LDL receptor protein is usually determined by the level of cellular LDL receptor mRNA levels (Brown and Goldstein, 1986; Rudling, 1992; Sanchez et al., 1995; Innerarity, 1991). The LDL receptor mRNA was increased 7-fold with 50 μM α–tocopherol, explaining the change in LDL receptor protein (5-fold) and binding activity (2-fold) in HepG2 cells. The small discrepancy between the amount of mRNA and the amount of protein could result if there was inefficient translation of the message or rapid degradation of the mRNA.

Effect of tocopherols on lipoprotein levels

There is no evidence in the current literature that shows that α–tocopherol can directly effect the LDL receptor. There is, however, some indirect evidence suggesting that the observed effect of α–tocopherol on plasma cholesterol levels may be related to effects on LDL receptor activity on the hepatic cell surface. Vitamin E deficiency is known to elevate

plasma cholesterol in rats (Shirai et al., 1980) and chicks (Dam et al., 1952). When rabbits were fed a vitamin E-free basal diet for 6 weeks, the total plasma cholesterol levels were 60% higher than that of control rabbits (Chupukcharoen et al., 1985). The increase in plasma cholesterol was found in LDL, VLDL and chylomicron fractions, but not in the HDL fraction of plasma lipoproteins. The elevation in plasma LDL with vitamin E deficiency indicates that the clearance of these particles is perturbed, probably by the downregulation of the hepatic LDL receptor. One can extrapolate that plasma LDL concentration would be diminished with vitamin E supplementation as LDL receptor activity would be induced.

Accumulating experimental evidence in rabbits (Wilson et al., 1978; Westrope et al., 1982; Wojcicki et al., 1991; Subramanyam et al., 1993), guinea pigs (Qiao et al., 1993), primates (Verlangieri and Bush, 1992) and birds (Donaldson, 1982) has shown that vitamin E supplementation can ameliorate the hyperlipidemia which was induced by cholesterol and/or lard feeding and prevent the development of atherosclerosis. When rabbits were fed for 10 weeks on an atherogenic diet containing a coconut oil diet ± 1% of vitamin E, total plasma cholesterol and the level of plasma LDL decreased by 50% in animals supplemented with vitamin E. Aortic and coronary atherosclerosis were less frequent in these animals, suggesting that vitamin E can inhibit atherogenesis by preventing hypercholesterolemia (Westrope et al., 1982). The prevention of hypercholesterolemia with vitamin E supplementation was probably a result of enhanced hepatic LDL receptor activity, thereby increasing the uptake of atherogenic LDL particles from the plasma.

Fruebis et al. (1994) and Kleinveld et al. (1994) recently demonstrated that the hyperlipidemia could not be ameliorated with vitamin E supplementation in the WHHL strain of rabbits that are LDL receptor deficient. The lack of an effect by vitamin E in the WHHL rabbits suggests that vitamin E ameliorates hypercholesterolemia by signalling the induction of the LDL receptor pathway. If the upregulation of the LDL receptor by vitamin E holds true *in vivo*, it would explain why cholesterol levels are lowered in normal rabbits with functioning LDL receptors.

Harats et al. (1990) and Muckle and Nazir, (1989) have shown that vitamin E supplementation in humans can lower their plasma LDL cholesterol levels. The study by Harats et al. (1990) found that plasma cholesterol could be lowered by 5% with 600 mg/day of vitamin E supplementation over a period of 4 weeks. However, other studies have observed no change or an increase in plasma LDL levels (Jialial et al., 1995; Mezzetti et al., 1995). These differing results may be related to the observations herein that the regulation of the LDL receptor is bimodal. If regulation is bimodal *in vivo*, it is possible that differences in plasma cholesterol response to vitamin E supplementation are due to differences in initial vitamin E concentrations of the subjects. Therefore, both the starting vitamin E concentrations and dosage used are important, and would be reflected in the response obtained.

Effects of α-tocopherol on as related to its oxidation properties

The LDL receptor gene promoter elements have been shown to be sensitive to oxidation products of cholesterol. These compounds, called oxysterols, are much more potent inhibitors than cholesterol itself in downregulating cholesterol biosynthesis and LDL receptor expression. As mentioned, α-tocopherol is one of most powerful natural lipid soluble antioxidants. It is conceivable that in its presence, the oxidation of cholesterol may be inhibited, thereby preventing the formation of oxysterols. With decreased concentration of oxysterol by increasing concentrations of α-tocopherol, LDL receptor gene transcription would be less restricted. The upregulation of the LDL receptor gene by α-tocopherol could be explained by the inhibition of oxysterol formation.

To elucidate whether the upregulation of the LDL receptor with α-tocopherol was associated with its high antioxidant capacity, different homologues of vitamin E possessing different antioxidant potential's were, therefore investigated. Although the data suggests that all three forms of vitamin E (α, γ and δ) were equally retained within the cells (Figure 6.7), γ and δ-tocopherol decreased LDL receptor expression (activity, protein, and mRNA) in contrast to α–tocopherol (Figure 6.10). The lower antioxidant potential of δ- and γ-tocopherol may be inadequate to prevent the oxidation of sterols and therefore transcription of the receptor is suppressed with these other vitamin E homologues. One can speculate that the upregulation of the LDL receptor by α–tocopherol maybe related to its higher antioxidant capacity compared to δ- and γ-tocopherol.

Recent evidence indicates that vitamin E can act as a pro-oxidant (Ingold et al., 1993). If the concentration of the vitamin E radical (tocopheroxy radical) becomes too high and other antioxidants, such as vitamin C and ubiquinol 10, are unavailable to regenerate the radical back to vitamin E, then the radical itself becomes a powerful oxidant promoting cell damage and oxidation of lipids. The decrease in LDL receptor expression at concentrations of α-tocopherol above 50 μM may be related to the adverse pro-oxidant effect of this vitamin at high concentrations. If the suppression of LDL receptor expression occurred *in vivo* because of high α-tocopherol concentrations, then the result could be an increase in plasma cholesterol levels.

A study by Kushi et al. (1996) investigated the affect of dietary vitamin E on the relative risk of developing CHD in post menopausal women. They found the risk of death from CHD in women was inversely associated with consumption of vitamin E that was derived from food. In contrast, when they examined the intake of α-tocopherol supplements, the relative risk of developing CHD actually increased as the α-tocopherol supplementation increased. Thus, the inverse association of CHD and vitamin E intake was not observed for the intake of vitamin E from supplementation. The adverse effects of supplemented vitamin E may be related to its pro-oxidant properties at high concentrations. Hense et al., 1993 have reported that there was a lack of an association between serum

vitamin E and myocardial infarction in a study population with high vitamin E levels. They suggested that the high vitamin E levels may not be preventative against CHD.

High α-tocopherol levels have been shown to have adverse effects in cellular processes. A study by Godfried et al. (1989) observed a potentiation of atherosclerotic lesions in rabbits fed a high dietary levels of vitamin E. The animals that were fed 10,000 mg vitamin E/kg diet (ie, 250 x requirement) showed significant plaque formation compared to controls, with plasma cholesterol levels reaching a very high level of 9000-14,000 mg/l. The authors suggested that this level of vitamin E may be atherogenic and may account for their observations.

Endothelial vasculature function has also been shown to vary with the amount of α-tocopherol. Low doses of α-tocopherol improved endothelial dependent arterial relaxation in cholesterol fed rabbits, but was impaired in animals receiving high doses of α-tocopherol (10-fold more, 1100 IU/day) (Keaney et al., 1993). This variation in endothelial function may be related to the antioxidant activity of α-tocopherol at low concentrations and pro-oxidant activity at high concentrations. These studies are indicative of the possible oxidant effects of elevated α-tocopherol concentrations on cellular processes. The suppression of LDL receptor mRNA levels (Figure 6.4) observed at high vitamin concentrations may also be due to its pro-oxidant effects.

The downregulation of the LDL receptor at higher α-tocopherol concentrations was unlikely due to toxic affects of this vitamin. Total cellular protein synthesis was estimated to ensure that vitamin E enrichment did not perturb cellular processes as a result of vitamin toxicity (see section 2.5). The amount of [^{14}C]-serine incorporated into cellular protein did not change at any concentration of vitamin E examined (data not shown).

Effects of α–tocopherol as related to its biological properties

Given that α-tocopherol has the highest antioxidant potential and biological capacity compared to the γ and δ-tocopherol, the specific increase in LDL receptor expression by α-tocopherol could be related to one of these properties (see below, Table 6.1). If the increase in LDL receptor expression by α-tocopherol was due to its antioxidant potential, then one would expect that δ-tocopherol (antioxidant potential of 50 compared to α-tocopherol, see below, Table 6.1) would also be able to increase the LDL receptor. However, since this is not the case, the LDL receptor may be increased by α-tocopherol due to its high biological activity compared to the other analogues. Both γ and δ-tocopherol species have substantially lower biological activities (see below, Table 6.1) compared to α-tocopherol, suggesting that the downregulation of the LDL receptor by these 2 tocopherols may be related to this property.

The lower biological activities of γ and δ-tocopherol is attributable to their inability to bind to hepatic α-tocopherol binding protein (TBP) in the liver (George Wolf, 1993; Kayden and Traber, 1993) (see 1.8.5.2). Therefore, unlike α-tocopherol, they are present in the free

form which allows them to be excreted out of the body. Perhaps the presence of unbound α-tocopherol would also cause the depression of the LDL receptor, as seen when the concentration of α-tocopherol exceeds 50μM. At higher concentrations of α-tocopherol, the TBP would presumably be saturated, and therefore, lead to the presence of unbound α-tocopherol. However, this idea does not account for why LDL receptor expression would be upregulated by the presence of 0-50 μM α-tocopherol, unless the TBP also has a role in the upregulating the LDL receptor.

One would expect from analysing the properties of the different tocopherol species, that the higher biological activity of α-tocopherol (compared to the other tocopherol species) would be responsible for the upregulation of the LDL receptor. However, there does appear to be any concrete evidence to support this idea, therefore other mechanisms have to explored.

Table 6.1: Antioxidant Potential and Biological Capacity of the different Vitamin E Analogues.

Vitamin E analogue	Antioxidant Potential	Biological Capacity
α	100	100
δ	50	10
γ	10	3

Coordinate regulation of LDL receptor and HMG-CoA reductase mRNA levels

HMG-CoA reductase mRNA levels were also found to be regulated in a bimodal manner, suggesting that α-tocopherol may effect the transcription of the LDL receptor and HMG-CoA reductase genes through similar mechanisms. It appears that under most conditions tested, these genes are coordinately regulated at the mRNA level (Rudling, 1992), suggesting a common mechanism may be responsible for controlling transcription of these two genes. This implies that if α-tocopherol induces changes in gene transcription of HMG-CoA reductase, then the same change will occur in LDL receptor gene transcription because they are coordinately regulated. Coordinate regulation would explain the similar pattern of biphasic gene transcription (Figure 6.4 and 6.6). However, the relationship may not hold beyond the mRNA level. Unlike the LDL receptor, HMG-CoA reductase can be affected at the transcriptional, translational as well as at the enzymatic level (Rudling, 1992). Therefore, the activity of this enzyme does not necessarily reflect the level at which it is transcribed. Thus, α-tocopherol may decrease HMG-CoA reductase activity (Table 6.2) even when HMG-CoA reductase gene transcription is upregulated (Figure 6.6).

The effect of tocopherols on LDL expression (activity, protein and mRNA) may be associated to their affects on cholesterol metabolism :

The upregulation of the LDL receptor by α-tocopherol may be explained by the effect of this vitamin on cholesterol metabolism. For example, the mechanisms responsible for the upregulation of the LDL receptor by α-tocopherol could be related to a change in intracellular cholesterol metabolism, ie. cholesterol synthesis and/or its excretion from the liver cells. There is evidence from a number of studies indicating that when animals are made vitamin E deficient, cholesterol synthesis and degradation are affected. Chupukcharoen et al. (1985) observed that vitamin E deficient rabbits exhibited a decrease in the liver microsome activity of 7-α-hydroxylase with a concomitant increase in tissue cholesterol, indicating a block in cholesterol conversion of cholesterol to bile acids or disposal in the liver. The increase in cellular cholesterol would result in the downregulation of the LDL receptor in the absence of vitamin E. This would imply that vitamin E supplementation could increase 7-α-hydroxylase activity and result in a shift towards the production of bile salts into this pathway, and hence, cause the depletion of cellular cholesterol levels. Under such circumstances, vitamin E could indirectly lead to an upregulation of the LDL receptor by increasing the activity of 7-α-hydroxylase.

In the same study, liver homogenates from vitamin E deficient-rabbits exhibited an increased ability to synthesise cholesterol from [^{14}C]-mevalonate, thereby increasing the amount of free cholesterol within the cell. These observations suggest that the addition of vitamin E could inhibit the cholesterol synthetic pathway and deplete the cell of cholesterol, and hence upregulate the LDL receptor. However, this may not necessarily mean that HMG-CoA reductase mRNA levels would also be decreased, as HMG-CoA reductase has been shown be to regulated post-transcriptionally (Rudling, 1992). Nevertheless, if an increase in 7-α-hydroxylase activity and a decrease in cholesterol synthesis both occur simultaneously with α-tocopherol enrichment in HepG2 cells, the resulting depletion of metabolic free cholesterol could result in the upregulation of the LDL receptor.

Shirai et al. (1980) suggested that α-tocopherol plays an important role in lipid synthesis and degradation by influencing key enzymes involved in these pathways. When rats were fed an α-tocopherol deficient diet for 16 weeks, they exhibited decreased arterial ACAT activity, lysosomal acid lipase, acid cholesteryl esterase, acyl CoA synthetase, and triacylglycerol synthesising enzyme (Shirai at al., 1980). Conversely, the presence of this vitamin may activate enzymes such as ACAT, thereby decreasing the free cholesterol content within the cell and leading to the induction of LDL receptor gene expression. The metabolic consequences of α-tocopherol on these enzymatic pathways could explain the observed increase in the LDL receptor in HepG2 cells. If the deficiency of α-tocopherol can lead to the suppression of ACAT activity, then the presence of α-tocopherol may result in its activation.

Since 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the key regulatory enzyme for cholesterol synthesis in the liver, any factor that affects the activity of this enzyme would eventually effect cholesterol metabolism and consequently, the LDL receptor. Parker et al. (1993) demonstrated that γ-tocotrienol can inhibit the rate of incorporation of [^{14}C] acetate into cholesterol in HepG2 cells by directly affecting the rate of HMG-CoA reductase activity. The principal mechanism by which this occurs is thought to be a post-translational process involving the accelerated degradation of the reductase protein. Furthermore, it was observed that the level of the LDL receptor protein was elevated in cells incubated with only 10 μM γ-tocotrienol. The decrease in the concentration of cellular cholesterol caused by the suppression of cholesterol synthesis with this tocotrienol most likely explains the upregulation of the LDL receptor.

Similarly, α-tocopherol may upregulate the LDL receptor in HepG2 cells if it alters the balance of cellular cholesterol by exerting an effect on HMG-CoA reductase synthesis. A recent study by Khor et al. (1995) reported that guinea pigs fed a diet supplemented with a range of 0-10 mg/day of tocotrienols (mainly α- and γ-tocotrienol) exhibited a dose dependent decrease in liver HMG-CoA reductase activity with increasing concentrations of tocotrienol up to 5 mg/day (Table 6.2). However, higher concentrations of tocotrienol supplementation in guinea pigs (>5 mg/ day) increased hepatic HMG-CoA reductase activity to control levels (Table 6.1). Interestingly, the content of α-tocopherol in the serum and liver also increased in linear fashion with increasing amounts of tocotrienol supplementation, indicating the bio-conversion of tocotrienols to tocopherols in the guinea pigs (Table 6.2). As an increase in tocotrienol supplementation led to an increase in the content of cellular α-tocopherol, it is possible that the increase in α-tocopherol content in the liver (and not the increase liver tocotrienol content) contributed to the initial decrease and the subsequent increase in HMG-CoA reductase activity.

Table 6.2: Liver HMG-CoA reductase activity in guinea pigs supplemented with increasing concentrations of tocotrienol (data taken from Khor et al., 1995).

Tocotrienol supplementation dosage (mg/day)	HMG-CoA reductase activity (pmol/min/mg protein)	% activity	α-tocopherol serum levels (μg/dL)	α-tocopherol liver levels (μg/g)
0	14.5 ± 0.5	100	14.26 ± 0.55	2.27 ± 0.05
5	7.3 ± 0.6	50	19.97 ± 0.58	3.67 ± 0.06
8	10.0 ± 0.7	70	32.00 ± 1.26	3.77 ± 0.16
10	13.2 ± 1.1	92	35.37 ± 1.07	4.05 ± 0.04

The bi-modal regulation of HMG-CoA reductase activity (Table 6.2) with increasing concentrations of tocotrienols resembles the biphasic expression of the LDL receptor with increasing concentrations of α-tocopherol (Figure 6.2, 6.3, 6.4). Hence, the effect of α-tocopherol on HMG-CoA reductase activity could be responsible for the biphasic regulation of LDL receptor activity observed in HepG2 cells. A decrease in HMG-CoA reductase activity and the subsequent decrease in cholesterol synthesis would lower the amount of total free cholesterol in the cell. The cell could then compensate for this decrease in cholesterol synthesis by increasing LDL receptor expression (activity, protein and mRNA) to try to maintain cholesterol homeostasis. Conversely, increased HMG-CoA reductase activity with higher tocotrienol supplementation would result in an elevation of cellular free cholesterol, potentially leading to the suppression of the LDL receptor. Therefore, the modulation of HMG-CoA reductase activity with increasing amounts of α-tocopherol could explain the bimodal regulation of the LDL receptor by this vitamin.

The action of α-tocopherol may imitate the behaviour of the HMG-CoA reductase inhibitors lovastatin, simvastatin and mevastatin. These are potent competitive inhibitors of the cholesterol biosynthetic pathway because their affinity for HMG-CoA reductase is approximately 1000-fold greater than its substrate (Endo, 1992). *In vivo* these compounds concentrate in the liver where they are metabolically converted to their active forms, inhibiting mevalonic acid production, and therefore inhibiting cholesterol synthesis. This depletes the supply of cholesterol to the liver required for bile acid production and assembly of VLDL. The deficit in liver cholesterol is compensated by enhanced uptake of LDL from the circulation by the liver, thereby effectively lowering the plasma cholesterol. Even though HMG-CoA activity is inhibited, treatment with these inhibitors results in a large increase in mRNA and protein for HMG-CoA reductase, HMG-CoA synthase, farnesyl pyrophosphate (FFP) and the LDL receptor. Therefore, the change in the level of cellular HMG-CoA reductase mRNA may not reflect the amount of HMG-CoA reductase protein or its activity. In fact, α-tocopherol may mimic the action of the HMG-CoA reductase inhibitors in that it may inhibit the activity of HMG-CoA reductase activity (suggested by the observations in Table 6.2), and in turn cause an upregulation of HMG-CoA reductase mRNA (observed in Figure 6.6). The resulting upregulation of the LDL receptor may then be a consequence of cholesterol depletion from the cell or because HMG-CoA reductase mRNA is coordinately regulated.

Independent regulation of the LDL receptor by α-tocopherol

Transcriptional control is believed to be the major modulator of LDL receptor production in human liver cells because the changes in the level of cellular mRNA parallel the level of LDL receptor protein expressed on the cell surface (Brown and Goldstein, 1986; Rudling, 1992; Sanchez et al., 1995; Innerarity, 1991). The regulation by α-tocopherol appears to be similar to the mechanisms of sterol regulation, since the expression of the LDL

receptor at the cell surface was determined by changes that occurred at the level of gene transcription (Figure 5.4). Since liver cells are incapable of synthesising α-tocopherol, their ability to meet their α-tocopherol requirement is through the exogenous supply carried by LDL. Therefore, by regulating the LDL receptor, the cell can control the amount of α-tocopherol entering the cell, and hence, fulfil the cells requirements for this vitamin. LDL receptor gene transcription, therefore, may be subject to feedback regulation, not only by cholesterol and fatty acids, but also by α-tocopherol in order to maintain α-tocopherol balance within the cell.

This alternative hypothesis in which α-tocopherol by itself can regulate LDL receptor gene transcription independent of other factors is based on the observation that LDL receptor regulation by α-tocopherol was observed irrespective of the extracellular cholesterol concentration. The upregulation of the LDL receptor by α-tocopherol was observed in the presence of both lipid depleted media (LPDS) (Figure 6.5) and lipid containing media (10% FCS) (Figure 6.2). Thus, α-tocopherol can override the effect of exogenous sterols, suggesting that the α-tocopherol effects may be entirely independent of intracellular cholesterol concentrations and/or the changes associated with intracellular cholesterol metabolism (ie, ACAT activity, cholesterol synthesis, cholesterol excretion). As the regulation of the LDL receptor by α-tocopherol occurs regardless of suppressive effects of cholesterol, these observations also support the above argument, in that the regulation of the LDL receptor by α-tocopherol presumably take place in order to ensure that the cellular α-tocopherol demands are met.

The mechanism of feedback regulation of LDL receptor gene transcription by cholesterol has been shown to be modulated by a sterol regulatory element binding protein (SREBP) that recognises the sterol regulatory element (SRE-1) (Sudhof et al., 1987b; Wang et al., 1994; Sanchez et al., 1995) in the LDL receptor promoter region (refer to section 1.7.5 for more detail). High concentrations of intracellular cholesterol silences the transcription of the LDL receptor gene by inhibiting the proteolysis of SREBP-1. Similarly, cellular α-tocopherol content may dictate the availability of SREBP-1. When cellular α-tocopherol concentrations are inadequate, gene transcription of the LDL receptor may be turned on by the cleavage of SREBP. Conversely, when the cell has accumulated sufficient amounts of α-tocopherol through the LDL receptor pathway, the vitamin may act to inhibit the proteolysis of SREBP. Hence, the biphasic regulation of LDL receptor expression (Figure 5.4) may reflect the cells need for α-tocopherol. The suppression of LDL receptor gene transcription at higher concentrations of α-tocopherol may reflect that cellular requirements have been satiated. α-tocopherol, therefore, may have a direct effect on the LDL receptor gene transcription system.

Given the causal relationship between the hepatic LDL receptor and plasma LDL levels (Goldstein and Brown, 1977; Brown and Goldstein, 1986) (discussed in section 1.7.1), the results herein, and previous animal studies discussed above, implicate that the

cell vitamin E status (and not the cellular cholesterol content) may be the factor which is responsible for modulating the LDL receptor activity and subsequently, plasma LDL levels. The theory is that vitamin E deficiency depresses LDL receptor activity, and supplementation enhances this activity regardless of whether cholesterol was present in the diet. For instance, the hypercholesterolemia in the cholesterol supplemented animals was alleviated when vitamin E was supplemented into the diet (Wilson et al., 1978; Westrope et al., 1982; Wojcicki et al., 1991; Subramanyam et al., 1993; Qiao et al., 1993; Verlangieri and Bush, 1992; Donaldson, 1982). One can speculate that the mechanism whereby vitamin E supplementation caused a decrease in plasma cholesterol concentrations was presumably due to its affect on the LDL receptor (upregulation). Thus, it appears from these studies that vitamin E can override the suppressive effects of cholesterol, and independently affect the LDL receptor.

SUMMARY

In conclusion, the work herein demonstrates that vitamin E upregulated LDL receptor binding activity when cells were incubated with increasing amounts up to 50 μM vitamin E, however, LDL receptor activity then declined with higher concentrations. Similar affects were observed with vitamin E on LDL receptor protein and mRNA levels. This is not surprising since no other post-transcriptional mechanisms are known to regulate the binding activity for this particular receptor (Goldstein and Brown, 1977; Brown and Goldstein, 1986; Rudling, 1992; Sanchez et al., 1995).

The observations that α-tocopherol can upregulate and downregulate the LDL receptor is a novel finding. To our knowledge, no other studies have investigated the effect of α-tocopherol, vitamins or other antioxidants on the LDL receptor. The downregulation of the LDL receptor by high concentrations of α-tocopherol may be related to its pro-oxidant properties. On the other hand, the upregulation of the LDL receptor by low amounts of α-tocopherol may be related to its high antioxidant capacity, suggesting a mechanism whereby antioxidants are antiatherogenic. Further investigation is required into whether these compounds affect other pathways in cellular cholesterol metabolism or lipoprotein metabolism. The work herein does show that there may be a direct effect on LDL receptor gene transcription. Our understanding of the process by which vitamin E affects LDL receptor metabolism clearly remains incomplete.

Chapter 7

7.0 THE EFFECT OF ANTIOXIDANTS ON THE LDL RECEPTOR

7.1 Introduction

The previous chapter described the specific upregulation by α-tocopherol and downregulation by δ- and γ-tocopherols of LDL receptor activity and gene expression. α-tocopherol has the highest biological activity and has the highest antioxidant potential compared to any of the other vitamin E species. This implies that the high antioxidant capacity of α-tocopherol could be the cause of the underlying effect on the LDL receptor. If the effect of α-tocopherol on LDL receptor expression is related to its antioxidant properties, other physiological antioxidants should similarly be able to upregulate LDL receptor expression. The major antioxidant micronutrients which seem to appear to alleviate oxidative stress are vitamin E (α-tocopherol), vitamin C, vitamin A and β-carotene (Muggli, 1994; Van Acker et al., 1993). Therefore, to test the hypothesis that the upregulation of LDL receptor is a general antioxidant phenomena, other well known vitamin antioxidants ß-carotene, vitamins A and vitamin C, two non-nutrient antioxidants green tea and red wine, were investigated.

These particular antioxidants were chosen as epidemiological studies have shown that high intakes of ß-carotene, vitamins A and C, green tea and red wine are associated with a decreased risk for coronary heart disease (CHD) (Gey et al., 1989; Mansion et al., 1993; Renaud, 1992; Kono et al., 1992). These seemingly chemically unrelated compounds have their own unique biological characteristics and functions, but they all act as antioxidants. It has been speculated that it is this inherent chemical property that enables them to be antiatherogenic.

ß-carotene

ß-carotene is an efficient quencher of singlet oxygen and can directly scavenge free radicals (Njus and Kelley, 1991; Muggli, 1994; Van Acker et al., 1993). It was initially thought that ß-carotene was antiatherogenic in humans because it is principally carried by LDL in the plasma and can protect the LDL from oxidation by reactive oxygen species (Ozhogina and Kasaikina, 1995) (see sections 1.8.4). However, the ability of ß-carotene to inhibit LDL oxidation *in vitro* is highly controversial and it has never been proven *in vivo*. Furthermore, ß-carotene can lose its antioxidant activity at high oxygen pressures and show an autocatalytic, pro-oxidant effect. This is particularly true at relatively high concentrations where it is unable to protect the LDL particle from lipid peroxidation (Burton and Ingold, 1984).

Shaish et al. (1995) found that ß-carotene had very little effect on LDL oxidation, even though ß-carotene supplementation inhibited arterial lesion formation in

hypercholesterolemic rabbits. These results imply that the effect of ß-carotene on atherosclerosis may be distinct from its ability to protect LDL from oxidation and that other mechanisms related to ß-carotene function and/or its antioxidant properties are responsible for preventing CHD. These mechanisms may involve the modulation of lipid and/or lipoprotein metabolism but, as yet, there is no substantial evidence linking ß-carotene to these actions.

Vitamin A

Retinol (vitamin A) appears to be just as effective as vitamin E in delaying the onset of lipid peroxidation, while retinyl palmitate is only utilised when all the other antioxidants are depleted. Although there is no direct evidence that vitamin A modulates LDL receptor expression, Nagasaki et al. (1994) found that vitamin A affected the expression of apolipoprotein AI and CIII genes in rats, linking this vitamin to lipid and lipoprotein metabolism. These apoproteins are major proteins of HDL and VLDL. Administration of vitamin A to normal and vitamin A deficient rats caused an increase in apo AI and CIII mRNA levels in the intestine. This effect of vitamin A on apoprotein gene expression could eventually lead to altered lipoprotein synthesis. Although it has not been shown, the effect of this vitamin on other genes involved in lipoprotein metabolism, such as the LDL receptor, may be due to its antioxidant properties and explain why vitamin A is antiatherosclerotic.

Vitamin C

Vitamin C (ascorbic acid), being water soluble, functions to scavenge reactive oxygen species in the hydrophilic environment (Njus and Kelley, 1991). Vitamin C is capable of reducing free radicals and tocopheroxyl radicals to tocopherol by readily donating an H atom, thereby making it a powerful antioxidant. However, the protective effect exerted by vitamin C against CHD does not seem to rely on its action on the arterial wall. Suarna et al. (1995) observed that there was no significant difference in the concentration of ascorbic acid in normal healthy arteries and advanced atherosclerotic plaques. Furthermore, the advanced plaques were shown not to be deficient in vitamins C or E despite the occurrence of massive lipid oxidation. This suggests that antiatherogenicity of vitamin C may lie elsewhere, perhaps in lipid and lipoprotein metabolism.

Recent studies have suggested that antioxidants may prevent atherogenesis by preventing hypercholesterolemia. Vitamin C deficiency has been shown to cause atherosclerotic lesions in guinea pigs and that these lesions regress after administration of this vitamin (Willis, 1957). Altman et al. (1980) reported delayed progression, and in some cases, regression of atherosclerotic lesions among hypercholesterolemic (elevated LDL levels) rats supplemented with vitamin C. The favourable action of vitamin C in reverting the process of atherogenesis is suggested by the authors to be as a consequence of cholesterol lowering. As LDL levels are affected by the absence or presence of vitamin C,

the underlying mechanism responsible for the changes in plasma LDL levels may be related to the modulation of hepatic LDL receptor activity.

Green tea

Green tea has been considered a crude medicine in China for over 4000 years, but it has only been recently recognised in the Western world as an antioxidant with antimutagenic and antiatherogenic properties (Fukuyo et al., 1986; Ikeda et al., 1992; Chiska et al., 1988). It is the polyphenolic tea tannins, called catechins, that are the active components of green tea. These have recently been demonstrated to have antioxidant properties. It has also been reported that these catechins have a hypocholesterolemic effect in experimental animals (Chisaka et al., 1988; Ikeda et al., 1992; Matsuda et al., 1986) and humans (Kono et al., 1992). The four main tea catechins are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg), and (-)-epigallocatechin gallate (ECGg). The antioxidant activity is in the following order: EC<ECg<EGC<EGCg. It has been demonstrated that the green tea catechin, EGCg, exerts the strongest hypocholesterolemic effect in animals compared to the other catechins, which may be attributable to its high antioxidant capacity (Fukuyo et al., 1986; Ikeda et al., 1992; Chiska et al., 1988).

Red wine

The French paradox describes the epidemiological observations that the French have one of the lowest rates of CHD (compared with other populations from developed countries), despite diets high in saturated fats and elevated plasma LDL cholesterol (Renaud, 1992; Criqui and Ringel, 1994). A possible explanation for the paradox was explained in a study that investigated alcohol, diet and mortality in 21 countries. The study found that the French had the highest red wine intake and the second lowest CHD mortality rate (Renaud, 1992). The authors suggested that it is the consumption of red wine by the French that reduces their risk of CHD.

Recently, it has also been shown that phenolic substances, such as quercetin, catechin, and resveratrol in red wine possess antioxidative characteristics which prevent lipid peroxidation *in vitro* (Fuhrman et al., 1995) and may prevent CHD *in vivo*. Red wine, but not white wine, has been shown to increase plasma HDL levels in humans (Lavy et al., 1994) and to possess anti-inflammatory activity (Muller and Fugelsang, 1994). These may contribute to the reduced CHD observed in red wine drinkers. Red wine contains a substantial amount of polyphenols in comparison with white wine (1.4 g quercetin equivalents/L in red wine compared with 0.25 g/l in white wine).

