n-3 PUFAs and Reperfusion Injury in Isolated Cardiomyocytes

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REQUIREMENTS FOR THE DEGREE OF
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Table of Contents

<u>Addendum – 20th November 2002</u>

(This addendum addresses all the points raised by both examiners. For continuity, these points are addressed in page order without specific reference to the particular examiner).

Page 12; section 1.4.4 Vitamin E, second sentence should read:
Alpha-tocopherol (α-tocopherol), one of the constituents of the fat-soluble vitamin E, is the most important chair breaking antioxidant present within membranes and lipoproteins (Halliwell, 1996).

Page 14; line 2,
Colon has been omitted.

Page 15; sentence 1 should read:
Animal fats are one of the major dietary sources of saturated fatty acids and include palmitic and stearic acids.

Page 15; lines 9 – 11 should read:
Dietary αLNA can be elongated and further desaturated to the longer chain n-3 PUFAs eicosapentaenoic acid (EP/ 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) (Figure 1.2).

Page 16; delete the first sentence; begin the second sentence with:
Arachidonic acid, which preferentially occupies the *sn*-2 position of cell membrane phospholipids can be metabolise via the lipoxygenase pathway…

Page 16 & 30; additional references to "Leaf and Weber 1988":
- Nieuwenhuys CM, Feijge MA, Offermans RF, Kester AD, Hornstra G, Heemskerk JW. (2001) Modulation of r: platelet activation by vessel wall-derived prostaglandin and platelet-derived thromboxane: effects of dietary fish o on thromboxane-prostaglandin balance. *Atherosclerosis* 154(2): 355-66.
- Calder, PC and Grimble, RF (2002) Polyunsaturated fatty acids, inflammation and immunity. *Eur. J Clin. Nutr.* 56 Suppl 3, S14-S19.

Page 27; additional reference to Stubbs and Smith 1984:
Feller SE, Gawrisch K, MacKerell AD Jr. (2002) Polyunsaturated fatty acids in lipid bilayers: intrinsic an environmental contributions to their unique physical properties. *J Am. Chem. Soc.* 124(2): 318-26.

Page 28; second-to-last line:
The word "anti-arrhythmic" should read "antiarrhythmic".

Page 53; hypothesis needs to be inserted after the aims. *(In relation to the point raised by this examiner, where chapter did not already contain a hypothesis, an appropriate hypothesis been added.)*
These aims were designed in order to test the hypothesis that exposure of cardiomyocytes to ROS would result in th generation of arrhythmic-type contractility, which would be reduced by the acute addition of n-3 PUFAs or by dietar fish oil supplementation.

Page 54; capitalisation:
The second instance of the word chapter should be capitalised.

Page 55; line 2, insert sentence:
This concentration is in the range of those used in studies examining the effect of n-3 PUFAs in neonatal and adult r: cardiomyocytes (Kang & Leaf, 1995; Leifert *et al.,* 2000).

Page 55; line 7, sentence should begin:
To further determine the effect of longer durations of incubation with higher concentrations of n-3 fatty acids, cell were…

Page 57; line 4:
To reduce ambiguity, the term in brackets "(a high-oleic sunflower oil)" should be omitted.

Page 58; insert after the last sentence:
The fish oil was an n-3 PUFA concentrate in the ethyl ester form obtained from the National Institutes of Health Bethesda, MD, USA.

Page 64; replace last sentence of Figure 3.3 legend with:
Control group, n=6; DHA, n=5; Vitamin E, n=3 rats.

Page 65; 4th line should read:
for vitamin E (n=3).

Page 65; paragraph 3, delete 4th sentence and replace with:
Compared with the OO group, proportions of 16:0 were significantly lower in the CO and FO groups (p<0.001), whilst the proportions of 18:0 were significantly higher in the CO and FO groups (p<0.05).

Page 67; First sentence of Figure 3.4 legend should read:
Cells were isolated from rats gavaged with olive oil (OO), canola oil (CO) or fish oil (FO), and exposed to G/GO (11 mM/0.15 U/ml) at time = zero min[4].

Page 68; line 1 word replacement:
The word "higher" should be replaced with "longer".

Page 68; symbols on graph:
Although the symbols on the legend to Figure 3.6 do correspond to those on the graph, for consistency with previous graphs, they should have appeared as a white square for the OO group (rather than filled symbols), a white triangle for the CO group and a black circle for the FO group. An amended figure with symbols consistent with previous graphs is included on the facing page.

Page 75; lines 6 & 7 should read:
...membranes of rats fed the 7% "low-ALA" Sunola oil diet (REF) were in fact lowered following feeding with the low-ALA diet, the sum of total n-3 PUFAs in these rats being...

Page 76; use of the term "compared to" or "compared with".
As the examiner did not insist on amendments regarding this point throughout the thesis, this was left unchanged.

Page 92; word replacement:
Replace "lard" with "saturated fat".

Pages 93, 126 and 170; tables:
The table on page 126 was incorrect (the SF data), which therefore caused significant variation between the respective tables. A corrected version of table 5.1 is included on the facing page.

Page 106; For clarity, delete sentence starting on line 4.
This did not allow for statistical analyses ... *(Confusion may have arisen due to the point that the number of cells from which synchronously contracting myocytes could be used varied due to the development of asynchrony. However there was sufficient asynchronously contracting myocytes for statistical analyses at the 20 min time point).*

Page 109; Figure A statistics.
The group values are not statistically different due to n=3 for the FO group versus n=6 for SF.

Page 114; typographical error on legend:
Replace "cell7s" with "cells"

Page 152; typographical error on legend:
*p<0.05 for CON vs FO.

Page 157; comment by examiner regarding the possibility that rat myocardium takes up long chain n-3 PUFAs from plasma lipoproteins as well as creating them within the myocardium. This point is acknowledged. Indeed, there is a delta 6 desaturase in rat myocardium: Lopez Jimenez JA, Bordoni A, Hrelia S, Rossi CA, Turchetto E, Zamora Navarro S, Biagi PL. (1993) Evidence for a detectable delta-6-desaturase activity in rat heart microsomes: aging influence on enzyme activity. *Biochem. Biophys. Res. Commun.* 192(3): 1037-41.

Page 167; delete sentence before aims, replace with:
It was therefore hypothesised that n-3 PUFAs, which become incorporated into membrane phospholipids of the myocardium may offer protection from cellular injury arising as a result of endogenously generated ROS.

List of Figures

List of Tables

List of Appendices

Abstract

Previous laboratory-based studies with experimental animals have indicated a protective effect of dietary fish oil (enriched with n-3 polyunsaturated fatty acids (PUFAs)) on reperfusion injury, which is believed to be induced by reactive oxygen species (ROS) and cellular Ca^{2+} overload. The broad aims of this thesis were to develop a cellular model for studying reperfusion injury, in order to investigate the reported protective effects of n-3 PUFAs, and to examine the underlying mechanisms associated with such protection. Cardiomyocytes were isolated from adult rat hearts and plated onto laminin-coated glass coverslips. Cells contracting under electrical field stimulation were induced to develop arrhythmic i.e. asynchronous contractile activity by the addition of ROS. Neither acute addition or overnight incubation of n-3 PUFAs (as the free fatty acid) conferred protection from ROS-induced asynchronous contractile activity in these cells. However, in cells isolated from rats supplemented with dietary fish oil (FO) for 3 weeks, a protective effect was observed in comparison to a saturated fat fed group. Dietary FO supplementation resulted in a significant increase in the proportion of n-3 PUFAs incorporated into myocardial membrane phospholipids. Basal membrane fluidity of cardiomyocyte membranes was not affected by diet. Similarly, under un-challenged conditions, diastolic and systolic $[Ca^{2+}]_i$ were not significantly different between cells isolated from rats fed FO versus saturated fat. However, following ROS addition, both diastolic and systolic $[Ca^{2+}]_i$ were significantly lowered in the FO group and the rate of rise in intracellular Ca^{2+} during contractions was lowered. There was no protective effect of dietary fish oil on reoxygenation injury in cardiomyocytes or reperfusion injury in isolated hearts. The mRNA levels of manganese-containing superoxide dismutase were significantly increased in the myocardium of rats fed FO compared with saturated fat and there was also a trend towards up-regulation of glutathione peroxidase and catalase. These results demonstrate the pleiotropic nature of the actions of fish oil fatty acids and indicate that for protection from reperfusion injury, incorporation of the fatty acids into the membrane phospholipids is required and this is associated with the enhancement of the antioxidant system and attenuation of the rise in intracellular Ca^{2+} during ROS exposure.

Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution associated with the work; and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited into the University Library, being made available for loan and photocopying.

Anisa Jahangiri
(September 2002)

"All our knowledge-- past, present, and future-- is nothing compared to what we will never know."

Konstantin Tsiolkovsky (1857-1935)

Acknowledgements

Publications and Abstracts Arising from this Thesis

#**Jahangiri, A.**, Leifert, W.R. and McMurchie, E.J. (2002) Omega-3 Polyunsaturated Fatty Acids: Recent Aspects in Relation to Health Benefits. *Food Australia* 54(3):74-77.

Jahangiri, A., Leifert, W.R. and McMurchie, E.J. (2002). The effect of dietary fish oil on antioxidant enzyme gene expression and intracellular calcium dynamics. *Proceedings of the Australian Society for Medical Research #O9.*

Jahangiri, A., Leifert, W.R. and McMurchie, E.J. (2002). Dietary fish oil reduces free radical damage in isolated cardiomyocytes. *Journal of Molecular and Cellular Cardiology* (in press).

#**Jahangiri, A.**, Kind, K.L., Leifert, W.R. and McMurchie, E.J. (2002) Enhancement of myocardial antioxidant capacity by dietary fish oil (To be submitted).

#**Jahangiri, A.**, Leifert, W.R. and McMurchie, E.J. (2002) Dietary fish oil supplementation reduces the severity of free radical damage in isolated cardiomyocytes (To be submitted).

Jahangiri, A., Leifert, W.R. and McMurchie, E.J. (2001). Membrane incorporation of dietary n-3 PUFAs protects cardiomyocytes from free radical damage. *Journal of Molecular and Cellular Cardiology* 33(6): A51.

#**Jahangiri, A.**, Leifert, W.R. Patten, G.S. and McMurchie, E.J. (2000) Termination of asynchronous contractile activity in rat atrial myocytes by n-3 polyunsaturated fatty acids. *Molecular and Cellular Biochemistry* 206: 33-41.

#Leifert, W.R., **Jahangiri, A.**, Saint, D.A and McMurchie, E.J. (2000) Effects of dietary n-3 fatty acids on contractility, Na^+ and K^+ currents in a rat cardiomyocyte model of arrhythmia. *Journal of Nutritional Biochemistry* 11(7-8): 382-392.

Jahangiri, A., Leifert, W.R. and McMurchie, E.J. (2000). Dietary fish oil protection from reperfusion injury. *Proceedings of the Nutrition Society of Australia* 24:99.

Jahangiri, A., Leifert, W.R. and McMurchie, E.J. (2000). Dietary n-3 PUFAs delay the development of free radical damage in rat cardiomyocytes. *Proceedings of the Australian Society for Medical Research #O11.*

#Leifert, W.R., **Jahangiri, A.**, and McMurchie, E.J. (1999) Antiarrhythmic fatty acids and antioxidants in animal and cell studies (Invited review). *Journal of Nutritional Biochemistry* 10: 252-267.

Jahangiri, A., Leifert, W.R. and McMurchie, E.J. (1999) Restoration of synchronous contractile activity by n-3 PUFAs in atrial cardiomyocytes. *Journal of Molecular and Cellular Cardiology* 31(1):A6.

Jahangiri, A., Leifert, W.R., Patten, G.S. and McMurchie, E.J. (1998) A Role for membrane fluidity in the prevention of asynchronous contractile activity by n-3 polyunsaturated fatty acids in rat atrial myocytes. *Proceedings of the Australian Society for Medical Research #24o.*

Full Paper

Academic Prizes and Travel Awards

Postgraduate All Round Achievement Prize, Department of Physiology, Adelaide University (2001).

Faculty of Health Sciences Postgraduate Travelling Fellowship, Adelaide University. To present at the World meeting of the International Society for Heart Research, Winnipeg, Canada (2001).

World body of the International Society for Heart Research (ISHR) Travel Award (2001).

Australasian ISHR Travel Award (2001).

Nutrition Trust of Australia Travel Grant (2001).

National Heart Foundation of Australia Travel Award (2001).

Walter and Dorothy Duncan Trust Award, Adelaide University (2000).

Nutrition Society of Australia Travel Award (2000).

Department of Physiology Postgraduate Publication Travel Award for publication in *Molecular and Cellular Biochemistry* (2000).

High Commendation Award for Student Presentation at the ISHR meeting, Australasian Section, Sydney (1998).

Conference Presentations

National

Australian Society for Medical Research (ASMR)- South Australian section, Adelaide, 30th May 2002. Oral presentation. "The effect of dietary fish oil on antioxidant enzyme gene expression and intracellular calcium dynamics".

Nutrition Society of Australia, Fremantle, Western Australia, 3rd - 6th December 2000. Oral Presentation. "Dietary fish oil protection from reperfusion injury".

International Society for Heart Research (ISHR) - Australasian section, Melbourne, Victoria, 5th - 7th August 2000. Poster presentation. "Dietary fish oil reduces free radical damage in isolated cardiomyocytes".

ASMR- South Australian section, Adelaide, 9th June 2000. Oral presentation. "Dietary n-3 PUFAs delay the development of free radical damage in rat cardiomyocytes".

ISHR- Australasian section, Sydney, NSW, 21st – 23rd August 1998. Poster presentation. "Restoration of synchronous contractile activity by n-3 PUFAs in atrial cardiomyocytes".

ASMR- South Australian section, Adelaide, 5th June 1998. Oral presentation. "A role for membrane fluidity in the prevention of asynchronous contractile activity by n-3 polyunsaturated fatty acids in rat atrial myocytes".

International

ISHR - World Congress Meeting, Winnipeg, Mannitoba, Canada, 6th – 11th July 2001. Poster presentation. "Membrane incorporation of dietary n-3 PUFAs protects cardiomyocytes from free radical damage".

Abbreviations

AA	arachidonic acid (20:4, n-6)
ALA / αLNA	alpha linolenic acid (18:3, n-3)
ANOVA	analysis of variance
BDM	2,3-butanedione monoxime
BSA	bovine serum albumin
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	intracellular calcium concentration
CAL	coronary artery ligation
CAT	catalase
CCD	charge coupled device
cDNA	complementary DNA
CHD	coronary heart disease
CM-DCFH	5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate
CM-DCF	ozidised CM-DCFH
CO	canola oil
CON	control (diet)
COX	cyclo-oxygenase
C_T	threshold cycle
CVD	cardiovascular disease
DCFH	2'7'-dichlorofluorescin diacetate
DCF	oxidized DCFH
DHA	docosahexaenoic acid (22:6, n-3)
DHE	dihydroethidium
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPA	docosapentaenoic acid (22:5, n-3)
EPA	eicosapentaenoic acid (20:5, n-3)
FAME	fatty acid methyl ester
FFA	free fatty acid
FO	fish oil
FRGS	free radical generating system
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GLC	gas liquid chromatography
GO	glucose oxidase
GPX	glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidised glutathione
GZFI-R	global zero-flow ischaemia-reperfusion
H_2O_2	hydrogen peroxide
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPLC	high pressure liquid chromatography
HR	heart rate
hr	hour
H-R	hypoxia-reoxygenation
IP_3	inositol (1,4,5)*tris*phosphate
I-R	ischaemia-reperfusion

LA	linoleic acid (18:2, n-6)
LDH	lactate dehydrogenase
mA	milli ampere
min	minute
MnSOD	manganese containing superoxide dismutase
mRNA	messenger RNA
MUFA	monounsaturated fatty acid
μM	micromoles per litre
n/d	not detectable
Na^+	sodium ion
$\cdot OH$	hydroxyl free radical
$O_2^{\cdot -}$	superoxide free radical
OO	olive oil
PCR	polymerase chain reaction
PGI_2	prostacyclin
PUFA	polyunsaturated fatty acid
REF	reference (diet)
RIAs	reperfusion-induced arrhythmias
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rRNA	ribosomal RNA
R_{ss}	steady-state fluorescence anisotropy
RT	reverse transcriptase
sec	second
SF	saturated fat
SFA	saturated fatty acid
SOD	superoxide dismutase
SR	sarcoplasmic reticulum
$T_{50\%}$	time taken until 50% of cells are asynchronously contracting
TBARS	thiobarbituric acid reactive substances
$Td_{50\%}$	the time to 50% decay of the systolic Ca^{2+} levels
T_m	melting temperature
TMAP-DPH	3-(diphenylhexatrienyl) propyltrimethylammonium *p*-toluene sulfonate
TXA_2	thromboxane A_2
TXA_3	thromboxane A_3
V	volt
VF	ventricular fibrillation
VT	ventricular tachycardia
XO	xanthine oxidase

CHAPTER ONE

Literature Review

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) continues to impose the largest burden on Australians in terms of morbidity and mortality, with the associated direct health care costs exceeding those for any other disease (AIHW, 2001b). CVD remains the leading cause of death in Australia, accounting for 40% of all deaths, with the single leading cardiovascular cause of death being coronary heart disease (CHD), accounting for 22% of all deaths in Australia (AIHW, 2001b). The major determinants of CHD risk are high blood pressure, smoking, obesity, diabetes mellitus, diet, age, gender, family history and physical inactivity (AIHW, 2001a). The underlying problem in CHD is atherosclerosis (AIHW, 2001b).

1.1.1 Atherosclerosis

Atherosclerosis starts with the accumulation of lipoprotein particles and their aggregates in the intima of the artery wall. This is likely to occur at sites where the endothelium is damaged. Monocytes/macrophages and T-lymphocytes from the blood circulation are attracted to these sites and localise subendothelially. The macrophages form into large foam cells due to lipid accumulation, and together with T-cells and smooth muscle, form a fatty streak. The fatty streak then progresses to a fibrofatty lesion and ultimately to a fibrous plaque. Plaques can become increasingly complex, with calcification, ulceration at the luminal surface, and haemorrhage from small blood vessels that grow into the lesion from the media of the blood vessel. Although advanced lesions can grow sufficiently large to block blood flow, the most important clinical complication is an acute occlusion (ischaemia) due to the formation of a thrombus or blood clot, resulting in myocardial

infarction or stroke (Ross, 1993; Lusis, 2000). Restoration of blood flow to the ischaemic region of the heart is termed reperfusion and clinical methods of reperfusion include acute by-pass surgery and lysis of occluding thrombi with fibrinolytic agents (Jennings & Reimer, 1983).

1.1.2 Reperfusion Injury

Although reperfusion of the ischaemic myocardium during the early stages is essential to prevent cardiac damage, reperfusion of the ischaemic heart after a certain period has been reported to have deleterious effects due to the generation of reactive oxygen species (ROS) (Dhalla *et al.*, 2000). In particular, contractile function and cardiac viability may become seriously compromised during the very early stages of reflow. Such dysfunctional changes are known as reperfusion injury and they encompass a spectrum of events including arrhythmias, myocardial stunning, microvascular damage and accelerated death of the more severely damaged cells (Opie, 1998). Reperfusion injury after a period of ischaemia is clinically relevant and can potentially occur under four clinical conditions (reviewed by Pierce & Czubryt (1995)). In the clinical setting, reperfusion occurring within seconds or minutes of ischaemia is associated with the relief of coronary artery spasm (Corr & Witkowski, 1983; Tzivoni *et al.*, 1983). The rhythmic consequence of reperfusion is the precipitation of serious reperfusion-induced arrhythmias, including ventricular tachycardia and ventricular fibrillation and may account for some cases of sudden cardiac death (Manning & Hearse, 1984). During aorto-coronary by-pass surgery, the heart is placed in low flow, ischaemic and hypothermic conditions for varying periods of time (minutes to hours (Manning & Hearse, 1984)) before normal blood flow is re-instituted to the reconstructed coronary vasculature (Pierce & Czubryt, 1995). This usually results in various forms of serious arrhythmias including ventricular fibrillation

(Manning & Hearse, 1984). Reperfusion after ischaemic intervals of hours or days occurs during balloon angioplasty of the coronary arteries which is performed to remove an atherosclerotic blockage enabling restoration of flow to a previously ischaemic region of the heart (Braunwald & Kloner, 1985). Thrombolytic therapy to dissolve a clot in the vessel will ultimately cause a reflow event (Braunwald & Kloner, 1985; Braunwald, 1985). Following angioplasty or thrombolytic therapy, certain forms of reperfusion arrhythmias such as those characterised by accelerated idioventricular rates and premature ventricular beats are often unwanted but useful indicators of successful flow restoration (Goldberg *et al.*, 1983).

1.1.3 Mechanisms of Reperfusion Injury

There are two main theories explaining the underlying basis of reperfusion injury. The calcium hypothesis proposes that ischaemia induces a defect in the ability of the cell to regulate intracellular levels of calcium such that upon reperfusion, the cell accumulates toxic levels of calcium. The second theory involves a role for free radicals and reactive oxygen species (ROS). This latter theory is based on the premise that partially reduced forms of molecular oxygen such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$) and the hydroxyl radical ($\bullet OH$) are produced at the time of reperfusion (Hess & Manson, 1984). Granger and colleagues proposed that during ischaemia, the breakdown of high-energy phosphate compounds results in the accumulation of the purine metabolites, hypoxanthine and xanthine (Figure 1.1). As the energy charge drops via ATP depletion, the cell no longer maintains normal ion gradients across various membranes and within intracellular compartments, resulting in a redistribution of calcium ions. The elevated intracellular calcium is believed to activate a protease capable of converting xanthine dehydrogenase to xanthine oxidase. At reperfusion, the sudden re-introduction of oxygen permits the

xanthine oxidase catalysed oxidation of hypoxanthine with the simultaneous reduction of oxygen to $O_2^{\bullet-}$ (Granger *et al.*, 1981; McCord, 1985). $O_2^{\bullet-}$ has an extremely short half life (Obata *et al.*, 1997) and rapidly undergoes dismutation yielding H_2O_2. The H_2O_2 can then form •OH via the Haber Weiss reaction with $O_2^{\bullet-}$, or by reaction with intracellular Fe^{2+} through the Fenton reaction (Halliwell & Gutteridge, 1990).

ATP

AMP

ISCHAEMIA

Adenosine

Xanthine dehydrogenase

Inosine Ca^{2+} protease

Xanthine oxidase

Hypoxanthine ⟶ $O_2^{\bullet-}$

O_2
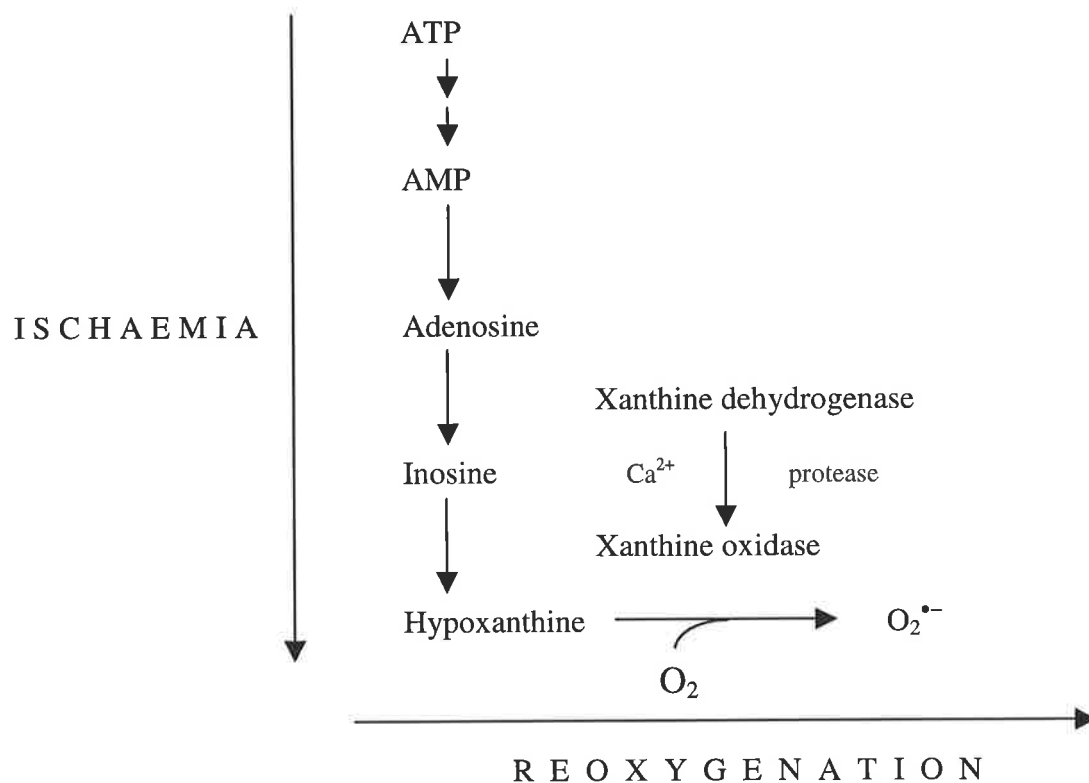
REOXYGENATION

FIGURE 1.1 Cellular mechanisms for reactive oxygen species generation during ischaemia-reperfusion (McCord, 1985).

1.1.4 Cellular Effects of ROS

Overproduction of oxygen-derived free radicals, occurring for example at reperfusion, may overload the cell's natural scavenging mechanisms, causing cellular damage (McCord, 1985). Indeed, a major feature of ROS-induced cellular injury is lipid peroxidation due to the effect of ROS on polyunsaturated fatty acids. As a result, the membrane lipid bilayer arrangement is disrupted which in turn affects its functional properties. Oxidation of membrane and other cellular proteins results in inactivation, and

hence, loss of their activity (Dhalla *et al.*, 2000). For example, in the heart, this may compromise membrane ion pump activity and promote local electrophysiological derangement(s) sufficient to trigger ventricular arrhythmias (Bernier *et al.*, 1986). In particular, it was suggested that reperfusion arrhythmias may occur as a result of sarcoplasmic reticular dysfunction due to excessive amounts of free radicals generated during reperfusion, leading to Ca^{2+} overload (Hearse, 1991). ROS have been reported to stimulate Ca^{2+} overload in cardiomyocytes through mechanisms involving inhibition of the sarcolemmal Na^+/K^+-ATPase (Kaminishi *et al.*, 1989), depression of the sarcolemmal Ca^{2+} extrusion pump (Kaneko *et al.*, 1989a) and inhibition of the ATP-dependent Ca^{2+} uptake process by the sarcoplasmic reticulum (Morris & Sulakhe, 1997). H_2O_2 has been shown to increase the amplitude of Ca^{2+} transients (Ward & Moffat, 1995), and activate L-type calcium channels in guinea pig myocytes (Thomas *et al.*, 1998). It therefore seems likely that $[Ca^{2+}]_i$ overload may be an important factor underlying ROS-induced arrhythmias. However, it is still debatable whether ROS damage to the sarcolemmal membrane and the resulting intracellular Ca^{2+} overload induces arrhythmias or whether free radicals result from high concentrations of cellular Ca^{2+}. Certainly hydroxyl radical generation has been demonstrated in response to Ca^{2+} overload after addition of ouabain (Obata *et al.*, 1997). However, most of the evidence supports the reverse situation. For example, in neonatal rat ventricular myocytes, the development of arrhythmic (asynchronous) contractile activity has been reported when intracellular Ca^{2+} is increased from nM to μM levels as a result of exposure to ROS (Burton *et al.*, 1990). In another study, in myocytes exposed to FRGS, it was shown that $[Ca^{2+}]_i$ overload, largely due to Ca^{2+} influx via voltage-gated Ca^{2+} channels was associated with $[Ca^{2+}]_i$ oscillations and after-contractions (Josephson *et al.*, 1991). These results are consistent with the view that oxidative stress may result in the occurrence of intracellular Ca^{2+} overload and subsequent

dysrhythmias, myocardial cell damage and cardiac dysfunction due to ischaemia-reperfusion injury (Dhalla *et al.*, 2000).

1.2 Free Radical Biology

A free radical is any species capable of independent existence that contains one or more unpaired electrons (Halliwell, 1987). Thus the ground state diatomic oxygen molecule (O_2) is itself a radical, containing two unpaired electrons with parallel electron spins (Hess & Manson, 1984). However ordinary molecular oxygen is relatively non-reactive (Hess & Manson, 1984). One way to increase the reactivity of oxygen is to move one of the unpaired electrons in a way that alleviates the spin restriction. This requires an input of energy and generates the singlet states of oxygen (Halliwell & Gutteridge, 1990). Singlet molecular oxygen ($^1O_2$) is highly reactive, can damage lipids and constituents of biological membranes and can lead to inactivation of many enzymes, DNA damage and oxidation of mitochondrial components (Kukreja & Hess, 1992). One electron reduction of oxygen produces the superoxide radical, $O_2^{\bullet-}$ (Halliwell & Gutteridge, 1990).

$$O_2 + e^- \longrightarrow O_2^{\bullet-} \quad \text{(Halliwell, 1991)}$$
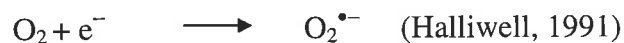
$O_2^{\bullet-}$ is formed in almost all aerobic cells, a major source being "leakage" of electrons onto O_2 from various components of the cellular electron transport chains such as those of mitochondria, chloroplasts and the endoplasmic reticulum (Halliwell & Gutteridge, 1990). $O_2^{\bullet-}$ is also produced during the respiratory burst of phagocytic cells, eg neutrophils, monocytes, macrophages and eosinophils (Halliwell & Gutteridge, 1986; Halliwell & Gutteridge, 1990; Halliwell, 1991). Under normal conditions, 3-5% of the oxygen taken up by the cell undergoes univalent reduction leading to the formation of free radicals (Singal *et al.*, 1998). The charged $O_2^{\bullet-}$ molecule cannot readily cross biological membranes and enter cells unless there is an anion channel through which it

can move (Freeman & Crapo, 1982). Superoxide dismutase (SOD) removes $O_2^{\bullet-}$ by catalysing a dismutation reaction involving oxidation of one $O_2^{\bullet-}$ to oxygen and reduction of another to hydrogen peroxide (H_2O_2).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2^{\bullet-} \text{ (Halliwell, 1991)}$$

However, H_2O_2 itself can be quite toxic to cells. Incubation of cells with H_2O_2 has been demonstrated to cause DNA damage, membrane disruption and release of Ca^{2+} ions within the cells, leading to activation of Ca^{2+} - dependent proteases and nucleases (Halliwell, 1991). At least some of this damage may be mediated by a reaction of H_2O_2 with $O_2^{\bullet-}$ in the presence of iron or copper ions, to form highly reactive radicals, one of which is the hydroxyl radical, ($\bullet$OH). The hydroxyl radical can be produced via the Haber-Weiss reaction, which is summarised as:

$$O_2^{\bullet-} + H_2O_2 \xrightarrow{\text{Fe/Cu}} OH^- + O_2 \text{ (Halliwell & Gutteridge, 1986)}$$

or by the Fenton reaction:

$$Fe^{2+} + H_2O_2 \longrightarrow \bullet OH + OH^- + Fe^{3+} \text{ (Halliwell & Gutteridge, 1990)}$$
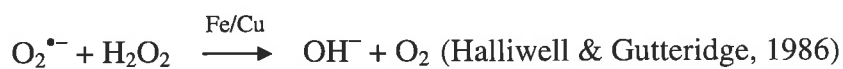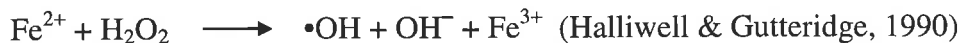
H_2O_2 has no unpaired electrons and is not a radical. Pure H_2O_2 has limited reactivity but can cross biological membranes. H_2O_2 can be removed by the action of catalase:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

or by glutathione peroxidase which removes H_2O_2 by using it to oxidise reduced glutathione (GSH) into oxidised glutathione (GSSG):

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O \text{ (Halliwell, 1991).}$$

The term reactive oxygen species (ROS) encompasses free radicals such as $O_2^{\bullet-}$ and $\bullet$OH as well as non-radical, but highly reactive oxygen species such as H_2O_2.

1.3 Reperfusion Injury and ROS

As discussed above, although the pathophysiological mechanisms responsible for reperfusion injury are not yet resolved, an attractive hypothesis is that this phenomenon is due to the rapid formation of ROS upon reperfusion. Because these species are extremely reactive and short lived, the majority of evidence of their involvement in reperfusion injury is indirect.

1.3.1 Human Studies

In patients undergoing open-heart surgery, vitamin E levels were reduced following the reperfusion procedure, indirectly indicating an oxidative stress on the myocardium (Barsacchi *et al.*, 1992). Another indirect account of oxidative stress in humans was reported by Mallat *et al.* (1998) showing that ventricular dilatation and symptomatic heart failure are associated with an increase in pericardial fluid levels of 8-iso-PGF$_{2\alpha}$, a free radical catalysed product of the oxidative modification of arachidonic acid. Similarly, the percentage molar ratio of the concentrations of 9,11-linolenic acid to 9,12-linolenic acid (the former being the diene conjugate derived by free radical interaction from its naturally occurring isomer 9,12-linolenic acid) were increased following recanalization of the coronary artery, indicating elevated free radical activity (Grech *et al.*, 1994). Furthermore, patients treated with an extract of *Ginkgo biloba* (with known antiradical activity) for a few days before undergoing coronary artery bypass surgery showed a significant improvement in indices of oxidative stress occurring in the systemic circulation during cardiopulmonary bypass (Pietri *et al.*, 1997). Collectively, these studies support the hypothesis of the generation of ROS in the clinical setting in humans.

1.3.2 Animal Studies

A commonly used model to study ischaemia-reperfusion in experimental animals is a working heart perfusion set up, perfusing with a physiological buffer such as Krebs-Henseleit. The left atrium is cannulated and the heart is subjected to 10 min coronary artery ligation (CAL) and 10 min reperfusion. This results in the development of reperfusion injury, commonly manifested as ventricular tachycardia (VT) and fibrillation (VF). A technique employing ascorbyl free radicals (AFR), detected by electron spin resonance spectroscopy, demonstrated an increase in the level of AFR following ischaemia and reperfusion in isolated rat hearts (Vergely *et al.*, 1998). A slow constant release of AFR occurred during low flow ischaemia, however upon reperfusion, there was a sudden and large burst of AFR liberation, which was further enhanced if the duration of ischaemia was increased from 20 min to 60 min. The increase in free radical generation was also associated with impairment of myocardial function. Using HPLC techniques, •OH was detected at reperfusion following 60 min global ischaemia in the isolated rat heart. The formation of •OH was associated with the development of ventricular fibrillation (Tosaki *et al.*, 1993). Reperfusion after 30 min of no-flow ischaemia in guinea-pig right ventricular walls was associated with premature action potentials and the development of tachycardias (Aiello *et al.*, 1995). Pre-treatment with SOD, CAT and mannitol (a free radical scavenger) for 20 min prior to ischaemia reduced the incidence of tachycardias and cell membrane damage, indicating that a combination of H_2O_2, $O_2^{\bullet-}$ and •OH are involved in the myocardial damage following ischaemia and reperfusion. In cultured neonatal ventricular myocytes undergoing ischaemia and reoxygenation, LDH release was increased with increasing hypoxic time (Qian *et al.*, 1997). The administration of SOD attenuated LDH release and the decrease in membrane fluidity induced by ischaemia and reperfusion. These results strongly support the role of free

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radicals in reperfusion injury and further suggest that free radical production at the time of reperfusion depends on the duration and extent of the preceding ischaemia.

1.4 Free-radical Scavengers and Antioxidants

Cellular mechanisms exist to counteract the effects of free radicals and these encompass several antioxidative enzymes and other cellular defense mechanisms. They include endogenous enzymes (including superoxide dismutase, catalase and glutathione peroxidase, endogenous factors (including glutathione, urate and coenzyme Q), and nutritional factors (principally the antioxidant nutrients, especially β-carotene and other carotenoids, vitamin C, vitamin E and selenium) (Temple, 2000). These antioxidants are necessary to prevent the formation of free-radicals and to inhibit some of the deleterious actions of reactive oxygen and nitrogen species which cause damage to DNA, lipids and proteins (Halliwell, 1996).

1.4.1 Superoxide Dismutase

The superoxide dismutases are thought to be the first line of antioxidant defence from oxygen toxicity. They exist as a family of three metaloproteins with copper and zinc (Cu/Zn-SOD), manganese (Mn-SOD) and iron (Fe-SOD) forms. The two intracellular subtypes of superoxide dismutase in mammalian cells are a cytosolic Cu/Zn-SOD and a mitochondrial Mn-SOD. These two enzymes catalyse the same reaction, namely the dismutation of two superoxide radicals ($O_2^{\bullet-}$) to yield hydrogen peroxide (H_2O_2) and oxygen. The resulting H_2O_2 is further scavenged by CAT and GPX (Ho *et al.*, 1991). Mn-SOD is a critical antioxidant enzyme in aerobic organisms because $O_2^{\bullet-}$ is mainly generated on the matrix side of the inner mitochondrial membrane. Thus, it is conceivable that increases in Mn-SOD activity may provide increased protection against oxidative

stress. Indeed, overexpression of mitochondrial Mn-SOD confers significant protection against ischaemia-reperfusion injury in Langendorff-perfused heart preparations (Chen *et al.*, 1998). Increased Mn-SOD mRNA expression was also associated with protection of cardiomyocytes from hypoxia-reoxygenation induced oxidative stress (Negoro *et al.*, 2001). Conversely, in heterozygous Mn-SOD knockout mice, Mn-SOD activity was decreased, and this was associated with higher mitochondrial $O_2^{\bullet-}$ and carbonyl content, concomitant with decreased glutathione levels and compromised mitochondrial function (Williams *et al.*, 1998). Transgenic mice overexpressing Cu/Zn-SOD in their coronary vascular cells were found to be more resistant to myocardial ischaemia-reperfusion injury (Chen *et al.*, 2000), and cardiac myocytes isolated from mice harbouring the human Cu/Zn-SOD transgene were also protected from the deleterious effects of prolonged hypoxia (Karliner *et al.*, 2000).

1.4.2 Glutathione Peroxidase

Glutathione peroxidase (GPX) also plays an important role as a cellular antioxidant defence mechanism by reducing H_2O_2 and various hydroperoxides using glutathione as a reducing agent to form water and the corresponding alcohols, respectively (Ho *et al.*, 1997). GPX is widely distributed in many tissues and organs such as liver, kidney, heart and red blood cells (Yoshimura *et al.*, 1988) and is present in both the cytosol and mitochondria (Ho & Howard, 1992). Transgenic mice overexpressing GPX exhibited a decreased content of peroxide in the brain following heat shock (Mirochnitchenko *et al.*, 1995) as well as an increased tolerance to myocardial injury induced by ischaemia-reperfusion (Yoshida *et al.*, 1996). It was also demonstrated that older (6-12 month) rat hearts were more susceptible to H_2O_2 induced arrhythmias compared to 3 month old

animals, and this was associated with lower myocardial levels of GPX and Mn-SOD (Abete *et al.*, 1999).

1.4.3 Catalase

Mammalian catalase (CAT) is found in virtually all aerobic cells, with highest levels in the liver, kidney and erythrocytes (Nakashima *et al.*, 1989). This enzyme catalyses the decomposition of H_2O_2 to oxygen and water (Nakashima *et al.*, 1989). Although low levels of CAT activity is present at in the heart, it is important in the detoxification of H_2O_2 in the myocardium (Li *et al.*, 1997). Overexpression of CAT in the heart was demonstrated to not only improve the recovery of the lessened contractile force after ischaemia-reperfusion, but also to reduce creatinine kinase release from the isolated Langendorff-perfused heart, and attenuate the concomitant myocardial infarction (Li *et al.*, 1997). Prior heat stress (20 min at 42°C) reduced the incidence and duration of ventricular tachycardia and/or fibrillation following 5 min occlusion and this was associated with an increase in CAT activity and expression of heat shock proteins 72 and 27 (Joyeux *et al.*, 1997).

1.4.4 Vitamin E

During extreme oxidative stress, the endogenous antioxidant system may be insufficient to scavenge all free radicals produced and consequently, diet-derived antioxidants are likely to play an important defensive role. Alpha-tocopherol (α-tocopherol), the major constituent of the fat-soluble vitamin E, is the most important chain-breaking antioxidant present within membranes and lipoproteins (Halliwell, 1996). This antioxidant inhibits lipid peroxidation by scavenging peroxyl radicals generated from polyunsaturated fatty acids in membrane phospholipids (Sies *et al.*, 1992). The resulting α-tocopherol radical

(although not completely unreactive) is much less reactive than the peroxyl radical, and therefore acts as a chain-breaking antioxidant (Halliwell, 1996). An increased requirement for vitamin E has been suggested to counteract the pro-oxidant effect of high PUFA consumption (Ibrahim *et al.*, 1999). Indeed, the inclusion of vitamin E with fish oil has been shown to be beneficial in reducing the extent of oxidative stress observed in various rat tissues (Suarez *et al.*, 1999; Ando *et al.*, 2000). Vitamin C is a potent antioxidant in extracellular fluids and efficiently scavenges $O_2^{\bullet-}$, H_2O_2, $\bullet OH$, hypochlorite ions, peroxyl radicals and singlet oxygen (Sies *et al.*, 1992). It may also facilitate the regeneration of α-tocopherol from the radical form (Halliwell, 1996).

1.4.5 Epidemiological Studies- Cardiovascular Disease and Antioxidants

Many studies have demonstrated an association between dietary and supplemental intake of antioxidant vitamins and decreased mortality and morbidity from CHD (Diaz *et al.*, 1997; van de Vijver *et al.*, 1997). Vitamin C, carotenoids and vitamin E, the three main dietary sources of antioxidants can each influence lipid peroxidation and may decrease atherosclerosis, thereby lowering the risk of CHD (Rimm & Stampfer, 1997). The evidence for a cardiovascular benefit of antioxidants is strongest for vitamin E (Rimm *et al.*, 1993; Stampfer *et al.*, 1993). Vitamins E and C supplemented together also lower the risk of total mortality in the elderly (Losonczy *et al.*, 1996). Clinical trials of β-carotene supplementation however, did not report any cardiovascular benefit (Rapola *et al.*, 1996). A cardioprotective role for the flavonoids was reported in a study from the Netherlands (Hertog *et al.*, 1993) in which an intake of 26 mg flavonoids/day was inversely associated with mortality from CHD. Numerous epidemiological, clinical and experimental studies have also confirmed an association between the consumption of fish oil (high in polyunsaturated fatty acids) and mortality from coronary heart disease (reviewed in

Leifert *et al.* (1999a)). Dietary n-3 polyunsaturated fatty acids act to prevent heart disease through a variety of actions. They prevent arrhythmias, have antiinflammatory properties, inhibit synthesis of cytokines and mitogens, stimulate endothelial-derived nitric oxide synthesis, are antithrombotic, have hypolipidemic properties with effects on triacylglycerols and very low density lipoproteins, and inhibit atherosclerosis (Connor, 2000).

1.5 Fatty Acid Metabolic Pathways

Fatty acids are typically classified into saturated, monounsaturated and polyunsaturated fatty acids (PUFAs).
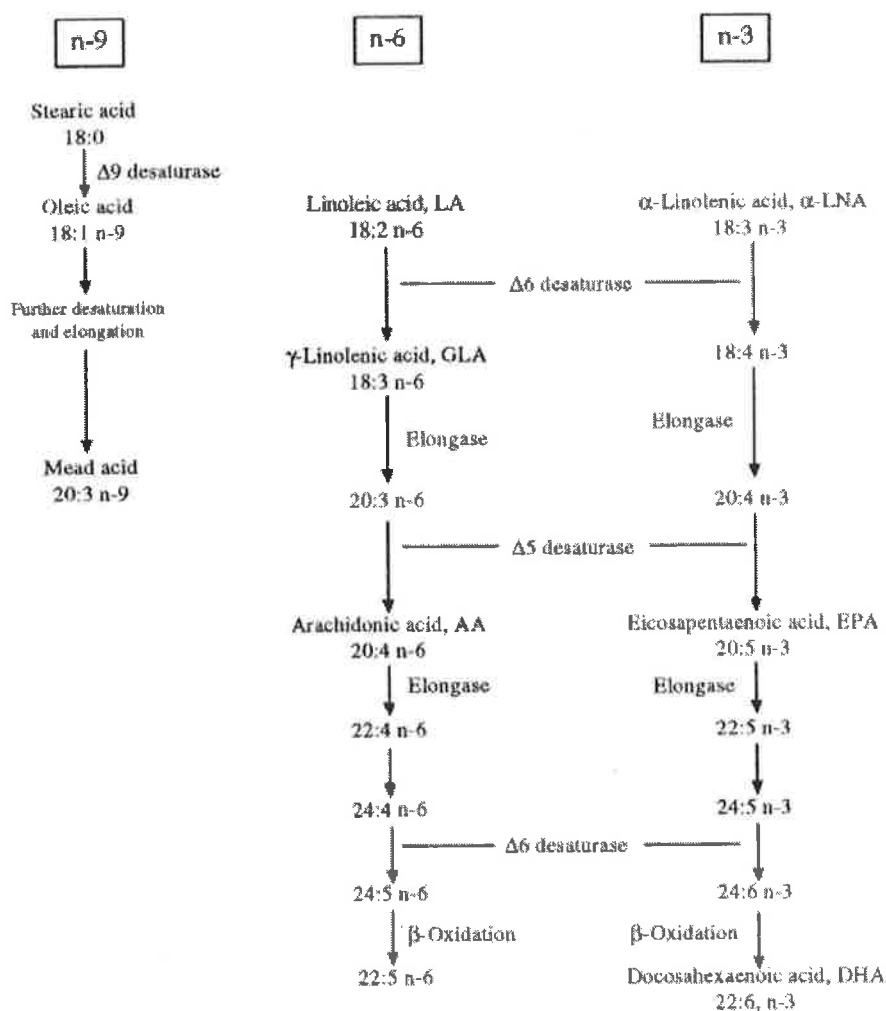
Figure 1.2: Fatty acid synthetic pathways (Leifert *et al.*, 1999a).

Animal fats are the major dietary source of saturated fatty acids and include palmitic and stearic acids. Oleic acid is a monounsaturated fatty acid found in most of the common edible oils such as olive oil. Mammals are able to synthesise all fatty acids *de novo* except the "essential" parent fatty acids, linoleic acid (LA, 18:2, n-6) and α-linolenic acid (αLNA, 18:3, n-3). These fatty acids are categorised as essential since humans lack the enzymes necessary to insert double bonds between the terminal methyl carbon and the 9th carbon atom. LA and αLNA must therefore be obtained from the diet. Linoleic acid is found in plant seed oils such as sunflower, safflower and cottonseed oil, and αLNA is found in canola, soybean and linseed oils. Dietary αLNA can be elongated and further desaturated to the longer chain n-3 PUFAs docosahexaenoic acid (DHA, 22:6, n-3) and eicosapentaenoic acid (EPA, 20:5, n-3) (Figure 1.2). Additionally, marine phytoplankton and zooplankton are rich sources of EPA and DHA. Since plankton is the ultimate food source for all marine fish, these fatty acids are found in high concentrations in the body fat of fish (Lees, 1990). The n-6 PUFAs have long been known to be essential dietary constituents for man. Specifically, arachidonic acid (AA) is essential for numerous physiological processes. AA can either be consumed directly in the diet or formed within the body from LA. The conversion of LA to AA competes with the conversion of ALA to EPA and then to DHA. Hence, a diet high in LA, but poor in ALA generates a relative excess of AA in the body and a relative deficiency of EPA and DHA. The excess AA generates increased levels of a series of highly active metabolites of AA, collectively termed 'eicosanoids', which are involved in a range of 'stress-related' disorders including cardiovascular and inflammatory conditions (Sargent, 1997).

1.5.1 Fatty Acids and Eicosanoids

During platelet activation, AA is released from the membrane phospholipids. It can then be metabolised via the lipoxygenase pathway to form the 4-series leukotrienes, or via the cyclooxygenase pathway to form eicosanoids of the 2-series, most importantly PGI_2 (prostacyclin) and TXA_2 (thromboxane A_2) (Lee *et al.*, 1985). TXA_2 has potent vasoconstrictive and platelet aggregatory effects, while PGI_2 is vasodilatory and anti-aggregatory (Leaf & Weber, 1988) and prevents adherence of platelets and leucocytes to blood vessel walls (Kinsella *et al.*, 1990). The balanced production of TXA_2 and PGI_2 is crucial in controlling the interactions between platelets and the endothelium, and with regard to haemodynamics and maintenance of coronary vascular tone (Kinsella *et al.*, 1990). Dietary EPA and DHA compete with AA by inhibiting its biosynthesis from linoleic acid, as well as competing for incorporation at the *sn*-2 position of membrane phospholipids, with the net result being decreased AA levels in plasma and cells. EPA competes with AA as a substrate for the cyclooxygenase enzyme, inhibiting the production of TXA_2 by platelets and promoting the production of TXA_3, the latter compound having negligible platelet aggregating ability. While EPA does not inhibit PGI_2 formation in endothelial cells, it does enhance the actions of PGI_2 by producing PGI_3 which is also vasodilatory and antiaggregatory (Leaf & Weber, 1988). These changes in eicosanoid synthesis result in a shift toward vasodilation and less tendency for platelet aggregation.

1.5.2 Epidemiological Studies

Interest in the cardioprotective effects of the n-3 PUFAs was first generated following epidemiological studies of Greenland Eskimos (Bang *et al.*, 1971; Bang & Dyerberg, 1972; Bang *et al.*, 1980). Eskimos with diets based on seal and whale had a lower intake

of saturated fats and higher intakes of monounsaturated and polyunsaturated fatty acids than their Danish counterparts (Bang *et al.*, 1976). It was also reported that this population had significantly lower levels of total cholesterol, triglycerides, low-density lipoproteins and increased levels of high-density lipoprotein cholesterol when compared with a Danish study population (Bang & Dyerberg, 1972). Importantly, the incidence of heart disease was found to be low in Greenland Eskimos (Bang & Dyerberg, 1972; Kromann & Green, 1980). Similarly, in Zutphen, a Dutch area included in the Seven Countries Study, an inverse correlation between fish consumption and coronary artery disease was reported in middle-aged men during 20 years of follow-up (Kromhout *et al.*, 1985). An association between consumption of sea-food and cardiovascular disease was strengthened by the findings of high proportions of long chained fatty acids in the plasma of the Greenland Eskimos. In particular, EPA was one of the major components of the esterified fatty acids in plasma, comprising 16% of the total (Dyerberg *et al.*, 1975), probably due to the high content of n-3 PUFAs present in the marine diet that they consumed (Bang *et al.*, 1976). Several intervention trials have since been carried out to confirm the beneficial effects of fish oil and n-3 PUFAs. The results of the Diet and Reinfarction Trial (DART), strongly support the role of fish and/or fish oil in decreasing total mortality and sudden death in patients with 1 episode of myocardial infarction (Burr *et al.*, 1989). Consumption of at least two 200-400g fatty fish portions per week was found to reduce mortality by 29% in men during the first two years after a myocardial infarct (Burr *et al.*, 1989). The Multiple Risk Factor Intervention Trial found an inverse association between αLNA and mortality from CHD, all CVD and all cause mortality (Dolecek, 1992). Similarly, in the Lyon Diet Heart Study, it was demonstrated that post infarct patients assigned to a Mediterranean αLNA-rich diet had a significant reduction in the rate of recurrence of cardiac events and overall mortality (de Lorgeril *et al.*, 1994).

Compared to no intake of dietary EPA and DHA, one fatty fish meal per week was associated with a 50% reduction in the risk of primary cardiac arrest, suggesting an antiarrhythmic effect from the ingestion of fish (Siscovick *et al.*, 1995). In a prospective, placebo-controlled, double-blind study of patients with spontaneous ventricular premature complexes (VPC's), supplementation with 2.4 g/day n-3 PUFAs for 16 weeks, raised EPA and DHA levels. The proportion of patients with a 70% reduction in VPC's was 44% in the fish oil group compared with 15% in the control group (Sellmayer *et al.*, 1995). A randomized, placebo-controlled trial of 1 year of treatment with fish oil (eicosapentaenoic acid, 1.08 g/day) and mustard oil (alpha-linolenic acid, 2.9 g/day) reported decreased total cardiac events, nonfatal infarctions, total cardiac arrhythmias, left ventricular enlargement, and angina pectoris in both treatment groups compared with the placebo group. The level of diene conjugated material was also significantly reduced in the fish oil and mustard oil groups, indicating that part of the benefit may be caused by the reduction in oxidative stress (Singh *et al.*, 1997). The GISSI-Prevenzione investigators studying post-infarction subjects, found that fish oil intake reduced the risk of both sudden and non sudden cardiac death (Valagussa *et al.*, 1999) and a recent study in Italy, demonstrated that n-3 PUFA and fish consumption are inversely associated with nonfatal acute myocardial infarction (Tavani *et al.*, 2001). These and numerous animal experimental studies provide compelling evidence for the cardioprotective actions of fish oil and the n-3 PUFAs. This evidence is such that the American Heart Association's dietary guidelines now include the recommendation for the consumption of at least 2 servings of fish per week (Lauber & Sheard, 2001).

1.5.3 Fish sources of n-3 PUFAs

Fish such as herring, mackerel, capelin, sardines, pilchards and anchovies, which are high in n-3 PUFAs, chiefly store their oil reserves in the fillet. In contrast, the oil reserves of fish such as cod, haddock and whiting are stored in the livers and not in the flesh. Therefore, cod fillets are not a rich source of oil rich in EPA and DHA, whereas cod liver is a rich source of these fatty acids, as are the fillets of herring, mackerel, sardines, etc (Sargent, 1997). In general, it is the cold-water fish that are high in n-3 PUFAs. When the temperature of the water decreases, as in the Polar Regions, the degree of unsaturation of fatty acids in the tissues of fish is increased to compensate for the reduction in fluidity of membranes (Uauy-Dagach & Valenzuela, 1996).

1.5.4 Cardiovascular effects of n-3 PUFAs

Different animal models of cardiac arrhythmia have been used for studies on the relationship between dietary lipids, cardiac membrane lipid composition, myocardial function and the biochemical mechanisms underlying antiarrhythmic effects in relation to nutritional components. A number of studies have investigated the protection afforded by various nutritionally-derived or related agents on arrhythmias induced in the isolated or ligated heart model using both dietary and acute addition of these putative antiarrhythmic compounds. These nutritional components range from the n-3 PUFAs through to polyphenolic compounds.

1.5.4.1 Animal Studies

McLennan and co-workers demonstrated that in long-term feeding studies with rats, diets supplemented with fish oils (high in n-3 PUFAs) significantly reduced the incidence and severity of arrhythmias, following both occlusion and reperfusion of the coronary arteries

in comparison to animals that consumed of vegetable oils or saturated fat (McLennan *et al.*, 1988; McLennan, 1993). Furthermore, in older rats maintained on a saturated fat diet for 9 months, fish oil feeding for a further 9 months reduced the severity of arrhythmias following occlusion and reperfusion of the coronary artery (McLennan *et al.*, 1990). In the marmoset monkey, an n-3 rich diet was also found to increase the threshold current necessary to induce VF (McLennan *et al.*, 1992). This antiarrhythmic effect of fish oil fatty acids was associated with increased amounts of EPA and especially DHA incorporation into the myocardial membrane phospholipids (McLennan *et al.*, 1993). In experiments with conscious dogs, an intravenous infusion of an emulsion comprised mainly of EPA and DHA in the free fatty acid form prevented ischaemia-induced VF. The infusion, which occurred 60 min prior to the ischaemic insult, prevented the arrhythmic episode (Billman *et al.*, 1994). Thus the authors concluded that the effect was independent of the need for the PUFAs to be incorporated into cardiac membrane phospholipids owing to the insufficient time for significant membrane incorporation to occur. These experiments therefore conflict somewhat with the results described by McLennan *et al.* in which extensive n-3 PUFA incorporation into membranes was associated with their antiarrhythmic actions. However, these disparate findings can be reconciled by the fact that n-3 PUFAs incorporated into cardiac membrane phospholipids during fish oil feeding could be liberated by phospholipase action during an arrhythmogenic insult (such as ischaemia) to provide sufficient free PUFA to prevent or terminate an arrhythmia (Nair *et al.*, 1997).

1.5.4.2 Cellular Studies

In neonatal rat ventricular myocytes, oleic, stearic, myristic, palmitic and lauric acids have no significant antiarrhythmic effects, while the n-3 (DHA, EPA and α-linolenic

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acid) and n-6 (linoleic and arachidonic acid) PUFAs have been shown to be antiarrhythmic. If the cells are superfused with the above-mentioned n-3 fatty acids prior to introduction of the arrhythmogenic stimulus, induction of arrhythmias is prevented (Kang & Leaf, 1995; Weylandt *et al.*, 1996; Kang & Leaf, 1996a). The effect of acutely added n-3 PUFAs occurs at low concentrations (1- 10 µM), however, the fatty acids are not antiarrhythmic when added in the triglyceride or ethyl ester form, but are active only as free fatty acids (FFA). However, in the dietary studies, animals consume n-3 PUFAs (EPA and DHA) in the triacylglycerol form (the usual form in which it is present in fish oil) yet antiarrhythmic effects are observed, as indicated by the animal feeding studies of McLennan and colleagues (McLennan *et al.*, 1988; McLennan *et al.*, 1993). The reason for this inconsistency may be explained by the fact that in the dietary studies, there is ample time for esterases in the gastrointestinal tract and in the plasma to liberate FFA (Leaf & Weber, 1988). Furthermore, as mentioned above, n-3 PUFAs incorporated into cardiac membrane phospholipids during fish oil feeding could be liberated by phospholipase action during an arrhythmogenic insult to provide sufficient free PUFA to prevent or terminate an arrhythmia (Nair *et al.*, 1997). Indeed, it has been reported that reperfusion *per se* results in a significant elevation of the myocardial non-esterified fatty acid content (van Bilsen *et al.*, 1989). Additionally, it was demonstrated that increasing the proportion of membrane phospholipids of neonatal rat ventricular myocytes with PUFAs (by culturing the cells in the presence of EPA or DHA in the medium) did not prevent the induction of arrhythmia upon stimulation with various arrhythmogens if the FFA was first removed from the cells with delipidated bovine serum albumin (BSA) (Weylandt *et al.*, 1996). BSA has high affinity binding sites for FFA and thus when cells are treated with BSA, it extracts the FFA partitioned into the phospholipids of the myocyte membranes, with the result that the only fatty acids present will be those

originally incorporated into the sarcolemmal membrane phospholipids. The investigators therefore concluded that fatty acids incorporated into the phospholipids of the cell membranes or bound covalently to membrane constituents are not directly antiarrhythmic. Were this to be the case, the arrhythmia would not recur when the FFA was removed, since it is only the FFA that is bound by the bovine serum albumin (Kang & Leaf, 1996b) and not the incorporated fatty acids. It therefore appears likely that in the superfusion experiments, the PUFAs infuse into the sarcolemmal membrane and intercalate within the phospholipids exerting an antiarrhythmic effect as FFA. In the dietary studies, there is sufficient time for incorporation into the membrane phospholipids, however, during an arrhythmogenic insult it is possible that phospholipase action releases the PUFAs from the membrane and they then exert their antiarrhythmic effects as FFAs.

1.6 n-3 PUFAs and Ischaemia-Reperfusion Injury

1.6.1 *In situ* and Isolated Heart Studies

Myocardial ischaemia results in the accumulation of lactate and a fall in intracellular pH (Woodcock *et al.*, 2001). Activation of the sodium-hydrogen exchanger probably represents the major mechanism for restoration of intracellular pH after ischaemia-induced acidosis. Although this activation is necessary for the restoration of normal pH, the exchange results in cellular Na^+ overload (Sack *et al.*, 1994) that can have serious deleterious effects on cellular function. This Na^+ accumulation may activate the sodium-calcium exchanger operating in the reverse mode (causing a sodium efflux and calcium influx), resulting in intracellular Ca^{2+} overload, which in turn leads to myocardial dysfunction and arrhythmogenesis (Sack *et al.*, 1994; Woodcock *et al.*, 2001). Noradrenaline release induced by ischaemic preconditioning concurrently results in inositol (1,4,5)*tris*phosphate (IP_3) release. IP_3 plays an important role in myocardial

arrhythmogenesis through its actions on releasing Ca^{2+} from intracellular stores (Woodcock *et al.*, 2001). Indeed, a 20 min period of ischaemia induced *in situ* by CAL followed by 2 min reperfusion, resulted in the generation of IP_3 and the development of arrhythmias, which were attenuated by inhibition of both the sodium-hydrogen exchanger (Harrison *et al.*, 2000) and the sodium-calcium exchanger operating in the reverse mode (with KB-R7943) (Woodcock *et al.*, 2001). Furthermore, an association was found between the inhibition of reperfusion-induced rises in intracellular IP_3 concentrations by dietary n-3 PUFAs and protection from reperfusion-induced arrhythmias (Anderson *et al.*, 1996) suggesting a possible role for n-3 PUFAs in modulating intracellular Ca^{2+} handling.

It was reported that following 15 min of ischaemia induced by *in situ* CAL, rats fed an olive oil supplemented diet (rich in monounsaturated fatty acids) for 12 weeks exhibited a higher incidence of ventricular fibrillation (VF) in comparison to rats fed a canola oil (containing the n-3 PUFA αLNA) enriched diet where no VF events and a lower arrhythmia score were recorded. If the duration of ischaemia was shortened to 5 min, the canola oil fed animals again exhibited a lower arrhythmia score, a tendency to fewer VF events and no fatal VF in comparison to olive oil fed animals (McLennan & Dallimore, 1995). Similarly, in electrically-paced, blood-perfused working hearts, rats fed a fish oil diet for 16 weeks were protected against the development of arrhythmias following ischaemia and reperfusion (Pepe & McLennan, 1996). The protection afforded by a fish oil diet (4 weeks) against arrhythmias induced *in situ* by coronary artery ligation and reperfusion was reported to be associated with reduced leukocyte infiltration in the left ventricular wall (Hock *et al.*, 1990). However, in isolated hearts, following reperfusion, a protective effect of fish oil independent of its effects on plasma was reported. Using a perfusate free of plasma and circulating cellular elements such as platelets and leukocytes,

reperfusion injury was lower in hearts from animals fed a fish oil diet for 5 days (Yang *et al.*, 1993). Although protection afforded by the n-3 PUFAs may be due to the coincident effect of the reduced saturated fatty acid content, it has been shown that animals fed a fish oil supplemented diet are consistently protected from developing arrhythmias compared with animals fed n-6 PUFA or saturated fatty acid diets, such that their effects do not correlate with the relative amounts of membrane saturated and polyunsaturated fatty acids *per se* (McLennan *et al.*, 1985; McLennan *et al.*, 1988; McLennan *et al.*, 1993). Additionally, canola oil, which like olive oil is composed mainly of oleic acid (which is not antiarrhythmic) but also contains approximately 8% αLNA, does offer significant antiarrhythmic protection (McLennan & Dallimore, 1995). The antiarrhythmic effect of the canola oil was not solely attributed to the presence of the n-3 PUFA, αLNA, since soybean oil, which contains similar concentrations of αLNA was not antiarrhythmic. The LA present in soybean oil probably competes with αLNA preventing its conversion to the longer chain n-3 PUFAs, which are potently antiarrhythmic, and this is not the case with canola oil.

1.6.2 Ischaemia-Reperfusion in Isolated Cells

Reactive oxygen species (ROS) have been reported to be generated during reperfusion following myocardial ischaemia in a number of experimental models (Hess & Manson, 1984; Khalid & Ashraf, 1993). Myocardial cells can be exposed to ROS circulating in the bloodstream or generated intracellularly.

1.6.2.1 Intracellularly Generated ROS

Using fluorescent probes to identify the ROS produced in chick cardiomyocytes during ischaemia and reperfusion, it was reported that during ischaemia the oxidation of both

2'7'-dichlorofluorescin diacetate (DCFH- oxidised by H_2O_2 and •OH) and dihydroethidium (DHE- oxidised by O_2•$^-$ and •OH) increased (Vandenhoek *et al.*, 1997). However, upon reperfusion, while DHE fluorescence levels fell rapidly, DCF fluorescence increased quickly within the first 5 min of reperfusion, indicating the presence of mainly H_2O_2 and •OH during the early reperfusion phase. Correspondingly, the use of 1,10-phenanthroline and mercaptopropylene glycol (a synthetic analog of glutathione) throughout ischaemia and reperfusion significantly reduced cell death during reperfusion in chick cardiomyocytes. It was also reported that these cardiomyocytes released significantly less lactate dehydrogenase during sustained ischaemia (4 hr) than during 1 hr ischaemia followed by 3 hr reperfusion (Vandenhoek *et al.*, 1996). Furthermore, increasing the duration of the ischaemic period (from 30 to 90 min) increased the extent of injury after 5 hours reperfusion, indicating increased ROS generation. In neonatal rat cardiomyocytes incubated with n-3 PUFAs for 4 days, the progressive loss of electrical activity induced by 2.5 hours hypoxia was accelerated in the n-3 PUFA group compared with the n-6 PUFA group, however the recovery of the resting potential during reoxygenation was faster in the n-3 PUFA group. Thus the contractile arrest may reduce the energy use of the substrate-deleted hypoxiated myocardial cells and therefore preserve the reoxygenation-induced damage (Durot *et al.*, 1997). Significant protection against hypoxia-reoxygenation-induced injury was demonstrated in cardiomyocytes isolated from rats supplemented with the dietary n-3 PUFAs, eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) for 4 weeks (Hayashi *et al.*, 1995) suggestive of protection from free radical damage. These effects were accompanied by an elevation in the proportion of EPA (but not DHA) in the myocardial membrane phospholipids. Since reoxygenation following anoxic conditions provokes oscillations in cytosolic Ca^{2+} (Siegmund *et al.*, 1997), the protective effects of n-3 PUFAs may in part

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be implicated in reoxygenation-induced hypercontracture by preventing oscillations of intracellular Ca^{2+} as well as concomitant free radical production during the early phase of reoxygenation (Obata *et al.*, 1997).

