Regulation of Indoleamine 2,3-dioxygenase (IDO-1) by fatty acids and their eicosanoid derivatives in THP-1 cells and human monocytes

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Dedication

This thesis is dedicated to the 1 year old boy whose cancerous THP-1 cells were generously donated by his parents for cancer research.

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Abbreviations

AA	Arachidonic acid
Ab	Antibody
AC	Adenylate cyclase
Ag	Antigen
AhR	Aryl hydrocarbon receptor
ALA	α-linolenic acid
AMP	Adenosine monophosphate
APCs	Antigen presenting cells
Ar	Arachidic acid
ATP	Adenosine triphosphate
Bin 1	BAR adaptor encoding protein
BMDCs	Bone marrow derived dendritic cells
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
CRTH2	chemoattractant receptor homologous molecule expressed on T-helper cells
CTLA-4	Cytotoxic T lymphocytes associated antigen-4
DCs	Dendritic cells
DHA	Docosahexaenoic acid (22:6n-3)
DP1	D-prostanoid receptor
DTT	Dithiothreitol
EPA	Eicosapentaenoic acid (20:5n-3)
Epac	Exchange protein activated by cAMP
ER	*p*-Dimethylaminobenzaldehyde (Ehrlich's reagent)
FAs	Fatty acids
Gα	G-protein subunit α
Gαi	Gαi inhibitory
Gαs	Gαs stimulatory
Gβ	G-protein subunit β
Gγ	G-protein subunit γ
GAS	Gamma interferon activated sequence
GCN2	General control nonderepressible 2
GDP	Guanosine-diphosphate
GPCR	G-protein coupled receptor
G-vs.-HD	Graft versus host disease
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
His	Histidine
HPLC	High performance liquid chromatography
IDO	Indoleamine 2,3-dioxygenase
IDO2	Indoleamine 2,3-dioxygenase like enzyme
IFN-α	Interferon alpha

IFN-β	Interferon beta
IFN-γ	Interferon gamma
IFN-γ R1	Interferon gamma receptor 1
IFN-γ R2	Interferon gamma receptor 2
IL	Interleukin
IM	Indomethacin
IRF-1	IFN regulatory factor 1
ISRE	Interferon stimulated response element
JAK	Janus kinase
Kyn	Kynurenine
LA	Linoleic acid
LBP	LPS binding protein
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
LX	Lipoxin
MAPK	Mitogen-activated protein kinase
mRNA	Messenger RNA
1-MT	1-Methyl tryptophan
mTOR	Mammalian target of rapamycin
MUFAs	Monounsaturated fatty acids
NaB	Sodium butyrate
NDGA	Nordihydroguaiaretic acid
NF-κB	Nuclear factor κB
NSAIDs	Non-steroidal anti-inflammatory drugs
PDE	Phosphodiesterase
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PLC	Phospholipases C
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl fluoride
PTMs	Posttranslational modifications
PUFAs	Polyunsaturated fatty acids
RAP	Ras-related protein
ROS	Reactive oxygen species
S^{727}	Serine ^{727}
SAFAs	Saturated fatty acids
SDS	Sodium dodecylsulphate
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TCA	Tricholoroacetic acid
TDO	Tryptophan 2,3-dioxygenase
TLR	Toll-like receptor

TNF-α	Tumour necrosis factor α
T-reg	T regulatory cells
tRNA	Transfer RNA
Trp	Tryptophan
Trp tRNA	Tryptophan tRNA
TX	Thromboxane
Tyr	Tyrosine
Tyr^{701}	Tyrosine701

Publications

1. **BASSAL, N. K**., HUGHES, B. P. & COSTABILE, M. 2012. Arachidonic acid and its COX1/2 metabolites inhibit interferon-gamma mediated induction of indoleamine-2,3 dioxygenase in THP-1 cells and human monocytes. *Prostaglandins Leukot Essent Fatty Acids,* 87**,** 119-26.