The antioxidant constituents of red wine have not been shown to modulate lipid or lipoprotein metabolism, but if they are able to upregulate hepatic LDL receptor activity due to their antioxidant properties, then it may explain why red wine consumption decreases mortality from CHD.

Many researchers have speculated that prevention of LDL oxidation by antioxidants is the most important mechanism whereby antioxidants inhibit the development of CHD. This theory, however, can not fully explain how such diverse compounds, such as vitamin A (which is mainly carried by retinol binding protein in plasma), water soluble vitamin C and the polyphenols of green tea protect LDL from oxidation as they are not directly incorporated into LDL. Alternative explanations as to how these antioxidants may prevent the development of atherosclerosis have to be considered.

Assuming that the upregulation of the LDL receptor observed by α-tocopherol was due to its antioxidant properties, then it can be hypothesised that any compound with a strong antioxidant potential, such as ß-carotene, vitamins A and C, green tea and red wine, would also be able to induce the transcription of the LDL receptor gene. It is this effect on LDL receptor activity that could be important for allaying CHD.

7.2 METHODS

HepG2 cells were grown at 37^{o}C in DMEM supplemented with 10 % fetal calf serum (FCS). Cells were subcultured from large flask 175 cm^3 into small 25 cm^3 flasks, and allowed to grow without disruption for 48 hr (or until approximately 80% confluent) in a background medium of DMEM supplemented with 10% FCS. HepG2 cells were incubated for 24 h with 50 µM and 100 µM of either vitamins A, C, or E or ß-carotene or green tea or red wine (see section 2.3) in a background media of DMEM supplemented with 10% FCS. The concentration of green tea extract needed to enrich the cells was based on the concentration of the major catechin, (-)-epigallocatechin gallate (Wang et al., 1992). Similarly, red wine concentration was calculated based on its quercertin (1508 mg/L) content.

After the 24 h incubation period, cells were harvested, analysed for vitamin A and ß-carotene content (see 2.3), and LDL receptor activity (see 2.6), protein (see 2.7) and mRNA levels (see 2.8) were measured.

7.3: RESULTS

7.3.1 Enrichment of HepG2 cells with antioxidants.

After incubation for 24 h in media containing from 0-100 μM vitamin A and ß-carotene, cultured HepG2 cells were analysed by HPLC for their vitamin content (see section 2.3). The analysis revealed that the cells had higher levels of vitamin A and ß-carotene levels compared to cells not supplemented with vitamins. Cells which were not incubated with vitamin A and ß-carotene had undetectable amounts of these vitamins. Cells incubated with 50 and 100 μM vitamin A contained 1.71 and 3.47 μg Vitamin A/mg cell protein, respectively. Similarly cells incubated with 50 and 100 μM ß-carotene contained 1.4 and 3.1 μg ß-carotene/mg cell protein, respectively. Therefore, HepG2 cells were able to effectively vitamin A and ß-carotene.

7.3.2 The effect of vitamin A, vitamin C and ß-carotene on LDL receptor activity.

The LDL receptor activity was measured in cells incubated for 24 h with 50 μM and 100 μM of either vitamins A, C, or E or ß-carotene (see section 2.3) in a background media of DMEM supplemented with 10% FCS.

LDL receptor activity was upregulated in cells when they were incubated with a concentration 50 μM of either vitamin A, vitamin C, or ß-carotene (Figure 7.1) compared to control cells (no antioxidants). As observed previously (see section 6.2), LDL receptor activity was significantly increased by 120% when cells were incubated with 50 μM vitamin E (α-tocopherol) compared to control cells incubated without antioxidants. However, in contrast to the suppression of LDL receptor activity by vitamin E at concentrations greater than 50 μM, a further enhancement of LDL receptor activity was observed when cells were incubated with 100 μM of vitamin A, vitamin C, or ß-carotene. LDL receptor activity was significantly increased in cells by approximately 178% with 100 μM vitamin A, 225% with 100 μM vitamin C, and 225% with 100 μM ß-carotene compared to control cells incubated without antioxidants (Figure 7.1).

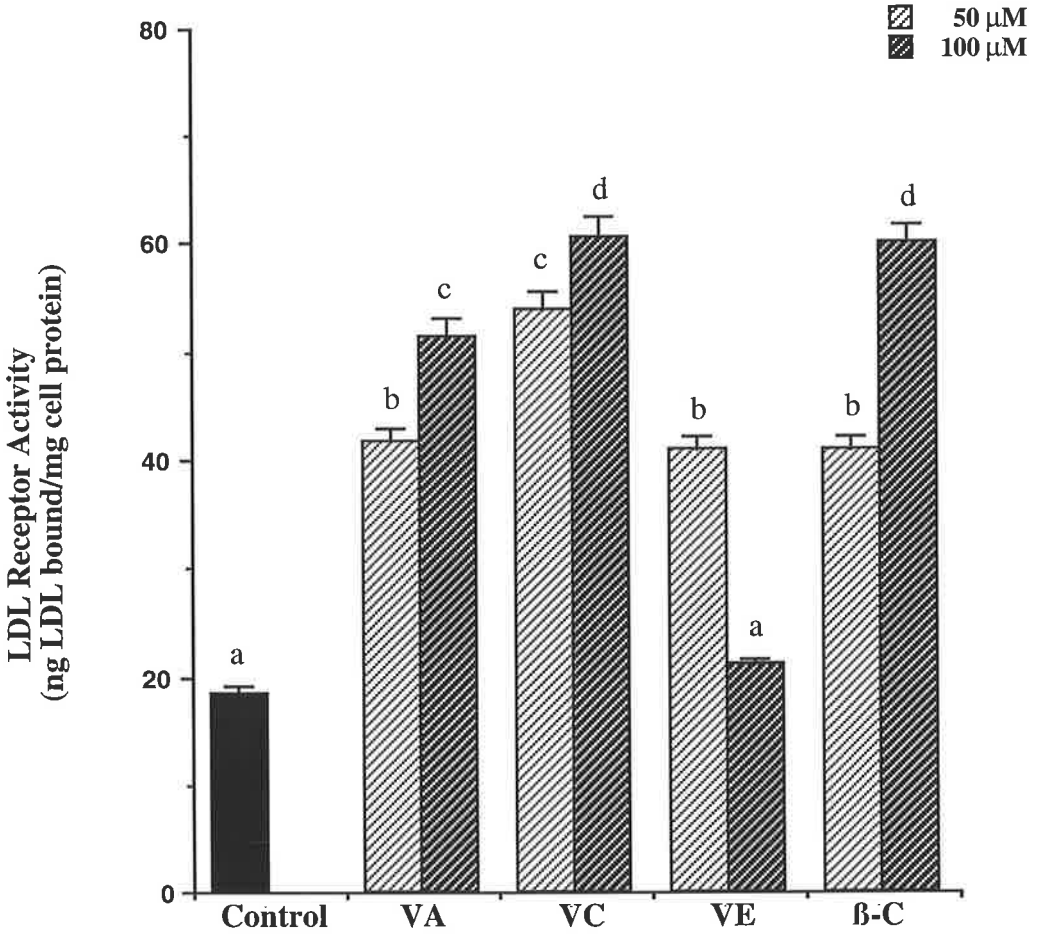


Figure 7.1: The effect of different antioxidants on LDL receptor activity in HepG2 cells. Cells were incubated for 24 h with 50 µM and 100 µM of various antioxidants in a background media (DMEM) containing 10% FCS. Control = no antioxidants, VA = vitamin A, VC = vitamin C, VE = vitamin E, ß-C = ß-carotene. LDL receptor binding activity was measured in triplicate using the LDL colloidal gold method described in section 2.6.3. Results represent specific LDL receptor binding activity (ng of LDL bound/mg cell protein) expressed as a mean ± SEM of 3 experiments. Means with the same letters above them are not significantly different at $P < 0.01$.

7.3.3 The effect of vitamin A, C and ß-carotene on LDL receptor protein.

To elucidate whether the effect of the antioxidants on LDL receptor activity was a result of their actions on protein synthesis, the LDL receptor protein was quantified. The LDL receptor protein was measured in cells incubated for 24 h with 50 μM and 100 μM of either vitamins A, C, E or ß-carotene (see section 2.7) in a background media of DMEM supplemented with 10% FCS.

LDL receptor protein increased in cells incubated with 50 μM vitamins A, C, or ß-carotene compared to control cells which were not supplemented with antioxidants (Figure 7.2). LDL receptor protein was also increased 4-fold when cells were incubated with 50 μM vitamin E, consistent with earlier findings (see section 6.3). However, in contrast to the effect of vitamin E on LDL receptor protein at concentrations greater than 50 μM, vitamin A, vitamin C and ß-carotene all enhanced LDL receptor activity at 100 μM compared to control cells without antioxidants. LDL receptor protein increased significantly to 200% with 100 μM vitamin A, 100% with 100 μM vitamin C, and 206% with 100 μM ß-carotene compared to control cells (Figure 7.2). The changes in LDL receptor protein were concomitant to changes observed in cellular LDL receptor activity when cells were incubated with these vitamins (Figure 7.1). This indicated that the effect of these antioxidants on LDL receptor activity could be explained by their effect on the amount of LDL receptor protein present in the cell.

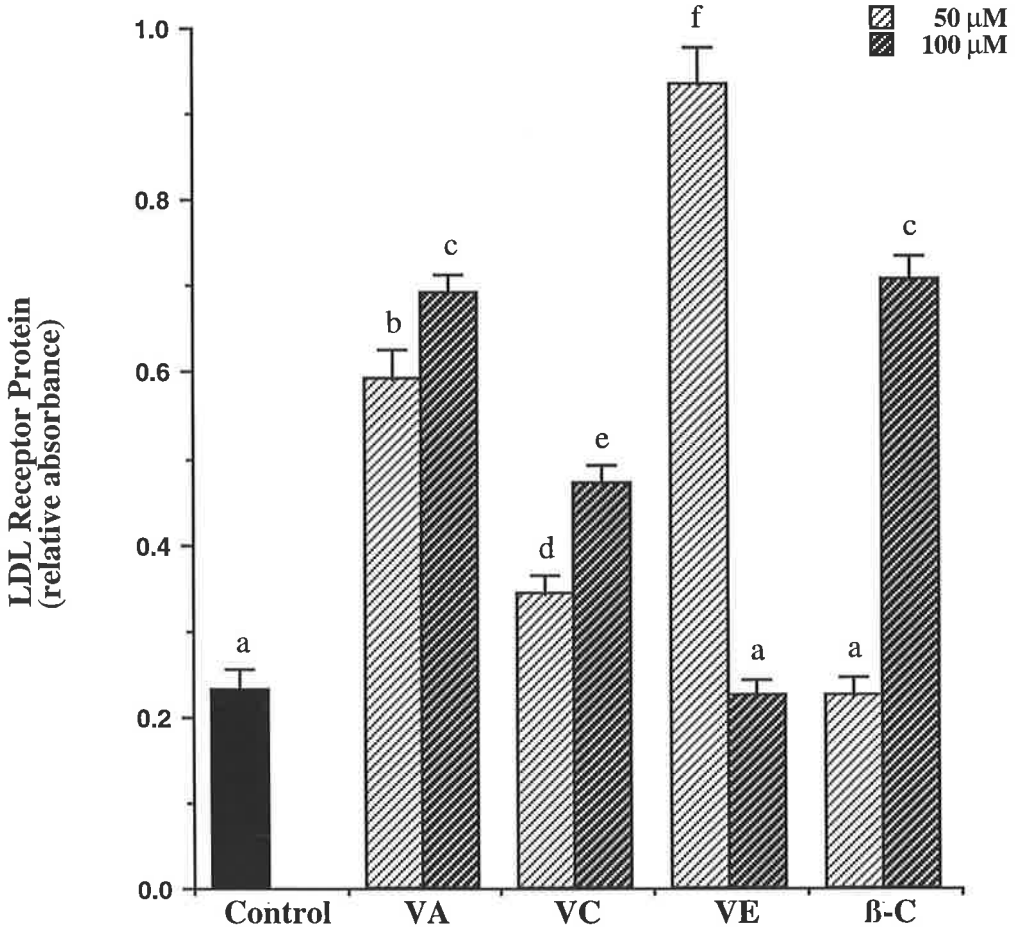


Figure 7.2: The effect of various antioxidants on LDL receptor protein in HepG2 cells. Cells were isolated after 24 h incubation with various antioxidants at two different concentrations (50 µM and 100 µM) in background media (DMEM) containing 10% FCS. Control = no antioxidants, VA = vitamin A, VC = vitamin C, VE = vitamin E, ß-C = ß-carotene. Cellular proteins were solubilised with Triton-X 100 detergent, electrophoresed, transferred to nitrocellulose and quantified by the ECL method as described in section 2.7. Experiments were performed in duplicate and the relative densities of the receptor band on the autoradiograph were quantified using laser densitometry. Results are expressed as relative absorbance and data represents the mean ± SEM of 2 experiments. Means with the same letters above them are not significantly different at $P< 0.01$.

7.3.4 The effect of vitamin A, vitamin C and ß-carotene on LDL receptor mRNA.

Since the LDL receptor has been shown previously to be regulated at the level of gene transcription by vitamin E, the upregulation of LDL receptor activity and protein by antioxidants could also be a result of a change in mRNA levels. The level of LDL receptor mRNA was quantified using RT-PCR (section 2.8) in cells incubated with 50 μM or 100 μM of either vitamins A, C, E or ß-carotene in a background media of DMEM supplemented with 10% FCS.

The amount of LDL receptor mRNA increased significantly in cells incubated with vitamin A, C, E or ß-carotene at 50 μM (Figure 7.3). LDL receptor mRNA abundance was further enhanced in cells incubated with a 100 μM of vitamin A, vitamin C or ß-carotene. At the higher antioxidant concentrations, LDL receptor mRNA amounts were significantly ($P < 0.05$) increased, approximately 13-fold with vitamin A and C, and 11-fold with ß-carotene. However, vitamin E depressed the amount of LDL receptor mRNA at this concentration, as observed previously (section 6.4).

The upregulation of LDL receptor gene transcription could explain the increase in LDL receptor protein and activity in cells incubated with the antioxidants, vitamin A, vitamin C and ß-carotene.

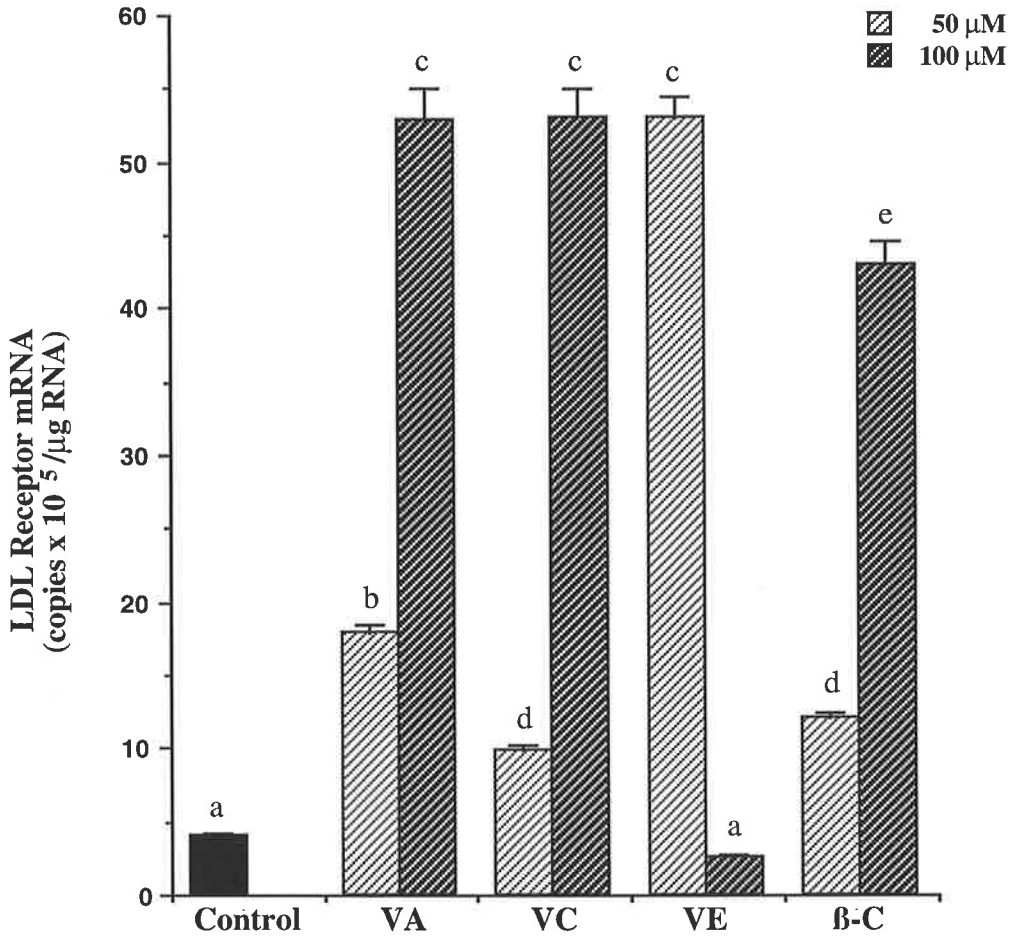


Figure 7.3: The effect of different antioxidants on LDL receptor mRNA levels in HepG2 cells. Cells were incubated with various antioxidants for 24 h in background media (DMEM) containing 10% FCS. Control = no antioxidants, VA = vitamin A, VC = vitamin C, VE = vitamin E, ß-C = ß-carotene. Total cellular mRNA was isolated, reverse transcribed and amplified using PCR with a synthetic piece of RNA used as an internal control (see section 2.8). The amplified products were electrophoresed, transferred to nitrocellulose and quantified by the ECL method as described in section 2.8. Cellular mRNA levels were measured relative to the internal control and expressed as the mean ± SEM of 3 experiments. Means with the same letters above them are not significantly different at $P< 0.01$.

7.3.5: The effect of green tea on LDL receptor activity.

The effect of green tea on LDL receptor was examined to determine whether other non-vitamin antioxidants would also regulate the LDL receptor. HepG2 cells were incubated with green tea extract for 24 h in a background media of DMEM supplemented with 10% FCS. The concentration of green tea extract, which was based on the content of (-)-epigallocatechin gallate (ECGg) (see section 2.3.2), was increased from 0-100 μM. Cells were harvested and LDL receptor activity was measured according to the LDL-gold method described in section 2.6.3.

When cells were incubated with increasing amounts of green tea extract, the LDL receptor binding activity progressively increased (Figure 7.4). LDL receptor activity increased to 300% above the control level with a concentration of green tea extract of 100 μM (Figure 7.4).

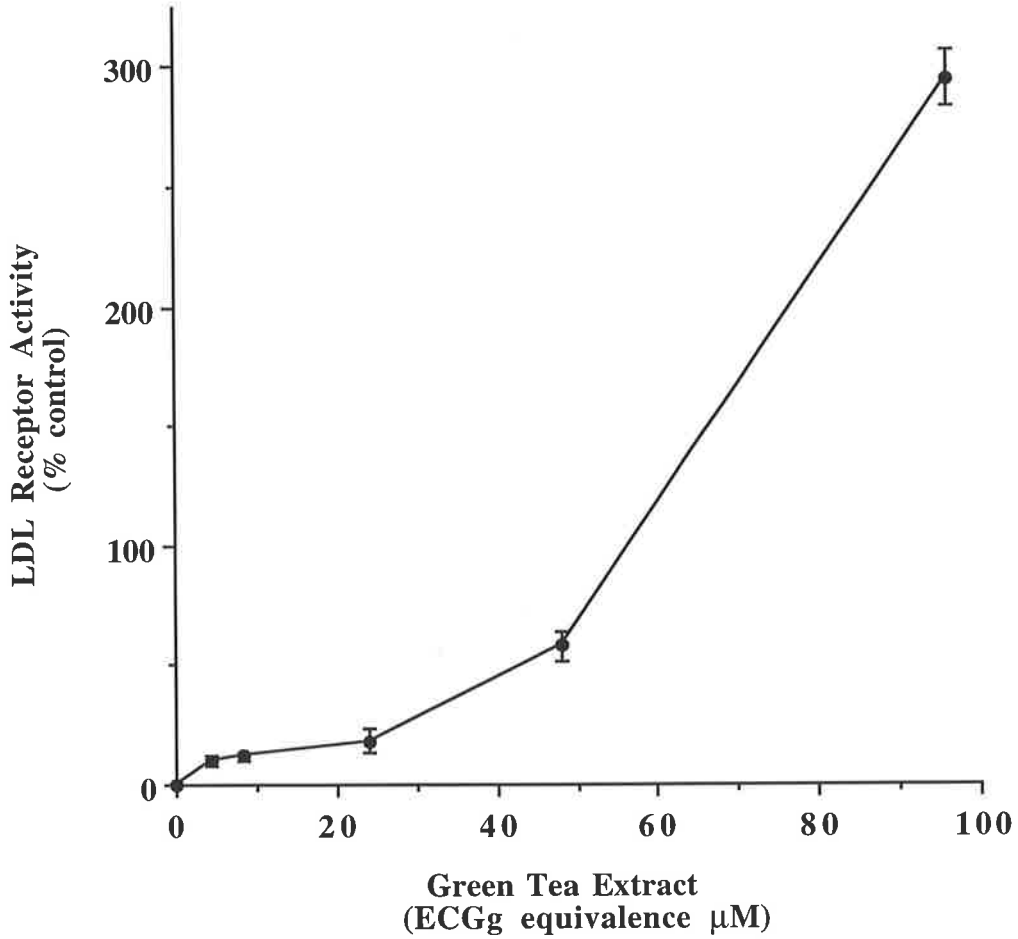


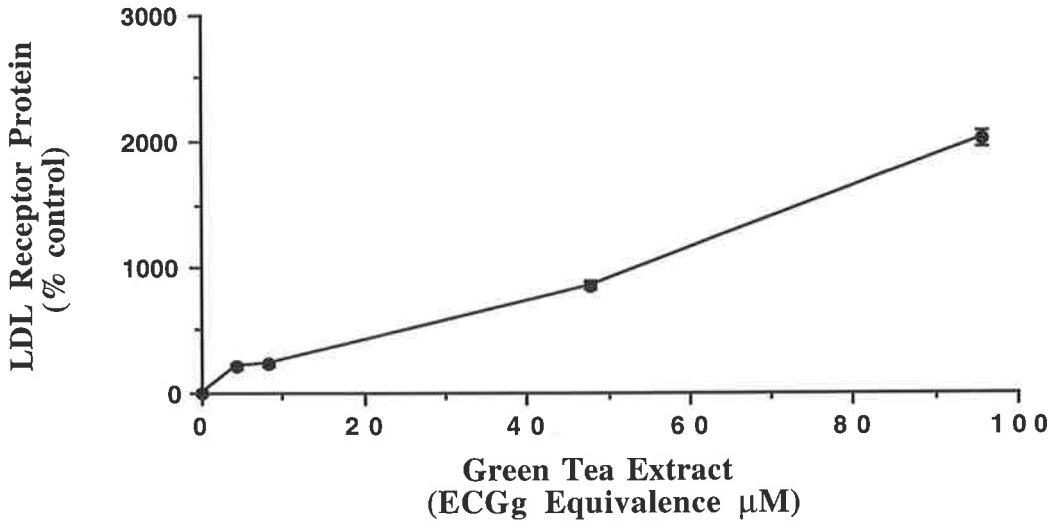
Figure 7.4: The effect of green tea on LDL receptor binding activity in HepG2 cells. Cells were incubated for 24 h with increasing concentrations of green tea extract (ECGg) up to 100 μM in a background medium of 10% FCS. LDL receptor binding activity was measured in triplicate using the colloidal-LDL gold method as described previously (section 2.6.3). Gold conjugates were incubated with 100 μg of cellular protein for 1 h at room temperature, either in the presence of $Ca(NO_3)_2$ to measure total binding or in the presence of EDTA to measure nonspecific binding. Specific binding was then calculated by taking the difference. Results represent specific LDL cell binding activity (ng of LDL/mg of cell protein). Data is a mean ± SEM of 2 experiments and is expressed as a percentage of the value in control cells (34 ± 3.5 LDL/mg of cell protein), which were not incubated with green tea extract.

7.3.6: The effect of green tea on LDL receptor protein.

Since changes in cellular LDL receptor binding activity usually reflect changes in the number of receptors, the LDL receptor protein was measured in HepG2 cells incubated for 24 h in media (supplemented with 10% FCS) containing 0-100 μM green tea extract (ECGg equivalence) (see section 2.3.2). Using a polyclonal antibody against the LDL receptor, a single band at 130 Kd was visualised corresponding to the LDL receptor.

The effect of green tea extract on LDL receptor protein was seen to be substantially enhanced when cells were incubated with increasing concentrations of green tea extract. The intensity of the LDL receptor band progressively increased 20-fold above control cells with increasing concentrations of green tea extract up to 100 μM (Figure 7.5). The changes observed in LDL binding activity in HepG2 cells are, therefore, attributable to changes in the amount of LDL receptor protein.

A)



B)

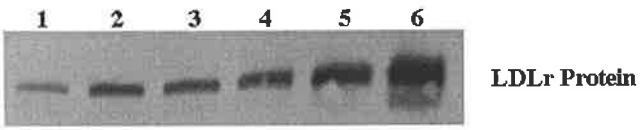


Figure 7.5: Dose dependent effect of green tea extract (ECGg equivalence) on LDL receptor protein. HepG2 cells were isolated after 24 h incubation with increasing concentrations of green tea extract (ECGg equivalence) in DMEM growth media supplemented with 10% FCS. Cellular proteins were solubilised with Triton-X detergent, electrophoresed, transferred to nitrocellulose and quantified by the ECL method as described section in 2.7. Experiments were performed in triplicate, and the relative densities of the receptor band were quantified using laser densitometry. **(A)** Data was a mean ± SEM of 2 experiments and was expressed as a percentage of the results obtained in control cells (0.3 ± 0.12 relative absorbance) which were not incubated with green tea extract. **(B)** ECL detection of LDL receptor protein, lanes : (1) control, no green tea (GT), (2) 5 μM GT, (3) 10 μM GT, (4) 25 μM GT, (5) 50 μM GT (6) 100 μM GT.

7.3.7: The effect of green tea on LDL receptor mRNA.

To elucidate whether the increase in LDL receptor activity and protein could be attributed to an upregulation at the level of gene transcription by the green tea extract (ECGg equivalence), the amount of LDL receptor mRNA was quantified. The LDL receptor mRNA was measured using RT-PCR (section 2.8) in cells incubated for 24 h with increasing amounts of green tea extract (section 2.3.2).

The amount of LDL receptor mRNA significantly increased up to 23-fold above control levels with increasing concentrations of the green tea extract (ECGg equivalence) up to 100 μM (see section 2.3.2) (Figure 7.6). Therefore, green tea extract is capable of changing LDL receptor activity and protein levels by altering the amount of LDL receptor mRNA.

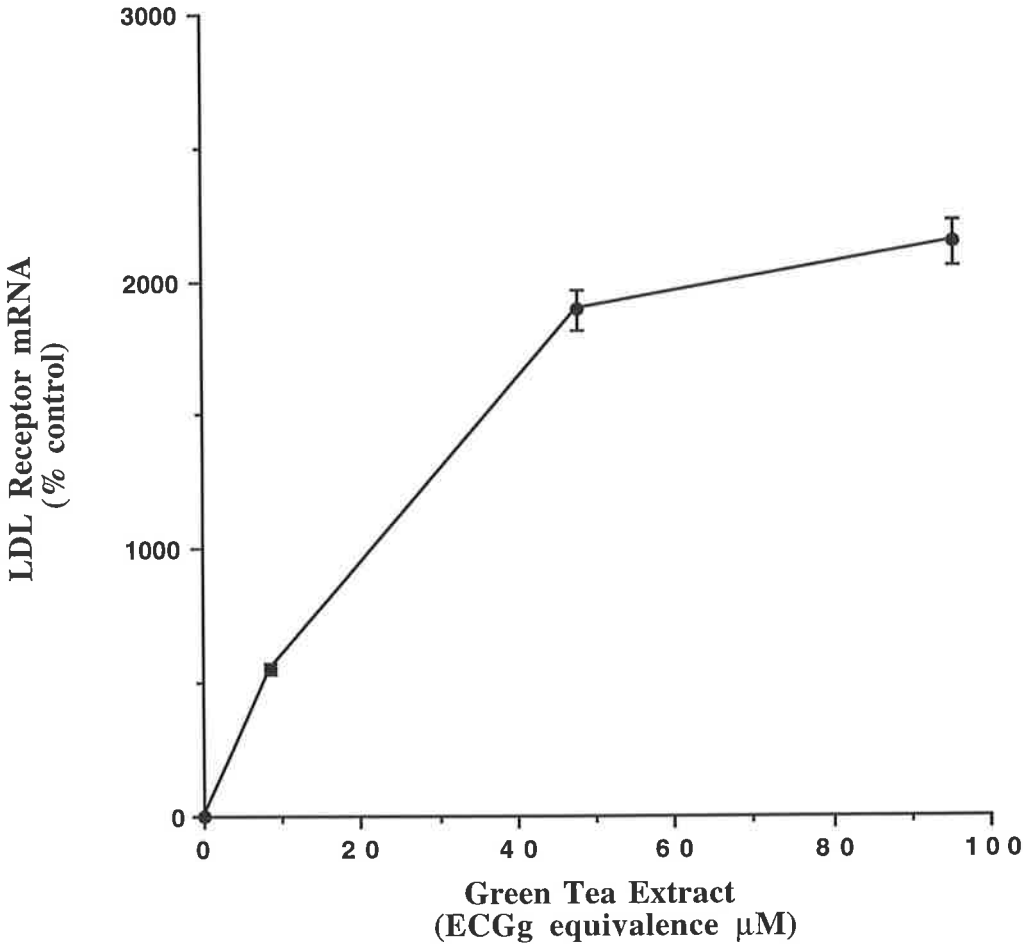


Figure 7.6: The effect of green tea extract (ECGg equivalence) on LDL receptor mRNA levels in HepG2 cells. Cells were incubated with increasing concentrations of green tea extract (ECGg equivalence) for 24 h in a background media of DMEM containing 10% FCS. Total cellular RNA was isolated, reverse transcribed and amplified using PCR, with a synthetic segment of RNA used as a quantitative internal control (section 2.8). Experiments were done in duplicate. Cellular mRNA levels were measured relative to the internal control and expressed as a percentage of the control cells (3.5 ± 0.2 copies x 10^{5}/μg RNA) incubated without green tea extract. Results represent mean ± SEM from 2 experiments.

7.3.8: The effect of green tea extract on HMG-CoA reductase mRNA.

Normally, the gene transcription of LDL receptor and HMG-CoA reductase are coordinately regulated. Therefore, the level of cellular HMG-CoA reductase mRNA was quantified in the presence of green tea extract.

The level of HMG-CoA reductase mRNA was measured in cells incubated for 24 h with increasing concentrations from 0-100 μM of green tea extract (ECGg equivalence) in a background media of DMEM containing 10% FCS (see section 2.3.2). The gene expression of HMG-CoA reductase was found to be linear with increasing concentrations of the green tea extract, similar to its effect on the LDL receptor gene expression (Figure 7.7). The level of mRNA increased up to 13-fold of 100 μM green tea extract, indicating that it may be coordinately regulating the expression of both the LDL receptor and HMG-CoA reductase genes.