1.6.2.2 Extrinsically Generated ROS

In isolated cells, addition of various free radical generating systems (FRGS) to simulate reperfusion injury causes contractile dysfunction, including arrhythmias, cessation of contractility and hypercontracture (Massey & Burton, 1990; Nakamura *et al.*, 1993; Courtois *et al.*, 1998). In response to a FRGS consisting of purine, xanthine oxidase and iron-loaded transferrin which generates $O_2^{\bullet-}$, H_2O_2 and $\bullet OH$, neonatal rat ventricular cardiomyocytes exhibited a decrease in the number of Ca^{2+} transients with eventual cessation of these transients. Further, the cells developed fibrillatory activity followed by a progressive rise in intracellular Ca^{2+} from nanomolar to micromolar levels. This latter event was also associated with blebbing of the cell membrane and hypercontracture (Burton *et al.*, 1990). Pre-treatment for 18 hours with α-tocopherol protected against the loss of contractile activity, the decreased lactate dehydrogenase (LDH) release and conjugated diene formation, as well as reducing the $[^3H]$ arachidonate release from neonatal rat cardiomyocytes occurring during exposure to ROS (Massey & Burton, 1990). At the time of writing, no studies investigating the effect of acute addition of n-3 PUFAs on ROS-induced cellular injury were found in the literature.

1.7 Mechanisms of Action of n-3 PUFAs

1.7.1 Effects on Cellular Biochemical/Biophysical Parameters

As one of the major effects of ROS is the disruption of the integrity of cellular membranes and damage via oxidation of their PUFA components, dietary membrane

enrichment with n-3 fatty acids may act in part, by providing a substrate for membrane repair (Horrobin, 1991). It is also possible that PUFAs present in plasma triacylglycerols may trap free radicals, thus protecting membrane phospholipid PUFAs from the effects of ROS (Uauy-Dagach & Valenzuela, 1996). The n-3 PUFAs have also been shown to be cardioprotective by virtue of reducing the myocardial O_2 consumption during ischaemia, hence increasing post-ischaemic recovery (Pepe & McLennan, 2002). Lysophosphatidylcholine decreases junctional conductance of cardiac cells and this is believed to be an important mediator of cardiac cell uncoupling, which contributes to conduction block and leads to re-entrant arrhythmias (Daleau, 1999). Acute addition of n-3 PUFAs prevented lysophosphatidylcholine induced arrhythmias in adult rat ventricular cardiomyocytes (Leifert *et al.*, 2000). The highly unsaturated nature of n-3 fatty acids could also be expected to alter the physicochemical properties of the lipid bilayer. Specifically, the degree of rotational motion about the fatty acyl chain (referring to membrane fluidity) is increased with increasing degrees of fatty acid unsaturation (Stubbs & Smith, 1984). Indeed, acute addition of n-3 PUFAs was shown to significantly increase cardiomyocyte membrane fluidity as measured by steady-state fluorescence anisotropy (Jahangiri *et al.*, 2000; Leifert *et al.*, 2000) and this was associated with a prevention of isoproterenol-induced cellular arrhythmias. Modifications in membrane fluidity may then alter the function of membrane receptors, enzymes and ion channels (McMurchie, 1988; Nalbone *et al.*, 1990; Fournier *et al.*, 1995; de Jonge *et al.*, 1996; Bastiaanse *et al.*, 1997).

1.7.2 Effects on Ion Channels

It has been suggested that the antiarrhythmic effect of n-3 PUFAs may be associated with their actions on blocking cardiac sodium channel currents (Xiao *et al.*, 1995; Leifert *et al.*, 1999b). Fatty acids are able to quickly partition into the membrane bilayer lipids and, as a

result, they may change the threshold voltage for the gating of sodium channels that initiate the action potential. As such, n-3 PUFAs are possible candidates for antiarrhythmic drug therapy due to the proarrhythmic nature of some of the current therapeutics that involve Na^+ channel blockade. The proarrhythmic actions of the antiarrhythmic drug mexiletine, was suggested to be due to its upregulation of cardiac sodium channel expression (Taouis *et al.*, 1991). EPA (shown to have antiarrhythmic effects) did not affect cardiac channel gene expression, but reduced the mexiletine induced increase in expression (Kang *et al.*, 1997).

The n-3 PUFAs, EPA and DHA have also been shown to interact with cardiac Ca^{2+} channels and modify inward Ca^{2+} fluxes (Hallaq *et al.*, 1992). Acute addition of the n-3 PUFAs, DHA and EPA, reduced the availability of Ca^{2+} for uptake and inhibited the Ca^{2+} release mechanism in rat SR (Negretti *et al.*, 2000) while dietary FO supplementation decreased SR Ca^{2+} uptake and reduced the Ca^{2+}-ATPase activity in rat hearts (Taffet *et al.*, 1993). Three days of incubation of neonatal cardiomyocytes with DHA impaired the α-adrenoceptor-induced positive inotropic effects and the induction of cellular arrhythmic activity concomitant with a decrease in IP_3 formation (Reithmann *et al.*, 1996). It is therefore likely that the n-3 PUFAs may be acting at many different sites in the various intracellular pathways responsible for Ca^{2+} handling. The effect of DHA and EPA on inhibition of the fast Na^+ and slow Ca^{2+} current, together with their actions on prolonging action potential duration (Macleod *et al.*, 1998) may, in part, explain their anti-arrhythmic effects.

1.7.3 Effects on Gene Expression

The effects of fatty acids on gene expression have received considerable attention as this represents a direct mechanism for fatty acids to regulate gene function (Jump & Clarke, 1999). The n-3 PUFAs have rapid effects on gene expression; changes in mRNAs encoding several lipogenic enzymes can be detected within hours of feeding animals diets enriched in n-3 PUFAs (Jump *et al.*, 1993; Jump, 2002b). Moreover, these effects are sustained as long as the n-3 PUFAs remain in the diet. In these cases, the fatty acid acts like a hormone to control the activity and/or abundance of key transcription factors (Jump, 2002b). A recent review has summarised the major effects of PUFAs on four families of transcription factors- the peroxisome proliferator activated receptor, liver X receptors, hepatic nuclear factor-4 alpha, and the sterol regulatory element binding proteins (Jump, 2002a).

1.8 Controversies and Risks

Although an inverse association between fish consumption and the risk of death from coronary heart disease has been found in many prospective studies (Kromhout *et al.*, 1985; Dolecek, 1992; Kromhout *et al.*, 1995), this was not the finding in similar investigations in Norway (Vollset *et al.*, 1985), among Japanese men in Hawaii (Curb & Reed, 1985), or among US physicians (Morris *et al.*, 1992). These inconsistencies may be explained by intrinsic differences in the lifestyle of these populations and also by other components within their diet that could have masked the effects of the fish oils. However, by far the majority of the evidence favours cardioprotective actions of fish oil and the n-3 polyunsaturated fatty acids. Since fish has always been a part of the Western diet, it is unlikely to be toxic, however, in high doses it may have some adverse effects. EPA and DHA are the main n-3 PUFAs in fish oil and as experimental animal studies have shown,

these fatty acids can result in a shift in the haemostatic balance towards one where there is greater vasodilation and less platelet aggregation. Thus there is concern that excessive bleeding may be associated with increased fish consumption; a characteristic exhibited by the Greenland Eskimos who bruise very easily and have prolonged bleeding times (Bang & Dyerberg, 1980). However, there have been no reports of major bleeding problems during clinical trials with fish-oil supplementation. Moreover, the bleeding times are less than those caused by the administration of aspirin (Leaf & Weber, 1988).

Immune reactivity is also decreased by the n-3 PUFAs (Leaf & Weber, 1988). This is likely to be beneficial for people with autoimmune diseases. Whether the n-3 PUFAs can suppress the immune system below its normal level of competence is not known. If this were the case, excessive dietary consumption of fish and fish oil may increase the susceptibility to certain infections. Certainly, at present, no adverse effects of marine n-3 PUFA on infection in humans have been reported (de Deckere *et al.*, 1998).

Fish oils rich in n-3 fatty acids are susceptible to oxidation because of their high degree of unsaturation (Alexander-North *et al.*, 1994; van Ginkel & Sevanian, 1994). Radical species can attack the double bonds of unsaturated lipids and initiate chain reactions leading to such end products as aldehydes, dialdehydes (eg malondialdehydye (MDA)) and short-chain hydrocarbons. Polyunsaturated (n-3) fatty acids are efficiently incorporated into tissue membrane phospholipids displacing arachidonic acid and linoleic acid. As a result of the n-3 PUFA incorporation, the unsaturation index is increased. Several reports show that high intakes of n-3 fatty acids lead to increased human plasma thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxide levels (Meydani *et al.*, 1991; Ando *et al.*, 1998). Similar results have been reported in animal studies

(Garrido *et al.*, 1989; Leibovitz *et al.*, 1990). The TBARS test is the most common assay used to measure oxidative stress, in particular lipid peroxidation. The basis for this test is the reaction of MDA, one of several low-molecular-weight end products formed from the decomposition of certain lipid peroxidation products, with TBA. However, there are numerous problems (and artefacts) associated with the assay (Ando *et al.*, 2000; Wander & Du, 2000). It has therefore been proposed that damage to protein carbonyl groups may be a better indicator of oxidative damage since this reaction evaluates damage to proteins in general as opposed to detecting the damage to specific amino acids (Wander & Du, 2000). Furthermore, damage to proteins may affect the function of such important compounds as receptors and enzymes, as well as contributing to the damage of other biomolecules. In fact, in a recent human study, 8 months supplementation with fish oil significantly increased plasma TBARS, but not protein carbonyls in plasma (Wander & Du, 2000). Similarly, mice fed for 4 weeks with 8% (w/w) MaxEPA® as an n-3 PUFA supplement had higher renal levels of TBARs and conjugated dienes, but not protein carbonyls, compared with rats supplemented with a lard diet not containing high levels of n-3 PUFAs (Ibrahim *et al.*, 1999). Studies such as these lend support for the non pro-oxidative nature of FO, or at least indicate that the levels of oxidative stress induced by FO do not reach levels that are severely damaging to cellular proteins. Studies have also indicated that inclusion of sufficient α-tocopherol will prevent any oxidative stress induced by FO (reviewed by Uauy-Dagach & Valenzuela (1996)). However, cells are not always passive to increased oxygen radical production, and moderate episodes of free radical stress (for example, induced by fish oil feeding) may in fact potentiate the defense system by stimulating the expression of the antioxidant enzymes (Vogt *et al.*, 1998; Csonka *et al.*, 2000; Perry *et al.*, 2000). For example, there is increasing evidence that

exercise-induced oxidative stress induces an adaptive response in skeletal muscle which serves to protect the tissue against further stress (Jackson, 1999).

1.9 Overall Objectives

The broad aims of this thesis were 1) to develop a cellular model of reperfusion injury, which would be used to determine the possible protective effects of dietary and acute n-3 PUFAs and 2) to examine the underlying mechanisms of n-3 PUFA action. The specific aims were to:

- Establish concentrations of, and duration of exposure to, various free radical generating systems (FRGS); a protocol of hypoxia-reoxygenation (H-R) in cardiac myocytes; and ischaemia-reperfusion (I-R) in isolated hearts in order to reproducibly induce asynchronous/arrhythmic contractile activity.

- Determine concentrations and duration of incubation of cells with PUFAs, as well as to develop appropriate diets and durations of feeding in rats for membrane incorporation of n-3 PUFAs to occur.

- Examine the susceptibility of adult rat cardiomyocytes to the development of myocardial arrhythmias induced by FRGS or H-R following acute addition of fatty acids or dietary lipid supplementation.

- Investigate the changes in arrhythmia susceptibility, cardiomyocyte membrane fluidity, intracellular ROS and Ca^{2+} dynamics induced by dietary lipid supplementation and FRGS.

- Analyse the changes in the gene expression of myocardial antioxidant enzymes following dietary lipid supplementation and whole heart I-R.

1.10 Research Plan

To develop a cellular model of reperfusion injury, isolated adult rat ventricular cardiomyocytes contracting in synchrony with electrical field stimulation were exposed to extracellularly generated free radicals, namely H_2O_2, $O_2^{\bullet-}$ and $\bullet OH$, in order to induce asynchronous contractile activity, mimicking arrhythmias at the organ level. This was termed the ROS-induced arrhythmia assay. To examine the protective effect of dietary fatty acids in myocardial reperfusion damage, rats were fed an n-3 PUFA rich diet and cells isolated from these animals were subjected to the protocols described above. The effects of the fish oil diet were compared to a saturated fat diet. Incorporation of fatty acids into membrane phospholipids was determined by gas chromatography. Isolated cells were also incubated for periods ranging from 30 min to overnight in medium supplemented with various fatty acids and antioxidants in order to investigate the differences in protection afforded by acute addition versus longer-term (non-dietary) supplementation (Chapter 3).

Fluorescent probes were used to monitor changes in intracellular calcium and ROS levels and the ROS-induced arrhythmia assay was used to assess myocardial injury during exposure to the free radical generating systems. The effect of dietary supplementation with n-3 PUFAs was tested for their ability to counteract these changes (Chapter 4).

In order to examine the effects of intracellularly generated free radicals, contracting cardiomyocytes underwent a protocol of hypoxia and reoxygenation. During hypoxia, cells were superfused with buffer lacking glucose and gassed with nitrogen (to replace oxygen) and reoxygenation was performed by changing to a solution containing glucose, gassed with oxygen (Chapter 6).

Finally, quantitative PCR was used to measure antioxidant enzyme gene expression in rat myocardium following dietary lipid supplementation (Chapter 5). There are many advantages to quantifying gene sequences using real-time PCR techniques, foremost being sensitivity and precision. This precision exists because quantitation of the PCR product amplification is performed during the exponential phase of the reaction, rather than endpoint measurement of accumulated PCR product (Bustin, 2000). Specificity is conferred by the requirement of two oligonucleotides to anneal to the DNA before any data are collected. Real-time quantitation eliminates post-PCR processing of PCR products, which not only increases throughput and reduces the chances for carryover contamination, but also removes post-PCR processing as a potential source of error. Furthermore, the advantage of the DNA binding dyes is that the same dye can be used to detect any amplified product (Applied Biosystems Technical Notes, 2001a).

The majority of the experiments in this thesis were performed on cardiomyocytes. To make some extrapolation between isolated cardiomyocytes and the situation in the whole heart, the fact that the imposition of arrhythmic stimuli, which induce cells to beat in a manner out of synchrony with an applied electrical stimulus, indicates that an isolated cardiomyocyte has the potential to develop all of the characteristics that would lead it, in association with neighbouring cells (were it within the heart itself), to give rise to a region of decremental conduction in the working myocardial fibres. Given the restriction that the whole heart cannot always provide an adequate experimental model for many of the approaches required, and the situation that certain re-entrant pathways in the heart occur as a result of damage that occurs to myocardial contractile tissue induced by arrhythmogenic agents or ischaemia-reperfusion, the isolated cardiomyocyte can offer advantages not available using the adult heart.

CHAPTER TWO

General Methods

2.1 Ethics and Animal Care

The animals used in the following studies were cared for according to the Australian National Health and Medical Council Guidelines for the Care and Use of Animals. All experimental procedures were subject to prior approval by the Adelaide University and CSIRO Health Sciences and Nutrition Animal Ethics Committees. Male Sprague Dawley rats were maintained in the CSIRO Health Sciences and Nutrition Animal Colony until use. Room temperature was maintained at 23°C with constant (55%) humidity, and lights were maintained on a 12 hour light (8am - 8pm)/dark cycle. Animals had free access to food and water at all times. For the dietary studies, groups of rats supplemented with the same diet were placed in cages (up to 6 rats per cage). The lipid-supplemented diets were changed every second day to minimise oxidation of the fatty acids. The animals were weighed weekly during the lipid supplementation.

2.2 Preparation of Diets

The composition of the standard laboratory rat chow is shown in Tables 2.1 and 2.2. This is the maintenance diet that was fed to the rats for the studies described in 3.2.2, 3.2.3 and 3.2.4. It was subsequently found that the composition of the standard commercial diet varied because of manufacturing practices and changes in the availability of ingredients. In particular the content of PUFAs was seasonally dependent. Hence, a diet low in PUFAs, particularly α-linolenic acid (ALA), termed the low-ALA diet was used in all subsequent studies.

Ingredients	Content (%)
Wheat	45.95
Oats	15.0
Soyabean Meal	12.37
Fish Meal	7.5
Lucerine Meal	5.5
Peas	5.0
Meat Meal	2.5
Tallow	2.0
Vitamins and Minerals	0.55
Salt	0.5
Choline Chloride	0.08
Lysine	0.05
Sunflower Oil	2.5
Blood meal	0.5

Vitamins and Minerals	Concentration (mg, unless specified)
Vitamin A	22,500 IU
Vitamin D_3	2,00 IU
Vitamin E	50 IU
Vitamin K	8.3
Vitamin B_{12}	120
Thiamine	58
Riboflavin	10
Piridoxine	12
Ca^{2+} Pantothenate	20
Niacin	18
Folic Acid	2
Biotin	110
Zinc	61
Iron	66
Copper	6
Manganese	102
Molybdenum	1
Iodine	0.6
Cobalt	120
BHA (antioxidant)	100

Table 2.1: Components of the Joint Stock Ration.
This was the standard laboratory rat chow fed to the rats *ad libitum*. The ration was purchased from Ridley Agriproducts (South Australia). The vitamins and minerals comprised 0.55% of the mix. The components of the vitamin and mineral mix are outlined in the second half of the table. Abbreviations: BHA, butylated hydroxy anisole.

Estimated Nutrient Specification	Content (%, unless specified)
Digestible Energy	3370 Kcal/kg
Metabolizable Energy	3000 Kcal/kg
Crude Protein	21
Crude Fat	7.5
Lysine	1.23
Methionine	0.39
Methionine and Cystine	0.71
Tryptophan	0.26
Isoleucine	0.87
Leucine	1.54
Threonine	0.76
Linoleic Acid	2.56
Calcium	0.74
Available Phosphorus	0.43

Table 2.2: Nutrient Specification of the Joint Stock Ration (Ridley Agriproducts, South Australia).

The low-ALA diet, used in the final dietary study of Chapter 3 (section 3.2.5) and in subsequent chapters, was purchased from Glen Forrest Stockfeeders (Western Australia) and is based on the AIN 93G diet, containing 7% (w/w) fat as Sunola oil (SO). For the lipid-supplemented diets, an additional 10% (w/w) fat (either SO, lard (saturated fat- SF) or fish oil (FO)) was added to the low-ALA base diet. The components of the low-ALA diet are given in Table 2.3.

Ingredient	Content (%)
Sucrose	10
Starch	39.75
Dextrinised starch	13.2
Cellulose	5
Casein (Acid)	20
Sunflower Oil	7
Methionine	0.3
AIN 93G Minerals	3.5
AIN 93G Vitamins	1
Choline Chloride 50% (w/w)	0.25

Table 2.3: Ingredients of the low-ALA diet.
This diet is based on the AIN 93G diet purchased from Glenforrest Stockfeeders (Western Australia).

The components of the Mineral Mix and the Vitamin Mix (as a percentage of the mix) are given in Table 2.4.

AIN 93G Minerals	*Concentration (%)*
Calcium Iodate	0.001
Copper Sulphate	0.065
Ferrous Sulphate	0.498
Magnesium Oxide	2.40
Manganese Oxide	0.032
Zinc Oxide	0.075
Selenium Premix (1% Se)	0.045
Calcium Carbonate	37.5
Sodium Chloride	7.40
Chromium Potassium Sulphate	0.055
Potassium Phosphate	19.60
Lithium Chloride	0.0017
Boric Acid	0.0082
Nickel Carbonate	0.0032
Ammonium Vanadate	0.0007
Potassium Sulphate	4.66
Potassium Citrate	7.08
Dextrose	20.58

AIN 93G Vitamins	*Concentration (%)*
Vitamin A 500 (500,000 IU/g)	0.08
Vitamin D 500 (500,000 IU/g)	0.02
Vitamin E (50% m/m)	1.50
Vitamin K (50% m/m)	0.02
Niacin	0.30
Riboflavin	0.06
Calcium Pantothenate	0.16
Vitamin B12 (1% m/m)	0.10
Thiamine	0.06
Pyrodoxine	0.07
Folic Acid	0.02
Biotin (2% m/m)	0.10
Sucrose	97.5

Table 2.4: Components of the mineral and vitamin mix present in the low-ALA diet.

For the studies of Chapter 4, 5 and 6, the low-ALA diet was made at CSIRO Health Sciences and Nutrition, based on the above formula. For the diet prepared at CSIRO Health Sciences and Nutrition, sucrose and dextrinised starch were purchased from Inpak Foods, Alberton, SA, Australia. Casein was from Marsh Dairy Products, Port Melbourne, VIC, Australia. Manganese oxide, calcium carbonate, potassium sulphate and potassium citrate were purchased from ACE Chemical Company, Camden Park, SA, Australia. All

other components were purchased from Sigma Chemical Company, Castle Hill, NSW, Australia.

2.3 The Gavaging Technique

Rats were gavaged between 3:00 pm to 5:00 pm each day. All oils were stored at $-20^{\circ}C$. Tubes containing the oil were warmed to about $32^{\circ}C$ until the oil reached a fluid consistency and 2 ml was withdrawn into a plastic syringe with a plastic feeding tube (40 cm long) attached to the end. The bottom 2 cm of the feeding tube was then lightly lubricated with glycerol before it was inserted into the rat's throat and gently pushed down the oesophagus until it reached the stomach. All feeding tubes had a mark (at the 20 cm point) that indicated the maximal extent to which they could be inserted. This gavaging technique involved holding the rat with the left hand firmly gripping either side of the animal's face, pulling the skin back towards the ear, which then allowed the mouth to open. Although this procedure probably did not cause any pain to the rats, performing the procedure as quickly as possible and ensuring that both the insertion and removal of the tube were done gently lessened any discomfort experienced by the animals. By the third day of gavage, the rats appeared to tolerate this protocol reasonably well, based on their lessened degree of agitation and movement during the gavaging.

2.4 Perfusion and Culture Media

Calcium-free Tyrode's perfusion media, contained (in mM) 137.7 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, 10.0 (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES), pH 7.40. $CaCl_2$ was added prior to use to give the appropriate concentrations indicated. Tyrode's solution was prepared using ultra-pure

(Milli-Q) water and was filtered through a 0.22 μm Millipore filter (Bedford, MA, USA) prior to use and gassed with 100% O_2. The pH was adjusted to 7.3 using NaOH.

Dulbecco's modified Eagle's medium (DMEM) with L-glutamine omitted and containing 4.5 g/L glucose was supplemented with 1 mM Ca^{2+}, 10 mM HEPES, 25 mM $NaHCO_3$, 100 U/ml Penicillin G, 100 mg/ml Streptomycin, 2 mM carnitine, 5 mM creatine, 5 mM taurine and 1 mg/ml delipidated bovine serum albumin (BSA). The solution was filtered prior to use and gassed with 95% O_2: 5% CO_2 to achieve a pH of 7.3. BSA was delipidated by successively washing in acetone, petroleum spirit and diethyl ether and dried by vacuum dessication.

2.5 Isolation of Adult Rat Ventricular Myocytes

Rats were anaesthetised with 60mg/kg (body weight) sodium pentobarbitone (and 2200U/kg (body weight) of heparin to prevent the blood from clotting) injected intraperitoneally. Following adequate anaesthesia, the heart was removed and placed into ice-cold, calcium-free Tyrode's solution. The heart was then mounted onto the cannula of a Langendorff apparatus and retrogradely perfused (non-recirculating) with Tyrode's solution containing 1 mM Ca^{2+} for 4 min. This was followed by 2 min perfusion with nominally Ca^{2+} free Tyrode's solution (non-recirculating). Buffers were maintained at 37°C and gassed with 100% O_2 during the Langendorff perfusion. The heart was further perfused for 20 min with Tyrode's solution containing 20 μM Ca^{2+}, 45 U/ml collagenase, 0.2 U/ml protease, 30 mM 2,3-butanedione monoxime (BDM) and 0.1% (w/v) bovine serum albumin (BSA) in a recirculating manner at 37°C. After perfusion with collagenase, the right ventricle was removed and gently minced with scissors in Tyrode's solution containing 40 μM Ca^{2+}, 30 mM BDM and 1.3% (w/v) BSA at room temperature to

liberate the isolated cells. The suspension was then filtered through a 250 μm nylon-mesh gauze and made to a final volume of 50 ml. The Ca^{2+} was increased to 1 mM in four stepwise increments (100, 250, 500, 1000 μM) with a 10 min interval between each addition. Cells were plated for 1 hour at room temperature onto 12 mm glass cover slips which had been coated with 50 μg/ml laminin (prepared in DMEM) one hour prior to use. Non-adhering cardiomyocytes were removed by gentle suction with a disposable plastic Pasteur pipette. Coverslips were washed twice with DMEM culture medium (containing 1 mM Ca^{2+}) pre-equilibrated with 5% CO_2:95% O_2 for 10 min. Cardiomyocytes were maintained in DMEM in a humidified incubator at 37°C, gassed with 5% CO_2 in air until use. Cells were used within 30 hours of isolation. For each experiment, cells of the same age (with respect to the length of time they were maintained in the incubator after isolation) were used. It was also important to be certain that the cellular damage due to the isolation procedures did not affect myocyte morphological viability during exposure to the ROS. Therefore two indexes of cell viability were examined for each isolation performed: 1) the percentage of quiescent rod-shaped cells viewed under the light microscope in 16 fields of 1 mm x 1 mm area, and 2) the percentage of rod-shaped cells responding to the electrical field stimulation. Cells considered viable and undamaged by these criteria were also found to exclude 0.4% trypan blue.

2.6 Cardiomyocyte Contractility

Cardiomyocytes adhering to laminin coated glass coverslips were superfused at 37°C with Tyrode's buffer, containing 2 mM Ca^{2+}, at a flow rate of 2 ml/min (using a Gilson minipump (Minipuls 2, Sydney, Australia)) and gassed with 100% O_2 for 3 min. Following this equilibration period, cells were stimulated to contract at a frequency of 1 Hz. The electrical impulses (square wave) were generated by an S4E stimulator (Grass

Instrument Co., Quincy, MA) and were delivered to the cells through a pair of platinum electrodes (separated by 10 mm) immersed in the perfusion solution, running parallel to the flow of the superfusing medium. The cells were stimulated at a voltage of between 30 – 40V as shown on the dial of the stimulator, however, the actual current reaching the cells is dependent on the size and geometry of the superfusion chamber, the volume of buffer in the chamber and the composition of the buffer. The current in the chamber was measured using a cathode ray oscilloscope and was found to be between 162 – 205 mA (Figure 2.1).
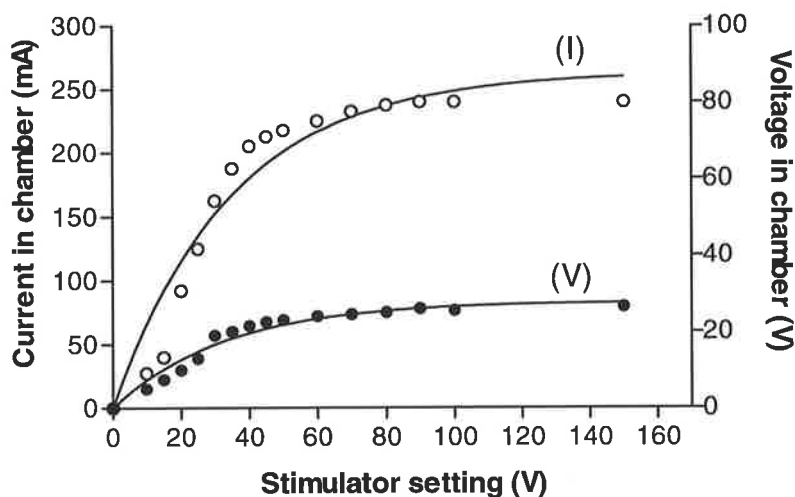
Figure 2.1: Stimulator settings versus actual superfusion chamber voltage and current measured using a cathode ray oscilloscope.
The clear circles represent the current and the coloured circles refer to the voltage.

2.7 Free Radical Generating System and Asynchrony Scoring

After 3 min equilibration, cells were stimulated to contract under electrical field stimulation for 2 min. To determine the contractile response of isolated cardiomyocytes to free radicals, cells were exposed to a free radical generating system (FRGS). The FRGSs were prepared by pre-incubating the necessary reagents together for 3-5.5 min prior to introducing the generated free radicals into the superfusion chamber. This was done both to allow the components to react together and also to allow time for the pH of the solution

to be adjusted back to 7.4. The contractile activity of the cells was continuously monitored following exposure to the FRGS by recording the number of cells developing asynchronous contractile activity. The term "asynchronous" was used to define cells not contracting in synchrony with the applied electrical field stimulation, for example, cells contracting at a rate above, or below that of the electrical field stimulation, irregular contractile activity, cells that had ceased contracting or hypercontracted cells. Cells behaving in this manner could easily be distinguished from cells displaying synchronous contractile activity under a light microscope. Three groups (ie coverslips) of approximately 20 cells were analysed per rat. The percentage of cells contracting in an asynchronous manner was counted every 2 min for 20 min to determine the time course for the development of asynchronously contracting cells.

2.8 Fatty Acid Analysis

Total lipids were extracted from the ventricular tissue, the standard colony diet and the dietary oils using a slight modification of the method of Bligh & Dyer (1959). Ventricular tissue (250-300 mg) was homogenised in 1 ml water using a Tenbroeck hand-held homogeniser (10 passes). Four ml of 2-propanol was added[1] and the mixture boiled for 30 sec. After cooling, 8 mL of chloroform was added, the mixture shaken, and the organic phase collected after overnight phase separation. After re-extracting the aqueous phase with a further 4 mL of chloroform, the organic phases were combined and evaporated to dryness under N_2 and then further dried under vacuum dessication. The phospholipids were separated from the other lipid classes by thin layer chromatography (TLC) on silica

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[1] For the fatty acid analysis of tissue from the study in Chapter 5, an ultra-turrax homogeniser was used. In that chapter, since the heart tissue had not been digested with collagenase (a Langendorff heart perfusion set up was used for ischaemia-reperfusion as opposed to collagenase digestion for isolating cells), a hand-held homogeniser was not effective in breaking down the tissue. The tissue was therefore homogenised with 1 ml water and 1 ml isopropanol in an ultra-turrax for approximately 40 sec or until the tissue was completely homogenised. A further 3 ml isopropanol was added, then the protocol was continued as described above.

Chapter Two

gel 150A-LK5D plates (Whatman, Clifton, NJ, USA), and developed in a solvent system of petroleum ether:acetone (3:1 [v/v]). The phospholipids remaining at the origin were scraped from the plate. Fatty acid methyl esters (FAMEs) were prepared by heating the samples at 50°C overnight in 1 % (v/v) H_2SO_4 in methanol. FAMEs were extracted using hexane and contaminants removed using a Biosil (silicic acid) column. FAMEs in hexane were evaporated to dryness under N_2 and redissolved in 50 µl iso-octane. All solvents used for lipid extraction, TLC, and preparation of FAMEs contained the antioxidant butylated hydroxytoluene (0.05% w/v). FAMEs (0.1 µl) from lipid extracts of the experimental oils, standard diet, and the myocardial phospholipid extracts, were analysed by GLC. GLC was performed using a Hewlett Packard HP 5710 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) fitted with a 50 metre BPX70 capillary column (Scientific Glass Engineering, Melbourne, Victoria, Australia). The FAMEs were separated using a carrier gas (hydrogen) flow of 35 cm/s with a temperature gradient of 130°C to 230°C at 4°C min. A cold on-column injector was used with the flame ionisation detector temperature set at 250°C. FAMEs were identified using authentic lipid standards (Nu-Chek-Prep Inc., Elysian, MN, USA) by GLC. The proportions of the total fatty acids were normalised to a value of 100%.

2.9 Vitamin E analysis

2.9.1 Sample preparation and extraction

The vitamin E content of the myocardial tissue was determined by a modification of the method of Katsanidis and Addis (Katsanidis & Addis, 1999). Briefly, 100 mg of ventricular tissue was homogenised in 5 ml of 10% (w/v) BHA in ethanol solution, for 1-2 min using an ultra turrex homogeniser. The mixture was saponified in 0.5 ml saturated KOH at 65°C for 30 min, vortexing the samples every 10 min for 30s. The reaction was

stopped by addition of 5 ml cold water and after cooling, 3 ml hexane was added, the mixture vortexed for 1 min. The added water increases the polarity of the aqueous phase and improves partitioning of vitamin E into the organic (hexane phase). The samples were centrifuged at 3000 rpm for 3 min at 10°C and the organic layer was collected. The original sample was re-extracted a further 2 times with hexane and the organic phases were combined and evaporated to dryness under N_2. The samples were then redissolved in 200 µl of the mobile phase and analysed by HPLC.

2.9.2 Standards

Initially, an effort was made to use α-tocopherol acetate (αTAc) as an internal standard to account for losses during the extraction process. However since αTAc is converted to α-tocopherol (αT) during the saponification stage, there would be no αTAc remaining in the sample to act as a standard. In preliminary experiments it was found that the fish oil contained the various isomers of tocopherol, therefore it would not be feasible to use any other form of tocopherol such as β, γ, or δ-tocopherol. Others have used tocol (rac-tocopherol; 2-methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol) as an internal standard; however, it is unstable in the saponification process with up to 30% loss to other compounds being reported for this compound (Hatam & Kayden, 1979). Thus it was decided to use αT itself to determine the recovery following extraction. In each set of samples analysed, 2 tubes containing 50 µg/ml α-tocopherol were extracted in the same way as the myocardial samples. The recovery of the known α-tocopherol samples was calculated and the average recovery of these samples was then used to calculate the actual levels of α-tocopherol in the experimental samples. Since the aim was to determine if there were *dietary* differences in the levels of myocardial vitamin E, the inter-sample

differences would not have a significant impact on the results. To calculate the concentration of α-tocopherol, a standard curve (0-200 µg/ml α-tocopherol) was used.

2.9.3 Chromatography

The HPLC consisted of an ICI LC 1500 pump and an ICI 206 PHD UV detector (GBC Scientific Equipment, Victoria, Australia). Samples (50 µl) were injected into a Spherisorb reverse phase column (C_{18}, 5-µm particle size, 4.6 mm ID × 25 cm). The mobile phase was (all (v/v)): 22% methanol, 55% acetonitrile, 11.5% dichloromethane and 11.5% hexane (HPLC grade). Ammonium acetate (2 ml of 5% (w/v) in methanol) was added (as a preservative) to each litre of the mobile phase. This solvent mixture was degassed by sonication for 10 min prior to use. The flow rate was 1 ml/min. The absorbance was monitored at a wavelength of 292 nm and data collected using the ICI DP 800 Chromatography Software. Alpha-tocopherol eluted within 6 min and a typical run lasted approximately 9 min.

2.10 Real-time Polymerase Chain Reaction (PCR)

2.10.1 The detection system

Quantitative real-time polymerase chain reaction (PCR) provides an accurate method for determination of levels of specific RNA and DNA sequences in tissue samples (Grove, 1999). It is based on detection of a fluorescent signal produced proportionally during amplification of a PCR product. The detection system consists of a 96-well thermal cycler connected to a laser and charge-coupled device (CCD) optics system. An optical fibre inserted through a lens is positioned over each well, and laser light is directed through the fibre to excite the fluorochrome in the PCR solution. Emissions travel through the fibre to the CCD camera, where they are analysed by the software's algorithms. Collected data

are subsequently sent to the computer. Emissions are measured every 7 seconds. The instrument used in the present study was the GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). The sensitivity of detection allows acquisition of data when PCR amplification is still in the exponential phase. This is determined by identifying the cycle number at which the reporter dye emission intensities rise above background noise; this cycle number being called the threshold cycle (C_T). The C_T is determined at the most exponential phase of the reaction and is therefore more reliable than end-point measurements of accumulated PCR products used by traditional PCR methods. The C_T is inversely proportional to the copy number of the target template; the higher the template concentration, the lower the threshold cycle measured (Grove, 1999).

2.10.2 Primer Design Using Primer Express® Software

The Primer Express® Software (Applied Biosystems, Foster City, CA) was used to generate appropriate primers for the PCR. Certain guidelines needed to be followed when designing primers. Amplicon length should be between 50-150 base pairs, since small amplicons promote high-efficiency assays. The G/C content should be in the 20-80% range since any regions with a G/C content in excess of this may not denature well during thermal cycling, leading to a less efficient reaction. In addition, G/C-rich sequences are susceptible to non-specific interactions that may reduce reaction efficiency and produce non-specific signal in SYBR® Green I assays. For this same reason, runs of four or more contiguous G bases should be avoided. A/T-rich sequences require longer primer sequences in order to obtain the recommended T_ms. The T_m of the primers should be 58 to 60°C. This is one of the factors that allows the use of universal thermal cycling parameters. The last five bases on the 3' end of the primers should contain no more than two C and/or G bases, which is another factor that reduces the possibility of non-specific

product formation. It is also preferable that the amplicon should span one or more introns to avoid amplification of the target gene in genomic DNA. When this rule is followed, if there is genomic contamination, it will not be completely amplified since it would result in a larger product. The short PCR cycles will not allow its amplification, hence eliminating the possibility of inaccuracies due to genomic DNA amplification. If the gene being studied does not have any introns, or a primer cannot be generated that spans an intron, it is necessary to run minus RT (reverse transcription) controls. The procedure for minus RT controls will be discussed in Chapter 5.

2.11 Materials

DMEM culture medium, BDM, BSA (fraction V), carnitine, creatine, taurine, protease (type XIV), laminin, purine, xanthine oxidase and isoproterenol were from Sigma Chemical Co. (Castle Hill, NSW, Australia). Collagenase was from Yakult Honsha Co., Ltd (Tokyo, Japan). Penicillin/Streptomycin was from GIBCO-BRL (Melbourne, Victoria, Australia). Fluorescent probes were purchased from Molecular Probes (Eugene, OR) and the reagents for the molecular biology experiments were purchased from GeneWorks Pty. Ltd. (Adelaide, Australia), Applied Biosystems (New Jersey, USA) or GensetOligos (Lismore, NSW, Australia) as outlined in Chapter 5. All other chemicals were of the highest grade available.

2.12 Statistical Analyses

Date are presented as mean ± SEM unless otherwise specified. Parametric statistical tests were used for all analyses, as described in each chapter. The most common tests used in this thesis were the two-tailed *t*-test with Welch's correction, and a one-way or two-way

analysis of variance (ANOVA) with the Bonferroni multiple comparisons (post-hoc) test. The criterion for significance was $p < 0.05$.

CHAPTER THREE

Dietary n-3 PUFAs and Reperfusion Injury in Isolated Cardiomyocytes

3.1 Introduction

Reactive oxygen species (ROS) have been implicated in the pathogenesis of tissue injury in a variety of organ systems. The role of ROS with regard to myocardial ischaemia and reperfusion has been of particular interest since advances in technology have now made it possible to intervene clinically in the process of acute myocardial infarction by means such as: lysing coronary arterial thrombi with thrombolytic agents, mechanically re-opening coronary artery obstructions with percutaneous transluminal coronary angioplasty, or by surgically bypassing the obstructions (Bolli, 1988). However, experimental studies have demonstrated that although early reperfusion limits (or even prevents) necrosis, this beneficial effect does not lead to immediate functional improvement; rather reperfusion is, in many cases, associated with damaging effects on the myocardium, possibly induced by the generation of various ROS (Hess & Manson, 1984; Manning & Hearse, 1984). Indeed, it is well documented that the exposure of isolated (intact) hearts and single myocytes to oxygen free radical generating systems (FRGSs) produces arrhythmias (Burton *et al.*, 1990) and electrophysiological alterations including membrane depolarisation, abnormal automaticity, action potential prolongation followed by shortening, early and late depolarisations, and triggered activity (Goldhaber & Weiss, 1992).

ROS are normally produced by the mitochondria under physiological conditions. Indeed, 2-4% of the oxygen consumed by the mitochondria is converted to superoxide ($O_2^{\bullet-}$) by the electron transport chain (Williams *et al.*, 1998) and hydrogen peroxide (H_2O_2) production by mitochondria represents 60-80% of the cellular production of ROS

(Gonzalez-Flecha & Boveris, 1995). However, the excessive production of ROS imposes an oxidant stress on the cell when the capacity of intracellular antioxidants or chain-breaking radical scavengers such as superoxide dismutase, catalase, glutathione peroxidase, glutathione, ascorbic acid or vitamin E to interrupt the sequential transfer of electrons, is exceeded (Simpson & Lucchesi, 1987). Most cellular components are susceptible to attack by oxygen-centred free radicals. In particular, highly (poly) unsaturated membrane lipids are prone to free radical attack leading to the formation of lipid peroxides, lipid hydroperoxides and aldehydes (Simpson & Lucchesi, 1987). The ability of free radicals to initiate lipid peroxidation of cellular membranes or oxidation of membrane protein sulfhydryl groups may potentially affect membrane permeability and the functioning of ion pumps. This will impact on the ability of the cell to maintain its ionic environment, especially in regard to homeostasis of Na^+ and Ca^{2+} flux (Burton *et al.*, 1990), ultimately contributing to electrophysiological abnormalities and contractile dysfunction (Goldhaber *et al.*, 1989).

Previous studies have indicated that dietary fish oil, high in n-3 polyunsaturated fatty acids (PUFAs), has a protective effect from reperfusion-induced arrhythmias (McLennan *et al.*, 1988; Hock *et al.*, 1990; Yang *et al.*, 1993). In light of the many studies demonstrating free radical production at reperfusion, either directly (Cavarocchi *et al.*, 1986) or indirectly (Barsacchi *et al.*, 1992), this would indicate a likely antioxidant as well as an antiarrhythmic role for dietary fish oil (FO). However, to date, no studies have linked ROS, reperfusion-type arrhythmias and n-3 PUFAs/FO. In atherosclerotic monkeys undergoing 1 hr ischaemia and 2 hr reperfusion *in situ*, a reduction of $O_2^{\bullet-}$ levels was shown in the coronary arteries and myocardium of animals supplemented for 12 months with FO (Supari *et al.*, 1995), however, no parameters relating to reperfusion

injury/arrhythmias were assessed. Ascorbyl free radical release at reperfusion was not different in hearts isolated from rats fed saturated fat (SF) or FO (Demaison *et al.*, 2001), however, since there was no significant physiological effects of FO relating to reperfusion injury, the relevance of this finding is questionable. Many studies of n-3 PUFA or free radical effects on the myocardium have been made in whole animal models or using isolated hearts. The experiments described in this chapter were designed to investigate the influence of polyunsaturated fatty acids on free radical-induced arrhythmias using an isolated cell model to determine the cellular and physiological consequences. Although whole animal or isolated hearts may be more physiologically relevant models, the use of isolated cardiomyocytes is appropriate for such an investigation because a cellular system has minimal potentially confounding neural and humoral influences. In particular, the use of cardiomyocytes isolated from adult animals is a more relevant model compared to cardiomyocytes isolated from neonatal animals which are commonly used, since aging increases the vulnerability of the heart to be triggered into arrhythmia. It has also been shown that the neonatal myocardium (Nishioka & Jarmakani, 1982) and embryo-derived cells (Byler *et al.*, 1994) are relatively more resistant to the effects of free radicals compared to the adult myocardium. Furthermore, contractility is regulated differently in adult cardiomyocytes than in neonatal cardiomyocytes in that neonatal cardiomyocytes have an underdeveloped sarcoplasmic reticulum and express different isoforms of contractile proteins and second messengers (eg protein kinase C) (Kanaya *et al.*, 1998).

Thus the aims of the present study were:

- To develop a cellular model of a reperfusion-type injury using a free radical generating system (FRGS) and isolated adult rat ventricular myocytes.

- To investigate the possible protective effects of acutely added n-3 fatty acids on cellular arrhythmias induced by a FRGS.

- To examine the effect of dietary supplementation with n-3 PUFAs on the response of isolated cardiomyocytes to free radical-induced arrhythmias.

3.2 Methods

3.2.1 A Cellular Model for Reperfusion Injury - The ROS Induced Arrhythmia Assay.

The procedure for isolation of adult rat ventricular myocytes from rat hearts and the protocol for equilibration and electrical field stimulation of cardiomyocytes has been described in detail in Chapter 2. Briefly, cardiomyocytes adhering to laminin coated glass coverslips were superfused at 37°C with Tyrode's buffer, containing 2 mM Ca^{2+}, and gassed with 100% O_2 for 3 min. Following this equilibration period, cells were field stimulated at a frequency of 1 Hz. After a further 2 min, cells were superfused with the FRGS. Three different FRGS were used to generate H_2O_2 alone, •OH alone, or a mixture of $O_2^{•-}$ and H_2O_2. The contractile activity of the cells was monitored following exposure to the FRGS by recording the number of cells displaying asynchronous contractile activity. As described in chapter 2, asynchronous activity was defined as cells contracting out of synchrony with the applied electrical field stimulation. Usually this was at a rate above that of the electrical stimulation and could easily be distinguished visually from synchronous contractile activity due to the fibrillatory nature of the contractions. The number of cells displaying asynchronous contractile activity was recorded for the duration of the exposure to the FRGS and plotted as "% Asynchronous" over time. This "ROS-induced arrhythmia assay" was a reproducible assay which could be used to determine the possible cardioprotective actions of various compounds including PUFAs. Data are expressed as mean ± SEM, determined from experiments performed in triplicate, ie three coverslips of cells were analysed per rat, with approximately 20 cells studied per coverslip. The 'n' value refers to the number of rats from which the data was derived. Significance was set at $p < 0.05$ and was determined using a t-test (with Welch's correction), a parametric one-way or two-way ANOVA as appropriate for the data set.

3.2.2 Acute Addition of ROS and the Effect of Free Fatty Acids

Cardiomyocytes contracting under electrical field stimulation were superfused with 15 μM DHA for 5 min prior to addition of 20 μM H_2O_2[2]. To investigate the effect of longer exposure to DHA, 15 μM DHA was added to the medium in which the cells were incubated (containing 0.1% (w/v) fatty acid free bovine serum albumin, BSA). Cells were then maintained at 37°C (in a CO_2 incubator) for periods of between 30 to 180 min and then superfused with the P/XO FRGS (2.3 mM purine plus 7 mU/ml xanthine oxidase[3]). To determine the effect of longer durations of incubation with n-3 fatty acids, cells were incubated with 25 μM DHA or 25 μM vitamin E (delivered to the cells with 0.1% (w/v) BSA) and maintained overnight in an incubator before being challenged with 20 μM H_2O_2 (superfusing). Cells were visually observed under a light microscope and assessed for arrhythmia induction by following the development of asynchronous contractile activity.

3.2.3 The Effect of 7 Days Gavage with Dietary Oils on ROS-Induced Arrhythmias

3.2.3.1 Animals and Feeding Protocol

To determine the effect of chronic n-3 PUFA supplementation, rats were fed fish oil, containing a high proportion of n-3 PUFAs for one week. Rats were obtained at 6-8 weeks of age and fed a normal laboratory rat chow *ad libitum*. At 9 weeks of age, they were further supplemented with 2 g/day dietary oils, consisting of olive oil (OO), canola oil (CO) or fish oil (FO) by gavage (described in Chapter 2). The OO and CO were obtained from Meadow Lea Foods (Ryde, NSW, Australia). The FO was from Nippon Suisan Kaisha Ltd. (Japan). Following one week of gavage, rats were sacrificed and

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[2] This experiment was performed after the first 21-day gavage study (3.2.4), hence the use of 20 μM H_2O_2 rather than G/GO as mentioned in 3.2.3.3.
[3] These experiments were performed after the second 21-day gavage study, which necessitated the use of higher concentrations of XO as outlined in 3.2.5.2.

Chapter Three

cardiomyocytes isolated as described in Chapter 2. Cells were maintained at 37°C in

DMEM (1 mM Ca^{2+}).

3.2.3.2 Fatty Acid Composition Analysis

The fatty acid analysis of the colony diet and dietary oils was carried out as described in

Chapter 2 (Bligh & Dyer, 1959). The composition is given in Table 3.1.

FAME[a]	REF	OO	CO	FO
14:0	1.79	n/d	0.07	6.03
14:1	0.40	n/d	n/d	0.21
16:0	18.73	11.86	4.67	8.93
16:1	1.37	0.39	0.19	11.10
18:0	8.08	1.78	1.80	0.97
18:1[b]	31.44	75.30	58.64	10.99
18:2 (n-6)	33.27	9.86	21.66	1.25
18:3 (n-3)	2.21	0.61	11.57	0.90
20:0	0.46	0.60	0.31	0.61
20:1	0.43	n/d	1.05	0.55
20:2	n/d	n/d	0.05	0.07
20:3 (n-6)	n/d	n/d	n/d	0.30
20:4 (n-6)	n/d	n/d	n/d	1.68
20:3 (n-3)	n/d	n/d	n/d	n/d
22:0	n/d	n/d	0.15	0.18
22:1	n/d	n/d	0.24	0.57
20:5 (n-3)	1.03	n/d	n/d	37.51
24:0	n/d	n/d	n/d	0.49
22:5 (n-3)	n/d	n/d	n/d	3.21
22:6 (n-3)	1.11	n/d	n/d	14.46
Σ SFA	29.06	14.24	7.00	17.20
Σ MUFA	33.64	75.69	60.12	23.42
Σ PUFA	37.61	10.47	33.27	59.38
Σ n-6	33.27	9.86	21.66	3.23
Σ n-3	4.35	0.61	11.57	56.07
n-6/n-3	7.65	16.08	1.87	0.06

Table 3.1: Fatty acid composition (wt%) of standard diet and dietary oils.
Data shown are means (n = 2). The shorthand notation for fatty acid structure is "a:b (n-c)" where "a" represents the total number of carbons in the fatty acyl chain, "b" the number of double bonds in the chain, and "c" represents the number of carbon atoms between the methyl end of the chain and the first double bond. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. aFAME, fatty acid methyl esters. b18:1 contains n-7 and n-9 isomers. Abbreviations: CO, canola oil; FO, fish oil; n/d not detectable; OO, olive oil; REF, reference group (normal laboratory chow).

The normal colony (REF) rat diet (containing 7.5% total fat) contained 29.1% saturated fatty acids (SFAs), 33.6% monounsaturated fatty acids (MUFAs) and 37.6% PUFAs. The PUFAs were mainly of the n-6 class. The OO contained mainly MUFAs, and like the REF diet, the PUFAs were mainly of the n-6 class. The CO (a high-oleic sunflower oil) was used as a source of short-chain n-3 PUFAs containing 11.6% α-linolenic acid (18:3 n-3, ALA) The main constituents of the FO (high in long-chain n-3 PUFAs) were EPA (37.5%) and DHA (14.5%).

3.2.3.3 ROS-Induced Asynchronous Contractile Activity

Following the normal procedure for equilibration and electrical field stimulation of cardiomyocytes (Chapter 2), cells were exposed to the FRGSs. Contractility and the development of asynchronous contractile behaviour was then monitored for 20 min. Approximately 20 cardiomyocytes were observed each time in triplicate (i.e. 3 coverslips) for each FRGS tested. H_2O_2 was generated by addition of 0.15 U/mL glucose oxidase (GO) to the Tyrode's buffer that contained 11 mM glucose. Glucose plus GO (G/GO) generates a constant flux of H_2O_2 of approximately 15 nmol/min/mL of reaction mix (Mohsen *et al.*, 1995). G/GO were pre-incubated together for 5.5 min prior to superfusing the cells. This duration was selected after testing a number of pre-incubation times and was chosen to give the highest $T_{50\%}$ (time until 50% of cells developed asynchronous contractile activity) with the shortest possible incubation time (for experimental efficiency). The reaction of 2.3 mM purine plus 0.01 U/mL xanthine oxidase was used to generate H_2O_2 and $O_2^{\bullet-}$. This reaction has also been shown to generate small amounts of •OH in isolated cardiomyocytes (Courtois *et al.*, 1998). Purine was used instead of xanthine as it has been reported to generate three times as much $O_2^{\bullet-}$ per mole compared with xanthine (Burton *et al.*, 1984). A similar FRGS containing 2 mM purine plus 0.02

U/ml xanthine was previously reported to yield $O_2^{\bullet-}$ generation at levels similar to in vivo $O_2^{\bullet-}$ production by activated neutrophils (cited by Massey & Burton (1990)). Hydroxyl radicals were generated via the Fenton reaction using 100 μM H_2O_2 plus 100 μM Fe^{2+}. The components of the •OH and the H_2O_2 plus $O_2^{\bullet-}$ generating systems were incubated together for 3 min prior to introducing them into the superfusion chamber. The results of this ROS-induced arrhythmia assay were plotted as the percentage of cells developing asynchronous activity over time. From this graph, the $T_{50\%}$ for the development of asynchronous contractile activity (arrhythmias) was determined.

3.2.4 The Effect of 21 Days Gavage with Dietary Oils on ROS-Induced Arrhythmias

3.2.4.1 Feeding Protocol and Fatty Acid Composition

Male Sprague Dawley rats were obtained at 8 weeks of age and maintained on a normal laboratory chow while they acclimatised in the CSIRO animal facility. At 9 weeks of age, they underwent 3 weeks of dietary lipid supplementation by gavage whilst still being fed the normal (REF) colony rat chow. Three weeks was chosen to optimise the incorporation of the n-3 PUFAs in the myocardial membrane phospholipids (based on the results of the previous study- 3.2.3). The lipid supplements (oils) used in this study were CO, FO and lard (saturated fat, SF). The complete fatty acid analysis of the REF diet and the supplemented oils is shown in Table 3.2.

FAME[a]	REF	SF	CO	FO
14:0	0.82	8.36	n/d	0.19
14:1	0.07	1.56	n/d	n/d
16:0	15.32	32.00	4.55	0.17
16:1	0.75	2.81	0.18	0.32
18:0	4.40	16.89	1.51	0.41
18:1[b]	23.60	35.71	58.80	4.87
18:2 (n-6)	46.20	1.34	21.76	n/d
18:3 (n-3)	4.57	0.68	12.06	0.50
20:0	0.47	0.65	0.17	0.16
20:1	0.39	n/d	1.06	10.70
20:3 (n-6)	0.04	n/d	n/d	0.15
20:4 (n-6)	0.02	n/d	n/d	1.71
20:3 (n-3)	n/d	n/d	n/d	n/d
22:0	0.30	n/d	n/d	n/d
22:1	0.12	n/d	n/d	0.73
20:5 (n-3)	0.96	n/d	n/d	50.47
24:0	0.22	n/d	n/d	0.88
24:1	0.06	n/d	n/d	n/d
22:5 (n-3)	0.15	n/d	n/d	1.28
22:6 (n-3)	1.18	n/d	n/d	27.54
Σ SFA	21.53	57.89	6.23	1.81
Σ MUFA	24.99	40.08	60.04	16.61
Σ PUFA	53.48	2.02	33.82	81.65
Σ n-6	46.46	1.34	21.76	1.86
Σ n-3	6.86	0.68	12.06	79.79
n-6/n-3	6.77	1.97	1.81	0.02

Table 3.2: Fatty acid composition (wt%) of standard (REF) diet and dietary oils.
Data shown are means (n=2). The shorthand notation for fatty acid structure was given in Table 3.1 legend. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-7 and n-9 isomers. Abbreviations: CO, canola oil; FO, fish oil; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acid; REF, Reference diet; SF, saturated fat; SFA, saturated fatty acids; n/d not detectable.

3.2.4.2 ROS-Induced Asynchronous Contractile Activity

In the previous study, the reaction of G/GO was used to generate H_2O_2 rather than superfusing with H_2O_2 alone, since G/GO has been reported to generate a constant flux of H_2O_2 (Mohsen *et al.*, 1995). However, G/GO resulted in quite severe cellular injury resulting in 100% asynchronous contractile activity by 10 min of superfusion with this FRGS. Thus it was necessary to prolong the $T_{50\%}$ in order to allow the fish oil to exert its

possible beneficial effects. If the damaging actions of the FRGS were too severe, any likely protective effect would not be able to be distinguished. Therefore, in this study, H_2O_2 alone was used, as it was more amenable to dose-response experiments compared with a two-component FRGS. A concentration of 20 µM was used, this value being determined from dose-response experiments (Figure 3.7). The concentrations of the components of the FRGS for generating H_2O_2 and $O_2^{\bullet-}$ were also altered based on dose-response experiments designed to raise the $T_{50\%}$ to a higher value (Figure 3.8). The final reaction consisted of 2.3 mM Purine and 0.005 U/ml xanthine oxidase to generate H_2O_2 and $O_2^{\bullet-}$. These concentrations are still in the range of those used in other studies, however, by lengthening the time scale of the induction of asynchronous contractile activity, they allowed the examination of the possible protective effect of the FO on the above parameter. It was possible that in the previous study, the free radical insult may have been too severe to be able to distinguish a protective effect, since the arrhythmias were developing very soon after the addition of the FRGS. The evaluation of arrhythmia was as described in the previous study.

3.2.5 The Effect of 21 Days Dietary Lipid Gavage Together With a Low-ALA Diet on ROS-Induced Arrhythmias.

3.2.5.1 Animals and Feeding Protocol

In this study, rats were obtained at 4 weeks of age and fed a modified AIN 93G diet (Chapter 2) formulated to contain minimal levels of α-linolenic acid (ALA). The final fatty acid analysis of this new "low-ALA" diet, containing 7% fat as Sunola oil (Table 3.3), indicated that the only n-3 PUFA present was ALA (0.7% of total fatty acids), in comparison to 4.57% ALA in the "high ALA" colony rat chow used in the previous study (Table 3.2).

Major FAME[a]	Low-ALA (n=2)	Sunola oil (n=2)	SF (n=3)	FO (n=3)
14:0	0.29	0.06	6.67 ± 0.14	8.07 ± 0.44
16:0	4.79	3.85	30.61 ± 0.21	15.50 ± 0.67
16:1	0.11	0.09	1.89 ± 0.80	10.13 ± 0.36
18:0	4.07	3.18	18.53 ± 0.21	2.64 ± 0.09
18:1[b]	75.25	75.29	35.97 ± 0.61	15.58 ± 0.74
18:2 (n-6)	13.27	15.95	1.78 ± 0.03	2.89 ± 0.11
18:3 (n-3)	0.68	0.47	0.65 ± 0.03	1.13 ± 0.14
20:4 (n-6)	n/d	n/d	0.06 ± 0.00	1.26 ± 0.05
20:5 (n-3)	n/d	n/d	0.07 ± 0.00	24.33 ± 0.48
22:5 (n-3)	n/d	n/d	n/d	2.28 ± 0.02
22:6 (n-3)	n/d	n/d	n/d	12.14 ± 0.07
Σ SFA	10.43	7.97	58.04 ± 0.54	27.31 ± 0.82
Σ MUFA	75.50	75.61	39.39 ± 0.57	28.16 ± 1.43
Σ PUFA	13.96	16.42	2.56 ± 0.09	44.57 ± 0.69
Σ n-6	13.27	15.95	1.84 ± 0.06	4.45 ± 0.14
Σ n-3	0.68	0.47	0.72 ± 0.05	40.00 ± 0.69
n-6/n-3	19.38	33.94	2.55 ± 0.10	0.11 ± 0.00

Table 3.3: Fatty acid composition of diets and oils.
The high ALA diet was the colony diet (Table 3.2), the Sunola oil was present in the low-ALA diet (7% w/w). The fatty acid analysis of the gavaged oils is shown in the two right hand columns (SF and FO). The shorthand notation for fatty acid structure was given in Table 3.1 legend. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains the n-7 and n-9 isomers. n/d not detectable. Abbreviations: FO, fish oil; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SF, saturated fat; SFA, saturated fatty acids. The SF supplement was the same as that used in the previous study (from Metro Quality Foods, Greenacres, NSW, Australia) and the FO was RoPUFA from Hoffmann La Roche (Basel, Switzerland).

At 9 weeks of age, rats were gavaged with approximately 2 g oil/day for 3 weeks in addition to consuming the low-ALA diet *ad libitum*. The gavage oils were FO and SF. A third group of rats were sham gavaged (reference group, REF) with a feeding tube coated in glycerol to control for any stress arising from the gavaging protocol.

3.2.5.2 ROS-Induced Asynchronous Contractile Activity

At the beginning of the study, it was found that 20 μM H_2O_2, which was used in the previous study, was not resulting in significant asynchronous contractile activity during

the 20 min duration of the study. This necessitated raising the H_2O_2 concentration to 30 µM, which reproducibly resulted in asynchronous contractile activity with a $T_{50\%}$ similar to that observed previously. Similarly, the FRGS for generating H_2O_2 and $O_2^{\bullet-}$ was adjusted to 2.3 mM purine and 7 mU/ml xanthine oxidase. The determination of arrhythmia was as used in the previous study.

3.2.6 Myocardial Vitamin E analysis

Vitamin E was analysed by a modification of the method of Katsanidis and Addis as described in Chapter 2 (Katsanidis & Addis, 1999).

3.3 Results

3.3.1 Acute Addition of ROS and Fatty Acids

Following isolation and plating onto laminin coated glass coverslips, myocytes were quiescent, rod shaped and displayed clear, cross-striations. When the cells were superfused with Tyrode's solution, containing 2 mM Ca^{2+}, they remained quiescent and did not display spontaneous contractile activity, indicating that they were calcium tolerant under these conditions. Following electrical field stimulation, greater than 90% of cells adhering to the coverslips displayed regular contractile activity, in synchrony with the stimulation. Cells contracted by about 2-5% of their resting cell length under basal conditions. As shown in Figure 3.1, after 2 min of exposure to 20 μM H_2O_2, cells began to develop asynchronous contractile activity. After 20 min of exposure to H_2O_2, 95.3 ± 2.9% of the control cells (superfused with Tyrode's buffer containing 0.05% ethanol (v/v)) were contracting in an asynchronous manner, compared to 85.7 ± 5.7% of cells that had been superfused for 5 min with DHA prior to the addition of H_2O_2 (n=7; ns, *t-test*). There was no cardioprotective effects of acute addition of DHA, with there being no difference in the time taken for 50% of the cells to develop asynchronous contractile activity ($T_{50\%}$ = 11.4 ± 0.4 min) compared to the control cells (12.5 ± 0.1 min), n=7.
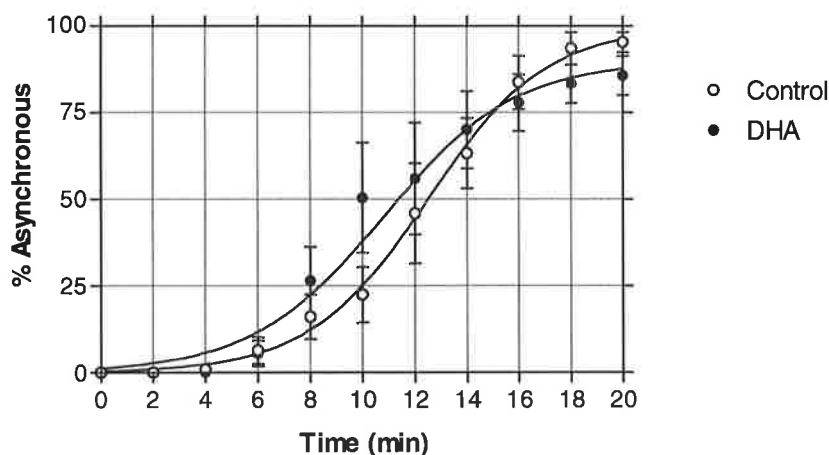
Figure 3.1: Development of asynchronous contractile activity in cells following 20 μM H_2O_2 addition.
Cells were superfused with Tyrode's solution containing 2 mM Ca^{2+}. H_2O_2 (hydrogen peroxide) was added to the cells at time = zero min. Data are averages from triplicate experiments consisting of approximately 20 cells per rat, n = 7 rats, as described in 3.2.1.