2. **Bassal, N. K.** Hughes, B. P., Costabile, M. 2012. Tryptophan Metabolism-Indolamine 2,3-Dioxygenase- Friend and Foe, *J Metabonomics Metabolites 1:2*

3. **BASSAL, N. K**., HUGHES, B. P., & COSTABILE, M. Prostaglandin D_2 and its metabolites inhibit indoleamine-2,3-dioxygenase (IDO-1) activity via the DP1/cAMP pathway. *In preparation.*

Abstract

Evasion of the normal immune response is essential for any cancer cell to persist. Cancer cells have developed many mechanisms to escape the immune response. One mechanism is the expression of high levels of the enzyme indoleamine 2,3-dioxygenase (IDO-1). Expression of elevated levels of IDO-1 is well accepted as being a mechanism of cancer-induced immunosupression and tolerance. Pharmacological inhibition of IDO-1 activity is thus a promising alternative in the treatment of cancer. IDO-1 catalyses the first and the rate-limiting step of tryptophan (Trp) metabolism through the kynurenine (Kyn) pathway. The combined depletion of Trp and increase in Kyn metabolites inhibits T-cell infiltration, induces T-cell cycle arrest, T-cell apoptosis and, through the aryl hydrocarbon receptor (AhR), the generation and activation of T regulatory (T-reg) cells, resulting in tumor evasion. A number of biologically relevant molecules such as fatty acids (FAs) and prostaglandins (PGs) have been shown to regulate IDO-1 activity. This research examined the regulation of IDO-1 by FAs and their eicosanoid derivatives in THP-1 cells and primary human monocytes.

Based on our previous data, a library of FAs was screened for their ability to modulate IDO-1 activity. Of these, 6 FAs significantly inhibited the IFN-γ mediated induction of IDO-1 activity in THP-1 cells. Saturated FAs showed no inhibitory activity while monounsaturated and polyunsaturated FAs (PUFAs) demonstrated inhibitory activity. The activity of individual FAs was affected by chain length, number of double bonds and bond configuration. Of these FAs, arachidonic acid (AA) was studied in greater detail. AA exerted its inhibitory effects in a time and dose dependent manner without any apparent toxicity. AA reduced both *INDO,* in THP-1 cells and human monocytes, and IDO-1 protein expression, in both cell lines, by its metabolism via the cyclooxygenase (COX1/COX2) pathway. To determine the active eicosanoid(s), a library of AA eicosanoid derivatives was screened for their ability to modulate IDO-1 activity in THP-1 cells and human monocytes. A total of 13 PGs displayed inhibitory activity of kynurenine (Kyn) production following IFN-γ mediated induction/activity of IDO-1 in both cells. A structure–function analysis revealed that the γ position of the cyclopentanone ring, double bond in the α-β position of the cyclopentenone ring, the presence of multiple OH groups in the side arm and the addition of the functional group ethanolamide, significantly increased the inhibitory activity of the PGs.

This research identified PGD_2 and its non-enzymatic metabolites as being principally responsible for the inhibitory induction/activity in both cells. PGD_2 inhibited in

a dose dependent manner without apparent toxicity. PGD_2 down-regulation of IDO-1 is at the transcriptional levels. PGD_2 mediated its effects via the G-protein coupled receptor D-prostanoid receptor (DP1) and not through chemoattractant receptor-homologous molecule expressed on T-helper–type 2 cells (CRTH2). DP1 agonists mimicked the PGD_2 inhibitory effects while CRTH2 agonists were devoid of inhibitory activity. The blockage of DP1 prevented the inhibitory activity of PGD_2. Direct activation of adenylate cyclase (AC) by forskolin inhibited Kyn production, as did exogenous modulation of cyclic adenosine monophosphate (cAMP). The inhibitory effects were mediated via protein kinase A (PKA) activation and completely reversed by its inhibitor, Rp-cAMP. Both PGD_2 and forskolin increased phosphorylation of the cAMP responsive element binding protein (CREB). Further, our results showed that PGD_2 could inhibit STAT1 phosphorylation at Tyr701. The PGD_2 cAMP/PKA/CREB pathway is a new negative regulatory mechanism controlling the IFN-γ JAK/STAT signalling pathway which modulates the induction of IDO-1 in both cells.