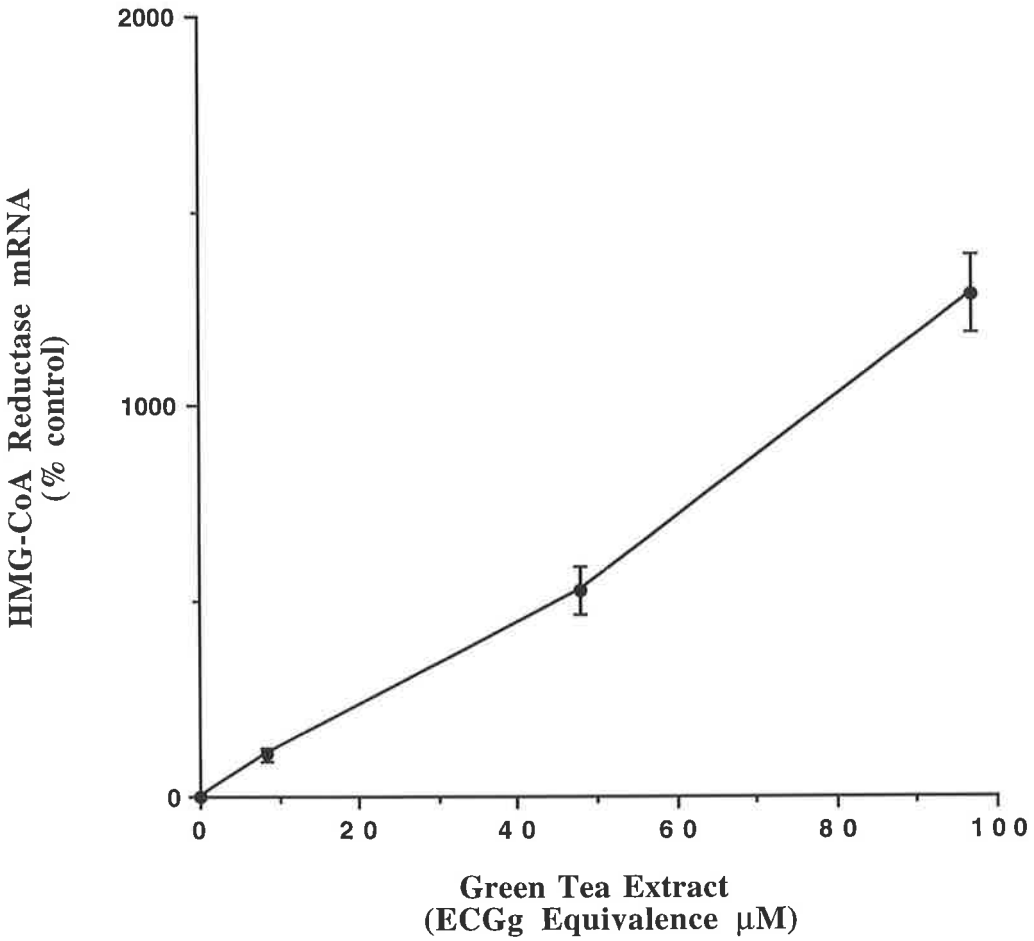


Figure 7.7: The effect of green tea extract (ECGg equivalence) on HMG-CoA reductase mRNA levels in HepG2 cells. Cells were incubated with increasing concentrations of green tea extract (ECGg equivalence) for 24 h in a background media of DMEM containing 10% FCS. Total cellular RNA was isolated, reverse transcribed and amplified using PCR, with a synthetic segment of RNA used as an internal control (section 2.8). Experiments were done in duplicate. Cellular mRNA levels were measured relative to the internal control and expressed as a percentage of the control cells ($4 \pm 0.3 \times 10^5$ copies/μg RNA) incubated without green tea extract. Data represents the mean ± SEM of 2 experiments.

7.3.9: The effect of red wine on LDL receptor activity and mRNA levels in HepG2 cells.

As red wine is also known to have an antioxidant capacity, its effect on LDL receptor activity was investigated. Cells were incubated with red wine 0-100 μM (based on the quercertin content of red wine) for 24 h in a background media of DMEM containing 10% FCS (see section 2.3.2). Cells were harvested and LDL receptor activity was measured according to the LDL-gold method described in section 2.6.3.

LDL receptor activity increased progressively to 160% above control levels with increasing concentrations of red wine (quercertin equivalence) up to 100 μM (Figure 7.8). Similarly, LDL receptor mRNA levels increased up to 350% when cells were incubated with increasing concentrations from 0-100 μM of red wine (Figure 7.9).

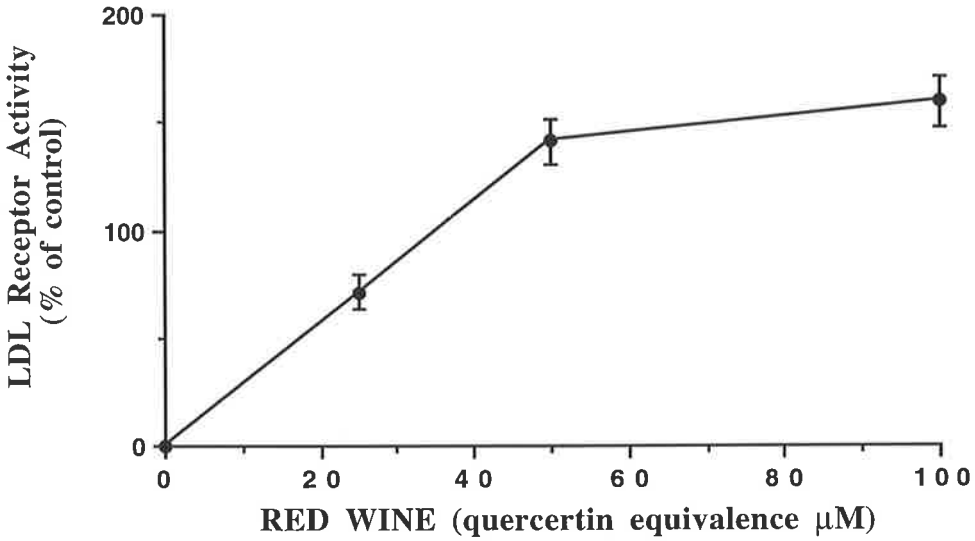


Figure 7.8: The effect of red wine (quercertin equivalence) on LDL receptor activity in HepG2 cells. Cells were incubated for 24 h with increasing concentrations of red wine (quercertin equivalence) up to 100 μM in a background medium of 10% FCS. LDL receptor binding activity was measured in triplicate using the colloidal-LDL gold method as described section 2.6.3. Gold conjugates were incubated with 100 μg of cellular protein for 1 h at room temperature, either in the presence of $Ca(NO_3)_2$ to measure total binding or in the presence of EDTA to measure non specific binding. Specific binding was then calculated by taking the difference. Results represent specific LDL cell binding activity (ng of LDL/mg of cell protein). Data represents a mean ± SEM of 2 experiments and is expressed as a percentage of the results obtained in control cells which were not incubated with red wine.

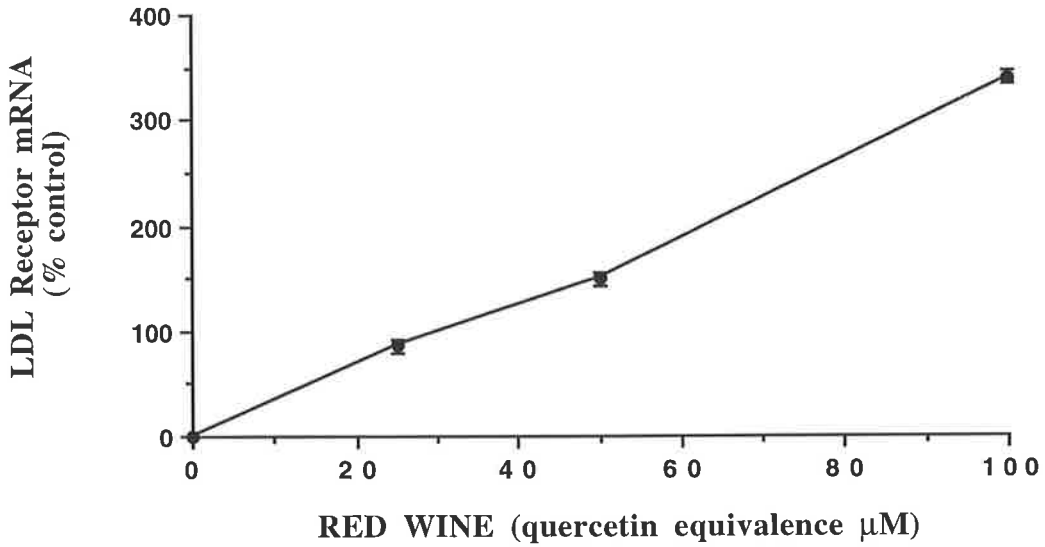


Figure 7.9: The effect of red wine (quercertin equivalence) on LDL receptor mRNA levels in HepG2 cells. Cells were incubated for 24 h with increasing concentrations of red wine (quercertin equivalence) up to 100 μM in a background medium of 10% FCS. Total cellular RNA was isolated, reverse transcribed and amplified by PCR, with a synthetic segment of RNA used as an internal control (section 2.8). Experiments were done in duplicate. Cellular mRNA levels were measured relative to the internal control and expressed as a percentage of the control cells ($3.6 \pm 0.26 \times 10^5$ copies/μg RNA) incubated without red wine. Data represents the mean ± SEM of 2 experiments.

7.4 DISCUSSION

The results described in this chapter clearly demonstrate that all the antioxidants examined were able to increase the LDL receptor at the transcriptional level, thus the upregulation of the LDL receptor appears to be a general antioxidant phenomenon. However, unlike the bimodal effect observed by α-tocopherol on LDL receptor expression, all the antioxidants examined further upregulated LDL receptor expression at higher concentrations. LDL receptor activity, mass and mRNA levels were coordinately upregulated suggesting that the increase by antioxidants on LDL receptor activity could be attributable to an increase in the level of gene transcription of the LDL receptor. These results indicate the upregulation of LDL receptor activity by these unrelated vitamin compounds may be due to their antioxidant properties and may occur through a common mechanism.

These results also imply that the modulation of LDL receptor activity at the cell surface is a direct consequence of the effect of the antioxidants on gene transcription. Even though there appeared to be a similar degree of change in LDL receptor activity with vitamin A, vitamin C and ß-carotene (approximately 200%) and protein (200%), there was a more marked increase in LDL receptor mRNA (approximately 10-13 fold increase with these antioxidants). The disparity between the amount of message and the amount of protein may be accounted for by the degradation of the mRNA,or protein (as discussed in detail in chapter 6, see section 6.4).

When HepG2 cells were incubated with 50 μM of α-tocopherol, LDL receptor activity (120% increase) and protein (300% increase) increased (Figure 7.2 and 7.3) to the same extent observed in earlier experiments (see Figure 6.2 and Figure 6.3). However, in comparison to the 7-fold increase in mRNA observed previously (Figure 6.4), in these experiments there was a 13-fold increase in the amount of mRNA when cells were incubated with 50 μM of α-tocopherol (Figure 7.3). Although the absolute values may vary for one experiment to the next, a similar biphasic trend was still observed with concentrations of α-tocopherol of 50 and 100 μM at the level of LDL receptor activity, protein and mRNA (Figure 7.1, 7.2 and 7.3). This exemplifies the variability that can occur from one cell batch to another grown at different times and may be due to small alterations in the growth conditions, such as temperature, pH, and exact time in the cell cycle when enrichment is initiated.

Green tea significantly upregulated the LDL receptor activity, protein, and mRNA levels in HepG2 cells with increasing concentrations. An increase of over 20-fold was observed in LDL receptor protein and mRNA levels at 100 μM of green tea extract compared to control cells. Although this degree of change was not detected in the LDL receptor binding activity, cells incubated with 100 μM of green tea extract had a significant increase of 300% in binding activity compared to control cells. The increase in LDL receptor binding activity in cells incubated with green tea can be accounted for by its effect on LDL receptor

gene transcription. Similarly, the amount of HMG-CoA reductase mRNA was found to be induced in a linear manner with green tea concentrations up to 100 μM. This is not surprising since the LDL receptor and HMG-CoA reductase genes are normally coordinately regulated (see section 1.7.5) and implies that the same factors may be responsible for the upregulation of both genes.

Red wine also enhanced the LDL receptor binding activity in HepG2 cells with increasing concentrations (Figure 7.8). LDL receptor binding activity reached 140% when cells were incubated with 50 μM of red wine (quercertin equivalence) and the activity had a small, but a significant, increase in binding activity at 100 μM (160% of control). Similarly, LDL receptor mRNA levels increased in cells incubated with higher amounts of red wine (Figure 7.9). Therefore, the effect of red wine on LDL receptor expression was similar to the effect observed with the other antioxidants investigated.

Despite the fact that all the antioxidants examined herein have different chemical structures and diverse physiological functions, they all had a similar effect on LDL receptor expression. This would imply that they may all act through a similar mechanism to upregulate the hepatic LDL receptor due to their common antioxidant property.

Vitamin A

Vitamin A is a generic term for retinoids that exhibit the biological activity of retinol (see section 1.8.5.4). After cellular uptake, retinol can be oxidised to retinoic acid (RA) which can exist in either 9-*cis* RA or all-*trans* RA. RA (9-*cis* and all-*trans*) has been shown to exert powerful effects on biological processes, such as cell differentiation and proliferation. These effects are believed to be mediated via their interactions with two families of nuclear transcription factors, their 9-*cis* retinoic acid receptors (RXRs) and the all-*trans* retinoic acid receptors (RARs). These retinoic acid receptors regulate the transcription of a particular set of target genes by binding to RA response elements (RAREs) in their gene promoters. All three subtypes (α, β, γ) of RAR and RXR belong to a gene superfamily of steroid hormone nuclear receptors. Retinoic acid has been found in cultured cells to regulate the expression of several proteins implicated in atherogenesis, including thrombomodulin, monocyte chemo-attractant protein-I and apoprotein (apo) AI (Haghpassand and Moberly, 1995; Nagasaki et al., 1994).

Haghpassand and Moberly (1995) observed that mRNA levels for apoAI increased 76% and apoAI secretion increased by 52% when HepG2 cells were exposed to 10 μM 9-*cis* RA. Similarly, Nagasaki et al. (1994) demonstrated that when either 9-*cis* RA or all-*trans* RA was administered intragastrically into RA deficient rats, hepatic expression of apoAI was higher compared to non-administered control rats, but intestinal expression of this gene was unaffected. On the other hand, vitamin A positively regulated apo CIII in the intestine, but not in the liver. A *cis* retinoic response element (RARE) has been identified in the apo AI

gene promoter. Such an element may be present in the LDL receptor gene, and if so, could account for the upregulation of the LDL receptor by vitamin A.

There is evidence to suggest that RAREs are not absolutely necessary for RA to specifically control gene transcription. RA has been shown to have a profound effect on growth and differentiation of epithelial cells, and this is thought to occur by its action on genes with no RAREs. Miller et al. (1996) found that retinol downregulated the expression of the transforming growth factor-α (TGF-α) at the transcriptional level in cultured epithelial cells. They observed a 30-fold difference in TGF-α mRNA levels between vitamin A treated and untreated cells. Further investigations indicated that RAREs were absent in the 5' flanking region of the human TGF-α gene, suggesting that the retinol metabolites (9-*cis* and all-*trans* RA) may be indirectly responsible for the downregulation of the TGF-α gene transcription. Even with the absence of RAREs, it is possible that RA may interact directly with a gene through an interaction of other unknown response elements.

Alternatively, vitamin A may indirectly induce the LDL receptor system by altering the levels of other transcription factors such as AP-1 which has been shown to be affected by vitamin A (see final discussion for more detail) (Schule et al., 1991). Although AP-1 has been shown to be able to bind to the LDL receptor, it only has one-tenth of the activity as Sp-1 (Smith et al., 1988). LDL receptor gene transcription induced by vitamin A may be also mediated by other nuclear transcription factors, such as Sp-1.

At this point it is difficult to ascertain by which mechanism vitamin A may be inducing the LDL receptor expression. No matter which pathway is involved, however, it would appear from the results herein that it would be a mode of regulation which is synonymous with its inherent antioxidant properties.

ß-carotene

The mechanism underlying the enhancement of LDL receptor expression by ß-carotene is difficult to explain. Even though its function as an antioxidant has been investigated, there is no evidence associating the involvement of ß-carotene in cholesterol or lipoprotein metabolism. However, ß-carotene has been implicated in modulating fatty acid metabolism in ruminants. Zhou et al. (1993) showed that the fatty acid composition of adipose tissue in healthy cattle was related to the yellowness of the tissue (which is correlated to its ß-carotene content). There was a significant increase in the total percentage of *cis* monounsaturated fatty acids and a decrease in saturated fatty acids as the total carotenoid content of the fat increased. This may occur due to the effect of ß-carotene on the fatty acid desaturating system in adipose tissue, as ß-carotene has been shown to affect the activity of Δ9-desaturase in rat liver microsomes (Alam et al., 1984). Rats supplemented with 100 mg/Kg diet of ß-carotene for 11 weeks had decreased levels of Δ9-desaturase and an increased proportion of phosphotidylethanolamine in the microsomal membrane. Although these findings in rat liver can not explain the increase in *cis* monounsaturated fatty acid in the

in adipose tissue, it does demonstrate that ß-carotene can influence the fatty acid composition by its effects on enzymes involved in fatty acid synthesis.

The modification of cellular fatty acids and the subsequent effect of LDL receptor expression in liver cells has been previously shown (see chapter 4 and 5). Whether the modification of the fatty acid composition of HepG2 cells occurs in the presence of ß-carotene, and is the underlying mechanism responsible for the enhanced LDL receptor expression by this antioxidant, is yet to be determined. As discussed earlier, ß-carotene may also exert its effects directly or indirectly through transcription factors in that, through the conversion to RA and retinol, it may mediate effects through RAREs.

Vitamin C

A study examining the relationship between cholesterol and plasma ascorbic acid (vitamin C) in humans reported a negative correlation between plasma ascorbic acid levels (PAA) and LDL cholesterol, but a positive correlation between PAA and HDL cholesterol levels in the group receiving vitamin C supplementation (Jaques, 1992). Similar observations have been found in a study by Gatto et al. (1996), which investigated the role of ascorbic acid supplementation on lipoprotein profiles in healthy young women. After a 4 week period of 1000 mg/day ascorbic acid supplementation, plasma LDL concentrations of the women were reduced by 16% compared to baseline, and there was a drop in total cholesterol concentrations. From these findings, it appears that vitamin C has a hypocholesterolemic effect. This cholesterol lowering effect of supplemented vitamin C could account for the observed protection of vitamin C against the development of CHD, but whether it is a consequence of its antioxidant capacity is uncertain (Stankova et al., 1984; Mezzeti et al., 1995; Gey et al., 1989, 1993; Jaques, 1992).

The lowering of plasma LDL level by vitamin C could be explained by an increased number of LDL receptors on the cell surface (as observed in HepG2 cells, Figure 7.3). The underlying mechanism by which vitamin C increases LDL receptor activity is not clear, although indirect evidence does suggest the involvement of ascorbic acid in lipid metabolism. The early studies of Ginter et al. (1971) and Holloway et al. (1981) showed that ascorbic acid deficient guinea pigs had increased total plasma cholesterol, reduced concentration of HDL cholesterol and reduced bile acid synthesis due to a reduction in the activity of 7-α hydroxylase. These studies imply that in the presence of ascorbic acid, bile acid synthesis increases, causing cholesterol to be depleted from the liver, and thereby, inducing the transcription of the LDL receptor gene. The alternate mechanism would invoke the direct effect of ascorbic acid on LDL receptor gene transcription. However, there is no evidence indicating an association of ascorbic acid with any nuclear transcriptional factors or inherent ascorbic acid responsive promoter elements.

Green Tea

Flavonoids are a class of plant phenylbenzopyrone derivatives with antioxidant properties. Catechins, an active component of green tea, and quercetin, a major component of red wine, are included in this class of flavonoids. Their powerful antioxidant capacity is due to their polyhydroxylation. Little is known about their pharmacokinetics, their levels in plasma, or whether they partition into LDL particles.

Green tea is considered to be a potential antiatherogenic agent as a number of studies have reported that its intake is associated with a lowering of blood cholesterol in humans and animals (Kono et al., 1992; Chisaka et al., 1988; Ikeda et al., 1992; Matsuda et al., 1986). The first epidemiological study done by Kono et al. (1992) compared serum cholesterol levels in 1,306 Japanese males drinking ≥ 9 cups/day to those who were consuming 0-2 cups/day of green tea. After adjustment for smoking, alcohol use, physical activity, and body protein index, total serum cholesterol was found to be inversely related to the consumption of green tea. There was no association, however, with serum triacylglycerols or HDL cholesterol. Consistent with these findings is the observed induction of LDL receptor gene expression in HepG2 cells when incubated with green tea extract (Figure 7.6). It can be speculated that the low plasma cholesterol could be due to an enhanced clearance of LDL cholesterol via the upregulation of the LDL receptor pathway.

Animal studies consistently show a decrease in plasma LDL levels with green tea extract administration (Chisaka et al., 1988; Ikeda et al., 1992; Matsuda et al., 1986). The observed decrease in plasma cholesterol through the upregulation of the LDL receptor pathway may be the mechanism by which green tea exerts its most significant antiatherogenic effect. Many of these animal studies have examined the individual components of green tea and their ability to induce hypocholesterolemia. Mice with an oral administration of (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (ECGg) not only had lower plasma cholesterol levels, but also lowered liver cholesterol concentrations. Chisaka et al. (1988) reported that the clearance of radiolabelled cholesterol from plasma was faster in rats fed ECGg than control. This suggests that the upregulation of the hepatic LDL receptor by this agent may have been responsible for the enhanced clearance of cholesterol.

Green tea seems to ameliorate the hypercholesterolemia induced by high fat and cholesterol feeding in many animal studies. Fukuyo et al. (1996) observed that ECGg had a profound effect on the reduction of hypercholesterolemia induced in rats by cholesterol feeding. The ECGg supplementation decreased plasma and LDL cholesterol concentrations, and increased plasma HDL cholesterol concentration. Total lipid, total cholesterol and triacylglycerol concentrations of the liver were increased in rats fed the high cholesterol diet, but the addition of ECGg decreased these parameters. It appears that ECGg is one of the active components of green tea that may be responsible for the hypocholesterolemic effect. As mentioned, ECGg has the highest antioxidant activity compared to the other catechins present in green tea and is presumably the most active component present. It can be

speculated that the upregulation of the LDL receptor expression in HepG2 cells with green tea extract could, in fact, be the result of ECGg.

There is no evidence, as yet, on the mechanism of action whereby green tea catechins induce the expression of LDL receptor gene transcription. Green tea may be involved in regulating LDL receptor gene transcription through the SREBP-1/SRE-1 system (see section 1.7.5). Both LDL receptor and HMG-CoA reductase genes have similar sterol response elements within their promoter regions (see section 1.7.5) and have been shown to be coordinately regulated (Rudling, 1992). The work described here showed that green tea had an identical effect on both HMG-CoA reductase and LDL receptor mRNA levels (Figure 7.6 and 7.7). Therefore, green tea could be simultaneously affecting the expression of both of these genes through a common transcriptional factor, such as Sp-1 or SREBP-1.

Green tea may be an HMG-CoA reductase inhibitor as it is a hypocholesterolemic agent. There is an increase in HMG-CoA reductase mRNA levels in HepG2 cells with green tea extract (Figure 7.7). HMG-CoA reductase inhibitors are usually described as agents which competitively inhibit HMG-CoA reductase activity (Endo, 1992) (see section 1.7.7). However, treatment with these inhibitors usually results in a large increase in the mRNA and protein for HMG-CoA reductase, the LDL receptor and other genes. This usually depletes the supply of cholesterol to the liver which is compensated for by the enhanced removal of LDL from the circulation by the liver. This effectively lowers the plasma cholesterol. The initial effects of green tea on HMG-CoA reductase activity may account for the substantial increase in LDL receptor and HMG-CoA reductase mRNA in HepG2 cells and explain why its intake reduces plasma cholesterol in humans and experimental animals. The inhibition of HMG-CoA reductase activity and the subsequent upregulation of HMG-CoA reductase mRNA levels by green tea may be result of its antioxidant properties. Although other antioxidants were not tested to see whether they could also affect HMG-CoA reductase gene transcription in this manner, it was previously demonstrated that α-tocopherol could also upregulate HMG-CoA reductase mRNA levels (Figure 6.6). Thus, the upregulation of HMG-CoA reductase mRNA levels could be a common "antioxidant phenomenon", which could either cause or be associated with the upregulation of the LDL receptor.

Red Wine

The metabolism and transport of polyphenolic flavonoids present in red wine have not been examined in detail thus far. However, these flavonoids have substantial antioxidant properties as they are able to prevent lipid peroxidation in LDL (Fuhrman et al., 1995). It can be speculated that these phenolic substances are absorbed by or bind to plasma LDL and are carried in the circulation by this lipoprotein. Fuhrman et al. (1995) isolated LDL from healthy male volunteers before and after the volunteers had consumed 400 ml/day of either red or white wine for 2 weeks. They compared the propensity of the LDL to undergo oxidation in the presence of copper ions. Those who consumed red wine appeared to have

significant protection of their LDL from lipid peroxidation (determined by thiobarbituric acid reactive substances), whereas LDL isolated from white wine consumers failed to be protected significantly against oxidation. One can imply from this study that in humans, the antioxidant phenolic substances in red wine are associated with LDL. It is conceivable that through this mode of transport these substances are also able to manipulate the LDL receptor in cells and confer protection against the development of CHD.

Even though red wine consumption protects LDL oxidation *in vitro*, it has never been shown *in vivo*. In addition, the effect of red wine consumption on plasma LDL concentrations has yet to be shown. However, red wine, but not white wine, has been discovered to increase plasma HDL concentrations (Fuhrman et al., 1995; Lavy et al., 1994). Similar effects have been shown with beer consumption. Nishiwaki et al. (1994) observed that men consuming alcohol (beer) for 4 weeks had a significant increase in their lipoprotein lipase activity and protein, along with a substantial rise in plasma HDL. The HDL raising effects by beer may be attributable to its high content of yeast vitamins (such as vitamins B and E), hops flavonoids, or even due to the alcoholic content. Thus, the antioxidant components of red wine may not be the only constituents that may be responsible for the enhanced expression of the hepatic LDL receptor, but the alcohol components of red wine also have to be considered as a factor.

It is still unknown whether red wine consumption has any impact on lipoprotein metabolism and/or cholesterol metabolism other than increasing plasma HDL levels. Based on the observations herein (Figure 7.9), it appears that red wine can have a profound effect on LDL receptor expression in HepG2 cells. Whether or not such affects on LDL receptor expression is due to the antioxidant capacity of red wine has to be further investigated. However, if the consumption of red wine has the same effect *in vivo* , it may explain why a decreased risk of CHD is observed with increased consumption.

SUMMARY

Antioxidants show great diversity in their structure, metabolism, function and mechanisms of action, though not necessarily in their presumed roles as physiological antioxidants. Given that these structurally distinct compounds similarly upregulate hepatic LDL receptor expression, it is conceivable that this effect may be linked to their common inherent antioxidant properties. Therefore, one could speculate that these antioxidants are powerful physiological modulators which may act through a common mechanism to upregulate gene transcription of the LDL receptor. However, evidence indicates that each antioxidant may have a unique pathway through which it can act either directly or indirectly to upregulate gene transcription of the LDL receptor. At this point, it is unclear by precisely which mechanism antioxidants exert their effects and whether it is a common mode of action for all these antioxidants. What is known from the work herein is that all these compounds are capable of upregulating the LDL receptor, mainly at the level of transcription. Thus, this

work supports the hypothesis that the upregulation of the LDL receptor is a common "antioxidant phenomenon".

Cells may be regulating the gene expression of the LDL receptor in order to fulfil antioxidant requirements. Certain compounds like vitamin E, ß-carotene and other fat soluble nutrients can only be transported to various cells throughout the body packaged in the LDL particle, which enters the cell via the LDL receptor pathway. Therefore, similar to the manner in which the cell maintains its cholesterol balance, the upregulation of the LDL receptor may also serve as the primary route in which the cell maintains its vitamin requirements. If this is the case, then the regulation of the LDL receptor by the antioxidant vitamins observed herein is not anomalous event.

Chapter 8

8.0. THE EFFECT OF POLYUNSATURATED FATTY ACIDS AND ANTIOXIDANTS ON THE LDL RECEPTOR IN HepG2 LIVER CELLS

8.1 Introduction

The earlier findings in HepG2 cells (Chapters 4 and 5) demonstrated that as LDL receptor expression (activity, protein and mRNA) increased, unsaturation of the fatty acid decreased. It is well established that the susceptibility to oxidation also increases as the unsaturation of the fatty acid increases (Slim et al., 1996). Hence, the PUFAs, linoleic acid and EPA, are more susceptible to oxidation than saturated or monounsaturated fatty acids. EPA has five double bonds and linoleic contains two double bonds. The possibility, therefore, existed that the observed suppression of the LDL receptor by linoleic acid and EPA was due to their oxidisation when they were incubated with the cells. As these fatty acids were incubated with the cells for 24 h, the temperature (37°C) and other conditions of the incubation media (95% air) could potentially be ideal for the formation of fatty acid oxidation products in that time period. Therefore, in order to address this issue, the effect of EPA and linoleic acid on the level of LDL receptor protein was examined in the presence of vitamin E (the major lipid soluble antioxidant). The results demonstrated that even in the presence of vitamin E, LDL receptor protein levels were effectively suppressed by EPA and linoleic acid (Chapter 5). This argued against the downregulation of the LDL receptor being due to oxidation of PUFAs during incubation.

These earlier experiments also revealed a very interesting observation, in that vitamin E alone was observed to be able to upregulate the level of LDL receptor protein (Chapter 5 and 6). When other antioxidants were examined (vitamin A, vitamin C and ß-carotene), they were also able to upregulate the LDL receptor (Chapter 7). Therefore, it was concluded that the upregulation of the LDL receptor could be a general "antioxidant phenomenon".

In summary, fatty acids and antioxidants individually can have a profound impact on the LDL receptor in HepG2 cells (Chapters 5, 6 and 7). In contrast to the suppressive nature of the PUFAs, EPA and linoleic acid, antioxidants appeared to be potent inducers of LDL receptor expression (activity, mass and mRNA). It was observed in one of the experiments (Chapter 5), that in the presence of vitamin E, the LDL receptor protein was not suppressed by EPA or linoleic to the extent observed with the fatty acids alone (Figure 5.7 and Figure 5.8). These findings suggested that EPA and vitamin E may be having separate effects on the LDL receptor. Based on the observations from earlier findings, it was hypothesised that vitamin E and fatty acids would have separate effects on the LDL receptor and similarly, it would follow that other antioxidants, such as vitamin A, vitamin C and ß-carotene, would also have independent effects on the LDL receptor in the presence of PUFAs. Therefore, the primary aim of the work described in this chapter was to investigate the independent effects of antioxidants and fatty acids on LDL receptor expression (activity, mass and mRNA).