Similarly, following longer incubations (up to 180 min) with DHA, there was no significant cardioprotection observed from ROS-induced asynchronous contractile activity (Figure 3.2).
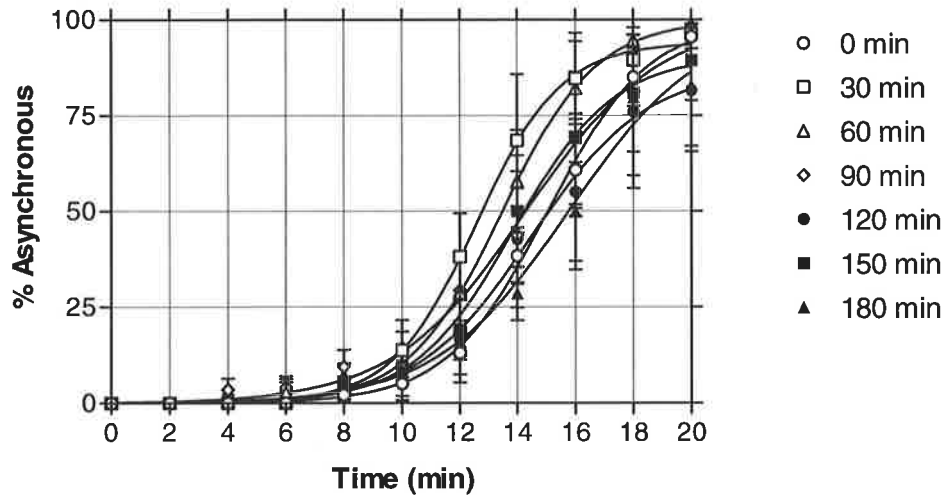
Figure 3.2: Development of asynchronous contractile activity in cells after addition of 2.3 mM purine plus 7 mU/ml xanthine oxidase.
Cells were incubated with 15 µM DHA (in culture medium containing 0.1% (w/v) BSA) for durations up to 3 hours prior to addition of the FRGS. Cells were superfused with Tyrode's solution containing 2 mM Ca^{2+}. Data are averages from approximately 20 cells per rat, performed in triplicate. n = 4-5 rats.

However, overnight incubation with 25 µM DHA (in 0.1% (w/v) fatty-acid free BSA) resulted in a significant right-shift of the asynchrony development curve following a challenge with 20 µM H_2O_2 (Figure 3.3).
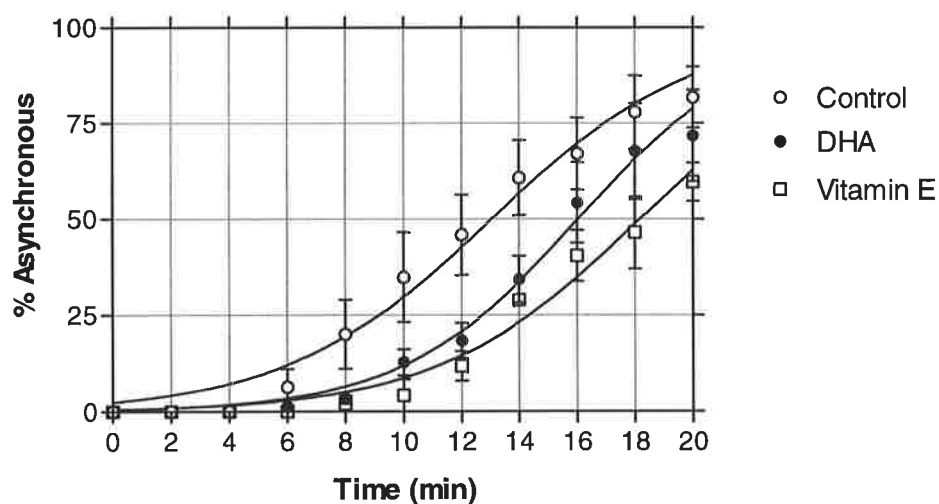
Figure 3.3: The effect of overnight incubation with DHA and Vitamin E on the development of asynchronous contractile activity following addition of 20 µM H_2O_2.
Cells were pre-incubated with 25 µM fatty acid/antioxidant overnight (in 0.1% (w/v) BSA) and superfused with Tyrode's buffer containing 2 mM Ca^{2+}. Data are averages from approximately 20 cells per rat, performed in triplicate. n = 3-6 rats.

Chapter Three

The effect of DHA was similar to that observed with 25 μM vitamin E. The $T_{50\%}$ was 13.0 ± 0.3 min for control cells (n=6) which was significantly shorter compared to 16.0 ± 0.2 min for cells incubated with DHA (n=5, p<0.05) and 18.2 ± 0.3 min (p<0.05) for vitamin E.

3.3.2 The Effect of 7 Days Gavage with Dietary Oils on ROS-Induced Arrhythmias

3.3.2.1 Body Weights

Following one week of dietary lipid supplementation by gavage, the body weights of the rats in the three lipid-supplemented groups were not significantly different (ANOVA). The rats gavaged with OO weighed 446.4 ± 18.6g (n=8) compared to 438.1 ± 10.4g (n=7) for rats gavaged with CO and 436.6 ± 21.5g (n=8) for the FO group.

3.3.2.2 Myocardial Fatty Acids

The fatty acid composition of myocardial total phospholipids from rats fed the OO, CO and FO diets are shown in Table 3.4. Following 7 days of gavage, there was no significant difference in the proportion of SFAs, MUFAs or PUFAs between the three groups. However, the dietary supplementation did induce changes in the proportions of some of the fatty acids within each class of fatty acid. Compared with the OO group, proportions of 16:0 and 18:0 were significantly higher in the CO and FO groups (p<0.01 and p<0.05 respectively). The proportion of LA in the CO group was significantly increased compared to the OO group (p<0.05). In the FO group, LA was significantly decreased compared to the OO group (p<0.05). Correspondingly, the fish oil group contained higher proportions of the n-3 PUFAs compared to both the OO group (p<0.001) and the CO group (p<0.001). This was attributable to relatively higher levels of EPA, DPA, and DHA present in the cardiac phospholipids of the FO group. The major effect of dietary FO

supplementation was apparent with regard to the ratio of the total n-6 to n-3 PUFAs, which was significantly decreased in the FO group compared to the OO group ($p<0.001$).

FAME[a]	OO (n=7)	CO (n=8)	FO (n=8)
14:0	0.24 ± 0.06	0.16 ± 0.02	0.35 ± 0.07
16:0	21.35 ± 1.20	18.64 ± 0.32[e]	17.42 ± 2.53[h]
18:0	21.69 ± 0.62	23.31 ± 0.15[c]	23.39 ± 0.44[f]
18:1[b]	13.92 ± 0.74	11.46 ± 0.32	11.03 ± 1.20
18:2 (n-6)	10.72 ± 0.62	12.99 ± 0.54[c]	8.66 ± 0.36[f,k]
18:3 (n-3)	1.40 ± 0.92	0.18 ± 0.03	0.09 ± 0.05
20:0	n/d	0.27 ± 0.07	0.22 ± 0.04
20:1	n/d	0.10 ± 0.04	0.03 ± 0.02
20:2	n/d	0.16 ± 0.06	0.11 ± 0.03
20:3 (n-6)	0.16 ± 0.06	0.38 ± 0.09	0.18 ± 0.04
20:4 (n-6)	19.93 ± 0.68	20.71 ± 0.37	19.83 ± 0.83
20:3 (n-3)	0.05 ± 0.05	0.04 ± 0.04	0.00 ± 0.00
22:0	0.02 ± 0.02	0.15 ± 0.06	0.16 ± 0.05
22:1	n/d	n/d	0.01 ± 0.01
20:5 (n-3)	0.04 ± 0.03	0.20 ± 0.02	2.05 ± 0.15[h,k]
24:0	0.10 ± 0.07	0.29 ± 0.03	0.25 ± 0.07
24:1	0.05 ± 0.05	0.09 ± 0.02	0.09 ± 0.04
22:5 (n-3)	1.38 ± 0.08	1.93 ± 0.11[d]	2.74 ± 0.08[h,j]
22:6 (n-3)	8.93 ± 0.25	8.95 ± 0.46	13.40 ± 0.49[h,k]
Σ SFA	43.41 ± 1.07	42.81 ± 0.20	41.80 ± 2.20
Σ MUFA	13.97 ± 0.72	11.65 ± 0.35	11.16 ± 1.19
Σ PUFA	42.61 ± 1.63	45.54 ± 0.43	47.04 ± 1.52
Σ n-6	30.81 ± 1.24	34.08 ± 0.33	28.66 ± 1.08[j]
Σ n-3	11.80 ± 0.80	11.30 ± 0.54	18.27 ± 0.52[h,k]
n-6/n-3	2.66 ± 0.16	3.07 ± 0.16	1.57 ± 0.04[h,k]

Table 3.4: Fatty acid composition of ventricular phospholipids from rats gavaged with OO, CO or FO Data shown are mean ± SEM for the number of animals indicated in brackets. The shorthand notation for fatty acid structure was given in the legend to Table 3.1. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. n/d not detected. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-7 and n-9 isomers. [c]$p<0.05$, [d]$p<0.01$, [e]$p<0.001$ for OO vs CO; [f]$p<0.05$, [g]$p<0.01$, [h]$p<0.001$ for OO vs FO; [i]$p<0.05$, [j]$p<0.01$, [k]$p<0.001$ for CO vs FO (by ANOVA with Bonferonni multiple comparison test). Abbreviations: CO, canola oil group; FO, fish oil group; OO, olive oil group.

3.3.2.3 ROS-Induced Asynchronous Contractile Activity

In response to a FRGS of G/GO, which generates H_2O_2, cells rapidly developed asynchronous contractile activity, and by 11 min after addition, 100% of cells isolated from animals in all 3 dietary groups were contracting in an asynchronous manner (Figure

3.4). There was no significant protective effect of the FO in delaying the onset of asynchronous contractile activity and thus the $T_{50\%}$ was not significantly different in the three groups.
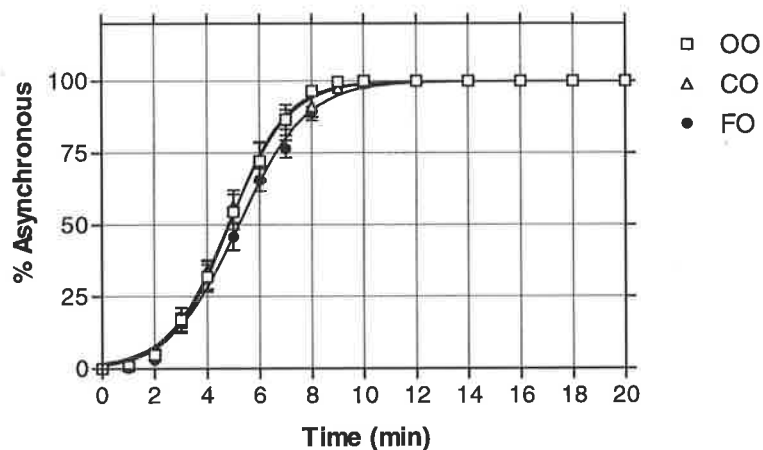
Figure 3.4: Development of asynchronous contractile activity in response to a G/GO FRGS.
Cells were isolated from rats gavaged with), or and exposed to G/GO (11 mM/0.15 U/ml) at time = zero min[4]. Each point is the average of approximately 20 cells, performed in triplicate. n = 7-8 rats. Abbreviations: CO, Canola oil; FO, fish oil; FRGS, free radical generating system; G/GO, glucose plus glucose oxidase; OO, olive oil.

Addition of the •OH generating system also induced a similar asynchrony curve (Figure 3.5), however, there was a trend towards a protective effect with FO evident by a right-shift in the asynchrony curve.
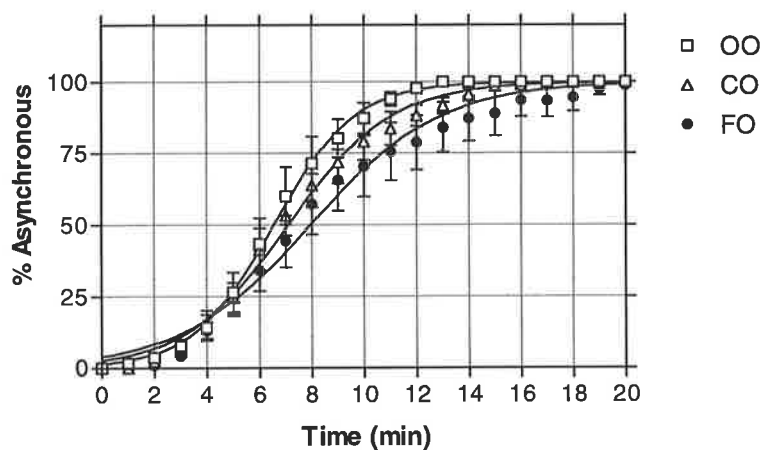
Figure 3.5: Development of asynchronous contractile activity in response to •OH.
The FRGS was 100 µM H_2O_2 plus 100 µM Fe^{2+}. Each point is the average of approximately 20 cells, performed in triplicate. n = 7-8 rats, ns (ANOVA). Abbreviations: CO, canola oil; FO, fish oil; OO, olive oil.

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[4] It should be noted that in this, and all subsequent Figures, time '0' (x-axis) refers to the point of introduction of the free radical generating system.
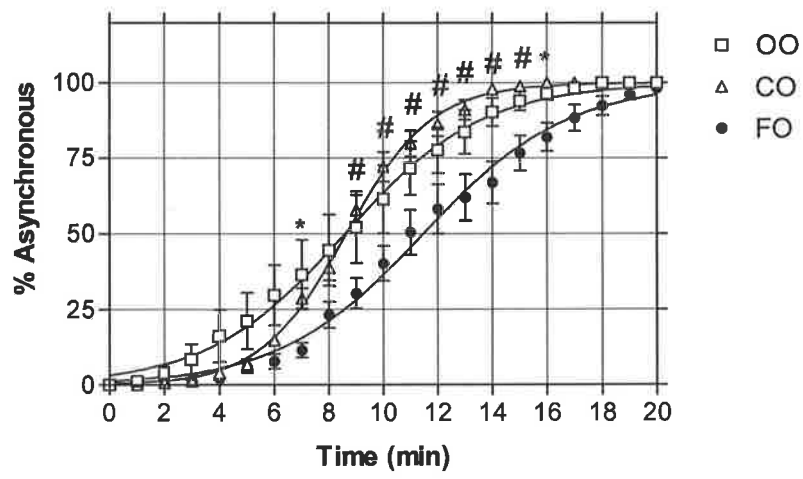
In the FO group[5], the $T_{50\%}$ was 7.9 ± 0.2 min (n=8), which was significantly higher than both the OO group (6.5 ± 0.1 min, n=8, p<0.001) and the CO group (7.1 ± 0.1 min, n=7, p<0.01). The response of cardiomyocytes to $O_2^{\bullet-}$ and H_2O_2 generated by the reaction of P/XO is shown in Figure 3.6. There was a protective effect of FO in delaying the development of asynchronous contractile activity indicated by a significant right-shift of the asynchrony curve. Between 9 and 15 min after addition of P/XO, the number of FO cells contracting in an asynchronous manner was significantly less than the OO group (p<0.001). Accordingly, the $T_{50\%}$ for the FO group (11.5 ± 0.1 min, n=8) was significantly longer than both the OO (8.6 ± 0.1 min, n=8) and CO groups (8.6 ± 0.05 min, n=7), p<0.001.
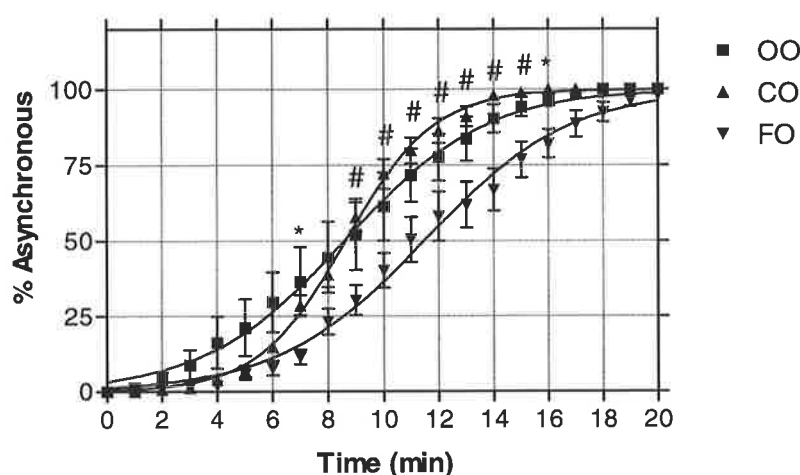
Figure 3.6: Development of asynchronous contractile activity in response to H_2O_2 and $O_2^{\bullet-}$.
The FRGS was 2.3 mM purine plus 0.01 U/ml xanthine oxidase. Each point is the average of approximately 20 cells, performed in triplicate, n = 7-8 rats. #p<0.001, *p<0.05 for FO vs OO (ANOVA). Abbreviations: CO, canola oil; FO, fish oil; OO, olive oil.

3.3.3 The Effect of 21 Days Gavage with Dietary Oils on ROS-Induced Arrhythmias

3.3.3.1 Body Weights

The body weights of the rats following three weeks lipid gavage was not significantly different between the three gavage groups. The average weight for the rats receiving SF

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[5] Henceforth the term CO group or CO cells will be used interchangeably to refer to cells isolated from rats fed CO. The same will apply for cells isolated from rats fed FO, OO or SF.

was 467.9 ± 11.1g (n=8) compared to 458.0 ± 14.0g (n=8) for the CO group and 460.1 ± 18.7g (n=7) for rats gavaged with FO.

3.3.3.2 Fatty Acid Composition of Rat Ventricular Phospholipids

The fatty acid composition of rat ventricular phospholipids after dietary lipid supplementation is shown in Table 3.5. The proportion of SFAs in the CO group was significantly lower than both the SF ($p < 0.01$) and FO groups ($p < 0.05$). The levels of MUFAs was lowest in the FO group.

FAME[a]	SF	CO	FO
14:0	0.18 ± 0.01	0.09 ± 0.02^d	0.11 ± 0.01^g
16:0	16.52 ± 0.36	14.81 ± 0.98	14.61 ± 0.77
17:0	0.41 ± 0.07	0.34 ± 0.06	0.44 ± 0.01
18:0	27.17 ± 0.43	26.13 ± 0.63	28.56 ± 0.47^i
18:1	5.90 ± 0.51	8.05 ± 0.53^c	4.71 ± 0.20^k
18:2 (n-6)	11.21 ± 0.23	12.11 ± 0.48	9.94 ± 0.70^i
18:3 (n-3)	0.07 ± 0.003	0.13 ± 0.01^e	0.06 ± 0.003^k
20:0	0.14 ± 0.003	0.43 ± 0.21	0.15 ± 0.01
20:1	n/d	0.14 ± 0.03^e	0.08 ± 0.01^h
20:2	0.18 ± 0.02	0.17 ± 0.01	0.14 ± 0.01
20:3 (n-6)	0.34 ± 0.02	0.40 ± 0.02	0.30 ± 0.02^j
20:4 (n-6)	20.59 ± 0.45	21.12 ± 0.63	19.73 ± 0.34
20:5 (n-3)	0.43 ± 0.04	0.57 ± 0.04	1.91 ± 0.14^{h,k}
24:0	0.29 ± 0.02	0.22 ± 0.02	0.26 ± 0.02
22:5 (n-3)	2.51 ± 0.06	2.35 ± 0.06	2.50 ± 0.08
22:6 (n-3)	14.13 ± 0.61	13.03 ± 0.58	16.56 ± 0.71^{f,j}
Σ SFA	44.64 ± 0.27	42.03 ± 0.74^d	44.12 ± 0.46^i
Σ MUFA	5.97 ± 0.51	8.09 ± 0.54^c	4.74 ± 0.19^k
Σ PUFA	49.37 ± 0.46	49.87 ± 0.77	51.14 ± 0.56
Σ n-6	32.15 ± 0.29	33.63 ± 0.60	29.98 ± 0.41^{f,k}
Σ n-3	17.11 ± 0.60	16.08 ± 0.60	21.03 ± 0.85^{g,k}
n-6/n-3	1.89 ± 0.08	2.11 ± 0.09	1.44 ± 0.08^{g,k}

Table 3.5: Fatty acid composition (wt%) of ventricular cardiomyocyte total phospholipids following 3 weeks gavage.
Data are mean ± SEM of 6 rats. The shorthand notation for fatty acid structure was given in Table 3.1 legend. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. n/d not detected. [a]FAME, fatty acid methyl esters, [b]18:1 contains n-7 and n-9 isomers. ^{c}p<0.05, ^{d}p<0.01, ^{e}p<0.001 for SF vs CO; ^{f}p<0.05, ^{g}p<0.01, ^{h}p<0.001 for SF vs FO; ^{i}p<0.05, ^{j}p<0.01, ^{k}p<0.001 for CO vs FO (by ANOVA with the Bonferroni multiple comparisons test). CO, canola oil; FO, fish oil; SF, saturated fat.

It must be noted that the n-3 PUFA content of the myocardial phospholipids of SF and CO dietary supplemented animals was very similar. This is in contrast to the very different fatty acid profiles of these two supplements (Table 3.2). Furthermore, the extent of increase in the n-3 PUFA content of ventricular phospholipids of the FO group, in comparison to the other dietary groups, although statistically significant, was well below that expected from the previous study. Additionally, it is important to note from this data that irrespective of dietary lipid supplement, the ventricular phospholipid DHA content was relatively high, and approximated the levels found in the myocardium of rats gavaged for one week with FO (Table 3.4). In view of this observation, the phospholipid fatty acid composition of hearts from rats maintained on the colony diet but *without* supplementation of any other oils was analysed. As shown in Table 3.6, the myocardial phospholipid fatty acid profile for rats on the colony diet was very similar to that observed for all dietary lipid supplemented groups of rats with regard to the proportion of n-3 PUFAs present, and in particular, the relatively high proportion of DHA. Furthermore, fatty acid analysis of the rat colony diet (Table 3.2) revealed that the ALA content of this diet was much higher than previously observed with such diets. This observation could therefore explain these results, since the elongation and desaturation of ALA could quite possibly give rise to high levels of DHA, which was indeed observed in the myocardial phospholipids.

FAME[a]	Colony rats
14:0	0.08 ± 0.08
16:0	14.21 ± 0.12
18:0	23.41 ± 0.45
18:1 (n-9)	3.27 ± 0.16
18:1 (n-7)	2.96 ± 0.12
18:2 (n-6)	19.97 ± 0.63
18:3 (n-3)	0.06 ± 0.06
20:2	0.75 ± 0.44
20:3 (n-6)	0.25 ± 0.01
20:4 (n-6)	18.78 ± 0.68
20:5 (n-3)	0.06 ± 0.06
22:5 (n-3)	1.83 ± 0.06
22:6 (n-3)	14.26 ± 0.30
Σ SFA	37.77 ± 0.73
Σ MUFA	6.23 ± 0.28
Σ PUFA	55.96 ± 0.49
Σ n-6	39.00 ± 0.06
Σ n-3	16.22 ± 0.11

Table 3.6: Fatty acid composition (wt%) of ventricular cardiomyocyte phospholipids from rats fed the reference (colony diet).
Data shown are mean ± SEM for 2 animals. The shorthand notation for fatty acid structure was given in the legend of Table 3.1. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. n/d not detected. [a]FAME, fatty acid methyl esters.

3.3.3.3 ROS-Induced Asynchronous Contractile Activity

The two FRGSs used in the present study were either 20 μM H_2O_2, or a combination of

purine (2.3 mM) and xanthine oxidase (5 mU/ml) to generate H_2O_2 and $O_2^{\bullet -}$.
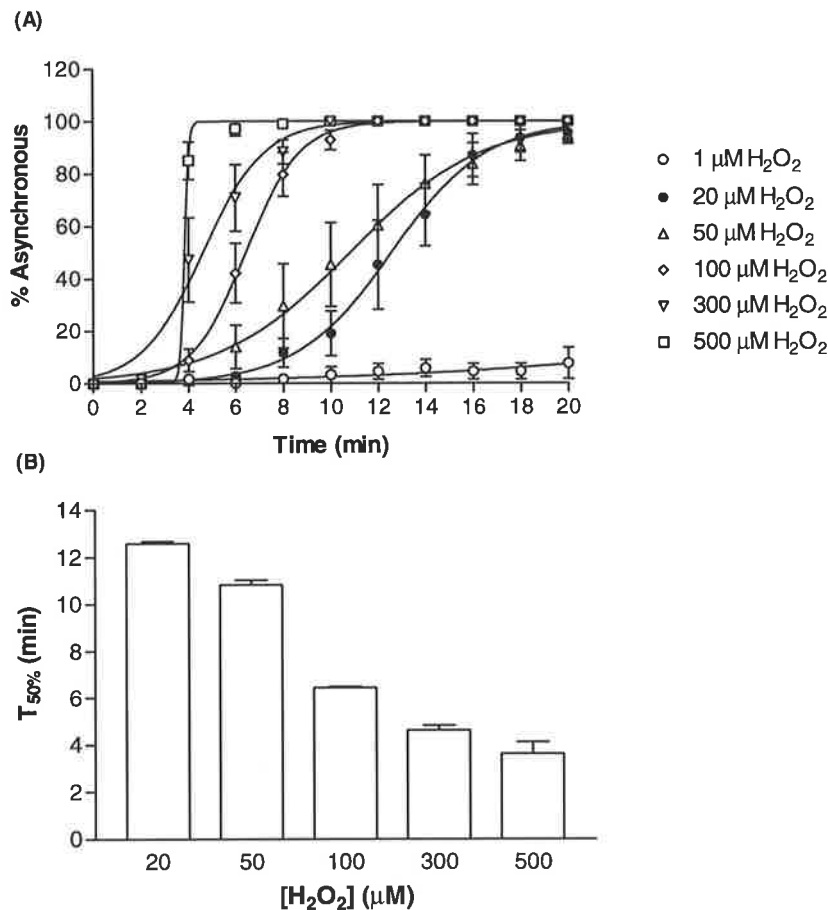
Figure 3.7: Dose response curves to various concentrations of H_2O_2.
Curves demonstrate (A) the different rates of development of asynchronous contractile activity in adult rat ventricular myocytes and (B) the $T_{50\%}$ in response to increasing concentrations of H_2O_2. Dilutions of H_2O_2 were prepared in Tyrode's buffer containing 2 mM Ca^{2+}. Each point is the average of approximately 20 cells, n=4-6 rats. Abbreviations: H_2O_2, hydrogen peroxide; $T_{50\%}$, time taken until 50% of cells are asynchronously contracting.

The concentrations of the FRGS were chosen based on data derived from dose curves (of

concentrations of reagents versus induction of asynchronous contractile activity) shown in

Figures 3.7 and 3.8. Both systems induced reproducible asynchronous contractile activity

in the cardiomyocyte bioassay system. As shown in Figure 3.9, there was no significant

difference in the development of asynchronous contractile activity or the $T_{50\%}$ value in

response to H_2O_2 between cells isolated from rats in the three dietary groups.

(A)

(B)
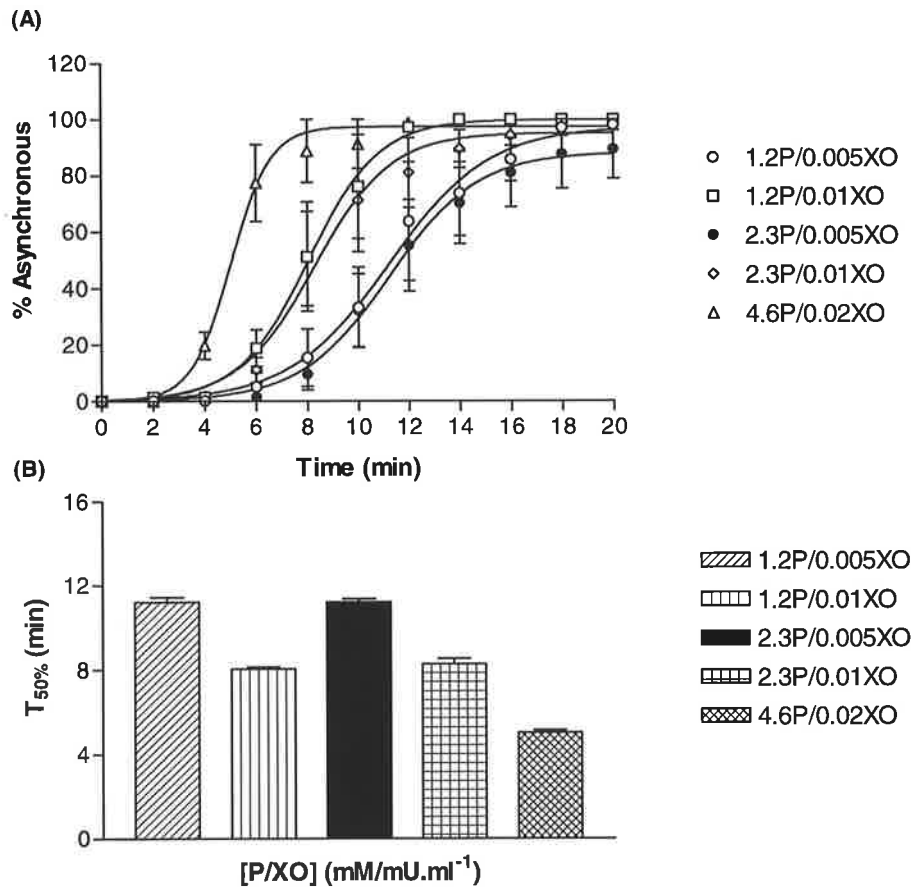
Figure 3.8: Dose curves of various combinations of P/XO.
Curves demonstrate the different rates of development of asynchronous contractile activity. Each point is the average of approximately 20 cells, n = 4-6 rats. Purine concentrations are in mM, xanthine oxidase concentrations are in mU/ml. Abbreviations P, purine; $T_{50\%}$, time taken until 50% of cells are asynchronously contracting; XO, xanthine oxidase.
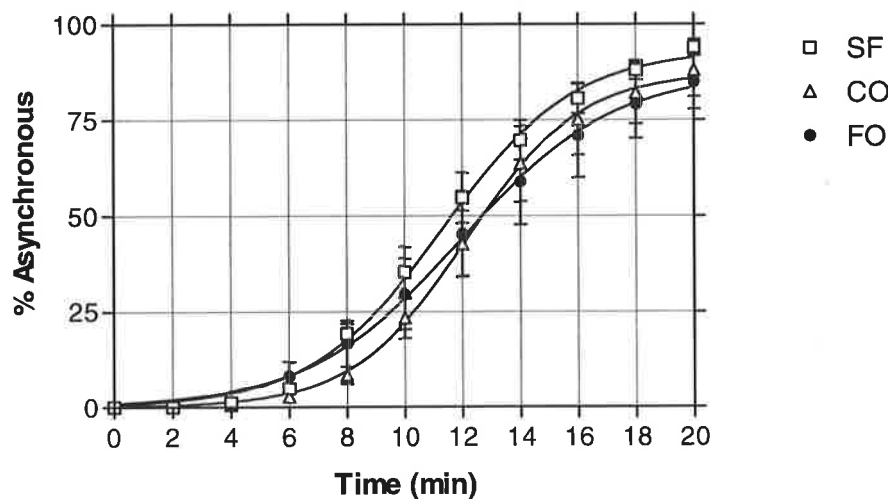
Figure 3.9: Development of asynchronous contractile activity in response to addition of 20 μM H_2O_2.
Each point is the average of approximately 20 cells, performed in triplicate. n = 8 rats, ns (ANOVA). Abbreviations: CO, canola oil; FO, fish oil; SF, saturated fat diet.

Following superfusion with P/XO (Figure 3.10), the $T_{50\%}$ was 14.0 ± 0.2 min for the FO

group, which was significantly longer compared with both the CO group (13.0 ± 0.2 min,

$p<0.01$) and the SF group (12.2 ± 0.2 min, $p<0.001$).
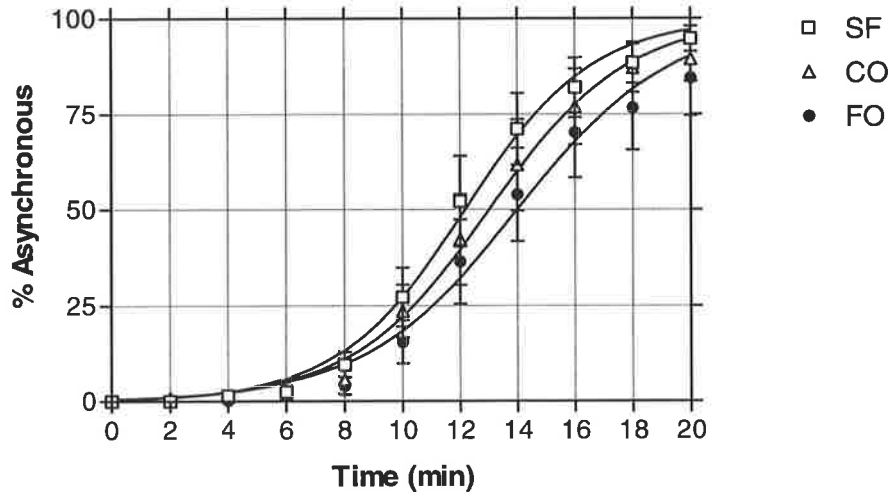
Figure 3.10: Development of asynchronous contractile activity in response to P/XO.
Cells were exposed to a FRGS of 2.3 mM purine (P) and 0.005 mU/ml xanthine oxidase (XO). Each point is the average of approximately 20 cells, in triplicate. n = 8 rats, ns (ANOVA). Abbreviations: CO, canola oil; FO, fish oil; SF, saturated fat diet.

3.3.4 The Effect of 21 Days Dietary Lipid Gavage and a Low-ALA Diet on ROS Induced Arrhythmias.

3.3.4.1 Body weights

The body weights of the rats were recorded throughout the 5 weeks of administration of

the low-ALA diet feeding and 3 weeks of lipid gavage.
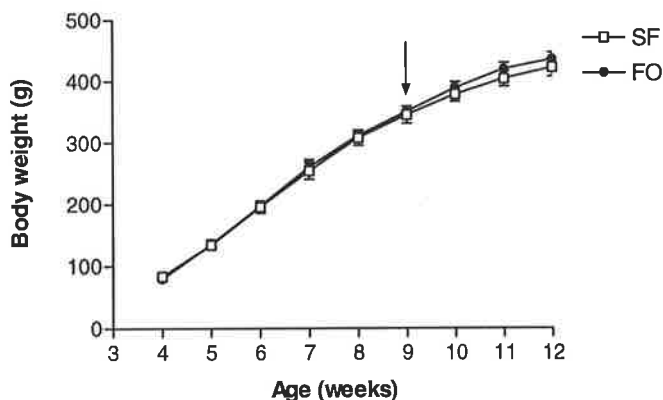
Figure 3.11: Changes in body weight of rats during growth (low-ALA diet) and lipid gavage.
The arrow indicates when the gavaging began. Rats were maintained on the low-ALA diet during the gavaging period. Data are mean $\pm$ SEM for 6-7 rats per dietary group.

Chapter Three

The rats were randomly assigned to receive a particular gavage oil, and as summarised in Figure 3.11, there was no significant difference in the rate of growth or final body weights in rats gavaged with SF compared with FO.

3.3.4.2 Fatty Acid Analysis of Total Ventricular Phospholipids

The fatty acid analysis of the myocardial ventricular phospholipids is shown in Table 3.7.

FAME[a]	REF	SF	FO
14:0	0.13 ± 0.02	0.27 ± 0.09	0.21 ± 0.02
14:1	0.07 ± 0.01	0.29 ± 0.12	0.15 ± 0.02
16:0	16.84 ± 0.34	16.06 ± 0.51	15.89 ± 0.39
16:1	0.32 ± 0.04	0.76 ± 0.47	0.25 ± 0.07
17:0	0.23 ± 0.01	0.36 ± 0.04[c]	0.41 ± 0.04[f]
18:0	24.75 ± 0.49	24.45 ± 0.71	25.28 ± 0.63
18:1[b]	15.38 ± 0.92	14.36 ± 0.56	10.13 ± 1.11[f,h]
18:2 (n-6)	6.29 ± 0.19	5.37 ± 0.16	3.72 ± 0.25[g,j]
18:3 (n-3)	0.34 ± 0.27	0.11 ± 0.05	n/d
20:0	0.19 ± 0.01	0.16 ± 0.02	0.21 ± 0.03
20:1	0.12 ± 0.01	0.10 ± 0.02	0.08 ± 0.001
20:2	0.63 ± 0.05	0.51 ± 0.04	0.06 ± 0.01[g,j]
20:3 (n-6)	0.19 ± 0.01	0.21 ± 0.03	0.21 ± 0.02
20:4 (n-6)	29.60 ± 0.36	28.66 ± 1.17	24.47 ± 1.34[e,h]
20:3 (n-3)	0.21 ± 0.01	n/d	0.20 ± 0.01
22:0	0.30 ± 0.01	0.15 ± 0.06	0.25 ± 0.06
20:5 (n-3)	0.18 ± 0.07	0.32 ± 0.17	3.25 ± 0.79[g,i]
24:0	0.76 ± 0.16	0.54 ± 0.08	0.18 ± 0.05[f]
22:5 (n-3)	0.41 ± 0.14	1.32 ± 0.22[d]	2.45 ± 0.07[g,j]
22:6 (n-3)	3.67 ± 0.65	6.70 ± 0.87	13.11 ± 0.53[g,j]
Σ SFA	43.12 ± 0.53	41.76 ± 0.85	42.29 ± 0.73
Σ MUFA	15.87 ± 0.93	15.38 ± 0.98	10.50 ± 1.13[f,h]
Σ PUFA	41.01 ± 0.48	42.85 ± 0.99	47.21 ± 1.53[f,h]
Σ n-6	36.08 ± 0.45	34.24 ± 1.17	28.36 ± 1.14[g,i]
Σ n-3	4.30 ± 0.82	8.10 ± 1.03[c]	18.84 ± 0.64[g,j]
n-6/n-3	8.39 ± 1.43	4.23 ± 1.05[c]	1.50 ± 0.06[g]

Table 3.7: Fatty acid composition (wt%) of ventricular cardiomyocyte total phospholipids following 3 weeks gavage.
Data are mean ± SEM of 6 rats. The shorthand notation for fatty acid structure was given in the legend of Table 3.1. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. n/d not detected. [a]FAME, fatty acid methyl esters, [b]18:1 contains n-7 and n-9 isomers, [c]$p<0.05$, [d]$p<0.01$ for REF vs SF; [e]$p<0.05$, [f]$p<0.01$, [g]$p<0.001$ for REF vs FO; [h]$p<0.05$, [i]$p<0.01$, [j]$p<0.001$ for SF vs FO (by ANOVA with the Bonferroni multiple comparisons test). Abbreviations: FO, fish oil; REF, reference diet (low-ALA diet) + sham gavage; SF, saturated fat.

The use of the "low-ALA" diet aimed to reduce, as much as possible, the elongation of dietary ALA to the longer chain n-3 PUFAs (DHA and EPA) which was observed in the previous dietary study (3.3.3.2) due to the relatively higher levels of ALA in the base diet. Hence, this enabled a better comparison of the possible protective effects of supplementation with dietary DHA. The levels of n-3 PUFAs in the myocardial membranes of rats fed the 7% ALA ("low-ALA") Sunola oil diet (REF) were in fact lowered following feeding with the ALA diet, the sum of total n-3 PUFAs in these rats being reduced to 4.3 ± 0.82%. The levels of SFAs were not significantly different in the myocardial phospholipids of rats in the three groups. However, the levels of MUFAs in the FO group were significantly lower than both the REF ($p < 0.01$) and the SF groups ($p < 0.05$). Correspondingly, there were significantly higher proportions of PUFAs in the FO group compared to the REF ($p < 0.01$) and the SF groups ($p < 0.05$).

3.3.4.3 Viability of Cells

The viability of cardiomyocytes isolated from the lipid-supplemented rats was not significantly different between the 3 dietary groups (ANOVA). For the REF group, 70.4 ± 1.7% of cells were rod shaped immediately after isolation (n=11) compared with 63.8 ± 3.3% (n=6) in the SF group, and 75.0 ± 3.8% of cells in the FO group (n=7).

3.3.4.4 ROS-Induced Asynchronous Contractile Activity

In this study, either 30 μM H_2O_2 or 2.3 mM purine plus 7 mU/ml xanthine oxidase were used as FRGSs. In response to H_2O_2, the cells developed asynchronous contractile activity (Figure 3.12), which, in the SF group, reached a level of 96.1 ± 1.2% (n=6) after 20 min. This was not significantly higher than the level of asynchrony developed in the FO group (81.8 ± 8.6%, n=7) or the REF group (90.76 ± 4.16%, n=6). However, there

was a significant right shift in the asynchrony curve induced by the FO diet. Hence, the

$T_{50\%}$ for cells isolated from the FO group (14.6 ± 0.2 min, n=7) was significantly longer

compared with both the REF (12.9 ± 0.2 min, n=6) and SF (12.2 ± 0.1 min, n=6) groups

(p<0.001).
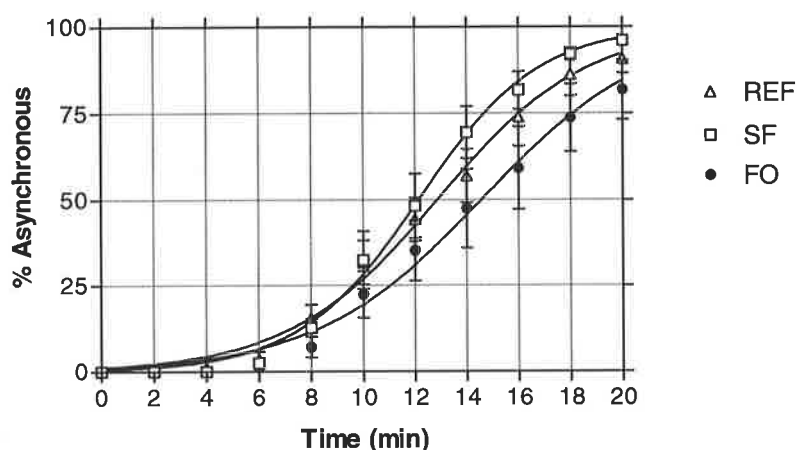
Figure 3.12: Development of asynchronous contractile activity following addition of H₂O₂.
Each point is the average of approximately 20 cells, performed in triplicate, n = 6-7 rats. Abbreviations: FO, fish oil; REF, reference diet (low-ALA); SF, saturated fat diet.

Following addition of the P/XO FRGS, cardiomyocytes from the FO group exhibited a

significant right shift in the asynchrony curve, indicating a delayed development of

asynchronous contractile activity in response to this FRGS. At every time point between

10 and 16 min after addition of P/XO, the FO group exhibited a lower number of

asynchronously contracting cells compared with the SF group (p<0.001), Figure 3.13. At

10 min and 12 min after addition of P/XO, a significantly higher percentage of cells from

the SF group were asynchronously contracting compared to the REF (p<0.01 and p<0.001

respectively). Similarly, the FO group had a lower number of asynchronously contracting

cells in comparison to the REF at 12 min, 14 min and 16 min following addition of P/XO

(p<0.01, p<0.001, p<0.05 respectively). Only the time points that were significantly

different between the SF and FO are indicated in Figure 3.13. The $T_{50\%}$ was higher for

cells isolated from the FO group, 14.2 ± 0.1 min (n=7) compared to both the SF (10.7 ± 0.1 min, n=6), and the REF (12.3 ± 0.1 min, n=9) groups, p<0.01.
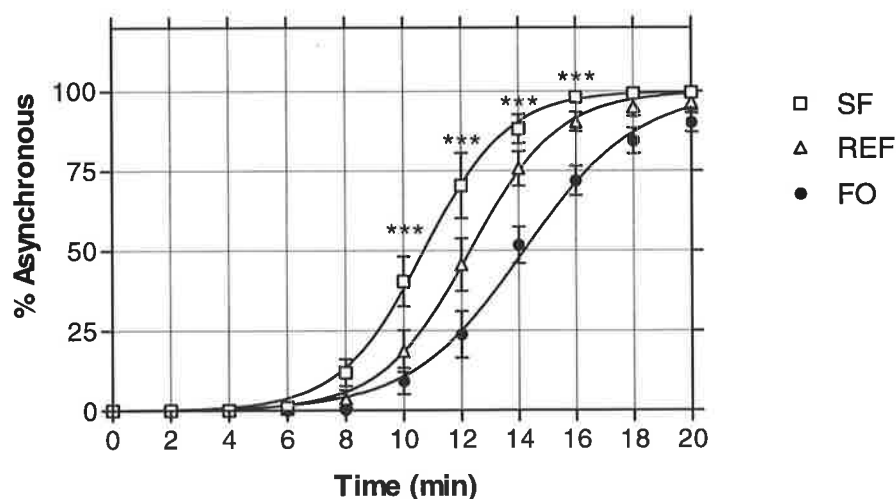
Figure 3.13: Development of asynchronous contractile activity following addition of P/XO.
Each point is the average of approximately 20 cells, in triplicate. n = 6-9 rats. Abbreviations: FO, fish oil; P, purine; XO, xanthine oxidase; SF, saturated fat diet; ***p<0.001 for SF vs FO (ANOVA).

3.3.5 Myocardial Vitamin E following dietary lipid supplementation

There was no significant difference between vitamin E levels in the myocardium of rats fed different dietary lipid supplements although there did appear to be a trend towards a lower vitamin E content in FO myocardium. The α-tocopherol content in REF rats was 46.22 ± 2.26 µg/g wet weight (n=6) compared to 43.92 ± 3.20 µg/g (n=6) for SF and 37.97 ± 1.74 µg/g (n=6) for the FO group.

3.4 Discussion

The first aim of this project was fulfilled by developing a ROS-induced arrhythmia assay. This assay involved superfusing isolated adult rat ventricular myocytes that were contracting under electrical field stimulation with a FRGS and monitoring the development of cellular asynchronous contractile activity. This model has permitted an examination of the direct consequences of free radicals on cardiomyocyte contractility and allowed an investigation of the effects on this parameter, of both acute addition of n-3 PUFAs as well as the effect of PUFAs incorporated into membrane phospholipids as a result of dietary supplementation. A cardiomyocyte model utilising freshly isolated adult cells was employed to study ROS-mediated cardiac injury since this model allows investigation of the direct effects of ROS on cell viability, independent of systemic hemodynamics and coronary circulation. Additionally, it indicates that the measured effects of ROS are intrinsic to the myocyte itself and are not due to the effects on other types of cells such as endothelial, smooth muscle or interstitial cells, which may, in the whole heart, indirectly affect contractile function of muscle cells. Finally, the use of myocytes enhances the access of solutions containing the FRGSs to the sarcolemma by eliminating possible interference from other cell types and connective tissue that could shield *in situ* cardiac cells from exogenous radicals.

The main finding of this chapter was that dietary supplementation, but not acute addition of the n-3 PUFAs had protective effects against free radical induced arrhythmias in isolated cardiomyocytes. Three different types of FRGSs were used to induce asynchronous contractile activity. Concentrations of reagents were based on the literature (Kaneko *et al.*, 1989a; Morris & Sulakhe, 1997; Courtois *et al.*, 1998) with modifications made according to dose-response curves, in order to refine the system to allow a

reproducible assay of cellular contractile damage. Under control conditions, cardiomyocytes exposed to a FRGS showed a time-dependent development of asynchronous contractile activity, which, with continued exposure, led to cessation of contractions and irreversible hypercontracture. Acute addition of DHA was not able to protect cardiomyocytes from free radical induced arrhythmias. There was a trend towards some protection with longer durations of incubation with this fatty acid prior to the free radical insult (statistical significance was achieved between the $T_{50\%}$ following overnight incubation), similar to those obtained with vitamin E. However, significant attenuation of the development of asynchronous contractile activity did not occur until the protocol was altered to that of dietary supplementation with fish oil (containing n-3 PUFAs, mainly DHA and EPA), suggesting a different mode of action of n-3 PUFAs when significant levels become incorporated into membrane phospholipids. DHA, when infused acutely, probably remains in the membrane as the free fatty acid, partitioning between the phospholipids, with its carboxyl group interacting with the polar phospholipid headgroups. In contrast, dietary n-3 PUFAs which are incorporated into membrane phospholipids, exist as esterified fatty acids. The membrane incorporation may induce other biochemical/biophysical changes in the cardiomyocyte membranes, which underlie the cardioprotective effects. These changes include, but are not necessarily limited to the following possibilities: effects on membrane fluidity and intracellular Ca^{2+} levels, enhancement of the antioxidant enzyme systems (including vitamin E) and a resultant decrease in intracellular free radicals. These principles are introduced in this discussion and will be addressed in greater detail in the following chapters.

In the first dietary study, the gavaging technique was used in order to ensure consistent dietary lipid intakes for each rat. This feeding protocol resulted in considerable

incorporation of the n-3 PUFAs into the myocardial membranes, however there was no major protective effect evident on the development of asynchronous contractile activity. There was a trend towards the FO cells being less sensitive to the effects of free radicals compared to cells isolated from the OO or CO groups, however this only reached statistical significance for the P/XO FRGS. It was necessary to firstly have a system to reproducibly induce arrhythmias in isolated cardiomyocytes, in order to determine the possible protective effects of acute and dietary supplementation of n-3 PUFAs. Secondly, since it was aim of this thesis to determine the underlying mechanism of antiarrhythmic/antioxidant action of the n-3 PUFAs (the subject of Chapters 4 and 5), a known dose and duration of feeding (of FO) was required in order to establish a model that could be used to investigate these mechanisms. Therefore, to optimise the system further, the feeding duration was increased to 3 weeks and a different fish oil (n-3 ethyl ester concentrate) was used. This FO contained higher proportions of n-3 PUFAs, in order to further increase the degree of incorporation of the n-3 PUFAs and potentially enhance the physiological effects of the FO. However, no significant cardioprotective effects were observed in this second dietary study. During the gavaging period of the study, the animals had been maintained on the standard laboratory chow (as in previous studies) which, due to seasonal variations in the composition of the constituent components used by the manufacturer to make the base diet, contained considerably higher levels of α-linolenic acid (ALA, 18:3 n-3). Thus the higher levels of n-3 PUFAs seen in the animals fed the base diet were probably due to this factor. It is likely that the ALA consumed by the rats had been elongated and desaturated *in vivo* to DHA in all the animals during the feeding period. Therefore, although the 3 weeks of gavage did raise the proportion of myocardial n-3 PUFAs significantly, the extent of increase, particularly of DHA, above the levels obtained in the myocardium of the CO and OO groups, was not to the levels

anticipated. A likely consequence of this was that the FO diet afforded no protection from free radical induced arrhythmias in this set of studies. Viewed in another light, it is also possible that by increasing the basal levels of n-3 PUFAs in the myocardium of rats fed OO and CO, the cardioprotective potential of these diets was raised. Hence, all three diets were equally effective in protecting from ROS-induced arrhythmia and the protective effects of FO could not be observed due to the lack of a negative effect of OO and CO supplementation. Whether the levels of n-3 PUFAs in the myocardium of rats in the FO group were raised to high enough proportions for cardioprotective effects to occur cannot be stated conclusively since the concentrations of FRGSs were different to the previous study and since there was no negative control group with which to compare the effects of FO feeding. For the next experiment, rats were not fed with the standard rat chow either before or during the gavage period. Instead, rats were maintained on a base diet prepared to exact specifications, containing 7% fat, as Sunola oil to give a final dietary preparation that contained 0.7% ALA as the sole n-3 PUFA source, compared with a level of 4.6% ALA in the base diet used in the previous study. When the FO was gavaged whilst the rats were maintained on this new, "low-ALA" diet, the basal levels of n-3 PUFAs in the reference group (REF) of rats (sham gavaged with glycerol) was 4.3% compared to 16.2% in the previous study. Although a different source of fish oil was used in this latter study which contained lower levels of n-3 PUFAs (the previous source was no longer available), the levels of n-3 PUFAs incorporated into cardiac phospholipid fatty acids were four times higher than observed in the reference rats. In this the third dietary study, the newly formulated low-ALA diet was used both as the base diet for all dietary groups during the lipid (and sham) gavage period, and it also acted as a reference diet to compare the effect of SF and FO supplementation. Comparison against such a "control" diet should be made with some care since rats on this diet only consumed 7% fat compared to

the experimental rats that received 17% total fat. Nevertheless, it is valuable to have a reference point in order to evaluate the effects of these experimental diets. Having a reference diet and two experimental diets allows the effects of both diets to be considered independently of each other, allowing the evaluation of a dietary supplemented animal against the "normal" colony animal. The use of the reference diet in the third dietary study did in fact demonstrate that the SF diet shifted the ROS-induced arrhythmia assay curve to the left while the FO diet caused a significant right shift indicating protective effects of FO feeding.

The observation that the "high-ALA" colony diet raised the proportion of n-3 PUFAs in the myocardial phospholipids has important implications which are discussed below. It was undesirable in the present study to have a high basal level of n-3 PUFAs in the membranes, since the effect of supplementation with FO (containing additional n-3 PUFAs) would be somewhat blunted as there would be little difference between myocardial n-3 PUFA levels in SF and CO treated groups compared with FO. However, the corollary is that this indicates that relatively low (4.6%) levels of ALA in the dietary supplement are effective in significantly raising the proportion of n-3 PUFAs present in rat myocardial phospholipids. As indicated in the results, the rats fed the "high-ALA" colony diet had a comparable myocardial n-3 PUFA profile to rats supplemented with FO. This finding has important implications if a similar effect can be demonstrated in humans, since it indicates that 18-carbon n-3 PUFAs obtained from sources other than fish oil (eg seed oils such as canola or flaxseed oil) are also able to raise DHA levels in the myocardium. A recent study has shown that rat astrocytes incubated with ALA were able to efficiently elongate this fatty acid to DHA under appropriate culture conditions (Williard *et al.*, 2001). However, the results of a human study were not as convincing,

indicating a limited efficiency of conversion of ALA to EPA (and therefore to DHA) (Pawlosky *et al.*, 2001). This area therefore needs further investigation.

In the final dietary study, significant attenuation by FO feeding was demonstrated for those arrhythmias (asynchronous contractile activity of cardiomyocytes) induced by P/XO but not H_2O_2. It is unlikely that FO is specifically an antioxidant for ROS generated by P/XO alone for at least two reasons. Firstly, P/XO generates both $O_2^{\bullet-}$ and H_2O_2 and these two ROS can quite likely react together to also form the highly reactive $\bullet OH$ through the Haber Weiss reaction (Halliwell & Gutteridge, 1990). Superfusion with H_2O_2 would result in the generation of $\bullet OH$ (by reaction with intracellular iron (Josephson *et al.*, 1991)) and H_2O_2 is itself permeable to cells (Freeman & Crapo, 1982) resulting in high intracellular H_2O_2 levels. Furthermore, the trend towards a right shift in the ROS-induced (H_2O_2) arrhythmia curve by the FO diet may indicate that the lack of statistical significance could be explained as a dose effect. (Indeed, in Chapter 4, rats fed the FO supplemented diet for 4 weeks do in fact show a significantly reduced sensitivity to the H_2O_2-induced arrhythmias). Although it would be preferable for any intervention in reperfusion arrhythmogenesis/injury to (almost) completely abolish the aberrant contractile activity which is occurring, in the majority of physiological systems, it is more likely for differing degrees of attenuation to be observed, as in the present study. Hence, the use of the $T_{50\%}$, enabled the comparative cardioprotective potential of the different diets to be examined in response to the different FRGSs. A lengthened $T_{50\%}$ indicates some degree of resistance to the effects of the ROS. Indeed, the time taken for 50% of the cells to develop asynchronous contractile activity was significantly higher in FO cells exposed to P/XO compared to cells derived from SF-fed rats. However the protective effects of FO do not appear to be due to a slower rate of development of asynchronous

contractile activity since the slopes of the ROS-induced arrhythmia curves were not significantly different between the dietary groups. Thus the difference may lie in the initial delay in the onset of asynchronous contractile activity. It is possible that this initial reduced susceptibility to the effects of the ROS may protect the myocardium by allowing it sufficient time to recruit other defences. In fact, a possible mechanism, which will be pursued in Chapter 5, is the hypothesis that the fish oil induces a mild oxidative stress, which in itself while not damaging, acts as a signal to enhance the cell's antioxidant mechanisms. If the antioxidant defences were upregulated by dietary FO containing n-3 PUFAs, it would be expected that the amount of ROS (when added exogenously) might not overwhelm the antioxidant capacity of the myocardium. This antioxidant upregulation has been suggested by others (Vogt *et al.*, 1998; Csonka *et al.*, 2000; Meilhac *et al.*, 2000) as a form of cellular adaptation, i.e. a secondary effect, to oxidative stress induced by the n-3 PUFA supplementation.

Another possible mechanism to explain the reduced susceptibility of contractile damage to the myocytes isolated from the FO dietary group following oxidative stress is the presence of a decreased level of intracellular free radicals following a ROS challenge which may or may not be related to the increase in ROS scavenging capacity mentioned above. Intracellular ROS levels were measured using a fluorescent probe approach during exposure to ROS, and the results are presented in Chapter 4. The effects of dietary fish oil in attenuating ROS-induced arrhythmias may also be in part attributed to their influence on Ca^{2+} handling, or effects on other channels. For example, in neonatal rat ventricular myocytes, the development of arrhythmic (asynchronous) contractile activity has been reported when intracellular Ca^{2+} is increased from nM to μM levels as a result of exposure to ROS (Burton *et al.*, 1990). Cells from the rats fed the FO could also be

protected from ROS effects due to reduced sarcolemmal membrane damage of the FO myocytes during the cell isolation procedures. However, this possibility appears unlikely given that there were no significant differences in the cell viability indexes of myocyte preparations between the SF and FO groups. Further, for all dietary groups, only single cardiac myocytes that were rod shaped with clear cross-striations, typical of the *in situ* myocardium, with no visible membrane blebs, and unattached to adjacent cells were analysed. Therefore, at least morphologically, the cells used were equivalent across the various dietary groups.

Most of the studies in the literature have emphasised the inclusion of sufficient vitamin E with fish oil supplementation to prevent the peroxidative effects of the PUFAs. Vitamin E is the major lipophilic antioxidant present in cell membranes and functions as a chain breaking antioxidant. The molecule contains an –OH group that can be readily deprotonated (removal of the H^+ ion), hence free radicals generated during lipid peroxidation preferentially combine with the antioxidant instead of an adjacent fatty acid in the membrane. This therefore terminates the chain reaction. In the absence of vitamin E, attack of the membrane fatty acids would generate another carbon-centred radical which continues the chain reaction and can convert many fatty acid side-chains into lipid hydroperoxides (Halliwell, 1989). This point has significance when the membrane contains a large percentage of unsaturated fatty acids, as occurred in the present study. The fish oils used in the present studies contained between 0.1-0.8% (w/w) α-tocopherol/g. The OO, CO and SF contained low or negligible levels of vitamin E, as measured by the manufacturers. The base diets contained a set level of vitamin E (100 IU/kg diet) and therefore, due to the added vitamin E in the oil, the rats consuming the FO would receive overall higher vitamin E levels. Nevertheless, it is unlikely that the

protective effects of FO would be attributed to the additional vitamin E since there was no significant difference in vitamin E levels in the myocardium of rats in the different dietary groups.

It may be argued that this system did not actually examine the effect of reperfusion *per se* since the cells had not undergone a period of ischaemia prior to reperfusion. The study described however, was not intended to be a model of ischaemia-reperfusion. Reperfusion injury is an independent event to the injury that arises as a result of ischaemia. Although the induction of reperfusion injury *in vivo* requires the preceding period of ischaemia, the mechanisms involved in reperfusion arrhythmogenesis are quite distinct to those involved in ischaemic arrhythmias (reviewed in Chapter 1). Hence, the isolation of reperfusion injury, in the present study was justified. Indeed, the "ROS-induced arrhythmia assay" was developed as a model of reperfusion injury in order to facilitate the further study of the mechanisms involved in the antiarrhythmic actions of n-3 PUFAs in reperfusion arrhythmogenesis.

In summary, this chapter established a reproducible system for inducing asynchronous contractile activity in isolated cardiomyocytes using a free radical generating system, which mimics reperfusion-induced arrhythmias. A formulation for a base diet was also established which resulted in low basal levels of n-3 PUFAs in the myocardial membranes. Acute addition of DHA did not protect against ROS- induced arrhythmias, however, dietary fish oil supplementation (with a high content of DHA) was able to significantly delay the development of free radical induced asynchronous contractile activity, independent of vitamin E levels. This latter effect was related to the higher proportions of n-3 PUFAs in the myocardial (membrane) phospholipids. Possible

mechanisms underlying the protective effects of dietary n-3 PUFAs which have been discussed include the requirement for the n-3 fatty acids to be incorporated into the membrane phospholipids in order to exert their protective effects, decreased intracellular levels of ROS and Ca^{2+} during oxidative stress in cardiomyocytes isolated from rats fed FO, and up-regulation of the myocardial antioxidant system. These points will be the focus of the following chapters.

CHAPTER FOUR

The Effect of Dietary n-3 PUFAs on Cardiomyocyte Membrane Fluidity, Intracellular ROS and Ca^{2+} Levels During Oxidative Stress.

4.1 Introduction

The effects of reactive oxygen species (ROS) on the contractility of adult rat ventricular myocytes were investigated in Chapter 3. A protective effect of delaying the onset of asynchronous contractile activity in response to ROS was demonstrated following dietary fish oil supplementation. To examine the mechanisms underlying this protective effect, fluorescent probe techniques were utilised to compare membrane fluidity, intracellular levels of Ca^{2+} and ROS during exposure to ROS in cardiomyocytes isolated from rats supplemented with fish oil and saturated fat.

ROS can be generated by a number of mechanisms including; the xanthine/xanthine oxidase (X/XO) system; the cyclooxygenase pathway involved with arachidonic acid metabolism (Kourie, 1998); the NADPH oxidase and myeloperoxide present in activated neutrophils (Nakamura *et al.*, 1993); and the electron transport system of mitochondria (Hoek *et al.*, 1997; Duranteau *et al.*, 1998). Although ROS are also produced during ischaemia (Vandenhoek *et al.*, 1997), they have been mainly shown to be generated at reoxygenation (Sharikabad *et al.*, 2000). There are various methods to determine oxidative damage to cells, for example measuring lactate dehydrogenase release or formation of lipid peroxidation products such as malondialdehyde and thiobarbituric acid reactive substances. However, none of these techniques directly evaluates cellular ROS levels. Following the first description of the use of 2',7'-dichlorofluorescin diacetate (DCFH-DA) as a fluorometric assay for hydrogen peroxide in 1965 (cited by Wang & Joseph (1999)), DCFH has been widely used as a probe to evaluate intracellular ROS

formation (Cathcart *et al.*, 1983; Bass *et al.*, 1983; Carter *et al.*, 1994; Hoek *et al.*, 1997; Vandenhoek *et al.*, 1997; Swift & Sarvazyan, 2000). A new, membrane-permeable derivative of DCFH, namely 5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate (CM-DCFH), reported to be better retained in cells than other derivatives (Xie *et al.*, 1999), was used in the present study. On entering the cell, the acetate group of CM-DCFH is cleaved by cellular esterases, trapping the non-fluorescent probe inside the cell. Subsequent oxidation by ROS yields the fluorescent product CM-DCF. Thus increases in CM-DCF fluorescence are suggestive of the presence of intracellular ROS.

ROS can alter the chemical and physical properties of cell membranes by modifying the composition, packing and distribution of the membrane lipids which in turn, determine fluidity (Solans *et al.*, 2000). The term 'membrane fluidity' in this context, is used to describe the motional freedom of lipid soluble molecular probes within a lipid bilayer (Bastiaanse *et al.*, 1997). The specific composition of individual biological membranes will influence the membrane fluidity. For example, if the acyl fatty acids of the phospholipids are mainly saturated, they will tend to stack together in a relatively tight manner, imparting a relatively rigid nature to the membrane. Addition of a double bond into the fatty acyl chain, introduces a bend into the molecule, increasing the degree of rotational motion about the chain (Stubbs & Smith, 1984). Thus membranes, or regions of membranes containing (poly) unsaturated fatty acids cannot pack as tightly as saturated fatty acyl chains; and this leads to an increase in the fluidity of the membrane. Cholesterol with its flat, stiff, ring structure reduces the rotational motion of the fatty acid chains and, this together with the length and degree of saturation of phospholipid fatty acyl chains within the membrane plays a major role in determining membrane fluidity (Solans *et al.*, 2000). Dietary fatty acids are absorbed from the small intestine and following

metabolism, can become incorporated into cell membranes throughout the body in a cell and tissue specific manner (Lund *et al.*, 1999). In fact, the levels of n-3 PUFAs, particularly DHA, are already relatively high in membrane phospholipids of the brain and retina (Leaf & Weber, 1988). Changes in the fatty acid composition or cholesterol content of the cell membranes can modify membrane fluidity and thereby alter the function of membrane receptors and membrane-bound enzymes (McMurchie *et al.*, 1983; Nalbone *et al.*, 1990; Fournier *et al.*, 1995; de Jonge *et al.*, 1996) and transporters for ions such as sodium and calcium (Bastiaanse *et al.*, 1997).