The biological activity of FAs as well as their COX1/COX2 derivatives highlights the important role of these molecules in the regulation of IDO-1 induction/activity *in vitro*. Our results with PGD_2 and its derivatives illustrate for the first time that eicosanoids, other than PGE_2, can affect IDO-1 induction/activity. The inhibitory activity of PGD_2, and the determination of key structural features important for inhibitory activity, may be used as the basis to develop new modulators of IDO-1.

Declaration

I declare that:

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university.

To the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged.

Nesrine Mohamed Kamal Bassal

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My husband, my love and my support. He encouraged me to start my PhD. He always believed that I can achieve great things and that I am lucky to work in the field that I love. Although he is overseas, he is always with me; especially in the last few months with his great support, wisdom and advice.

My family for being there for me, encouraging and supporting me through the tough days.

My grandchildren, whom I love so much, now we can have more time to play! I look forward to when I will read their PhD theses too.

Finally I would like to send special thanks to my MUM the strongest person I have ever met and to my Aunty the most selfless person I have ever known.

So to you all, Thanks.

Chapter 1

Introduction

1.1 Introduction

Expression of elevated levels of the immunoregulatory enzyme Indoleamine 2,3-dioxygenase (IDO) has been implicated as an immunosuppressive and tolerogenic mechanism contributing to cancer induced tolerance and helping cancer cells evade the immune response.

A number of biologically relevant molecules have been shown to regulate IDO activity. Recently, we have shown that the polyunsaturated fatty acid (PUFA) arachidonic acid (AA) can inhibit interferon gamma (IFN-γ) mediated induction of IDO-1 in THP-1 cells (Bassal 2008; Bassal, Hughes & Costabile 2012). Prostaglandin E_2 (PGE_2), an AA derived cyclooxygenase (COX) metabolite, has been shown to increase both IDO-1 mRNA and protein levels in matured dendritic cells (DCs) *in vitro* (von Bergwelt-Baildon et al. 2006), while another study showed that PGE_2 inhibited IDO in DCs (Jung et al. 2010). COX2 inhibitors inhibited IDO production (Basu et al. 2006) and sodium butyrate (NaB) also has been shown to inhibit IDO activity (Jiang et al. 2010). Lipopolysaccharide (LPS) also induces IDO activity in monocytes/macrophages, and the degree of activation is further enhanced byIFN-γ stimulation (Werner-Felmayer et al. 1990).

Fatty acids (FAs) have many important roles in both health and disease. FAs have both pro-inflammatory and anti-inflammatory effects with documented effects on cancer promotion and/or inhibition. However, little is known about the regulation of IDO by FAs and their eicosanoid derivatives, the focus of this research.

This research examined the regulation of IDO activity by FAs and their eicosanoid derivatives using THP-1 cells, an acute monocytic leukeamic cell line, as a model of cancer cells, human monocytes as normal control cells and IFN-γ as a physiological inducer of IDO. This model has been used by others to study the effects of colchicine and atorvastatin on their ability to modulate induction of Trp degradation by IDO (Altindag et al. 1997; Neurauter et al. 2003).

1.1.1 Tumor formation and evasion of the immune response

Tumor formation can occur during normal cell division through genetic mutation as a result of chemical, radiation or pathogen exposure. Tumor cells can express abnormal antigens to which the immune system, especially high avidity T cells, can respond efficiently to remove and destroy. This process, named immune surveillance, protects the host against tumor formation (Van den Eynde & van der Bruggen 1997; Lennerz et al. 2005; Dunn, Koebel & Schreiber 2006; van der Bruggen & Van den Eynde 2006).