Earlier observations indicated that fatty acid peroxides of EPA and linoleic acid did not appear to be responsible for the suppression of the LDL receptor (Chapter 5). Another objective of work described in this chapter was to further test this possibility using different antioxidants. These experiments should also verify the previous findings and add further support to the theory that fatty acid peroxides of EPA and linoleic acid are not responsible for the decrease in LDL receptor expression (activity, mass and mRNA) seen in the cell culture experiments described in this thesis.

8.2 METHODS

The cell experiments were designed to investigate the effect of antioxidants and fatty acids on LDL receptor expression when HepG2 cells were incubated in the presence of both. Cells were subcultured from large stock flasks into smaller flasks (see section 2.1.2) and grown in DMEM containing 10% FCS for 24 h (day 1). The media was then replaced on day 2 with DMEM containing 10% LPDS and cells were grown for a further 24 h in this media. On day 3, cells received various treatments of either fatty acids or antioxidants depending on the design of experiments as indicated below (section 8.3). These agents were added to cells incubated in DMEM supplemented with 10% LPDS and therefore differ from previous experiments (Chapter 6 and 7), which were carried out in DMEM supplemented with 10% FCS. At the end of the 24 h incubation period, cells were harvested and LDL receptor activity (section 2.6), protein (section 2.7) and mRNA levels (section 2.8) were measured. When measurements were made in 2 treatment groups only, statistical evaluation was done using Students t-test. When measurements were made in more than 2 treatment groups, statistical evaluation was done using a one way analysis of variance (ANOVA), and the pooled variance was then used to determine the least significant difference when comparing individual groups. Significance was defined at $P \leq 0.01$.

8.3 RESULTS

8.3.1 The effect of EPA and vitamin E on LDL receptor protein in HepG2 cells.

The initial objective of the work described in this chapter was to confirm the results from earlier work (Figure 5.7), which had demonstrated the independent effects of vitamin E and EPA on LDL receptor expression.

HepG2 cells were subcultured and grown until day 3 according to the method indicated above (section 8.2). On day 3, HepG2 cells were incubated simultaneously with 50 μM vitamin E and 250 μM EPA for 24 h in a background medium of DMEM containing 10% LPDS.

In the presence of 50 μM vitamin E and 250 μM EPA, LDL receptor protein was decreased by 40% compared to cells incubate with no vitamin E or EPA (Figure 8.1). These observations are consistent with those observed in earlier findings (Figure 5.7) in which EPA, in the presence of vitamin E, was unable to inhibit the LDL receptor to the extent observed without vitamin E. The LDL receptor protein content has been observed to be decreased by 75% in cells incubated with 100 μM EPA alone compared to cells incubated without fatty acids or vitamin E (Figure 5.4). Thus, the presence of vitamin E, in conjunction with EPA, increases LDL receptor activity from the suppressed level observed with EPA alone. Therefore, it appears that both EPA and vitamin E are exerting an affect on the LDL receptor protein, where the final level results from the combined effects of both agents.

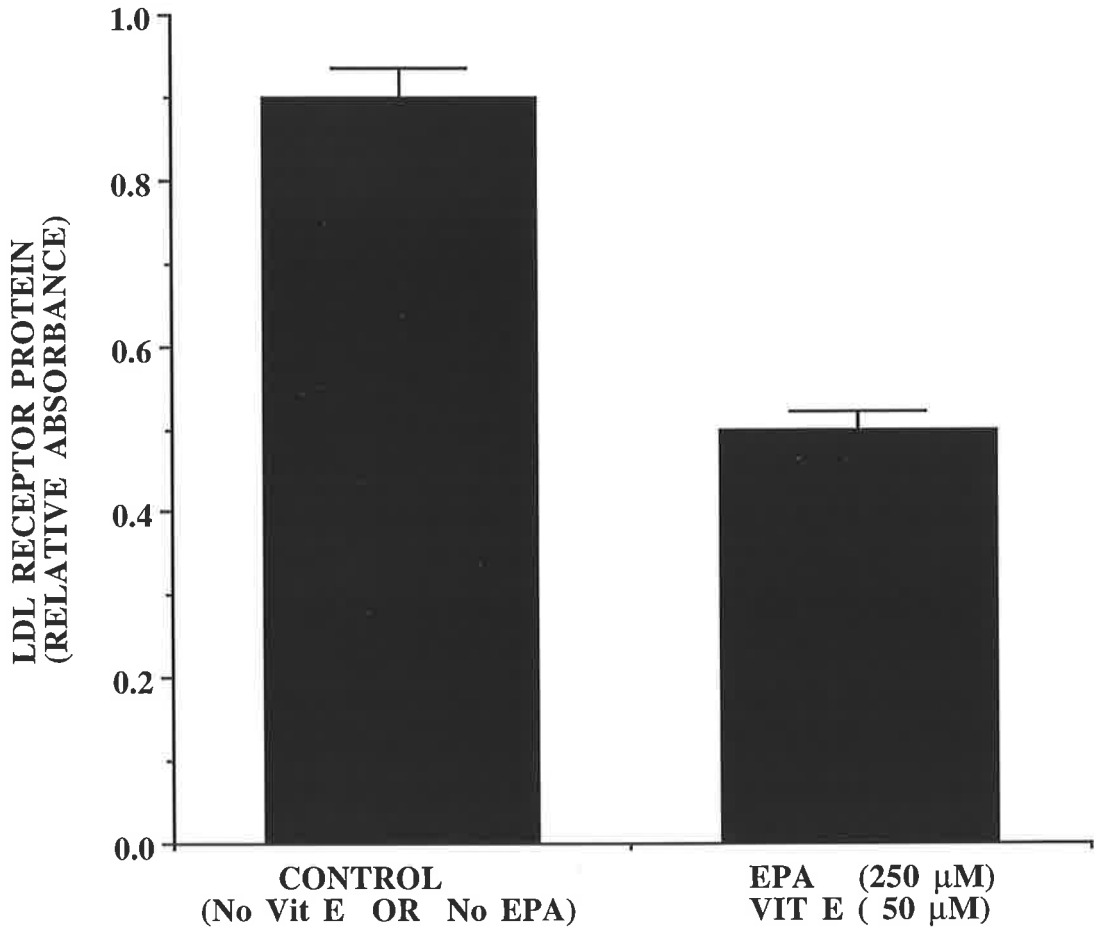


Figure 8.1 The effect of EPA and vitamin E on LDL receptor protein in HepG2 cells. Cells were isolated after 24 h incubation with 50 μM EPA and 250 μM vitamin E. Cellular proteins were solubilised with Triton X-100 detergent, electrophoresed, transferred to nitrocellulose and quantified by the ECL method as described in section 2.7. Treatments were performed in duplicate and the relative densities of the receptor band were quantified by laser densitometry. Results are relative absorbance. Data represents the mean ± SEM of 3 experiments.

8.3.2 The effect on LDL receptor protein mass with increasing concentrations of EPA in the presence of 50 μM vitamin E.

The LDL receptor expression (activity, protein and mRNA) in HepG2 cells has been shown to be downregulated in the presence of EPA (Chapter 5) and upregulated in the presence of 50 μM vitamin E (Chapter 6). In addition, it appears that when both of these agents are present in cells at the same time, they each have an impact on LDL receptor protein (Figure 8.1) However, it is unclear whether one agent has a greater effect than the other. Although this question can be addressed in a number of ways, the initial experiment was designed to elucidate whether the increased LDL receptor protein by vitamin E could be overridden by EPA. Thus, HepG2 cells were incubated with increasing concentrations of EPA in the presence of a fixed concentration of 50 μM vitamin E.

HepG2 cells were subcultured into 10 flasks, and grown until day 3 according to the method indicated above (section 8.2). On day 3, 5 flasks of HepG2 cells (containing DMEM supplemented with 10% LPDS) were incubated with 50 μM vitamin E and with various concentrations of EPA up to 250 μM. Another 5 flasks (containing DMEM supplemented with 10% LPDS) received various amounts of EPA only. Cells were then incubated for a further 24 h, and subsequently harvested.

In the absence of vitamin E, LDL receptor protein decreased in a curvilinear manner in HepG2 cells incubated with increasing concentrations of EPA (dotted line, Figure 8.2). This decrease in cellular LDL receptor protein with increasing concentrations of EPA was consistent with earlier experiments (Figure 5.6). The gradual decrease in cellular LDL receptor protein with increasing amounts of EPA was also observed in the presence of 50 μM vitamin E in the background media (Figure 8.2). Compared to cells incubated without EPA but with 50 μM vitamin E, LDL receptor protein decreased 78% and 88% when cells were incubated with 100 μM and 250 μM EPA, respectively (Figure 8.2, Table 8.1). Similarly, compared to cells incubated with no EPA and no vitamin E, LDL receptor protein decreased 77% and 90%, when cells were incubated with 100 μM and 250 μM EPA, respectively (Figure 8.2, Table 8.1). Thus, the percentage decrease in LDL receptor protein with increasing amounts of EPA was similar at each concentration of EPA tested, with or without vitamin E and regardless of the absolute amount of LDL receptor protein present in the cells (refer to Table 8.1).

As the amount of LDL receptor protein was initially increased with vitamin E in the absence of EPA compared to cells incubated without vitamin E and EPA, the decrease in LDL receptor protein with increasing concentrations of EPA started at an elevated level when vitamin E was in the background media (Figure 8.2). The upregulation of LDL receptor protein with 50 μM vitamin E seen here was consistent with earlier findings (Figure 6.3). Interestingly, LDL receptor protein was effectively increased by vitamin E to approximately the same extent at every concentration of EPA tested (Table 8.2), indicating that vitamin E

has the same effect on LDL receptor protein whether or not EPA is present. The total amount of LDL receptor protein in cells incubated with 50 μM vitamin E was approximately 4-5 times higher than cells incubated without 50 μM vitamin E at any concentration of EPA (dotted line in Figure 8.2, Table 8.2).

Although the starting absolute value of LDL receptor protein changed when cells were exposed to one of the agents, the percentage increase with vitamin E or percentage decrease with EPA was the same regardless of the presence of the other agent. Thus, these results indicate that both EPA and vitamin E exerted their own independent effects on LDL receptor protein and that neither agent could influence the effect of the other.

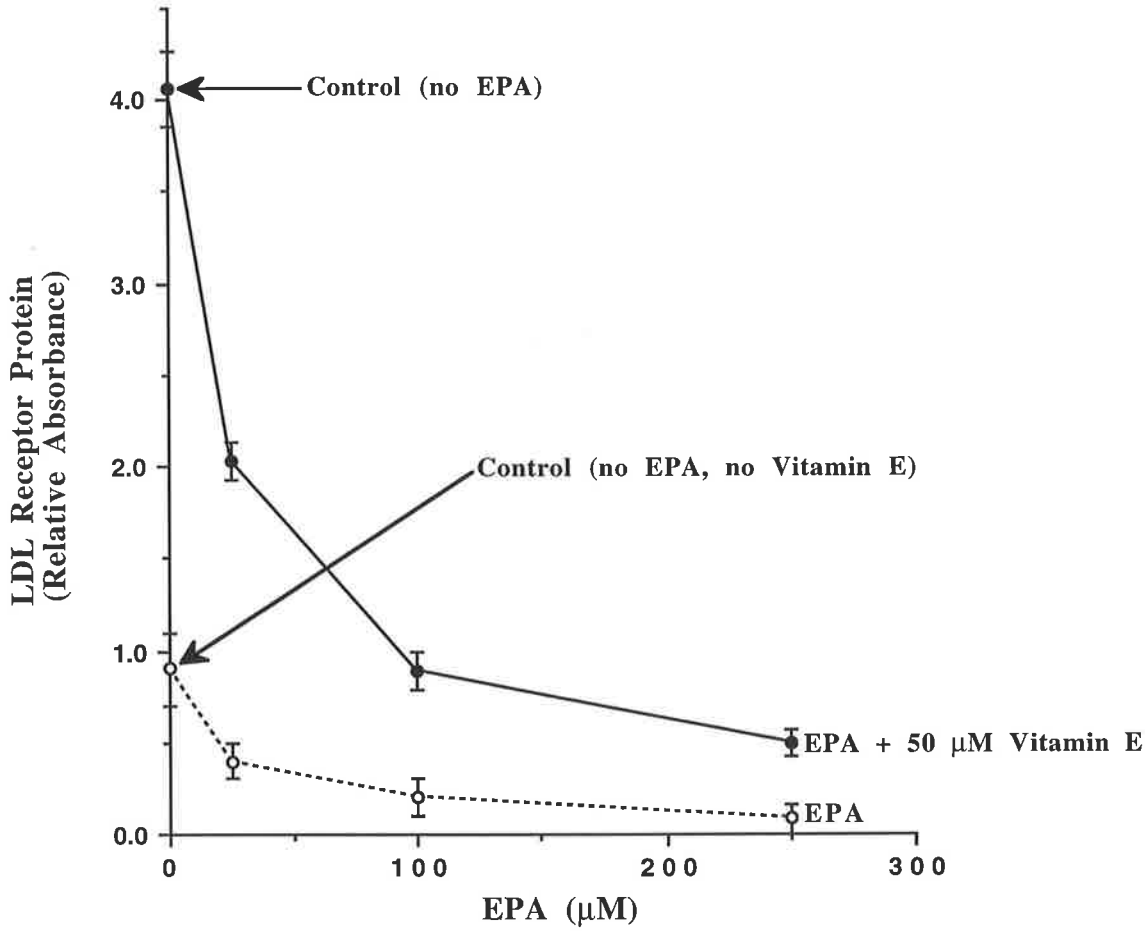


Figure 8.2 The effect on LDL receptor protein of increasing concentrations of EPA in the presence and absence of 50 μM vitamin E. Cells were incubated with either increasing concentrations of EPA and 50 μM vitamin E (solid line) or increasing concentrations of EPA without vitamin E (dotted line) in a background media of DMEM supplemented with 10% LPDS. After a 24 h incubation period, cellular proteins were solubilised with Triton X-100, electrophoresed, transferred to nitrocellulose and LDL receptor protein was quantified using the ECL method (see section 2.7). Treatments were performed in duplicate and the relative densities of the receptor bands were quantified using laser densitometry. Results represent the relative absorbance and are the mean ± SEM of 2 experiments.

Table 8.1: Percentage decrease in LDL receptor activity with increasing concentrations of EPA in the presence and absence of vitamin E.

EPA (μM)	**EPA plus vitamin E** (% decrease in LDL receptor protein from 0 μM EPA)	**EPA minus vitamin E** (% decrease in LDL receptor protein from 0 μM EPA)
25	50	55
100	78	77
250	88	90

Table 8.2: Percentage increase of LDL receptor protein in cells incubated with 50 μM vitamin E compared to cells incubated with EPA alone.

EPA (μM)	% increase in LDL receptor protein in cells incubated with EPA + vitamin E compared to cells incubated with EPA alone (dotted line)
0	350
25	407
100	345
250	485

8.3.2 The effect on LDL receptor protein of increasing concentrations of vitamin E in the presence of 250 μM EPA.

Given that when cells were incubated with increasing concentrations of EPA in the presence of 50 μM vitamin E (Figure 8.2), both agents were observed to independently influence LDL receptor protein levels, the reverse experiment was designed. Cells were incubated with increasing concentrations of vitamin E in the presence of 250 μM EPA.

The HepG2 cells were subcultured into 14 flasks, and grown until day 3 (section 8.2). On day 3, 7 flasks of HepG2 cells (containing DMEM supplemented with 10% LPDS) were incubated with 250 μM EPA and various concentrations of vitamin E up to 100 μM. Another 7 flasks received only increasing amounts of vitamin E. Cells were incubated for another period of 24 h.

A biphasic expression of the LDL receptor with increasing concentrations of vitamin E was observed with and without the presence of 250 μM EPA in the background media (Figure 8.3A). In the presence of 250 μM EPA, LDL receptor protein increased by 310% in the presence of 50 μM vitamin E and then decreased from this maximum level as the concentration of vitamin E was increased (Figure 8.3 A). It is interesting to note that even though the LDL receptor was elevated over 300% with 50 μM vitamin E in the presence of EPA, the absolute value of LDL receptor protein (relative absorbance = 0.730) did not even reach the absolute value of LDL receptor protein in cells incubated in the absence of vitamin E and EPA (relative absorbance = 0.9) (Figure 8.3 A). Moreover, the distinct bimodal effect of vitamin E in the presence of 250 μM EPA on LDL receptor is not seen clearly when plotted on the same graph as vitamin E alone (Figure 8.3 A). When these curves are normalised, however, the percent increase in LDL receptor protein by vitamin E was approximately the same whether or not EPA was absent or present in the background media (Figure 8.3B). These identical curves indicate that the effect of vitamin E on LDL receptor expression is not influenced by the presence of EPA.

Interestingly, since the amount of LDL receptor protein was initially decreased by 80% by 250 μM EPA in the absence of vitamin E compared to cells incubated without vitamin E and EPA, the subsequent increase in LDL receptor protein with increasing vitamin E concentrations in the presence of 250 μM EPA started at a lower level of LDL receptor protein (Figure 8.3 A). Also, at any given concentration of vitamin E, LDL receptor protein was approximately 85% lower in cells with 250 μM EPA in the background media than without EPA (Table 8.3). Thus, the effect of EPA on LDL receptor protein is the same, regardless of the presence or absence of vitamin E.

Thus, the starting absolute value of LDL receptor protein changed when cells were exposed to EPA, but the percentage increase with vitamin E was the same, regardless of the presence or absence of EPA. Both EPA and vitamin E, therefore, exerted their own independent effects on LDL receptor protein such that the absolute amount of LDL receptor protein present in the cell results from the combined effects of both agents.

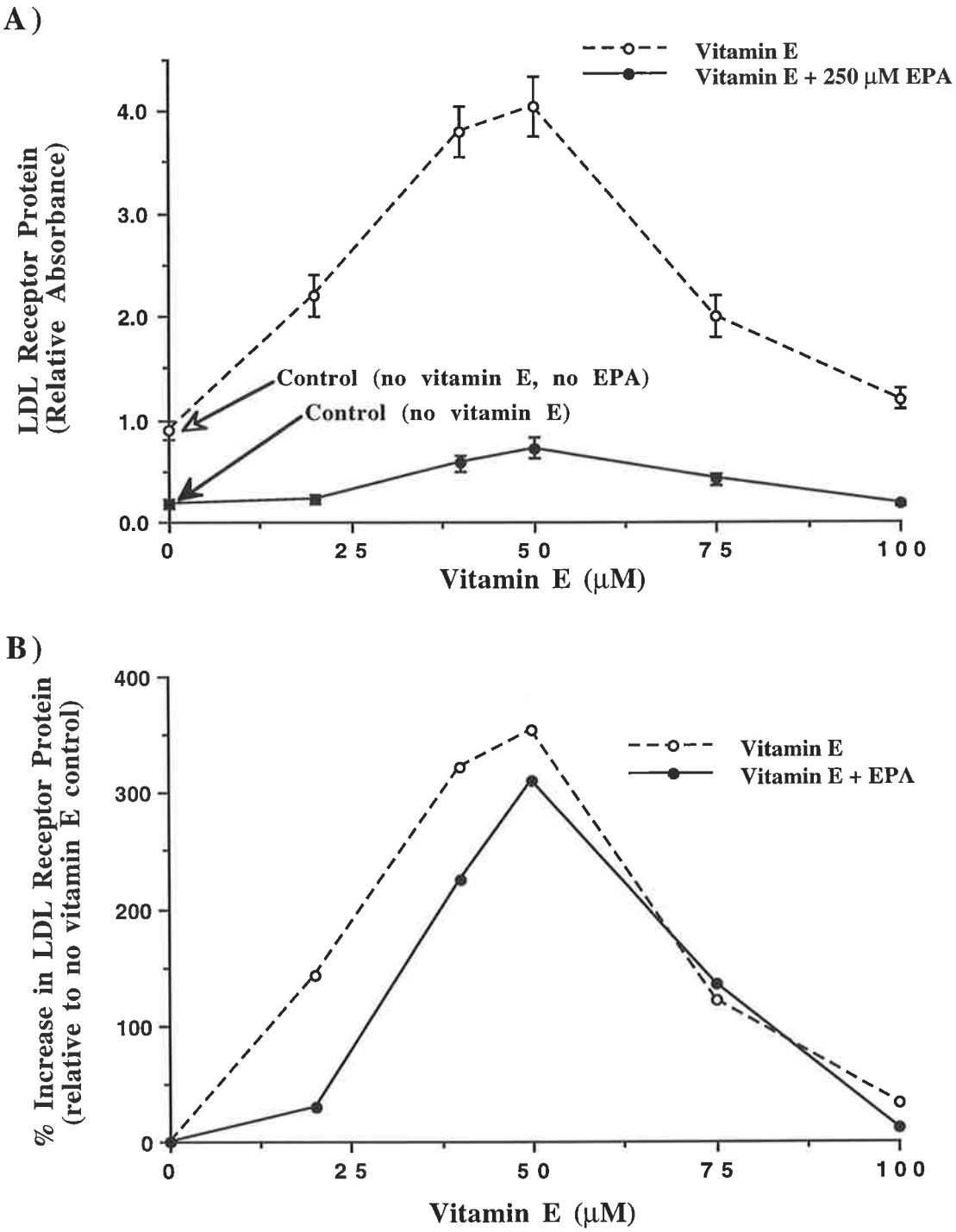


Figure 8.3 The effect of LDL receptor protein with increasing concentrations of vitamin E in the presence of 250 μM EPA. Cells were incubated in a background media of DMEM supplemented with 10% LPDS and (1) with increasing concentrations of vitamin E and a fixed concentration of 250 μM EPA (solid line), or (2) increasing concentrations of vitamin E without EPA (dotted line). After a 24 h incubation period, cellular proteins were solubilised with Triton X-100, electrophoresed, transferred to nitrocellulose and LDL receptor protein was quantified using the ECL method (see section 2.7). Treatments were performed in duplicate and the relative densities of the receptor band were quantified by laser densitometry. Results are expressed as **(A)** relative absorbances (absolute values) and data represents the mean ± SEM of 2 experiments, or **(B)** relative change compared to their appropriate no vitamin E control value.

Table 8.3: Percent decrease of LDL receptor protein in cells incubated with vitamin E+ EPA compared to cells incubated with vitamin E alone.

Vitamin E concentration (μM)	% decrease in LDL receptor protein in cells incubated vitamin E + EPA compared to cells incubated with vitamin E alone
0	80.0
20	89.5
40	84.0
50	82.0
75	79.0
100	85.0

8.3.3 The effect on LDL receptor activity, protein and mRNA levels in HepG2 cells supplemented at different times with EPA and vitamin E.

The results so far imply that neither vitamin E nor EPA can overpower the other as they both appear to contribute to the final level of the LDL receptor protein. However, it was not known whether this effect would still be apparent when cells are exposed to these agents sequentially, rather than simultaneously. The next experiment, therefore, was designed such that the second agent was added to the incubation media 24 h after the incubation with the first agent.

HepG2 cells were subcultured into 7 flasks and grown until day 3 according to the method indicated above (section 8.2). On day 3, flasks 1 to 3 were incubated with DMEM supplemented with 10% LPDS (without EPA or vitamin E), flasks 4 and 6 were incubated with 50 μM vitamin E (in DMEM containing 10% LPDS), and flasks 6 and 7 were incubated with 250 μM EPA (in DMEM containing 10% LPDS) (Table 8.4). After 24 hours (day 4), the media was decanted in all the flasks, and flask 1 was incubated with only DMEM containing 10% LPDS (without EPA or vitamin E), flasks 2, 4 and 7 were supplemented with 50 μM vitamin E, and flasks 3, 5, and 6 were supplemented with 250 μM EPA (Table 8.4). Cells were then incubated for another 24 h.

Table 8.4: Experimental Design.

Flask #	Day 1 (DMEM +)	Day 2 (DMEM +)	Day 3	Day 4
1	10% FCS	10% LPDS	10% LPDS	10% LPDS
2	10% FCS	10% LPDS	10% LPDS	10% LPDS+VitE
3	10% FCS	10% LPDS	10% LPDS	10% LPDS+EPA
4	10% FCS	10% LPDS	10%LPDS+Vit E	10% LPDS+VitE
5	10% FCS	10% LPDS	10%LPDS+EPA	10% LPDS+EPA
6	10% FCS	10% LPDS	10% LPDS+VitE	10% LPDS+EPA
7	10% FCS	10% LPDS	10%LPDS+EPA	10% LPDS+VitE

LDL receptor activity

Compared to cells in flask 1 (no vitamin E and no EPA), LDL receptor activity was depressed by 62% when cells were incubated with EPA for one day (flask 3) and by 96% when incubated with EPA for two days (flask 5) (Figure 8.4). This presumably occurred because the incorporation of EPA into cells/cell membranes is slow and had not reached its maximum within 24 h. In contrast, LDL receptor activity was upregulated by 122% and

110% when cells were incubated with vitamin E for 24 h (flask 2) or 48 h (flask 4), respectively, compared to control cells (flask 1) (Figure 8.4). LDL receptor activity was not further augmented after 48 h with vitamin E, suggesting that uptake of vitamin E by cells is fast and had reached its maximum by 24 h.

When vitamin E was introduced 24 h prior to the addition of EPA, LDL receptor activity was only increased slightly compared to cells which were incubated with EPA alone for one day (flask 3) (Figure 8.4). In this case, the presence of vitamin E for 24 h before the addition of EPA did not appear to influence the effect of EPA on LDL receptor activity. This suggests that vitamin E was quickly cleared from the cells and was no longer present in the cells after this time period.

In contrast, when EPA was introduced to the cells before the addition of vitamin E (flask 7), LDL receptor activity was suppressed compared to cells incubated with vitamin E for one day (flask 2). In this case, the addition of vitamin E did not upregulate the receptor activity to the level observed when cells were incubated with vitamin E alone (flask 2). This demonstrates that the effect of EPA was still apparent even after 48 h, and suggests that the clearance this fatty acid out of the cells was slow.

LDL Receptor protein and mRNA

The response of cellular LDL receptor protein levels to vitamin E and EPA at different times was similar to the effect of these agents on LDL receptor activity (Figure 8.5). LDL receptor protein was inhibited by 92% (flask 3) and 96% (flask 5) by EPA when the cells were supplemented for 24 h or 48 h, respectively, compared to cells incubated without vitamin E and EPA (flask 1).(Figure 8.5). In contrast, when cells were exposed to vitamin E alone for 24 h (flask 2) or 48 h (flask 4), LDL receptor protein levels increased 280% above control cells (flask 1, no vitamin E and no EPA).

When cells were incubated with EPA, after a 24 h incubation with vitamin E (flask 6), LDL receptor protein levels were slightly elevated compared to cells incubated with EPA alone (flask 2) (Figure 8.5). Conversely, when EPA was added to cells 24 h prior to the addition of vitamin E (flask 7), LDL receptor protein levels were still depressed compared to cells incubated with vitamin E alone (flask 2). This again suggested EPA was still present in cell, even after they were incubated in the absence of the fatty acid for 24 h.

The level of LDL receptor mRNA in response to vitamin E after the introduction of EPA (24 h prior) and the response to EPA after the prior addition of vitamin E (Figure 8.6) parallelled the effect of these agents on LDL receptor protein (Figure 8.5) and LDL receptor binding activity (Figure 8.4). Therefore, vitamin E and EPA are exerting their effects at the level of gene transcription, and this can explain the observations at the level of LDL receptor protein and binding activity.

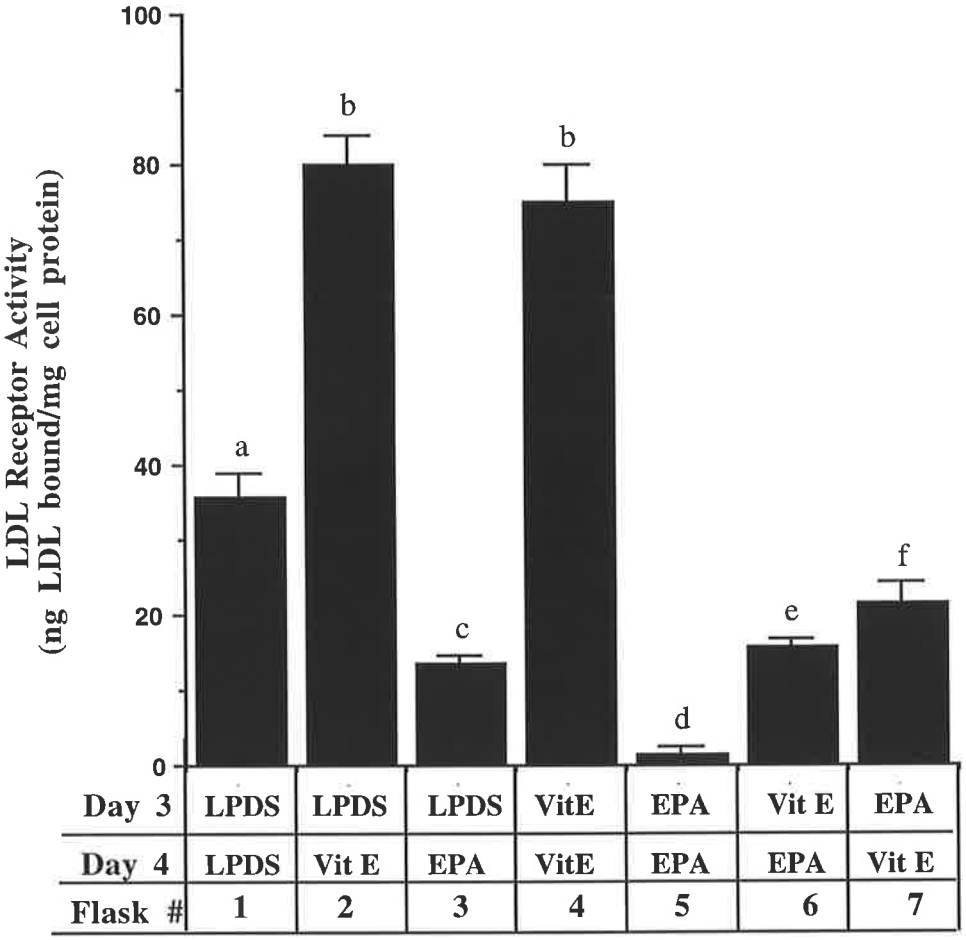


Figure 8.4 The effect on LDL receptor activity in HepG2 cells supplemented at different times with EPA and vitamin E. HepG2 cells were subcultured into small flasks containing DMEM + 10% FCS (day 1). On day 2, cells were incubated with DMEM + 10% LPDS. On day 3 and day 4, cells were incubated with the various agents (Table 8.4) in a background media containing DMEM + 10 % LPDS. The specific agents were added to the flasks on 2 consecutive days (day 3 agent/day 4 agent). Flask 1 = LPDS/LPDS (control flask, without EPA or vitamin E), flask 2 = LPDS/vitamin E, flask 3 = LPDS/EPA, flask 4 = vitamin E/vitamin E, flask 5 = EPA/EPA, flask 6 = vitamin E/EPA and flask 7 = EPA/vitamin E. After 24 h (day 5), cells were harvested and LDL receptor binding activity was measured in triplicate using the LDL colloidal gold method described in section 2.6.3. Results represent specific LDL receptor binding activity (ng of LDL bound/mg cell protein) which expressed as a mean ± SEM of 2 experiments. Means with different letters above them are significantly different at $P < 0.01$.