Intracellular calcium ($[Ca^{2+}]_i$) is an important second messenger system in the myocardial cycle of excitation-contraction coupling. ROS-induced functional abnormalities in cardiac muscle are thought to give rise to increases in $[Ca^{2+}]_i$. In cardiac tissue, the elevation of cytosolic Ca^{2+} (which may lead to Ca^{2+} overload) associated with ROS-induced tissue damage during ischaemia-reperfusion, is linked to various abnormalities such as contractile dysfunction and ventricular arrhythmia (Goldhaber & Weiss, 1992). ROS can specifically damage ion pumps, exchangers and channels, including the Ca^{2+}, Na^+ and K^+ channels, as extensively reviewed in Kourie (1998). Based on the findings of Chapter 3, it was hypothesised that the n-3 PUFAs may be exerting their protective effects on free radical induced asynchronous contractile activity by their influence on the Ca^{2+} dynamics, and that this may occur as a result of delaying or preventing Ca^{2+} overload.

Thus the aims of the present study were therefore to:

- Examine various Ca^{2+} handling parameters in cardiomyocytes from rats fed fish oil (FO) or saturated fat (SF) supplemented diets following exposure to ROS.

- Find and validate a suitable free radical probe to use to compare intracellular ROS levels in cells isolated from FO or SF supplemented rats following exposure to ROS.

- Determine the effect of dietary FO supplementation and ROS exposure on cardiomyocyte cell membrane fluidity.

4.2 Methods

4.2.1 The Effect of Dietary Lipid Supplementation on Intracellular Ca^{2+} Handling and ROS levels in Ventricular Cardiomyocytes.

4.2.1.1 Animals and Feeding Protocol

Rats were obtained at 4 weeks of age and fed a reference (REF) low-ALA diet, containing 7% (w/w) fat, as Sunola oil. This was the same low-ALA diet as used in the final study described in Chapter 3, however, for this study, the dietary oils were incorporated into the pellets rather than being administered by gavage. The food intake of young adult rats is approximately 20 g/day, therefore 10% (w/w) added fat was calculated to provide a comparable amount of (added) lipid as in the previously described studies. At 9 weeks of age, the animals were given the lipid supplemented diets for a period of 4 weeks[6]. As shown in Table 4.1, the low-ALA diet was used as the base diet, to which either lard (beef and mutton fat), or fish oil (RoPUFA30) were added, providing a total of 17% (by weight) fat. The control group (CON) were fed 17% (w/w) fat as Sunola oil.

Diet	Base Diet	Added Fat
REF	Low-ALA (7% SO)	Nil
CON	Low-ALA (7% SO)	10% SO
SF	Low-ALA (7% SO)	10% lard
FO	Low-ALA (7% SO)	10% fish oil

Table 4.1: Composition of dietary supplements fed to the rats.
Abbreviations: ALA, α-linolenic acid (18:3 n-3), CON, control; FO, fish oil; REF, reference; SF, saturated fat, SO, Sunola oil. All dietary additions were as weight/weight.

4.2.1.2 Dietary Fatty Acid Analysis

The fatty acid analysis of the respective diets, carried out according to the methods in Chapter 2 is shown in Table 4.2.

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[6] Due to technical problems with the fluorescent microscope and fluorescent probes beyond my control, rats could not be used at 3 weeks (as in previous studies) and were therefore maintained on the same diets for a further week.

FAME[a]	REF	CON	SF	FO
14:0	0.10	0.08	3.80	4.31
16:0	3.94	3.84	19.56	11.71
16:1	0.09	0.11	1.20	5.75
17:0	0.04	n/d	0.83	0.25
18:0	3.93	3.79	13.91	3.63
18:1[b]	80.36	80.14	54.12	44.20
18:2 (n-6)	9.29	9.89	4.68	5.40
18:3 (n-3)	0.38	0.35	0.55	0.84
20:0	0.36	0.33	0.39	0.37
20:1	0.34	0.33	0.22	2.22
20:2	n/d	0.01	0.08	0.12
20:3 (n-6)	n/d	n/d	n/d	0.14
20:4 (n-6)	n/d	n/d	n/d	0.92
22:0	0.92	0.85	0.36	0.47
22:1	n/d	n/d	n/d	2.03
20:5 (n-3)	n/d	0.05	0.05	10.02
24:0	0.25	0.24	0.10	0.13
22:5 (n-3)	n/d	n/d	0.04	1.09
22:6 (n-3)	n/d	0.02	0.05	5.93
Σ SFA	9.55	9.13	38.95	20.87
Σ MUFA	80.79	80.59	55.60	54.66
Σ PUFA	9.66	10.30	5.45	24.46
Σ n-6	9.29	9.89	4.68	6.46
Σ n-3	0.38	0.41	0.68	17.88
n-6/n-3	24.64	24.30	6.84	0.36

Table 4.2: Fatty acid composition (wt%) of the low-ALA reference diet (7% Sunola oil) with and without a further added 10% dietary fat (as Sunola oil, lard or fish oil).
The fatty acid analysis of the lipid supplements (oils) was shown in the right hand columns of Table 3.3 (Chapter 3). The shorthand notation for fatty acid structure was given in the legend to Table 3.1. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains the n-7 and n-9 isomers. n/d not detectable. All percentages are as w/w. Only a single aliquot was analysed in duplicate for each type of diet except for the CON diet in which 2 aliquots were analysed in duplicate.

4.2.1.3 ROS-Induced Asynchronous Contractile Activity in Cardiomyocytes

Following 4 weeks dietary lipid supplementation, rats were sacrificed and cells isolated as described in Chapter 2. Isolated cardiomyocytes were loaded with the fluorescent probes Fura-2 or CM-DCFH in order to measure intracellular Ca^{2+} transients or ROS generation respectively. Mitochondrial compartmentalisation of cytosolic probes is a well-known phenomenon that can be reduced by decreasing the time or altering the temperature of

probe loading (Swift & Sarvazyan, 2000). Hence, cells were loaded with the probe at 25°C. However, at this temperature, which has been used in many other studies, the contractility of the cells was significantly slower and no arrhythmias were observed in the period that the cells were studied (results not shown). Therefore 25°C was used for loading and 32°C was chosen as a midpoint temperature (compromise between 25°C and 37°C) for contractility experiments. The protocol for this experiment involved 3 min of equilibration with Tyrode's solution containing 2 mM Ca^{2+} followed by electrical field stimulation (at a frequency of 0.5 Hz) for 2 min. Cells were then superfused under these conditions with 30 μM H_2O_2, and the development of asynchronous contractile activity monitored for 20 min. It was found that asynchronous contractile activity could be reproducibly induced under these conditions.

4.2.1.4 Fluorescence Microscope Set-up

The set-up of the fluorescent microscope (Nikon, Tokyo, Japan) is given in Appendix 9.1, indicating the light path and filter locations (numbers). For each probe, the relevant filter block, as specified in the SDR Clinical Technology handbook, was used. Filters and dichroics were purchased from Omega Optical Inc. (VT, USA). Data was acquired by the *AcqKnowledge* software (SDR Clinical Technology, Sydney, NSW, Australia) and processed in a Microsoft Excel spreadsheet program.

4.2.1.5 Measurement of $[Ca^{2+}]_i$ Using Fura-2

The dual excitation, single emission probe, Fura-2 was used to monitor intracellular Ca^{2+} changes during ROS exposure. Isolated cardiomyocytes plated onto laminin coated coverslips (#0 glass) were transferred to a Petri dish containing Tyrode's solution supplemented with 2 mM Ca^{2+}, 1.3% (w/v) BSA, 30 mM 2,3-butane-dione monoxime and 5

µM Fura-2 (acetomethoxy form) at 25°C for 15 min in the dark. The coverslips were then transferred to a custom-designed superfusion chamber (#0 glass base) located on the stage of an inverted epifluorescence microscope (Nikon, Tokyo, Japan) and superfused at 2.5 ml/min with Tyrode's buffer containing 2 mM Ca^{2+} at 32°C. This superfusion rate also enabled the washout of any probe that had not been incorporated into the cells. After 3 min equilibration, cells were stimulated at 0.5 Hz for 2 min, using two platinum wire electrodes placed within the superfusion chamber and connected to a DS9A Digitimer stimulator (Digitiman Ltd, Welwyn Garden City, Herts, England). At this point, baseline calcium transients were measured. Following this 2 min equilibration period, 30 µM H_2O_2 was introduced into the superfusion chamber and Ca^{2+} transients were measured every 4 min for 20 min, with the actual fluorescence readings being made for a period of 20 sec. For fluorescence measurements of Ca^{2+} transients, cells were illuminated sequentially at 340 ± 10 nm and 380 ± 10 nm with a UV light source (175 W Xenon lamp) and Lambda DG-4 high-speed filter changer (Sutter Instrument Company, Novato, CA, USA) with a signal time resolution of 2 msec. The emitted fluorescence was detected with a photomultiplier at an emission wavelength of 510 ± 40 nm. An adjustable rectangular diaphragm in the light-path preceding the photomultiplier restricted measurement of fluorescence to a single cell. Although Ca^{2+} ratios were being compared between cells and not absolute intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$)[7], the aperture was adjusted such that the same size area of all cells was studied. The signals from the photomultiplier were fed through an A-D converter (BIOPAC Systems, Santa Barbara, CA, USA), digitised at 200 Hz and passed through a low-pass filter and output to a computer for real-time

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[7] $[Ca^{2+}]_i$ concentrations are calculated according to the formula: $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times \beta$ where R is the ratio of the sample at 340 nm and 380 nm; R_{max} and R_{min} represent the ratios for Fura-2 at the same wavelengths in the presence of saturating calcium and (effectively) zero calcium, respectively; β is the ratio of fluorescence of Fura-2 at 380 nm in zero and saturating calcium; and K_d is the dissociation constant of Fura-2 for calcium (Grynkiewicz *et al.*, 1985).

analysis. After subtraction of the background signal, the ratio of fluorescence signals at 340 nm/380 nm was calculated. The purpose of this study was not to analyze actual $[Ca^{2+}]_i$, but rather, to compare Ca^{2+} dynamics between cells isolated from rats fed SF and FO supplemented diets. Since the relative values of ratio elevation were enough for the purpose of this study, $[Ca^{2+}]_i$ calibration was not performed. Many previous studies have also reported only fluorescence Ca^{2+} ratios as opposed to actual $[Ca^{2+}]_i$ (Koyama *et al.*, 1991; Siegmund *et al.*, 1992; Rohn *et al.*, 1997; Siegmund *et al.*, 1997; Ladilov *et al.*, 2000). Indeed, it would appear that there is a preference for the use of fluorescence ratios rather than absolute Ca^{2+} concentrations (Tamada *et al.*, 1998; Hattori *et al.*, 2000; Kotsanas *et al.*, 2000; Ren & Bode, 2000), since dye leakage from the cells (Di Virgilio *et al.*, 1990) and non-cytosolic compartmentalisation of the probe (Roe *et al.*, 1990) may result in inaccuracies in determining actual $[Ca^{2+}]_i$. Further, in single cell studies, one cannot use the lysis method to achieve R_{max} as the dye leaks from the field of view (Roe *et al.*, 1990). The majority of investigators still use the original K_d as determined by (Grynkiewicz *et al.*, 1985) in their calculations of $[Ca^{2+}]_i$, whereas it was shown that K_d should be determined specifically for the conditions of the experiment (Uto *et al.*, 1991). Hence, in the present study, the 340/380 nm Ca^{2+} ratios are presented. However, the term $[Ca^{2+}]_i$ will be used when discussing systolic or diastolic Ca^{2+} ratios for ease of explanation, as has been used by previous investigators. Fluorescence data for the Ca^{2+} transient studies were imported into the Acqknowledge computer program and at least five consecutive Ca^{2+} transients were ensemble averaged to obtain the Ca^{2+} transient parameters for each time point. The following parameters were calculated: the end-diastolic Ca^{2+} ratio, (an indication of the resting $[Ca^{2+}]_i$ between successive contractions); the systolic Ca^{2+} ratio (the maximum Ca^{2+} level reached during a contraction); the rate of rise in intracellular Ca^{2+} during a contraction; and the rate of relaxation (tau (τ), the time

constant for the Ca^{2+} transient decay). To determine the time constant for the decay of Ca^{2+} transients (τ), exponential rates of decay were fitted to a single exponential function, defined as:

$$R = ((R_{c\text{-}max}-R_{c\text{-}min})/e^{(t/\tau)}) + R_{c\text{-}min}$$

where $R_{c\text{-}max}$ is the systolic $[Ca^{2+}]_i$, $R_{c\text{-}min}$ is the diastolic $[Ca^{2+}]_i$, t is the time (sec) and τ is the time constant of Ca^{2+} transient decay (measured in seconds). An example of an ensemble averaged Ca^{2+} transient is given in Figure 4.1, indicating the parameters that were measured.
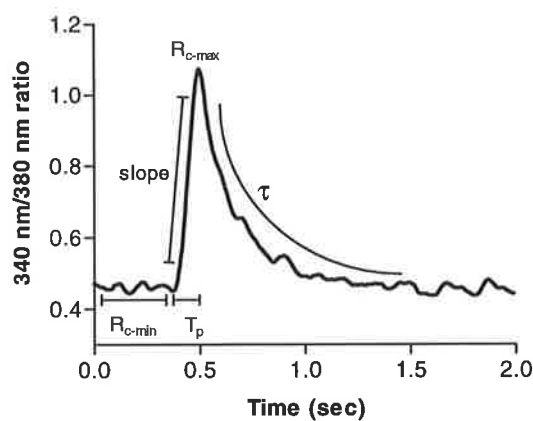
Figure 4.1: An ensemble averaged Ca^{2+} transient representing eight consecutive Ca^{2+} transients. Abbreviations: $R_{c\text{-}min}$, end diastolic Ca^{2+} ratio (340 nm/380 nm); $R_{c\text{-}max}$, systolic Ca^{2+} ratio; slope, upstroke velocity (rate of influx of Ca^{2+} into cytoplasm); Tp, time to peak Ca^{2+} ratio; τ, time constant of Ca^{2+} transient decay (rate of Ca^{2+} removal from cytoplasm).

During asynchronous contractile activity, 5 individual transients were analyzed during the 20 sec recording period of the indicated time points and the average measurements were used to obtain the Ca^{2+} transient parameters. The following parameters were calculated: end diastolic Ca^{2+} ratio; systolic Ca^{2+} ratio; the time to 50% decay of the systolic Ca^{2+} levels ($Td_{50\%}$); the area under the curve (ie asynchronous transient as shown in Figure 4.2- a measure of the amount of calcium cycling during asynchronous contractile activity); and the integral of the Fura-2 signal during asynchronous contractions. The rationale behind measuring both the area and the integral, was based on the fact that it may be possible for cells from both the SF and the FO supplemented groups to exhibit the same

degree of Ca^{2+} cycling during asynchronous contractile activity (i.e. area under the curve), however, if in one dietary group, the accumulated levels of intracellular Ca^{2+} were higher, the integral would detect this difference. A representative "asynchronous" Ca^{2+} transient is shown in Figure 4.2, indicating the parameters that were measured.
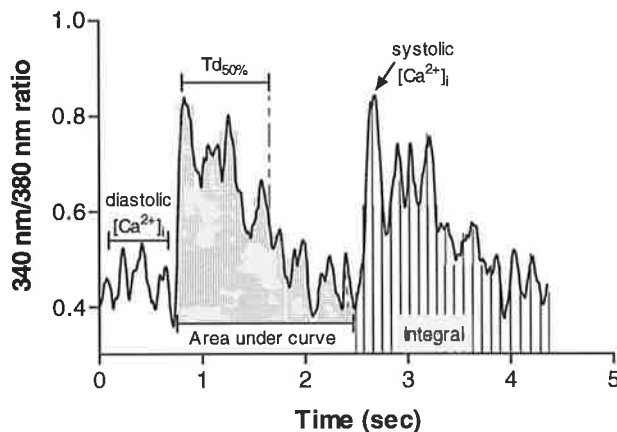
Figure 4.2: Ca^{2+} transient parameters measured during asynchronous contractile activity.
Two representative Ca^{2+} transient tracings during asynchronous contractile activity are shown. Five such transients were analysed for each cell at each time point during asynchronous contractile activity and the average values obtained. Abbreviations are explained in the text.

4.2.1.6 Detection of Intracellular ROS with Fluorescent Probes

A 5 mM stock solution of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCFH, Molecular Probes, Eugene, OR, USA) was prepared in ethanol. Freshly prepared cardiomyocytes from young adult rats attached to glass coverslips, were loaded with 10 μM probe diluted in Tyrode's solution, containing 2 mM Ca^{2+}, 1.3% (w/v) BSA, pH 7.3, for 15 min at 25°C in the dark. A coverslip containing the cells was then placed in the superfusion chamber and cells were superfused with the same solution excluding the probe and BSA, at 32°C. Production of intracellular ROS was measured by changes in fluorescence intensity due to the oxidation of CM-DCFH to the fluorescent product CM-DCF by ROS. CM-DCF fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 535 nm, using a neutral density filter to lower the light intensity to only 1% transmission. The difference of the fluorescence reading at baseline and following 20 min superfusion with H_2O_2 was

converted to a percentage increase in fluorescence in order to compare differences between intracellular ROS levels in cells from rats fed a FO diet compared to a SF diet.

4.2.2 The Effect of Three Weeks Dietary Lipid Supplementation on Ventricular Cardiomyocyte Membrane Fluidity.

4.2.2.1 Animals and Feeding Protocol

The cells used in this study were from the animals used in the experiments detailed in Chapter 6. Therefore animals, feeding protocol and dietary fatty acid analysis will be described in detail in that chapter. Briefly, cells were isolated from rats fed a lipid supplemented diet for 3 weeks. The fish oil and lard were the same as that used in Chapter 3 (Table 3.3), with the difference being that these supplements were mixed into the low-ALA diet prepared in house. Lipid-supplemented diets were stored at minus 20°C until use and diets in the rat feeding trays were changed every second day to minimize oxidation. A summary of the dietary fatty acid analysis is presented in Table 4.3.

FAME[a]	Saturated Fat	Fish Oil
Σ SFA	36.37 ± 0.28	18.58 ± 0.25
Σ MUFA	55.12 ± 0.91	44.03 ± 0.33
Σ PUFA	8.50 ± 0.63	37.39 ± 0.08
Σ n-6	7.14 ± 0.46	7.80 ± 0.04
Σ n-3	1.37 ± 0.17	29.39 ± 0.16
n-6/n-3	5.26 ± 0.32	0.27 ± 0.00

Table 4.3: Fatty acid composition (wt%) of the lipid supplemented diets.
Data shown are mean ± SEM (n=3). The shorthand notation for fatty acid structure was given in the legend to Table 3.1. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-7 and n-9 isomers. Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids. The fatty acid analysis of the oils was given in Chapter 3.

4.2.2.2 Measurement of Cardiomyocyte Membrane Fluidity

Membrane fluidity was determined by measuring the steady-state fluorescence anisotropy (R_{SS}) of the probe 3-(diphenylhexatrienyl) propyltrimethylammonium *p*-toluene sulfonate

(TMAP-DPH) according to a modification of a method described previously (de Jonge *et al.*, 1996; Leifert *et al.*, 1999b). This probe readily partitions into the cell membrane and is positioned by the TMAP moiety in the membrane lipid bilayer (Lentz, 1989; Beck *et al.*, 1993). Freshly isolated adult rat ventricular cardiomyocytes attached to laminin-coated glass coverslips were washed in Tyrode's buffer containing 2 mM Ca^{2+}. Cardiomyocytes were then loaded with 1 μM TMAP-DPH for 15 min at 37°C in the dark. Cells on coverslips were then placed in a glass cuvette and R_{SS} values were measured according to the following formula as described previously (Lentz, 1989):

$$R_{SS} = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH})$$

where I_{VV} and I_{VH} represent the fluorescence intensity parallel and perpendicular to the excitation plane (when set vertically), respectively. G is a correction factor for the difference in the transmission efficiency for vertically and horizontally polarised light, and is calculated by I_{HV}/I_{HH}. Relative membrane fluidity is inversely related to the R_{SS}. Decreased R_{SS} levels indicate a higher level of anisotropy of TMAP-DPH within the bilayer lipids and hence likely reflect increased cell membrane fluidity. Measurements were obtained using a spectrofluorometer (Hitachi, Tokyo, Japan, model 650-10S), provided with vertical and horizontal polarisation filters (Polaroid, Castle Hill, NSW, Australia). The excitation and emission monochromators were set at wavelengths of 350 nm and 430 nm respectively, with slit width set to 10 nm for both excitation and emission modes. Readings were corrected for both background fluorescence of TMAP-DPH and intrinsic light scatter by the cardiomyocyte preparation. Measurements were taken every 5 min for a total of 20 min. To investigate the effects of free radicals on cardiomyocyte membrane fluidity, 2.3 mM purine plus 7 mU/ml xanthine oxidase were added to the cuvette 3 min prior to the first measurement being taken on the spectrofluorometer.

4.3 Results

The aim of the studies carried out in this chapter was to compare the effects of dietary FO and SF supplementation on Ca^{2+} handling dynamics, intracellular ROS levels and membrane fluidity of isolated ventricular cardiomyocytes. Cardiomyocytes used in the experiments were isolated from adult rats fed either an SF or a FO diet. For brevity, hereafter, these cells will be termed SF cells or FO cells.

4.3.1 The Effect of Four Weeks Dietary Lipid Supplementation on Intracellular Ca^{2+} Handling and ROS levels in Ventricular Cardiomyocytes.

4.3.1.1 Body weights

The body weight of rats fed the lipid supplemented diets were recorded during the 4 weeks of lipid supplementation (feeding began at 9 weeks of age). As shown in Figure 4.3, there were no significant changes differences in body weights of the animals between the two dietary groups (ANOVA).
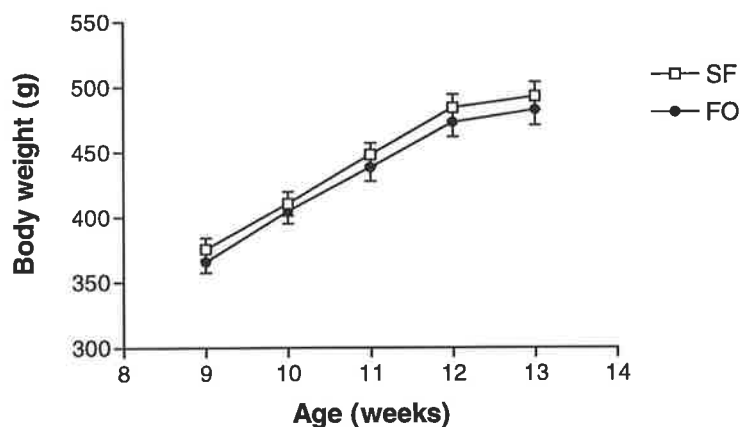
Figure 4.3: Changes in body weights of rats during lipid supplementation with SF or FO for 4 weeks. Abbreviations: FO, fish oil, n=10; SF, saturated fat, n=13. The number of rats indicated on this graph is greater than those used in each of the fluorescent probe studies, due to the fact that it was not always possible to perform all the experimental procedures on cells of one rat. Data are shown as mean ± SEM for the indicated number of animals in each group. The "control" rats fed the 17% (w/w) Sunola oil diet (CON) were not used in the experimental phase of the present study, however the fatty acid composition of the group was used for statistical comparisons in analysing the effect of dietary SF and FO supplementation on myocardial fatty acid composition. Hence, only the body weights of the experimental animals is graphed.

4.3.1.2 Myocardial Fatty Acids

The fatty acid composition of myocardial total phospholipids from rats fed the SF and FO

diets are shown in Table 4.4.

FAME[a]	CON (n=6)	SF (n=8)	FO (n=8)
14:0	0.08 ± 0.01	0.14 ± 0.02	0.12 ± 0.03
16:0	7.44 ± 1.27	8.37 ± 1.22	9.82 ± 0.54
16:1	0.11 ± 0.03	0.12 ± 0.03	0.31 ± 0.06[i,f]
17:0	0.12 ± 0.03	0.38 ± 0.02[e]	0.35 ± 0.04[h]
18:0	25.42 ± 0.17	25.29 ± 0.39	23.33 ± 0.24[h,k]
18:1[b]	14.46 ± 1.09	12.73 ± 0.35	10.31 ± 0.26[h,i]
18:2 (n-6)	11.19 ± 0.59	10.33 ± 0.79	5.85 ± 0.37[h,k]
18:3 (n-3)	0.04 ± 0.01	0.04 ± 0.003	0.05 ± 0.003
20:0	0.18 ± 0.04	0.14 ± 0.04	0.18 ± 0.04
20:1	0.15 ± 0.01	0.10 ± 0.01	0.24 ± 0.02[g,k]
20:2	0.35 ± 0.02	0.78 ± 0.03[e]	0.18 ± 0.03[g,k]
20:3 (n-6)	0.29 ± 0.01	0.49 ± 0.03[e]	0.37 ± 0.02[j]
20:4 (n-6)	30.50 ± 1.74	29.30 ± 0.88	17.54 ± 0.24[h,k]
22:0	0.30 ± 0.16	0.34 ± 0.02	0.18 ± 0.07
22:1	n/d	n/d	0.38 ± 0.02
20:5 (n-3)	0.05 ± 0.01	0.08 ± 0.004	2.79 ± 0.14[h,k]
24:0	1.09 ± 0.09	0.87 ± 0.06[c]	0.12 ± 0.01[h,k]
24:1	0.07 ± 0.01	0.10 ± 0.01	0.10 ± 0.04
22:5 (n-3)	0.28 ± 0.05	0.95 ± 0.15[d]	2.89 ± 0.12[h,k]
22:6 (n-3)	8.12 ± 0.77	10.12 ± 0.96	25.11 ± 0.73[h,k]
Σ SFA	34.47 ± 0.99	35.31 ± 1.28	34.07 ± 0.52
Σ MUFA	14.72 ± 1.09	13.01 ± 0.35	11.14 ± 0.26[g]
Σ PUFA	50.81 ± 2.00	51.67 ± 1.58	54.78 ± 0.75
Σ n-6	41.98 ± 1.35	40.13 ± 0.76	23.77 ± 0.32[h,k]
Σ n-3	8.48 ± 0.73	11.16 ± 1.00	30.84 ± 0.93[h,k]
n-6/n-3	4.95 ± 0.31	3.76 ± 0.27[d]	0.78 ± 0.03[h,k]

Table 4.4: Fatty acid composition of ventricular phospholipids from rats supplemented with SF or FO diets for 4 weeks.
Data shown are mean ± SEM for the number of animals indicated in brackets. The shorthand notation for fatty acid structure was given in the legend to Table 3.1. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. n/d not detected. [a]FAME, fatty acid methyl ester, [b]18:1 contains the n-7 and n-9 isomers, [c]p<0.05, [d]p<0.01, [e]p<0.001 for CON vs SF; [f]p<0.05, [g]p<0.01, [h]p<0.001 for CON vs FO; [i]p<0.05, [j]p<0.01, [k]p<0.001 for SF vs FO (by ANOVA with the Bonferroni multiple comparisons test). CON, control diet, SF, saturated fat, FO, fish oil diet.

The total levels of PUFAs were not significantly different between the three dietary

groups, however, the proportion of n-6 PUFAs was lower and the n-3 PUFAs higher in

the FO group (p<0.001 compared to SF and CON). Hence the n-6/n-3 ratio was lower in the FO group (p<0.001 compared to SF and CON). There was no significant difference in the levels of SFAs between the CON and the SF or FO supplemented groups, however the FO group contained significantly lower proportions of MUFAs (p<0.01 versus CON).

4.3.1.3 Cell Viability

The viability of cells isolated from the dietary lipid supplemented rats was not significantly different between the two groups. For the SF group, 77.5 ± 1.5% of cells were rod shaped immediately after isolation (n=8) compared with 76.1 ± 1.1% rod-shaped cells (n=7) in the FO dietary group.

4.3.1.4 Free Radical Induced Asynchronous Contractile Activity of Cardiomyocytes

In response to superfusion with 30 μM H_2O_2, cardiomyocytes developed asynchronous contractile activity when electrically stimulated at 0.5 Hz. As shown in Figure 4.4, FO feeding significantly shifted the asynchrony curve to the right, indicating that the cells derived from the FO supplemented rats were less sensitive to the effects of exogenous H_2O_2, with comparatively less damage occurring over time. At all time points between 10-18 min following the addition of H_2O_2, there was a consistently lower percentage of cells from the FO group contracting in an asynchronous manner compared to SF cells (p<0.05 and p<0.01). Furthermore, the $T_{50\%}$ was significantly higher in the FO group (p<0.001), i.e. a longer time for the development of asynchronous contractile activity following addition of H_2O_2 in the FO group.
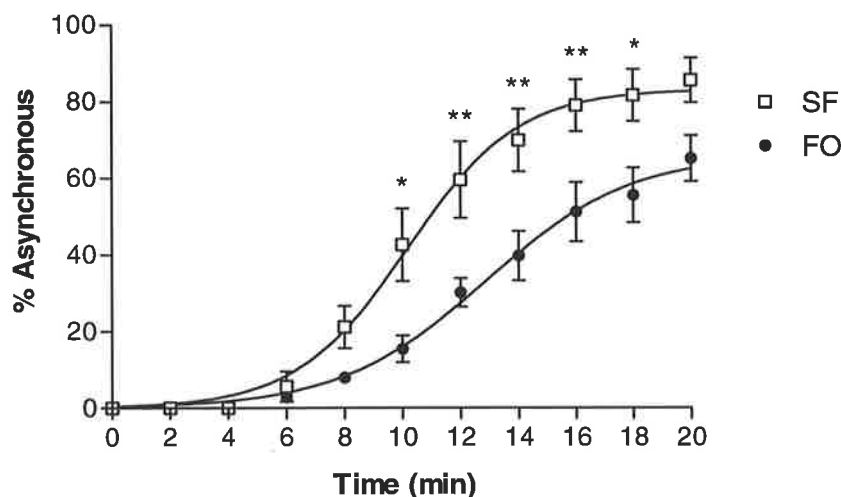
Figure 4.4: Development of asynchronous contractile activity following addition of 30 μM H₂O₂.
Data are shown as the mean ± SEM with each point being the average of approximately 20 cells, in triplicate. n = 4-5 rats. Abbreviations: FO, fish oil; SF, saturated fat diet; *p<0.05; **p<0.01.

4.3.1.5 Ca^{2+} Handling, Fish Oil and H_2O_2

The 340 nm/380 nm Ca^{2+} ratio was used as a comparative $[Ca^{2+}]_i$ indicator (as previously discussed). As shown in Figure 4.5, the baseline diastolic and systolic $[Ca^{2+}]_i$ levels were not significantly different between cells from the SF and FO dietary groups.
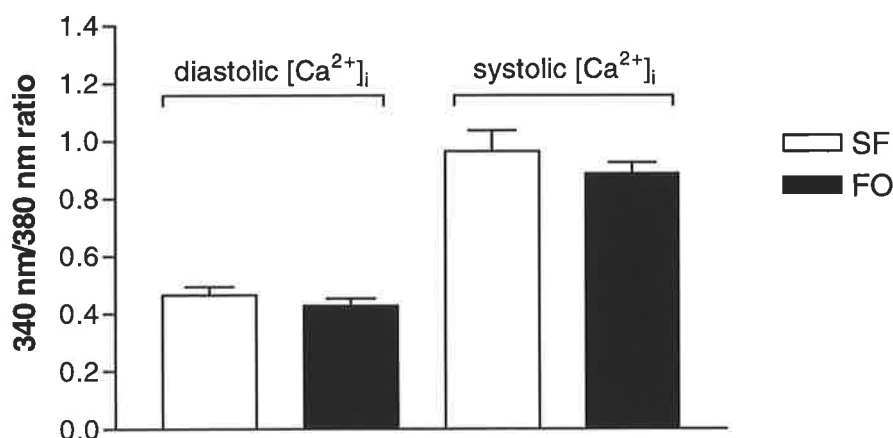
Figure 4.5: Diastolic and Systolic $[Ca^{2+}]_i$ of cardiomyocytes under resting cell conditions.
Cells were isolated from rats fed SF or FO supplemented diets for 4 weeks, loaded with 5 μM Fura-2 for 15 min at 32°C in Tyrode's solution containing 2 mM Ca^{2+}, 1.3% (w/v) BSA and 30 mM BDM. The experiment was carried out at 25°C with cells stimulated at 0.5 Hz in Tyrode's containing 2 mM Ca^{2+}. Data are presented as mean ± SEM of the 340/380 nm Fura-2 ratio for 3-4 cells from each of 5-6 rats. Abbreviations: BDM, 2,3-butane-dione monoxime; BSA, bovine serum albumin; FO, fish oil supplemented diet; SF, saturated fat supplemented diet.

During superfusion with H_2O_2, the 340/380 nm Fura-2 ratio in the SF group gradually

increased, reflecting an increase in the diastolic $[Ca^{2+}]_i$, while the diastolic $[Ca^{2+}]_i$ in the

FO group decreased (Figure 4.6A). Hence, at 16 min following the addition of H_2O_2 the

diastolic $[Ca^{2+}]_i$ of the SF group was 1.36 times higher than in the FO group ($p<0.05$).

Similarly, following 16 min superfusion with H_2O_2, the systolic $[Ca^{2+}]_i$ for cells from the

SF group was 1.36 times higher than those from the FO group ($p<0.01$, Figure 4.6B).

A
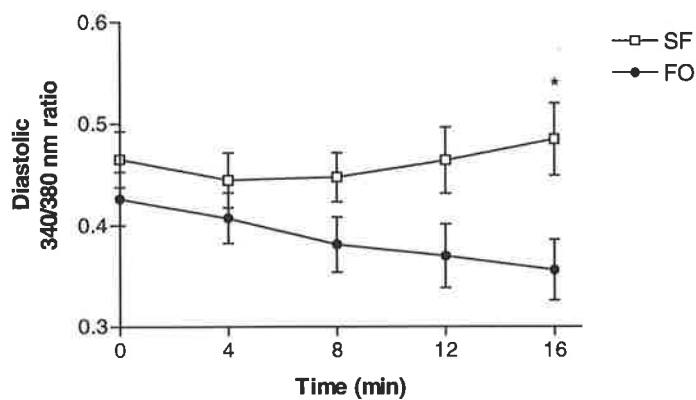
B
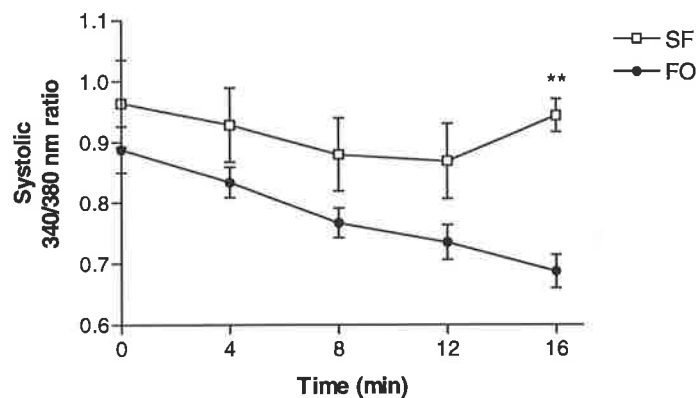
Figure 4.6: Changes in diastolic (A) and systolic (B) $[Ca^{2+}]_i$ during H_2O_2 superfusion in myocytes from the SF and FO dietary groups.
At time = 0, 30 μM H_2O_2 was added, and the 340/380 nm Fura-2 ratio was measured. A large proportion of cells developed asynchronous contractile activity after 20 min, hence only the results up to 16 min are presented in this and subsequent figures. Loading and superfusion conditions were as presented in the legend to Figure 4.5. *$p<0.05$; **$p<0.01$ for SF vs FO at 16 min (ANOVA), data are presented as mean ± SEM for n=3-4 cells from each of 5-6 rats per dietary group. [SF: time 0 vs 16 min for both diastolic and systolic $[Ca^{2+}]_i$ (ns). FO: systolic $[Ca^{2+}]_i$ for time 0 vs 16 min ($p<0.01$), not shown on graphs].

The number of values for the measured parameters at the 20 min time point (following addition of H_2O_2) was n=3 or lower than the other time points, due to the large percentage of cells asynchronously contracting at that time. This did not allow for statistical analyses to be carried out for this time point. Measurements at the 20 min time point are presented in the section detailing the results of the asynchronously contracting cells. As shown in Figure 4.7, the developed Ca^{2+}, ie the difference in systolic and diastolic $[Ca^{2+}]_i$ was significantly lower in cells of the FO group compared to the SF group (p<0.05) following 16 min exposure to H_2O_2.
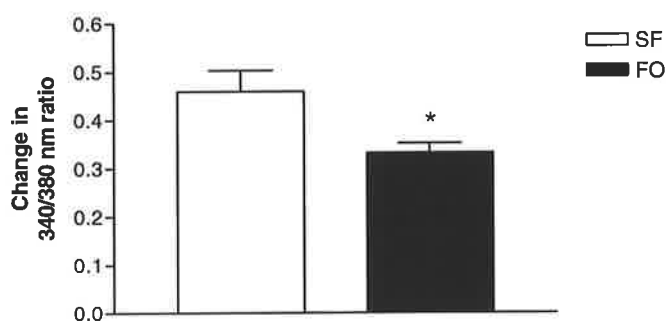
Figure 4.7: The developed Ca^{2+} in SF vs FO cells.
Ratios were measured following 16 min superfusion with 30 μM H_2O_2. Loading and superfusion conditions were as presented in the legend to Figure 4.5. *p<0.05 (t-test); data are presented as mean ± SEM for n=3-4 cells from each of 5-6 rats per dietary group.

The rate of rise in intracellular Ca^{2+} was measured as the slope of the upstroke of the ensemble averaged Ca^{2+} transient (as schematically shown in Figure 4.1).
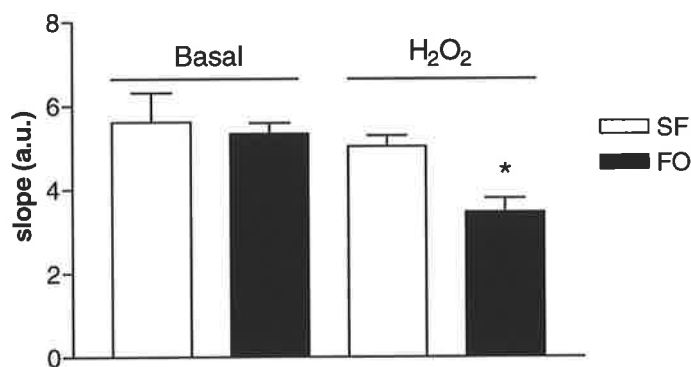
Figure 4.8: The rate of rise in $[Ca^{2+}]_i$ influx (slope) at rest and following exposure to H_2O_2 in SF and FO cells.
Loading and superfusion conditions were as presented in the legend to Figure 4.5. *p<0.05 for SF vs FO (H_2O_2) ANOVA. Data are presented as mean ± SEM for n=3-4 cells from each of 5-6 rats per dietary group.

The basal slope (expressed as arbitrary units) was not significantly different between cells in the FO or SF group. However, following exposure to 30 μM H_2O_2, the rate of rise of Ca^{2+} was slower in the FO cells (p<0.05) while in the SF group, it remained unchanged (Figure 4.8). The time constant of Ca^{2+} transient decay (indicating the rate of Ca^{2+} removal from the cytoplasm, ie cell relaxation) was not significantly different between cells from the two dietary groups of rats either before or after the addition of H_2O_2. However, within the FO group itself, following 16 min of exposure to H_2O_2, there was a significant decrease in the value of the time constant (p<0.05 vs basal, Figure 4.9).
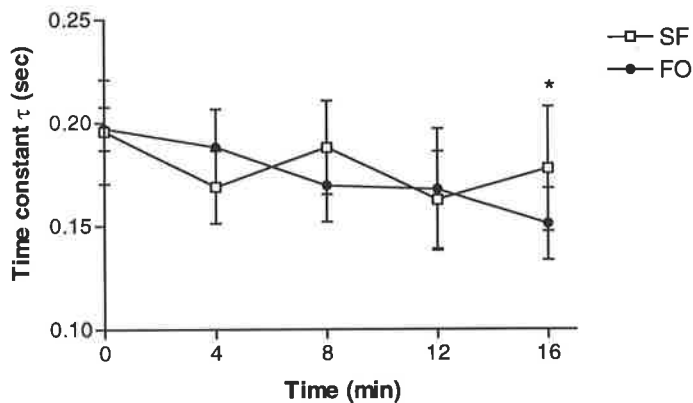
Figure 4.9: The effect of 30 μM H_2O_2 on the time constant of Ca^{2+} transient decay.
Loading and superfusion conditions were as presented in the legend to Figure 4.5. Data are presented as mean ± SEM for n=3-4 cells from each of 5-6 rats per dietary group. *p<0.05 for FO cells at the 16 min time point versus baseline.

4.3.1.6 Ca^{2+} Handling During Asynchronous Contractile Activity

During asynchronous contractile activity (i.e. at 20 min after addition of 30 μM H_2O_2) the diastolic $[Ca^{2+}]_i$ in SF cells was 1.4 times higher than the FO group (p<0.01; Figure 4.10A). There was also a trend towards a higher systolic $[Ca^{2+}]_i$ in the SF group, which had a value 1.3 times higher than the FO, however this did not reach statistical significance (Figure 4.10B).
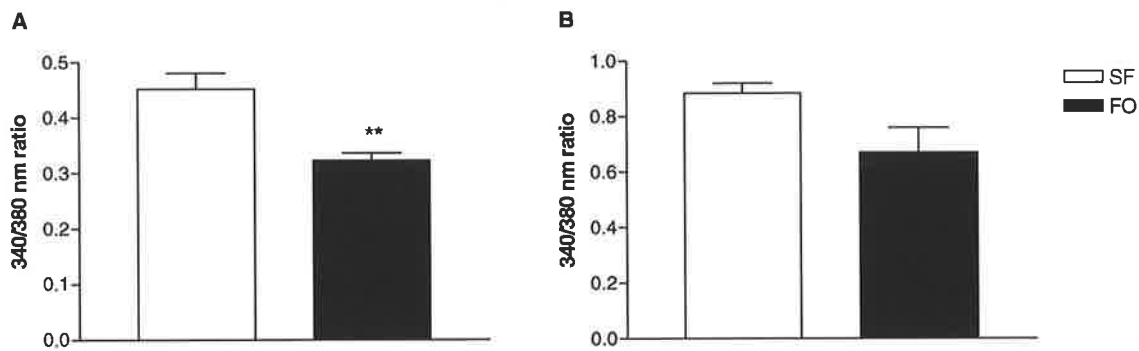
Figure 4.10: Diastolic (A) and systolic (B) [Ca²⁺]ᵢ in asynchronously contracting SF and FO cells.
Ratios were measured at 20 min following 30 µM H_2O_2 addition. Loading and superfusion conditions were as presented in the legend to Figure 4.5. Data are presented as mean ± SEM for n=3-4 cells from each of 5-6 rats per dietary group, **p<0.01.

The time to 50% decay of the Ca^{2+} transient ratio ($Td_{50\%}$) was used as a measure of the size of the Ca^{2+} transients during asynchronous contractile activity, with a longer $Td_{50\%}$ indicating a broader transient and likely representing a greater degree of Ca^{2+} cycling due to the delayed removal of Ca^{2+} from the cell (as schematically depicted in Figure 4.2). During acquisition of the data, it was noticed that the "wider" transients corresponded to fibrillatory contractile activity. There was a trend towards a shorter $Td_{50\%}$ in the FO cells at 20 min after the addition of H_2O_2, ie reflecting a less broader transient, which would be suggestive of reduced Ca^{2+} cycling within the cells, however, this change did not reach statistical significance (Figure 4.11A). Similarly, neither the area under the curve (Figure 4.11B), nor the integral (Figure 4.11C) were significantly different between the SF and FO groups during asynchronous contractile activity. It is possible that statistical significance was not achieved due to the large variation in the data obtained from cells isolated from SF fed animals.

A

B

C
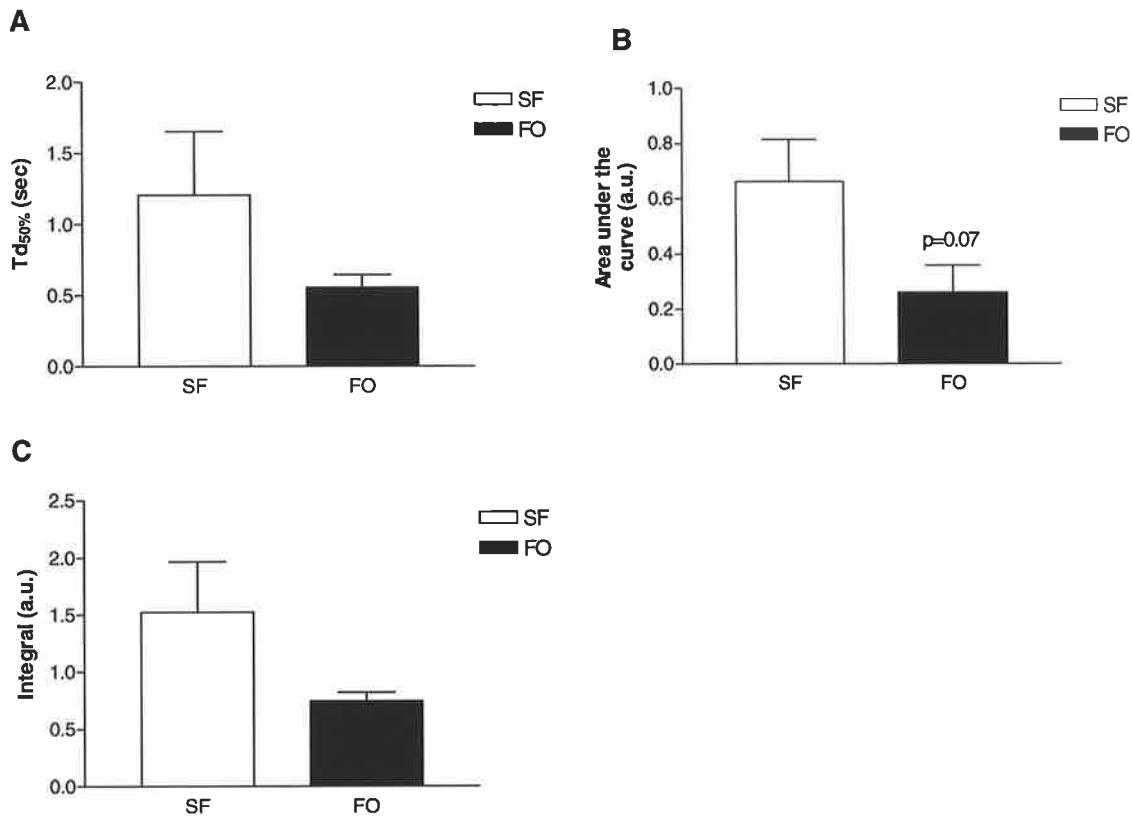
Figure 4.11: Changes in (A) Td$_{50\%}$, (B) area under the Ca^{2+} transient and (C) integral of the Ca^{2+} transient during H$_2$O$_2$ superfusion, for cells isolated from rats fed SF or FO diets.
Loading and superfusion conditions were presented in the legend to Figure 4.5. Data are presented as mean ± SEM for n=3-4 cells from each of 5-6 rats per dietary group.

4.3.1.7 Measurements of Intracellular ROS Production by Fluorescence Microscopy

The basal levels of ROS in cells from the SF group were not significantly different to those from the FO group, with the fluorescence intensity of CM-DCF (arbitrary units) in SF cells being 1.28 ± 0.17 (n=6) compared with 0.92 ± 0.07 (n=6) for the FO cells (results not shown). Following 30 μM H$_2$O$_2$ superfusion, the fluorescence of CM-DCF increased over the 20 min period of the study. As shown in Figure 4.12, the percentage increase in fluorescence intensity following 30 μM H$_2$O$_2$ addition was not significantly different between cells derived from the two dietary groups (ANOVA).
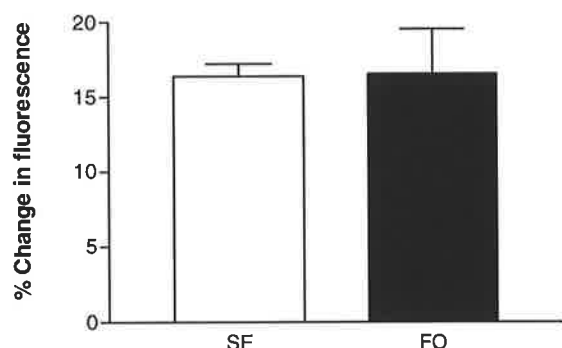
Figure 4.12: Change in fluorescence intensity following H$_2$O$_2$ addition.
Cardiomyocytes were loaded with 10 μM CM-DCFH for 15 min at 25°C in the dark, followed by exposure to 30 μM H$_2$O$_2$ for 20 min. CM-DCF fluorescence intensity was measured at an excitation wavelength of 490 nm and an emission wavelength of 535 nm. Fluorescence is presented as the mean ± SEM for n=3-4 cells from each of 6 rats for both the FO (fish oil) and SF (saturated fat) dietary groups.

4.3.2 The Effect of Three Weeks Dietary Lipid Supplementation on Ventricular Cardiomyocyte Membrane Fluidity.

4.3.2.1 Fatty Acid Composition of Rat Ventricular Phospholipids

The fatty acid composition of the ventricular cardiomyocyte phospholipids following 3 weeks dietary lipid supplementation is presented in Table 6.4 and discussed extensively in that chapter. Briefly, there was no significant difference between the levels of SFAs or total PUFAs in the myocardial total phospholipids of SF and FO fed rats. There were significantly lower proportions of MUFAs (p<0.001) and n-6 PUFAs (p<0.01) in the FO group while the proportions of n-3 PUFAs were significantly elevated (p<0.001) in comparison with the SF group (Table 4.5).

FAME[a]	SF	FO
Σ SFA	37.65 ± 2.41	35.99 ± 0.70
Σ MUFA	14.87 ± 0.64	10.93 ± 0.48[b]
Σ PUFA	47.48 ± 2.69	53.08 ± 0.87
Σ n-6	40.14 ± 2.43	26.72 ± 1.73[c]
Σ n-3	6.88 ± 0.90	26.23 ± 0.94[b]
n-6/n-3	6.21 ± 0.60	1.04 ± 0.10[b]

Table 4.5: Fatty acid composition (wt%) of ventricular cardiomyocyte total phospholipids following 3 weeks dietary lipid supplementation.
Data are mean ± SEM of 6 rats. The shorthand notation for fatty acid structure was given in the legend to Table 3.1. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. n/d, not detected. [a]FAME, fatty acid methyl esters, [b]p<0.001, [c]p<0.01 for SF vs FO (Unpaired t-test with Welch's correction). Abbreviations: FO, fish oil diet; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SF, saturated fat; SFA, saturated fatty acids.

4.3.2.2 Membrane Fluidity

As shown in Figure 4.13, the membrane fluidity of cardiomyocytes isolated from rats fed

the two dietary lipid supplements was not significantly different.
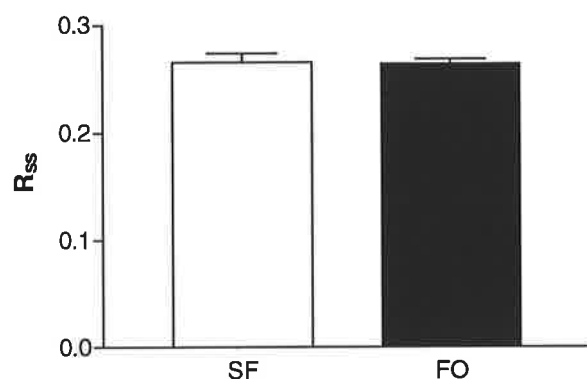
Figure 4.13: Cardiomyocyte membrane fluidity of cells isolated from SF or FO- fed rats.
Fluidity was measured using the fluorescent probe TMAP-DPH at 37°C. Cells were attached to glass coverslips, with approximately 70-90% of the surface of each coverslip covered with a layer of cells. Coverslips were placed into a quartz cuvette containing Tyrode's solution with 2 mM Ca^{2+}, thus all cells on the coverslip were included in the measurements. FO: n=8 rats, 3 coverslips per rat; SF: n=6 rats, 3 coverslips per rat.

It was noted upon probe addition, under basal conditions (no ROS addition), that the R_{SS}

value significantly decreased in cells from both the SF and FO fed rats ($p<0.05$ and

$p<0.01$ respectively), indicating a relative increase in cell membrane fluidity over time

during the first 20 min (Figure 4.14A). However, in the cells treated with ROS (P/XO) the

change was even more significant ($p<0.01$ for SF and $p<0.001$ for FO) over the 20 min

incubation with ROS (Figure 4.14B). Thus, in order to analyse the changes induced by the

ROS and to determine if the membrane fluidity was further influenced by dietary lipid

supplementation, (i.e. changes above this gradual increase in fluidity), the percentage

increase in fluidity after 20 min (in both control and ROS stimulated cells) was compared

between the SF and FO derived cells.

(A)

(B)
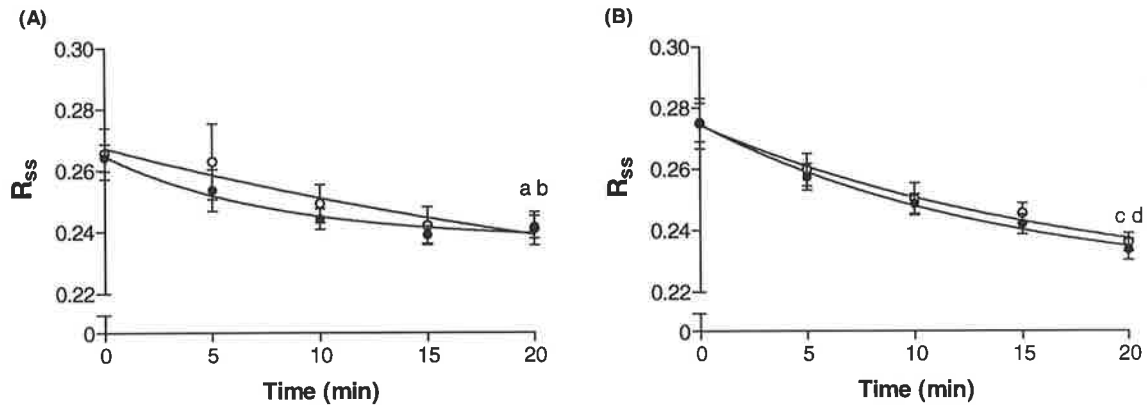
Figure 4.14: (A) Basal membrane fluidity and (B) fluidity following P/XO addition.
Cardiomyocytes were loaded with 2 μM TMAP-DPH for 15 min and R_{SS} values measured for 20 min. Fluidity is inversely related to R_{SS}. Open circles represent data from the SF group, closed circles refer to the FO group. ^{a}p<0.05 and ^{c}p<0.01 for SF, 0 min vs 20 min; ^{b}p<0.01 and ^{d}p<0.001 for FO, 0 min vs 20 min.

As shown in Figure 4.15, the percent increase in fluidity for untreated cells (no ROS addition) from the SF group was 9.0 ± 2.6% compared with an increase of 13.9 ± 1.8% following 20 min incubation with ROS (n=6, ns). In the FO group, addition of ROS caused an increase in fluidity of 14.9 ± 1.6% compared to 8.4 ± 1.9% in the cells not exposed to the ROS (p<0.05). Therefore, while the dietary lipid supplementation alone did not influence the overall (basal) membrane fluidity as measured by steady-state fluorescence polarisation, treatment of cardiomyocytes with ROS led to an increase in cell membrane fluidity (ie a decrease in R_{SS} value) which only reached significance in the FO group.
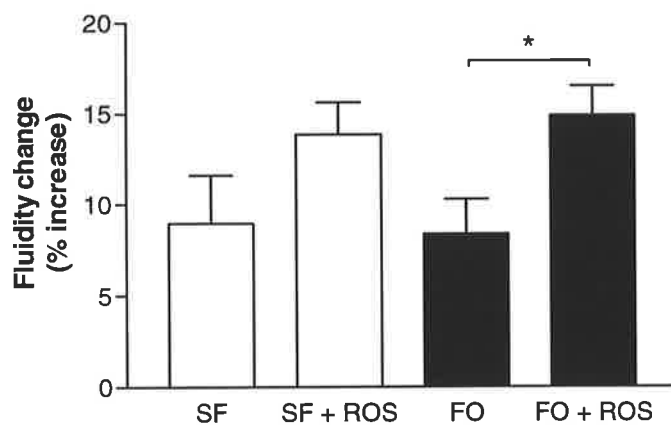
Figure 4.15: Percentage changes in membrane fluidity after ROS addition.
The data presented in Figure 4.14 (at the 20 min time point) was converted into the percentage increase in fluidity in order to compare possible differences between the two dietary groups. SF, saturated fat diet; FO, fish oil diet; ROS, reactive oxygen species; *p<0.05 for FO vs FO + ROS.

4.4 Discussion

The aims of this chapter were to investigate the possible mechanisms underlying the protective effects of the n-3 PUFAs on ROS-induced asynchronous contractile activity as demonstrated in Chapter 3. Such cellular dysfunction is considered to be associated with reperfusion-type arrhythmias, with the two most relevant hypotheses explaining the cellular events involved in reperfusion damage being those in which Ca^{2+} overload or ROS-induced cellular damage occur (Opie, 1989). These aims were designed in order to test the hypothesis that dietary FO delays the development of reperfusion-induced arrhythmias, in part, by attenuating the ROS-induced intracellular Ca^{2+} or ROS overload. It was demonstrated in this chapter, that cardiomyocytes isolated from rats supplemented with FO had higher proportions of phospholipid n-3 PUFAs, were less sensitive to the arrhythmogenic effects of ROS and did not develop Ca^{2+} overload in response to H_2O_2. Furthermore, FO cells did not exhibit significantly different basal membrane fluidity or intracellular ROS levels in comparison to cells isolated from SF fed rats, however following ROS exposure, membrane fluidity was increased.

Several lines of evidence support a role for ROS in reperfusion injury (McCord, 1985; Zweier *et al.*, 1987; Bolli *et al.*, 1989a; Bolli *et al.*, 1989b; Onodera & Ashraf, 1991). In the present study, adult rat ventricular myocytes were challenged with H_2O_2 in order to induce cellular arrhythmias considered characteristic of reperfusion type injury. Cells isolated from rats fed a FO supplemented diet exhibited a delayed response with regard to the development of asynchronous contractile activity; ie they were less sensitive to the damaging actions of H_2O_2. As hypothesized in Diagram 4.1A, it is possible that the FO cells may have been protected from arrhythmias by accumulating lower levels of intracellular ROS (shaded regions) in response to the H_2O_2 challenge (since H_2O_2 has

been shown to generate the highly reactive •OH radical by its reaction with intracellular iron (Josephson *et al.*, 1991)). Indeed, a recent study showed that the n-3 PUFA EPA inhibited the γ-irradiation-induced ROS accumulation in hippocampal tissue from rats (Lonergan *et al.*, 2002). However, following 20 min of superfusion with H_2O_2, the overall increase in fluorescence intensity, indicative of increased intracellular ROS accumulation, was not significantly different between cells isolated from FO fed rats compared with rats supplemented with SF.
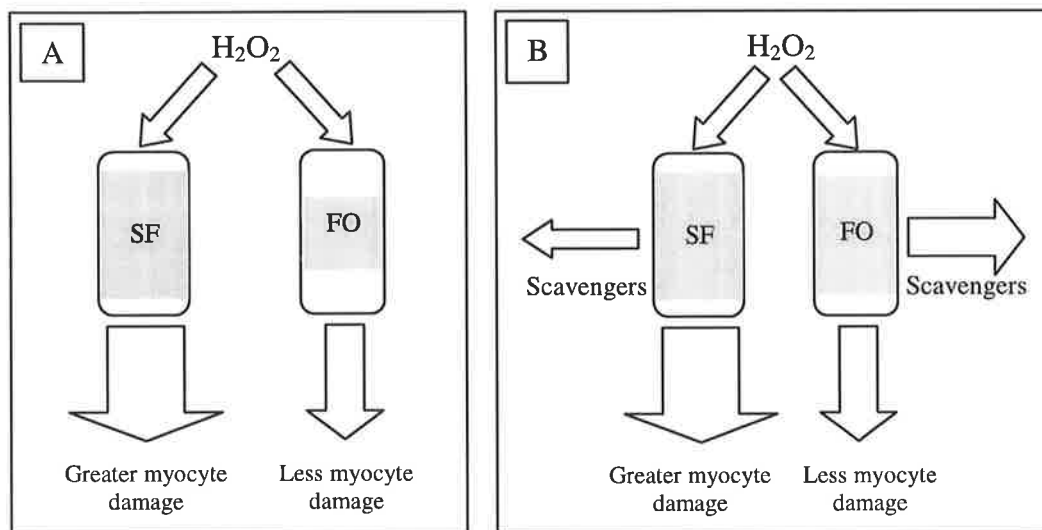
Diagram 4.1: Hypothesized mode of action of dietary FO in reperfusion injury in terms of antioxidant defences.
Although both SF and FO cells receive the same dose of H_2O_2, the FO cell7s are less damaged, possibly due to reduced ROS accumulation intracellularly in the FO cells (A). This may be due to greater antioxidant defences (scavengers) in the FO group (B) which minimise the damaging effects of the ROS, since the measured intracellular levels of ROS (shaded regions of cells) were not significantly between the SF and FO cells.

Alternatively, as hypothesized in Diagram 4.1B, the protective effect of dietary FO, in the presence of comparable intracellular levels of ROS, could also be explained by a more efficient scavenging system in the cardiomyocytes isolated from the FO fed rats which removes or nullifies the damaging effects of free radicals on cellular function to a relatively greater extent. The results thus far support the notion that the FO supplementation enhances the endogenous myocardial antioxidant defences, since in response to the same dose of H_2O_2, the FO cells developed similar intracellular levels of

ROS as the SF cells, with a concomitant lower percentage of cells developing asynchronous contractile activity. It is likely that as a consequence of the strengthened antioxidant defences in the FO myocardium, a lower proportion of ROS would act via the damaging pathway. In contrast, in the SF cells, a smaller proportion of ROS may be scavenged by the antioxidant system, allowing most of the ROS to act through the damaging pathway resulting in a greater degree of arrhythmogenesis. This hypothesis is further investigated in Chapter 5.

ROS have been implicated in the pathogenesis of myocardial ischaemia-reperfusion injury possibly via a mechanism which involves damage and perturbation to cell membranes (Bagchi *et al.*, 1989). These effects may underlie their arrhythmia induction. To investigate this further, cell membrane fluidity was measured, before and after the addition of ROS. One means by which ROS might damage cell membranes may be by their actions on membrane phospholipid fatty acids, e.g. causing fatty acid oxidation and cross linking. Since fatty acids are significant determinants of cell membrane fluidity, it would be likely that under such circumstances, membrane fluidity would be altered. Previous studies have demonstrated a significant fluidising effect of acute addition of the n-3 PUFA DHA in endothelial cells (Hashimoto *et al.*, 1999) and in cardiomyocytes (Jahangiri *et al.*, 2000; Leifert *et al.*, 2000). The known fluidising agent, benzyl alcohol also increased membrane fluidity, whilst saturated fatty acids were without effect (Jahangiri *et al.*, 2000). It is important to note in this context, that this reported effect of DHA on endothelial cells and cardiomyocytes occurs immediately following acute addition to such cells, and is not reliant on actual incorporation of exogenously added fatty acids into membrane phospholipids. The effect of acute n-3 PUFAs on increasing cardiomyocyte membrane fluidity may be explained by the disordering effect that such

highly polyunsaturated, free fatty acids would have on the acyl fatty acid chains of the membrane phospholipids. However, in the present study, there was no change in overall cardiomyocyte membrane fluidity following dietary lipid supplementation measured using the probe TMAP-DPH, despite a considerable increase in incorporation of DHA and EPA into the total myocardial phospholipids. This finding is supported by the data of Vognild *et al.* (1998) in which it was reported that in humans, consumption of 15 ml of cod liver oil per day, did not alter platelet membrane fluidity. Similarly, despite enrichment of membrane phospholipids with n-3 PUFAs, no increase in membrane fluidity was reported following DHA feeding in rats (Hagve *et al.*, 1998) and in another study, 5 weeks feeding with FO was also reported to be without effect on rat hepatocyte plasma membrane fluidity (Clamp *et al.*, 1997).