The ability of the immune system to reject a cancerous cell is defined as tumor immunity. Tumor immunity creates a selective cellular pressure that promotes tumor cells to escape the immune system, referred to as immunoediting (Katz, Muller & Prendergast 2008). The first step of immunoediting is the response of the immune system against a nascent tumor (immune surveillance). At this stage, there is no clinical evidence of disease and the host immune system is able to effectively eradicate any transformed cells. The second stage begins when tumor cells evolve the ability to colonise the host tissue. At this stage, the tumor cells continue to divide and the immune system is still able to contain the tumor cells (immune equilibrium) with no clinical manifestation of the disease. The final stage of immunoediting occurs when tumor cells effectively evade, suppress and overcome the control of the immune system (immune escape/evasion). At this stage, the clinical disease has been manifested in the host and has been diagnosed and detected (Katz, Muller & Prendergast 2008). The pathological interactions between tumor and host immune cells within the tumor microenvironment create an immunosuppressive network that promotes tumor growth while simultaneously protecting the tumor from immune attack (Rodriguez, Zea & Ochoa 2003; Drake et al. 2006).

So for any cancer cells to persist, they have to evade the immune response. In order to do so, tumors have developed many mechanisms (Ercolini et al. 2005; Gajewski et al. 2006). For example, tumor cells can decrease the expression or change the physical structure of their surface epitopes so that they are no longer detectable by immune cells (Marincola et al. 2000; Pawelec et al. 2000; Medema et al. 2001). One novel mechanism cancer cells use to evade the immune response and induce immune tolerance is by expressing high levels of IDO and the hyper metabolism of the essential amino acid, tryptophan.

1.2 Tryptophan (Trp)

IDO catalyses the initial and rate-limiting step in the metabolism of the essential amino acid Trp along the kynurenine (Kyn) pathway. Increased expression of IDO leads to the depletion of Trp in the local microenvironment and the accumulation of Kyn metabolites which directly suppress infiltrating T cells, and increase the number and activity of local T-reg immunosuppressive cells (Munn & Mellor 2006, 2007; Katz, Muller & Prendergast 2008). Most tumors express high levels of IDO compared to normal cells of the same stroma (Werner-Felmayer et al. 1989b; Uyttenhove et al. 2003). Even tumor cells that do not constitutively express IDO can be stimulated by inflammatory cytokines, for

example IFN-γ, to produce IDO, which can consequently increase the number of tumors using IDO as an escape mechanism (Munn et al. 1999; Kudo et al. 2001).

1.2.1 Structure

Trp is one of nine essential amino acids (ILKMFTWVH) that must be provided in the diet (McGaha et al. 2012). Most absorbed Trp is used by cells for protein synthesis and cell division, while the remainder is metabolised through the Kyn pathway and excreted in the urine (Amirkhani et al. 2002). Trp is a monobasic aromatic amino acid with a heterocyclic structure. Trp has an indole ring that is attached to the α-carbon atom via a methyl group. The Trp R-group provides the amino acid with both hydrophobic and hydrophilic properties. Apart from being an essential amino acid, Trp is also the least abundant amino acid in the body and, consequently, cells are highly sensitive to changes in its levels (Snider, McGarry & Hanson 2006; King & Thomas 2007). As Trp is metabolised in the body through the Kyn pathway it produces important molecules including melatonin, serotonin and nicotinamide adenine dinucleotide, which have a wide range of activities in both physiological and pathological conditions (Amirkhani et al. 2002).

1.2.2 Metabolism

Trp metabolism through the Kyn pathway can result in Trp depletion, which suppresses T cell proliferation and results in immune suppression or anti-microbial effects (MacKenzie et al. 2007). The pathway is involved in diverse biological processes, from immune modulation in the pregnant female allowing the foetus to be tolerated (Munn et al. 1998), to activation of a number of central nervous system disorders including AIDS-related dementia and cerebral malaria (Heyes et al. 1998; Sanni et al. 1998). Trp metabolites (Figure 1-1) are known to be involved in many neurological diseases including Alzheimer's (Baran, Jellinger & Deecke 1999; Amirkhani et al. 2002), Parkinson's (Ogawa et al. 1992), schizophrenia (Erhardt et al. 2001) epilepsy (Yamamoto, S et al. 1995; Yamamoto, T et al. 1995) and depression (Jenny et al. 2009).