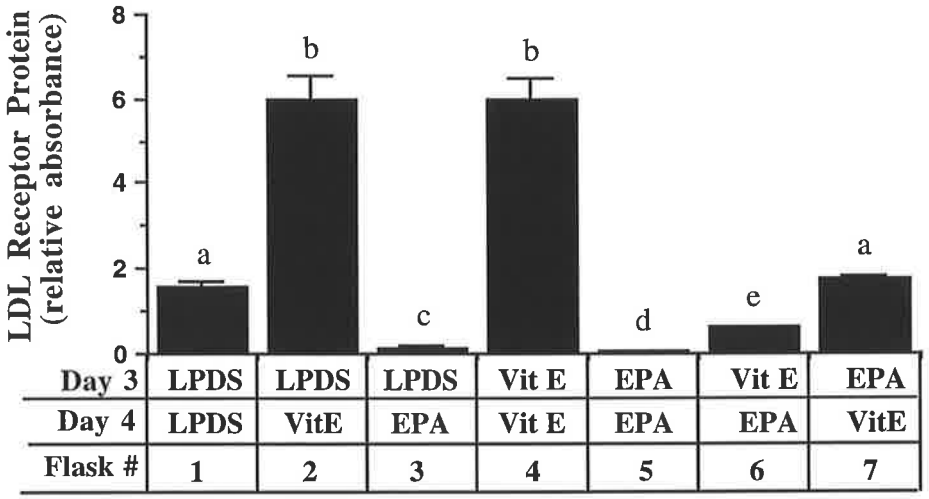


Figure 8.5 The effect on LDL receptor protein levels in HepG2 cells supplemented at different times with EPA and vitamin E. HepG2 cells were subcultured into small flasks containing DMEM + 10% FCS (day 1). On day 2, cells were incubated with DMEM + 10% LPDS. On day 3 and day 4, cells were incubated with the various agents (Table 8.4), in a background media containing DMEM + 10 % LPDS. The specific agents were added to the flasks on 2 consecutive days (day 3 agent/day 4 agent). Flask 1 = LPDS/LPDS (control flask, without EPA or vitamin E), flask 2 = LPDS/vitamin E, flask 3 = LPDS/EPA, flask 4 = vitamin E/vitamin E, flask 5 = EPA/EPA, flask 6 = vitamin E/EPA and flask 7 = EPA/vitamin E. After 24 h (day 5), cells were harvested and cellular proteins were solubilised with Triton X-100, electrophoresed, transferred to nitrocellulose and LDL receptor protein was quantified using the ECL method (section 2.7). Treatments were performed in duplicate and the relative densities of the receptor band were quantified by laser densitometry. Results are expressed as relative absorbances and data represents the mean ± SEM of 2 experiments. Means with different letters above them are significantly different at $P<0.01$.

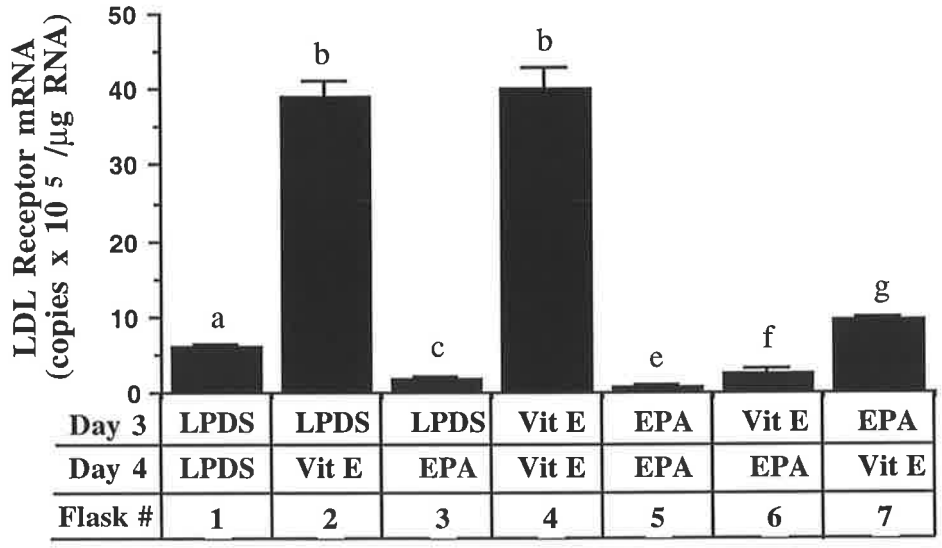


Figure 8.6 The effect on LDL mRNA levels in HepG2 cells supplemented at different times with EPA and vitamin E. HepG2 cells were subcultured into small flasks containing DMEM + 10% FCS (day 1). On day 2, cells were incubated with DMEM +10% LPDS. On day 3 and day 4, cells were incubated with the various agents (table 8.4), in a background media containing DMEM + 10 % LPDS. The specific agents were added to the flasks on 2 consecutive days (day 3 agent/day 4 agent). Flask 1 = LPDS/LPDS (control flask, without EPA or vitamin E), flask 2 = LPDS/vitamin E, flask 3 = LPDS/EPA, flask 4 = vitamin E/vitamin E, flask 5 = EPA/EPA, flask 6 = vitamin E/EPA and flask 7 = EPA/vitamin E. After 24 h (day 5), cells were harvested, total cellular RNA was isolated, reverse transcribed and amplified by PCR, with a synthetic piece of RNA used as an quantitative internal control (section 2.8). Cellular mRNA levels were measured relative to the internal control and expressed as mean ± SEM of 2 experiments. Means with different letters above them are significantly different at $P < 0.01$.

8.3.4 The effect on LDL receptor activity, protein and mRNA levels in HepG2 cells supplemented with antioxidants and EPA or linoleic acid.

Given that LDL receptor protein levels were observed to be independently influenced by vitamin E and EPA when they were simultaneously incubated with cells (Figure 8.3 and Figure 8.3), it was of interest to see if the same would happen when other fatty acids or when other antioxidants were present. The independent effects of fatty acids and antioxidants were further explored by examining whether the antioxidants vitamin A, vitamin C or ß-carotene could still influence LDL receptor expression in the presence of EPA or linoleic acid and *vice versa.* LDL receptor activity, protein and mRNA levels were measured in HepG2 cells incubated simultaneously with the antioxidants vitamin A, vitamin C or ß-carotene in the presence of either 250 µM EPA or linoleic acid.

HepG2 cells were subcultured and grown until day 3 (section 8.2). On day 3, all flasks (containing DMEM with 10% LPDS) were supplemented with either one of the antioxidants (100 µM vitamin A, vitamin C or ß-carotene) alone or with a fatty acid (250 µM EPA or linoleic acid) plus 100 µM vitamin A, vitamin C or ß-carotene. The cells were then further incubated for a 24 h period.

EPA and Antioxidants

In the absence of fatty acids, LDL receptor activity (Figure 8.7A), protein (Figure 8.8A) and mRNA (Figure 8.9A) levels were increased by vitamin A, vitamin C and ß-carotene in cells incubated in DMEM containing 10% LPDS. The increase in LDL receptor at these three levels (activity, protein and mRNA) was to the same extent observed in earlier experiments (Figure 7.1-7.3) in which cells were incubated with the same antioxidants but in DMEM supplemented with 10% FCS.

Consistent with earlier observations (Chapter 5), LDL receptor activity (Figure 8.7B), protein (Figure 8.8B) and mRNA levels (Figure 8.9B) were decreased by 75%, 88% and 70%, respectively, in the presence of EPA compared to cells incubated without EPA. In addition, compared to LPDS control cells (no fatty acid and no antioxidant), the antioxidants upregulated the LDL receptor activity, protein and mRNA in the presence of EPA (Figures 8.7B, 8.8B, 8.9B).

As previously observed for vitamin E (Figure 8.3 A), it appeared that in the presence of EPA, the antioxidants vitamin A, vitamin C and ß-carotene were unable to upregulate the LDL receptor to the same level observed in the presence of the antioxidants alone (Figures 8.7A, 8.8A, 8.9A). For example, LDL receptor was increased 300% with the antioxidants alone compared to the LPDS control cells. However, in the presence of EPA, vitamin A, vitamin C and ß-carotene only increased LDL receptor activity by 30% compared to control cells (no antioxidant or fatty acid). The absolute level of LDL receptor activity was, therefore, reduced when both agents were present together compared to when antioxidants were present alone.

Although it appears that these antioxidants were unable to upregulate the LDL receptor in the presence of EPA (Figures 8.7B, 8.8B, 8.9B) to the level observed with the antioxidants alone (Figures 8.7A, 8.8A, 8.9A), the extent to which LDL receptor activity was increased by these antioxidants was identical even in the presence of EPA when compared to the appropriate control (EPA alone) (Figures 8.7B, 8.8B, 8.9B). For example, compared to cells incubated with EPA alone, LDL receptor activity increased 323%, 349% and 291% when cells were incubated with EPA plus vitamin A or vitamin C or ß-carotene, respectively. The percentage increase in LDL receptor activity is consistent with the 300% increase in cellular LDL receptor activity exhibited when antioxidants are present alone compared to LPDS control cells (Figure 8.7A). As for the vitamin E and EPA experiments, the only difference between the results in Figures 8.7A and Figure 8.7B is that the absolute level of LDL receptor activity was lower when EPA and antioxidants were present together than when the antioxidants were present alone.

Even though it appears from the absolute values that the LDL receptor activity was not decreased by EPA when it is present in the cell together with the antioxidants compared to the LPDS control (Figure 8.7 B), the independent effect of EPA can be observed if one compares the absolute values in Figures 8.7 A and B (Table 8.5). For example, LDL receptor activity in cells incubated with vitamin A alone was 75 ng LDL bound/mg cell protein (Figure 8.7 A), whereas LDL receptor activity was 29 ng LDL bound/mg cell protein in cells incubated with both vitamin A and EPA (Figure 8.7 B). Therefore, the difference between these two values is an estimate of the percentage decrease in LDL receptor activity by EPA alone (Table 8.5). Thus, the decrease in cellular LDL receptor activity by EPA was similar in the absence or presence of antioxidants. The results herein clearly show that EPA and the antioxidants are having independent effect on LDL receptor activity when incubated with cells, simultaneously.

Table 8.5 % Decrease in LDL Receptor Activity by EPA.

Antioxidants	% Decrease in LDL Receptor Activity in cells incubated with EPA plus antioxidants compared to cells incubated with antioxidants alone (Figure 8.7 A versus B)
Vitamin A	61%
Vitamin C	60%
ß-Carotene	63%

Concomitantly, LDL receptor protein was also increased 313% by vitamin A, 200% by vitamin C and 313% by ß-carotene compared to LPDS control cells (no antioxidants) (Figure 8.8A). Similarly, the LDL receptor protein was increased 13-fold with vitamin A, 331% with vitamin C, and 737% with ß-carotene compared to the cells incubated with EPA alone (Figure 8.8B). Although there was a difference in the percentage increase, the antioxidants were still able to upregulate LDL receptor activity in the presence and absence of EPA. Conversely, the percent decrease in LDL receptor protein in cells incubated with EPA plus vitamin A, or vitamin C or ß-carotene compared to cells incubated with antioxidants alone was 54%, 79%, and 72%, respectively. This indicated that the relative amount of LDL receptor protein was decreased due to the presence of this fatty acid regardless of whether antioxidants were present or absent.

The LDL receptor mRNA levels were increased approximately 13-fold when the antioxidants were present alone compared to the LPDS control (Figure 8.9A). In the presence of EPA, LDL receptor mRNA levels were also increased significantly by 564%, 300% and 478% in cells incubated with vitamin A or vitamin C or ß-carotene, respectively, compared to cells incubated with EPA alone (without antioxidants) (Figure 8.9B). Although in this case, a 13-fold increase was not observed with the antioxidants when they were incubated with EPA, the independent affect of the antioxidants was still observed. Conversely, the LDL receptor mRNA was decreased in cells incubated with antioxidants plus EPA (Figure 8.9 B) compared to cells incubated with antioxidants alone (Figure 8.9 A). The percentage decrease in mRNA levels by EPA was approximately 85% in all cases.

The increase in LDL receptor activity, protein and mRNA levels due to antioxidants clearly shows that all the antioxidants could upregulate LDL receptor expression even in the presence of 250 μM EPA in the background media. Conversely, the suppression of the LDL receptor by EPA was also observed in the absence or presence of the antioxidants. These experiments demonstrated that antioxidants and EPA independently influence the LDL receptor expression in which the overall result is a combined effect of both agents. In addition, the independent effect of EPA in the presence of the antioxidants further suggests that fatty acid peroxides of EPA were not responsible for the observed decrease in LDL receptor expression.

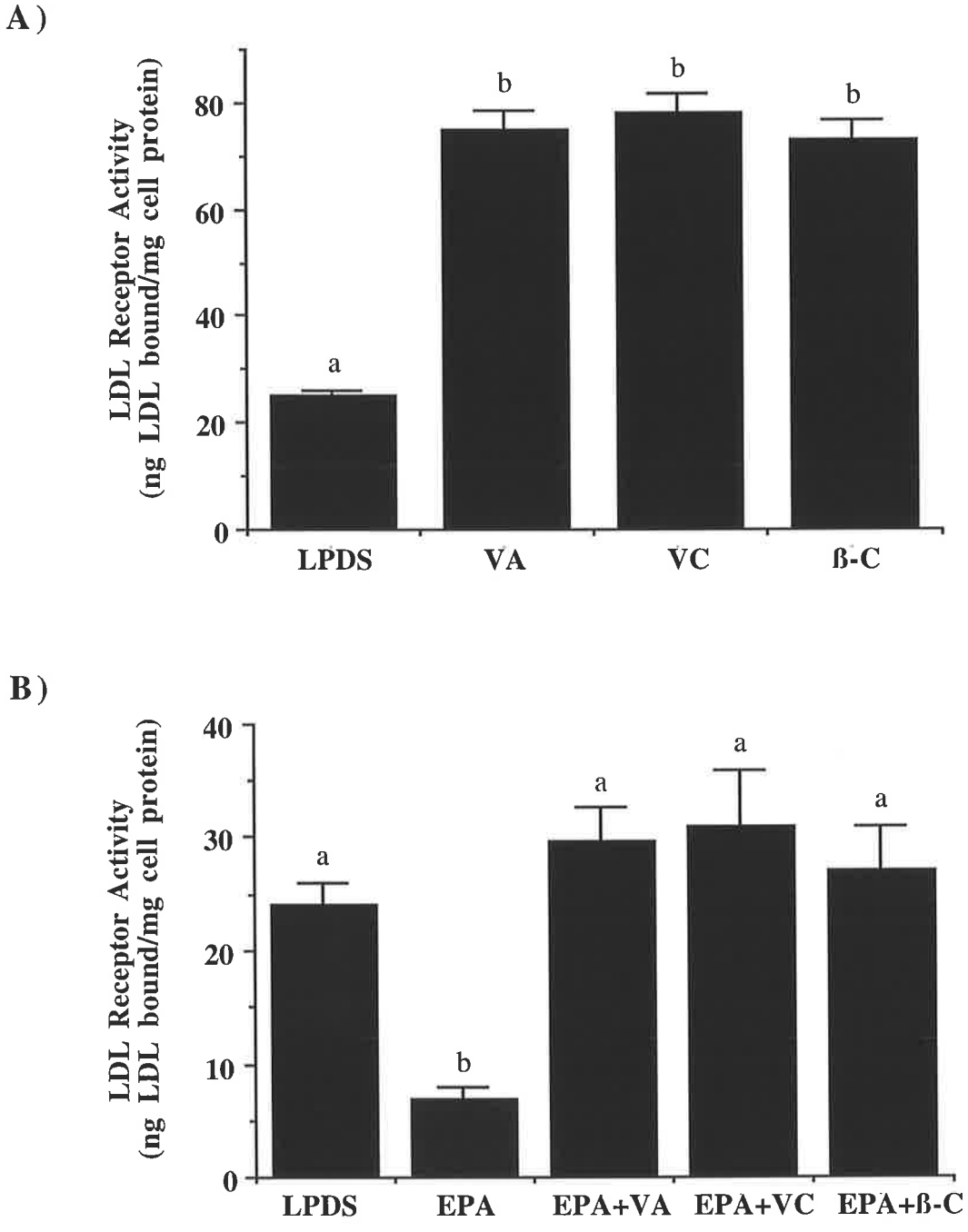


Figure 8.7 The effect on LDL receptor activity in HepG2 cells supplemented with antioxidants in the absence or presence of EPA. HepG2 cells were incubated with DMEM containing 10% LPDS after 2 days of subculturing. On day 3, cells were supplemented with either **(A)** no antioxidants (indicated as LPDS) or 100 μM vitamin A, vitamin C or ß-carotene in a background media containing 10% LPDS or with **(B)** no antioxidants and no EPA (indicated as LPDS), or 250 μM EPA alone, or 250 μM EPA and 100 μM vitamin A, vitamin C or ß-carotene in a background media containing 10% LPDS. Cells were further incubated for a period of 24 h, they were harvested (day 4), and LDL receptor binding activity measured in triplicate using the LDL colloidal gold method described in section 2.6.3. Results represent specific LDL receptor binding activity (ng of LDL bound/mg cell protein) which are expressed as the mean ± SEM of 2 experiments. Means with different letters above them are significantly different at $P < 0.01$.

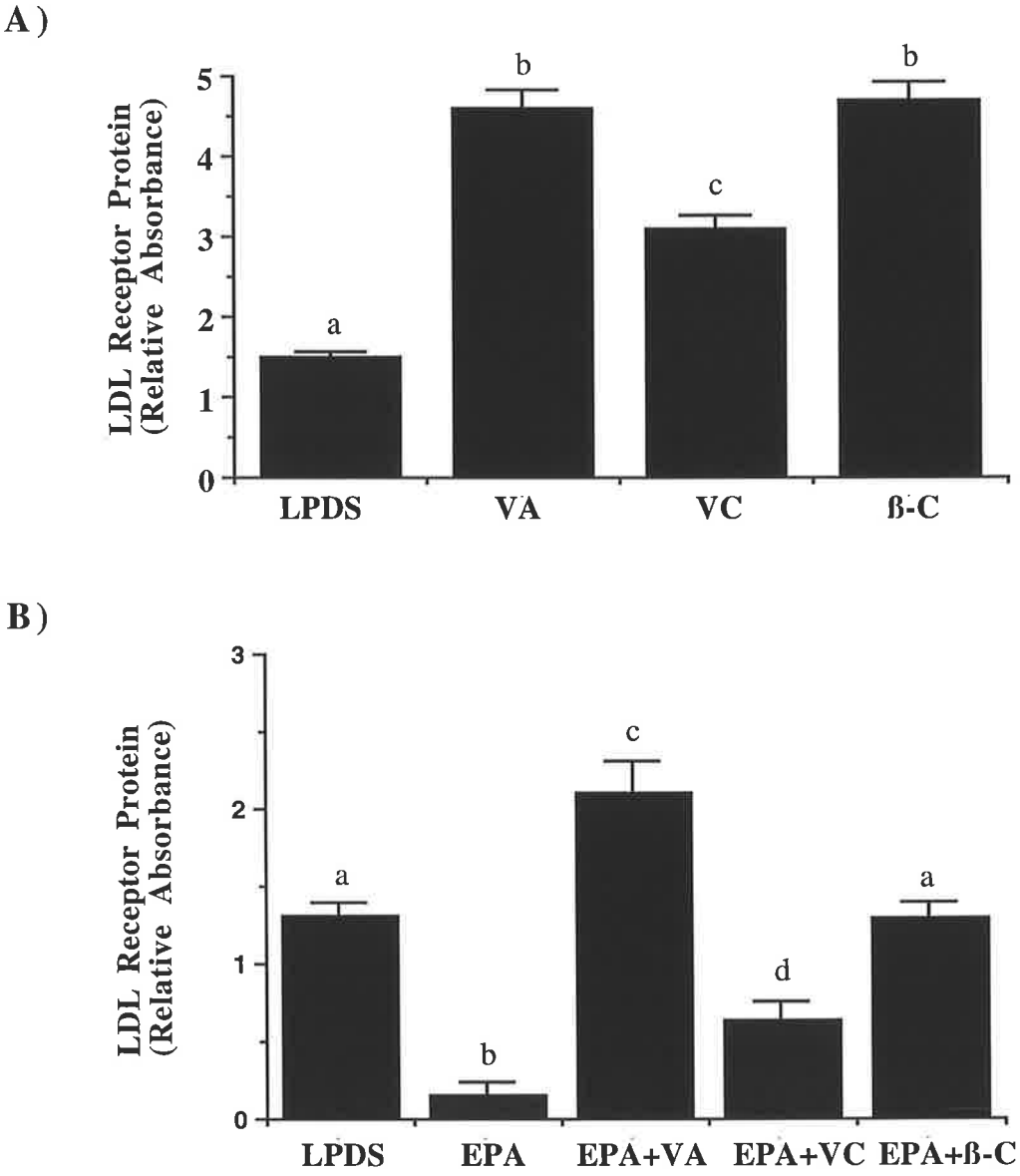
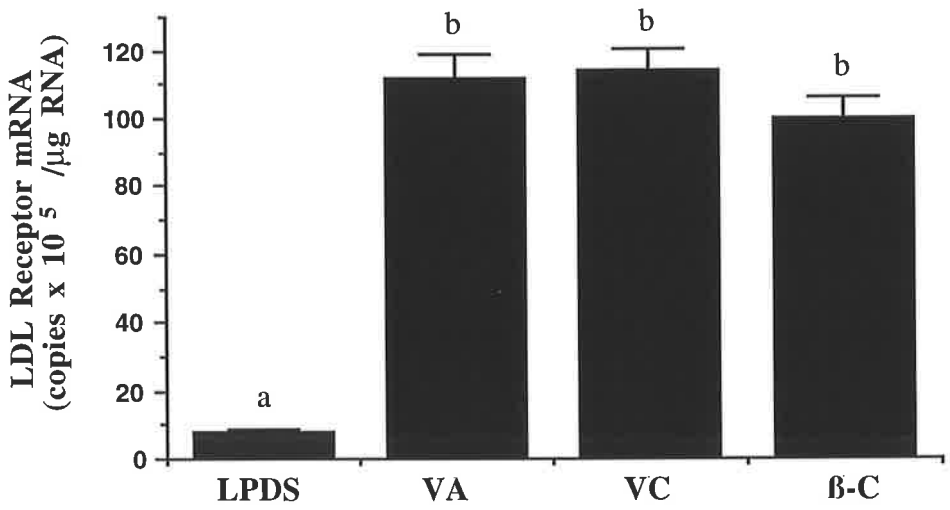


Figure 8.8 The effect on LDL receptor protein levels in HepG2 cells supplemented with antioxidants in the absence or presence of EPA. HepG2 cells were incubated with DMEM containing 10% LPDS after 2 days of subculturing. On day 3, cells were supplemented with either **(A)** no antioxidants (indicated as LPDS), or 100 µM vitamin A, vitamin C or ß-carotene in a background media containing 10% LPDS or with **(B)** no antioxidants and no EPA (indicated as LPDS), or 250 µM EPA alone, or 250 µM EPA and 100 µM vitamin A, vitamin C or ß-carotene in a background media containing 10% LPDS. Cells were further incubated for a period of 24 h and subsequently harvested (day 4). LDL receptor cellular proteins were solubilised with Triton X-100, electrophoresed, transferred to nitrocellulose and LDL receptor protein was quantified by the ECL method (section 2.7). Treatments were performed in duplicate and the relative densities of the receptor band were quantified by laser densitometry. Results are expressed as relative absorbances and data represents the mean ± SEM of 2 experiments. Means with different letters above them are significantly different at $P < 0.01$.

A)



B)

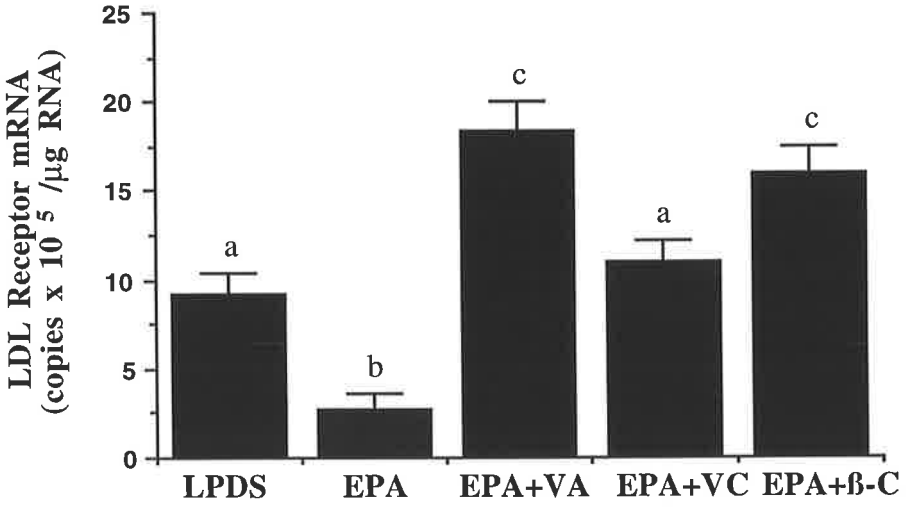


Figure 8.9 The effect on LDL receptor mRNA levels in HepG2 cells supplemented with antioxidants in the absence or presence of EPA. HepG2 cells were incubated with DMEM containing 10% LPDS after 2 days of subculturing. On day 3, cells were supplemented with either **(A)** no antioxidants (indicated as LPDS), or 100 µM vitamin A, vitamin C or ß-carotene in a background media containing 10% LPDS or with **(B)** with no antioxidants and no EPA (indicated as LPDS), or 250 µM EPA alone, or 250 µM EPA and 100 µM vitamin A, vitamin C or ß-carotene in a background media containing 10% LPDS. Cells were further incubated for a period of 24 h after which they were harvested (day 4). Total cellular RNA was isolated, reverse transcribed and amplified using PCR with a synthetic piece of RNA used as an quantitative internal control (section 2.8). Cellular mRNA levels were measured relative to the internal control and expressed as the mean ± SEM of 2 experiments. Means with different letters above them are significantly different at $P < 0.01$.

Linoleic Acid and Antioxidants

When cells were incubated with linoleic acid, LDL receptor activity (Figure 8.10), protein (Figure 8.11) and mRNA (Figure 8.12) levels decreased 36%, 35% and 40%, respectively, compared to the LPDS control cells. These findings were consistent with those observed in earlier experiments (Chapter 5).

Even with linoleic acid in the background media, LDL receptor activity was increased by 110% with vitamin A, 100 % with vitamin C and 123% with ß-carotene, compared to cells incubated with linoleic acid alone (Figure 8.10). Conversely, the absolute level of LDL receptor activity was decreased by the presence of linoleic acid plus antioxidants (Figure 8.10) compared to antioxidants alone (Figure 8.7A). The percentage decrease in LDL receptor activity by linoleic acid was approximately 60% in all cases.

The antioxidants increased the level of LDL receptor protein (Figure 8.11) and mRNA (Figure 8.12) even with the presence of linoleic acid in the background media (DMEM supplemented with 10% LPDS). LDL receptor protein was augmented 180% by vitamin A, 110% with vitamin C, and 90% with ß-carotene compared to the cells incubated with linoleic acid and no antioxidants (Figure 8.11). Although these antioxidants increased the amount of LDL receptor protein, linoleic acid exerted its effects simultaneously on the LDL receptor. This is reflected in the absolute amount of LDL receptor protein (Figure 8.11) which was lower than the level observed when antioxidants were present alone (Figure 8.8A).

LDL receptor mRNA levels were increased 167%, 160% and 167% with linoleic acid and vitamin A, vitamin C and ß-carotene, respectively, compared to cells incubated with linoleic acid and no antioxidants (Figure 8.12). The decrease in the absolute amount of mRNA levels by the presence of linoleic acid was estimated by the difference between cells incubated with antioxidants in the presence and absence of linoleic acid. The decrease was observed to be approximately 80%.

Thus, the results herein demonstrate that the antioxidants vitamin A, vitamin C and ß-carotene exert separate effects on LDL receptor activity even though linoleic acid is present at the same time in the background media and *vice versa*. The final level of LDL receptor expression is a result of combined effects of both antioxidants and linoleic acid. In addition, the independent effect of linoleic acid in the presence of the antioxidants also suggests that oxidation products of this fatty acid were not responsible for the observed decrease in LDL receptor expression.

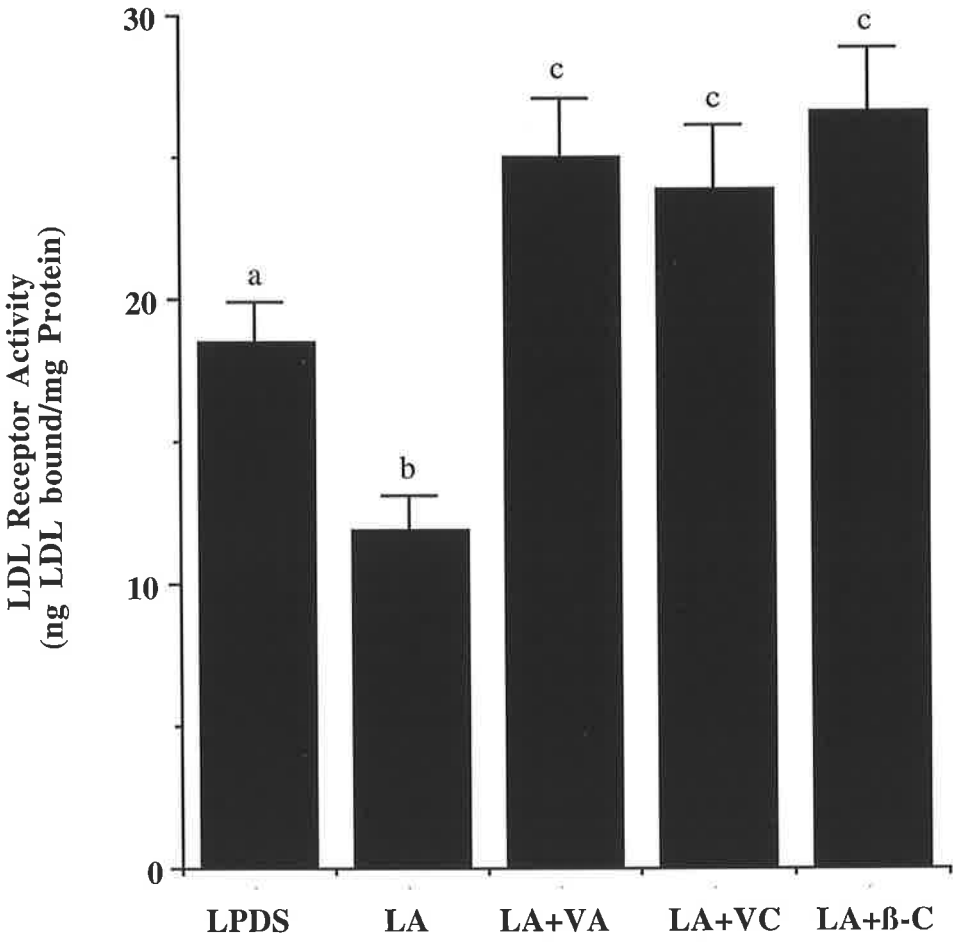


Figure 8.10 The effect on LDL receptor activity in HepG2 cells supplemented with antioxidants and linoleic acid. HepG2 cells were incubated with DMEM containing 10% LPDS after 2 days of subculturing. On day 3, cells were supplemented with either no antioxidant and no linoleic acid (indicated as LPDS), or 250 µM linoleic acid alone, or 250 µM linoleic acid and 100 µM vitamin A, vitamin C or ß-carotene in a background media containing 10% LPDS. Cells were further incubated for a period of 24 h after which they were harvested (day 4). LDL receptor binding activity was measured in triplicate using the LDL colloidal gold method described in section 2.6.3. Results represent specific LDL receptor binding activity (ng of LDL bound/mg cell protein), which are expressed as the mean ± SEM of 2 experiments. Means with different letters above them are significantly different at $P < 0.01$.