The failure to significantly alter baseline membrane fluidity despite large changes in the acyl fatty acid profile of cellular membranes, as outlined above, is likely due to a homeostatic phenomenon termed homeoviscous adaptation (reviewed in McMurchie (1988)). Homeostatic mechanisms within the cell act to re-establish normal fluidity levels following changes in and perturbations to the membrane phospholipids, such as an increase in n-3 PUFA proportions. This could be achieved by a number of compensatory mechanisms such as alterations in the cholesterol to phospholipid ratio, the phospholipid class distribution, the type of fatty acids present, with regard to the chain length and degree of unsaturation, and the combination of these acyl fatty acids on the membrane phospholipids (McMurchie, 1988). Indeed, studies have demonstrated a significantly increased level of incorporation of cholesterol following incubation of cells with acutely added n-3 PUFAs (de Jonge *et al.*, 1996; Hashimoto *et al.*, 1999) or dietary supplementation with n-3 PUFAs (Igal & Gomez Dumm, 1997; Lund *et al.*, 1999). It is

also likely that the lipid fluidity in certain regions of the membrane may have been increased by n-3 PUFA supplementation without affecting the overall membrane fluidity in the local region being interrogated by the particular fluidity probe. Local fluidity changes may also be responsible for the effects on ion current activity observed following the acute addition of n-3 PUFAs (Leifert *et al.*, 1999b), and the effects of fish oil feeding on the activities of the Ca^{2+}-ATPase (Taffet *et al.*, 1993), alkaline phosphatase (Stenson *et al.*, 1989), 5'-nucleotidase and Mg^{2+}-ATPase (Hashimoto *et al.*, 2001). The manipulation of the membrane lipid environment may also modulate membrane-associated enzyme activity by the interaction of the enzyme with specific membrane lipids *per se,* rather than by changes in the physicochemical state of the membrane lipids in the micro-domain or environment around the probe (Stenson *et al.*, 1989). Since the probe used in the present study only reports on relative changes in the bulk membrane fluidity, local changes (as opposed to bulk lipid fluidity) would be unlikely to be distinguished. Future studies could evaluate whether local membrane fluidity changes are induced by FO feeding and whether such changes affect the functioning of channels or enzyme systems involved in the genesis of free radical-induced arrhythmias.

As to the question of which membranes were influenced by the parameters employed in this study, the fluidity probe is probably limited to reporting on the state of the sarcolemmal membrane. This coincides with the region of the cell into which acutely added fatty acids would intercalate. However, fatty acids obtained in the diet would likely be incorporated into all the cellular membranes. Exogenously added ROS are likely exerting effects on the sarcolemmal membrane, inducing asynchronous contractile activity. Hence, although the probe is not measuring the fluidity of all membranes, the results coincide with the membranes affected by lipid supplementation and ROS attack.

The present study demonstrated an increase in cardiomyocyte (sarcolemmal) membrane fluidity following ROS addition (which was only significant in the FO group). This is in contrast to previous studies reporting a rigidifying effect (decrease in fluidity) of free radicals on membrane phospholipids (Bagchi *et al.*, 1989; Chen & Yu, 1994). The decrease in fluidity induced by ROS reported by others, has been attributed to the presence of lipid peroxidation products interacting with membrane components, enhancing cross-linking among the lipid and protein moieties of the membrane, thereby causing restriction in the freedom and mobility of acyl fatty acid chains in the membrane bilayer. It has also been demonstrated that lipid-comprising structures such as lipoproteins exhibit a relatively lower fluidity when the constituent lipids become oxidised (Bonnefont-Rousselot *et al.*, 1995). The present findings of a relative increase in fluidity induced by ROS may be explicable in terms of the activity of phospholipase A_2. Phospholipase A_2 has been shown to be activated during ischaemia and other cellular stresses (reviewed in Gross (1992)). On activation, this enzyme acts on the *sn*-2 acyl group of cellular phospholipids to liberate the unsaturated fatty acids that are normally preferentially incorporated in that position (reviewed in Stubbs & Smith, 1984; McMurchie, 1988). Therefore it is likely that in response to the ROS insult, the action of phospholipase A_2 liberated the n-3 PUFAs from the membrane phospholipids of the FO cells and subsequently, these released PUFAs could now act as free fatty acids to induce an increase in the membrane fluidity as was the case in the studies cited above with acute fatty acid addition. This idea is also supported by the finding that fish oil feeding *per se* increases the activity of phospholipase A_2 in rat mitochondria (Malis *et al.*, 1990). However, it remains to be elucidated whether the free fatty acids *per se* provide protective effects from ROS or whether the membrane incorporation leads to other changes responsible for the antioxidant-like effects of FO. The increase in membrane fluidity may

itself result in changes in the activity/functioning of various cardiac enzymes, receptors or ion channels.

Reperfusion arrhythmias are mainly non re-entrant in nature, likely involving an abnormal form of automaticity or triggered activity (Pogwizd & Corr, 1987; Priori *et al.*, 1990). Indeed, delayed afterdepolarisations and triggered activity occur when cytosolic Ca^{2+} is increased, for example at reperfusion (Pogwizd & Corr, 1987). Both oxygen free radicals and metabolic inhibition can increase intracellular Ca^{2+} levels and induce myocardial Ca^{2+} overload, which in turn can trigger hypercontracture, arrhythmias and other electrophysiological abnormalities (Josephson *et al.*, 1991; Goldhaber & Weiss, 1992). The ability of free radicals to alter the function of the Na^{+}-K^{+}-ATPase (Kim & Akera, 1987) and Ca^{2+}-ATPases (Kaneko *et al.*, 1989b) may also account for some of the alterations observed in the Ca^{2+} transients after exposure of cardiomyocytes to ROS such as H_2O_2. One mechanism of action which was proposed in this study in relation to the antiarrhythmic effect of the n-3 PUFAs, was with regard to their possible effects on intracellular Ca^{2+} dynamics.

The studies of this chapter were aimed at determining whether a reduced level of Ca^{2+} cycling was occurring within the FO cells during the ROS challenge. The measure of developed Ca^{2+} (the difference between systolic and diastolic $[Ca^{2+}]_i$) was used as an indication of Ca^{2+} cycling during contractions in the presence of H_2O_2. Baseline diastolic and systolic $[Ca^{2+}]_i$ were not found to be different between cells isolated from FO or SF diet fed animals. However, following exposure to H_2O_2, $[Ca^{2+}]_i$, as inferred from the fluorescent ratio measurements, was significantly lower in cells isolated from FO-fed rats. This resulted in the developed Ca^{2+} being 1.4 times lower in cells isolated from the FO

dietary group. This significant finding may indicate that whilst under basal conditions FO feeding may not alter Ca^{2+} dynamics, during free radical stress, it prevents cells from overloading with Ca^{2+} by reducing the extent of Ca^{2+} influx. Consistent with this was the finding of a significantly shallower slope (i.e. slower rate of rise in $[Ca^{2+}]_i$ in the FO cells) and a shorter time constant of Ca^{2+} transient decay (i.e. faster sequestration of Ca^{2+} into the sarcoplasmic reticulum (SR) and/or efflux from the cell) following H_2O_2 superfusion. This finding is consistent with published reports that acute addition of the n-3 PUFAs, DHA and EPA, reduced the availability of Ca^{2+} for uptake and inhibited the Ca^{2+} release mechanism in rat SR (Negretti *et al.*, 2000). Neonatal cardiomyocytes incubated for 3-5 days with EPA, were also protected from developing Ca^{2+} overload in response to 0.1 mM ouabain (Hallaq *et al.*, 1990).

It was not in the scope of the present study to investigate which ion channels and transporters were affected by the dietary lipid supplementation and ROS, however, there may be a role for either the SR or sarcolemmal calcium ion transporters in mediating some aspects of the dietary n-3 PUFA effects following cellular stress from ROS as they represent the main pathways responsible for raising $[Ca^{2+}]_i$. The contribution of Ca^{2+} buffering (Delbridge *et al.*, 1996) was not examined in the present study, however, it is likely that this factor could influence the cellular Ca^{2+} handling, and the diets themselves may affect Ca^{2+} buffering to different extents. In the present study, even in the SF cells, which were shown to be relatively more prone than the FO cells to the arrhythmogenic effects of ROS, exposure to H_2O_2 did not actually induce Ca^{2+} overload although there was a slight (but insignificant) increase in systolic $[Ca^{2+}]_i$ in the SF cells. However, the present findings indicate that under conditions of the same ROS stress, in comparison to the SF cells, the FO cells responded by lowering $[Ca^{2+}]_i$ and increasing Ca^{2+} efflux (or

sequestration into the SR), thus reducing the likelihood of Ca^{2+} overload that may occur with prolonged exposure to ROS. At the same time, these findings indicate that the asynchronous contractile activity observed in response to H_2O_2 is not solely due to Ca^{2+} overload, as both groups of cells developed arrhythmias in the absence of Ca^{2+} overload. On the other hand, it may have been that the concentrations of ROS employed, although sufficient to induce asynchronous contractile activity, did not present a severe enough stress to result in Ca^{2+} overload, as manifested by the fluorescence Ca^{2+} ratios. Indeed, 50 μM H_2O_2 did not induce Ca^{2+} uptake in rat myocytes until after 22 min exposure, reaching maximum levels 45-60 min after addition of H_2O_2 (Clague & Langer, 1994). Similarly, with regard to the ROS fluorescent probes (which also did not detect large increases in intracellular ROS levels in the present study), a 40% increase in DCF fluorescence intensity was demonstrated following a 30 min exposure to 30 μM H_2O_2 compared to a 150% increase using 1 mM H_2O_2 (Wang & Joseph, 1999). It is therefore likely that the use of higher H_2O_2 concentrations would have resulted in greater DCF fluorescence than that observed in the present study. In fact, significant increases in fluorescence intensity were not achieved until concentrations of H_2O_2 of greater than 100 μM were used (Vandenhoek *et al.*, 1997). However, rather than employ excessively high concentrations of oxidant, the concentration of H_2O_2 chosen in this study was such as to be effective in inducing an oxidative injury in a reasonable time frame for study. The aim of the present study was not to determine the effect of H_2O_2 on Ca^{2+} transient dynamics and/or actual intracellular concentrations of ROS and Ca^{2+}, but to develop a system where ROS-induced damage in myocytes did occur, and to compare the responses of cells isolated from rats fed FO and SF supplemented diets. Such high concentrations of H_2O_2 (up to 4 mM) as reportedly used in some other studies (Duan & Moffat, 1992) are useful for mechanistic studies, but would have caused such rapid injury that the investigation of

a possible protective effect of the n-3 PUFAs may not have been evident by the experimental techniques employed in this study.

In summary, the proportions of n-3 PUFAs in myocardial membrane phospholipids was significantly increased following dietary FO supplementation. Although this did not alter membrane fluidity levels under resting conditions, an increase in fluidity resulted following exposure to ROS. Cells from FO-fed rats exhibited a reduced sensitivity to the arrhythmogenic effects of H_2O_2, co-incident with reduced diastolic and systolic $[Ca^{2+}]_i$ and a reduced rate of Ca^{2+} influx into the myocytes. Basal and ROS-induced intracellular levels of ROS were not significantly different between cells isolated from rats fed SF and FO diets indicating that other pathways are likely also involved in the antiarrhythmic and antioxidant effects of n-3 PUFAs. One such mechanism, that of enhanced antioxidant enzyme gene expression induced by dietary FO will be investigated in the following chapter.

CHAPTER FIVE

The Effect of Dietary Fish Oil Supplementation on Antioxidant Enzyme Gene

Expression in Rat Myocardium.

5.1 Introduction

Free radical induced oxidative damage has been implicated in the pathogenesis of a number of injury and disease states. While constantly being subjected to oxidative stress, aerobic organisms are normally protected against oxidative damage by a variety of antioxidant systems. Major antioxidant mechanisms include: (i) interaction of ascorbic acid and reduced glutathione (GSH) with oxidants and oxidising agents; (ii) scavenging of free radicals and singlet oxygen by vitamin E, ascorbic acid, β-carotene, and superoxide dismutase; (iii) reduction of hydroperoxides by glutathione peroxidase and catalase; (iv) binding of transition metals by various chelators; and (v) repair of resultant damage via metabolic activities (Ibrahim *et al.*, 2000). Oxidative damage, however, may occur when antioxidant potential is decreased and/or when oxidative stress is increased.

The superoxide dismutases[8] (SOD, EC 1.15.1.1) are thought to be the first line of antioxidant defence from oxygen toxicity. They catalyse the dismutation of two superoxide radicals ($O_2^{\bullet-}$) to yield hydrogen peroxide (H_2O_2) and oxygen. The resulting H_2O_2 is further scavenged by catalase and glutathione peroxidase (Ho *et al.*, 1991). The mitochondrial Mn-SOD is a critical antioxidant enzyme in aerobic organisms because $O_2^{\bullet-}$ is mainly generated on the matrix side of the inner mitochondrial membrane. Thus, it would be expected that increases in Mn-SOD activity may provide increased protection against oxidative stress. Indeed, over-expression of Mn-SOD confers significant protection against ischaemia-reperfusion injury in Langendorff perfused heart

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[8] The antioxidant enzymes were described in detail in Chapter one, however, a brief summary is given again to introduce the topic.

preparations (Chen *et al.*, 1998). Increased Mn-SOD mRNA expression was also associated with protection of cardiomyocytes from hypoxia-reoxygenation induced oxidative stress (Negoro *et al.*, 2001).

Glutathione peroxidase (GPX, GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) also plays an important role as a cellular antioxidant defence mechanism by reducing H_2O_2 and various hydroperoxides using glutathione as a reducing agent to form water and the corresponding alcohols, respectively (Ho *et al.*, 1997). GPX is present in both the cytosol and mitochondria (Ho & Howard, 1992). Transgenic mice over-expressing GPX exhibited an increased tolerance to myocardial injury induced by ischaemia-reperfusion (Yoshida *et al.*, 1996), while GPX knockout mice had 100% mortality in response to oxidative stress induced by the compound diquat (Fu *et al.*, 1999).

Mammalian catalase (CAT, hydrogen peroxide:hydrogen peroxide oxidoreductase; EC 1.11.1.6) is found in virtually all aerobic cells, with highest levels in the liver, kidney and erythrocytes. This enzyme catalyses the decomposition of H_2O_2 to oxygen and water (Nakashima *et al.*, 1989). Although CAT is present at low levels of activity in the heart, it is important in the detoxification of H_2O_2 in the myocardium (Li *et al.*, 1997). Overexpression of CAT in the heart was demonstrated to not only improve the recovery of the lessened contractile force after ischaemia reperfusion, but also to reduce creatinine kinase release from the isolated Langendorff-perfused heart, and attenuate the concomitant myocardial infarction (Li *et al.*, 1997).

Previous studies have advanced the idea that incorporation of n-3 PUFAs into membrane phospholipids following dietary supplementation would bring about damaging effects to

cellular membranes by virtue of the susceptibility of the highly unsaturated acyl fatty acids to peroxidation (Gardner, 1989; Alexander-North *et al.*, 1994; van Ginkel & Sevanian, 1994). If this heightens the level of oxidative stress, then the antioxidative capacity of the heart may be diminished, and this in turn, may raise the requirement for nutritional antioxidants such as vitamin E (L'Abbe *et al.*, 1991). However, it is also possible that the n-3 PUFA induced oxidative stress, may function to potentiate the defense system and stimulate the upregulation of the antioxidant enzymes themselves (Perry *et al.*, 2000). Thus the aim of the present study was to determine whether dietary fish oil supplementation is associated with increased expression of the antioxidant enzymes Mn-SOD, CAT and GPX. This was achieved using molecular biology techniques, specifically real-time polymerase chain reaction (PCR) technology.

The specific aims of this chapter were to:

- Set up a quantitative real-time PCR method for analysis of the abundance of mRNA for antioxidant enzymes in myocardial tissue.

- Compare the relative abundance of mRNA of three antioxidant enzymes in the myocardium of rats fed fish oil and saturated fat versus the control animals.

These aims were designed in order to test the hypothesis that antioxidant enzyme gene expression will be enhanced following dietary fish oil supplementation.

FAME[a]	CON	SF	FO
14:0	0.18	3.88	4.66
14:1	0.01	0.59	0.16
16:0	4.81	20.15	13.06
16:1	0.02	0.18	0.03
17:0	0.03	0.81	0.39
18:0	3.85	13.75	3.81
18:1[b]	77.78	53.41	47.54
18:2 (n-6)	11.25	5.25	6.26
18:3 (n-3)	0.27	0.54	0.99
20:0	0.33	0.58	0.29
20:1	0.27	0.06	1.36
20:2	0.01	0.05	0.13
20:3 (n-6)	n/d	n/d	0.16
20:4 (n-6)	n/d	n/d	n/d
20:3 (n-3)	n/d	0.03	0.56
22:0	0.89	0.52	0.48
22:1	n/d	0.06	0.56
20:5 (n-3)	0.01	0.03	11.14
24:0	0.23	0.07	0.08
24:1	0.02	n/d	0.07
22:5 (n-3)	n/d	0.03	1.11
22:6 (n-3)	0.01	n/d	7.17
Σ SFA	10.33	39.77	22.77
Σ MUFA	78.10	54.30	49.72
Σ PUFA	11.57	5.93	27.51
Σ n-6	11.25	5.25	6.41
Σ n-3	0.30	0.63	20.97
n-6/n-3	37.42	8.33	0.31

5.2 Methods

5.2.1 Animals and Feeding Protocol

Male Sprague Dawley rats were obtained at 4 weeks of age and fed a modified AIN 93G

diet (low-ALA diet).

FAME[a]	CON	SF	FO
14:0	0.18	3.33	4.66
14:1	0.01	0.51	0.16
16:0	4.81	20.96	13.06
16:1	0.02	0.20	0.03
17:0	0.03	0.86	0.39
18:0	3.85	15.01	3.81
18:1[b]	77.78	57.05	47.54
18:2 (n-6)	11.25	0.08	6.26
18:3 (n-3)	0.27	0.64	0.99
20:0	0.33	0.68	0.29
20:1	0.27	0.11	1.36
20:2	0.01	0.05	0.13
20:3 (n-6)	n/d	n/d	0.16
20:4 (n-6)	n/d	n/d	n/d
20:3 (n-3)	n/d	n/d	0.56
22:0	0.89	0.38	0.48
22:1	n/d	n/d	0.56
20:5 (n-3)	0.01	n/d	11.14
24:0	0.23	0.07	0.08
24:1	0.02	n/d	0.07
22:5 (n-3)	n/d	0.06	1.11
22:6 (n-3)	0.01	n/d	7.17
Σ SFA	10.33	41.31	22.77
Σ MUFA	78.10	57.87	49.72
Σ PUFA	11.57	0.82	27.51
Σ n-6	11.25	0.08	6.41
Σ n-3	0.30	0.70	20.97
n-6/n-3	37.42	0.11	0.31

Table 5.1: Fatty acid composition (wt%) of the diets used for supplementation.
The shorthand notation for fatty acid structure was given in the legend to Table 3.1. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-7 and n-9 isomers. Abbreviations: CON, control group; FO, fish oil; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SF, saturated fat; SFA, saturated fatty acid, n/d not detectable.

The base diet contained 7% (w/w) fat, present as sunola oil and rats were maintained on this diet until 9 weeks of age. For a further 4 weeks[9], animals were fed a lipid supplemented diet, containing the base diet with an additional 10% (w/w) fat added as either sunola oil (control- CON), lard (saturated fat, SF) or fish oil (FO). Eight rats were used in each dietary group. The source of fish oil used in Chapters 3 and 4 was no longer available and therefore the fish oil supplement, Fishaphos (marketed by Felton Grimwade & Bickford Pty Ltd, Australia) was used. This FO is a natural fish oil concentrate containing n-3 PUFA triglycerides, with a slight lemon taste. In a pilot experiment, it was determined that the lemon flavour did not affect the amount of diet consumed by the rats (results not shown). The fatty acid analysis of the diets was carried out as described in Chapter 2, and the composition is presented in Table 5.1.

5.2.2 Myocardial Tissue Storage

The myocardial tissue used in this study was obtained following Langendorff perfusion of rat hearts. This is the subject of Chapter 6 where the effect of lipid supplementation on reperfusion-induced arrhythmias was investigated. A separate group of control hearts from rats fed the CON, SF and FO diets that did not undergo ischaemia-reperfusion were perfused with Tyrode's solution for the duration of the study (70 min). Following the perfusion protocol, these hearts were minced into approximately 20 mg pieces and then randomly chosen pieces totalling approximately 100 mg were snap frozen in liquid nitrogen and maintained at minus 80°C until further processing was required. The rationale for mincing the myocardial tissue into 20 mg pieces was to ensure that the sections analysed were representative of the whole heart. Another method could have

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[9] The myocardial tissue samples used in this study were those obtained from rats used in the isolated Langendorff heart studies described in Chapter 6. Due to technical difficulties with the experimental protocol, the execution of the study needed to be delayed by one week; hence it was necessary to maintain the rats on the lipid supplements for an additional week.

been to pulverise the whole heart in liquid nitrogen and 100 mg of the slurry be aliquoted into sterile tubes. However, this method was found to be too cumbersome and if not performed quickly, it was noted that the tissue thawed before aliquoting was carried out. Preliminary experiments with the minced tissue indicated that the chosen process was not damaging the tissue since the quality and yield of RNA isolated was comparable to that extracted from un-minced tissues.

5.2.3 RNA Isolation

RNA was isolated using the RNAzol™ B reagent (GeneWorks Pty. Ltd., Adelaide, Australia), with modifications of the manufacturer's instructions which are based on the method of Chomczynski & Sacchi (1987). The initial method for isolating RNA used was that supplied with the RNAzol™ B reagent. At all times, sterile techniques were employed. This involved the use of autoclaved water[10], autoclaved pipettes, glassware and other instruments, spraying all surfaces with RNase ZAP® (GeneWorks Pty. Ltd., Adelaide, Australia), and the use of sterile RNase free tubes. Using this method, intact RNA was observed following gel electrophoresis, however, although the gels did not show significant degradation of the RNA (Figure 5.4), the 260/280 nm ratio (an indicator of the purity of the RNA preparation) was below the recommended value of 1.9, and a low yield was also recorded. Various parameters of the RNA isolation protocol were modified in order to increase the yield and quality of the RNA including the use of frozen tissue compared with fresh tissue. Both methods gave similar yields and purity of RNA. The final protocol chosen, which optimised yield and purity, is described below. The most important adjustment to the original protocol (Chomczynski & Sacchi, 1987) was

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[10] Initially diethyl pyrocarbonate (DEPC)-treated autoclaved water was used, however, in subsequent experiments, its omission did not affect the quality of the RNA isolated, hence autoclaved water was used for all experiments.

that most of the extraction steps were performed at room temperature rather than on ice, which greatly lowered the protein contamination, believed to be a major source of the low A_{260}/A_{280} ratios.

Myocardial samples (approximately 100 mg pieces) that had been stored at minus 80°C in sterile 5 ml tubes were allowed to thaw on ice and 1 ml of RNAzol™ B was added to each tube. Sterile scissors were used to mince the tissue in the tube, then the tissue was homogenised using an ultra-turrax T25 (IKA Werke, Staufen, Germany) for approximately 30 sec or until the tissue appeared to be completely homogenised. Longer durations of homogenisation did not improve the quality or yield of the RNA. Mincing the tissue versus pulverising in liquid nitrogen did not affect the yields, and the results obtained with Tri-reagent (Sigma, Castle Hill, Australia) or Trizol (Invitrogen Australia Pty. Ltd., Melbourne, Australia) were not different to those obtained using RNAzol™ B. The homogenisation probe was washed with water and 1 M NaOH between samples. The homogenised tissue in RNAzol™ B was transferred to a labelled eppendorf tube and centrifuged at 4°C for 10 min at 11,000*g*. This centrifugation step is an additional step to the standard protocol and was found to greatly increase the quality of RNA isolated since it precipitates glycogen and fat, which interfere with RNA extraction. The supernatant was aspirated with a pipette into a new eppendorf tube and allowed to stand for 10 min at room temperature. This differs from the original RNA extraction method where incubations are on ice (Chomczynski & Sacchi, 1987). This change was based on the protocol for Tri-reagent and was found to be extremely important for obtaining a good yield of high quality RNA. At room temperature, the nucleoprotein complexes completely dissociate and therefore this step removes a significant portion of the protein contamination (Sigma-Aldrich, 1999). To the supernatant, 200 µl chloroform was added

and thoroughly mixed by inversion (not vortexing) for 15 sec. After a further 15 min at room temperature, the tubes were centrifuged at 4°C for 15 min at 11,000*g*. At this point, the homogenate forms two phases: the lower, blue phenol-chloroform phase and the upper colourless aqueous phase. RNA is present in the aqueous phase whereas DNA and proteins are in the interphase and the phenol phase. The clear aqueous phase (approximately 450 μl) was transferred to a fresh Eppendorf tube and 500 μl isopropanol was added and the sample was shaken vigorously for 20 sec. Tubes were left at room temperature for 10 min and centrifuged at 4°C for 10 min at 11,000*g* to precipitate the RNA pellet. Increasing the duration of incubation times with chloroform and isopropanol were not particularly advantageous. The supernatant was carefully removed and 1 ml ethanol added. After a further centrifugation step at 4°C (5 min at 11,000*g*), all of the supernatant was removed and the pellet allowed to air dry at room temperature for 5-10 min. When the pellet was sufficiently dry, 30 μl autoclaved water was added and gently mixed to dissolve the RNA. The sample was frozen at minus 80°C for 30 min to help dissolve larger pellets. This was another important step which was found to increase the yield of RNA. Samples were diluted into sterile water and the absorbance measured between 200-300 nm. For the real-time PCR experiments, it was necessary to remove any contaminating DNA from the RNA preparations and therefore the samples were DNase-treated prior to the reverse transcription step.

5.2.4 DNase treatment

The DNA-*free*™ Kit (produced by Ambion, distributed by Geneworks, Adelaide, Australia) was used to remove contaminating DNA from the total RNA preparations. To each RNA sample (approximately 30 μl of 1 μg/ml RNA), 3 μl of 10x DNase I buffer and 2 units (1 μl) of DNase I were added, mixed gently and incubated at 37°C for 30 min.

Five µl of DNase inactivation reagent was added and incubated at room temperature for 2 min. The tube was then centrifuged for 5 min at 13,000g to pellet the DNase inactivation reagent. At this point, 30 µl of the DNA- free RNA preparation was aspirated carefully, without disturbing the inactivation reagent and stored in a new RNAse-free tube. From this sample, 2 µl RNA was removed and diluted with 498 µl H$_2$O for spectrophotometric analysis. The remainder of the sample was stored at minus 80°C.

5.2.5 Gel Electrophoresis

DNase treated RNA (10 µg) was electrophoresed in 1.5% (w/v) agarose to confirm the presence of intact (undegraded) RNA. Prior to electrophoresis, the gel tank, comb and gel mould were soaked in 0.5% SDS for 10 min, rinsed in water and then rinsed briefly with 2% H$_2$O$_2$ (v/v). To prepare the gel, 750 mg of agarose was dissolved in 50 ml of 1 x TAE buffer (2M Tris-(hydroxymethyl)-aminomethane, 1M Glacial acetic acid and 50mM EDTA (Ethylenediaminetetra-acetic acid)) and heated in a microwave. Ethidium bromide (0.1 µg/ml) was added when the agarose was completely dissolved and then heated again until the ethidium bromide was completely mixed with the agarose solution. The gel was allowed to set for 15 min and the comb pulled out to expose the wells. The mould was placed in the gel tank, covered with approximately 250 ml of the 1 x TAE buffer and allowed to stand for 30 min prior to loading the samples (prepared with 1.5 µl of loading buffer). The samples were electrophoresed for 1½ hours at 60V at room temperature. Following the electrophoresis, RNA bands were briefly visualised under ultraviolet illumination. In no case was there any evidence of significant degradation of the RNA.

5.2.6 Reverse Transcription

RNA (2 μg) was reverse transcribed using a Corbett FTS-1 Thermal Sequencer (Mort Lake, NSW, Australia) in a 50 μl reaction volume. Table 5.2 indicates the components of the reverse transcription reaction mix.

Component	Volume/Tube (μl)	Final Concentration
10 x RT Buffer	5	1x
25 mM MgCl$_2$	11	5.5 mM
Deoxy NTP's mixture	10	500 μM of each
Random hexamers	2.5	2.5 μM
RNase inhibitor	1	0.4 U/μl
MultiScribe reverse transcriptase (50 U/μl)	1.25	1.25 U/μl
RNA sample in RNase-free H$_2$O	19.25	2 μg
Total	**50**	-

Table 5.2: The reverse transcription mix.
The volume and final concentration of the reagents in the reverse transcription protocol are indicated. The procedure is outlined in the text.

All components were purchased as part of the TaqMan® Reverse Transcription Reagents from Applied Biosystems (New Jersey, USA). The non-enzymatic components were dispensed into a 0.5 ml PCR tube and briefly vortexed. The RNA was then added and heated at 75°C for 3 min to remove RNA secondary structures. Once initiated by a primer, synthesis of cDNA usually proceeds to the 5' terminus of the RNA template. However, regions of the template that are rich in secondary structure may cause the reverse transcriptase to pause or stop, resulting in cDNAs that are less than full length (Sambrook *et al.*, 1989). Therefore to increase the efficiency of the reverse transcription, secondary structures were first removed by heat-treatment. Following the heating procedure, the mixture was cooled on ice and the reverse transcriptase and RNAse inhibitor were added. The components were mixed and tubes were transferred to the thermal cycler block. The reverse transcription was performed with the following incubations: 10 min at 25°C, 30 min at 48°C and 5 min at 95°C. At the end of the reaction, the tubes were immediately

cooled on ice, and aliquots were dispensed into sterile 0.5 ml PCR tubes and stored at minus 20°C. For each RNA preparation, a tube was included in which the reverse transcriptase enzyme (RT) was omitted from the reaction, providing an additional control in each PCR reaction (in addition to the no template control). This is referred to as the minus RT control (see 5.2.8).

5.2.7 Primer Design

The gene sequences for all the antioxidant genes were obtained from the Genbank website (http://www.ncbi.nlm.nih.gov/Genbank/index.html) based on published rat sequences. For catalase, the accession number was NM_012520, version GI:6978606 (Furuta *et al.*, 1986). The accession number for glutathione peroxidase was X12367, version GI:288455 (Reddy *et al.*, 1988). The accession number for Mn-SOD was NM_017051, version GI:8394330 (Ho *et al.*, 1991). The sequence for 18S rRNA was sourced from (Chan *et al.*, 1984), accession number X01117 K01593, version GI:57149. Primers for GAPDH were not designed since they have previously been validated and published, hence sequences from the literature were used (Schoenfeld *et al.*, 1998). The complete Genbank mRNA sequences for the four primers designed are included in Appendices 9.2-9.5. Primers were designed according to the rules detailed in Chapter 2. Following primer design, the sequences were aligned with all DNA sequences entered in the databases (using BLAST programmes) and checked for similarities with repetitive sequences or other loci elsewhere in the genome. None of the designed primers cross-reacted with any other gene. Oligonucleotide primers were synthesised by GensetOligos (Lismore, NSW, Australia), based on the designed sequences and supplied as 100 µM solutions that were diluted to 20 µM with sterile water and stored at minus 20°C. Prior to experimentation, the primers were tested in order to detect amplicon independent amplification (eg primer-

dimer formation). The sequence detection system was also set up to generate a dissociation (melting) curve for each primer after the final amplification cycle. This is done by slowly increasing the temperature above the melting temperature (T_m) of the amplicon and measuring the fluorescence. As the T_m of the amplicon depends markedly on its nucleotide composition, it is possible to identify the signal obtained from the correct product. A characteristic melting peak at the T_m of the amplicon (approximately 80°C) will distinguish it from amplification artefacts (eg primer-dimers) that melt at lower temperatures (75°C) in broader peaks. Primer-dimers are most prevalent in the no template control (see 5.2.8) wells and sample wells containing low concentrations of template since the primers tend to bind each other due to low levels (or non existence) of template.

5.2.8 Controls

Many controls were used in this study. The main control was to DNase-treat all RNA prior to reverse transcription to lower the possibility of DNA contamination. This technique is reported by the manufacturer to remove DNA contamination to levels undetectable by PCR. The GeneAmp® 5700 sequence detection system also uses a passive reference dye (ROX) that does not participate in the PCR reaction. This reference provides an internal reference to which the SYBR® Green (see 5.2.9.1) double stranded DNA complex signal can be normalised during data analysis. Normalisation is necessary to correct for fluorescent fluctuations caused by changes in concentration or volume (Applied Biosystems Technical Notes, 2001a). In each PCR reaction, one no template control (NTC) was also used, which was a tube containing water instead of cDNA. Since there was no template in this tube, any fluorescence detected would be indicative of DNA contamination. A minus RT tube acted as a control for genomic DNA contamination. The

minus RT sample was prepared in the same manner as the cDNA, however, the reverse transcriptase enzyme was omitted from the reverse transcription mix. In this way, only RNA should be present in the tube, and no cDNA. Thus, following the real-time PCR procedure, there should be no amplification products in this tube. Finally, since fluorescent contaminants can interfere with SYBR® Green I assays and give false positive results, a No Amplification Control tube was included that contained sample but no enzyme. If the absolute fluorescence of the No Amplification Control is greater than that of the No Template Control after PCR, it indicates that fluorescent contaminants may be present in the sample or in the heat block of the thermal cycler. The absence of fluorescent contaminants was confirmed in a preliminary experiment prior to the start of the study.

5.2.9 Real-time PCR

5.2.9.1 The PCR reaction

The Perkin Elmer Applied Biosystems GeneAmp® 5700 sequence detection system (Applied Biosystems, Foster City, CA, USA) was used in the present study. A description of this system was given in Chapter 2. The PCR is programmed to perform 40 cycles of amplification and the formation of product is measured and graphed at the end of each cycle. Figure 5.1 shows representative amplification plots graphing R_n versus cycle number. There are 4 techniques that can detect the amplified product. The present study used the simplest method, which involves detection of the binding of a fluorescent dye (SYBR® Green) to double-stranded DNA produced during the PCR amplification. The unbound dye exhibits little fluorescence in solution, but during elongation, increasing amounts of dye bind to the nascent double-stranded DNA. The SYBR® Green dye present in the SYBR® Green master mix (containing all the PCR reagents) is excited at 488 nm

and the fluorescence emitted is detected at 520 nm. Fluorescence measurements at the end

of the elongation step of every PCR cycle are performed to monitor the increasing amount

of amplified DNA (Bustin, 2000).
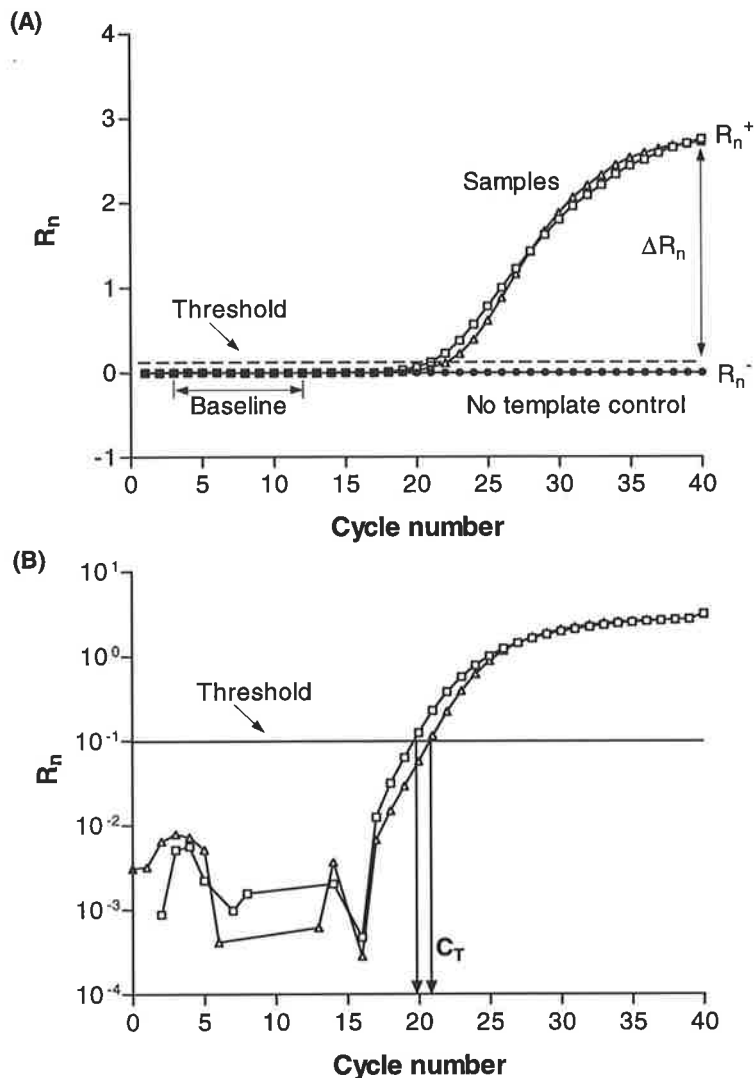
Figure 5.1: Model of a representative amplification plot for cDNA during real-time PCR.
In (A) linear and (B) log transformation. All parameters and symbols are explained in the text.

Normalisation of the fluorescent signal is accomplished by dividing the emission intensity

of the SYBR® Green by the emission intensity of the passive reference to obtain a ratio

defined as the R_n (normalised reporter). The R_n value of a reaction containing all

components including the template is referred to as the R_n^+. The R_n value of an un-reacted

sample is the R_n^-. ΔR_n is the magnitude of the signal generated by the PCR reaction, i.e.

$[(R_n^+) - (R_n^-)]$. The baseline fluorescence is set using the average fluorescence during cycles 3 to 12, and an increase in fluorescence above this baseline indicates the detection of accumulated PCR product that occurs with increasing PCR cycles when SYBR® Green binds to double stranded DNA. Quantitation involves the use of the cycle threshold (C_T) value from each amplification. This value is calculated based on the time (measured in PCR cycle numbers) at which the reporter fluorescent emission increases beyond the set baseline. The C_T value is correlated to input target mRNA levels; a greater quantity of input mRNA target results in a lower C_T value, as a result of requiring fewer PCR cycles for reporter fluorescent emission intensity to reach the threshold. The advantage of real-time PCR is that quantitation is done during the exponential phase of the PCR before any of the reaction components become limiting, leading to more accurate comparisons. Following normalisation to an appropriate housekeeping gene, levels of expression between the experimental groups under study can be compared. In the present study, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA were both used as housekeeping/internal control genes.

5.2.9.2 Preliminary Optimisation Experiments

To optimise the concentrations of forward and reverse primers, a comprehensive assay was carried out, testing all possible combinations of forward and reverse primers that could be generated from 50, 300 and 900 nM primers with a set template (cDNA) concentration (Table 5.3). The purpose of optimising primer concentrations was to determine the minimum primer concentrations giving the lowest C_T and maximum ΔR_n value whilst minimising non-specific amplification. This experiment was performed in detail for the catalase primers.

Reverse Primer (nM)	Forward Primer (nM)		
	50	**300**	**900**
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900

Table 5.3: The combinations of forward and reverse primers tested in the primer optimisation experiment for catalase.
The template concentration (cDNA) was equivalent to a starting RNA concentration of 20 ng.

When the SYBR® Green system is used, the primer optimisation matrix should also be challenged with NTC's (Applied Biosystems Technical Notes, 2001b). In this case, the primer concentrations selected should provide a low C_T and high ΔR_n when the target template is analysed, but should also minimise non-specific product formation when challenged with NTC's. Significant cDNA amplification was achieved using the highest primer concentrations without the formation of primer-dimer artefacts (Figure 5.2).
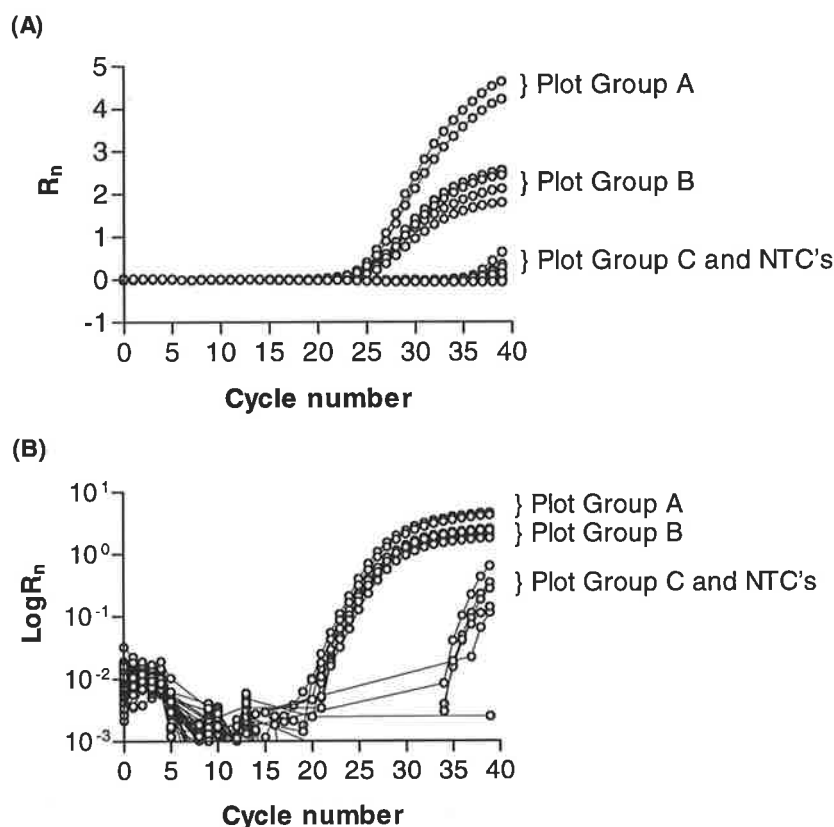
Figure 5.2: Primer optimisation experimental results showing amplification plots of all primer combinations in the primer optimisation matrix for the catalase primers.
(A) linear and (B) log transformation. Plot Group A: combinations that contain a 900 nM concentration of forward and reverse primer. Plot Group B: combinations that contain a 300 nM concentration of forward or reverse primer. Plot Group C: combinations that contain at least a 50 nM concentration of forward or reverse primer. This group also contains all the NTC's.

An additional experiment using 500 nM of both forward and reverse primers resulted in curves midway between the 300/300 nM and 900/900 nM (results not shown). This combination was then tested for all other primers and found to result in significant DNA amplification, and an R_n value comparable to that obtained with the highest primer concentrations, with no primer-dimer formation. Thus 500 nM forward and reverse primer concentrations were used in the final experiments for all primers.

5.2.9.3 Standard Curves

In order to determine the relative gene expression of the antioxidant enzymes in the myocardium of lipid supplemented animals, semi-quantitative methods were employed. This involved the use of standard curves for each gene with normalisation to the housekeeping genes. To generate a standard curve, the C_T was plotted versus the log of the initial RNA amount. The standard curves covered a range of RNA equivalent amounts from 1 ng to 200 ng (a range from 0.1 ng to100 ng was used for 18S rRNA due to its relatively high abundance) which resulted in a linear response. Since 18S is abundantly expressed in mammalian tissues, a lower amount of cDNA was also used for the experiments with this primer. The standard curve was performed using a reference cDNA. This cDNA was a mixture containing equi-proportions of cDNA from all three dietary groups and was used so that it would incorporate the entire range of C_T's exhibited by the three dietary groups.

5.2.9.4 The Real-time PCR Procedure

Based on the standard curve, cDNA equivalent to a starting RNA concentration of 20 ng of RNA was used in the experiments. This template concentration resulted in a C_T value, which, in all of the genes studied, lay in the middle of the linear amplification range. The

cDNA prepared for each dietary group was diluted and 12 µl aliquots of cDNA at 3.3 ng/µl (RNA equivalents) were maintained at minus 20°C. To prevent repeated freeze-thawing, small aliquots were prepared and therefore only thawed once. One reference sample that had been previously freeze-thawed was also amplified with each experimental run to compare the effect of freeze thawing. This sample was named "Th-ref". The real-time PCR was performed in a 20 µl reaction volume. The cDNA from all three dietary groups of rats was amplified on the same day for each gene (to minimise any variability) and a standard curve for each gene was also generated in the same run. For a typical experiment, the 96-well plate (tubes were used in the well positions) was set up as indicated in Table 5.4. Each of the tubes in the PCR reaction contained the SYBR® Green PCR Master Mix (PE Applied Biosystems, CA, USA) (1x concentration), 500 nM of forward primer, 500 nM of reverse primer and cDNA generated from 20 ng total RNA (except for the 18S rRNA experiment, where the cDNA was used at 5 ng RNA equivalent). All experiments were performed in duplicate.

Row	Gene (SOD, CAT, GPX, GAPDH or 18S rRNA)							
A	C1	C1	C2	C2	C3	C3	C4	C4
B	C5	C5	C6	C6	C7	C7	C8	C8
C	SF1	SF1	SF2	SF2	SF3	SF3	SF4	SF4
D	SF5	SF5	SF6	SF6	SF7	SF7	SF8	SF8
E	FO1	FO1	FO2	FO2	FO3	FO3	FO4	FO4
F	FO5	FO5	FO6	FO6	FO7	FO7	FO8	FO8
G	200	200	100	100	50	50	20	20
H	10	10	1	1	-RT	NTC	Th-ref	Th-ref

Table 5.4: Set up of the PCR 96-tube plate.
Rows A and B represent the cDNA samples (in duplicate) from the control (C) group of rats fed the 17% Sunola diet, in duplicate. Rows C and D represent the samples from the saturated fat (SF) fed rats (in duplicate) and row D and E are samples from the FO animals. All tubes contain cDNA generated from 20 ng total RNA. Row G and H are the reference cDNAs used for the standard curve and the numbers refer to the amount of RNA equivalents (ng). The -RT is the reference (RNA) sample without any reverse transcriptase and therefore should not contain any cDNA. NTC is the no template control and contains water instead of cDNA. Th-ref is the freeze-thawed reference cDNA sample as outlined in the text, n=8.

The universal thermal cycling parameters that were used for the quantitative SYBR® Green I assay consisted of an initial 10 min hold at 95°C followed by 40 cycles of melting

and annealing. The melting duration was 15 sec at 95°C followed by 1 min annealing at 60°C. The 10 min hold at 95°C is required for AmpliTaq® DNA Polymerase activation. The hot start PCR technique, as used in the present study, enables the reaction to only be started at elevated temperatures. This thermal activation of the enzyme ensures that active enzyme is generated only at temperatures where the DNA is fully denatured. This is an important step, since if the enzyme is added to the reaction mixture at room temperature, the inactive enzyme is not capable of primer extension. Therefore any low-stringency mispriming events that may have occurred will not be enzymatically extended and subsequently amplified. The extraction of RNA, the DNase procedure, reverse transcription and real-time PCR were all performed within 6 weeks of cardiac tissue sampling and storage in order to minimise the breakdown of RNA and to ensure stability of the cDNA.

5.2.9.5 Relative Quantitation

5.2.9.5.1 The Standard Curve Method

Standard curves of C_T versus log RNA input were generated using the reference cDNA. The abundance of mRNA for each gene relative to the reference cDNA, was determined for all of the experimental samples using the C_T value and the following formula:

$$\text{Input amount} = 10^{(C_T - c)/(m)}$$

Where "m" and "c" are obtained from the standard curve for each gene, with "m" being the slope of the standard curve and "c" the y-intercept. The same procedure was applied to the "housekeeping genes". The experimental samples were then normalised to each of the housekeeping genes (separately) to determine the relative expression of the gene, by using the following formula:

$$\text{Expression} = \frac{\text{amount (gene)}}{\text{amount (housekeeping gene)}}$$

The graphs were normalised using the C_T values obtained from both the GAPDH and the 18S rRNA amplifications.

5.2.9.5.2 The $\Delta\Delta$ C_T Method

An alternative method for obtaining relative gene expression data is the $\Delta\Delta$ C_T method, previously described by (Applied Biosystems Technical Notes, 1997). The $\Delta\Delta C_T$ method uses a single sample, termed the calibrator sample, for comparison of every unknown sample's gene expression level. The calibrator sample is analysed on every assay plate with the unknown samples of interest. The formula that can be used is:

$$\text{Fold induction} = 2^{-[\Delta\Delta\,CT]}$$

Where $\Delta\Delta$ C_T = [C_T GI (unknown sample) − C_T (housekeeping gene of unknown sample)] − [C_T GI (calibrator sample) − C_T (housekeeping gene of calibrator sample)]. GI is the gene of interest (ie Mn-SOD, CAT or GPX). The calibrator sample can be any sample chosen to represent 1x expression of the gene of interest (ie the CON group). The formula is based on the assumption that the rate of C_T change versus the rate of target copy change is identical for the gene of interest and the housekeeping gene and that a doubling of target results in a one cycle decrease in the measured C_T (Winer *et al.*, 1999). Although these assumptions will rarely be exact, they do closely proximate relative expression levels. However, the advantage of this method is that sample throughput is increased since standard curves are not analysed for gene targets on each plate.

5.2.10 DNA Microarray Analysis

For gene screening in response to dietary lipid supplementation, DNA microarray analysis was used. For these studies, an Atlas™ Glass Rat 1.0 Microassay was utilised (Clontech, CA, USA). This microarray consists of 1081 rat DNA fragments, nine

housekeeping genes as well as negative and positive controls immobilized on a glass slide. The Atlas™ Glass Fluorescent Labeling Kit (Clontech, CA, USA) was used for synthesizing and purifying fluorescently labeled cDNA probes for hybridization to the glass microarrays. First strand cDNA was synthesized from 20 µg DNAse-treated total RNA, and labeled probes were synthesized using the cDNA Synthesis (CDS) Primer Mix. The cDNA from FO supplemented rats was bound with Cy5 (fluoresces red) and the cDNA from the SF group was bound with Cy3 (fluoresces green). Probes were hybridized to the Atlas Glass Microarray, which was then washed and dried prior to scanning. Slides were scanned using the GenePix 4000 microarray scanner (Axon Instruments Inc., Foster City, CA, USA) and the GenePix Pro 3.0 analysis software (Axon Instruments). The scanner contained 2 lasers that excite cyanine dyes at approximate wavelengths of 635 nm (Cy5) and 532 nm (Cy3), respectively. Data sets were extracted into a Microsoft Excel spreadsheet. The data sets consisted of signal and background intensity, and ratio of media and or mean or total intensity including flags. A macroprogram in Microsoft Excel was used to perform normalization, which corrects the intensity of each spot for variations in the overall intensity of the image with respect to a control image. This normalized ratio was used to classify genes in decreasing order of upregulation by FO.

5.3 Results

5.3.1 Body Weights of Rats Following Lipid Supplementation

The body weights of the rats in the three dietary groups were not significantly different

following 4 weeks dietary lipid supplementation as shown in Table 5.5.

	Age of rats	
Diet/Body Weight (g)	9 weeks	13 weeks
CON	356.4 ± 8.9	500.4 ± 13.8
SF	349.3 ± 6.9	494.8 ± 10.8
FO	355.5 ± 9.8	498.5 ± 6.1

Table 5.5: Changes in body weights of the rats during dietary supplementation.
Rats were obtained at 4 weeks of age and maintained on the low-ALA diet. At 9 weeks of age, they were fed the lipid supplemented diet which contained an additional 10% (w/w) added fat, present as either Sunola oil (CON), fish oil (FO), or saturated fat (SF) for 4 weeks.

5.3.2 Myocardial Fatty Acids

The dietary lipid supplements were 17% total fat, present as 7% (w/w) Sunola oil in the

base diet with an additional 10% (w/w) fat, either as Sunola oil (CON), saturated fat (SF)

or fish oil (FO). The fatty acid analysis of the ventricular phospholipids is shown in Table

5.6. Dietary lipid supplementation resulted in significant changes to the fatty acid profile.

The most important change was that the proportion of n-3 PUFAs in the FO group[11] was

29.1 ± 1.2%, which was 2.7 times higher than that in the SF group ($p < 0.001$) and 3.5

times higher than that in the CON ($p < 0.001$). Consequently the n-6/n-3 PUFA ratio was

significantly lower in the FO group in comparison to both the SF and CON groups

($p < 0.001$).

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[11] In this chapter, where reference is made to the fatty acid composition or the expression of genes in the myocardium of rats fed the different lipid-supplemented diets, for simplicity, these groups will be termed the CON, SF or FO groups.

FAME[a]	CON	SF	FO
14:0	0.12 ± 0.00	0.21 ± 0.02^d	0.23 ± 0.01^g
16:0	10.84 ± 0.32	12.22 ± 0.41	13.41 ± 0.51^f
16:1	0.20 ± 0.01	0.17 ± 0.02	0.25 ± 0.03
17:0	0.15 ± 0.02	0.40 ± 0.02^d	0.40 ± 0.01^g
18:0	25.27 ± 0.26	25.15 ± 0.23	23.97 ± 0.39^{e,h}
18:1[b]	12.74 ± 0.40	12.14 ± 0.30	9.28 ± 0.22^{g,j}
18:2 n-6	8.57 ± 0.67	8.66 ± 0.34	5.97 ± 0.65^{e,h}
18:3 n-3	n/d	n/d	n/d
20:0	0.19 ± 0.01	0.19 ± 0.00	0.19 ± 0.01
20:1	0.13 ± 0.01	n/d	0.13 ± 0.00
20:2	0.22 ± 0.03	0.47 ± 0.02^d	0.12 ± 0.00^{e,j}
20:3 n-6	0.20 ± 0.02	0.29 ± 0.01^c	0.29 ± 0.03^e
20:4 n-6	27.42 ± 0.66	26.95 ± 0.37	15.90 ± 0.87^{g,j}
22:0	0.38 ± 0.02	0.24 ± 0.01^d	0.32 ± 0.02^h
22:1	n/d	n/d	0.32 ± 0.01
20:5 n-3	n/d	0.14 ± 0.03	3.32 ± 0.07^j
24:0	5.22 ± 0.50	2.06 ± 0.13^d	0.30 ± 0.04^{g,i}
24:1	n/d	n/d	0.14 ± 0.01
22:5 n-3	0.41 ± 0.03	0.87 ± 0.04^d	2.52 ± 0.05^{g,j}
22:6 n-3	8.03 ± 0.18	9.90 ± 0.43	23.29 ± 1.21^{g,j}
Σ SFA	42.09 ± 0.54	40.48 ± 0.48	38.55 ± 0.87^f
Σ MUFA	13.07 ± 0.40	12.33 ± 0.29	10.08 ± 0.21^{g,j}
Σ PUFA	44.84 ± 0.56	47.19 ± 0.66	51.37 ± 0.87^{g,i}
Σ n-6	36.18 ± 0.43	35.91 ± 0.50	22.16 ± 0.61^{g,j}
Σ n-3	8.44 ± 0.20	10.82 ± 0.43	29.13 ± 1.24^{g,j}
n-6/n-3	4.29 ± 0.08	3.35 ± 0.15^d	0.77 ± 0.06^{g,j}

Table 5.6: Fatty acid composition (wt%) of the ventricular cardiomyocyte total phospholipids following dietary lipid supplementation.
Data are mean ± SEM for 6 rats. The shorthand notation for fatty acid structure was given in the legend to Table 3.1. Computational parameters (Σ and n-6/n-3) were derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-7 and n-9 isomers. [c]$p<0.05$, [d]$p<0.001$ for CON vs SF; [e]$p<0.05$, [f]$p<0.01$, [g]$p<0.001$ for CON vs FO; [h]$p<0.05$, [i]$p<0.01$, [j]$p<0.001$ for SF vs FO (by ANOVA with the Bonferroni multiple comparisons test). Abbreviations: CON, control diet (17% sunola oil); SF, saturated fat diet; FO, fish oil diet; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n/d not detectable.

5.3.3 RNA Isolation

The absorbance of the total RNA samples was determined between the wavelengths of 200 and 300 nm. A representative scan is shown in Figure 5.3.
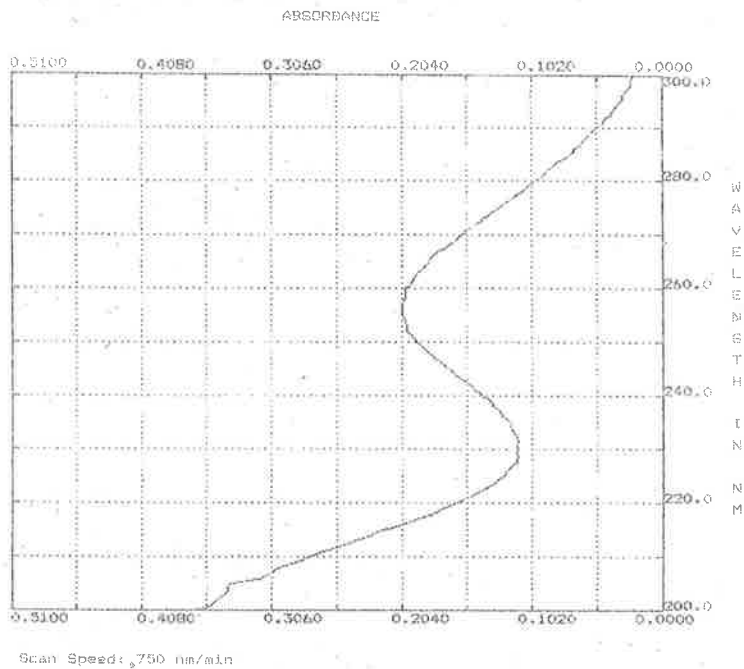
ABSORBANCE

Figure 5.3: A representative scan of RNA extracted from rat heart.
The absorbance (horizontal axis) of the RNA was scanned at a speed of 750 nm/min and plotted between 200 and 300 nm (vertical axis) using a Beckman DU-65 spectrophotometer. Measured parameters are given in Table 5.5.

The 260/280 nm ratio was used as an indicator of the purity of the isolated RNA and was also used to calculate the concentration and therefore the yield of RNA from the myocardial samples. A wavelength of 260 nm is used for the detection of nucleic acids (RNA and DNA), and at a wavelength of 280 nm proteins are detected, based on the presence of aromatic amino acids. The concentration of RNA was determined by measuring the OD_{260} of an aliquot of the final preparation. A solution whose $OD_{260} = 1$, contains approximately 40 µg of RNA per millilitre in a 1-cm path length (Sambrook *et al.*, 1989). The RNA concentration was calculated using the following formula:

concentration (µg/µl) = (OD_{260} x dilution factor x RNA constant)/1000

where the RNA constant is the above-mentioned value of 40 µg/ml. None of the parameters measured were significantly different between the dietary groups (Table 5.7).

DIET	CON	SF	FO
Tissue weight (mg)	106.13 ± 2.41	106.13 ± 1.60	106.25 ± 0.80
OD 260nm	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01
OD 280nm	0.07 ± 0.01	0.08 ± 0.00	0.08 ± 0.01
260/280 ratio	2.08 ± 0.05	2.05 ± 0.06	2.08 ± 0.06
[RNA] (µg/µl)	1.45 ± 0.12	1.64 ± 0.05	1.64 ± 0.13
Total RNA (µg)	43.43 ± 3.56	49.09 ± 1.35	49.09 ± 3.86
Yield (µg/mg heart)	0.41 ± 0.04	0.46 ± 0.02	0.46 ± 0.03

Table 5.7: Summary of RNA extractions indicating various parameters measured.
The first data row indicates the weight of heart tissue from which RNA was extracted (n=8 per dietary group). The absorbance readings were obtained following a 1 in 250 dilution of the original RNA sample (which was made in 30 µl of sterile water). Abbreviations: CON, control diet (17% sunola oil); FO, fish oil diet; OD, optical density; SF, saturated fat diet.

Following DNase treatment, the 260/280 nm ratio was lowered in all three dietary groups indicating that DNA contamination had contributed to the 260 nm absorbance readings. The 260/280 nm ratio was lowered to 1.89 in the CON group, 1.97 in the SF group and 1.93 for the FO group (not significant between RNA samples isolated from the three dietary treatment groups). DNase treated RNA (10 µg) was electrophoresed on a 1.5% (w/v) agarose gel to confirm the presence of intact (ie. undegraded RNA). A representative gel is shown in Figure 5.4.
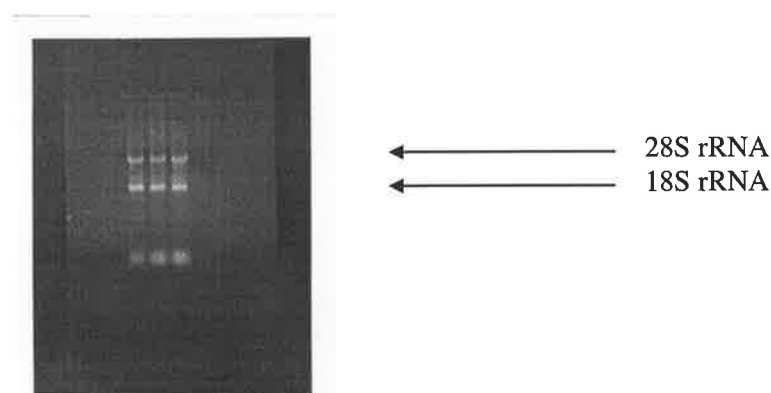
← 28S rRNA
← 18S rRNA

Figure 5.4: A representative gel of RNA extracted from rat myocardium.
The 28S and 18S rRNA bands are marked. The third (lowest) band likely contains both 5S rRNA and transfer RNAs. Following DNase treatment of the total RNA, 10 µg RNA was run on a 1.5% (w/v) agarose gel at 60V. The gel contained 0.1 µg/ml ethidium bromide to facilitate visualisation of the bands under UV illumination.

5.3.4 Primers

The sequences of the designed primers is given in Table 5.8. The amplicon length (in base pairs) for the sequences were as follows: Mn-SOD, 63 base pairs (bp); CAT, 70 bp; GPX, 66 bp; 18S rRNA, 69 bp. A published sequence was used for GAPDH which generates a 171 bp product (Schoenfeld *et al.*, 1998).

Gene	Primer Length	T_m (°C)	GC content (%)	Primer Sequence (5' → 3')
Mn-SOD- fwd primer	20	60	65	GCCTCCCTGACCTGCCTTAC
MnSOD- rev primer	20	59	50	GCATGATCTGCGCGTTAATG
CAT- fwd primer	21	58	48	CCCAGAAGCCTAAGAATGCAA
CAT- rev primer	21	60	52	GCTTTTCCCTTGGCAGCTATG
GPX- fwd primer	21	58	52	GTGTTCCAGTGCGCAGATACA
GPX- rev primer	21	59	52	GGGCTTCTATATCGGGTTCGA
18S- fwd primer	21	58	52	AGTCCCTGCCCTTTGTACACA
18S- rev primer	21	59	57	GATCCGAGGGCCTCACTAAAC
GAPDH- fwd primer	Published sequence[*]			ATGTTCCAGTATGACTCCACTCACG
GAPDH- rev primer	Published sequence[*]			GAAGACACCAGTAGACTCCACGACA

Table 5.8: Sequences and parameters for primers designed using the Primer Express software.
The primers were designed according to the rules set out in Chapter 2. Primer lengths are in base pairs. Abbreviations: CAT, catalase; fwd, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; Mn-SOD, manganese containing superoxide dismutase; rev, reverse; [*](Schoenfeld *et al.*, 1998).

None of the designed primers exhibited significant amplification or primer dimer formation in the minus RT control samples. The dissociation curves generated for each of the designed primers also did not indicate any primer-dimer formation. The PCR amplification products of the designed primers were electrophoresed in 4% (w/v) agarose to confirm the presence of only one product. All of the genes resulted in only one band, corresponding to the correct size product (results not shown). Figure 5.5 shows a typical amplification plot obtained with the designed primers. In the example shown, the highest amount of template (200 ng cDNA) was amplified the earliest (ie. exhibiting the lowest C_T). The late fluorescence (highest C_T) occurring in the minus RT sample most likely

reflects non-specific primer dimer formation rather than actual product, particularly with regard to the data shown in Figure 5.6 which establishes the absence of primer-dimer artefacts by the non-appearance of a second peak at 75°C.

(A)

(B)
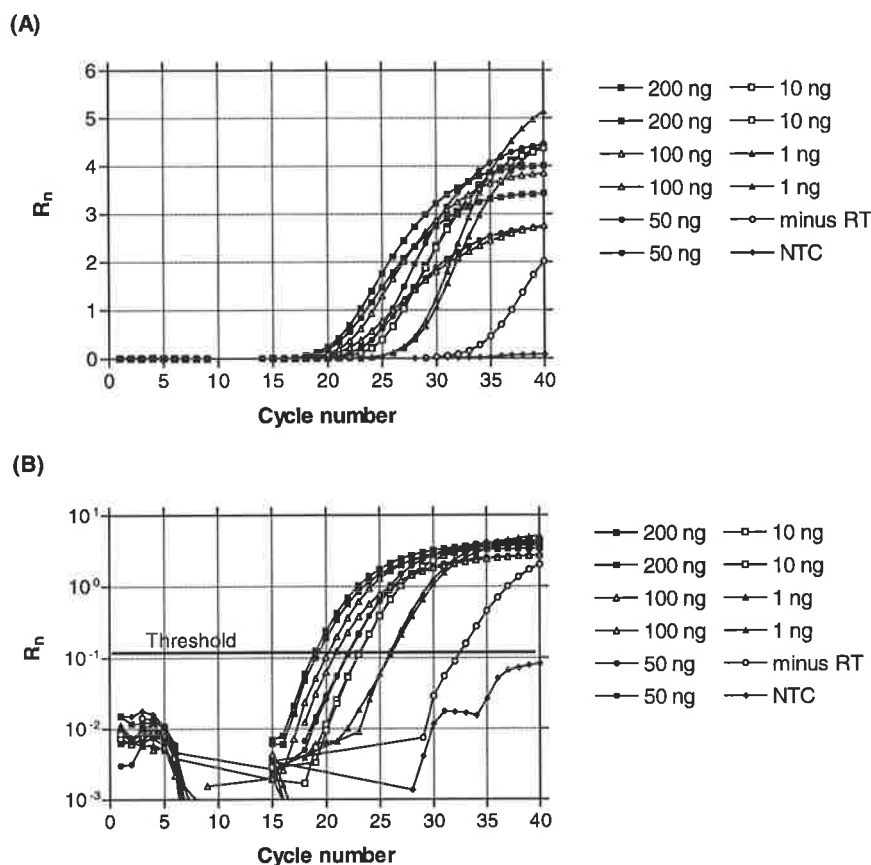
Figure 5.5: Amplification plot for glutathione peroxidase.
(A) linear and (B) log transformation plots indicating the position of the threshold (the threshold was set as outlined in 5.2.9.1). The C_T was determined as the cycle number where the threshold meets the amplification plot. The PCR was performed under the conditions described in (5.2.9.4) using duplicate tubes of a range of RNA equivalent concentrations (ng). The minus RT sample is the RNA sample where the reverse transcriptase enzyme was omitted during reverse transcription (described in 5.2.6). NTC, non template control (contains sterile water instead of cDNA).