The first and rate-limiting step in the Kyn pathway is the oxidation of Trp to N-formyl Kyn and then Kyn. Since the 1970s, the first and rate-limiting step in the Kyn pathway was believed to be performed by one of two enzymes, hepatic tryptophan 2,3-dioxygenase (TDO) or extra hepatic IDO. However in 2007, a gene with homology to IDO was discovered (Ball et al. 2007; Ball et al. 2009). This new enzyme was named

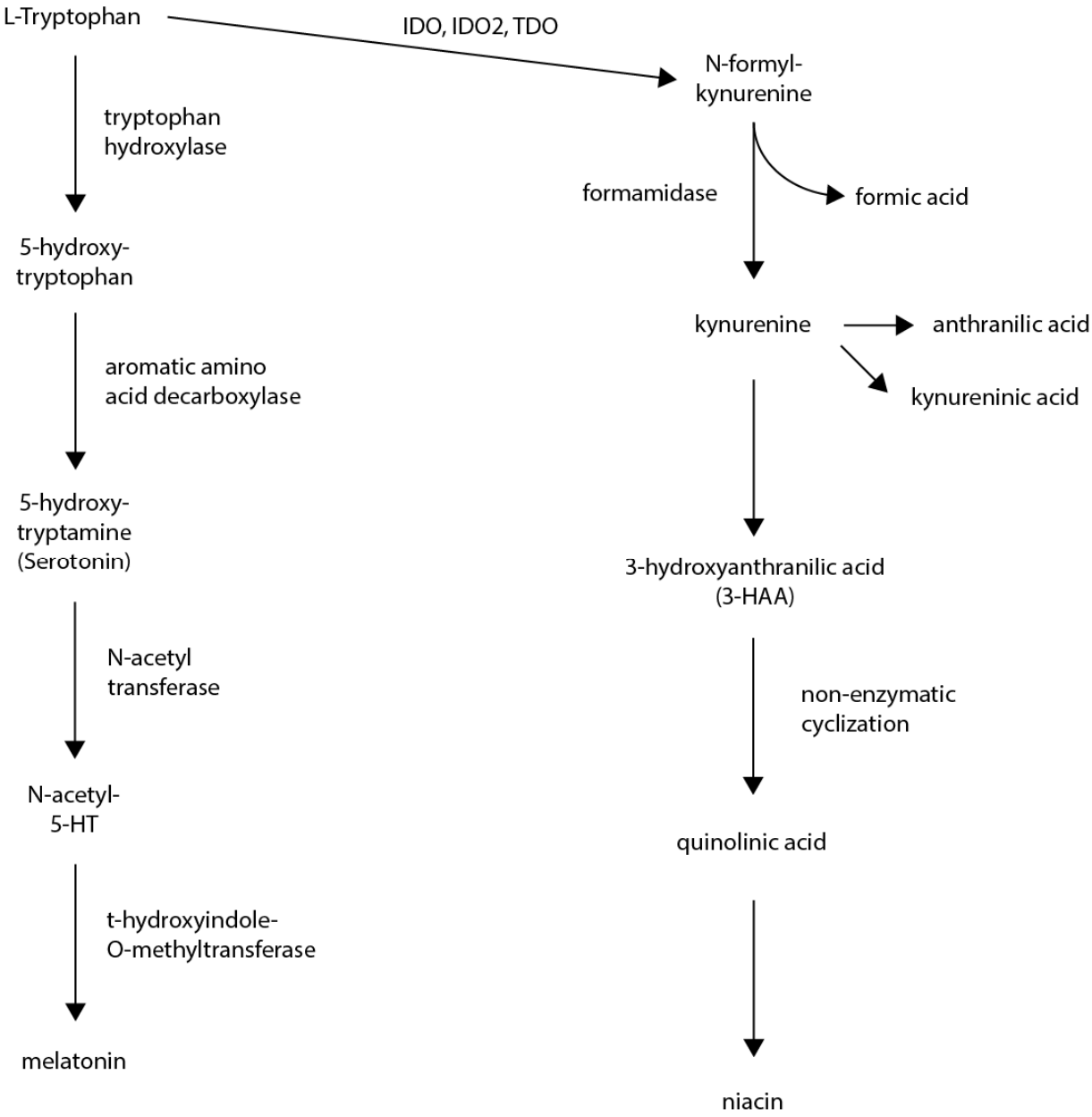


Figure 1-1 Diagrammatic illustration of Trp metabolism via the Kyn pathway

Trp that is not required for protein synthesis is metabolised along the Kyn pathway. The initial and rate-limiting step is catalysed by TDO, tryptophan 2,3-dioxygenase; IDO-1, indoleamine 2,3-dioxygenase and IDO-2, Indoleamine 2,3-dioxyogenase like enzyme. Binding of Trp to the enzyme active site leads to the oxidation of the pyrrole ring to form N-formyl Kyn, which is further converted to Kyn and formic acid. Kyn can be further metabolised to various derivatives that can inhibit immunological, neurological or antioxidant processes (Thomas & Stocker 1999; Mellor & Munn 2004; Curti et al. 2009) (Adapted fromThomas & Stocker 1999).

indoleamine 2,3-dioxygenase like enzyme or IDO-2 due to its similarity with IDO (IDO-1). Both IDO-1 and IDO-2 have different biochemical characteristics and different physiological functions (Austin et al. 2010). The two enzymes, IDO-1 and IDO-2, are expressed by a similar genomic structure located adjacent to each other on the human and mousechromosome 8, and are most likely a result of gene duplication (Ball et al. 2007; Ball et al. 2009).