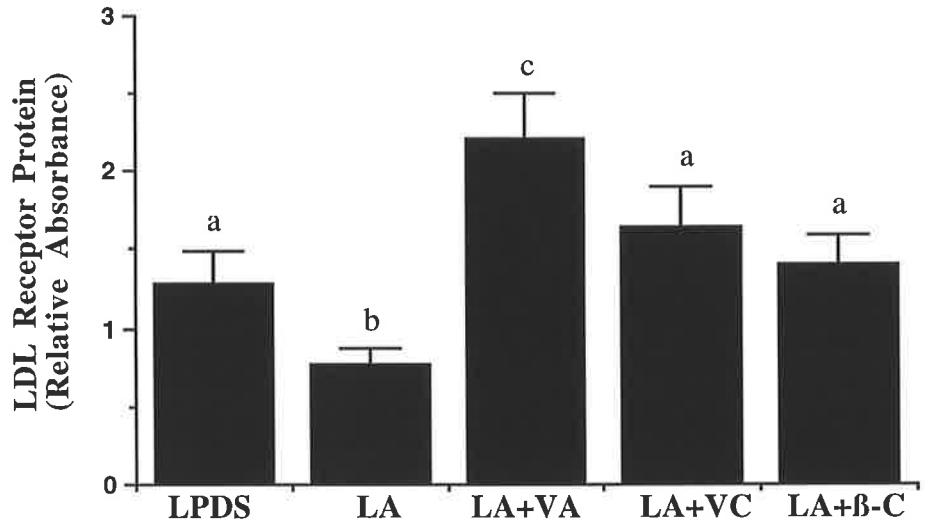


Figure 8.11 The effect on LDL receptor protein levels in HepG2 cells supplemented with antioxidants and linoleic acid. HepG2 cells were incubated with DMEM containing 10% LPDS after 2 days of subculturing. On day 3, cells were supplemented with either no antioxidant and no linoleic acid (indicated as LPDS), or 250 µM linoleic acid alone, or 250 µM linoleic acid and 100 µM vitamin A, vitamin C or ß-carotene in a background media containing 10% LPDS. Cells were further incubated for a period of 24 h and subsequently harvested (day 4). LDL receptor cellular proteins were solubilised with Triton X-100 detergent, electrophoresed, transferred to nitrocellulose and LDL receptor protein was quantified using the ECL method (section 2.7). Treatments were performed in duplicate and the relative densities of the receptor band were quantified by laser densitometry. Results are expressed as relative absorbances and data represents the mean ± SEM of 2 experiments. Means with different letters above them are significantly different at $P < 0.01$.

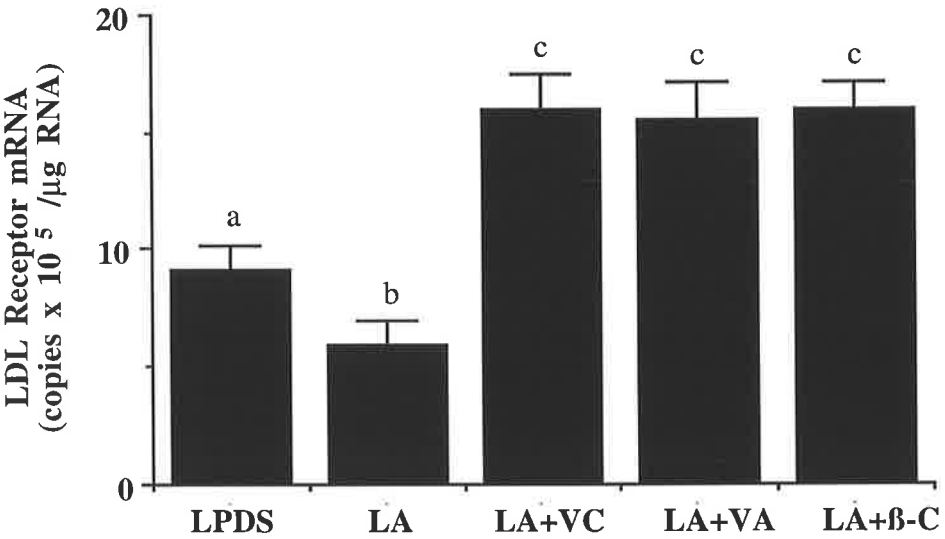


Figure 8.12 The effect on LDL receptor mRNA levels in HepG2 cells supplemented with antioxidants and linoleic acid. HepG2 cells were incubated with DMEM containing 10% LPDS after 2 days of subculturing. On day 3, cells were supplemented with either no antioxidant and no linoleic acid (indicated as LPDS), or 250 µM linoleic acid alone, or 250 µM linoleic acid and 100 µM vitamin A, vitamin C or ß-carotene in a background media containing 10% LPDS. Cells were further incubated for a period of 24 h after which they were harvested (day 4). LDL receptor mRNA levels were quantified according to the method in section 2.8. Cellular mRNA levels were measured relative to the internal control and expressed as the mean ± SEM of 2 experiments. Means with different letters above them are significantly different at $P < 0.01$.

8.4 DISCUSSION

The bimodal effect on LDL receptor protein was observed with increasing concentrations of vitamin E in the presence or absence of 250 μM EPA in the background media (Figure 8.2). Conversely, in the presence or absence of vitamin E, LDL receptor protein was found to decrease in a curvilinear manner with increasing concentrations of EPA (Figure 8.3). This indicated that both EPA and vitamin E were able to exert independent effects on LDL receptor expression when they were present in the cell simultaneously.

However, when vitamin E and EPA were introduced to the cells sequentially (Figures 8.4, 8.5, 8.6), there appeared to be a "carry over" effect with EPA but not with vitamin E. This suggested that the clearance of EPA out of the cell was much slower than vitamin E. The implication is that the intracellular concentration of vitamin E and EPA was dictated by their own unique intracellular metabolism which subsequently determined their effects on the LDL receptor.

The antioxidants vitamin A, vitamin C and ß-carotene were also able to increase LDL receptor expression (activity, protein and mRNA) either in the presence of EPA or linoleic acid and *vice versa* (Figure 8.7-8.12). Although the absolute level of LDL receptor expression changed, the percentage increase (with antioxidants) or decrease (with EPA or linoleic acid) in LDL receptor expression from the appropriate controls was similar to that observed with these agents alone.

In summary, the results herein support the hypothesis that antioxidants and fatty acids have independent effects on LDL receptor expression even when both are present simultaneously. The findings indicate that fatty acid peroxides are not responsible for the suppression of the LDL receptor. If fatty acid peroxides were responsible for the suppression of the LDL receptor, then the antioxidants would have been expected to inhibit the effect of EPA and linoleic acid. Hence, the results also support the second hypothesis that fatty acid peroxides are not responsible for the observed decrease in LDL receptor expression when HepG2 cells are incubated with linoleic acid and EPA.

The effect of increasing concentrations of EPA and vitamin E on LDL receptor in the presence and absence of the other agent

In a fixed concentration of 50 μM vitamin E, LDL receptor protein decreased curvilinearly as the concentration of EPA increased (Figure 8.2). The degree to which LDL receptor protein decreased in the presence of EPA plus vitamin E was similar to that observed with an increasing concentration of EPA in the absence of vitamin E (Figure 8.2). These results were consistent with the previous findings in Chapter 5 which showed that LDL receptor protein was depressed by 75% with 100μM EPA (Figure 5.5 and 5.6) and 90% with 250 μM EPA (Figure 5.4) compared to the control cells (10% LPDS). Both agents are, therefore, having a separate effect on LDL receptor expression, and the final level of the LDL receptor protein is the resultant effect of both agents.

A bimodal effect of the LDL receptor protein was observed when HepG2 cells were incubated with a fixed concentration of 250 μM EPA and an increasing concentration of vitamin E in a cholesterol free media (DMEM supplemented with 10% LPDS) (Figure 8.3A). This effect of vitamin E on cellular LDL receptor expression was consistent with earlier findings (Chapter 6, Figures 6.2-6.4), in which LDL receptor protein increased with higher amounts of vitamin E up to 50 μM, but decreased at higher concentrations. However, the quantity of LDL receptor protein at all concentrations of vitamin E tested was significantly lower by 85% (refer to table 8.3) in cells which were exposed to 250 μM EPA than in cells exposed to vitamin E alone (Figure 8.3A). This presumably occurred because the LDL receptor protein initially decreased in cells by 80% in the presence of 250 μM EPA without vitamin E compared to control cells (no vitamin E and no EPA). Thus, the subsequent response of cellular LDL receptor protein when vitamin E was introduced occurred from this lower level of LDL receptor protein (Figure 8.3 A). Nevertheless, with 50 μM vitamin E plus 250 μM EPA, LDL receptor protein in HepG2 cells increased 310% compared to appropriate control (cell incubated in EPA only) (Figure 8.3B). Even with this massive increase of LDL receptor protein with 50 μM vitamin E, the relative the amount of LDL receptor protein did not reach the level observed in LPDS alone (no vitamin E or EPA) (Figure 8.3A). These results clearly show that both EPA and vitamin E are simultaneously, but independently affecting LDL receptor expression. LDL receptor protein levels appear to be increased by vitamin E, while at the same time they are decreased by the presence of EPA. The final effect on LDL receptor protein appears to be a combined effect of the two agents, instead of a predominant effect of one compound.

Based on these results, it would appear that the response of LDL receptor protein to these two agents is rapid and independent. Such effects may be explained by changes in intracellular sterol metabolism or the direct effect of these agents on gene transcription, rather than by changes through time dependent alterations in membrane composition (see Chapter 5). As discussed earlier, the effect of fatty acids on intracellular cholesterol metabolism, such as cholesterol esterification, cholesterol secretion *via* VLDL, and cholesterol biosynthesis, are all possible mechanisms by which EPA could affect the "metabolically active cholesterol pool" (Grundy, 1991) (Chapter 5, section 5.4). Vitamin E has also been found to affect these various pathways, but in a manner opposing that observed by EPA (Chapter 6, section 6.4). For example, cholesterol esterification has been shown to be suppressed by EPA (Pal and Davis, 1991), whereas vitamin E has been suggested to increase ACAT activity (Shirai et al., 1980). Thus, the manner in which these two agents affect LDL receptor expression may be a result of their opposing effects on cholesterol esterification, synthesis, bile acid conversion and VLDL synthesis/secretion. Although no supportive evidence is available, one can also speculate that the independent affect of both agents on LDL receptor protein levels may arise from two distinct cellular events at the level of LDL receptor gene transcription. If both influence different transcription factors, such as

SREBP-1 and Sp-1, in an opposing manner, then transcription of the LDL receptor can be mediated, but only to a limited extent.

The effect on LDL receptor in cells supplemented sequentially with EPA and vitamin E

The observations described above clearly showed that the LDL receptor could not be preferentially influenced by either vitamin E or EPA when present together (Figures 8.2, 8.3 a and b). Further experiments were designed to address whether vitamin E and EPA would still have the same effect if they were introduced to the cell in a sequential manner.

These experiments suggested that the effect of vitamin E and EPA on LDL receptor was time dependent (Figures 8.4, 8.5, 8.6). When EPA is added 24 h after the addition of the vitamin, the final effect on the LDL receptor more closely resembles the effect of EPA alone (flask 3) than the effect of vitamin E alone (flask 2). This suggested that vitamin E must be rapidly cleared out of the cell as its influence on the LDL receptor does not persist 24 h after it was removed from the media and replaced by EPA. In contrast, when EPA is added 24 h prior to vitamin E, the suppression of the LDL receptor was still evident. This suggested that clearance of EPA out of the cell could be quite slow. In this case, it seems that EPA has been integrated into the cell and can not readily removed, even though it was removed from the media and replaced with vitamin E (flask 7). Therefore, the effect of these agents appear to be dependent on their clearance from the cells.

The difference between the LDL receptor activities over a 2 day time period when cells are incubated with either EPA (flask 3 and 5) or vitamin E (flask 2 and 4) is consistent with the idea that the incorporation and retention of EPA and vitamin E in cells vary. It is interesting to note that the suppression of the LDL receptor activity, protein and mRNA levels by EPA was significantly higher after a 48 h incubation (flask 5) compared to a 24 h incubation period (flask 3). However, there was no difference in the increase in LDL receptor activity, protein or mRNA levels between cells incubated with vitamin E for 24 h or 48 h. These observations further indicate that the effects of EPA and vitamin E on the LDL receptor differ with time.

The effect of EPA on LDL receptor activity appeared to be quite strong even after the cell media was removed and subsequently replaced with vitamin E media (Figures 8.4-8.6). It would be expected that EPA would take longer to incorporate into the cells and membrane structures as EPA is normally metabolised into phospholipids, cholesterol esters, triacylglycerols and eicosanoids (prostaglandins, thromboxanes, prostacyclins, leukotrienes, lipoxins) (Vance and Vance, 1985). Thus, the metabolism of EPA provides the cell with a store of this fatty acid which can not be rapidly emptied. Only when the lipid stores are slowly broken down and the fatty acid released do they provide the cell with free EPA which may affect cellular processes as before. Therefore, the rate at which the fatty acids are released from these stores will determine how long the fatty acids will affect processes such as LDL receptor expression. For example, EPA release from phoshotidylcholine (PC) is

dependent on the actions of phospholipase A_2 (Vance and Vance, 1985). Thus, with regards to EPA, the rate of release must be quite slow as LDL receptor activity was still suppressed even 24 h after the fatty acid had been washed from the cells and the media replaced. Therefore, the introduction of vitamin E after the addition and removal of EPA (flask 6) has an effect similar to that seen in the presence of EPA in the media (flask 3) (Figure 8.4).

On the other hand, vitamin E, unlike EPA, does not go through elaborate metabolic processes for storage. It can simply be stored in the membrane in its free form or be present in the cell bound to TBP (α-tocopherol binding protein) (George Wolf, 1993; Kayden and Traber, 1993). Compared to EPA, the residency time of α-tocopherol in the liver is quite short. TBP binds to the vitamin and aids its incorporation into VLDL, thus ensuring rapid transport of vitamin E out of the cell. Vitamin E, therefore, is not considered to be toxic in high doses, unlike other fat soluble vitamins, since it is not stored in the liver. If vitamin E is easily cleared from the HepG2 cells, it may explain why its effects on LDL receptor expression do not continue after its removal from cell culture (Figure 8.4-8.6). Thus, the difference in the metabolism of EPA and vitamin E may explain their different influences on LDL receptor expression (activity, protein and mRNA).

The results herein provide a very important clue in the mechanism by which these two agents are influencing the LDL receptor. The experiments indicate that the amount of vitamin E or EPA present in the cell media was unimportant compared to their intracellular concentration. It follows that the final level of LDL receptor expression is determined by the agent present in the highest concentration intracellularly. Thus, the effect of EPA and vitamin E on the LDL receptor may be determined by the manner in which these two compounds are metabolised in the cell. The carry-over effect of one agent from day 3 to day 4 would presumably depend on the clearance of that agent out of the cell because it has been removed from the cell media. Therefore, the metabolism of these agents would dictate how long vitamin E and EPA would be present in the cell, and consequently, how long they would have an effect on LDL receptor expression.

The effect on LDL receptor by other antioxidants and EPA or linoleic acid

To further investigate the independent effects of antioxidants and fatty acids on LDL receptor expression, a range of antioxidants were examined in the presence of EPA and linoleic acid. All the antioxidants examined increased the expression of the LDL receptor in the presence of linoleic acid and EPA, although the absolute level of LDL receptor expression had been reduced due to the presence of the fatty acids (Figure 8.7-8.12).

The LDL receptor activity in HepG2 cells was significantly increased by approximately 300% by vitamin A, vitamin C and ß-carotene in a background media containing 10% LPDS (Figure 8.7A). The ability of antioxidants to exert their own effects on LDL receptor expression (activity, protein and mRNA) was also observed in chapter 7 (Figure 7.1, 7.2, 7.3) in that the antioxidants were capable of overriding the suppressive

effects of cholesterol. The LDL receptor activity was increased approximately 200% with these antioxidants compared to the control cells without antioxidants in a background media containing 10% FCS (similar to cells incubated with 10% LPDS). Thus, the effect of the antioxidants on LDL receptor activity was independent of the cholesterol content of the background media.

The experiments herein revealed that vitamin A, vitamin C and ß-carotene and EPA independently influenced LDL receptor expression (activity, protein and mRNA). Similarly, the independent affect of antioxidants and fatty acids on LDL receptor expression was also observed when the background fatty acid was linoleic acid (Figure 8.10, 8.11 and 8.12). It can be suggested that both fatty acids and all the antioxidants examined are having a separate effect on the LDL receptor, but the overall effect on LDL receptor expression is a combination of both agents.

LDL receptor activity was increased by vitamin A, vitamin C and ß-carotene to the same extent with or without EPA (Figure 8.7A and B). However, it is interesting to note that this trend was not observed at the level of LDL receptor protein (Figure 8.8B). In this case, LDL receptor protein was not increased by vitamin C to the same extent observed by the other antioxidants with or without EPA (Figure 8.8). Similar observations were found with vitamin C at the level of LDL receptor mRNA when antioxidants and EPA were present in the cells together (Figure 8.9B). This was in spite of the fact that all the antioxidants increased LDL receptor mRNA levels to the same extent when EPA was absent (Figure 8.9A). The distinct effect of vitamin C on LDL receptor expression suggests that it may regulate the LDL receptor differently compared to the other antioxidants. This may be related to the fact that vitamin C is a water soluble antioxidant while vitamin A, vitamin E and ß-carotene are lipid soluble antioxidants. There appears to be an effect of vitamin C on the distribution of the LDL receptor, in that the LDL receptor gene does not need to be transcribed to the same extent in order to produce the same level of LDL receptor activity on the intact cell surface as for the other antioxidants. It is possible that vitamin C may increase the rate of LDL receptor recycling such that more LDL receptors are shifted to the cell membrane even though there are fewer LDL receptors present in the cell. However, this unique effect of vitamin C on the LDL receptor was not observed in the presence of linoleic acid (Figures 8.10, 8.11, 8.12).

Relevance of fatty acids and antioxidants in physiological systems

The relationship between the fatty acids and the antioxidants in the cell has been shown to be important in influencing a number of cellular processes in addition to their effects on the LDL receptor (see below). Conceivably, there could be an interaction between lipid soluble antioxidants and fatty acids because of their similar locality within the membranes of the cell (eg. plasma membrane, endoplasmic reticulum, golgi bodies). For example, most of the vitamin E is situated in the membranes of the cells, adjacent to

unsaturated fatty acids that are vulnerable to free radical attack (Muggli, 1994; Van Acker et al., 1993). It has been suggested that vitamin E stabilises the cell membrane through the interaction of its phytyl side chains with the polyunsaturated fatty acyl groups of phospholipids (Muggli, 1994; Van Acker et al., 1993). Thus, the presence of a compound in the same vicinity could easily determine and influence the actions of the other, in that, the amount and type of fatty acid could determine the amount and type of antioxidants and *vice versa*. The interaction between these two compounds, however, may have either positive or negative outcomes.

A number of possibilities exist regarding the interaction of PUFAs and antioxidants. For example, since PUFAs are more susceptible to oxidation than saturated or monounsaturated fatty acids (Slim et al., 1996), the relationship between antioxidants and PUFAs may be more biologically relevant than saturated and monunsaturated fatty acids. PUFAs, such as linoleic acid and EPA, are of major concern, as linoleic acid contains two double bonds and EPA has five double bonds. As the antioxidants preserve the structure of PUFAs, one could imagine that in a high PUFA environment, the antioxidant content could easily be depleted. Thus, most studies investigating the interaction between these two compounds have been limited to examining the requirements of antioxidants needed to counteract the damaging effects of lipid peroxidation associated with high PUFA intake in animals and humans (Slim et al., 1996). Nevertheless, the biological action of these antioxidants ultimately depends on the concentration of these compounds and reactive species within the target tissue. This, in turn, will also be determined by the amount and type of dietary PUFAs, and becomes especially significant at the crucial point in the pathophysiologic process that determines atherogenesis.

When human diets or cells in tissue culture are enriched in PUFAs, they are generally incorporated into membrane phospholipids (Pal and Davis, 1990; Rapp et al., 1991). It has been found that the requirement of antioxidants in humans or animals is usually dependent on the amount and the degree of unsaturation of PUFA in the diet (Alexander-North et al., 1994). It would be expected that below a certain antioxidant threshold, PUFAs would be vulnerable to peroxidation.

Although the epidemiological studies have shown that an increased consumption of PUFAs is associated with reduced plasma cholesterol and CHD, they may also be detrimental due to their increased susceptibility to oxidation. It has been shown that after ingestion of fish oils, PUFAs accumulate in human coronary arteries (Rapp et al., 1991). Felton et al. (1994) analysed the effect of long-term intake of essential fatty acids, and compared the fatty acid composition of aortic plaques with that of post-mortem serum and adipose tissue. They found a positive association between the content of plaque and serum content of 18:3, 20:4, 20:5, and 22:6 fatty acids. Dietary PUFAs may reduce the risk of thrombosis and lower certain atherogenic lipoprotein levels in plasma. On the other hand, they promote LDL oxidation and other adverse effects which are associated with fatty acid

peroxides. One could argue, therefore, that the antioxidant environment would be extremely important in the arterial wall to prevent lipid peroxidation of PUFAs. By preserving the structural integrity of PUFAs, antioxidants may indirectly promote the beneficial effects of these long chain fatty acids in the arterial wall, rather than deleterious effects which induce the atherosclerotic process. Therefore, the interaction of fatty acids and antioxidants may be closely linked to the disease process, and impies that adequate antioxidant concentrations are essential for proper fatty acid function.

Oxidised fatty acids, however, are not always deleterious to the cell, but are essential for some cellular processes to occur. For example, several investigators have reported that PUFAs, such as EPA and DHA, inhibit the proliferation of cells in culture through lipid peroxidation (Shiina et al., 1993). It is thought that the oxidation products of these fatty acids suppress vascular smooth muscle cell proliferation by decreasing the production of platelet derived growth factor (PDGF). This process, however, has been shown to be reversed by vitamin E. An inhibition in cell proliferation would be advantageous in the artery wall to prevent plaque formation.

Recent evidence suggests that PUFAs and antioxidants may regulate the response of immune cells in a counteracting manner. The PUFAs are immunomodulators via the regulation of cytokines and other factors (Endres and von Schacky, 1996). It appears that ω-6 and ω-3 PUFAs suppress the capacity of cells to synthesise the cytokines interleukin (IL)-1 and tumour necrosis factor (TNF), which are principal mediators of inflammation (Endres and von Schacky, 1996; Riener et al., 1993). Whereas, recent evidence suggests that antioxidants vitamin A, vitamin E and β-carotene may, in fact, increase cytokine production (Boland, personal communication). Regulating cytokine production may be important in determining LDL receptor expression since IL-1 and TNF have been shown to upregulate the LDL receptor (Hamaka et al., 1992). Thus, it may be through IL-1 and /or TNF that both fatty acids and antioxidants modulate the LDL receptor *in vivo*.

It might be speculated that the oxidative products of fatty acids may be involved in the suppression of LDL receptor gene transcription. This would be consistent with the fact that the oxidative products of cholesterol, such as 27-hydroxy cholesterol, are responsible for regulating LDL receptor gene transcription (Bjorkhem et al., 1994; Carpenter et al., 1995). Although fatty acid peroxides of EPA and linoleic acid could be responsible for inducing the suppression of the LDL receptor, this idea, however, was not supported by the results herein. The suppression of the LDL receptor activity, protein and mRNA levels was influenced by EPA and linoleic acid similarly either in the presence or absence of the antioxidants, vitamin E (Figure 8.1-8.6), vitamin C, vitamin A and ß-carotene (Figure 8.7-8.12).

Effects on fatty acid composition by antioxidants

Changes in fatty acid composition is a possible mechanism whereby antioxidants and fatty acids manipulate LDL receptor expression. *Diabetes mellitus* induces alterations of fatty acid patterns in various tissues in animals (Douillet and Ciavatti, 1995). Increases in linoleic acid are associated with a decrease of oleic acid and arachidonic acid, which are caused by a depression of Δ9, Δ6 and Δ5 desaturase activities in diabetes. Douillet and Ciavatti (1995) have shown that vitamin E supplementation in diabetic rats corrects Δ9, Δ6 and Δ5 desaturase activity and consequently, increases monounsaturated fatty acids in all tissues.

Consistent with this finding above, compared to the control cells (no fatty acids), incorporation of linoleic acid and EPA in cells increased at the expense of arachidonic acid and monounsaturated fatty acids (Table 5.2; Palozza et al., 1995). In contrast, however, antioxidants (vitamins E, A, C and ß-carotene) may be correcting the fatty acid induced changes in HepG2 cells by affecting enzyme activity, thereby ameliorating the suppression of LDL receptor gene transcription induced by EPA or linoleic acid. A recent study by Jenkins and Atwal (1995) showed that phenolic acid antioxidants (such as quercetin, morin and ferulic acid) had marked effects on the fatty acid composition of tissue lipids. When consumed by chicks, these antioxidants reduced 18:1 and 20:3 (ω-9 fatty acids), increased 18:2 and 20:4 (ω-6 fatty acids), and promoted the production and/or conservation of EPA. They suggested that the main effect of the antioxidants was on the desaturase and elongase enzymes involved in fatty acid metabolism. The contradictory studies make it difficult to elucidate whether antioxidants have the potential to influence fatty acid membrane composition, and if it is through this mechanism whereby vitamin A, vitamin C, vitamin E and ß-carotene modulate LDL receptor activity.

One approach to address whether antioxidants are affecting membrane fatty acids would be to examine the fatty acid composition of HepG2 cells in the presence of the antioxidants. For example, in the presence of EPA and vitamin E, enzymes may be activated which increase saturated and monounsaturated fatty acids, resulting in the elevation of LDL receptor activity even in the presence of EPA. In addition, one could determine if the cellular LDL receptor activity could be upregulated by palmitic acid or oleic acid subsequent to an EPA induced suppression of the LDL receptor, similar to the effect of vitamin E in cells after a 24 h prior incubation with EPA. However, the response of LDL receptor expression to vitamin E and EPA is not likely to be due to their indirect effects on membrane composition as these modifications are time dependent. As LDL receptor expression is affected by these agents quite rapidly, it would suggest that more direct mechanisms are involved.

SUMMARY

The results described herein suggest that vitamin E and EPA both exert independent effects on the LDL receptor when present together. Similarly, independent effects on the LDL receptor were observed with either vitamin A, vitamin C and ß-carotene in the presence or absence of either EPA or linoleic acid. Based on the findings, it is difficult to discern whether the antioxidants and fatty acids are exerting their effects on the LDL receptor through similar or different pathways. These agents may be affecting the LDL receptor through transcription factors or through their effects on intracellular cholesterol concentrations (see Chapter 9).

The results herein also suggest that fatty acid peroxides of EPA and linoleic acid were not responsible for the effect of these fatty acids on the LDL receptor. The range of antioxidants used in combination with EPA and linoleic acid did not influence the suppression of LDL receptor activity, protein and mRNA induced by EPA or linoleic acid. If fatty acids peroxides were responsible for the reduction in LDL receptor expression, then the suppression by EPA or linoleic acid would have been prevented by at least one or all of the different antioxidants examined. Thus, the results further support the hypothesis that fatty acid peroxides of linoleic and EPA were not responsible for the downregulation of LDL receptor expression.

Chapter 9

9.0 FINAL DISCUSSION

The individual effects of saturated, monounsaturated and polyunsaturated fatty acids (PUFAs) on LDL receptor activity in a rat liver, a human liver and two monocytic cell lines were observed (Chapters 4 and 5). Based on the findings of previous studies (Wong and Nestel, 1987; Roach et al., 1987), it was hypothesised that LDL receptor activity in human and rat liver cells would be suppressed by PUFAs compared to monounsaturated and saturated fatty acids. In both the human and rat liver cell lines, LDL receptor activity was suppressed when the cells were incubated with PUFAs compared to cells incubated with saturated and monounsaturated fatty acids. Hence, the hypothesis was supported. In general, as the polyunsaturation of the fatty acid increased, the LDL receptor activity in the cells decreased for both liver cell lines. Since LDL receptor activity is similarly affected by fatty acids in both liver cell lines, it suggests that at the cellular level, human and rat liver respond to fatty acids in the same manner.

The trend between fatty acid unsaturation and LDL receptor activity was not, however, observed in the two human monocytic cell lines. This indicated that there may be a tissue specific response to fatty acids even within one species. Such differences between cells could be expected as different cells have diverse functions and distinct characteristics that set them apart from each other.

The results described in chapters 4 and 5, therefore, demonstrate two important points: (1) the LDL receptor in cells of the same tissue, but from two different species, responds to fatty acids in a like manner, and (2) the LDL receptor in cells from different tissues, but from the same species, does not necessarily respond to fatty acids in the same manner, presumably due to the functional specialisation of the cells. These results may help to explain why there are inconsistencies observed in both animal studies and immortalised cell line studies which examine the effects of the different fatty acids on LDL receptor binding activity. This makes the task of comparing studies very difficult.

To elucidate whether the action of fatty acids on LDL receptor activity was due to effects at the level of gene transcription and/or protein translation, LDL receptor protein and mRNA levels were quantified in HepG2 cells (Chapter 5). In parallel to the effects on LDL receptor activity, both LDL receptor protein and mRNA were decreased as the degree of fatty acid unsaturation increased. Regression analysis revealed that the correlation between LDL receptor activity and protein levels, protein and mRNA levels, and activity and mRNA levels in cells treated with the various fatty acids were highly significant. Thus, the effect of the fatty acids on LDL receptor activity in HepG2 cells were attributable to their effects on gene transcription. This is consistent with the well documented regulation of the LDL receptor at this level (Goldstein and Brown, 1977; Brown and Goldstein, 1986; Rudling, 1992; Sanchez et al., 1995).

A feature of PUFAs is that they are more susceptible to being oxidised to lipid peroxides than monounsaturated and saturated fatty acids (Slim et al., 1996). To elucidate whether fatty acid peroxides could be responsible for the observed suppression of LDL receptor activity by PUFAs, vitamin E (α-tocopherol) was co-incubated with EPA and linoleic acid (Chapter 5). These experiments not only demonstrated that fatty acid peroxides were probably not responsible for downregulating the LDL receptor, but they also revealed, by serendipity, that vitamin E was able to upregulate the LDL receptor.

A hypothesis that vitamin E could upregulate the LDL receptor gene was formulated based on these results (chapter 5). Testing the hypothesis showed that the α-tocopherol form of vitamin E had, in fact, a bimodal effect on LDL receptor expression (activity, protein and mRNA levels) (Chapter 6). The LDL receptor expression increased with α-tocopherol concentrations up to 50 μM and then decreased at higher concentrations. The same bimodal regulation of HMG-CoA reductase mRNA gene was also observed with increasing concentrations of α-tocopherol: the HMG-CoA reductase gene transcription increased with α-tocopherol up to 50 μM, and then decreased from this level with higher concentrations. These results demonstrated the coordinate regulation (induction and repression) of the LDL receptor and HMG-CoA reductase genes by non-sterol agents. This is an important finding which is consistent with the coordinate regulation of these genes by sterols (Goldstein and Brown, 1977; Rudling, 1992).