The NTC did not result in any appreciable fluorescence confirming absence of RNA or DNA contamination in the samples. The amplification of the Th-ref sample was comparable to the non freeze-thawed cDNA, hence indicating that freeze-thawing the cDNA once did not cause significant cDNA damage to influence the amplification. The C_T values were determined (following setting of a threshold) and a standard curve was

constructed using the C_T values. This process of setting a threshold, obtaining a C_T and

generation of a standard curve was performed for all 5 primers.
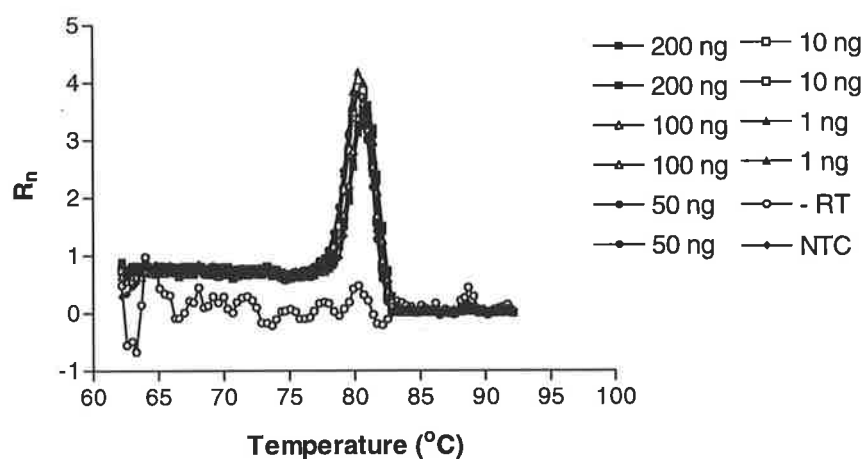
Figure 5.6: Dissociation curve for glutathione peroxidase.
Following the 40 cycles of PCR, the dissociation curve was generated. The –RT is the RNA sample that did not have reverse transcriptase included in the reaction. NTC, no template control (contains sterile water instead of cDNA).

5.3.5 Gene Expression

5.3.5.1 The Standard Curve Method of Quantitation

The amplification plots for all 5 genes of the dietary study are shown in Appendices 9.6-

9.8. The C_T for all duplicate measurements was determined from the amplifications as

described above. The standard curves for all 5 genes, obtained using the reference cDNA

is plotted in Figure 5.7.
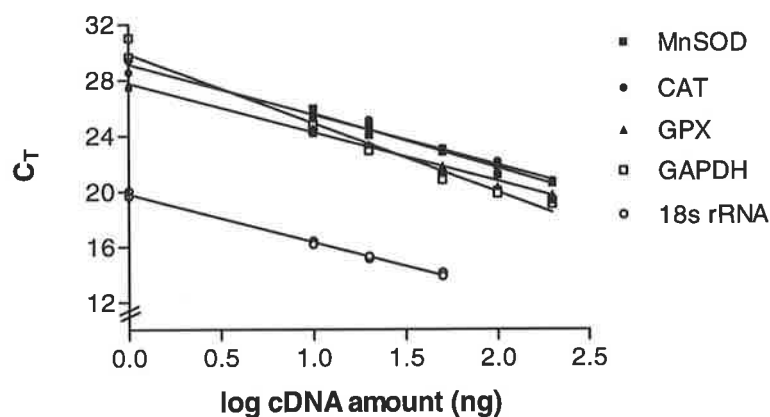
Figure 5.7: Standard curves generated using reference cDNA.
These curves were generated in the same run as the experimental samples. Abbreviations: CAT, catalase; C_T, cycle threshold; GAPDH, glyceraldehye-3-phosphate dehydrogenase; GPX, glutathione peroxidase; Mn-SOD, manganese containing superoxide dismutase; 18S rRNA, ribosomal RNA. The equations and R^2 values for each gene is presented in Table 5.9.

The equations and R^2 values are tabulated in Table 5.9. The dissociation curves resulted in a single product for each of the 3 genes, giving a T_m between 79-82°C, with no evidence of products with lower T_m's, ie indicating the absence of the formation of primer dimmers (results not shown).

Gene	Equation	R^2
Mn-SOD	y = -3.9x + 29.5	0.9756
CAT	y = -3.6x + 29.0	0.9887
GPX	y = -3.5x + 27.7	0.9871
GAPDH	y = -4.9x + 29.9	0.9783
18S rRNA	y = -3.3x + 19.6	0.9963

Table 5.9: Standard curve parameters for reference cDNA.
These data relate to the standard curves shown in Figure 5.7. CAT, catalase; GPX, glutathione peroxidase; GAPDH, glyceraldehye phosphate dehydrogenase; Mn-SOD, manganese containing superoxide dismutase; 18S rRNA, ribosomal RNA.

Table 5.10A shows the C_T values obtained following the real-time PCR amplification, prior to normalisation to the housekeeping genes.

(A)

	C_T's		
	CON	**SF**	**FO**
MnSOD	25.21 ± 0.33	25.14 ± 0.34	23.99 ± 0.23 *
CAT	24.49 ± 0.43	24.31 ± 0.56	23.46 ± 0.35
GPX	25.57 ± 0.40	25.20 ± 0.28	24.81 ± 0.16
GAPDH	24.13 ± 0.53	24.62 ± 0.46	24.23 ± 0.23
18s rRNA	20.03 ± 0.29	19.95 ± 0.12	20.00 ± 0.25

(B)

	Amount (relative units)		
	CON	**SF**	**FO**
MnSOD	14.27 ± 2.36	15.30 ± 3.29	27.75 ± 3.45 *
CAT	23.71 ± 4.74	29.91 ± 6.76	44.67 ± 9.47
GPX	5.26 ± 1.14	6.09 ± 1.05	7.26 ± 0.80
GAPDH	17.67 ± 4.04	13.19 ± 2.32	14.77 ± 1.73
18s rRNA	0.83 ± 0.16	0.79 ± 0.07	0.83 ± 0.15

Table 5.10: Summary of (A) C_T values and (B) Amount of PCR product following real-time PCR amplification.
The data is presented as mean ± SEM for n=8 rats. The "amount" was derived from the standard curves according to the formula described in 5.2.9.5.1 and is therefore relative to the reference cDNA. All data followed a normal (Gaussian) distribution and hence parametric analyses were used. Abbreviations: Mn-SOD, manganese containing superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; GAPDH, glyceraldehye-3-phosphate dehydrogenase; rRNA, ribosomal RNA; CON, control diet; SF, saturated fat, FO, fish oil; * $p < 0.05$ for FO versus CON and SF (ANOVA with the Bonferroni multiple comparisons test).

The amount of amplified product was calculated from the standard curves relative to the reference cDNA, and is displayed in Table 5.10B, without correction for any housekeeping genes. These results followed a normal (Gaussian) distribution and therefore they were analysed by parametric one-way ANOVA. The amount of Mn-SOD in the FO myocardium was 1.8 times higher than both the CON and SF groups (p<0.05). There was no significant difference in the (uncorrected) amounts of the mRNA for CAT, GPX, GAPDH or 18S rRNA in the 3 dietary groups. Normalisation of the gene expression of the three antioxidant enzymes to each of the housekeeping genes revealed significant changes in the expression of the enzymes following the dietary lipid supplementation. Normalisation to both housekeepers is presented, since different results (in terms of statistical significance) were obtained following correction for GAPDH or 18S rRNA. Data are presented as the amount of mRNA of the antioxidant gene divided by the housekeeping gene (arbitrary units). These data also followed a Gaussian distribution, hence parametric analyses were performed.
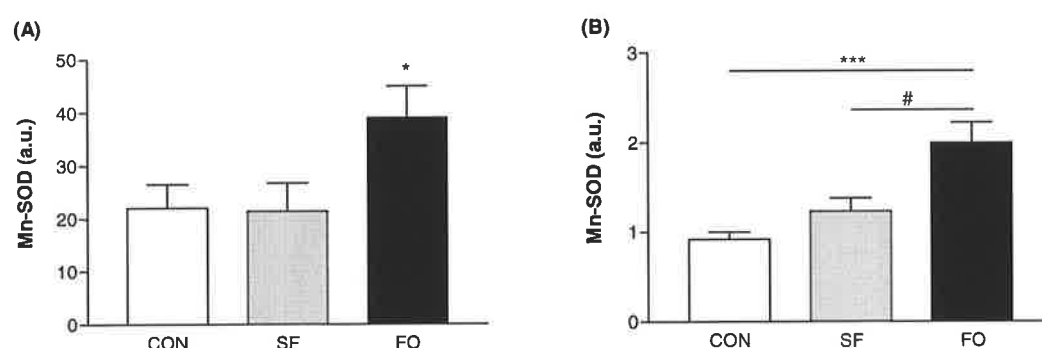
Figure 5.8: Expression of Mn-SOD mRNA in the myocardium of dietary lipid-supplemented rats.
Data are normalised to 18S rRNA (A) or GAPDH (B). Abbreviations: CON, control diet (Sunola oil); FO, fish oil supplemented diet; GAPDH, glyceraldehye-3-phosphate dehydrogenase; Mn-SOD, manganese containing superoxide dismutase; rRNA, ribosomal RNA; SF, saturated fat supplemented diet;. *p<0.05 for SF vs CON; ***p<0.001 for CON vs FO; #p<0.01 for SF vs FO (ANOVA with the Bonferroni multiple comparisons test), n=8.

As shown in Figure 5.8A, the amount of Mn-SOD mRNA in the FO myocardium normalised to the amount of 18S rRNA in the FO myocardium was 1.8 times higher than the expression of Mn-SOD in both the CON and SF group (p<0.05). Similarly, if the data was corrected to GAPDH, the expression of Mn-SOD in the FO group was 1.6 times

higher than the SF group (p<0.01) and 2.2 times higher than the CON (p<0.001, Figure

5.8B). The reason for the varying degrees of statistical significance achieved when

normalising the data to 18S rRNA compared with GAPDH cannot be attributed to

changes in expression of the housekeeping genes *per se*. As indicated in Figure 5.9, the

expression of both housekeeping genes remained constant following dietary
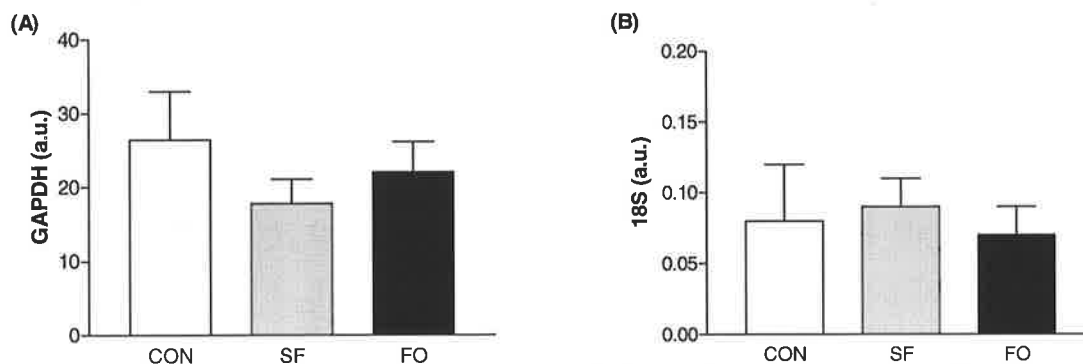
supplementation.

Figure 5.9: Expression of GAPDH and 18S rRNA in lipid supplemented rat myocardium.
Data are normalised to 18S rRNA (A) or GAPDH (B). Abbreviations: CON, control diet (Sunola oil); FO, fish oil supplemented diet; GAPDH, glyceraldehye-3-phosphate dehydrogenase; rRNA, ribosomal RNA; SF, saturated fat supplemented diet. There was no significant differences between the expression of either GAPDH or 18S rRNA in the different dietary groups (ANOVA), n=8.

The expression of catalase (CAT) was not significantly different between the three lipid

supplemented groups when normalised to 18S rRNA (Figure 5.10A). However, there was

a trend towards a higher expression of CAT in the FO group (p=0.06) when the data was

normalised to GAPDH (Figure 5.10B).
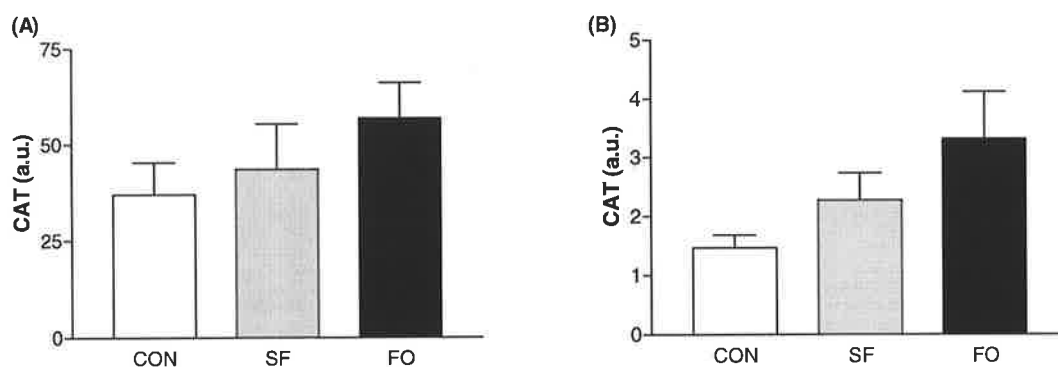
Figure 5:10: Expression of CAT mRNA in lipid supplemented rat myocardium.
Data are normalised to 18S rRNA (A) or GAPDH (B). Abbreviations: CON, control diet (Sunola oil); CAT, catalase; FO, fish oil supplemented diet; GAPDH, glyceraldehye-3-phosphate dehydrogenase; rRNA, ribosomal RNA; SF, saturated fat supplemented diet. n=8. In (B), the data followed a normal distribution, however the standard deviations were significantly different, hence the data was log transformed prior to performing a one-way ANOVA with the Bonferroni multiple comparisons test.

Similarly, the expression of glutathione peroxidase (GPX) was not significantly different

between the three dietary groups when normalised to 18S rRNA (Figure 5.11A), however,

the expression of GPX was significantly higher in the FO group when normalised against

GAPDH ($p<0.05$ vs CON, Figure 5.11B).
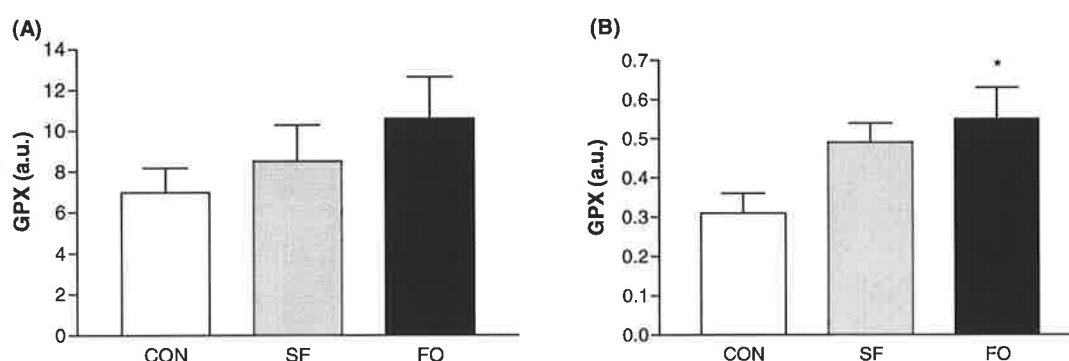
Figure 5.11: Expression of GPX mRNA in lipid supplemented rat myocardium.
Data are normalised to 18S rRNA (A) or GAPDH (B). Abbreviations: CON, control diet (Sunola oil); FO, fish oil supplemented diet; GAPDH, glyceraldehye-3-phosphate dehydrogenase; GPX, glutathione peroxidase; rRNA, ribosomal RNA; SF, saturated fat supplemented diet. *$p<0.05$ vs CON (ANOVA with the Bonferroni multiple comparisons test), n=8.

5.3.5.2 The $\Delta\Delta C_T$ Method of Quantitation

Table 5.12 summarises the data obtained using the standard curve and $\Delta\Delta$ C_T methods.

(A)

Normalisation to GAPDH- Comparison of Standard Curve and $\Delta\Delta C_T$ Methods

	CON		SF		FO	
	$\Delta\Delta C_T$	Std Curve	$\Delta\Delta C_T$	Std Curve	$\Delta\Delta C_T$	Std Curve
MnSOD	1x	1x	1.46	1.34	2.46	2.16
CAT	1x	1x	1.36	1.55	1.78	2.26
GPX	1x	1x	1.75	1.58	1.74	1.77

(B)

Normalisation to 18S rRNA- Comparison of Standard Curve and $\Delta\Delta C_T$ Methods

	CON		SF		FO	
	$\Delta\Delta C_T$	Std Curve	$\Delta\Delta C_T$	Std Curve	$\Delta\Delta C_T$	Std Curve
MnSOD	1x	1x	0.97	0.97	2.14	1.77
CAT	1x	1x	0.90	1.18	1.55	1.53
GPX	1x	1x	1.16	1.22	1.52	1.52

Table 5.11: Comparison of the standard curve method and the $\Delta\Delta C_T$ method.
Comparison of fold increase over CON in SF and FO myocardium for Mn-SOD, CAT and GPX normalised to (A) GAPDH or (B) 18S rRNA. Abbreviations: CAT, catalase; CON, control group; C_T, threshold cycle; FO, fish oil; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; Mn-SOD, manganese containing superoxide dismutase; rRNA, ribosomal RNA; SF, saturated fat;.

The CON group was designated as the target. The values presented are the fold increases over the target expression and the results of the $\Delta\Delta C_T$ method of interpreting gene expression data are comparable to the standard curve-derived results normalised to GAPDH (Table 5.12A) and 18s rRNA (Table 5.12B).

5.3.6 MicroArray Analysis

A representative scan is shown in Figure 5.12. To compare the gene expression between the two groups, the ratio of the signal generated from Cy5/Cy3 was calculated i.e. a red signal indicated a higher expression in the FO group whilst the green signal indicated a higher expression in the SF group. The ratios of the 20 genes that had the highest ratio (indicating up-regulation by FO) are summarised in Table 5.13. Only c-jun and interferon induced protein were among the 20 most up regulated genes in both microarray analyses. It is impossible to make any conclusions following the scanning of only 2 slides, however, further analyses were not possible due to the high costs and set-up time. Nevertheless, that 3 of the antioxidant genes (glutathione-S-transferase, Cu/Zn-SOD, phospholipid hydroperoxide GPX) were up regulated in the FO was a positive finding.
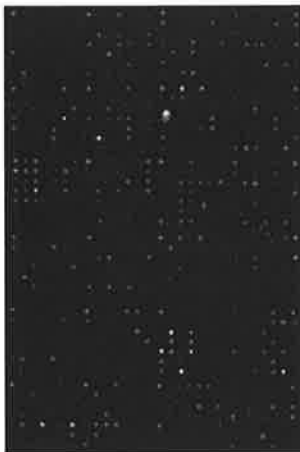
Figure 5.12: Representative microarray scan of myocardial cDNA from rats supplemented with SF versus FO.
The glass microarray was analysed using the GenePix scanner. Red spots indicate genes upregulated by the FO diet, green spots specify the SF. Yellow spots are co-regulated genes.

Array #1	Array #2
Glutathione S-transferase, subunit 5	G protein-coupled receptor 27; gustatory
Cytochrome P-450 4F1, hepatic tumour	ADP-ribosylation factor 5 (ARF5)
Sodium channel protein 6	*Interferon induced protein*
Liver carboxylesterase 10 precursor	Ribosomal protein S29 40S subunit
Ribosomal protein L13	Vasopressin V1b receptor
c-jun proto-oncogene; transcription factor	Fibroblast growth factor receptor
Fatty acid-binding protein	Ear-3; V-erbA related protein
Phospholipid hydroperoxide GPX	Cytochrome c oxidase, subunit VIIIh L+
Organic cation transporter 2	GTP-binding protein (G-alpha-8)
Fructose-bisphosphate aldolase A	RIN1; interacts directly with Ras and co
Adipocyte fatty acid-binding protein	NVP-2; neural visinin-like Ca2+-binding
D(4) dopamine receptor	Kidney oligopeptide transporter; peptide
ATP synthase lipid-binding protein P1	Mitochondrial aldehyde dehydrogenase 2
Cu/Zn-SOD	Glutamate (NMDA) receptor subunit
Interferon induced protein	Guanine nucleotide-binding protein G(O)
Neurodegeneration associated protein 1	*c-jun proto-oncogene; transcription factor*
Ribosomal protein S4	Vasopressin V2 receptor
Somatostatin receptor	Polyubiquitin
LIM, muscle	Neuromedin B receptor
Ribosomal protein L12	c-ErbA oncogene; thyroid hormone receptor

Table 5.12: Differential Gene Expression in rat myocardium by n-3 PUFAs.
Results presented are the top 20 expressed genes in the 2 replicate glass microarrays scanned. The genes in bold case are antioxidant enzymes. The genes in italics are those that were found to be among the 20 most up-regulated genes analyzed in both arrays.

5.4 Discussion

The major finding of this study was that rat myocardium exhibited enhanced antioxidant gene expression following dietary fish oil supplementation. This was associated with an enrichment of the membrane phospholipids of rat myocardium with the n-3 PUFAs, particularly EPA, DPA and DHA following fish oil feeding.

Following dietary lipid supplementation, the proportion of n-3 PUFAs in the myocardium of rats fed FO was higher than the SF and the CON groups. The myocardial levels of ALA were below the levels of detection in all three dietary groups, indicating that the rat myocardium has the ability to efficiently elongate and desaturate this fatty acid to the longer chain n-3 PUFAs (Williard *et al.*, 2001). Interestingly, although the SF diet contained higher proportions of SFAs and less PUFA than the FO diet, there was no significant difference between myocardial levels of SFA in the SF and FO fed rats. Similar observations were made in the previous chapters and these results are in agreement with other investigators (McMurchie *et al.*, 1983; Luostarinen *et al.*, 1997; Nair *et al.*, 1999). Thus it appears that the myocardial membranes of rats maintain constant proportions of saturated and unsaturated FAs regardless of the type of dietary fat intake, although changes in fatty acid subclasses can be induced by dietary lipid supplementation.

The study of mammalian gene expression is often carried out at the level of mRNA. In such studies, it is necessary to correct for both variations in the amount of starting material between samples and for other variations occurring as part of the PCR processes. The accepted method for minimising these errors and correcting for sample-to-sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an

internal reference against which other RNA values can be normalised (Bustin, 2000). GAPDH was one of the housekeeping genes used in the present study and its expression was not found to be altered with dietary lipid supplementation. However, despite its widespread use as a normalisation control, studies have shown that GAPDH mRNA levels are modulated under many circumstances, both *in vitro* and *in vivo* (Graven *et al.*, 1994; Zhong & Simons, 1999; Bustin, 2000; Suzuki *et al.*, 2000). Conversely, a 24 hr incubation of neonatal myocytes with 3 mU/ml glucose oxidase (which generates 2.5 mmol/min H_2O_2) did not change the mRNA levels for GAPDH (Lai *et al.*, 1996) and 30 min perfusion with 25 μM H_2O_2 did not affect GAPDH mRNA levels in rat hearts (Csonka *et al.*, 2000). Whether or not fish oil supplementation induces a severe enough oxidative stress to increase GAPDH mRNA remains to be elucidated. Certainly the data obtained in the present study does not indicate any up- or down-regulating effects of fish oil on GAPDH expression and this is consistent with the findings of others who also used GAPDH as a housekeeping gene in dietary n-3 PUFA studies (Chandrasekar & Fernandes, 1994; Venkatraman *et al.*, 1994). Ribosomal RNAs (rRNAs), which constitute 85-90% of total cellular RNA, are useful internal controls, as the various rRNA transcripts are generated by a distinct polymerase and their levels are less likely to vary under conditions that influence the expression of mRNAs (reviewed in Bustin (2000)). Indeed, the use of a less regulated gene, such as 18S rRNA, may be more appropriate since it also reflects the total amount of RNA in the sample. In view of these considerations, the present study used two different (but widely used) housekeeping genes, since the similarity in the results using both genes lends credibility to their use and to the accuracy of the normalised data. Thus the fact that normalisation to GAPDH, but not 18S rRNA, resulted in statistically significantly higher expression of the antioxidant enzymes in the FO group is intriguing. Neither housekeeping gene was shown to be up or

down regulated by diet, hence justifying their use for normalisation. This lack of complete agreement of antioxidant expression following correction to both housekeeping genes indicates that further studies are required to clarify the appropriateness of these housekeeping genes. Nevertheless, regardless of the housekeeping gene used to normalise the data obtained in the present study, there was at minimum, a trend towards higher expression of the antioxidant enzymes following dietary fish oil supplementation. Furthermore, the results of the gene expression prior to normalisation to the housekeeping genes also showed a similar pattern.

Fish oils rich in n-3 fatty acids are susceptible to oxidation because of their high degree of unsaturation (Alexander-North *et al.*, 1994; van Ginkel & Sevanian, 1994). Radical species can attack the double bonds of unsaturated lipids and initiate chain reactions leading to such end products as aldehydes, dialdehydes (eg malondialdehydye, MDA) and short-chain hydrocarbons. Polyunsaturated (n-3) fatty acids are efficiently incorporated into tissue membrane phospholipids and as a result of the PUFA incorporation, the unsaturation index is increased. Many studies have demonstrated a greater extent of oxidative stress in tissues from humans and animals supplemented with n-3 PUFAs (Garrido *et al.*, 1989; Meydani *et al.*, 1991). However, cells are not always passive to increased oxygen radical production, and moderate episodes of free radical stress (for example, induced by fish oil feeding) may in fact potentiate the defense system by stimulating the expression of the antioxidant enzymes (Vogt *et al.*, 1998; Csonka *et al.*, 2000; Perry *et al.*, 2000). Indeed, significantly higher activities of hepatic CAT, GPX and Cu/Zn-SOD were measured in FO supplemented rats (Ruiz-gutierrez *et al.*, 1999). Similarly, supplementation with FO raised Mn-SOD, CAT and GPX activities of spleen homogenates (Avula & Fernandes, 1999). In humans, FO supplementation increased GPX

activities in erythrocytes and platelets (Bellisola *et al.*, 1992). However, an increase in gene expression does not necessarily translate into an increased enzyme activity, and at the time of writing, no studies to our knowledge have investigated the effects of FO supplementation on both enzyme activity and gene expression in rat *myocardium*. In autoimmune-prone female mice, FO feeding increased hepatic mRNA expression (assessed by Northern Blotting techniques) and activities of SOD, CAT and GPX (Venkatraman *et al.*, 1994). Conversely, feeding with EPA and DHA (1500 mg/d/kg) had no effect on the activity and mRNA levels of SOD, glutathione reductase, GPX and glutathione-S-transferase (Vaagenes *et al.*, 1998). And in garlic-supplemented rats, renal and hepatic CAT activity and protein content was reported to be decreased without any changes in mRNA levels (Pedraza-Chaverri *et al.*, 2001).

The majority of studies as reviewed above, have demonstrated an increase in activity of antioxidant enzymes following fish oil supplementation. However, other studies in rats have reported a lowering of antioxidant enzyme activity following FO supplementation (Schimke *et al.*, 1997; Nieto *et al.*, 1998; Yuan *et al.*, 1998). These results indicate the requirement for further investigations in this area with well-controlled experiments, particularly with regard to the level of dietary vitamin E levels and the type of tissue analysed. It is also necessary to perform enzyme activity assays together with analysis of mRNA and protein levels within the same study. As has been the case of the shortcomings of previous studies in the literature, the present study investigated antioxidant mRNA expression without examining changes in antioxidant activity and protein levels (due to time constraints). The present study was further limited by only studying antioxidant enzyme gene expression in the myocardium. It has been previously demonstrated that the expression of antioxidant enzymes is differentially regulated in

various organs in response to different treatments. For example, copper deprivation was shown to significantly decrease the activity, expression and protein levels of CAT in liver, but the CAT activity, mRNA abundance and immunoreactive protein levels in heart were increased following this treatment (Lai *et al.*, 1995).

In the present study, it was hypothesized that the fish oil supplementation, by acting as a mild oxidative stress, induced the expression of the antioxidant defences, i.e. acting as a preconditioning stimulus. This then enabled the myocardium of the FO-fed rats to be better equipped to withstand the experimental ROS challenge. Consistent with this idea, is the finding that incubation of neonatal myocytes for 12 h with 3 mU/ml glucose oxidase (a mild oxidative stress) resulted in a 100% increase in the level of CAT mRNA (Lai *et al.*, 1996). Preconditioning of myocytes either with exogenous $O_2^{\bullet-}$ or 2 cycles of 5 min anoxia and 5 min reoxygenation was protective from a more severe oxidative stress of 60 min anoxia and 60 min reoxygenation and this was associated with increased Mn-SOD activity (Zhou *et al.*, 1996). Supporting the findings of the present study, autoimmune lupus-prone mice supplemented with 10% FO from birth to age 6.5 months, exhibited significantly higher expression of the antioxidant enzymes CAT, SOD and GPX in kidneys (Chandrasekar & Fernandes, 1994) and liver (Venkatraman *et al.*, 1994) as assessed by Northern blotting techniques. To date, these appear to be the only studies that have determined antioxidant enzyme gene expression following dietary FO supplementation.

Several mechanisms probably contribute to the enhanced gene expression of antioxidant enzymes following n-3 PUFA supplementation. One such mechanism may be summarised under the category of nutrient-gene interactions, which covers the influence

of nutrient intake on gene expression and protein synthesis. Potentially the effects of nutrition can be exerted at different stages between transcription of the genetic sequence from DNA into RNA and synthesis of the final active protein product from the mRNA sequence. The activity of the gene itself in producing RNA transcripts is regulated in part by the promoter region to which specific transcription factors can bind (Hesketh *et al.*, 1998). It has been suggested that the direct effects of the n-3 PUFAs EPA and DHA on gene expression are most probably mediated by their ability to bind to positive and/or negative regulatory transcription factors (Fernandes *et al.*, 1998). It was recently demonstrated that DHA can activate the retinoic X receptor (RXR, a nuclear receptor that functions as a ligand activated transcription factor RXR) and bind directly to the ligand-binding domain of this nuclear receptor (de Urquiza *et al.*, 2000). It has also been proposed that PUFAs (or a PUFA metabolite) are transferred to the nucleus, where they function as ligands or modifiers of a nuclear fatty acid-receptor binding protein (PUFA-BP). Following PUFA-dependent modification of the PUFA-BP, the PUFA-BP interacts with a *cis*-acting element in the target gene that governs transcription of particular genes (Clarke & Jump, 1994). PUFAs have also been demonstrated to induce the transcription of genes encoding proteins involved in lipid oxidation and to suppress the expression of genes encoding proteins involved in lipid synthesis (Xu *et al.*, 2001). Genes encoding the oxidative enzymes appear to be regulated by a common PUFA-activated transcription factor- peroxisome proliferator-activated receptorα (PPARα) (Xu *et al.*, 2001). In particular, the n-3 PUFAs have been shown to function as ligand activators for PPARα. The n-3 PUFAs also simultaneously suppress the expression and nuclear localisation of sterol regulatory element-binding protein-1, the nuclear factor responsible for the transcription of lipogenic genes (Price *et al.*, 2000). The effects of oxidative stress are mediated through numerous intracellular signalling molecules. Amongst them is nuclear

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factor kappaB (NF-κB). This transcription factor has been shown to be activated in response to H_2O_2 (Vogt *et al.*, 1998) and ouabain (Xie *et al.*, 1999), during myocardial reperfusion (Chandrasekar *et al.*, 2000) and following cerebral ischaemia (Gabriel *et al.*, 1999). FO feeding, combined with food restriction prolonged the life span of autoimmune prone mice and this was associated with a blunting of the age-dependent increase of NF-κB concomitant with increased SOD and CAT activity (Jolly *et al.*, 2001). This finding appears to not only provide support for the non pro-oxidative effect of FO, but also to demonstrate its antioxidant-like activity. Clarke and associates (reviewed in Clarke & Jump (1996)) demonstrated that PUFAs decrease hepatic fatty acid synthase mRNA levels by inhibiting the rate of gene transcription. They concluded that the rapidity of the effect (<3 h) strongly suggests that PUFAs directly modulate gene transcription rather than by exerting their influence by modifying membrane fatty acid composition and altering hormone release or signalling (Clarke & Jump, 1996). In view of the many studies demonstrating an increase in activity of antioxidant enzymes following FO feeding, it is possible that the action of fish oil is predominantly at the level of transcription. Indeed it has been shown that PUFAs activate multiple mechanisms to influence hepatic gene transcription (reviewed in (Jump *et al.*, 1999)). However, fatty acids can also modulate gene expression indirectly by altering the membrane fatty acid composition or by influencing the generation of intracellular lipid second messengers, eg diacylglyceryol and ceramide (Fernandes *et al.*, 1998). The classic effect of FO feeding is a decrease in the n-6 fatty acid arachidonic acid and a concomitant increase in the phospholipid n-3 fatty acids EPA and DHA. Once incorporated into the phospholipids, n-3 PUFAs can affect a vast array of intracellular signalling pathways leading to altered gene expression (Fernandes *et al.*, 1998).

In contrast to the studies mentioned above, suggesting induction of oxidative stress by dietary fish oil, an alternative mechanism in which it would be just as likely for SF dietary lipid supplementation to promote oxidative stress, could also be proposed. This may also explain the mechanism of the protective effects of FO. The basis for this alternative mechanism is related to arachidonic acid (AA). It has been demonstrated that ROS are generated from AA (Cocco *et al.*, 1999) through the action of cyclooxygenase (COX) (Didion *et al.*, 2001). Since in all of the studies described in this thesis, the levels of AA were significantly higher in the myocardium of SF-supplemented rats compared with the FO group, this would indicate that the SF diet would be likely to generate more ROS than the FO diet. Furthermore, AA, but not the n-3 PUFA, EPA, was shown to activate NF-κB, the transcription factor that regulates the genetic expression of several genes critical for immuno-inflammatory reactions (Camandola *et al.*, 1996). Therefore this additional mechanism to explain the protection of the cells from the FO group, may relate to the lowered availability of AA in the membranes of FO-fed rats that can activate NF-κB. The role of 5-lipoxygenase in mediating ROS generation has been previously confirmed using fluorescent techniques DCF (Woo *et al.*, 2000). Since the n-3 PUFAs inhibit the activity of 5-lipoxygenase and COX (Leaf & Weber, 1988; Kinsella *et al.*, 1990), it is likely that by such mechanisms, SF diets could promote increased free radical production while dietary PUFAs would result in decreased free radical generation. Indeed, saturated fatty acids (SFAs) have been shown to activate NF-κB and induce COX-2 expression, actions that were inhibited by PUFAs (Lee *et al.*, 2001). A final mechanism by which the FO may be exerting protective effects may be by the phospholipid n-3 PUFAs acting as an oxidisable buffer and therefore preventing the peroxidation of other molecules. They may also reduce the deleterious effects of increased free radical activity by replacement of membrane PUFA damaged by peroxidation (O'Farrell & Jackson, 1997).

In summary, the results of this chapter indicated that dietary FO supplementation raised n-3 PUFA proportions in rat myocardium and this was associated with enhanced antioxidant enzyme gene expression. Although indices of oxidative stress were not directly measured in any rat organs or cells, it is unlikely that the FO diet resulted in significant long-term oxidative stress since the results of Chapters 3, 4 and 6 indicate that the viability and contractility of isolated cells and whole hearts was not compromised by the FO diet. Furthermore, if the FO did initially result in oxidative stress, then the myocardium rapidly adapted and increased some of its antioxidant defences as evidenced in this chapter. This may have been responsible for the protective effects against exogenously added free radicals that were observed in earlier chapters.

CHAPTER SIX

The Effect of Dietary Lipids on Ischaemia-Reperfusion Injury in Rat Myocardium.

6.1 Introduction

The mechanisms and consequences of reperfusion-induced arrhythmias have received much attention since their first description (Tennant & Wiggers, 1935). Ventricular arrhythmias occurring upon reperfusion of the acutely ischaemic myocardium are one of the major manifestations of reperfusion injury and such arrhythmias do not occur unless the ischaemic myocardium is reperfused (Tanguy *et al.*, 1996). As discussed in the previous chapters, reperfusion injury and quite possibly reperfusion-induced arrhythmias are believed to be precipitated by the generation of ROS and cellular Ca^{2+} overload. Experimental models of ischaemia-reperfusion (I-R) injury have utilised a variety of protocols such as *in situ* coronary artery ligation (CAL) in small laboratory animals; CAL in isolated hearts; global zero-flow ischaemia and reperfusion (GZFI-R) or perfusion with a hypoxic solution in order to induce reperfusion injury and associated arrhythmias. A protective effect of dietary fish oil has been demonstrated in experimental models of I-R injury induced by CAL in rats *in situ* (McLennan *et al.*, 1988) and in isolated hearts (Yang *et al.*, 1993; Isensee & Jacob, 1994). In the results described in Chapter 3, adult rat ventricular cardiomyocytes were exposed to extracellularly generated ROS in order to mimic *in vivo* reperfusion injury - ie that injury which occurs upon exposure of myocardial cells to ROS present in the blood (generated through the action of xanthine oxidase, neutrophils or other mechanisms) (Sussman & Bulkley, 1990). Addition of the various FRGS to adult rat ventricular myocytes induced asynchronous contractile activity in these cells, which was attenuated by dietary n-3 PUFA supplementation but not by acute addition of DHA (as the free fatty acid) to isolated cells. It has been demonstrated that ROS are generated in isolated hearts (Zweier *et al.*, 1987; Tritto *et al.*, 1998;

Demaison *et al.*, 2001), endothelial cells (Zulueta *et al.*, 1997) and cardiomyocytes (Vandenhoek *et al.*, 1997; Maddaford *et al.*, 1999; Sharikabad *et al.*, 2000) at reoxygenation subsequent to a period of hypoxia (H-R). It is therefore possible that n-3 PUFAs, which become incorporated into membrane phospholipids of the myocardium may offer protection from injury arising as a result of endogenously generated ROS. Thus the aims of the study were:

- To develop suitable protocols for H-R in field stimulated adult rat cardiomyocytes so as to induce damage to these cells particularly following reoxygenation.

- To determine whether n-3 PUFA incorporation confers protective effects from reoxygenation-induced injury by using cardiomyocytes isolated from hearts of rats supplemented with FO containing relatively high levels of n-3 PUFAs.

- To expose isolated hearts from rats supplemented with FO to a protocol of I-R.

The purpose of this final aim was two-fold. Firstly, it served as a whole organ model comparison to the cellular studies undertaken earlier. Secondly, the myocardial tissue derived from these experiments would be used for the gene expression studies (results presented in Chapter 5), to determine the effect of dietary lipid supplementation on antioxidant enzyme gene expression and the added effect of I-R on gene expression.

6.2 Methods

6.2.1 The Effect of dietary lipids on Cellular Hypoxia and Reoxygenation (H-R) Injury

6.2.1.1 Procedure for Inducing H-R Injury in Isolated Cells

Cardiomyocytes were isolated as described in Chapter 2. Cells attached to laminin-coated glass coverslips were placed into a superfusion chamber on the stage of a Zeiss inverted microscope (Germany) and continuously perfused with a buffer containing (in mM): 140 NaCl, 6 KCl, 6 HEPES, 1 $MgCl_2$, 10 glucose and 1.25 Ca^{2+}, pH 7.3, gassed with 100% O_2 (equilibrating and reoxygenation buffer). Following 3 min equilibration at 37°C at a flow rate of 2 ml/min, cells were field stimulated at a frequency of 1 Hz at 40V (205 mA) for 2 min. Cardiomyocytes then underwent simulated ischaemia which involved superfusion with a "hypoxic solution". The duration of hypoxia was 5, 10 or 15 min. The composition of the hypoxia solution was (in mM): 140 NaCl, 8 KCl, 6 HEPES, 1 $MgCl_2$, 5 lactate and 1.25 Ca^{2+}, pH 6.0, gassed with 100% N_2 (based on a modification of the methods of Maddaford *et al.* (1999)). The frequency and voltage of stimulation were not altered during hypoxia, however the flow rate was reduced to 1 ml/min to simulate low-flow ischaemia. The hypoxic and reoxygenation buffers were gassed with N_2 and O_2 respectively, for 45 min prior to use, to ensure complete saturation of the solutions with the appropriate gasses. During hypoxia, the Perspex unit surrounding the microscope was gassed with N_2. A glass slide was placed over the superfusion chamber, with an inlet in order to gently gas the area above the solution in the superfusion chamber with N_2. In this way, both the buffer superfusing the cell and the external environment would be saturated as much as possible with N_2. Following the hypoxic period, the external N_2 was turned off, the cover of the superfusion chamber was removed, cells were perfused with the "reoxygenation buffer" and the flow rate was increased to 2 ml/min. Arrhythmias (i.e.

asynchronous contractile activity) did not represent the main manifestation of reoxygenation injury. This is similar to findings in neonatal myocytes in which the spontaneous electromechanical activity resumed following reoxygenation, without the development of dysrhythmias (Ponsard *et al.*, 1999). Upon reoxygenation, the cells "hypercontracted" briefly, then either partially recovered to form shortened, square-shaped forms capable of contracting, or rounded up rapidly to irreversibly hypercontracted forms. Cells were classified as rod-, square- or round-shaped (Haworth *et al.*, 1981; Hayashi *et al.*, 1995). The ratios of cell length to cell width were divided into those more than 3 for the rod-shaped cell, and less than 3 for the square-shaped cells (Nasa *et al.*, 1998). Square cells continued contracting in synchrony with the electrical field stimulation. Such cells have been reported to have distinct sarcomeres and are therefore considered viable (Stern *et al.*, 1985). Rounded cells were distinctly round and could be easily distinguished from the other categories used to define cell morphology. The numbers of rod and square shaped cells combined, at reoxygenation, represented the percentage of surviving cells.
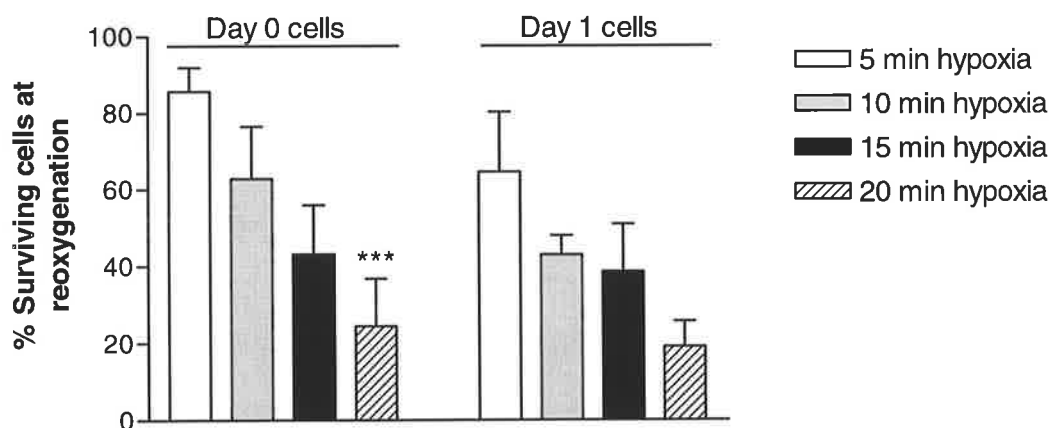
Figure 6.1: Comparison of responses of cells to increasing durations of hypoxia.
Day 0 cells were used on the same day as isolation. Day 1 cells were maintained in the incubator overnight prior to use. Reoxygenation duration was 20 min. Data are averages of approximately 20 cells per rat, n=3-6 rats. ***p<0.001 vs 5 min hypoxia.

As shown in Figure 6.1, preliminary experiments indicated that reoxygenation following hypoxia reduced the percentage of surviving cells. The degree of damage was increased with longer durations of ischaemia. Day 0 cells (used on the same day as isolation) were used for the dietary experiment since day 0 cells were used in the previous dietary studies, and this would therefore allow comparison between the two modes of induction of reperfusion injury.

6.2.1.2 Animals and Dietary Lipid Supplements

Male Sprague Dawley rats were obtained at 4 weeks of age and maintained on the low-ALA diet containing 7% (w/w) fat as Sunola oil, until 9 weeks of age when they were fed the lipid supplemented pellets for a further 3 weeks. The supplements were 10% (w/w) lard and 10% (w/w) fish oil (RoPUFA 30). The supplements were incorporated into the pellets at the time of preparation. Fatty acids were analysed as outlined in Chapter 2 and the analysis of the diets is given in Table 6.1. Following lipid supplementation, rats were sacrificed and cardiomyocytes were isolated. The H-R protocol was carried out as described above.

FAME[a]	Saturated Fat	Fish Oil
14:0	4.32 ± 0.19	4.80 ± 0.15
14:1	0.68 ± 0.04	0.14 ± 0.01
16:0	19.89 ± 0.30	10.56 ± 0.11
16:1 (n-9)	1.09 ± 0.81	6.68 ± 0.01
18:0	10.63 ± 0.26	2.63 ± 0.04
18:1[b]	53.07 ± 0.01	36.67 ± 0.31
18:2 (n-6)	7.14 ± 0.46	6.65 ± 0.03
18:3 (n-3)	1.22 ± 0.16	0.88 ± 0.03
20:0	0.68 ± 0.04	0.18 ± 0.02
20:1	0.23 ± 0.15	0.48 ± 0.03
20:4 (n-6)	n/d	0.98 ± 0.01
22:0	0.16 ± 0.03	0.18 ± 0.01
20:5 (n-3)	n/d	17.83 ± 0.38
22:5 (n-3)	0.15 ± 0.01	1.70 ± 0.07
22:6 (n-3)	n/d	8.94 ± 0.17
Σ SFA	36.37 ± 0.28	18.58 ± 0.25
Σ MUFA	55.12 ± 0.91	44.03 ± 0.33
Σ PUFA	8.50 ± 0.63	37.39 ± 0.08
Σ n-6	7.14 ± 0.46	7.80 ± 0.04
Σ n-3	1.37 ± 0.17	29.39 ± 0.16
n-6/n-3	5.26 ± 0.32	0.27 ± 0.00

Table 6.1: Fatty acid composition (wt%) of the lipid supplemented diets.
Data shown are mean ± SEM (n=3). The shorthand notation for fatty acid structure was given in the legend to Table 3.1. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. n/d not detected. [a]FAME, fatty acid methyl esters. [b]18:1 contains n-7 and n-9 isomers. Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids. The fatty acid analysis of the oils was given in Chapter 3.

6.2.2 Ischaemia-Reperfusion (I-R) in Isolated Hearts

6.2.2.1 Langendorff Heart Perfusion

Rats were injected with 60mg/kg sodium pentobarbitone and 500 IU heparin (to prevent the blood from clotting in the coronary vessels) as a single intraperitoneal injection. Following adequate anaesthesia, the heart was excised and the beating arrested by placing the heart into ice-cold Ca^{2+}-free Tyrode's solution for 10-30 seconds until it ceased contracting. The heart was then rapidly mounted onto the cannula of the Langendorff apparatus and the aorta firmly tied with suture. Tyrode's buffer, containing 2.5 mM Ca^{2+} (pH 7.4) was used to perfuse the heart to wash the blood from the heart and the associated vasculature and to initiate autonomous contractile activity. When the contractile activity stabilised, two small ECG needles were inserted, one in the left atrium and one in the left ventricle, near the apex of the heart. A pressure transducer level with the aorta measured aortic pressure. The heart was maintained in a warm, and humidified environment during the entire perfusion by the positioning of a large glass chamber around the heart. Buffers were maintained at 37°C and gassed with 100% O_2 during the entire superfusion period.

6.2.2.2 I-R Injury Induced by H_2O_2 Perfusion

In order to reproduce the free radical-induced arrhythmias that were observed in isolated cardiomyocytes in response to the addition of various ROS, isolated hearts were perfused with H_2O_2 in the Langendorff mode. Hearts were first perfused with Tyrode's solution containing 2 mM Ca^{2+} and gassed with 100% O_2 for 10 min (in the non working-heart mode). Following this equilibration period, hearts were perfused for 30 min with the same solution also containing 30 μM H_2O_2. Upon perfusion with H_2O_2, the force of the contractions became stronger, however, no arrhythmic activity was observed. Using 50-200 μM H_2O_2 in the perfusing medium, arrhythmias were only observed in 4 out of 13

experiments, the most common endpoint being the occurrence of cardiac contracture or "stone heart" which describes the cardiac stiffening resulting from intracellular calcium overload (Bello-Klein *et al.*, 1997).

6.2.2.3 Global Zero-Flow Ischaemia-Reperfusion in Isolated Hearts

Due to the lack of arrhythmias occurring in response to perfusion of isolated hearts with H_2O_2, a modified protocol of ischaemia-reperfusion (I-R) was used. Hearts were initially equilibrated for 10 min by perfusion with Tyrode's buffer, pH 7.4. This was then followed by 30 min of global zero-flow ischaemia and 30 min reperfusion (GZFI-R). Control hearts underwent 10 min equilibration followed by a further 60 min of perfusion with the same buffer. Global ischaemia was achieved by turning off the flow of buffer to the heart. At this time, the aortic pressure was 0 mmHg and the ECG matched the contractility of the heart, i.e. the contractions slowed down over a period of 3-5 min and ceased by 20 min of ischaemia. Two min prior to reperfusion, a second tap, situated at the base of the Langendorff chamber was opened to allow the cold buffer remaining in the tubing to run out so that at reperfusion, the heart would only be receiving warm, oxygenated buffer. Global ischaemic periods of 5 – 20 min were not sufficiently severe to result in reperfusion-induced arrhythmias. However, following 30 min global ischaemia, 2 out of 6 hearts developed ventricular fibrillation (VF) within 60 sec of reperfusion, 1 heart developed persistent ventricular tachycardia (VT) and the remaining 3 hearts had intermittent periods of VT and regular contractions.

6.2.2.4 The Effect of Dietary Lipid Supplementation on I-R Injury in Isolated Hearts

Male Sprague Dawley rats were obtained at 4 weeks of age and maintained on the low-ALA diet until 9 weeks at which time they were fed the lipid supplemented pellets. The

supplements were CON (control diet, a further 10% (w/w) sunola oil), SF (10% (w/w) lard) and FO (10% (w/w) fish oil, Fishaphos (marketed by Felton Grimwade & Bickford Pty Ltd, Australia)) and were fed for 4 weeks[12]. The myocardial tissue from these experiments was used in the gene expression studies described in Chapter 5, with the fatty acid analyses of the diets and hearts presented in that chapter. A protocol of 30 min global zero-flow ischaemia and reperfusion (GZFI-R) was used in these dietary experiments. Since a further aspect of this study was to use the tissue from these experiments for gene expression analysis (Chapter 5), after 30 min reperfusion the heart was removed from the Langendorff apparatus and immediately placed into a large Petri dish filled with ice-cold Ca^{2+} free Tyrode's solution. When the heart ceased contracting, it was cut into small (approximately 20 mg) pieces (in a sterile environment) and then pieces were chosen at random and placed into a pre-weighed sterile, RNAse free tube to make a combined total of approximately 100 mg of heart tissue. Tubes containing myocardial tissue were immediately snap frozen in liquid nitrogen and stored at minus 80°C for RNA isolation and fatty acid analysis.

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[12] As explained in Chapter 5, due to technical difficulties with the Langendorff system and the experimental protocol for I-R, the implementation of the study was delayed by one week. Hence, it was necessary to maintain the rats on the lipid supplements for an additional week.

6.3 Results

6.3.1 Dietary Lipid Supplementation and H-R Injury in Isolated Cells

6.3.1.1 Fatty Acid Composition of the Membrane Phospholipids

The fatty acid composition of rat ventricular phospholipids following dietary lipid supplementation is shown in Table 6.2.

FAME[a]	SF	FO
16:0	9.36 ± 0.90	11.92 ± 0.48[c]
16:1	0.22 ± 0.05	0.16 ± 0.02
17:0	0.32 ± 0.04	0.31 ± 0.05
18:0	25.94 ± 1.26	23.10 ± 0.44
18:1[b]	14.53 ± 0.61	10.24 ± 0.57[e]
18:2 (n-6)	5.90 ± 1.10	3.69 ± 0.64
18:3 (n-3)	n/d	n/d
20:0	1.01 ± 0.83	0.20 ± 0.03
20:1	0.14 ± 0.01	0.17 ± 0.04
20:2	0.47 ± 0.13	0.16 ± 0.04
20:3 (n-6)	0.62 ± 0.29	0.80 ± 0.35
20:4 (n-6)	33.62 ± 2.43	22.23 ± 1.97[d]
22:0	0.27 ± 0.06	0.26 ± 0.02
22:1	n/d	0.40 ± 0.07
20:5 (n-3)	n/d	3.24 ± 0.14
24:0	0.72 ± 0.06	0.16 ± 0.03[e]
24:1	n/d	0.31 ± 0.13
22:5 (n-3)	0.85 ± 0.11	2.51 ± 0.12[e]
22:6 (n-3)	5.99 ± 0.81	20.47 ± 0.88[e]
Σ SFA	37.65 ± 2.41	35.99 ± 0.70
Σ MUFA	14.87 ± 0.64	10.93 ± 0.48[e]
Σ PUFA	47.48 ± 2.69	53.08 ± 0.87
Σ n-6	40.14 ± 2.43	26.72 ± 1.73[d]
n-3	6.88 ± 0.90	26.23 ± 0.94[e]
n-6/n-3	6.21 ± 0.60	1.04 ± 0.10[e]

Table 6.2: Fatty acid composition (wt%) of ventricular cardiomyocyte total phospholipids following 3 weeks dietary lipid supplementation.
Data are mean ± SEM of 6 rats. The shorthand notation for fatty acid structure was given in the legend to Table 3.1. Computational parameters (Σ and n-6/n-3) are derived from the full fatty acid set. n/d, not detected. [a]FAME, fatty acid methyl esters, [b]18:1 contains n-7 and n-9 isomers. [c]p<0.05, [d]p<0.01, [e]p<0.001 for SF vs FO (Unpaired t-test with Welch's correction). Abbreviations: FO, fish oil diet; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SF, saturated fat.

Although there was no significant difference between the two dietary groups with respect to the total proportion of all PUFAs, the dietary supplementation did induce changes in the proportions of the different PUFA classes. The SF group had a higher proportion of n-6 PUFAs compared to the FO group (p<0.01), mainly due to a higher proportion of arachidonic acid. The total proportion of n-3 PUFAs in the FO myocardium was almost four times that observed in the SF group (p<0.001). This increase was mainly attributed to the higher 22:6 (n-3) proportions (p<0.001). The SF group did not exhibit detectable levels of 20:5 (n-3), whilst the FO group exhibited a proportion of 3.2 ± 0.1%. Docosapentaenoic acid (DPA, 22:5 n-3) was also present in higher proportions in the FO group (p<0.001).

6.3.1.2 Body Weights and Cell Viability

Following 3 weeks lipid supplementation, rats fed the SF diet weighed 474.3 ± 10.3g (n=8), which was not significantly different to those in the FO group, weighing 477.9 ± 11.4g (n=9). The viability of cells isolated from hearts of the rats fed the two different diets was also not significantly different with 73.4 ± 3.9% rods in the SF group and 75.6 ± 2.3% rod-shaped cells isolated from rats in the FO group.

6.3.1.3 Contractile Responses to Ischaemia-Reperfusion (I-R) in Isolated Cardiomyocytes

A protocol of H-R in isolated myocytes was used to mimic *in vivo* I-R injury. Immediately upon superfusion with the hypoxic solution, contractile activity ceased (in the presence of electrical field stimulation) and stimulation at higher voltages did not restore contractile activity. Between 11 and 15 min of hypoxia, weak contractile activity resumed in some cells, however, although reproducible, this was not significantly

different between the cells isolated from the two dietary groups of rats. There were no incidences of asynchronous contractile activity occurring during the hypoxic period. At reoxygenation, contractile activity resumed, beginning with slow contractile waves passing through the cell until strong synchronous activity was re-established. The majority of cells did not develop asynchronous contractile activity and as discussed in the preliminary studies, the main manifestations of reoxygenation-induced injury were either the occurrence of hypercontracture (rounded cells) or the development of "square" cell morphology. Hence, the classification of cells at reoxygenation was chosen as "percentage surviving cells" which included rod and square shaped cells contracting in synchrony with the electrical field stimulation. The responses of cells derived from both the SF and FO supplemented groups at reoxygenation followed a similar trend, with the percentage of surviving cells decreasing primarily within the first 3 min of reoxygenation. After 10 min, no further damage manifested as altered cell morphology or contractile activity. There was no significant difference in the percentage of surviving cells (rod and square-shaped cells) occurring at any time point during the reoxygenation phase between cells from the SF and FO groups.
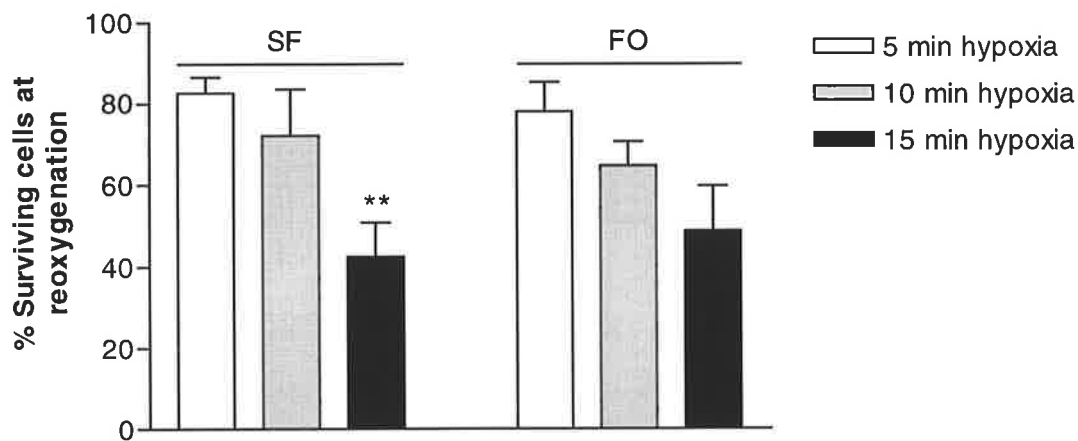
Figure 6.2: Reoxygenation induced injury in rat cardiomyocytes.
Rats were fed a diet supplemented with saturated fat (SF) or fish oil (SF) for 3 weeks. Cells underwent 5 (circular symbols), 10 (squares) or 15 (triangle) min hypoxia followed by 10 min reoxygenation. The percentage of surviving cells (rod cells plus square shaped cells) after 10 min reoxygenation is graphed. Data are averages of approximately 20 cells per rat, performed in triplicate, n=7-8 rats. **p<0.01 for SF 5 min vs 15 min.

Figure 6.2 compares the total percentage of surviving cells (rods plus squares) at 10 min of reoxygenation. Within the SF group, as the hypoxic time increased, the percentage of surviving cells decreased, reaching significance for a hypoxic period of 15 min ($p < 0.01$). There was a similar trend in the FO group ($p = 0.07$). There was no significant difference between the responses of cells between the two dietary groups for the different durations of hypoxia. Analysis of the type of surviving cells revealed that there was a trend towards a lower percentage of square-shaped cells in the FO group, however, this did not reach statistical significance (Figure 6.3).
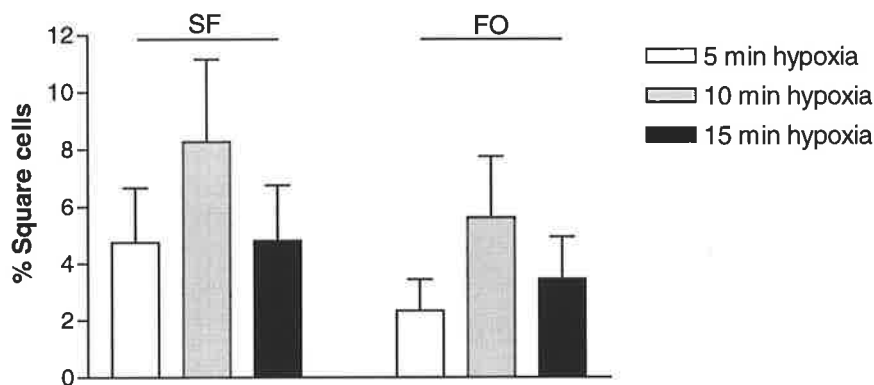
Figure 6.3: Hypoxia-reoxygenation injury in cardiomyocytes from lipid-supplemented rats.
Comparison of the percentages of square shaped cells following 10 min reoxygenation subsequent to various periods of hypoxia. Rats were fed a diet supplemented with saturated fat (SF) or fish oil (SF) for 3 weeks. Data are averages of approximately 20 cells per rat, n=7-8 rats per dietary group.

6.3.2 Dietary Lipid Supplementation and I-R Injury in Isolated Hearts

6.3.2.1 Fatty Acid Analyses

The fatty acid analysis of diets and the myocardial tissue for this particular study was given in Table 5.1 and Table 5.6 respectively. A brief summary of the major myocardial phospholipid fatty acids of interest is presented in Table 6.3. This data is the composition analysis of the hearts undergoing control perfusion (i.e. not GZFI-R).

FAME[a]	CON	SF	FO
Σ SFA	42.09 ± 0.54	40.48 ± 0.48	38.55 ± 0.87[c]
Σ MUFA	13.07 ± 0.40	12.33 ± 0.29	10.08 ± 0.21[d,f]
Σ PUFA	44.84 ± 0.56	47.19 ± 0.66	51.37 ± 0.87[d,e]
Σ n-6	36.18 ± 0.43	35.91 ± 0.50	22.16 ± 0.61[d,f]
Σ n-3	8.44 ± 0.20	10.82 ± 0.43	29.13 ± 1.24[d,f]
n-6/n-3	4.29 ± 0.08	3.35 ± 0.15[b]	0.77 ± 0.06[d,f]

Table 6.3: Fatty acid composition (wt%) of the ventricular myocardial total phospholipids following dietary lipid supplementation.
Data are mean ± SEM for 6 rats per dietary group. The shorthand notation for fatty acid structure was given in the legend to Table 3.1. Computational parameters (Σ and n-6/n-3) were derived from the full fatty acid set. [a]FAME, fatty acid methyl esters. [b]$p < 0.001$ for CON vs SF; [c]$p < 0.01$, [d]$p < 0.001$ for CON vs FO; [e]$p < 0.01$, [f]$p < 0.001$ for SF vs FO (by ANOVA with the Bonferroni multiple comparisons test). Abbreviations: CON, control diet (17% (w/w) Sunola oil); FO, fish oil diet; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SF, saturated fat diet.

6.3.2.2 Body Weights

The body weights of the animals in the three dietary groups used in the control perfusion and the GZFI-R experiments, both before and following four weeks lipid supplementation were not significantly different between the dietary groups. The data is presented in Table 6.4.

Treatment	Body Weight (g)	CON	SF	FO
Control perfusion	Starting	356.4 ± 8.9	349.3 ± 6.9	355.5 ± 9.8
	Final	500.4 ± 13.8	494.8 ± 10.7	498.5 ± 6.1
GZFI-R	Starting	361.4 ± 6.7	351.9 ± 9.3	356.5 ± 5.2
	Final	496.6 ± 9.3	486.3 ± 13.8	471.1 ± 12.1

Table 6.4: Rat body weights prior to and after lipid supplementation.
The control perfusion hearts that did not undergo GZFI-R were perfused for 70 min with oxygenated buffer. The GZFI-R hearts underwent 10 min equilibration followed by 30 min global, zero flow ischaemia and 30 min reperfusion. Starting body weight refers to the weight of the rats at 4 weeks of age. Abbreviations: CON, control dietary group; FO, fish oil; GZFI-R, global zero-flow ischaemia-reperfusion; SF, saturated fat.