While Trp can be metabolized by three enzymes, TDO, IDO-1 and IDO-2 (Table 1-1) (Bender 1986; King & Thomas 2007; Katz, Muller & Prendergast 2008), many aspects relating to these enzymes still require investigation. Historically, TDO was believed to be only present in the liver regulating homeostatic levels of Trp. Recently, Platten and colleagues (2012) demonstrated that TDO was also expressed by malignant glioma cells and is responsible for breaking down Trp leading to tumor progression and metastasis. IDO has been shown to be more widely expressed in the body with increased expression primarily in antigen presenting cells (APCs) (Mellor & Munn 2004).

1.3 Indoleamine 2,3-dioxygenase (IDO-1)

1.3.1 Discovery and Structure

Indoleamine 2,3-dioxygenase (IDO-1, EC 1.13.11.17) was discovered in rabbit intestine as a dioxygenase enzyme with broad substrate specificity for various indolamines (Higuchi & Hayaishi 1967; Takikawa 2005). IDO-1 is a monomeric protein that consists of two alpha-helical domains (one small and one large) connected by a heme loop and histidine (His364) at the proximal site (Rafice et al. 2009).The heme component of IDO-1 is its sole prosthetic group and is essential for its enzymatic activity (King & Thomas 2007). IDO-1 is encoded by *INDO1* which is located on human chromosome 8p12. IDO-1 is widely expressed in the body (Munn et al. 1998; Katz, Muller & Prendergast 2008). Human *INDO1* comprises 10 exons spanning 15 kbp of DNA and encodes 403 amino acids with a molecular weight of ~45 kDa (Kadoya et al. 1992; King & Thomas 2007; Austin et al. 2010). Sixteen alternatively spliced variants of IDO-1 have been identified with two single nucleotide polymorphisms (SNPs) found located in exons 1 and 3. The exon 1 SNP leads to a non-functional allele of *INDO1*, while the exon 3 SNP encodes a protein completely devoid of enzymatic activity. These variations can explain the difference in IDO-1 activity between different individuals (Arefayene et al. 2009).