In contrast to α-tocopherol, δ- and γ-tocopherol analogues of vitamin E decreased LDL receptor activity, protein and mRNA levels at all concentrations examined (Chapter 6). This suggested that the effect of α-tocopherol on the LDL receptor was either due to its high antioxidant potential or its high biological activity compared to the other forms of tocopherols.

To test the hypothesis that the upregulation of the LDL receptor is a general antioxidant phenomenon, the effects of vitamin A, vitamin C, ß-carotene, green tea extract and red wine were examined (Chapter 7). These compounds are highly diverse in their structure, metabolism, function and mechanism of action, but are similar in that they are all potent antioxidants. Vitamin A, vitamin C, ß-carotene, green tea extract and red wine all increased LDL receptor activity, mass and mRNA levels at the concentrations examined (Chapter 7). HMG-CoA reductase mRNA levels were also increased when cells were incubated with green tea extract.

All of these chemically diverse compounds were, therefore, able to upregulate the LDL receptor, presumably because they are all antioxidants. If this common property is the reason these compounds affect LDL receptor expression (activity, protein and mRNA), then the upregulation of the LDL receptor may be a "general antioxidant phenomenon" mediated through a common pathway. However, the nature of this pathway and whether it is associated with effects on intracellular sterol metabolism or specific transcription factors, is yet to be determined.

Early experiments (Chapter 5) revealed that vitamin E and EPA appeared to have opposing effects on the LDL receptor no matter whether the other was present or absent. For example, in the presence of vitamin E, the amount of LDL receptor protein was decreased by EPA to the same extent (on a percentage basis) as observed when EPA was used alone. Thus, the initial experiments suggested that the PUFA and the antioxidant were having independent effects on the LDL receptor.

The interrelationship between antioxidants and fatty acids on the LDL receptor was, therefore, further investigated in HepG2 cells (Chapter 8). Confirming the initial results (Chapter 5), LDL receptor activity, mass and mRNA levels were again observed to be independently controlled by vitamin E and EPA. Similarly, independent effects were also seen with the other antioxidants tested (namely, vitamin A, vitamin C and ß-carotene) in the presence and absence of the PUFAs, EPA and linoleic acid.

Overall, it was found that PUFAs can downregulate the LDL receptor when incubated with liver cells in culture. This is unlikely to be related to their susceptibility to oxidation because antioxidants could not prevent the effect of the PUFAs. The experiments also led to the novel and important discovery that antioxidants can upregulate the LDL receptor in HepG2 cells. Furthermore, the opposing effects of the PUFAs and the antioxidants appeared to be independent of each other.

9.1 Effect of PUFAs on the LDL receptor.

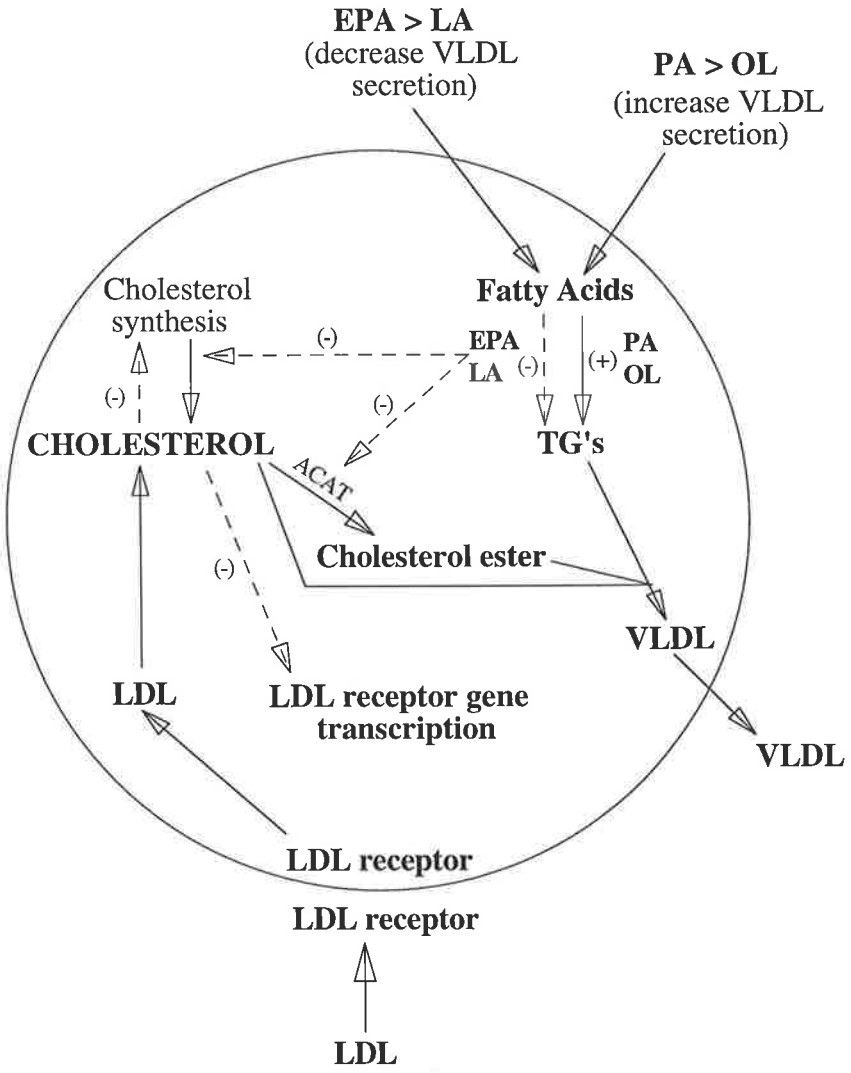
The PUFAs, EPA and linoleic acid, decreased the LDL receptor compared to saturated and monounsaturated fatty acids in rat H-35 and human HepG2 liver cells. This effect by PUFAs did not appear to be due to their increased susceptibility to oxidation as the decrease in LDL receptor expression was still observed in the presence of several antioxidants. A number of mechanisms by which these PUFAs could potentially affect the LDL receptor have been previously discussed (Chapters 4 and 5). Based on available experimental evidence, however, it would appear that their effects on intracellular cholesterol metabolism may be the primary mechanism by which these PUFAs mediate the suppression of the LDL receptor gene (see below).

9.1.1 Potential mechanisms.

The major driving force that leads to the downregulation of cellular LDL receptor gene transcription is an increase in intracellular free cholesterol (Goldstein and Brown, 1977; Brown and Goldstein, 1986). It follows, therefore, that if fatty acids could affect an aspect of hepatic cholesterol metabolism which gives rise to an overall increase in free cholesterol, then this would be the most likely scenario by which fatty acids regulate LDL receptor gene transcription. There is strong evidence to suggest that PUFAs can affect either cholesterol esterification, cholesterol biosynthesis, or lipoprotein metabolism (as discussed in Chapters 4 and 5, see Figure 9.1). It may be through their effects on these pathways that PUFAs can

regulate the intracellular cholesterol concentration or the putative "metabolic pool of cholesterol" (see Chapter 1 for more detail). Thus, modification of intracellular cholesterol levels through any of these pathways would trigger the appropriate response in LDL receptor gene transcription.

Figure 9.1: The effect of fatty acids on intracellular cholesterol metabolism. (The dotted lines with (-) represent inhibitory effects and (+) represents a stimulatory effect on reactions. EPA = eicosapentaenoic acid, LA = linoleic acid, PA = palmitic acid, OL = oleic acid, TG's = triacylglycerols, LDLr = LDL receptor, VLDL = very low density lipoprotein, LDL = low density lipoprotein).



Two of the potential mechanisms by which PUFAs could increase intracellular cholesterol are through the inhibition of cholesterol esterification and/or VLDL secretion (see Figure 9.1). Inhibition of PUFAs on both these pathways could result in an increase in "metabolically active cholesterol" in the cell, thereby causing inhibition of LDL receptor gene transcription.

There are numerous studies showing that PUFAs can increase the intracellular cholesterol content by affecting free cholesterol esterification. Studies have documented that human fibroblasts, rat liver cells and human intestinal CaCo-2 cells enriched with EPA exhibit lower ACAT activity compared to cells enriched with palmitic, oleic and linoleic acid (Pal and Davis, 1991; Rustan et al., 1988; Murthy et al., 1988). In addition, cells incubated with linoleic acid have decreased ACAT activity compared to cells incubated with saturated fatty acids (Mathur et al., 1983; Pal, 1990). Therefore, the build up of free cholesterol, through the suppression of cholesterol esterification, may contribute to the feedback inhibition of LDL receptor gene transcription observed with PUFAs (Figure 9.1).

The rate of VLDL secretion has to be considered as a major factor which can affect the intracellular cholesterol concentration in the liver. It is well documented that EPA can dramatically inhibit the synthesis of triacylglycerols and the secretion of VLDL and apoprotein B in HepG2 cells (Wong and Nestel, 1987; Wong et al., 1989; Nestel et al., 1984). Decreased cholesterol ester synthesis with EPA may also contribute to a decreased synthesis and secretion of VLDL (see above and Figure 9.1). These effects may explain why fish oil consumption causes a substantial reduction in plasma VLDL and triacylglycerols in humans and animals (Harris et al., 1983; Spady, 1993; Wiener et al., 1986; Harris, 1989; Land, 1986; Nestel, 1990; Rich et al., 1989). There is also evidence that linoleic acid inhibits VLDL synthesis and secretion by cells (Chait et al., 1974; Cortesse et al., 1983), and may explain why linoleic acid causes a reduction in plasma VLDL level. It appears that the decreased synthesis and secretion of triacylglycerols by PUFAs contributes to the diminished synthesis and secretion of the VLDL particles (Wong and Nestel, 1987; Wong et al., 1989; Lang and Davis, 1990).

Consistent with this idea, the study of Schafer and Kattermann (1992) has shown that cholesterol biosynthesis and secretion decreased in HepG2 cells incubated with 200 μM linoleic acid, and even further decreased with 200 μM EPA compared to cells incubated with 200 μM palmitic or oleic acid. Thus, in addition to the effect of PUFAs on VLDL secretion, the decrease in cholesterol secretion by PUFAs could further compound the accumulation of cholesterol in the cell.

Cholesterol and cholesterol esters are important constituents of VLDL. Therefore, a decrease in VLDL secretion out of the cell will lead to less demand on cholesterol and a rise in unincorporated cholesterol. The increase of unincorporated cholesterol can lead to an overall increase in hepatic free cholesterol, and therefore, cause a feedback downregulation on both cholesterol synthesis (HMG-CoA reductase) and on the LDL receptor. Thus, a

block in VLDL secretion by PUFAs could be the mechanism which causes an increase in hepatic free cholesterol and the consequent suppression of the LDL receptor (see Figure 9.1).

9.1.2 Implications of the *in vitro* results of PUFAs on *in vivo* studies.

i) Linoleic acid (ω-6 PUFA)

The hypothesis that ω-6 PUFAs lower plasma cholesterol by upregulating the LDL receptor was not supported by the results herein. This can be interpreted in two ways: (1) ω-6 PUFAs downregulate the LDL receptor *in vitro* and *in vivo,* but the decrease in plasma cholesterol is due to other reasons, or (2) ω-6 PUFAs do lower plasma cholesterol by upregulating the LDL receptor *in vivo,* but the effect on the receptor *in vivo* is via indirect mechanisms not generated in the cell culture system.

(1) If ω-6 PUFAs have the same effect on the LDL receptor *in vivo* as *in vitro,* then a decreased LDL receptor could not be the reason why plasma LDL cholesterol is lowered by these fatty acids. In this case, there must be other pathways affected by ω-6 PUFAs, which cause the cholesterol lowering.

It is clear from experimental results obtained from a variety of human and animal studies that the steady state of plasma LDL cholesterol is determined by the rate of entry of LDL into the plasma (or in other words, the production rate of LDL) relative to the rate of its removal from the circulation mainly via the hepatic LDL receptor (Woolett and Dietschy, 1994; Dietschy et al., 1993). Therefore, the secretion of VLDL would have a major impact on plasma LDL cholesterol as this lipoprotein is the precursor of LDL.

In light of this, one can assume that the effect of ω-6 PUFAs on VLDL in humans is more likely to be the mechanism by which LDL levels are decreased rather than their effects on LDL receptor activity. It has been documented that linoleic acid decreases VLDL synthesis and secretion from liver cells (Chait et al., 1974; Cortesse et al., 1983) (see above and Figure 9.1). This effect of linoleic acid may be the cause the reduction in VLDL levels observed *in vivo*. Thus, the reduction of VLDL secretion by ω-6 PUFAs could be one of the major mechanisms whereby LDL cholesterol is lowered, even when LDL receptor activity is reduced by these fatty acids.

In many species, including humans, it has been shown that under certain conditions, the production of LDL from VLDL may have a bigger impact on the steady state levels of LDL cholesterol than LDL receptor activity (Woolett and Dietschy, 1994; Dietschy et al., 1993). Inhibition of cholesterol synthesis by statins (HMG-CoA reductase inhibitors) have a pronounced lowering effect on plasma cholesterol, although changes in LDL receptor are not observed (Roach and Smith, personal communication). This suggests that upregulation of the LDL receptor is not a prerequisite for the cholesterol lowering effect of statins. By affecting VLDL production, linoleic acid may be able to lower plasma cholesterol levels even if the LDL receptor is downregulated. However, it is difficult to know whether this occurs

in humans because lipoprotein production by the liver *in vivo* in humans has not been studied as suitable techniques are not available.

(2) There is the possibility that linoleic acid really does lower plasma cholesterol by upregulating the LDL receptor *in vivo*. In such a case, the effect of linoleic acid on hepatic LDL receptor activity *in vivo* may be as a consequence of extra hepatic factors which are activated or produced as a result of the linoleic acid intake. These factors, in turn, may cause an increase in LDL receptor activity in the liver and overpower the original effect as seen *in vitro*. For example, the plasma levels of certain factors, such as insulin (Wade et al., 1988) or tumour necrosis factor-α (TNF-α), have been shown to be altered by PUFA intake (Hamaka et al., 1992). Interestingly, LDL receptor activity has also been shown to be increased by these factors. This suggests that the effect of PUFAs on the LDL receptor may really represent an effect of extra hepatic factors. Such factors could potentially mask the original suppression of the LDL receptor by the linoleic acid, resulting in a rise in plasma LDL. Although this idea has been raised throughout the work herein, it is difficult to decipher between the direct effect of the fatty acids and the indirect effect of these extra hepatic factors on the LDL receptor *in vivo*.

ii) EPA (ω-3 PUFAs)

The downregulation of the LDL receptor seen with EPA in HepG2 cells (Figure 5.2) is consistent with the rise in plasma LDL levels observed in humans consuming this fatty acid (Harris et al., 1983, 1988; Nestel, 1986; Illingworth et al., 1984) (discussed earlier in section 1.8.3.5). These results are also supported by the study of Wong and Nestel (1987) in that LDL receptor activity was suppressed when human HepG2 cells were enriched with EPA compared to cells enriched with oleic acid. As in humans, dietary fish oils can also cause an increase in plasma cholesterol levels in some animals, such as hamsters (Surette et al., 1992) and cynomolgus monkeys (Manning et al., 1992). Downregulation of the hepatic LDL receptor is believed to be the reason for this rise in plasma cholesterol in these studies, but an increased conversion of VLDL to LDL may also occur. Huff and Telford (1989) have reported that fish oil feeding to miniature pigs caused an increase in LDL receptor mediated clearance of LDL and also resulted in the secretion of a VLDL particle that was preferentially converted to LDL compared to corn oil fed pigs.

The downregulation of the LDL receptor by EPA was also observed in the rat liver cell line H-35 (Chapter 4). Consistent with this *in vitro* result, others have observed similar effects in rat hepatic membranes isolated from rats fed 8% fish oils (Roach et al., 1987). However, in contrast to humans, ω-3 PUFAs generally lower total plasma cholesterol levels in rats even though the hepatic LDL receptor of these two species responds similarly to ω-3 PUFAs. One explanation would be that the LDL receptor is downregulated in rats *in vivo,* and the decline in total cholesterol is due to other reasons.

The changes in total cholesterol levels in the rat may due to some other effect of these fatty acids on cholesterol or lipoprotein metabolism. In most species, such as rats, mice, hamsters, and dogs, HDL (not LDL) is the major transporter of cholesterol. Only humans are unique with respect to their high LDL to HDL ratio (Harris, 1996). In humans, 50-100% of the VLDL is converted to LDL, whereas in these animals most of the VLDL is cleared from the circulation before being converted to LDL (Grundy, 1991; Grundy and Denke, 1990). Since only 10-20% of VLDL is converted to LDL, most of the surface components of VLDL end up in HDL. Therefore, it follows that factors that inhibit VLDL synthesis and secretion in the liver could also affect HDL synthesis. As mentioned earlier, EPA has been shown to inhibit VLDL synthesis (see above), and consequently, this may be the reason why the synthesis of HDL cholesterol would be decreased in these animal species. The resulting decrease in HDL synthesis by EPA would cause a lowering in total plasma cholesterol.

Thus, it would be expected that fatty acids, such as EPA, would affect cholesterol levels differently in various species, such as the rat, as the level would presumably depend on the lipoprotein metabolism to a large extent. That is, the distinct effect of fatty acids on cholesterol levels in various species may be a result of differences in lipoprotein metabolism between animal species. For instance, swine are usually unaffected by fish oils (Huff and Telford, 1989), while monkeys (Davis et al., 1987) and rats (Spady, 1993) show a significant lowering of plasma LDL cholesterol, and LDL cholesterol is highly variable in rabbits (Aldstein et al., 1992).

Given the differences in lipoprotein metabolism and the response to dietary fatty acids between humans and rats, it is remarkable that at the cellular level the LDL receptor activities in rats and humans liver cells were identical when incubated with a variety of fatty acids (Figure 4.10 and Figure 5.2). The effects of the fatty acids were even identical in the absence or presence of cholesterol, in that LDL receptor activity was affected similarly in both cell lines by the interaction of cholesterol and oleic acid. It may be that most species respond in a similar manner to fatty acids at the cellular level due to gene conservation seen across species (eg. similar promoters, transcription factors). If this is the case, then many species should be influenced similarly at the molecular and cellular level in the same tissue.

9.1.3 Relevance of ω-3 PUFAs to CHD.

Although the changes in plasma levels in LDL cholesterol induced by PUFA feeding may not always correspond to the changes they produce in hepatic LDL receptor activity, these alterations in LDL receptor activity could still be pertinent in determining the progression of CHD. The beneficial effect of ω-3 fatty acids against the development of coronary artery disease has been demonstrated in many human studies and confirmed in several controlled animal studies even though ingestion of fish oil ω-3 PUFAs can increase LDL cholesterol (Davis et al., 1987; Wiener et al., 1986). The reduction in cardiovascular

mortality by these fatty acids is, therefore, most likely unrelated to the rise in plasma cholesterol concentration observed when these fatty acids are consumed, and more likely to be related to their effects on the LDL receptor (see below).

Studies have implied that a decrease in CHD is directly related to their effects on the hepatic LDL receptor. This hypothesis is supported by the fact that fish oils have failed to protect LDL receptor deficient Watanabe heritable hyperlipidemic rabbits (WHHL) against atherosclerosis. Both Clubb et al. (1989) and Rich et al. (1989) showed that feeding ω-3 fatty acids to WHHL rabbits compared to chow fed WHHL rabbits did not reduce plaque thickness or have any effect on the levels of serum cholesterol, triacylglycerol, lipid peroxides and aortic atherosclerosis. Whereas, similar studies done in other animals including pigs, hamsters and rabbits possessing a normal functioning receptor, have consistently shown that ω-3 fatty acids protect against the development of coronary artery disease (Davis et al., 1987; Thiery and Seidel, 1987; Wiener et al., 1986). The lack of an effect of ω-3 fatty acids in the WHHL rabbits suggests that these fatty acids require the involvement of the LDL receptor pathway in conferring protection against atherosclerosis. The effect of fatty acids on plasma lipoprotein levels, therefore, may be irrelevant in the mechanism whereby these fatty acids confer protection against CHD. Thus, correlation or the lack of correlation between LDL receptor activity and plasma lipoprotein levels seen with PUFA consumption may be inconsequential in the action of these fatty acids against the progression of this disease.

9.2 Effect of antioxidants on the LDL receptor.

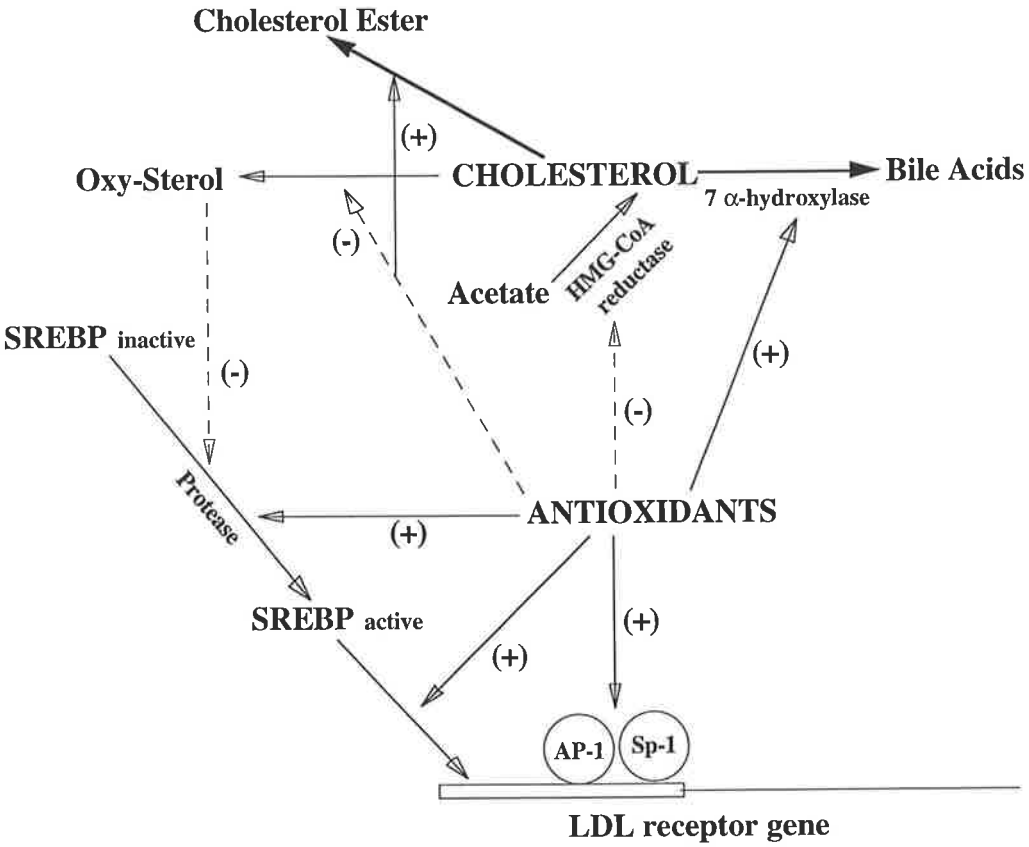
The work herein has demonstrated that a wide range of vitamin and non-vitamin compounds which are chemically and functionally diverse were able to upregulate the LDL receptor. The chemical structure of these compounds divides them into 2 categories: fat soluble or water soluble. Given that these structurally distinct compounds upregulate the hepatic LDL receptor, it is conceivable that this effect may be linked to the common inherent antioxidant properties of these compounds. The results herein support the hypothesis that the upregulation of the LDL receptor is a common "antioxidant phenomena".

9.2.1 Possible mechanisms.

Thus, the question that follows from these observations is whether these powerful physiological vitamin and non-vitamin antioxidants act through a common mechanism to upregulate the LDL receptor. It is unclear by precisely which mechanism antioxidants exert their effects and whether it is a common mode of action for all these antioxidants (Figure 9.2). It would seem likely, however, that these compounds would have one common mode of action because of their common antioxidant property, rather than each acting through its own unique pathway. This potential pathway(s) may be associated with or entirely

independent of intracellular sterol metabolism (Figure 9.2). If the antioxidants are not involved in any of the sterol metabolic pathways, then they could be acting exclusively through transcription factors which are involved in LDL receptor gene transcription.

Figure 9.2: The effect of antioxidants on LDL receptor gene transcription. (Where the dotted lines with (-) represent inhibitory effects and (+) represent stimulatory effects on reactions indicated with solid lines. The effects of antioxidants on the sterol regulatory binding protein (SREBP), cholesterol to oxysterol, and the transcription factors, AP-1 and Sp-1, are speculative).



Thus, there are clearly quite a number of pathways that can be affected by antioxidants (Figure 9.2). It is difficult to discriminate whether all of these pathways are simultaneously affected by antioxidants or whether one pathway is more important than the others. As these pathways are interconnected, at face value, it would appear that the antioxidants could affect several pathways rather than acting through just one.

The major factor leading to an upregulation of LDL receptor gene transcription has been shown to be a decrease in free cholesterol (Brown and Goldstein, 1986). Therefore, based on this evidence, the antioxidants are more likely to regulate the LDL receptor through the pathways that determine intracellular cholesterol concentrations. This highlights the

importance of the effect of antioxidants on bile acid secretion and cholesterol esterification as the main mechanism by which antioxidants may regulate LDL receptor gene transcription (Figure 9.2). If antioxidants interfere with any of these pathways such that intracellular cholesterol is depleted, then presumably the induction of the LDL receptor would result.

Although the effect of the antioxidants on cholesterol metabolism appears to be the most likely mechanism whereby the LDL receptor is upregulated, antioxidants could potentially regulate the LDL receptor through other mechanisms. Discussed below are some other possible mechanisms by which antioxidants, as a whole, may act to upregulate LDL receptor gene expression (also see Figure 9.2).

Mechanism #1; Mimicking HMG-CoA inhibitors

Antioxidants may be mimicking the actions of HMG-CoA reductase inhibitors (Figure 9.2). Even though treatment with these inhibitors competitively inhibit HMG-CoA reductase activity, they also result in a large increase in the mRNA and protein for HMG-CoA reductase, the LDL receptor and other genes. This usually depletes the supply of cholesterol to the liver which is compensated by the enhanced uptake of LDL from the circulation by the liver (Endo, 1992) (see section 1.7.7). A recent study by Khor et al. (1995) reported that guinea pigs fed a diet supplemented of tocotrienols (mainly α- and γ-tocotrienol) exhibited a dose dependent decrease in liver HMG-CoA reductase activity. Although HMG-CoA reductase activity was not measured in HepG2 cells incubated with the various antioxidants, it was demonstrated that α-tocopherol and green tea extract could upregulate HMG-CoA reductase mRNA levels (Figures 6.6 and 7.7). Thus, the upregulation of HMG-CoA reductase and LDL receptor mRNA levels could be a common "antioxidant phenomena" caused by an inhibition of HMG-CoA reductase activity.

Mechanism #2: Sterol oxidation

The antioxidant properties of the vitamin and non-vitamin compounds examined herein may be a feature of their ability to prevent intracellular sterol oxidation. Sterol balance within the cell has been shown to be an important determinant of LDL receptor activity in HepG2 cells (Grundy, 1991; Brown and Goldstein, 1988; Rudling, 1992; Sanchez et al., 1995), in which the regulatory effects of oxidised cholesterol or related oxysterols on the LDL receptor is largely at the transcriptional level (Kandutsch et al., 1973, 1974; Kreiger et al., 1978). This is further exemplified in experiments where different vitamin E analogues with high and low antioxidant potentials were examined for their ability to upregulate the LDL receptor (Chapter 5). The δ- and γ-tocopherol analogues of vitamin E, which have lower antioxidant potentials compared to α-tocopherol, were unable to upregulate the LDL receptor. This would strongly support the idea that the high antioxidant capacities of a compound is necessary for LDL receptor upregulation. In support of this theory, it has been demonstrated that ECGg (one of the active components of green tea which has the highest

antioxidant activities compared to the other catechins) also lowers plasma LDL levels (presumably due to an increase in LDL receptor activity) better than any of the other catechins (Chiska et al., 1988: Ikeda et al., 1992). The antioxidant properties, therefore, may explain why ECGg causes hypocholesterolemia when this component is supplemented. Therefore, the role of antioxidants in the prevention of oxysterol formation must be considered as a potential mode of action whereby LDL receptor is regulated by vitamin and non-vitamin antioxidants.

Mechanism #3: Activating transcription factors

Antioxidants could be regulating the LDL receptor through their effects on nuclear transcription factors. In order for LDL receptor gene transcription to take place, transcription factors, such as Sp-1, have to bind directly to specific regions of the gene (Sudhof et al., 1987; Wang et al., 1994) (see section 1.7.5). Thus, expression of the LDL receptor gene could occur if antioxidants are able to induce the production of transcription factors or stimulate the binding of these transcription factors to the LDL receptor gene. Recent reports have indicated that antioxidants increase the transcription factor AP-1 (Pinkus et al., 1996; Azzi et al., 1995). AP-1 is a ubiquitous regulatory protein complex which interacts with AP-1 binding sites of target genes to regulate transcription in response to environmental stimuli. Azzi et al. (1995) reported that vitamin E was able to inhibit smooth cell proliferation in a dose dependent manner by upregulating the transcription factor AP-1. Although it has also been implicated as a antioxidant response factor, it is thought that AP-1 can be induced in response to oxidative stress as well (Pinkus et al., 1996). Although AP-1 has been shown to bind to a region of the LDL receptor gene, transcription of the gene is weak compared to factors such as Sp-1 (Smith et al., 1988).

Like AP-1, Sp-1 could be activated by vitamin E and other antioxidants, and this therefore, may explain how most antioxidants upregulate LDL receptor transcription (see chapter 1.7.5 and Figure 9.2). Further support for this idea comes from the work described here showing that green tea and vitamin E had an identical effect on both HMG-CoA reductase and LDL receptor mRNA levels (Figures 6.5, 6.6, 7.6 and 7.7). Thus, it is possible that the simultaneous upregulation of these genes by antioxidants could be mediated through a common transcription factor, such as Sp-1 (Figure 9.2).

Although there is no evidence as yet, antioxidants could also be involved in regulating LDL receptor gene transcription through the SREBP-1/SRE-1 system (see Figure 9.2). If the effect on the LDL receptor is independent on sterol concentrations, then antioxidants may be enhancing LDL receptor gene transcription by competing with cholesterol and facilitating the cleavage of SREBP through a direct action on the proteolytic enzymes. However, these proteolytic enzymes have not been identified yet.

Alternatively, the regulation of the LDL receptor at the molecular level by antioxidants can occur independently, but still in conjunction with cholesterol. For example,

antioxidants may bind directly to the regulatory region of the LDL receptor gene and induce transcription even though SREBP-1 cleavage has been inhibited by cholesterol. There is the possibility that antioxidants may also be upregulating the LDL receptor through its effects on both SREBP and Sp-1. In this case, SREBP-1 may be activated when cholesterol is depleted in the cell due to antioxidant effects on the cholesterol metabolism (see below), and Sp-1 binding may be simultaneously activated by the antioxidants. This presents a case where antioxidants can exert their effects on the LDL receptor by regulating cholesterol metabolism and transcription factors at the same time.