6.3.2.3 Heart Rates and Contractility

The heart rate (HR) was determined from the ECG traces. As indicated in Table 6.5, the HR at the start of the perfusion was not significantly different between the three dietary groups, both for the control perfusion and the GZFI-R group (ANOVA).

	Heart Rate (beats per minute)		
	CON	**SF**	**FO**
Control perfusion	280.5 ± 20.7	249.8 ± 24.2	301.5 ± 12.7
GZFI-R	217.4 ± 24.4	250.5 ± 23.6	269.6 ± 29.6

Table 6.5: Heart rates for dietary animals in both isolated heart perfusion groups.
Abbreviations and explanations as in Table 6.4

Under normal perfusion conditions, all three dietary groups exhibited a gradual slowing down of the HR, which stabilised at approximately 135 beats per minute (bpm) after 70 min (Figure 6.4A). Although this was not expected, based on previous reports of the maintenance of steady heart rates during Langendorff perfusions (Watts & Maiorano, 1999; Mcdonald *et al.*, 1999), it occurred reproducibly in all three groups. For most of the experiments described in the literature for H-R or I-R studies in isolated cells or whole hearts, a Krebs-Henseleit buffer has been used. The present experiments utilised a modified Tyrode's buffer so as to maintain consistency with the earlier experiments described in Chapters 3 and 4. The main difference between the Tyrode's solution and the Krebs-Henseleit buffer is that the latter contains 118 mM bicarbonate, while there is no bicarbonate present in Tyrode's buffer. In order to determine whether this difference in buffers contributed to the decreasing heart rate during Langendorff perfusion, following completion of this dietary study, GZFI-R experiments were carried out using another group of non lipid-supplemented rats with the perfusion being carried out using Krebs-Henseleit buffer. The HR was found to decrease in the same manner as which occurred with Tyrode's buffer, thus eliminating the type of buffer as a reason for the decrease in HR. For the purposes of analysis, the classification was therefore adjusted such that if the HR at 30 min reperfusion reached the same level as in the normally (non-ischaemic) perfused hearts (at 70 min), they were considered as "recovered". This was justified due to the shifting baseline (decreasing HR) throughout perfusion. The buffers were

oxygenated continuously throughout the entire perfusion and the pressure was maintained at 100 cm of H_2O, so it is unlikely that the HR decreased due to damage resulting from insufficient pressure or oxygen. Further, the HEPES based Tyrode's buffer maintained the pH at 7.4 throughout the experiment, confirmed by continuous determination of the pH of aliquots of coronary perfusate during the protocol.
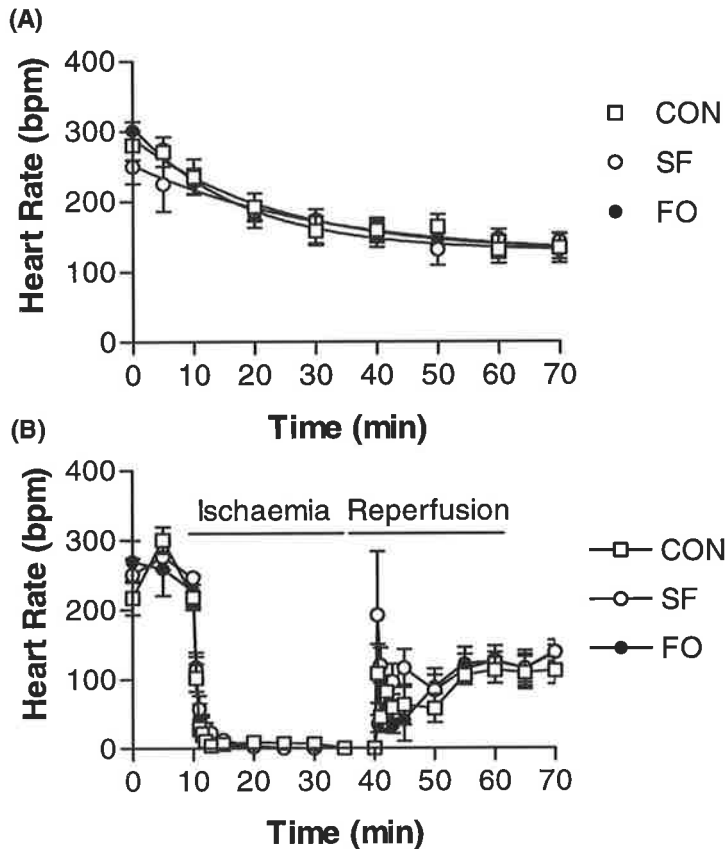
Figure 6.4: The effect of ischaemia-reperfusion on myocardial heart rate.
(A) Isolated hearts were perfused with Tyrode's solution for 70 min or (B) following a 10 min equilibration period, hearts underwent 30 min global zero-flow ischaemia followed by 30 min reperfusion. Rats were supplemented with 17% (w/w) Sunola oil (CON), lard (SF) or fish oil (FO), n=8 rats per dietary group.

Following 30 min reperfusion, the HR of the CON hearts was 112.0 ± 18.0 bpm (n=6) which was not significantly different to the HR after 70 min normal perfusion (133.9 ± 20.9 bpm, n=7, Figure 6.4B). The HR of the SF group recovered to 138.7 ± 17.2 bpm (n=6) in comparison to the control perfusion HR of hearts in this group (142.3 ± 11.5 bpm, n=7, ns). The HR of the FO group at 30 min reperfusion was 137.1 ± 12.0 bpm

(n=7), which was not significantly higher than FO hearts that were perfused under control conditions (129.8 ± 11.3 bpm, n=8). By the above-mentioned criterion, following GZFI-R, all three dietary groups showed an equivalent recovery of HR. There were no occurrences of ventricular fibrillation (VF) or tachycardia (VT) in hearts from rats in any of the dietary groups undergoing the "control" perfusion (ie no ischaemia). In all control perfusion groups however, there were instances of missed beats and irregular rhythms that lasted less than 20 sec, with hearts always recovering spontaneously (results not shown). Hearts that underwent 30 min GZFI-R either fibrillated upon reperfusion, or after brief periods of irregular rhythms/tachycardia (not significantly different between the dietary groups- results not shown), resumed regular contractile activity. In the CON and SF dietary groups, 2 out of 8 hearts developed irreversible VF at reperfusion. In the FO group, 1 out of 8 hearts developed reperfusion-induced VF.

6.4 Discussion

The main finding of the present study was that dietary FO supplementation enriched rat myocardial phospholipids with n-3 PUFAs, however no protection was afforded from I-R in perfused hearts or H-R in isolated cardiomyocytes.

Dietary lipid supplementation had a dramatic effect on myocardial fatty acid composition, confirming that the heart is one of the most responsive tissues to changes in dietary PUFA content (Kramer, 1980; al Makdessi *et al.*, 1994). Previous studies have shown that DHA is the major n-3 PUFA incorporated into rat myocardial membranes following FO feeding (Pepe & McLennan, 1996; Sergiel *et al.*, 1998) even for those fish oil preparations in which EPA is the predominant fatty acid. Retro-conversion of DHA to EPA does not appear to be a significant metabolic process as far as the heart is concerned (Sergiel *et al.*, 1998). Therefore EPA given in the diet appears to be readily elongated and desaturated to DHA which is subsequently incorporated into the myocardial phospholipid fraction. The results of the present study support this concept in as much that while the FO diets contained twice as much EPA than DHA, the membrane phospholipids contained about 20% DHA and 3% EPA (of total fatty acids) following FO feeding. This predominant incorporation of DHA suggests that DHA plays an important role in cardiac function.

One consequence of the biological role of DHA is with regard to protection from arrhythmogenesis. Indeed, supplementation with DHA alone was shown to inhibit experimentally-induced ischaemic arrhythmias in the *in situ* rat heart (McLennan *et al.*, 1996). However, in the present study, despite significant enrichment of the myocardial membranes with DHA, there was no significant protection afforded from injury induced by a protocol of H-R or I-R. Furthermore, it was an unexpected finding that the frequency

of reperfusion arrhythmias was not higher in the CON and SF groups compared to the FO supplemented group. It is important to note however, that the protocol of GZFI-R failed to induce *any* significant damage to the rat heart. After 30 min reperfusion following global zero-flow ischaemia, a regular contractile rhythm was observed for most hearts from all three dietary groups. This may have been explicable by the type of ischaemia protocol (global versus regional) that was employed.

The majority of previous studies on reperfusion arrhythmias have used a coronary artery ligation protocol (regional ischaemia) applied either *in situ* (al Makdessi *et al.*, 1995; Lepran & Szekeres, 1992; McLennan *et al.*, 1985; McLennan *et al.*, 1988; McLennan *et al.*, 1993; McLennan & Dallimore, 1995), or in isolated hearts perfused in the working mode (Walker *et al.*, 1998). Both of the above protocols may have induced a greater ischaemic stress than a GZFI-R procedure (Rietz *et al.*, 1993; Pepe & McLennan, 1996). Support for this concept comes from a study by (Jaeschke *et al.*, 1988) in which a GZFI-R protocol was applied to isolated rat livers. Release of oxidised glutathione from cells was used as an index of oxidative stress and was found to be unaltered during I-R, suggesting that ROS were not generated by this protocol. This suggests that the use of GZFI-R in the present study may not have been a severe enough stressor to induce damage to the myocardium significant enough to elicit arrhythmias[13]. For the present study, it was not possible for a variety of reasons to set up a working heart perfusion with cannulation through the atrium; hence the Langendorff perfusion protocol was used. However, in preliminary studies, this procedure did result in the induction of reperfusion-induced arrhythmias (RIAs), initially justifying the use of this protocol. Another factor that may

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[13] Although the results of the I-R experiments were unexpected, the myocardial tissue was viable and therefore it was used for the gene analysis studies described in Chapter 5. However, since significant dietary differences were not seen with the I-R protocol in terms of arrhythmogenesis, only the tissue from the "control perfusion" experiments was used for the gene expression studies so that only basal values for expression of antioxidant enzymes could be determined.

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account for the lessened severity of the ischaemic stress in the present study, may relate to the duration of ischaemia (30 min). Longer times for ischaemia may be less damaging since the heart may be able to adjust its cellular metabolism to afford greater protection during the ischaemic period, hence enabling a better recovery following reperfusion (Lepran & Szekeres, 1992). Kane *et al.* (1984) demonstrated a bell-shaped response for ischaemic damage in which the most severe arrhythmias occurred after an occlusion period of 5 or 15 min. Shorter or longer periods of occlusion outside this time frame, reduced the number of RIAs and no periods of VF occurred.

The choice of a 30 min period of ischaemia in the present study was based on consideration of a number of factors. Firstly a GZFI-R as opposed to CAL protocol for ischaemia was being used, and secondly, a different physiological solution compared to the literature was also being used to perfuse the rat hearts (Tyrode's rather than Krebs-Henseleit buffer). However, preliminary studies using this protocol (GZFI-R plus Tyrode's) resulted in 50% of hearts developing VF or VT upon reperfusion. Such a value was comparable to the values obtained by Kane *et al.* (1984), and was considered adequate justification for the use of this protocol in the present study. Furthermore, a protocol of 30 min global ischaemia has been used by other investigators (Ray *et al.*, 1999; Csonka *et al.*, 2000) and demonstrated to result in 100% VF and VT at reperfusion (Tosaki *et al.*, 1988). Finally, a third factor underlying the choice of a 30 min ischaemia protocol was with regard to enabling sufficient time for changes in gene expression to occur, which was a further aim of this study. Therefore, based on the study by Csonka *et al.* (2000), in which it was demonstrated that changes in spontaneously hypertensive rat myocardial antioxidant enzyme gene expression were apparent following 30 min

perfusion with H_2O_2 or global ischaemia, a 30 min GZFI-R protocol was used in the present study to generate tissue that could be used for gene expression analysis.

The above-mentioned aim of this study to compare the mRNA levels of several antioxidant enzymes in the myocardium of animals supplemented with different dietary lipids under basal conditions and after I-R, was in order to determine whether the gene expression was altered at reperfusion. This would have aided in the interpretation of the results presented in Chapter 3 and given some further insight into the antiarrhythmic mechanism of FO and the n-3 PUFAs in relation to possible effects of these PUFAs on free radical status. However, the finding that FO feeding increased basal antioxidant enzyme gene expression levels (as shown in Chapter 5) was sufficient evidence to partly explain the antiarrhythmic action of FO by effects related to their attenuation of ROS-induced arrhythmias. Furthermore, due to the failure in the present study to significantly increase the incidence of RIAs by the protocols employed, the antioxidant gene expression in cardiac tissue following GZFI-R was not investigated and the study was therefore limited to those effects attributed to dietary lipid supplementation. It is not believed that the lowered induction of RIAs in the present study are due to the methods employed; ie the use of a protocol to maintain consistency with earlier experiments in this thesis (the use of Tyrode's buffer) and an experimental duration and protocol (ie. 30 min GZFI-R) known to induce changes in myocardial antioxidant gene expression (Csonka *et al.*, 2000), since the preliminary findings were in accordance with the results of Kane *et al.* (1984) regarding the ability to induce RIAs.

Many authors have reported that dietary fish oil attenuates reperfusion-induced arrhythmias (McLennan *et al.*, 1988; Yang *et al.*, 1993; McLennan, 1993; Pepe &

McLennan, 1996). In contrast, although FO feeding increased n-3 PUFA incorporation into the myocardial membrane phospholipids, it failed to have any beneficial effect on creatinine kinase release (indicator of cellular damage) following hypoxia-reoxygenation in isolated hearts (O'Farrell & Jackson, 1997). Interestingly, it was reported that while 0.1 μM EPA present in the perfusing buffer improved the contractile function of the ischaemic-reperfused rat heart, 4 weeks dietary supplementation with n-3 PUFAs did not significantly influence myocardial function following an I-R protocol (Takeo *et al.*, 1998). Similarly, VF did not occur at reperfusion following GZFI-R even when perfusing with Krebs-Henseleit buffer under working heart conditions (Sergiel *et al.*, 1998). In that study, only VT's were observed at reperfusion following 17 min ischaemia, and the extent of VT's were not significantly different between the groups fed n-3 PUFAs or n-6 PUFAs. The authors suggested that the absence of VF was probably related to the homogeneity of the reperfused myocardium due to global ischaemia (Sergiel *et al.*, 1998). Since the incidence and duration of arrhythmias are known to depend on the severity of ischaemic cell damage, it seems that in the present study, the ischaemic cell damage occurred to the same extent in all dietary groups. Furthermore, since greater than 50% of non-dietary hearts fibrillated within 2 min of reperfusion following 30 min of ischaemia (preliminary experiments), this suggests that both diets were able to provide some protection from I-R injury.

Generation of intracellular ROS, detected by fluorescent probes, has been shown to occur in chick cardiomyocytes following H-R (Zweier *et al.*, 1987; Vandenhoek *et al.*, 1997; Demaison *et al.*, 2001). Hence, based on the results presented in Chapter 3 showing that dietary FO delayed the development of reperfusion injury induced by exogenous ROS, it was hypothesized that the FO diet could also afford some protection from endogenously

generated ROS following H-R. However, there was no significant difference in the percentages of surviving cells after 10 min reoxygenation between cells isolated from rats supplemented with SF or FO. The lack of protection by the FO diet from H-R injury in the present study was unexpected in light of the findings presented in Chapter 5 that MnSOD gene expression was up-regulated in the myocardium of rats in the FO group, and the report by others of a reduction of $O_2^{\bullet-}$ production in monkey myocardium and coronary arteries following FO supplementation (Supari *et al.*, 1995). One explanation could be due to the existence of considerable variability in the raw data (not shown). In this regard, Ralenkotter *et al.* (1997) also found that the time taken for guinea pig ventricular myocytes to go into rigor contracture varied between 10-90 min, which was attributed to varying levels of ATP and glycogen stores between cells. In the present study, reoxygenation following a period of hypoxia reduced the percentage of surviving cells. Despite the fact that there was no difference in surviving cells between the two groups, there was a trend towards a lower percentage of square-shaped cells in the FO group. Although square-shaped cells *are* viable, they are believed to represent an early stage of hypercontracture. In agreement with the present study, in cardiomyocytes isolated from rats fed FO rich and FO deprived diets, H-R reduced the percentage of viable cells, however the difference was not significant between the FO rich and FO deprived groups (Nasa *et al.*, 1998). In a study of neonatal rat cardiomyocytes incubated for 24 hr with n-3 PUFAs, despite the higher proportion of n-3 PUFAs in the cellular phospholipid fraction, there were no differences in the biochemical parameters measured between cells enriched with n-3 PUFAs compared to n-6 PUFAs. For example, lactate release, levels of ATP, ADP and AMP, levels of SOD, GPX and the extent of LDH release, were all affected by H-R to the same extent in both PUFA groups (Chevalier *et al.*, 1990). In the present study, the effect of acute n-3 PUFAs was not determined for

isolated cells or hearts following various H-R/I-R protocols. It has been shown however, that both acute addition of 10 µM EPA, and dietary supplementation with EPA attenuated the morphological deterioration of cardiomyocytes following hypoxia-reoxygenation (Takeo *et al.*, 1998). Similarly, 1g/kg EPA or DHA supplemented by gavage, has been reported to significantly increase the survival of rat cardiomyocytes after 15 min reoxygenation following 150 min substrate-free hypoxia (Hayashi *et al.*, 1995).

Although there is considerable inconsistency between the results of studies investigating the effect of the n-3 PUFAs in the setting of myocardial I-R or H-R, the majority of studies do appear to support a protective role for the n-3 PUFAs. The variations in the results may be attributed to different experimental models, eg CAL versus GZFI-R, isolated versus *in situ* hearts, adult versus neonatal cardiomyocytes, or due to the use of electrically stimulated versus quiescent or spontaneously contracting cells. The type and composition of the fish oil/n-3 PUFA concentrate may also be an important factor, as could the duration of dietary lipid supplementation or the conditions for incubation of cells with fatty acids. Despite the varying results reported in the literature and the non-positive findings of this chapter, a possible protective role for the n-3 PUFAs in attenuating reperfusion injury should not be dismissed. Certainly the animal studies reported for experimental animal models to date and the results of Chapters 3, 4 and 5 justify the use of n-3 PUFAs/dietary fish oil in clinical studies of reperfusion injury to determine their potential use.

CHAPTER SEVEN

General Discussion

7.1 Aims

The broad aims of this thesis were 1) to develop a cellular model of reperfusion injury, which would be used to determine the possible protective effects of dietary and acute n-3 PUFAs on reperfusion injury; and 2) to examine the underlying mechanisms of n-3 PUFA action with regard to any antiarrhythmic effects.

7.2 Summary of Results

In Chapter 3, a reproducible system was established for inducing asynchronous contractile activity in isolated cardiomyocytes. This type of aberrant contractility mimics *in vivo* reperfusion-induced arrhythmias, one of the manifestations of reperfusion injury. A FRGS of H_2O_2/iron, purine/xanthine oxidase or H_2O_2 alone was used, with the major finding of that chapter being that dietary fish oil delayed the development of ROS-induced arrhythmias in isolated cardiomyocytes. This was in contrast to the observation that acute addition of the n-3 PUFA DHA was not protective. However, a trend towards cardioprotection from ROS-induced arrhythmias was observed following overnight incubation of cells with DHA. Another finding from the work described in that chapter was the significant contribution of small levels of dietary α-linolenic acid (ALA) on the extent of DHA incorporation into the myocardial membranes. This was found to be the result of variations in the levels of ALA present in the commercially acquired laboratory rat chow. As a consequence, the level of ALA in the base diet was carefully controlled and maintained at low levels during the preparation of the base diet. Three weeks of dietary lipid supplementation was determined to be appropriate for significant incorporation of n-3 PUFAs into the myocardial phospholipids to occur. This was over

and above that which was normally present in the myocardial membrane prior to specific n-3 PUFA supplementation. The accompanying effect of increased DHA incorporation was one of delaying the development of ROS-induced asynchronous contractile activity, and this was independent of myocardial vitamin E levels.

In Chapter 4, it was demonstrated that the protection afforded to cells isolated from rats fed FO diets enriched in n-3 PUFAs was not due to accumulation of lower intracellular levels of ROS. Furthermore, it did not appear related to differences in resting $[Ca^{2+}]_i$. However, when challenged with a FRGS, cells isolated from FO-fed rats developed lower systolic and diastolic levels of Ca^{2+}, which was possibly related to the slower rate of rise of intracellular Ca^{2+} and faster efflux of Ca^{2+} from the cytoplasm. These results therefore suggested that preventing Ca^{2+} overload in response to ROS might be a possible mechanism of cardioprotective action of dietary FO. The fluorescent probe TMAP-DPH was used to measure cardiomyocyte membrane fluidity. Under the experimental conditions of the study undertaken, it is likely that the TMAP-DPH was only probing the sarcolemmal membranes and not the other (inner cellular) membranes such as those associated with the mitochondria or sarcoplasmic reticulum. In contrast, the total phospholipid fatty acid analysis represented the fatty acid composition of all the membrane phospholipids of the cell, including the sarcolemmal fatty acids. Indeed, the sarcolemmal membrane would be expected to contribute a relatively minor amount of the total phospholipids isolated from rat myocardial tissue due to consideration of the relative membrane surface area contributed by the different organelles of the cell. Only some 3% of the phospholipids in cardiac myocytes have been reported to be in the sarcolemma of cardiac myocytes (Gross, 1992). Nevertheless, it would be expected that since this technique measures the fluidity of the sarcolemmal membrane, it would report on the

effect of n-3 PUFA incorporation on the physical properties of only those membranes. The sarcolemmal membrane fluidity under resting conditions was not different between cells isolated from the SF or FO groups despite significantly greater incorporation of n-3 PUFAs in the myocardium of FO-fed rats. However, membrane fluidity increased following the addition or generation of ROS. This increase reached significance only in the FO dietary group, possibly due to a membrane disordering effect induced by some effect of ROS on membrane phospholipid (poly)unsaturated fatty acids related to their physicochemical properties. A release of free fatty acids (FFAs) and subsequent intercalation of these FFAs between the membrane phospholipids may have contributed to this effect. In view of the protective effects of dietary FO supplementation on ROS-induced arrhythmias demonstrated in Chapters 3 and 4, it is also possible that this disordering effect may be influencing ion channels and/or receptors in the membrane in a manner which imparts some form of cardioprotection. In contrast to its effects on cellular arrhythmias, the exposure of myocytes to H_2O_2 only modestly increased intracellular ROS levels and this was not significantly different between cells isolated from SF versus FO-fed rats.

The results of Chapter 5 demonstrated the effect of FO feeding on enhancing the gene expression of the antioxidant enzymes Mn-SOD, CAT and GPX. This may be one possible reason to explain the inability of acutely added n-3 PUFAs to exert cardioprotective effects in contrast to FO feeding and concomitant incorporation of n-3 PUFAs into membrane phospholipids. It is likely that changes in the antioxidant gene expression patterns may not have occurred within the very short period over which acute n-3 PUFA studies were conducted. If future studies can demonstrate that the increased pattern of gene expression by FO feeding does indeed lead to the enhancement of the

Chapter Seven

antioxidant enzyme system (i.e. increased protein levels and activity) *in vivo*, it would suggest a role for FO in the treatment of I-R injury. This is particularly so in light of findings reporting a down regulation of the antioxidant system during I-R (Singh *et al.*, 1993).

During reperfusion injury *in vivo*, the myocardial cells are exposed to ROS present in the circulatory system. The cardiomyocytes can also generate ROS intracellularly following a period of hypoxia-reoxygenation. The aim of Chapter 6 was to determine whether cardiomyocytes isolated from FO-fed rats were also protected from intracellularly generated ROS. Buffers that were "hypoxic" or "reoxygenated" were prepared and used in a protocol that resulted in reoxygenation-induced hypercontracture of cardiomyocytes. However, dietary FO supplementation had no significant effect on reducing cardiomyocyte injury following a protocol of H-R. In order to extrapolate the "cellular studies" of this thesis to the organ level, hearts from control (non lipid-supplemented) rats were perfused with H_2O_2. However, these hearts did not fibrillate, but rather developed what has been termed "stone heart" in the literature, and which is considered characteristic of myocardial hypercontracture (Bello-Klein *et al.*, 1997). A Langendorff heart perfusion was set up to induce global ischaemia and reperfusion in isolated hearts, which, in pilot experiments, resulted in irreversible reperfusion-induced arrhythmias. However, in hearts isolated from rats on a lipid-supplemented diet, this effect was not observed. Due to insufficient time, this aspect was not further investigated.

7.3 General Discussion

The n-3 PUFAs have been shown to have numerous beneficial effects in preventing, delaying and/or treating various diseases, many of which are of a chronic nature. Despite

practical therapeutic effects of dietary FO in various settings, there has always been concern over the instability of the very long chain fatty acids and their susceptibility to oxidation. Although indices of oxidative stress were not directly measured in any rat organs or cells in the present study, it is unlikely that the FO diet caused significant long-term oxidative stress since the viability and contractility of isolated cells and whole hearts did not appear compromised in animals maintained on the FO diet. Furthermore, if the FO diet did initially result in oxidative stress, then the myocardium rapidly adapted and increased some of its antioxidant defences as evidenced by some of the results presented in this thesis. Such a mechanism may have been responsible for some of the protective effects on cardiomyocyte arrhythmias induced by exogenously added ROS. Another mechanism to explain the action of FO is that it may be acting at the membrane as an inhibitor of lipid peroxidation by breaking peroxyradical chain reactions of membrane lipids in a manner similar to vitamin E. Recent experimental findings support the hypothesis that lipid peroxidation inhibitors protect the myocardium from injury induced by ischaemia and reperfusion (Nagy *et al.*, 1998; Shimizu *et al.*, 1998). Similarly, by being a target of oxidation, the fish oil would be acting to protect various cellular components such as lipids and proteins from oxidative stress.

It was not always possible to maintain the duration of dietary supplementation to exactly equivalent periods, nor was it feasible to use the same commercial source of fish oil in all the dietary experiments relating to the studies aimed at investigating the underlying mechanisms of action of the n-3 PUFAs. However, regardless of the source of fish oil or the duration of n-3 PUFA feeding, the fatty acid analysis of the rat myocardial phospholipids consistently revealed significantly higher proportions of n-3 PUFAs in the membrane phospholipids of the FO-fed rats compared to the other experimental dietary

groups. All the effects of fish oil feeding observed in this thesis, have been attributed to the actions of the n-3 PUFAs, as these fatty acids make up the major proportion of the fish oil concentrate. It cannot be definitively stated that the effects reported were solely due to the n-3 PUFAs and not the fish oil *per se*, or to any other component not categorised as an n-3 PUFA. However, based on other studies cited in the literature (Billman *et al.*, 1994; McLennan *et al.*, 1996), it is quite likely that the n-3 PUFAs were the active components on the experimental parameters examined.

In this thesis, protective effects of dietary FO were demonstrated when a FRGSs was used as the experimental protocol in contrast to a protocol of H-R, which also presumably damages cells by generation of ROS. There are probably a number of explanations for this difference when using alternative protocols. The simplest concept may be that the H-R challenge, by generating high levels of ROS, overwhelmed the intrinsic antioxidant defence system of the cells. This may also explain the inability of this protocol to result in asynchronous contractile activity. The ROS stress may have been so severe that the cells hypercontracted in response to the Ca^{2+} overload induced by the loss of cardiomyocyte membrane integrity. Due to time constraints, ROS levels were not measured during H-R, however, preliminary measurements of $[Ca^{2+}]_i$ indicated a rise in $[Ca^{2+}]_i$ during H-R. Another mechanism by which the FO cells may have been protected from exogenous ROS but not from endogenously generated ROS could be due to the site of action of the ROS. The cell may have a better defence from the actions of ROS generated from extracellular sources compared with intracellular ROS generation, with the cell membrane providing a barrier that regulates the entry of ROS as a "first line of defence". Hence in this manner the cell's defence system would not be overwhelmed. The production of ROS at reoxygenation was reported in isolated hearts using quenchers of singlet oxygen and

•OH (Gauduel & Duvelleroy, 1984). The authors further demonstrated the release of CAT from the myocardium during ischaemia, which was further exacerbated following reoxygenation. It has also been reported that during ischaemia, the levels of glutathione, GPX and SOD are reduced (Opie, 1989); hence their ability to protect from the effects of ROS at reperfusion would likely be diminished. Although the gene expression levels of the antioxidant enzymes was not determined during I-R in the present study, there was a trend towards up-regulation of the expression of SOD, CAT and GPX following FO supplementation under un-challenged conditions, suggesting a possible role for FO in providing additional antioxidant reserve at reperfusion. Hence, the negative results of the cellular H-R experiments of the present study may be explained as a dose effect, perhaps requiring a higher dietary intake of FO to enhance the antioxidant defenses. Consistent with this idea was the fact that cardiac myocytes from hypertrophied hearts were reported to be less damaged by H-R compared with "normal" hearts, and this was associated with an increase in endogenous antioxidant reserve, shown by increases in SOD and GPX enzyme activities in hypertrophied hearts (Kirshenbaum *et al.*, 1995).

The present study in combination with numerous studies in the relevant literature have focussed on the potentially destructive role of ROS, however, it must be stressed that ROS are being continually produced in a vast number of tissues, albeit in small, controlled amounts. ROS play a pivotal role in many physiologic reactions, such as cytochrome P450-mediated oxidations, regulation of smooth muscle tone and in many tissues they also form an important part of the cellular defence arsenal (Bast *et al.*, 1991). Furthermore, a growing body of data suggests that ROS generated by the mitochondria may activate intracellular signalling cascades involved in protective responses (Vandenhoek *et al.*, 1998). Recent reports have also reported that ROS stimulate signal

transduction for transcription factor activation, gene expression, muscle contraction, cell growth and chemotaxis (Suzuki *et al.*, 1997).

In summary, a system was established for reproducibly inducing arrhythmia (evident as asynchronous contractile activity) in isolated cells, which was significantly delayed in cells isolated from rats supplemented with FO. FO supplementation did not alter basal fluidity, intracellular ROS or Ca^{2+} levels; however, an increase in the gene expression levels of the antioxidant enzymes was measured in the myocardium of rats fed FO. Following exposure to ROS, an increase in fluidity, and changes in Ca^{2+} handling properties was demonstrated. These findings are consistent with the fact that cardiomyocytes are highly adaptable to changes in their milieu and can respond in many ways to ensure maintenance of cellular homeostasis. However, these results also demonstrate that while FO supplementation does not alter various basal physiological parameters, myocytes isolated from rats fed FO do appear to be better able to tolerate subsequent ROS challenges. For example, the basal $[Ca^{2+}]_i$ was not different between SF and FO cells, however, in response to H_2O_2 addition, the cells isolated from FO-fed rats were able to reduce both systolic and diastolic $[Ca^{2+}]_i$, to prevent Ca^{2+} overload. Similarly, bulk membrane fluidity was not altered following FO feeding despite an enrichment of the myocardial membranes with n-3 PUFAs. However after ROS addition, an increase in fluidity was observed, which might have contributed to a mechanism whereby n-3 PUFAs were liberated from the membrane phospholipids in order to exert beneficial effects.

7.4 Future Directions

The results of this thesis have provided some novel and interesting findings, and confirm the pleiotropic effects of the n-3 PUFAs. However, many unanswered questions remain. Many aspects that this thesis touched upon, should be further addressed. Firstly, it would be practical to extend the molecular biology aspects to further identify the mechanisms by which FO (itself highly susceptible to oxidation), paradoxically protects cells from ROS. In particular, the preliminary results of the microarray experiments warrant further investigation. Although it was not possible in the present study to investigate the effect of dietary FO supplementation on gene expression using DNA microarray technology in great detail, this technology has enormous potential to rapidly screen differential gene expression patterns for a large number of genes. Genes that are indicated as being altered following lipid supplementation by such techniques could then be studied in detail using quantitative PCR techniques. It has already been reported using colon cancer cells, that 48 hr incubation with DHA altered the expression of several transcription factors as assessed by oligonucleotide arrays (Narayanan *et al.*, 2001). That the microarray analysis and real-time PCR techniques used in the present study both demonstrated up regulation of antioxidant defences by FO was a positive finding, however, the microarray analysis should be investigated with greater rigor in future studies. Further, the results of the real-time PCR studies may be strengthened by either using another housekeeping gene such as β-actin or by refining the technique by using the more specific TaqMan® assay with two primers and a probe. Normalisation to an appropriate housekeeping gene may further clarify the effects of dietary FO on antioxidant enzyme gene expression levels. It would also be advantageous to determine the protein levels and enzyme activities of SOD, CAT and GPX in order to better delineate their role in the action of the n-3 PUFAs on cellular antioxidant status and protection from ROS. The molecular biology investigations could

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also be extended to study the effect of dietary FO supplementation on the genes involved in Ca^{2+} homeostasis, for example the sodium-calcium exchanger, the SR Ca^{2+} release channel and the SR Ca^{2+}-ATPase. It would also be worthwhile to analyse the myocardial antioxidant gene expression in FO fed rats following a period of *in situ* CAL to determine whether there is an association between antioxidant enzyme gene expression and protection from reperfusion arrhythmias. Indeed, it would be helpful to compare the effects of *in situ* CAL, regional ischaemia induced by CAL in isolated hearts, and GZFI-R type protocols to further understand the mechanisms of reperfusion injury and the actions of the n-3 PUFAs.

Clearly the present findings, although interesting, can have no physiological relevance until they are reproduced in human cells or in clinical trials. The ROS-induced arrhythmia assay developed in this thesis could be used not only to test the possible protective effects of other compounds on cellular arrhythmias, but also for testing the effects of compounds, including fatty acids on human-derived cells. The advantage of this cardiomyocyte system is that small quantities of compounds can be tested, and confounding effects of nerves, hormones and other factors in the blood are removed.