Table 1-1 Comparison between TDO, IDO-1 and IDO-2

Characteristic	TDO	IDO-1	IDO-2	Reference
Cellular location	Liver, placenta brain malignant Glioma cells	intestine, lung placenta monocytes epididymis endocrine glands thymus central nervous system	kidney, epididymis liver	(Suzuki et al. 2001; Hansen et al. 2004; Ball et al. 2007; Katz, Muller & Prendergast 2008; Austin et al. 2010; Platten, Wick & Van den Eynde 2012)
Gene location	Chromosome 4q31-q32	Chromosome 8p12	Chromosome 8p12	(Katz, Muller & Prendergast 2008)
Size (MW)	47 kDa	45 kDa	47 kDa	(Katz, Muller & Prendergast 2008; Austin et al. 2010)
Size (aa)	406	403	420	(Katz, Muller & Prendergast 2008)
Reaction catalysed	Trp to Kyn	Trp to Kyn	Trp to Kyn	(Munn & Mellor 2007; Katz, Muller & Prendergast 2008)
Physiological inducers	Trp, tyrosine, histidine, gluco-corticoids Kyn.	IFN-γ, LPS, PGE_2, IL-1, TNF	IFN-γ (in mouse)	(Taylor & Feng 1991; Katz, Muller & Prendergast 2008)
Reduction by	Ascorbic acid borohydride	Methylene blue cytochrome b_5	cytochrome b_5	(Vottero et al. 2006; Maghzal et al. 2008; Austin et al. 2010)
Substrate	L-Trp	L or D-Trp, serotonin, Tryptamine, 5 hydroxytryptophan	L-Trp	(Katz, Muller & Prendergast 2008)

Key characteristics of TDO, IDO and IDO2. The table outlines some differences between TDO, IDO-1 and IDO-2. aa, amino acid; D-Trp, D-tryptophan; IFN-γ, interferon gamma; IL-1, interleukin 1; L-Trp, L-tryptophan; LPS, lipopolysaccharide; MW, molecular weight; PGE_2, prostaglandin E_2; TNF, tumor necrosis factor.

1.3.2 Activation

The heme group within IDO is essential for its enzymatic activity. IDO is purified in an inactive state with the heme present as ferric-iron. Activation requires the single electron reduction of ferric-iron (Fe^{3+}) to ferrous-iron (Fe^{2+}) that facilitates the binding of Trp and oxygen to the enzyme active site (Figure 1-2) (Thomas & Stocker 1999). Trp binds firstly in close proximity, but not directly, to the distal heme iron, followed secondly by the binding of oxygen to the vacant sixth iron coordination position. The intracellular reducing co-factors required for IDO heme-iron reduction are unknown. Hence, *in vitro* enzyme assays utilise artificial reducing co-factors, commonly ascorbic acid plus the redox-active dye, methylene blue. Putative candidates for the intracellular reducing co-factor(s) include superoxide anion radical, dihydroflavin mononucleotide, tetrahydrobiopterin and cytochrome reductase (Thomas & Stocker 1999; Takikawa 2005; Maghzal et al. 2008).

1.3.3 Gene expression

IDO can be expressed in a constitutive or inducible manner. Constitutively, it is expressed in the placenta, lung, stomach, intestine, liver, spleen, kidney, brain and many types of cancers (Uyttenhove et al. 2003; King & Thomas 2007). IDO is inducible in cells of the myeloid lineage, for example DCs, monocytes, macrophages and eosinophils, and in non-lymphoid cells such as epithelial and endothelial cells, fibroblasts and vascular smooth muscles. IDO is also expressed in a great number of tumor cell lines compared to the normal cells of the same stroma (Grohmann et al. 2003a; Uyttenhove et al. 2003; Maghzal et al. 2008).