Mechanism #4 :Cholesterol Metabolism

Although the mechanisms described above are all possible, there is no direct evidence to support these theories. As mentioned earlier, cholesterol is the major regulator of the LDL receptor, and therefore, the direct link of antioxidants with cholesterol metabolism may be a key to understanding the mechanism of action by these compounds. As upregulation of the receptor requires the decrease in the level of cellular cholesterol, the depletion of cellular cholesterol by antioxidants could occur by the following pathways:

i) It has been suggested that antioxidants can induce the activity of 7α-hydroxylase, thereby promoting the depletion of cholesterol from the liver (Ginter et al., 1971; Holloway et al., 1981; Chupukcharoen et al., 1985). This pathway could potentially be the main mechanism whereby antioxidants upregulate the LDL receptor (see Figure 9.2). Vitamin C and vitamin E have both been found to modulate hepatic 7-α hydroxylase (Ginter et al., 1971; Holloway et al., 1981; Chupukcharoen et al., 1985). Animals deficient in these vitamins have increased total plasma cholesterol and reduced bile acid synthesis due to a reduction in 7-α hydroxylase activity with a concomitant decrease in tissue cholesterol. These studies suggest that in the presence of vitamin C or vitamin E, cholesterol conversion to bile acids would increase. Thus, cholesterol would be depleted in the liver, inducing the transcription of the LDL receptor gene and leading to a decrease in plasma cholesterol. Such may be the case, as Chisaka et al. (1988) found that rats fed green tea extract had a decrease in hepatic cholesterol and plasma cholesterol. Similarly, total lipid, total cholesterol and triacylglycerol concentrations of the liver increased in rats fed a high cholesterol diet, but the addition of ECGg to the diet decreased these parameters (Fukuyo et al., 1986).

The 7α-hydroxylase is thought to be the rate limiting enzyme for bile acid (cholic acid) synthesis. Interestingly, it not only converts cholesterol to 7α-hydroxycholesterol which then is converted to bile acids, but also hydroxylates other oxysterols, such as 25- and 27-hydroxycholesterol (previously known as 26-hydroxycholesterol). It has been shown that as much as 46% of the administered 27-hydroxycholesterol can be metabolised into cholic acid (Ayaki et al., 1989). The 27-hydroxycholesterol is a naturally occurring sterol found in human plasma and has been implicated in being the major physiological oxysterol

regulator of the LDL receptor (Ayaki et al., 1989). The enzymes responsible for the formation of oxysterols from cholesterol have not been completely characterised, although it is apparent that they are members of the cytochrome P450 family. Thus, the increase in 7α-hydroxylase activity may promote the conversion of 27-hydroxycholesterol, and therefore, less of this oxysterol will be present in the cell to inhibit the LDL receptor (see Figure 9.2).

ii) The decrease in intracellular cholesterol could be related to the effect of antioxidants on ACAT activity (Figure 9.2). Although other antioxidants were not examined, Shirai et al. (1980) reported that when rats were fed a vitamin E deficient diet for 16 weeks, they exhibited decreased arterial ACAT activity. This suggests that feeding this vitamin may increase cholesterol esterification storage and lead to a decrease in free cholesterol.

iii) Cholesterol depletion may also result if there was a decrease in cholesterol synthesis. Indirect evidence has shown that antioxidants can affect the cholesterol biosynthetic pathway as vitamin E deficient-rabbits exhibited an increased ability to synthesise cholesterol from [^{14}C]-mevalonate, thereby increasing the amount of free cholesterol within the cell. These observations suggest that the addition of vitamin E could inhibit the cholesterol synthetic pathway and deplete the cell of cholesterol. A study by Khor et al. (1995) also suggested that vitamin E could decrease cholesterol content in the liver by downregulating HMG-CoA reductase activity (Figure 9.2).

Thus, depletion of cellular cholesterol by antioxidants through one or all of these pathways could be the major mechanism whereby antioxidants upregulate the LDL receptor.

9.2.2 Relevance of antioxidants to CHD

Numerous studies suggesting that antioxidants decrease the risk of CHD are now emerging (see Chapter 1 for detail). It has been proposed that the decreased risk of CHD associated with antioxidant supplementation is due to their ability to prevent LDL oxidation. Since the role of antioxidants in the prevention of LDL oxidation has not been proven *in vivo*, it may not be the primary mechanism whereby antioxidants inhibit the process of atherosclerosis as has been suggested. The decreased risk in CHD could be more closely related to the antioxidant effects on the hepatic LDL receptor.

A decrease in plasma cholesterol is a commonly reported effect associated with antioxidant supplementation of animal or human diets. Gatto et al. (1996) reported vitamin C supplementation in women reduced plasma LDL concentrations by 16% compared to baseline. Animal and human studies also consistently show a decrease in plasma LDL levels with green tea extract administration, and an enhanced clearance of plasma cholesterol has been demonstrated (Chisaka et al., 1988; Ikeda et al., 1992; Matsuda et al., 1986; Kono et al., 1992). Similarly, Harats et al. (1990) and Muckle et al. (1989) have shown that vitamin

E supplementation in humans can lower their plasma LDL cholesterol levels. One can speculate that the observed decrease in plasma cholesterol through the upregulation of the LDL receptor pathway may be the mechanism by which antioxidants exert at least some of their antiatherogenic effect.

9.3 The combined effects of fatty acids and antioxidants on the LDL receptor.

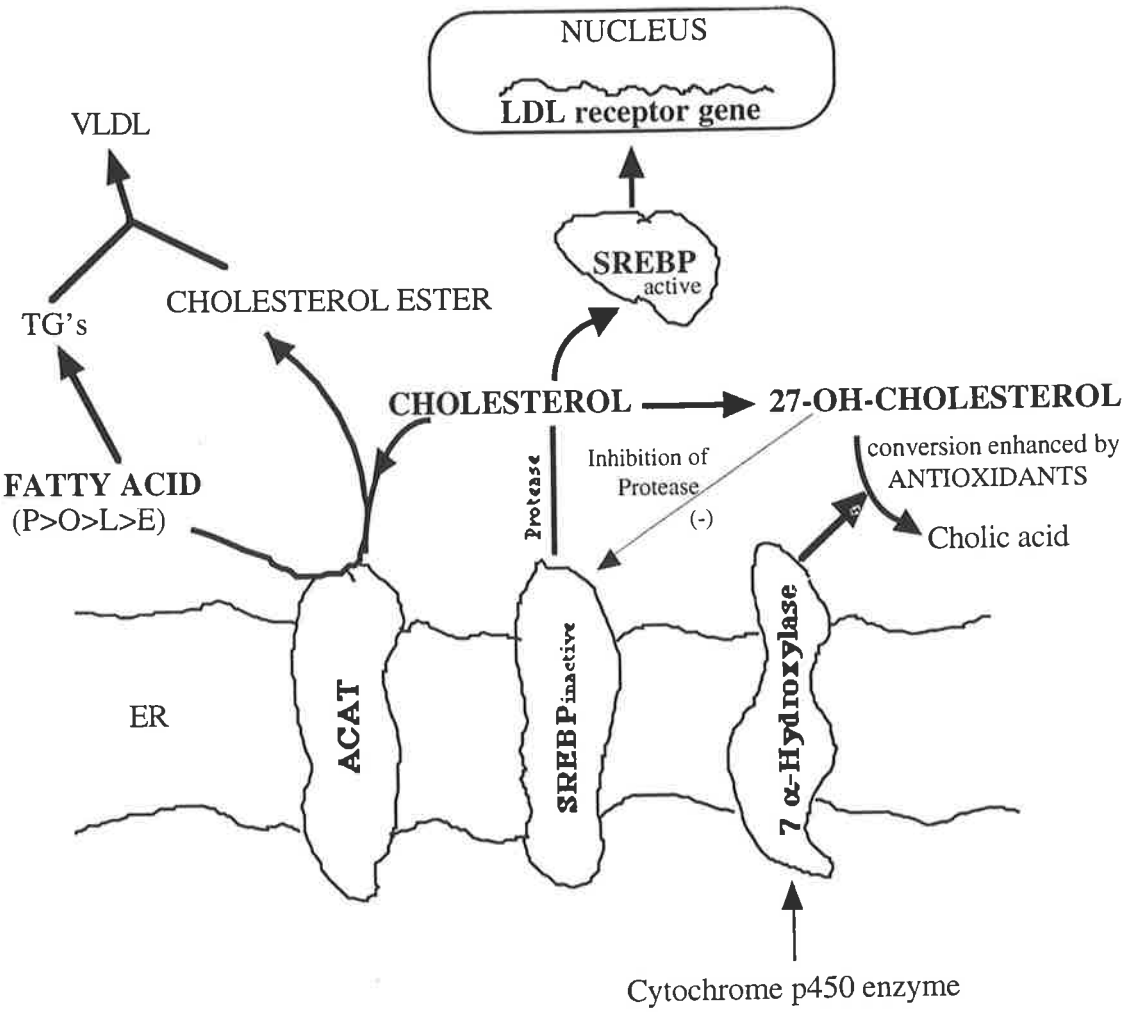
9.3.1 Possible mechanisms.

What is evident from the results herein is that all antioxidants examined are capable of upregulating LDL receptor gene expression, while PUFAs are able to downregulate this gene. The same effect is observed even when both agents are present in the cell together (Chapter 8), indicating that fatty acids and antioxidants have an independent influence on the LDL receptor. Given that LDL receptor gene expression is regulated by antioxidants and fatty acids independently, the next issue is to determine what are the possible mechanisms by which these two agents simultaneously effect LDL receptor gene expression in an opposing manner. Are their actions through identical pathways or do they effect LDL receptor gene transcription through different pathways? On the surface, the answer to these questions is difficult to ascertain as there is evidence to support both theories.

There are many potential pathways that can be affected by antioxidants and fatty acids in which the end result would cause an appropriate response in LDL receptor gene expression (Figure 9.3). As previously discussed, however, the major mechanism involved in regulating LDL receptor gene expression, which has been well investigated and accepted, is the one which involves cholesterol metabolism. Figure 9.3 illustrates two potential pathways that antioxidants and fatty acids may be acting through to regulate the LDL receptor independently. In this figure, antioxidants are shown to deplete intracellular cholesterol by enhancing bile acid formation. On the other hand, cholesterol concentrations could be increased due to inhibition of VLDL secretion by PUFAs. In another example, antioxidants may be depleting cellular cholesterol by inducing 7α-hydroxylase activity and causing an increase in the conversion of cholesterol into bile acids. On the other hand, PUFAs may be increasing the free cholesterol pool by inhibiting ACAT activity (Figure 9.3).

These routes provide an opportunity for both of these agents to act independently on cholesterol metabolism, but the subsequent changes in cholesterol levels are dictated by both. Therefore, it would follow that the final level of the LDL receptor would be a result of the action of both agents on intracellular cholesterol (see below).

Figure 9.3: Proposed pathway whereby fatty acids and antioxidants may be exerting their effects simultaneously, but independently. (Where E= eicosapentaenoic acid, L= linoleic acid, P= palmitic acid, O= oleic acid, TG's= triglycerides).



The dilemma still remains whether the two agents have opposing effects on the same pathway. For example, hepatic cholesterol esterification has been shown to be decreased by ω-3 PUFAs in cell culture and animal experiments (Pal and Davis, 1991; Rustan et al., 1988; Murthy et al., 1988). In contrast, recent evidence suggests that cholesterol esterification may be increased by vitamin E (Shirai et al., 1980) (Figure 9.3). In another example, both agents could also be simultaneously affecting the conversion of cholesterol to bile acids. It has been shown that animals that are fed a PUFA rich diet have a very low conversion of cholesterol to bile acids compared to animals fed a diet supplemented with saturated coconut fat. (Berr et al., 1993). Inhibition of this pathway by ω-3 fatty acids can lead to an increase in the concentration of cellular cholesterol in liver cells. However, as discussed above, antioxidants can increase the conversion of cholesterol to bile acids, thereby depleting cell

cholesterol. In this case, the fatty acids and antioxidants influence the same pathway, but have opposite effects.

Cholesterol is the only driving force that has been consistently documented to regulate the LDL receptor. The mechanisms postulated above all involve cholesterol metabolism, ie. cholesterol esterification, cholesterol synthesis, VLDL synthesis and secretion, and/or bile acid secretion. Thus, the mechanism of action with which both fatty acids and antioxidants regulate the LDL receptor could be through their opposing effects on any pathway(s) that determine intracellular cholesterol concentrations (Figure 9.3).

9.3.2 Future experiments in this area.

The work herein has provided many interesting and novel findings. However, there are still many questions to be addressed which can be catagorised into two groups: i) those clarifiying the physiological relevance of the *in vitro* results, and ii) those defining possible mechanisms whereby fatty acids and antioxidants are exerting their effects.

i)Testing the physiological relevance of the fatty acids and antioxidant effects

A number of avenues could be pursued to modify the experiments *in vitro* so they better mimick *in vivo* conditions. For instance, similar experiments to those described herein could be performed using chylomicrons to deliver the fatty acids of interest. Since liver cells *in vivo* usually acquire dietary fatty acids from chylomicrons, this method may be more physiologically relevant. Therefore, in order to mimic the *in vivo* conditions, individuals could be fed a bolus drink of the fatty acid of interest, a blood sample can be taken after a period of time (4 h) and chylomicrons isolated. A study by Lindsey et al. (1992) did a similar study but isolated LDL from humans fed fish oils. They observed a decrease in LDL receptor binding activity in HepG2 cells incubated with LDL isolated from individuals fed fish oils compared to cells incubated with LDL isolated from the same individuals before the fish oil feeding. Similar experiments can be undertaken with the antioxidants of interest. In order for these cell culture experiments to be more physiologically relevant, the fat soluble antioxidants could be presented to the cell incorporated into chylomicrons. It is possible that their effect on the LDL receptor could be different when presented to the cell in this manner.

To complete the fatty acid experiments, combinations of fatty acids may be examined. For example, the effect of saturated fatty acids or monounsaturated fatty acids in the presence of either linoleic acid or EPA could be tested. It would be interesting to determine whether the saturated fatty acids or PUFAs predominate in determining the final level of LDL receptor activity. This situation could be more physiologically significant as humans normally consume a diet of mixed fatty acid composition. Another experiment would be to investigate the effect of palmitic acid on LDL receptor activity after a 24 h incubation period with EPA and *vice versa*. This would determine whether palmitic acid could normalise LDL receptor activity after EPA induced suppression.

Animal experiments could be designed to correspond to the antioxidant effects on the LDL receptor in cell experiments. Increasing concentrations of individual antioxidants could be supplemented in the diet of guinea pigs (since LDL levels in guinea pigs respond to fatty acids similarly as in humans). After 8 weeks, their plasma lipoprotein levels, liver lipid levels (cholesterol ester, free cholesterol and TG) and hepatic LDL receptor expression (activity, protein and mRNA levels) could be measured, and compared to untreated animals. It would be interesting to observe if plasma lipoprotein levels, specifically LDL, are altered and whether the hepatic expression of the LDL receptor resembled *in vitro* results.

Whether the effect of several antioxidants on LDL receptor activity is additive when present in the cell at the same time needs to be investigated as these effects could be more physiologically relevant than testing antioxidants alone. Of particular interest would be to examine the effect of a combination of antioxidants, like vitamin E and vitamin C on LDL receptor activity. For example, can vitamin C prevent the suppression of the LDL receptor seen with 100 μM vitamin E? Furthermore, the effect of vitamin C, in the presence of increasing concentrations of vitamin E could be studied. In this case, if the bimodal effect of vitamin E on LDL receptor gene expression was still observed in the presence of vitamin C, but at a higher level, it would demonstrate that different antioxidants have independent effects on the LDL receptor. To follow the cell work, animal experiments (similar to those described above) could be designed to examine the effect of several antioxidants concurrently on lipid and lipoprotein levels.

Since cells are normally exposed to both fatty acids and antioxidants *in vivo*, the interrelationship of their effects should be further explored to clarify some of the experiments herein. In the future, the experiments should be repeated with additional control flasks: EPA could be added on day 3 and LPDS on day 4 and similarly, vitamin E added on day 3 and LPDS on day 4. These controls are more relevant in that it would enable more appropriate comparisons to be made with regards to the EPA and vitamin E effects. One could determine quantitatively, how much vitamin E is able to increase LDL receptor activity after it has been suppressed by EPA and *vice versa*.

ii) Testing possible mechanisms

Although the effect of PUFAs on cholesterol metabolism appears to be the more likely mechanism whereby fatty acids may affect LDL receptor gene transcription, there is a chance that PUFAs may bind to the promoter region of the LDL receptor gene and inhibit transcription. To eliminate this possibility, one could examine whether PUFAs can drive LDL receptor gene transcription using transient transfection. Others have investigated the role of non-sterol regulators in LDL receptor gene expression by transiently transfecting HepG2 cells with a reporter plasmid bearing a genetic element believed to be responsible for the sterol-mediated regulation of the LDL receptor gene. Transient transfections, using the luciferase gene as the reporter, have shown that growth hormone and mevinolin were able to

drive the induction of a reporter gene if this element is present (Pak et al., 1996). Sudhof et al., 1987 (b), using cells transfected with a large fragment of the LDL receptor promoter, have also shown that growth hormone and other factors could drive CAT (chloramphenicol acyl transferase) gene expression. Thus, it appears that non-sterol factors are capable inducing LDL receptor gene transcription. PUFAs could be used in such a system to examine whether they can drive or inhibit CAT expression.

As suggested, the effect of fatty acids on the LDL receptor could be related to its effects on synthesis and VLDL secretion. Therefore, one could examine whether EPA can downregulate the LDL receptor in the presence of an agent which stimulates VLDL synthesis and secretion in HepG2 cells. Fatty acids, such as oleic acid, have been shown to enhance the synthesis and secretion of VLDL (Wong and Nestel, 1987). HepG2 cells can be incubated with oleic acid and EPA simultaneously. In theory, if VLDL secretion is stimulated in the presence of these compounds and the LDL receptor is downregulated, then it may suggest that EPA suppresses the LDL receptor through mechanisms other than those related to VLDL synthesis and secretion.

Increased bile acid conversion by antioxidants could be a general mechanism whereby these compounds deplete intracellular free cholesterol and cause an upregulation of the LDL receptor. To test this, HepG2 cells could be incubated with increasing concentrations of different antioxidants, and subsequently, 7α-hydroxylase activity could be measured. It would be interesting to examine whether there would be a linear correlation between 7α-hydroxylase activity and the amount of antioxidant. To follow these experiments, HepG2 cells could be incubated with ACAT inhibitors in the presence and absence of these antioxidants. Inhibition of ACAT activity would result in an increase in free cholesterol, therefore, leading to an inhibition of the LDL receptor. Thus, if the LDL receptor is upregulated in the presence of antioxidants and ACAT inhibitors, then (1) the antioxidants are most likely not acting by increasing ACAT activity, and (2) there is a stronger possibility that antioxidants are likely to be increasing 7-α hydroxylase activity. Furthermore, inhibitors of ACAT and 7-α hydroxylase can be used simultaneously in the absence and presence of antioxidants. Using such inhibitors provides a method of defining the precise mechanisms(s) whereby these compounds are affecting the LDL receptor.

The idea has been raised that PUFAs may suppress the LDL receptor by causing an increase intracellular cholesterol levels (see Figure 9.1), which can then feedback to inhibit LDL receptor gene transcription. To test whether an increase in intracellular cholesterol is responsible for the inactivation of SREBP, cells can be incubated in the presence of PUFAs and chenodeoxycholic acid (CDCA). CDCA has been shown to affect sterol metabolism by inhibiting the sterol mediated inactivation of SREBP, and to induce the LDL receptor mRNA level 4-fold (Kawabe et al., 1995). Thus, if PUFAs are able to suppress the LDL receptor in the presence of this compound, then it may suggest that PUFAs can directly bind the receptor gene and prevent transcription. On the other hand, if PUFAs are not able to

suppress the LDL receptor gene in the presence of this compound, then it would suggest that they regulate the LDL receptor through their effects on modulating cholesterol levels (ie. by inhibiting ACAT activity, inhibiting VLDL synthesis, etc.).

A simple way to determine whether antioxidants and fatty acids affect the LDL receptor through the activation of SREBP is to measure the amount of SREBP protein (active and inactive forms) in the HepG2 cells. Antibodies have been raised in rabbits against SREBP and are now available (Wang et al, 1994). After cells are incubated with the various agents of interest, cell proteins can be solubilised, electrophoresed and transferred to nitrocellulose (see section 2.7) and immunoblot analysis with anti-SREBP can be performed. The amount of mature SREBP (65 kD) can be compared with the inactive form (120 kD) in cells incubated with antioxidants or fatty acids.

Clearly, many other experiments could be considered. However, the experiments described herein would help further define the effects of fatty acids and antioxidants on the LDL receptor. One of the most important points that comes from the work herein is the finding that in the presence of antioxidants, the fatty acid effect on the LDL receptor can be completely altered (Chapter 8). This finding is of great relevance in examining the effect of fatty acids, especially PUFAs, on different lipid and lipoprotein parameters in all animal and human studies. It is common practice to include antioxidants in fatty acid preparations to preserve the fatty acid structure. However, by doing so, one may be dramatically altering the outcome of the fatty acid effect, and the results, therefore, can be very misleading. One can easily investigate whether such anomalies are occurring by using proper controls. Fatty acid supplementation should be performed with and without the presence of antioxidants and in the presence of antioxidants alone.

9.4 Final Conclusions.

The work in this thesis demonstrates that PUFAs downregulate LDL receptor expression (activity, protein, mRNA) when incubated with liver cells in culture. These experiments also led to the unique and important discovery that antioxidants can upregulate the LDL receptor in HepG2 cells. When liver cells are incubated with antioxidants and fatty acids simultaneously, the final level of LDL receptor expression is a result of effects of both agents. However, the opposing effects of the PUFAs and the antioxidants appear to be independent of each other.

The findings described in this thesis have significant implications for future studies that address complex issues of regulation of the LDL receptor gene by non sterol compounds. While there is a possibility that these agents may act directly on the LDL receptor gene or indirectly though transcription factors to regulate the LDL receptor, the major mechanism involved is most likely to be related to their effects on the pathways that determine the "metabolically active pool of cholesterol".

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ERRATUM

p2 2nd line: substitute "gender" for "sex".
p6: ACAT is defined as Acyl coenzyme A: cholesterol acyl transferase.
p12, Table 1.1: "trace" is referred to as minor apoproteins present in the lipoprotein particles.
p14,1.5.2: VLDL is defined as very low density lipoproteins.
p16, para3: Turnover studies suggest that the rate of turnover of VLDL-apo B can account for most of the LDL apoB synthesised.
p16,1,3; referred to.
p22,1 23; and a half.
p25, Fig 1.11 (Hatched domains show regions reacting with monoclonal antibodies that block LDL binding only partially. Dark domains show regions containing epitopes of monoclonal antibodies that totally block the binding of LDL to the LDL receptor.)
p30, para 3: potent oxysterols refers to those compounds with high biological activity.
p37, 15: affecting
p41, last line: "others" refers to other investigators
p50 Figure 1.18: Taken from Dutta-Roy, AK, Gordon, MJ, Campbell, FM, Duthie, GG, and James, WP (1994). Vitamin E requirements, transport, and metabolism: Role of α-tocopherol binding protein. *J.Nutr.Biochem.* 5, 562-570.
p59: LPDS was fatty acid free. Ethanol (in the culture media) was present as a carrier at a concentration of < 0.01% (this is an acceptable amount when used as a carrier).
p74, accurate repeatability means experiments can be reproduced where significance is defined at $p<0.01$.
p74, para6: normal cholesterol response is defined by an increase in LDL receptor activity in cells in the absence of cholesterol and a decrease in LDL receptor activity in the presence of cholesterol.
p75 para 4 16: As
p75, 16: pravastatin is obtained from Bristol Myers Squibb
p75, 118: As
p75,115: a number of experiments was carried.
p81 heading 3.4.5, para 2 9, para 3 5: Chemiluminescent
p89,124:EPA do affect
p89, 125 same probably occur
p99 para 4, p100,101,102: Table of ANOVA not required, significance differences between treatments are described using letters on figures 4.9-4.12 .
p99, para 4; p100, para 2: Significance is defined as $p<0.01$
p106,11: "no fatty acid control:" refers to cells incubated in medium not supplemented with fatty acids.
p107,130; p176,12; p180,116; p240,18: effect.
p108,13; p108,110: may be.
p108,122; delete in higher
p112,13: addition of cholesterol
p115, para 2: TNF (tumour necrosis factor), IL (interleukin)
p115, para 3; p116 para 2: Lindsey.
p121, Table 5.2: minor fatty acids have been excluded, the sums of each of the columns are similar. The change in the relative percentage of fatty acid compared to control cells was assessed (see discussion).
p123, Figure 5.2 statistical tests used are outlined in the methods section.
p130, para 1: There is no detectable vitamin E in the LPDS or FCS.
p131, Figure 5.7: Units are incorrect, should be 10-fold less.
p134,110: delete: is as a consequence.
p135, 15 : delete: slight 25%
p136, Effects
p141, 12: is a factor

p 162, figure 6.5:

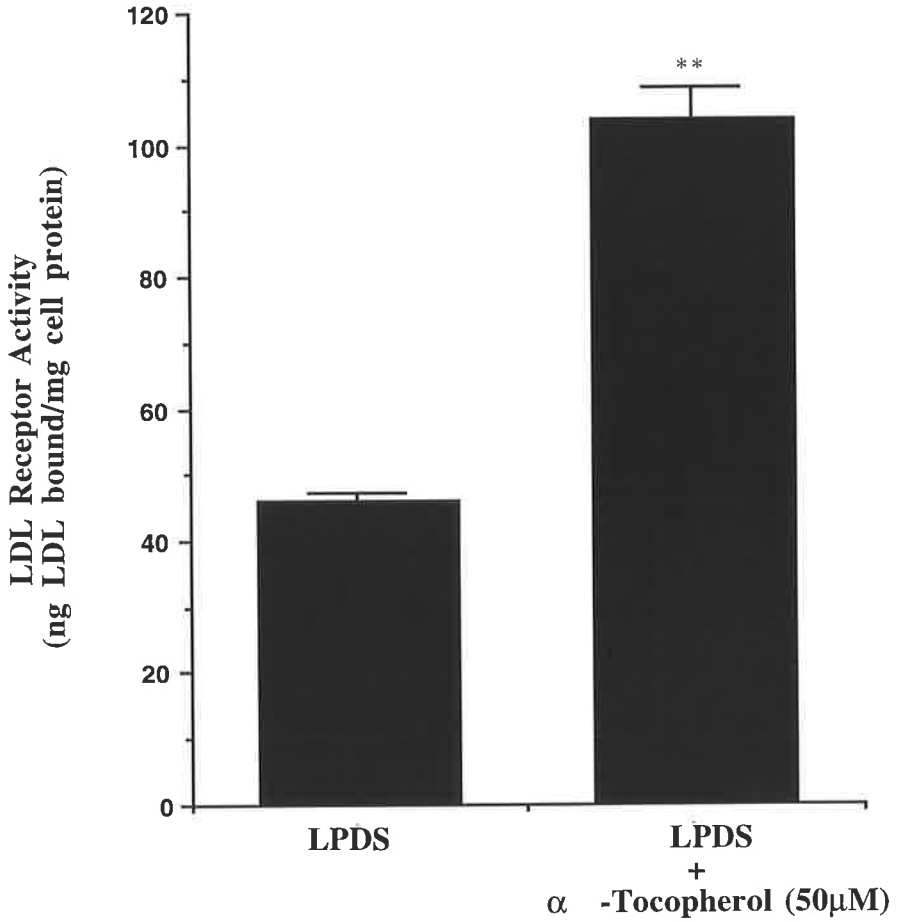

Figure 6.5: The effect of α-tocopherol on LDL receptor binding in HepG2 cells incubated in culture medium without cholesterol. Cells were incubated for 24 h with 50 μM α-tocopherol in a background medium of 10% LPDS. LDL receptor binding activity was measured in triplicate using the colloidal gold-LDL method as described section 2.6.3. Gold conjugates were incubated with 100 μg of cellular protein for 1 h at room temperature, either in the presence of $Ca(NO_3)_2$ to measure total binding or in the presence of EDTA to measure non specific binding. Specific binding was then calculated by taking the difference. Results represent specific LDL cell binding activity (ng of LDL/mg of cell protein) which is the mean ± SEM of 2 experiments.

p167, There is no evidence to suggest that there is a difference in solubility between delta and gamma. Both analogues appear to be taken up by the cells in a similar manner (see Figure 6.7).
p169, Figure 6.9: Concentrations of plasma vitamin E in man can vary between 10-60 μM due to the absence or presence of vitamin E supplementation. Thus, the concentration of vitamin E added to the HepG2 cells in this study does fall within a physiological range. The optimal range for man would be difficult to determine as cells receive vitamin E *in vivo* via LDL and not in the free form as in these experiments.
p173 heading: Effects of α-tocopherol on LDL receptor expression as related to its oxidation properties.
p175 para 2 13: However, there does not appear...
p179, 15: cells'
p182, para: Vitamin A is an antioxidant (see reference Livrea et al, 1995).

p184, 14: crude medicine refers to green tea being used as a herbal medicine by the Chinese.
p186: As catechins are considered to be one of the major components of green tea, the other compounds of green tea were not addressed in these experiments. Catechin concentrations were only used as a means whereby amount of green tea added to the cells could be quantified. Further experiments are needed to elucidate which specific components of green tea are responsible for the upregulation of the LDL receptor. Ethanol was added to the control cells to match the alcohol concentration in cells treated with red wine.
p186, 110: p202 Fig 7.8 axis and legend; Fig 7.9 legend: p204,18: quercetin
p187 para 1 last line: Therefore, HepG2 cells were able to effectively take up vitamin E and ß-carotene.
p210 last line: "....is not an anomalous event"
p219 Table8.1, 8.2; p222, Table 8.3: p228, Table 8.5: the data in these tables are only rough calculations of the percentage change in LDL receptor activity with the various treatments (see chapters 5 and 6 for significance levels for each treatment).
p227: Additional effect on LDL receptor expression was not observed when cells were incubated with more than 100 µM antioxidant. Therefore, in order to compare the effect of the antioxidants and fatty acids on LDL receptor expression with previous observations, 100 µM of antioxidant was used (see chapter 7). Since LDL receptor expression was observed to be regulated by the fatty acids similarly in the presence or absence of antioxidants, and *vice versa*, the amount of antioxidants did not appear to make a difference. Nevertheless, the amount of antioxidant needed to protect cells from oxidation by EPA and LA may be different.
p238: para 3 19 : "...readily be removed,..."
p238 para 5 14: "..EPA is incorporated into phospholipids...'
p238: phosphatidylcholine
p242 para 1 line 6: implies
p252, last para: 8% w/w
p255, Figure 9.2 . Where is the evidence that antioxidants stimulate the production of bile acids? Antioxidants, such as vitamin C and vitamin E, have been suggested to increase 7-α hydroxylase (a rate limiting enzyme in bile acid synthesis), refer to Ginter et al., 1971; Holloway et al., 1981: Chupukcharoen et al., 1985.
p259 last para: A reduction in plasma cholesterol as a result of antioxidant supplementation is not a commonly reported effect.
p267, Adelstein, title: .....dietary n-3 fatty acid supplementation....
p272: Goh, title:....triglyceride.
p274: Havel et al ,1955, p1345-1352
p276 Keough and Davis, 1979, title: Gel to liquid crystalline phase transitions in water dispersions of saturated mixed acid phosphatidylcholines.
p276: Kestin
p277: Lands, WE (1986), title: Renewed questions about polyunsaturated fatty acids.
p277 Lang and Davis, (1990), p 2079-2086
p281: Parker, Pearce, Clarke, Gordon and Wright, vol 268
p283: Shaish et al., 1995, J.Clin. Invest.
p286: Wang et al., 1993, p 1380-1389