CHAPTER EIGHT

Appendices
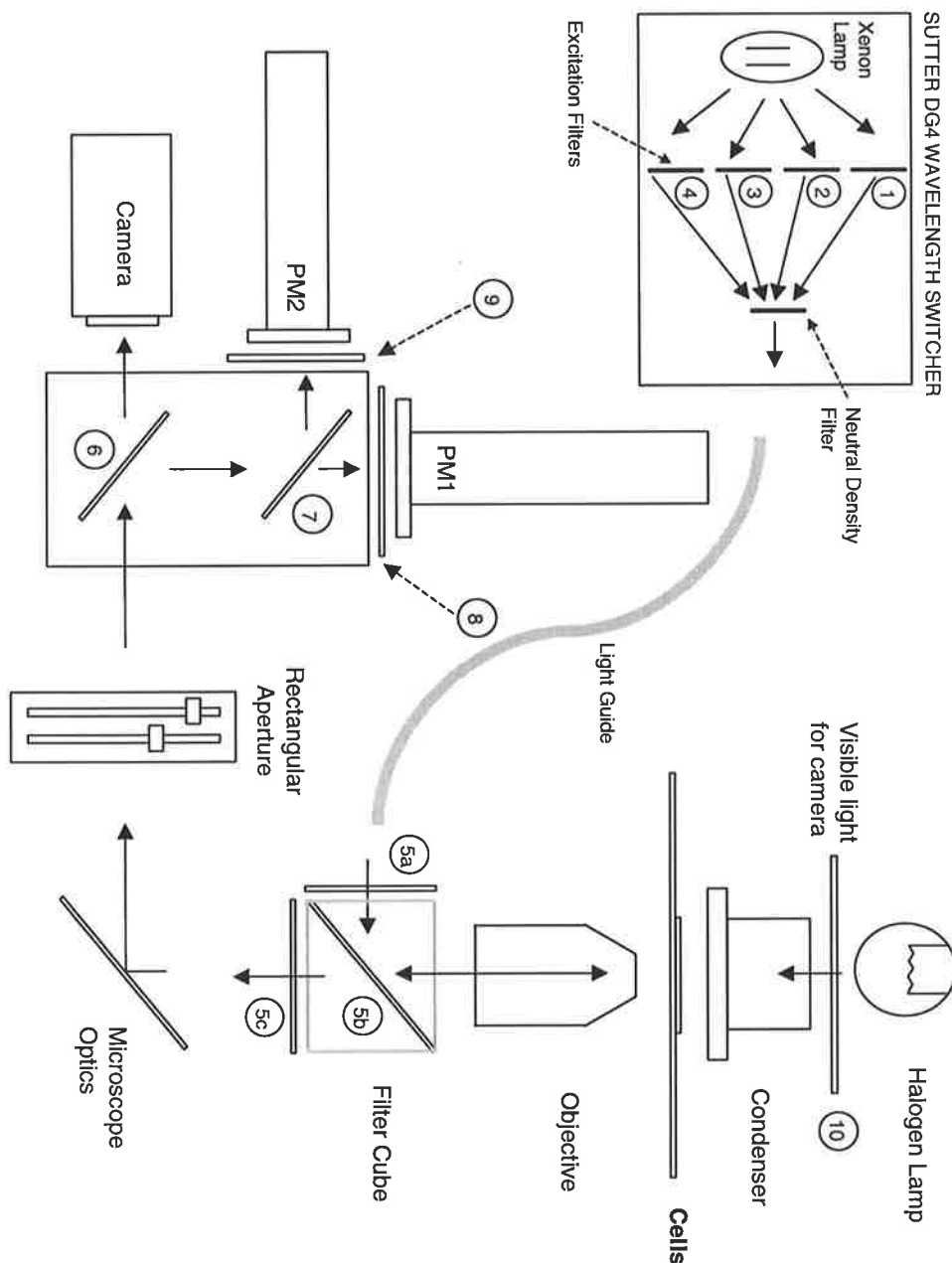
Appendix 9.1: Light Path and Filter Locations for the Fluorescent Microscope.
Filter numbers 1 – 4 refer to the excitation filters installed in the Sutter DG4 Wavelength Switcher (SDR Clinical Technology, Sydney, NSW, Australia). Light from the Switcher's internal UV lamp was directed through one of these filters upon program control. Positions 5a, b and c refer to components of the filter cube, fitted in a slider below the objective turret. A specific filter cube was used for each probe. Component 6 is a dichroic mirror mounted on the photomultiplier block, which separates emission wavelengths for detection by the photomultipliers from red light used to image the cells on the video camera. Component 7 is another dichroic mirror (which was not used in any of the studies described in this thesis). Components 8 and 9 are interference filters mounted immediately in front of each of the two photomultipliers to ensure that each photomultiplier receives only the relevant emission wavelength, minimising the effects of stray light. Component number 10 was a low-pass filter inserted into the visible light path before the microscope condenser, to provide illumination (red) for viewing the myocytes by eye or by the video camera. At low levels of illumination, this signal did not interfere with the fluorescence signals from the cells.

```
   1 gagcagacgc gcggctgcta gcgaacggcc gtgttctgag gagagcagcg gtcgtgggcg
  61 cctcagcaat gttgtgtcgg gcggcgtgca gcgcgggcag aagactgggc cccgcggcca
 121 gtaccgcggg ctcccggcac aagcacagcc tccctgacct gccttacgac tatggcgcgc
 181 tggagccgca cattaacgcg cagatcatgc agctgcacca cagcaagcac cacgcgacct
 241 acgtgaacaa tctgaacgtc accgaggaga agtaccacga ggcgctggcc aagggagatg
 301 ttacaactca ggttgctctt cagcctgcac tgaagttcaa tggcgggggc catatcaatc
 361 acagcatttt ctggacaaac ctgagcccta agggtggtgg agaacccaaa ggagagttgc
 421 tggaggctat caagcgtgac tttgggtctt ttgagaagtt taaggagaaa ctgacagctg
 481 tgtctgtggg agtccaaggt tcaggctggg gctggcttgg cttcaataag gagcaaggtc
 541 gcttacagat tgccgcctgc tctaatcagg acccactgca aggaaccaca ggccttattc
 601 cactgctggg gattgatgtg tgggagcacg cttactatct tcagtataaa aacgtcagac
 661 ctgactatct gaaagccatt tggaatgtaa tcaactggga gaatgttagc caaagataca
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 781 gtagtagtgt agagcattgc agcactgtgg ctgagctgtt gtaatcttca ttgatgccta
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1201 tatttatggc cacagtttca cagaaacatc atttttcac ttgaaacgtg taactaggct
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1441 ctattaaaac cgactgtgta ttgtatgaaa gtgctcaaga tggacaaagt at
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Appendix 9.2: mRNA sequence for Rattus norvegicus Mn-SOD.
Accession: NM_017051. Reference: Ho,Y.S., Howard, A.J. and Crapo, J.D. (1991) Molecular structure of a functional rat gene for manganese-containing superoxide dismutase *Am. J. Respir. Cell Mol. Biol.* 4 (3), 278-286.

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 121 agcagtggaa ggagcagcgg gcccctcaga aacccgatgt cctgaccacc ggaggcggga
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 241 aagatgtggt tttcaccgac gagatggcac actttgacag agagcggatt cctgagagag
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 361 gatactccaa ggcaaaggtg tttgagcata ttgggaagag gactcctatt gccgtccgat
 421 tctccacagt cgctggagag tcaggctcag ctgacacagt tcgtgaccct cgtgggtttg
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 721 atggctatgg ctcacacacc ttcaagctgg ttaatgcgaa tggagaggca gtgtactgca
 781 agttccatta caagactgac cagggcatca aaaacttgcc tgttgaagag gcaggaagac
 841 ttgcacagga gacccggat tatggcctcc gagatctttt caatgccatc gccagtggca
 901 attacccatc ctggactttt tacatccagg tcatgacttt caaggaggca gaaaccttcc
 961 catttaatcc atttgacctg accaaggttt ggcctcacaa ggactaccct cttataccag
1021 ttggcaaact ggtcttaaac agaaatcctg ctaattattt tgctgaagtt gaacagatgg
1081 cttttgaccc aagcaacatg ccccctggca ttgagcccag cccggacaag atgctccagg
1141 gccgcctttt tgcttaccca gacactcacc gccaccgcct gggaccaaac tatctgcaga
1201 tacctgtgaa ctgtccctac cgtgctcgcg tggccaacta ccagcgcgat ggccccatgt
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1381 acagtgctaa tgaagacaac gtcactcagg tgcggacatt ctatacgaag gtgttgaatg
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Appendix 9.3: mRNA sequence for Rattus Norvegicus Catalase.
Accession: NM_012520. Reference: Furuta, S., Hayashi, H., Hijikata, M., Miyazawa, S., Osumi, T. and Hashimoto, T. (1986) Complete nucleotide sequence of cDNA and deduced amino acid sequence of rat liver catalase. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 313-317.

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Appendix 9.4: mRNA sequence for Rattus Norvegicus Glutathione Peroxidase.

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Appendix 9.5: mRNA sequence for Rattus Norvegicus 18s rRNA.
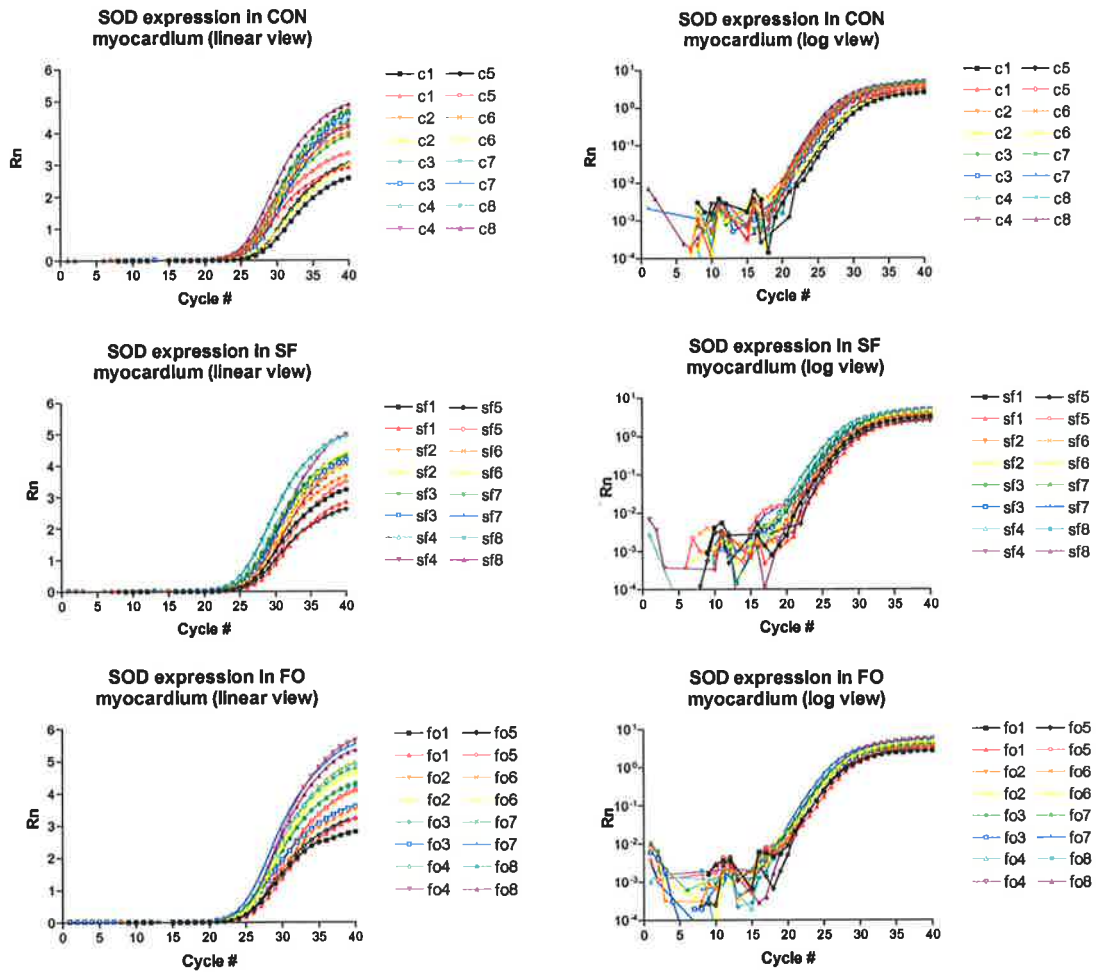
Appendix 9.6: Amplification Curves for SOD in Rat Myocardium.
Raw Data for CON, SF and FO Groups.
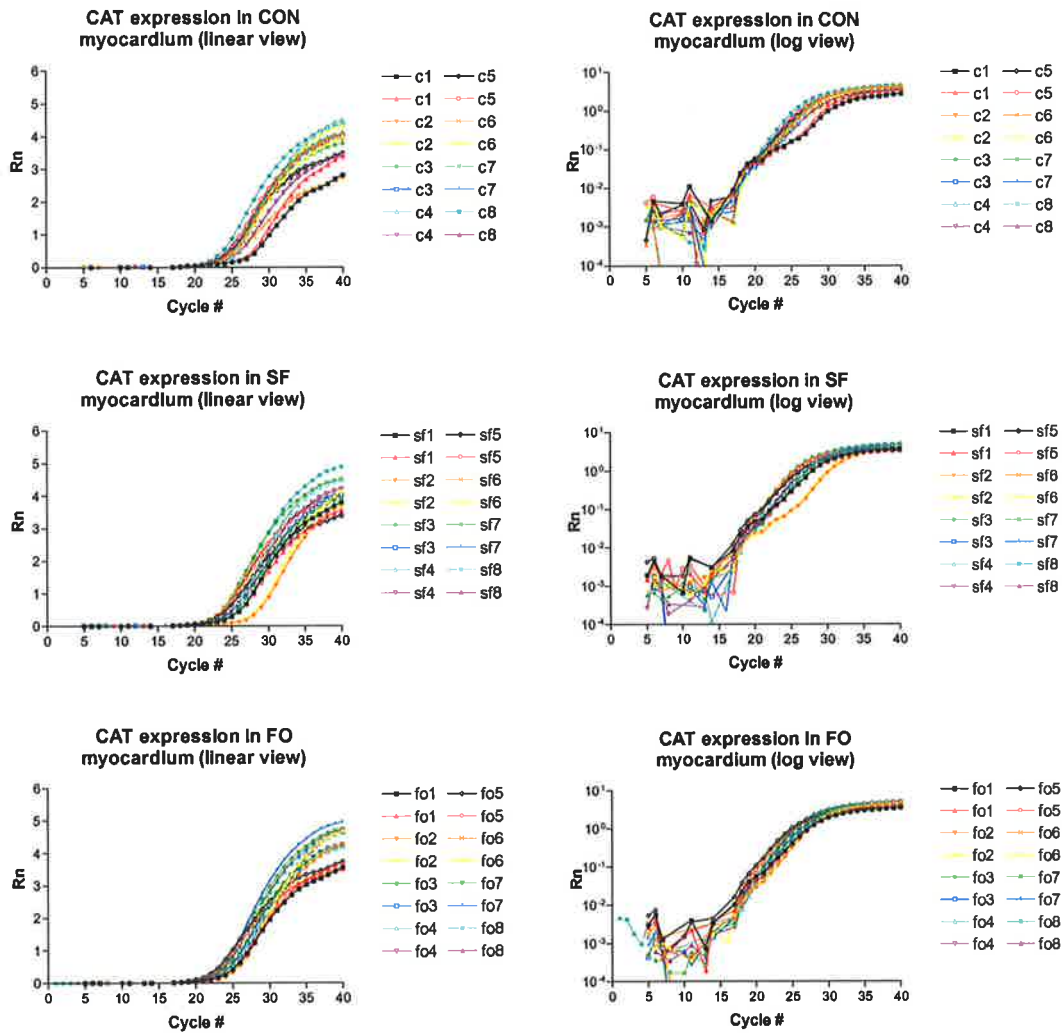
Appendix 9.7: Amplification Curves for CAT in Rat Myocardium.
Raw Data for CON, SF and FO Groups.
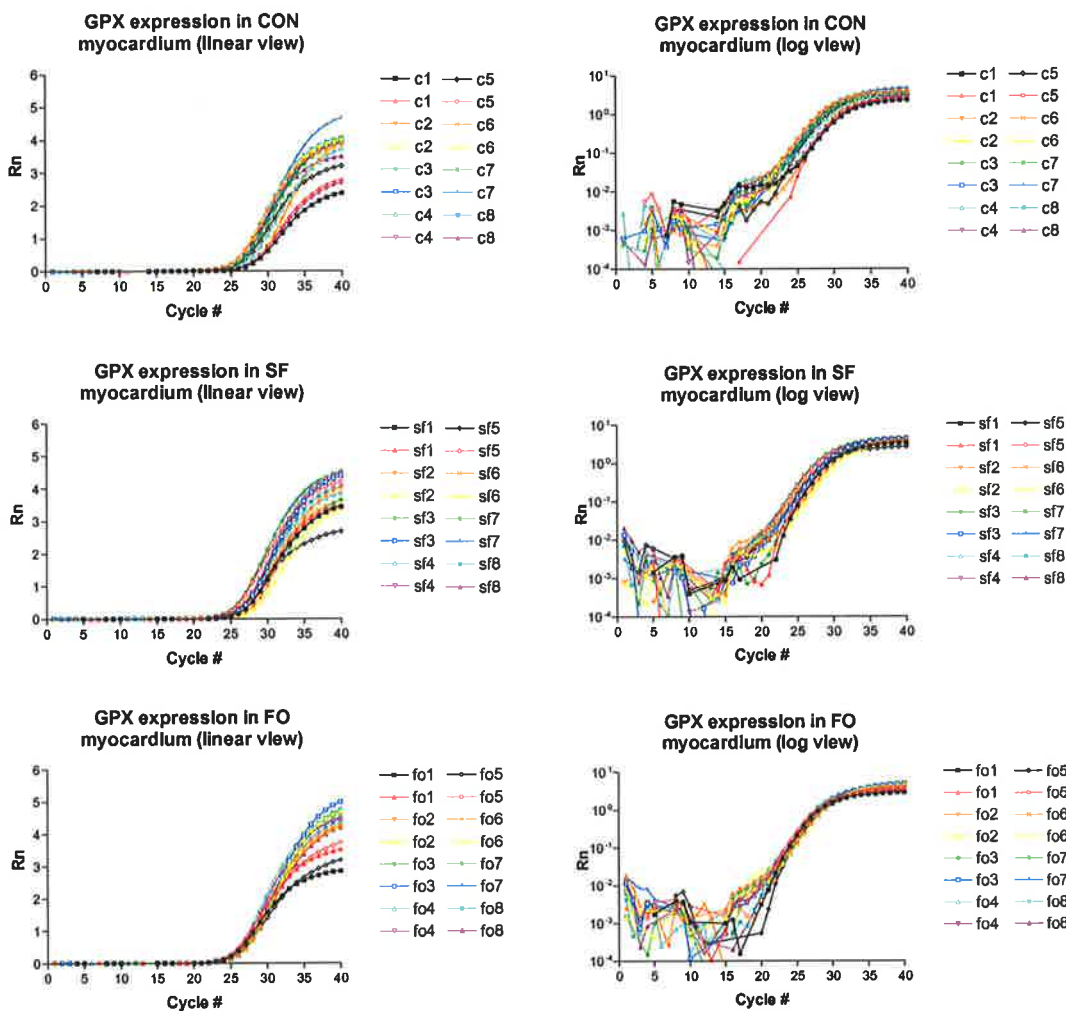
Appendix 9.8: Amplification Curves for GPX in Rat Myocardium.
Raw Data for CON, SF and FO Groups.

CHAPTER NINE

References

Abete, P., Napoli, C., Santoro, G., Ferrara, N., Tritto, I., Chiariello, M., Rengo, F. & Ambrosio, G. (1999). Age-related decrease in cardiac tolerance to oxidative stress. *J. Mol. Cell Cardiol.* **31**, 227-236.

Aiello, E. A., Jabr, R. I. & Cole, W. C. (1995). Arrhythmia and delayed recovery of cardiac action potential during reperfusion after ischemia. Role of oxygen radical-induced no-reflow phenomenon. *Circ. Res.* **77**, 153-162.

AIHW. Chronic diseases and associated risk factors in Australia 2001. Australian Institute of Health and Welfare. AIHW Cat. No. PHE 33, http://www.aihw.gov.au/publications/phe/cdarfa01/index.html. 2001a.

AIHW. Heart, Stroke and Vascular Diseases- Australian facts 2001. Australian Institute of Health and Welfare. AIHW Cat. No. CVD 13. 2001b. National Heart Foundation of Australia, and National Stroke Foundation of Australia.

al Makdessi, S., Brandle, M., Ehrt, M., Sweidan, H. & Jacob, R. (1995). Myocardial protection by ischemic preconditioning: the influence of the composition of myocardial phospholipids. *Mol. Cell Biochem.* **145**, 69-73.

al Makdessi, S., Sweidan, H. & Jacob, R. (1994). n-3 versus n-6 fatty acid incorporation into the phospholipids of rat heart sarcolemma. A comparative study of four different oil diets. *J. Mol. Cell. Cardiol* **26**, 23-29.

Alexander-North, L. S., North, J. A., Kiminyo, K. P., Buettner, G. R. & Spector, A. A. (1994). Polyunsaturated fatty acids increase lipid radical formation induced by oxidant stress in endothelial cells. *J. Lipid Res.* **35**, 1773-1785.

Anderson, K. E., Du, X. J., Sinclair, A. J., Woodcock, E. A. & Dart, A. M. (1996). Dietary fish oil prevents reperfusion Ins(1,4,5)P3 release in rat heart: possible antiarrhythmic mechanism. *Am. J. Physiol.* **271**, H1483-90.

Ando, K., Nagata, K., Beppu, M., Kikugawa, K., Kawabata, T., Hasegawa, K. & Suzuki, M. (1998). Effect of n-3 fatty acid supplementation on lipid peroxidation and protein aggregation in rat erythrocyte membranes. *Lipids.* **33**, 505-512.

Ando, K., Nagata, K., Yoshida, R., Kikugawa, K. & Suzuki, M. (2000). Effect of n-3 polyunsaturated fatty acid supplementation on lipid peroxidation of rat organs. *Lipids.* **35**, 401-407.

Applied Biosystems Technical Notes (1997). *User Bulletin #2* PE Applied Biosystems, Foster City, CA, USA.

Applied Biosystems Technical Notes (2001a). *DNA/RNA Real-Time Quantitative PCR* PE Applied Biosystems, Foster City, CA, USA.

Applied Biosystems Technical Notes (2001b). *Sequence Detection Systems Quantitative Assay Design and Optimisation.* PE Applied Biosystems, Foster City, CA, USA.

Avula, C. P. & Fernandes, G. (1999). Modulation of antioxidant enzymes and apoptosis in mice by dietary lipids and treadmill exercise. *J. Clin. Immunol.* **19**, 35-44.

Bagchi, M., Prasad, M. R., Engelman, R. M. & Das, D. K. (1989). Effects of free radicals on the fluidity of myocardial membranes. *Free Radic. Res. Commun.* **7**, 375-380.

Bang, H. O. & Dyerberg, J. (1972). Plasma lipids and lipoproteins in Greenlandic west coast Eskimos. *Acta. Med. Scand.* **192**, 85-94.

Bang, H. O. & Dyerberg, J. (1980). The bleeding tendency in Greenland Eskimos. *Dan. Med. Bull.* **27**, 202-205.

Bang, H. O., Dyerberg, J. & Hjoorne, N. (1976). The composition of food consumed by Greenland Eskimos. *Acta. Med. Scand.* **200**, 69-73.

Bang, H. O., Dyerberg, J. & Nielsen, A. B. (1971). Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos. *Lancet.* **1**, 1143-1145.

Bang, H. O., Dyerberg, J. & Sinclair, H. M. (1980). The composition of the Eskimo food in north western Greenland. *Am. J. Clin. Nutr.* **33**, 2657-2661.

Barsacchi, R., Pelosi, G., Maffei, S., Baroni, M., Salvatore, L., Ursini, F., Verunelli, F. & Biagini, A. (1992). Myocardial vitamin E is consumed during cardiopulmonary bypass: indirect evidence of free radical generation in human ischemic heart. *Int. J. Cardiol.* **37**, 339-343.

Bass, D. A., Parce, J. W., Dechatelet, L. R., Szejda, P., Seeds, M. C. & Thomas, M. (1983). Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J. Immunol.* **130**, 1910-1917.

Bast, A., Haenen, G. R. & Doelman, C. J. (1991). Oxidants and antioxidants: state of the art. *Am. J. Med.* **91**, 2S-13S.

Bastiaanse, E. M., Hold, K. M. & Van der, L. A. (1997). The effect of membrane cholesterol content on ion transport processes in plasma membranes. *Cardiovasc. Res.* **33**, 272-283.

Beck, A., Heissler, D. & Duportail, G. (1993). Influence of the length of the spacer on the partitioning properties of amphiphilic fluorescent membrane probes. *Chem. Phys. Lipids* **66**, 135-142.

Bellisola, G., Galassini, S., Moschini, G., Poli, G., Perona, G. & Guidi, G. (1992). Selenium and glutathione peroxidase variations induced by polyunsaturated fatty acids oral supplementation in humans. *Clin. Chim. Acta* **205**, 75-85.

Bello-Klein, A., Oliveira, A. R., Miranda, M. F., Irigoyen, M. C., Homem-de-Bittencourt, P. I., Jr., Llesuy, S. & Bello, A. A. (1997). Effect of trolox C on cardiac contracture induced by hydrogen peroxide. *Braz. J Med Biol. Res.* **30**, 1337-1342.

Bernier, M., Hearse, D. J. & Manning, A. S. (1986). Reperfusion-induced arrhythmias and oxygen-derived free radicals. Studies with "anti-free radical" interventions and a free radical-generating system in the isolated perfused rat heart. *Circ. Res.* **58**, 331-340.

Billman, G. E., Hallaq, H. & Leaf, A. (1994). Prevention of ischemia-induced ventricular fibrillation by omega 3 fatty acids. *Proc. Natl. Acad. Sci. USA.* **91**, 4427-4430.

Bligh, E. C. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917.

Bolli, R. (1988). Oxygen-derived free radicals and postischemic myocardial dysfunction ("stunned myocardium"). *J. Am. Coll. Cardiol.* **12**, 239-249.

Bolli, R., Jeroudi, M. O., Patel, B. S., Aruoma, O. I., Halliwell, B., Lai, E. K., McCay & PB. (1989a). Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "stunning" is a manifestation of reperfusion injury. *Circ. Res.* **65**, 607-622.

Bolli, R., Jeroudi, M. O., Patel, B. S., DuBose, C. M., Lai, E. K., Roberts, R., McCay & PB. (1989b). Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. *Proc. Natl. Acad. Sci. USA.* **86**, 4695-4699.

Bonnefont-Rousselot, D., Motta, C., Khalil, A. O., Sola, R., La Ville, A. E., Delattre, J. & Gardes-Albert, M. (1995). Physicochemical changes in human high-density lipoproteins (HDL) oxidized by gamma radiolysis-generated oxyradicals. Effect on their cholesterol effluxing capacity. *Biochim. Biophys. Acta* **1255**, 23-30.

Braunwald, E. (1985). The aggressive treatment of acute myocardial infarction. *Circulation* **71**, 1087-1092.

Braunwald, E. & Kloner, R. A. (1985). Myocardial reperfusion: a double-edged sword? *J. Clin. Invest.* **76**, 1713-1719.

Burr, M. L., Fehily, A. M., Gilbert, J. F., Rogers, S., Holliday, R. M., Sweetnam, P. M., Elwood, P. C. & Deadman, N. M. (1989). Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet.* **334**, 757-761.

Burton, K. P., McCord, J. M. & Ghai, G. (1984). Myocardial alterations due to free-radical generation. *Am. J. Physiol.* **246**, H776-83.

Burton, K. P., Morris, A. C., Massey, K. D., Buja, L. M. & Hagler, H. K. (1990). Free radicals alter ionic calcium levels and membrane phospholipids in cultured rat ventricular myocytes. *J. Mol. Cell. Cardiol* **22**, 1035-1047.

Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **25**, 169-193.

Byler, R. M., Sherman, N. A., Wallner, J. S. & Horwitz, L. D. (1994). Hydrogen peroxide cytotoxicity in cultured cardiac myocytes is iron dependent. *Am. J. Physiol.* **266**, H121-7.

Camandola, S., Leonarduzzi, G., Musso, T., Varesio, L., Carini, R., Scavazza, A., Chiarpotto, E., Baeuerle, P. A. & Poli, G. (1996). Nuclear factor kB is activated by arachidonic acid but not by eicosapentaenoic acid. *Biochem. Biophys. Res. Commun.* **229**, 643-647.

Carter, W. O., Narayanan, P. K. & Robinson, J. P. (1994). Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J. Leukoc. Biol.* **55**, 253-258.

Cathcart, R., Schwiers, E. & Ames, B. N. (1983). Detection of picomole levels of hydroperoxides using a fluorescent dichlorofluorescein assay. *Anal. Biochem.* **134**, 111-116.

Cavarocchi, N. C., England, M. D., Schaff, H. V., Russo, P., Orszulak, T. A., Schnell, W. A., Jr., O'Brien, J. F. & Pluth, J. R. (1986). Oxygen free radical generation during cardiopulmonary bypass: correlation with complement activation. *Circulation* **74**, III130-III133.

Chan, Y. L., Gutell, R., Noller, H. F. & Wool, I. G. (1984). The nucleotide sequence of a rat 18 S ribosomal ribonucleic acid gene and a proposal for the secondary structure of 18 S ribosomal ribonucleic acid. *J. Biol. Chem.* **259**, 224-230.

Chandrasekar, B., Colston, J. T., Geimer, J., Cortez, D. & Freeman, G. L. (2000). Induction of nuclear factor kappaB but not kappaB-responsive cytokine expression during myocardial reperfusion injury after neutropenia. *Free Radic. Biol. Med* **28**, 1579-1588.

Chandrasekar, B. & Fernandes, G. (1994). Decreased pro-inflammatory cytokines and increased antioxidant enzyme gene expression by omega-3 lipids in murine lupus nephritis. *Biochem. Biophys. Res. Commun.* **200**, 893-898.

Chen, J. J. & Yu, B. P. (1994). Alterations in mitochondrial membrane fluidity by lipid peroxidation products. *Free. Radic. Biol. Med.* **17**, 411-418.

Chen, Z., Oberley, T. D., Ho, Y., Chua, C. C., Siu, B., Hamdy, R. C., Epstein, C. J. & Chua, B. H. (2000). Overexpression of CuZnSOD in coronary vascular cells attenuates myocardial ischemia/reperfusion injury. *Free Radic. Biol. Med.* **29**, 589-596.

Chen, Z., Siu, B., Ho, Y. S., Vincent, R., Chua, C. C., Hamdy, R. C. & Chua, B. H. (1998). Overexpression of MnSOD protects against myocardial ischemia/reperfusion injury in transgenic mice. *J. Mol. Cell Cardiol.* **30**, 2281-2289.

Chevalier, A., Demaison, L., Grynberg, A. & Athias, P. (1990). Influence of phospholipid polyunsatured fatty acid composition on some metabolic disorders induced in rat cardiomyocytes by hypoxia and reoxygenation. *J. Mol. Cell. Cardiol* **22**, 1177-1186.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Clague, J. R. & Langer, G. A. (1994). The pathogenesis of free radical-induced calcium leak in cultured rat cardiomyocytes. *J. Mol. Cell Cardiol.* **26**, 11-21.

Clamp, A. G., Ladha, S., Clark, D. C., Grimble, R. F. & Lund, E. K. (1997). The influence of dietary lipids on the composition and membrane fluidity of rat hepatocyte plasma membrane. *Lipids.* **32**, 179-184.

Clarke, S. D. & Jump, D. B. (1994). Dietary polyunsaturated fatty acid regulation of gene transcription. *Annu. Rev. Nutr.* **14**, 83-98.

Clarke, S. D. & Jump, D. B. (1996). Polyunsaturated fatty acid regulation of hepatic gene transcription. *Lipids.* **31 Suppl**, S7-11.

Cocco, T., Di Paola, M., Papa, S. & Lorusso, M. (1999). Arachidonic acid interaction with the mitochondrial electron transport chain promotes reactive oxygen species generation. *Free Radic. Biol. Med* **27**, 51-59.

Connor, W. E. (2000). Importance of n-3 fatty acids in health and disease. *Am. J. Clin. Nutr.* **71**, 171S-175S.

Corr, P. B. & Witkowski, F. X. (1983). Potential electrophysiologic mechanisms responsible for dysrhythmias associated with reperfusion of ischemic myocardium. *Circulation* **68**, I16-24.

Courtois, M., Maupoil, V., Fantini, E., Durot, I., Javouhey-Donzel, A., Athias, P, Grynberg, A. & Rochette, L. (1998). Correlation between direct ESR spectroscopic measurements and electromechanical and biochemical assessments of exogenous free radical injury in isolated rat cardiac myocytes. *Free. Radic. Biol. Med.* **24**, 121-131.

Csonka, C., Pataki, T., Kovacs, P., Muller, S. L., Schroeter, M. L., Tosaki, A. & Blasig, I. E. (2000). Effects of oxidative stress on the expression of antioxidative defense enzymes in spontaneously hypertensive rat hearts. *Free Radic. Biol. Med* **29**, 612-619.

Curb, J. D. & Reed, D. M. (1985). Fish consumption and mortality from coronary heart disease. *New. Engl. J. Med.* **313**, 821.

Daleau, P. (1999). Lysophosphatidylcholine, a metabolite which accumulates early in myocardium during ischemia, reduces gap junctional coupling in cardiac cells. *J. Mol. Cell. Cardiol.* **31**, 1391-1401.

de Deckere, E. A., Korver, O., Verschuren, P. M. & Katan, M. B. (1998). Health aspects of fish and n-3 polyunsaturated fatty acids from plant and marine origin. *Eur. J Clin. Nutr.* **52**, 749-753.

de Jonge, H. W., Dekkers, D. H., Bastiaanse, E. M., Bezstarosti, K., van der Laarse, A & Lamers, J. M. (1996). Eicosapentaenoic acid incorporation in membrane phospholipids modulates receptor-mediated phospholipase C and membrane fluidity in rat ventricular myocytes in culture. *J. Mol. Cell. Cardiol* **28**, 1097-1108.

Delbridge, L. M., Bassani, J. W. & Bers, D. M. (1996). Steady-state twitch Ca^{2+} fluxes and cytosolic Ca^{2+} buffering in rabbit ventricular myocytes. *Am. J. Physiol* **270**, C192-C199.

de Lorgeril, M., Renaud, S., Mamelle, N., Salen, P., Martin, J. L., Monjaud, I., Guidollet, J., Touboul, P. & Delaye, J. (1994). Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. *Lancet.* **343**, 1454-1459.

de Urquiza, A. M., Liu, S., Sjoberg, M., Zetterstrom, R. H., Griffiths, W., Sjovall, J. & Perlmann, T. (2000). Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science.* **290**, 2140-2144.

Demaison, L., Moreau, D., Vergely-Vandriesse, C., Gregoire, S., Degois, M. & Rochette, L. (2001). Effects of dietary polyunsaturated fatty acids and hepatic steatosis on the functioning of isolated working rat heart under normoxic conditions and during post-ischemic reperfusion. *Mol. Cell Biochem.* **224**, 103-116.

Dhalla, N. S., Temsah, R. M. & Netticadan, T. (2000). Role of oxidative stress in cardiovascular diseases. *J. Hypertens.* **18**, 655-673.

Di Virgilio, F., Steinberg, T. H. & Silverstein, S. C. (1990). Inhibition of Fura-2 sequestration and secretion with organic anion transport blockers. *Cell. Calcium.* **11**, 57-62.

Diaz, M. N., Frei, B., Vita, J. A. & Keaney, J. F., Jr. (1997). Antioxidants and atherosclerotic heart disease. *New. Engl. J. Med.* **337**, 408-416.

Didion, S. P., Hathaway, C. A. & Faraci, F. M. (2001). Superoxide levels and function of cerebral blood vessels after inhibition of CuZn-SOD. *Am. J. Physiol Heart Circ. Physiol* **281**, H1697-H1703.

Dolecek, T. A. (1992). Epidemiological evidence of relationships between dietary polyunsaturated fatty acids and mortality in the multiple risk factor intervention trial. *Proc. Soc. Exp. Biol. Med.* **200**, 177-182.

Duan, J. & Moffat, M. P. (1992). Potential cellular mechanisms of hydrogen peroxide-induced cardiac arrhythmias. *J. Cardiovasc. Pharmacol.* **19**, 593-601.

Duranteau, J., Chandel, N. S., Kulisz, A., Shao, Z. & Schumacker, P. T. (1998). Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. *J. Biol. Chem.* **273**, 11619-11624.

Durot, I., Athias, P., Oudot, F. & Grynberg, A. (1997). Influence of phospholipid long chain polyunsaturated fatty acid composition on neonatal rat cardiomyocyte function in physiological conditions and during glucose-free hypoxia- reoxygenation. *Mol. Cell. Biochem.* **175**, 253-262.

Dyerberg, J., Bang, H. O. & Hjorne, N. (1975). Fatty acid composition of the plasma lipids in Greenland Eskimos. *Am. J. Clin. Nutr.* **28**, 958-966.

Fernandes, G., Troyer, D. A. & Jolly, C. A. (1998). The effects of dietary lipids on gene expression and apoptosis. *Proc. Nutr. Soc.* **57**, 543-550.

Fournier, A., Fantini, E., Sergiel, J. P., Athias, P. & Grynberg, A. (1995). Influence of the phospholipid content in docosahexaenoic acid on electrophysiology and contraction of rat heart muscle cells. *Cardioscience.* **6**, 71-78.

Freeman, B. A. & Crapo, J. D. (1982). Biology of disease: free radicals and tissue injury. *Lab. Invest.* **47**, 412-426.

Fu, Y., Cheng, W. H., Porres, J. M., Ross, D. A. & Lei, X. G. (1999). Knockout of cellular glutathione peroxidase gene renders mice susceptible to diquat-induced oxidative stress. *Free Radic. Biol. Med* **27**, 605-611.

Furuta, S., Hayashi, H., Hijikata, M., Miyazawa, S., Osumi, T. & Hashimoto, T. (1986). Complete nucleotide sequence of cDNA and deduced amino acid sequence of rat liver catalase. *Proc. Natl. Acad. Sci. U. S. A* **83**, 313-317.

Gabriel, C., Justicia, C., Camins, A. & Planas, A. M. (1999). Activation of nuclear factor-kappaB in the rat brain after transient focal ischemia. *Brain Res. Mol. Brain Res.* **65**, 61-69.

Gardner, H. W. (1989). Oxygen radical chemistry of polyunsaturated fatty acids. *Free Radic. Biol. Med* **7**, 65-86.

Garrido, A., Garrido, F., Guerra, R. & Valenzuela, A. (1989). Ingestion of high doses of fish oil increases the susceptibility of cellular membranes to the induction of oxidative stress. *Lipids.* **24**, 833-835.

Gauduel, Y. & Duvelleroy, M. A. (1984). Role of oxygen radicals in cardiac injury due to reoxygenation. *J. Mol. Cell. Cardiol* **16**, 459-470.

Goldberg, S., Greenspon, A. J., Urban, P. L., Muza, B., Berger, B., Walinsky, P. & Maroko, P. R. (1983). Reperfusion arrhythmia: a marker of restoration of antegrade flow during intracoronary thrombolysis for acute myocardial infarction. *Am. Heart. J.* **105**, 26-32.

Goldhaber, J. I., Ji, S., Lamp, S. T. & Weiss, J. N. (1989). Effects of exogenous free radicals on electromechanical function and metabolism in isolated rabbit and guinea pig ventricle. Implications for ischemia and reperfusion injury. *J. Clin. Invest* **83**, 1800-1809.

Goldhaber, J. I. & Weiss, J. N. (1992). Oxygen free radicals and cardiac reperfusion abnormalities. *Hypertension.* **20**, 118-127.

Gonzalez-Flecha, B. & Boveris, A. (1995). Mitochondrial sites of hydrogen peroxide production in reperfused rat kidney cortex. *Biochim. Biophys. Acta.* **1243**, 361-366.

Granger, D. N., Rutili, G. & McCord, J. M. (1981). Superoxide radicals in feline intestinal ischemia. *Gastroenterology* **81**, 22-29.

Graven, K. K., Troxler, R. F., Kornfeld, H., Panchenko, M. V. & Farber, H. W. (1994). Regulation of endothelial cell glyceraldehyde-3-phosphate dehydrogenase expression by hypoxia. *J. Biol. Chem.* **269**, 24446-24453.

Grech, E. D., Bellamy, C. M., Jackson, M. J., Muirhead, R. A., Faragher, E. B. & Ramsdale, DR. (1994). Free-radical activity after primary coronary angioplasty in acute myocardial infarction. *Am. Heart. J.* **127**, 1443-1449.

Gross, R. W. (1992). Myocardial Phospholipiases A_2 and Their Membrane Substrates. *Trends Cardiovasc Med* **2**, 115-121.

Grove, D. S. (1999). Quantitative Real-Time Polymerase Chain Reaction for the Core Facility Using TaqMan and the Perkin-Elmer/Applied Biosystems Division 7700 Sequence Detector. *http://www. abrf. org/JBT/1999/March99/mar99grove. html.*

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450.

Hagve, T. A., Woldseth, B., Brox, J., Narce, M. & Poisson, J. P. (1998). Membrane fluidity and fatty acid metabolism in kidney cells from rats fed purified eicosapentaenoic acid or purified docosahexaenoic acid. *Scand. J. Clin. Lab Invest* **58**, 187-194.

Hallaq, H., Sellmayer, A., Smith, T. W. & Leaf, A. (1990). Protective effect of eicosapentaenoic acid on ouabain toxicity in neonatal rat cardiac myocytes. *Proc. Natl. Acad. Sci. USA.* **87**, 7834-7838.

Hallaq, H., Smith, T. W. & Leaf, A. (1992). Modulation of dihydropyridine-sensitive calcium channels in heart cells by fish oil fatty acids. *Proc. Natl. Acad. Sci. USA.* **89**, 1760-1764.

Halliwell, B. (1987). Oxidants and human disease: some new concepts. *FASEB. J.* **1**, 358-364.

Halliwell, B. (1989). Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *Br. J Exp. Pathol.* **70**, 737-757.

Halliwell, B. (1991). Reactive oxygen species in living systems: source, biochemistry, and role in human disease. *Am. J. Med.* **91**, 14S-22S.

Halliwell, B. (1996). Antioxidants in human health and disease. *Annu. Rev. Nutr.* **16**, 33-50.

Halliwell, B. & Gutteridge, J. M. (1986). Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* **246**, 501-514.

Halliwell, B. & Gutteridge, J. M. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. *Methods. Enzymol.* **186**, 1-85.

Harrison, S. N., Du, X. J., Arthur, J. F. & Woodcock, E. A. (2000). Activation of the $Na^{(+)}/H^{(+)}$ exchanger is required for reperfusion- induced Ins(1,4,5)P(3) generation. *J. Mol. Cell Cardiol.* **32**, 1851-1858.

Hashimoto, M., Hossain, M. S., Shimada, T., Yamasaki, H., Fujii, Y. & Shido, O. (2001). Effects of docosahexaenoic acid on annular lipid fluidity of the rat bile canalicular plasma membrane. *J. Lipid Res.* **42**, 1160-1168.

Hashimoto, M., Hossain, S., Yamasaki, H., Yazawa, K. & Masumura, S. (1999). Effects of eicosapentaenoic acid and docosahexaenoic acid on plasma membrane fluidity of aortic endothelial cells. *Lipids.* **34**, 1297-1304.

Hatam, L. J. & Kayden, H. J. (1979). A high-performance liquid chromatographic method for the determination of tocopherol in plasma and cellular elements of the blood. *J. Lipid Res.* **20**, 639-645.

Hattori, Y., Matsuda, N., Kimura, J., Ishitani, T., Tamada, A., Gando, S., Kemmotsu, O. & Kanno, M. (2000). Diminished function and expression of the cardiac $Na^{+}-Ca^{2+}$ exchanger in diabetic rats: implication in Ca^{2+} overload. *J. Physiol* **527 Pt 1**, 85-94.

Haworth, R. A., Hunter, D. R. & Berkoff, H. A. (1981). Contracture in isolated adult rat heart cells. Role of Ca^{2+}, ATP, and compartmentation. *Circ. Res.* **49**, 1119-1128.

Hayashi, M., Nasa, Y., Tanonaka, K., Sasaki, H., Miyake, R., Hayashi, J., Takeo & S. (1995). The effects of long-term treatment with eicosapentaenoic acid and docosahexaenoic acid on hypoxia/rexoygenation injury of isolated cardiac cells in adult rats. *J. Mol. Cell. Cardiol* **27**, 2031-2041.

Hearse, D. J. (1991). Reperfusion-induced injury: a possible role for oxidant stress and its manipulation. *Cardiovasc. Drugs Ther.* **5 Suppl 2**, 225-235.

Hertog, M. G., Feskens, E. J., Hollman, P. C., Katan, M. B. & Kromhout, D. (1993). Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet.* **342**, 1007-1011.

Hesketh, J. E., Vasconcelos, M. H. & Bermano, G. (1998). Regulatory signals in messenger RNA: determinants of nutrient-gene interaction and metabolic compartmentation. *Br. J. Nutr.* **80**, 307-321.

Hess, M. L. & Manson, N. H. (1984). Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. *J. Mol. Cell. Cardiol* **16**, 969-985.

Ho, Y. S. & Howard, A. J. (1992). Cloning and characterization of the rat glutathione peroxidase gene. *FEBS Lett.* **301**, 5-9.

Ho, Y. S., Howard, A. J. & Crapo, J. D. (1991). Molecular structure of a functional rat gene for manganese-containing superoxide dismutase. *Am. J. Respir. Cell Mol. Biol.* **4**, 278-286.

Ho, Y. S., Magnenat, J. L., Bronson, R. T., Cao, J., Gargano, M., Sugawara, M. & Funk, C. D. (1997). Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. *J. Biol. Chem.* **272**, 16644-16651.

Hock, C. E., Beck, L. D., Bodine, R. C. & Reibel, D. K. (1990). Influence of dietary n-3 fatty acids on myocardial ischemia and reperfusion. *Am. J. Physiol.* **259**, H1518-26.

Hoek, T. L. V., Shao, Z. H., Li, C. Q., Schumacker, P. T. & Becker, L. B. (1997). Mitochondrial electron transport can become a significant source of oxidative injury in cardiomyocytes. *J. Mol. Cell. Cardiol.* **29**, 2441-2450.

Horrobin, D. F. (1991). Is the main problem in free radical damage caused by radiation, oxygen and other toxins the loss of membrane essential fatty acids rather than the accumulation of toxic materials? *Med. Hypotheses* **35** , 23-26.

Ibrahim, W., Lee, U. S., Szabo, J., Bruckner, G. & Chow, C. K. (1999). Oxidative stress and antioxidant status in mouse kidney: effects of dietary lipid and vitamin e plus iron. *J. Nutr. Biochem.* **10**, 674-678.

Ibrahim, W., Lee, U. S., Yen, H. C., St Clair, D. K. & Chow, C. K. (2000). Antioxidant and oxidative status in tissues of manganese superoxide dismutase transgenic mice. *Free Radic. Biol. Med.* **28**, 397-402.

Igal, A. & Gomez Dumm, N. T. (1997). Dietary n-3 fatty acids influence the lipid composition and physical properties of liver microsomal membranes in diabetic rats. *Prostaglandins Leukot. Essent. Fatty Acids* **56**, 245-252.

Isensee, H. & Jacob, R. (1994). Differential effects of various oil diets on the risk of cardiac arrhythmias in rats. *J. Cardiovasc. Risk* **1**, 353-359.

Jackson, M. J. (1999). Free radicals in skin and muscle: damaging agents or signals for adaptation? *Proc. Nutr. Soc.* **58**, 673-676.

Jaeschke, H., Smith, C. V. & Mitchell, J. R. (1988). Reactive oxygen species during ischemia-reflow injury in isolated perfused rat liver. *J. Clin. Invest.* **81**, 1240-1246.

Jahangiri, A., Leifert, W. R., Patten, G. S. & McMurchie, E. J. (2000). Termination of asynchronous contractile activity in rat atrial myocytes by n-3 polyunsaturated fatty acids. *Mol. Cell. Biochem* **206**, 33-41.

Jennings, R. B. & Reimer, K. A. (1983). Factors involved in salvaging ischemic myocardium: effect of reperfusion of arterial blood. *Circulation* **68**, I25-36.

Jolly, C. A., Muthukumar, A., Avula, C. P., Troyer, D. & Fernandes, G. (2001). Life span is prolonged in food-restricted autoimmune-prone (NZB x NZW)F(1) mice fed a diet enriched with (n-3) fatty acids. *J. Nutr.* **131**, 2753-2760.

Josephson, R. A., Silverman, H. S. , Lakatta, E. G., Stern, M. D. & Zweier, J. L. (1991). Study of the mechanisms of hydrogen peroxide and hydroxyl free radical- induced cellular injury and calcium overload in cardiac myocytes. *J. Biol. Chem.* **266**, 2354-2361.

Joyeux, M., Ribuot, C., Bourlier, V., Verdetti, J., Durand, A., Richard, M. J., Godin-Ribuot, D. & Demenge, P. (1997). In vitro antiarrhythmic effect of prior whole body hyperthermia: implication of catalase. *J. Mol. Cell Cardiol.* **29**, 3285-3292.

Jump, D. B. (2002a). Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr. Opin. Lipidol.* **13**, 155-164.

Jump, D. B. (2002b). The Biochemistry of n-3 Polyunsaturated Fatty Acids. *J. Biol. Chem.* **277**, 8755-8758.

Jump, D. B. & Clarke, S. D. (1999). Regulation of gene expression by dietary fat. *Annu. Rev. Nutr.* **19**, 63-90.

Jump, D. B., Clarke, S. D., MacDougald, O. & Thelen, A. (1993). Polyunsaturated fatty acids inhibit S14 gene transcription in rat liver and cultured hepatocytes. *Proc. Natl. Acad. Sci. U. S. A* **90**, 8454-8458.

Jump, D. B., Thelen, A. & Mater, M. (1999). Dietary polyunsaturated fatty acids and hepatic gene expression. *Lipids.* **34 Suppl**, S209-S212.

Kaminishi, T., Matsuoka, T., Yanagishita, T. & Kako, K. J. (1989). Increase vs. decrease of calcium uptake by isolated heart cells induced by H2O2 vs. HOCl. *Am. J. Physiol.* **256**, C598-607.

Kanaya, N., Zakhary, D. R., Murray, P. A. & Damron, D. S. (1998). Differential effects of fentanyl and morphine on intracellular Ca^{2+} transients and contraction in rat ventricular myocytes. *Anesthesiology* **89**, 1532-1542.

Kane, K. A., Parratt, J. R. & Williams, F. M. (1984). An investigation into the characteristics of reperfusion-induced arrhythmias in the anaesthetized rat and their susceptibility to antiarrhythmic agents. *Br. J. Pharmacol.* **82**, 349-357.

Kaneko, M., Beamish, R. E. & Dhalla, N. S. (1989a). Depression of heart sarcolemmal Ca^{2+}-pump activity by oxygen free radicals. *Am. J. Physiol.* **256**, H368-74.

Kaneko, M., Elimban, V. & Dhalla, N. S. (1989b). Mechanism for depression of heart sarcolemmal Ca^{2+} pump by oxygen free radicals. *Am. J. Physiol.* **257**, H804-11.

Kang, J. X. & Leaf, A. (1995). Prevention and termination of beta-adrenergic agonist-induced arrhythmias by free polyunsaturated fatty acids in neonatal rat cardiac myocytes. *Biochem. Biophys. Res. Commun.* **208**, 629-636.

Kang, J. X. & Leaf, A. (1996a). Protective effects of free polyunsaturated fatty acids on arrhythmias induced by lysophosphatidylcholine or palmitoylcarnitine in neonatal rat cardiac myocytes. *Eur. J. Pharmacol.* **297**, 97-106.

Kang, J. X. & Leaf, A. (1996b). The cardiac antiarrhythmic effects of polyunsaturated fatty acids. *Lipids.* **31 Suppl**, S41-4.

Kang, J. X., Li, Y. & Leaf, A. (1997). Regulation of sodium channel gene expression by class I antiarrhythmic drugs and n - 3 polyunsaturated fatty acids in cultured neonatal rat cardiac myocytes. *Proc. Natl. Acad. Sci. USA.* **94**, 2724-2728.

Karliner, J. S., Honbo, N., Epstein, C. J., Xian, M., Lau, Y. F. & Gray, M. O. (2000). Neonatal mouse cardiac myocytes exhibit cardioprotection induced by hypoxic and pharmacologic preconditioning and by transgenic overexpression of human Cu/Zn superoxide dismutase. *J. Mol. Cell Cardiol.* **32**, 1779-1786.

Katsanidis, E. & Addis, P. B. (1999). Novel HPLC analysis of tocopherols, tocotrienols, and cholesterol in tissue. *Free Radic. Biol. Med.* **27**, 1137-1140.

Khalid, M. A. & Ashraf, M. (1993). Direct detection of endogenous hydroxyl radical production in cultured adult cardiomyocytes during anoxia and reoxygenation. Is the hydroxyl radical really the most damaging radical species? *Circ. Res.* **72**, 725-736.

Kim, M. S. & Akera, T. (1987). O2 free radicals: cause of ischemia-reperfusion injury to cardiac Na^{+}- K^{+}-ATPase. *Am. J. Physiol* **252**, H252-H257.

Kinsella, J. E., Lokesh, B. & Stone, R. A. (1990). Dietary n-3 polyunsaturated fatty acids and amelioration of cardiovascular disease: possible mechanisms. *Am. J. Clin. Nutr.* **52**, 1-28.

Kirshenbaum, L. A., Hill, M. & Singal, P. K. (1995). Endogenous antioxidants in isolated hypertrophied cardiac myocytes and hypoxia-reoxygenation injury. *J. Mol. Cell Cardiol.* **27**, 263-272.

Kotsanas, G., Delbridge, L. M. & Wendt, I. R. (2000). Stimulus interval-dependent differences in Ca^{2+} transients and contractile responses of diabetic rat cardiomyocytes. *Cardiovasc. Res.* **46**, 450-462.

Kourie, J. I. (1998). Interaction of reactive oxygen species with ion transport mechanisms. *Am. J. Physiol.* **275**, C1-24.

Koyama, T., Temma, K. & Akera, T. (1991). Reperfusion-induced contracture develops with a decreasing [Ca^{2+}]i in single heart cells. *Am. J. Physiol* **261**, H1115-H1122.

Kramer, J. K. (1980). Comparative studies on composition of cardiac phospholipids in rats fed different vegetable oils. *Lipids.* **15**, 651-660.

Kromann, N. & Green, A. (1980). Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. *Acta. Med. Scand.* **208**, 401-406.

Kromhout, D., Bosschieter, E. B. & de Lezenne Coulander, C. (1985). The inverse relation between fish consumption and 20-year mortality from coronary heart disease. *New. Engl. J. Med.* **312**, 1205-1209.

Kromhout, D., Feskens, E. J. & Bowles, C. H. (1995). The protective effect of a small amount of fish on coronary heart disease mortality in an elderly population. *Int. J. Epidemiol.* **24**, 340-345.

Kukreja, R. C. & Hess, M. L. (1992). The oxygen free radical system: from equations through membrane-protein interactions to cardiovascular injury and protection. *Cardiovasc. Res.* **26**, 641-655.

L'Abbe, M. R., Trick, K. D. & Beare-Rogers, J. L. (1991). Dietary (n-3) fatty acids affect rat heart, liver and aorta protective enzyme activities and lipid peroxidation. *J. Nutr.* **121**, 1331-1340.

Ladilov, Y., Schafer, C., Held, A., Schafer, M., Noll, T. & Piper, H. M. (2000). Mechanism of Ca^{2+}) overload in endothelial cells exposed to simulated ischemia. *Cardiovasc. Res.* **47**, 394-403.

Lai, C. C., Huang, W. H., Askari, A., Klevay, L. M. & Chiu, T. H. (1995). Expression of glutathione peroxidase and catalase in copper -deficient rat liver and heart. *J. Nutr. Biochem.* **6**, 256-262.

Lai, C. C., Peng, M., Huang, L., Huang, W. H. & Chiu, T. H. (1996). Chronic exposure of neonatal cardiac myocytes to hydrogen peroxide enhances the expression of catalase. *J. Mol. Cell. Cardiol* **28**, 1157-1163.

Lauber, R. P. & Sheard, N. F. (2001). The American Heart Association Dietary Guidelines for 2000: a summary report. *Nutr. Rev.* **59**, 298-306.

Leaf, A. & Weber, P. C. (1988). Cardiovascular effects of n-3 fatty acids. *New. Engl. J. Med.* **318**, 549-557.

Lee, J. Y., Sohn, K. H., Rhee, S. H. & Hwang, D. (2001). Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J. Biol. Chem.* **276**, 16683-16689.

Lee, T. H., Hoover, R. L., Williams, J. D., Sperling, R. I., Ravalese, J. 3., Spur, B. W., Robinson, D. R., Corey, E. J., Lewis, R. A. & Austen, K. F. (1985). Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. *New. Engl. J. Med.* **312**, 1217-1224.

Chapter Nine

Lees, R. S. (1990). Impact of Dietary Fat on Human Health. In *Omega-3 Fatty Acids in Health and Disease.*, eds. Lees, R. S. & Karel, M., Marcel Dekker Inc, New York.

Leibovitz, B. E., Hu, M. L. & Tappel, A. L. (1990). Lipid peroxidation in rat tissue slices: effect of dietary vitamin E, corn oil-lard and menhaden oil. *Lipids.* **25**, 125-129.

Leifert, W. R., Jahangiri, A. & McMurchie, E. J. (1999a). Antiarrhythmic fatty acids and antioxidants in animal and cell studies (Review). *J. Nutr. Biochem.* **10**, 252-267.

Leifert, W. R., Jahangiri, A. & McMurchie, E. J. (2000). Membrane fluidity changes are associated with the antiarrhythmic effects of docosahexaenoic acid in adult rat cardiomyocytes. *J. Nutr. Biochem.* **11**, 38-44.

Leifert, W. R., McMurchie, E. J. & Saint, D. A. (1999b). Inhibition of cardiac sodium currents in adult rat myocytes by n-3 polyunsaturated fatty acids. *J. Physiol.* **520**, 671-679.

Lentz, B. R. (1989). Membrane "fluidity" as detected by diphenylhexatriene probes. *Chem. Phys. Lipids* **50**, 171-190.

Lepran, I. & Szekeres, L. (1992). Effect of dietary sunflower seed oil on the severity of reperfusion-induced arrhythmias in anesthetized rats. *J. Cardiovasc. Pharmacol.* **19**, 40-44.

Li, G., Chen, Y., Saari, J. T. & Kang, Y. J. (1997). Catalase-overexpressing transgenic mouse heart is resistant to ischemia-reperfusion injury. *Am. J. Physiol.* **273**, H1090-5.

Liu, R., Buettner, G. R. & Oberley, L. W. (2000). Oxygen free radicals mediate the induction of manganese superoxide dismutase gene expression by TNF-alpha. *Free Radic. Biol. Med* **28**, 1197-1205.

Lonergan, P. E., Martin, D. S., Horrobin, D. F. & Lynch, M. A. (2002). Neuroprotective Effect of Eicosapentaenoic Acid in Hippocampus of Rats Exposed to gamma -Irradiation. *J. Biol. Chem.* **277**, 20804-20811.

Losonczy, K. G., Harris, T. B. & Havlik, R. J. (1996). Vitamin E and vitamin C supplement use and risk of all-cause and coronary heart disease mortality in older persons: the Established Populations for Epidemiologic Studies of the Elderly. *Am. J. Clin. Nutr.* **64**, 190-196.

Lund, E. K., Harvey, L. J., Ladha, S., Clark, D. C. & Johnson, I. T. (1999). Effects of dietary fish oil supplementation on the phospholipid composition and fluidity of cell membranes from human volunteers. *Ann. Nutr. Metab* **43**, 290-300.

Luostarinen, R., Wallin, R. & Saldeen, T. (1997). Dietary (n-3) fatty acids increase superoxide dismutase activity and decrease thromboxane production in the rat heart. *Nutr. Res.* **17**, 163-175.

Lusis, A. J. (2000). Atherosclerosis. *Nature.* **407**, 233-241.

Macleod, J. C., Macknight, A. D. C. & Rodrigo, G. C. (1998). The electrical and mechanical response of adult guinea pig and rat ventricular myocytes to omega-3 polyunsaturated fatty acids. *Eur. J. Pharmacol.* **356**, 261-270.

Maddaford, T. G., Hurtado, C., Sobrattee, S., Czubryt, M. P. & Pierce, G. N. (1999). A model of low-flow ischemia and reperfusion in single, beating adult cardiomyocytes. *Am. J. Physiol.* **46**, H788-H798.

Maixent, J. M., Gerbi, A., Barbey, O., Lan, C., Jamme, I., Burnet, H., Nouvelot, A., Levy, S., Cozzone, P. J. & Bernard, M. (1999). Dietary fish oil promotes positive inotropy of ouabain in the rat heart. *Am. J. Physiol* **277**, H2290-H2297.

Malis, C. D., Weber, P. C., Leaf, A. & Bonventre, J. V. (1990). Incorporation of marine lipids into mitochondrial membranes increases susceptibility to damage by calcium and reactive oxygen species: evidence for enhanced activation of phospholipase A2 in mitochondria enriched with n-3 fatty acids. *Proc. Natl. Acad. Sci. USA.* **87**, 8845-8849.

Mallat, Z., Philip, I., Lebret, M., Chatel, D., Maclouf, J. & Tedgui, A. (1998). Elevated levels of 8-iso-prostaglandin F2alpha in pericardial fluid of patients with heart failure: a potential role for in vivo oxidant stress in ventricular dilatation and progression to heart failure. *Circulation* **97**, 1536-1539.

Manning, A. S. & Hearse, D. J. (1984). Reperfusion-induced arrhythmias: mechanisms and prevention. *J. Mol. Cell. Cardiol* **16**, 497-518.

Massey, K. D. & Burton, K. P. (1990). Free radical damage in neonatal rat cardiac myocyte cultures: effects of alpha-tocopherol, Trolox, and phytol. *Free. Radic. Biol. Med.* **8**, 449-458.

McCord, J. M. (1985). Oxygen-derived free radicals in postischemic tissue injury. *New. Engl. J. Med.* **312**, 159-163.

Mcdonald, M. C., Zacharowski, K., Bowes, J., Cuzzocrea, S. & Thiemermann, C. (1999). Tempol reduces infarct size in rodent models of regional myocardial ischemia and reperfusion. *Free. Radic. Biol. Med* **27**, 493-503.

McLennan, P., Howe, P., Abeywardena, M., Muggli, R., Raederstorff, D., Mano, M., Rayner, T. & Head, R. (1996). The cardiovascular protective role of docosahexaenoic acid. *Eur. J. Pharmacol.* **300**, 83-89.

McLennan, P. L. (1993). Relative effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on cardiac arrhythmias in rats. *Am. J. Clin. Nutr.* **57**, 207-212.

McLennan, P. L., Abeywardena, M. Y. & Charnock, J. S. (1985). Influence of dietary lipids on arrhythmias and infarction after coronary artery ligation in rats. *Can. J Physiol. Pharmacol.* **63**, 1411-1417.

McLennan, P. L., Abeywardena, M. Y. & Charnock, J. S. (1988). Dietary fish oil prevents ventricular fibrillation following coronary artery occlusion and reperfusion. *Am. Heart. J.* **116**, 709-717.

McLennan, P. L., Abeywardena, M. Y. & Charnock, J. S. (1990). Reversal of the arrhythmogenic effects of long-term saturated fatty acid intake by dietary n-3 and n-6 polyunsaturated fatty acids. *Am. J. Clin. Nutr.* **51**, 53-58.

McLennan, P. L., Bridle, T. M., Abeywardena, M. Y. & Charnock, J. S. (1992). Dietary lipid modulation of ventricular fibrillation threshold in the marmoset monkey. *Am. Heart. J.* **123**, 1555-1561.

McLennan, P. L., Bridle, T. M., Abeywardena, M. Y. & Charnock, J. S. (1993). Comparative efficacy of n-3 and n-6 polyunsaturated fatty acids in modulating ventricular fibrillation threshold in marmoset monkeys. *Am. J. Clin. Nutr.* **58**, 666-669.

McLennan, P. L. & Dallimore, J. A. (1995). Dietary canola oil modifies myocardial fatty acids and inhibits cardiac arrhythmias in rats. *J. Nutr.* **125**, 1003-1009.

McMurchie, E. J. (1988). Dietary Lipids and the Regulation of Membrane Fluidity and Function. In *Physiological Regulation of Membrane Fluidity*, eds. Aloia, R. C., Curtain, C. C. & Gordon, L. M., pp. 189-237. Alan R. Liss Inc., New York.

McMurchie, E. J., Abeywardena, M. Y., Charnock, J. S. & Gibson, R. A. (1983). Differential modulation of rat heart mitochondrial membrane- associated enzymes by dietary lipid. *Biochim. Biophys. Acta.* **760**, 13-24.

Meilhac, O., Zhou, M., Santanam, N. & Parthasarathy, S. (2000). Lipid peroxides induce expression of catalase in cultured vascular cells. *J. Lipid Res.* **41**, 1205-1213.

Meydani, M., Natiello, F., Goldin, B., Free, N., Woods, M., Schaefer, E., Blumberg, J. B. & Gorbach, S. L. (1991). Effect of long-term fish oil supplementation on vitamin E status and lipid peroxidation in women. *J. Nutr.* **121**, 484-491.

Mirochnitchenko, O., Palnitkar, U. , Philbert, M. & Inouye, M. (1995). Thermosensitive phenotype of transgenic mice overproducing human glutathione peroxidases. *Proc. Natl. Acad. Sci. U. S. A* **92**, 8120-8124.

Mohsen, M., Pinson, A., Zhang, R. & Samuni, A. (1995). Do nitroxides protect cardiomyocytes from hydrogen peroxide or superoxide? *Mol. Cell. Biochem.* **145**, 103-110.

Morris, M. C., Manson, J. E., Bernard, R., Buring, J. E., Willett, W. C. & Hennekens, C. H. A Prospective Study of Fish Consumption on Cardiovascular Disease. Circulation 86[Suppl 1], I:-463. 1992.

Morris, T. E. & Sulakhe, P. V. (1997). Sarcoplasmic reticulum Ca^{2+}-pump dysfunction in rat cardiomyocytes briefly exposed to hydroxyl radicals. *Free. Radic. Biol. Med.* **22**, 37-47.

Nagy, A., Valen, G., Ek, B., Sellei, P., Sjoquist, P. O. & Vaage, J. (1998). Effects of a novel, low-molecular weight inhibitor of lipid peroxidation on ischemia-reperfusion injury in isolated rat hearts and in cultured cardiomyocytes. *Free. Radic. Biol. Med* **24**, 1462-1469.

Nair, S. S., Leitch, J. W., Falconer, J. & Garg, M. L. (1997). Prevention of cardiac arrhythmia by dietary (n-3) polyunsaturated fatty acids and their mechanism of action. *J. Nutr.* **127**, 383-393.

Nair, S. S. D., Leitch, J., Falconer, J. & Garg, M. L. (1999). Cardiac (n-3) non-esterified fatty acids are selectively increased in fish oil-fed pigs following myocardial ischemia. *J. Nutr.* **129**, 1518-1523.

Nakamura, T. Y., Goda, K., Okamoto, T., Kishi, T., Nakamura, T. & Goshima, K. (1993). Contractile and morphological impairment of cultured fetal mouse myocytes induced by oxygen radicals and oxidants. Correlation with intracellular Ca^{2+} concentration. *Circ. Res.* **73**, 758-770.

Nakashima, H., Yamamoto, M., Goto, K., Osumi, T., Hashimoto, T. & Endo, H. (1989). Isolation and characterization of the rat catalase-encoding gene. *Gene* **79**, 279-288.

Nalbone, G., Grynberg, A., Chevalier, A., Leonardi, J., Termine, E. & Lafont, H. (1990). Phospholipase A activity of cultured rat ventricular myocyte is affected by the nature of cellular polyunsaturated fatty acids. *Lipids.* **25**, 301-306.

Narayanan, B. A., Narayanan, N. K. & Reddy, B. S. (2001). Docosahexaenoic acid regulated genes and transcription factors inducing apoptosis in human colon cancer cells. *Int. J. Oncol.* **19**, 1255-1262.

Nasa, Y., Hayashi, M., Sasaki, H., Hayashi, J. & Takeo, S. (1998). Long-term supplementation with eicosapentaenoic acid salvages cardiomyocytes from hypoxia/reoxygenation-induced injury in rats fed with fish-oil-deprived diet. *Jpn. J Pharmacol.* **77**, 137-146.

Negoro, S., Kunisada, K., Fujio, Y., Funamoto, M., Darville, M. I., Eizirik, D. L., Osugi, T., Izumi, M., Oshima, Y., Nakaoka, Y., Hirota, H., Kishimoto, T. & Yamauchi-Takihara, K. (2001). Activation of signal transducer and activator of transcription 3 protects cardiomyocytes from hypoxia/reoxygenation-induced oxidative stress through the upregulation of manganese superoxide dismutase. *Circulation* **104**, 979-981.

Negretti, N., Perez, M. R., Walker, D. & Oneill, S. C. (2000). Inhibition of sarcoplasmic reticulum function by polyunsaturated fatty acids in intact, isolated myocytes from rat ventricular muscle. *Journal Of Physiology-London* **523**, 367-375.

Nieto, N., Fernandez, M. I., Torres, M. I., Rios, A., Suarez, M. D. & Gil, A. (1998). Dietary monounsaturated n-3 and n-6 long-chain polyunsaturated fatty acids affect cellular antioxidant defense system in rats with experimental ulcerative colitis induced by trinitrobenzene sulfonic acid. *Dig. Dis. Sci.* **43**, 2676-2687.

Nishioka, K. & Jarmakani, J. M. (1982). Effect of ischemia on mechanical function and high-energy phosphates in rabbit myocardium. *Am. J. Physiol* **242**, H1077-H1083.

O'Farrell, S. & Jackson, M. J. (1997). Dietary polyunsaturated fatty acids, vitamin E and hypoxia/reoxygenation-induced damage to cardiac tissue. *Clin. Chim. Acta* **267**, 197-211.

Obata, T., Tamura, M. & Yamanaka, Y. (1997). Evidence of hydroxyl free radical generation by calcium overload in rat myocardium. *J. Pharm. Pharmacol.* **49**, 787-790.

Onodera, T. & Ashraf, M. (1991). Detection of hydroxyl radicals in the post-ischemic reperfused heart using salicylate as a trapping agent. *J. Mol. Cell. Cardiol* **23**, 365-370.

Opie, L. H. (1989). Reperfusion injury and its pharmacologic modification. *Circulation* **80**, 1049-1062.

Pawlosky, R. J., Hibbeln, J. R., Novotny, J. A. & Salem, N., Jr. (2001). Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans. *J. Lipid Res.* **42**, 1257-1265.

Pedraza-Chaverri, J., Granados-Silvestre, M. D., Medina-Campos, O. N., Maldonado, P. D., Olivares-Corichi, I. M. & Ibarra-Rubio, M. E. (2001). Post-transcriptional control of catalase expression in garlic-treated rats. *Mol. Cell Biochem.* **216**, 9-19.

Pepe, S. & McLennan, P. L. (1996). Dietary fish oil confers direct antiarrhythmic properties on the myocardium of rats. *J. Nutr.* **126**, 34-42.

Pepe, S. & McLennan, P. L. (2002). Cardiac membrane fatty acid composition modulates myocardial oxygen consumption and postischemic recovery of contractile function. *Circulation* **105**, 2303-2308.

Perry, G., Raina, A. K., Nunomura, A., Wataya, T., Sayre, L. M. & Smith, M. A. (2000). How important is oxidative damage? Lessons from Alzheimer's disease. *Free Radic. Biol. Med* **28**, 831-834.

Pierce, G. N. & Czubryt, M. P. (1995). The contribution of ionic imbalance to ischemia/reperfusion-induced injury. *J. Mol. Cell. Cardiol* **27**, 53-63.

Pietri, S., Seguin, J. R., d'Arbigny, P., Drieu, K. & Culcasi, M. (1997). Ginkgo biloba extract (EGb 761) pretreatment limits free radical- induced oxidative stress in patients undergoing coronary bypass surgery. *Cardiovasc. Drugs Ther.* **11**, 121-131.

Pogwizd, S. M. & Corr, P. B. (1987). Electrophysiologic mechanisms underlying arrhythmias due to reperfusion of ischemic myocardium. *Circulation* **76**, 404-426.

Ponsard, B., Durot, I., Delerive, P., Oudot, F., Cordelet, C., Grynberg, A. & Athias, P. (1999). Cross-influence of membrane polyunsaturated fatty acids and hypoxia-reoxygenation on alpha- and beta-adrenergic function of rat cardiomyocytes. *Lipids.* **34**, 457-466.

Price, P. T., Nelson, C. M. & Clarke, S. D. (2000). Omega-3 polyunsaturated fatty acid regulation of gene expression. *Curr. Opin. Lipidol.* **11**, 3-7.

Priori, S. G., Mantica, M., Napolitano, C. & Schwartz, P. J. (1990). Early afterdepolarizations induced in vivo by reperfusion of ischemic myocardium. A possible mechanism for reperfusion arrhythmias. *Circulation* **81**, 1911-1920.

Qian, Z. M., Xu, M. F. & Tang, P. L. (1997). Superoxide dismutase does protect the cultured rat cardiac myocytes against hypoxia/reoxygenation injury. *Free. Radic. Res.* **27**, 13-21.

Ralenkotter, L., Dales, C., Delcamp, T. J. & Hadley, R. W. (1997). Cytosolic [Ca^{2+}], [Na^+], and pH in guinea pig ventricular myocytes exposed to anoxia and reoxygenation. *Am. J. Physiol.* **272**, H2679-85.

Rapola, J. M., Virtamo, J., Haukka, J. K., Heinonen, O. P., Albanes, D., Taylor, P. R. & Huttunen, J. K. (1996). Effect of vitamin E and beta carotene on the incidence of angina pectoris. A randomized, double-blind, controlled trial. *JAMA.* **275**, 693-698.

Ray, P. S., Maulik, G., Cordis, G. A., Bertelli, A. A., Bertelli, A. & Das, D. K. (1999). The red wine antioxidant resveratrol protects isolated rat hearts from ischemia reperfusion injury. *Free Radic. Biol. Med* **27**, 160-169.

Reddy, A. P., Hsu, B. L., Reddy, P. S., Li, N. Q., Thyagaraju, K., Reddy, C. C., Tam, M. F. & Tu, C. P. (1988). Expression of glutathione peroxidase I gene in selenium-deficient rats. *Nucleic Acids Res.* **16**, 5557-5568.

Reithmann, C., Scheininger, C., Bulgan, T. & Werdan, K. (1996). Exposure to the n-3 polyunsaturated fatty acid docosahexaenoic acid impairs alpha 1-adrenoceptor-mediated contractile responses and inositol phosphate formation in rat cardiomyocytes. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **354**, 109-119.

Ren, J. & Bode, A. M. (2000). Altered cardiac excitation-contraction coupling in ventricular myocytes from spontaneously diabetic BB rats. *Am. J. Physiol Heart Circ. Physiol* **279**, H238-H244.

Rietz, B., Isensee, H., Strobach, H., Makdessi, S. & Jacob, R. (1993). Cardioprotective actions of wild garlic (allium ursinum) in ischemia and reperfusion. *Mol. Cell Biochem.* **119**, 143-150.

Rimm, E. B. & Stampfer, M. J. (1997). The role of antioxidants in preventive cardiology. *Curr. Opin. Cardiol* **12**, 188-194.

Rimm, E. B., Stampfer, M. J., Ascherio, A., Giovannucci, E., Colditz, G. A., Willett & WC. (1993). Vitamin E consumption and the risk of coronary heart disease in men. *New. Engl. J. Med.* **328**, 1450-1456.

Roe, M. W., Lemasters, J. J. & Herman, B. (1990). Assessment of Fura-2 for measurements of cytosolic free calcium. *Cell. Calcium.* **11**, 63-73.

Rohn, T. T., Sauvadet, A., Pavoine, C. & Pecker, F. (1997). Xanthine affects $[Ca^{2+}]_i$ and contractile responses of ventricular cardiocytes to electrical stimulation. *Am. J. Physiol.* **273**, C909-17.

Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* **362**, 801-809.

Ruiz-gutierrez, V., Perez-espinosa, A., Vazquez, C. M. & Santa-maria, C. (1999). Effects of dietary fats (fish, olive and high-oleic-acid sunflower oils) on lipid composition and antioxidant enzymes in rat liver. *Br. J. Nutr.* **82**, 233-241.

Sack, S., Mohri, M., Schwarz, E. R., Arras, M., Schaper, J., Ballagi-Pordany, G., Scholz, W., Lang, H. J., Scholkens, B. A. & Schaper, W. (1994). Effects of a new Na^+/H^+ antiporter inhibitor on postischemic reperfusion in pig heart. *J. Cardiovasc. Pharmacol.* **23**, 72-78.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning- A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Sargent, J. R. (1997). Fish oils and human diet. *Br. J. Nutr.* **78 Suppl 1**, S5-13.

Schimke, I., Haberland, A., Wirth, M., Papies, B., Moritz, V. & Baumann, G. (1997). Influence of long-term supplementation with alpha-linolenic acid on myocardial lipid peroxidation and antioxidative capacity in spontaneously hypertensive rats. *Prostaglandins Leukot. Essent. Fatty Acids* **57**, 545-550.

Schoenfeld, J. R., Vasser, M., Jhurani, P., Ng, P., Hunter, J. J., Ross, J., Jr., Chien, K. R. & Lowe, D. G. (1998). Distinct molecular phenotypes in murine cardiac muscle development, growth, and hypertrophy. *J. Mol. Cell Cardiol.* **30**, 2269-2280.

Sellmayer, A., Witzgall, H., Lorenz, R. L. & Weber, P. C. (1995). Effects of dietary fish oil on ventricular premature complexes. *Am. J. Cardiol.* **76**, 974-977.

Sergiel, J. P., Martine, L., Raederstorff, D., Grynberg, A. & Demaison, L. (1998). Individual effects of dietary epa and dha on the functioning of the isolated working rat heart. *Can. J. Physiol. Pharmacol.* **76**, 728-736.

Sharikabad, M. N., Hagelin, E. M., Hagberg, I. A., Lyberg, T. & Brors, O. (2000). Effect of calcium on reactive oxygen species in isolated rat cardiomyocytes during hypoxia and reoxygenation. *J. Mol. Cell Cardiol.* **32**, 441-452.

Shimizu, M., Wang, Q. D., Sjoquist, P. O. & Ryden, L. (1998). The lipid peroxidation inhibitor indenoindole H290/51 protects myocardium at risk of injury induced by ischemia-reperfusion. *Free. Radic. Biol. Med.* **24**, 726-731.

Siegmund, B., Schlack, W., Ladilov, Y. V., Balser, C. & Piper, H. M. (1997). Halothane protects cardiomyocytes against reoxygenation-induced hypercontracture. *Circulation* **96**, 4372-4379.

Siegmund, B., Zude, R. & Piper, H. M. (1992). Recovery of anoxic-reoxygenated cardiomyocytes from severe Ca^{2+} overload. *Am. J. Physiol* **263**, H1262-H1269.

Sies, H., Stahl, W. & Sundquist, A. R. (1992). Antioxidant functions of vitamins. Vitamins E and C, beta-carotene, and other carotenoids. *Ann. N. Y. Acad. Sci.* **669**, 7-20.

Sigma-Aldrich, T. N. (1999). *Tri-Reagent Instructions*. Sigma Aldrich, St. Louis, MO, USA.

Simpson, P. J. & Lucchesi, B. R. (1987). Free radicals and myocardial ischemia and reperfusion injury. *J. Lab. Clin. Med.* **110**, 13-30.

Singal, P. K., Khaper, N., Palace, V. & Kumar, D. (1998). The role of oxidative stress in the genesis of heart disease. *Cardiovasc. Res.* **40**, 426-432.

Singh, I., Gulati, S., Orak, J. K. & Singh, A. K. (1993). Expression of antioxidant enzymes in rat kidney during ischemia- reperfusion injury. *Mol. Cell Biochem.* **125**, 97-104.

Singh, R. B., Niaz, M. A., Sharma, J. P., Kumar, R., Rastogi, V. & Moshiri, M. (1997). Randomized, double-blind, placebo-controlled trial of fish oil and mustard oil in patients with suspected acute myocardial infarction - the indian experiment of infarct survival. *Cardiovasc. Drugs. Ther.* **11**, 485-491.

Siscovick, D. S., Raghunathan, T. E., King, I., Weinmann, S., Wicklund, K. G., Albright, J., Bovbjerg, V., Arbogast, P., Smith, H., Kushi, L. H., Cobb, L. A., Copass, M. K., Psaty, B. M., Lemaitre, R., Retzlaff, B., Childs, M. & Knopp, R. H. (1995). Dietary intake and cell membrane levels of long-chain n-3 polyunsaturated fatty acids and the risk of primary cardiac arrest. *JAMA.* **274**, 1363-1367.

Solans, R., Motta, C., Sola, R., La Ville, A. E., Lima, J., Simeon, P., Montella, N., Armadans-Gil, L., Fonollosa, V. & Vilardell, M. (2000). Abnormalities of erythrocyte membrane fluidity, lipid composition, and lipid peroxidation in systemic sclerosis: evidence of free radical- mediated injury. *Arthritis Rheum.* **43**, 894-900.

Stampfer, M. J., Hennekens, C. H., Manson, J. E., Colditz, G. A., Rosner, B., Willett & WC. (1993). Vitamin E consumption and the risk of coronary disease in women. *New. Engl. J. Med.* **328**, 1444-1449.

Stenson, W. F., Seetharam, B., Talkad, V., Pickett, W., Dudeja, P. & Brasitus, T. A. (1989). Effects of dietary fish oil supplementation on membrane fluidity and enzyme activity in rat small intestine. *Biochem. J.* **263**, 41-45.

Stern, M. D., Chien, A. M., Capogrossi, M. C., Pelto, D. J. & Lakatta, E. G. (1985). Direct observation of the "oxygen paradox" in single rat ventricular myocytes. *Circ. Res.* **56**, 899-903.

Stubbs, C. D. & Smith, A. D. (1984). The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim. Biophys. Acta.* **779**, 89-137.

Suarez, A., Ramirez-Tortosa, M., Gil, A. & Faus, M. J. (1999). Addition of vitamin E to long-chain polyunsaturated fatty acid-enriched diets protects neonatal tissue lipids against peroxidation in rats. *Eur. J. Nutr.* **38**, 169-176.

Supari, F., Ungerer, T., Harrison, D. G. & Williams, J. K. (1995). Fish oil treatment decreases superoxide anions in the myocardium and coronary arteries of atherosclerotic monkeys. *Circulation* **91**, 1123-1128.

Sussman, M. S. & Bulkley, G. B. (1990). Oxygen-derived free radicals in reperfusion injury. *Methods. Enzymol.* **186**, 711-723.

Suzuki, T., Higgins, P. J. & Crawford, D. R. (2000). Control selection for RNA quantitation. *Biotechniques* **29**, 332-337.

Suzuki, Y. J., Forman, H. J. & Sevanian, A. (1997). Oxidants as stimulators of signal transduction. *Free Radic. Biol. Med* **22**, 269-285.

Swift, L. M. & Sarvazyan, N. (2000). Localization of dichlorofluorescin in cardiac myocytes: implications for assessment of oxidative stress. *Am. J. Physiol Heart Circ. Physiol* **278**, H982-H990.

Taffet, G. E., Pham, T. T., Bick, D. L., Entman, M. L., Pownall, H. J. & Bick, R. J. (1993). The calcium uptake of the rat heart sarcoplasmic reticulum is altered by dietary lipid. *J. Membr. Biol.* **131**, 35-42.

Takeo, S., Nasa, Y., Tanonaka, K., Yabe, K., Nojiri, M., Hayashi, M., Sasaki, H., Ida, K. & Yanai, K. (1998). Effects of long-term treatment with eicosapentaenoic acid on the heart subjected to ischemia/reperfusion and hypoxia/reoxygenation in rats. *Mol. Cell. Biochem.* **188**, 199-208.

Tamada, A., Hattori, Y., Houzen, H., Yamada, Y., Sakuma, I., Kitabatake, A. & Kanno, M. (1998). Effects of beta-adrenoceptor stimulation on contractility, $[Ca^{2+}](i)$, and Ca^{2+} current in diabetic rat cardiomyocytes. *Am. J. Physiol* **43** , H1849-H1857.

Tanguy, S., Boucher, F. R., Malfroy, B. & de Leiris, J. G. (1996). Free radicals in reperfusion-induced arrhythmias: study with EUK 8, a novel nonprotein catalytic antioxidant. *Free. Radic. Biol. Med.* **21**, 945-954.

Taouis, M., Sheldon, R. S. & Duff, H. J. (1991). Upregulation of the rat cardiac sodium channel by in vivo treatment with a class I antiarrhythmic drug. *J. Clin. Invest* **88**, 375-378.

Tavani, A., Pelucchi, C., Negri, E., Bertuzzi, M. & La Vecchia, C. (2001). n-3 Polyunsaturated fatty acids, fish, and nonfatal acute myocardial infarction. *Circulation* **104**, 2269-2272.

Chapter Nine

Temple, N. J. (2000). Antioxidants and Disease: More Questions than Answers. *Nutr. Res.* **20**, 449-459.

Tennant, R. & Wiggers, C. J. (1935). The effect of coronary occlusion on myocardial contraction. *Am. J. Physiol.* **112**, 351-361.

Thomas, G. P., Sims, S. M., Cook, M. A. & Karmazyn, M. (1998). Hydrogen peroxide-induced stimulation of L-type calcium current in guinea pig ventricular myocytes and its inhibition by adenosine A1 receptor activation. *J. Pharmacol. Exp. Ther.* **286**, 1208-1214.

Tosaki, A., Bagchi, D., Pali, T., Cordis, G. A. & Das, D. K. (1993). Comparisons of ESR and HPLC methods for the detection of OH. radicals in ischemic/reperfused hearts. A relationship between the genesis of free radicals and reperfusion arrhythmias. *Biochem. Pharmacol.* **45**, 961-969.

Tosaki, A., Balint, S. & Szekeres, L. (1988). Protective effect of lidocaine against ischemia and reperfusion- induced arrhythmias and shifts of myocardial sodium, potassium, and calcium content. *J. Cardiovasc. Pharmacol.* **12**, 621-628.

Tritto, I., Duilio, C., Santoro, G., Elia, P. P., Cirillo, P., De Simone, C., Chiariello, M. & Ambrosio, G. (1998). A short burst of oxygen radicals at reflow induces sustained release of oxidized glutathione from postischemic hearts. *Free Radic. Biol. Med* **24**, 290-297.

Tzivoni, D., Keren, A., Granot, H., Gottlieb, S., Benhorin, J. & Stern, S. (1983). Ventricular fibrillation caused by myocardial reperfusion in Prinzmetal's angina. *Am. Heart. J.* **105**, 323-325.

Uauy-Dagach, R. & Valenzuela, A. (1996). Marine oils: the health benefits of n-3 fatty acids. *Nutr. Rev.* **54**, S102-S108.

Uto, A., Arai, H. & Ogawa, Y. (1991). Reassessment of Fura-2 and the ratio method for determination of intracellular Ca^{2+} concentrations. *Cell. Calcium.* **12**, 29-37.

Vaagenes, H., Muna, Z. A., Madsen, L. & Berge, R. K. (1998). Low doses of eicosapentaenoic acid, docosahexaenoic acid, and hypolipidemic eicosapentaenoic acid derivatives have no effect on lipid peroxidation in plasma. *Lipids.* **33**, 1131-1137.

Valagussa, F., Franzosi, M. G., Geraci, E., Mininni, N., Nicolosi, G. L., Santini, M., Tavazzi, L., Vecchio, C., et al. (1999). Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the gissi- prevenzione trial. *Lancet.* **354**, 447-455.

van Bilsen, M., van der Vusse, G. J., Willemsen, P. H., Coumans, W. A., Roemen, T. H. & Reneman, R. S. (1989). Lipid alterations in isolated, working rat hearts during ischemia and reperfusion: its relation to myocardial damage. *Circ. Res.* **64**, 304-314.

van de Vijver, L. P., Kardinaal, A. F., Grobbee, D. E., Princen, H. M. & van Poppel, G. (1997). Lipoprotein oxidation, antioxidants and cardiovascular risk: epidemiologic evidence. *Prostaglandins. Leukot. Essent. Fatty Acids.* **57**, 479-487.

van Ginkel, G. & Sevanian, A. (1994). Lipid peroxidation-induced membrane structural alterations. *Methods. Enzymol.* **233**, 273-288.

Vandenhoek, T. L., Becker, L. B., Shao, Z. H., Li, C. Q. & Schumacker, P. T. (1998). Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *J. Biol. Chem.* **273**, 18092-18098.

Vandenhoek, T. L., Li, C. Q., Shao, Z. H., Schumacker, P. T. & Becker, L. B. (1997). Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. *J. Mol. Cell. Cardiol.* **29**, 2571-2583.

Vandenhoek, T. L., Shao, Z., Li, C., Zak, R., Schumacker, P. T. & Becker, L. B. (1996). Reperfusion injury on cardiac myocytes after simulated ischemia. *Am. J. Physiol.* **270**, H1334-41.

Venkatraman, J. T., Chandrasekar, B., Kim, J. D. & Fernandes, G. (1994). Effects of n-3 and n-6 fatty acids on the activities and expression of hepatic antioxidant enzymes in autoimmune-prone NZBxNZW F1 mice. *Lipids.* **29**, 561-568.

Vergely, C., Maupoil, V., Benderitter, M. & Rochette, L. (1998). Influence of the severity of myocardial ischemia on the intensity of ascorbyl free radical release and on postischemic recovery during reperfusion. *Free. Radic. Biol. Med.* **24**, 470-479.

Vognild, E., Elvevoll, E. O., Brox, J., Olsen, R. L., Barstad, H., Aursand, M. & Osterud, B. (1998). Effects of dietary marine oils and olive oil on fatty acid composition, platelet membrane fluidity, platelet responses, and serum lipids in healthy humans. *Lipids.* **33**, 427-436.

Vogt, M., Bauer, M. K., Ferrari, D. & Schulze-Osthoff, K. (1998). Oxidative stress and hypoxia/reoxygenation trigger CD95 (APO-1/Fas) ligand expression in microglial cells. *FEBS Lett.* **429**, 67-72.

Vollset, S. E., heuch, I. & Bjelke, E. (1985). Fish consumption and mortality from coronary heart disease. *New. Engl. J. Med.* **313**, 820-821.

Walker, M. K., Vergely, C., Lecour, S., Abadie, C., Maupoil, V. & Rochette, L. (1998). Vitamin E analogues reduce the incidence of ventricular fibrillations and scavenge free radicals. *Fundam. Clin. Pharmacol.* **12**, 164-172.

Wander, R. C. & Du, S. H. (2000). Oxidation of plasma proteins is not increased after supplementation with eicosapentaenoic and docosahexaenoic acids. *Am. J. Clin. Nutr.* **72**, 731-737.

Wang, H. & Joseph, J. A. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic. Biol. Med.* **27**, 612-616.

Ward, C. A. & Moffat, M. P. (1995). Role of protein kinase C in mediating effects of hydrogen peroxide in guinea-pig ventricular myocytes. *J. Mol. Cell. Cardiol* **27**, 1089-1097.

Watts, J. A. & Maiorano, P. C. (1999). Trace amounts of albumin protect against ischemia and reperfusion injury in isolated rat hearts. *J. Mol. Cell. Cardiol.* **31**, 1653-1662.

Weylandt, K. H., Kang, J. X. & Leaf, A. (1996). Polyunsaturated fatty acids exert antiarrhythmic actions as free acids rather than in phospholipids. *Lipids.* **31**, 977-982.

Williams, M. D., Van Remmen, H., Conrad, C. C., Huang, T. T., Epstein, C. J. & Richardson, A. (1998). Increased oxidative damage is correlated to altered mitochondrial function in heterozygous manganese superoxide dismutase knockout mice. *J. Biol. Chem.* **273**, 28510-28515.

Williard, D. E., Harmon, S. D., Kaduce, T. L., Preuss, M., Moore, S. A., Robbins, M. E. & Spector, A. A. (2001). Docosahexaenoic acid synthesis from n-3 polyunsaturated fatty acids in differentiated rat brain astrocytes. *J. Lipid Res.* **42** , 1368-1376.

Winer, J., Jung, C. K., Shackel, I. & Williams, P. M. (1999). Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal. Biochem.* **270**, 41-49.

Woo, C. H., Eom, Y. W., Yoo, M. H., You, H. J., Han, H. J., Song, W. K., Yoo, Y. J., Chun, J. S. & Kim, J. H. (2000). Tumor necrosis factor-alpha generates reactive oxygen species via a cytosolic phospholipase A_2-linked cascade. *J. Biol. Chem.* **275**, 32357-32362.

Woodcock, E. A., Arthur, J. F., Harrison, S. N., Gao, X. M. & Du, X. J. (2001). Reperfusion-induced Ins(1,4,5)P(3) generation and arrhythmogenesis require activation of the $Na(^+)/Ca(^{2+})$ exchanger. *J. Mol. Cell Cardiol.* **33**, 1861-1869.

Xiao, Y. F., Kang, J. X., Morgan, J. P. & Leaf, A. (1995). Blocking effects of polyunsaturated fatty acids on Na^+ channels of neonatal rat ventricular myocytes. *Proc. Natl. Acad. Sci. USA.* **92**, 11000-11004.

Xie, Z., Kometiani, P., Liu, J., Li, J., Shapiro, J. I. & Askari, A. (1999). Intracellular reactive oxygen species mediate the linkage of Na^+/K^+- ATPase to hypertrophy and its marker genes in cardiac myocytes. *J. Biol. Chem.* **274**, 19323-19328.

Xu, J., Teran-Garcia, M., Park, J. H., Nakamura, M. T. & Clarke, S. D. (2001). Polyunsaturated fatty acids suppress hepatic sterol regulatory element- binding protein-1 expression by accelerating transcript decay. *J. Biol. Chem.* **276**, 9800-9807.

Yang, B., Saldeen, T. G., Nichols, W. W. & Mehta, J. L. (1993). Dietary fish oil supplementation attenuates myocardial dysfunction and injury caused by global ischemia and reperfusion in isolated rat hearts. *J. Nutr.* **123**, 2067-2074.

Yoshida, T., Watanabe, M., Engelman, D. T., Engelman, R. M., Schley, J. A., Maulik, N., Ho, Y. S., Oberley, T. D. & Das, D. K. (1996). Transgenic mice overexpressing glutathione peroxidase are resistant to myocardial ischemia reperfusion injury. *J. Mol. Cell Cardiol.* **28**, 1759-1767.

Yoshimura, S., Takekoshi, S., Watanabe, K. & Fujii-Kuriyama, Y. (1988). Determination of nucleotide sequence of cDNA coding rat glutathione peroxidase and diminished expression of the mRNA in selenium deficient rat liver. *Biochem. Biophys. Res. Commun.* **154**, 1024-1028.

Yuan, Y. V., Kitts, D. D. & Godin, D. V. (1998). Variations in dietary fat and cholesterol intakes modify antioxidant status of SHR and WKY rats. *J. Nutr.* **128**, 1620-1630.

Zhong, H. & Simons, J. W. (1999). Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. *Biochem. Biophys. Res. Commun.* **259**, 523-526.

Zhou, X., Zhai, X. & Ashraf, M. (1996). Direct evidence that initial oxidative stress triggered by preconditioning contributes to second window of protection by endogenous antioxidant enzyme in myocytes. *Circulation* **93**, 1177-1184.

Zulueta, J. J., Sawhney, R., Yu, F. S., Cote, C. C. & Hassoun, P. M. (1997). Intracellular generation of reactive oxygen species in endothelial cells exposed to anoxia-reoxygenation. *Am. J. Physiol.* **272**, L897-902.

Zweier, J. L., Flaherty, J. T. & Weisfeldt, M. L. (1987). Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proc. Natl. Acad. Sci. USA.* **84**, 1404-1407.