INDO1 is induced at the transcriptional level in response to specific inflammatory stimuli. The main physiological inducer of *INDO1* is IFN-γ, both *in vitro* and *in vivo*. The *INDO1* promoter contains multiple sequence elements that confer responsiveness to IFN-γ. These are the interferon stimulated response element (ISRE) homolog-binding and gamma interferon activated sequence (GAS) binding elements in the *INDO1* promoter that recognize IRF-1 and the activated signal transducer and activator of transcription (STATlα) (King & Thomas 2007). Other inflammatory stimuli also induce *INDO1*, for example IFN-α, IFN-β, and LPS, although they are markedly less effective than IFN-γ (Thomas & Stocker 1999; Neurauter et al. 2003; Mellor & Munn 2004). IDO is also induced by interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) (Austin et al. 2010) and cytotoxic T lymphocyte associated antigen-4 (CTLA-4) in a manner that may be dependent

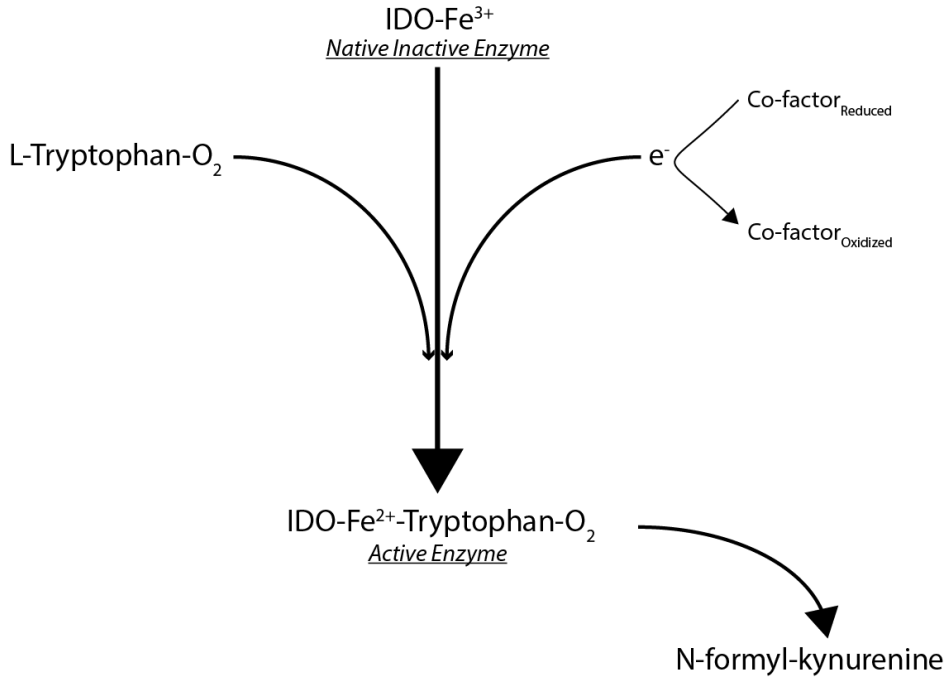


Figure 1-2 IDO activation and Trp binding

IDO activation involves the single electron reduction of heme-iron from the ferric (IDO-Fe^{3+}) to ferrous (IDO-Fe^{2+}) state which facilitates the binding of Trp to the active enzyme site. (Adapted from Thomas & Stocker 1999)

or independent of nuclear factor kappa B (NF-κB) regulated autocrine synthesis of IFN-γ (Mellor & Munn 2004). IDO expression is also modulated by IL-4, IL-6, IL-10, transforming growth factor beta (TGFβ), prostaglandin E_2 (PGE_2), CD40, suppressor of cytokine signalling 3 (SOCS3), BAR adapter encoding protein (Bin1) and DAP12. Cellular infection with microbial agents, influenza viruses and other intracellular parasites such as *Toxoplasma gondii*, *Chlamydia psittaci* and *Chlamydia trachomatis* (Taylor & Feng 1991), can induce IDO in certain cell types (Thomas & Stocker 1999).

The expression of IDO is well regulated, especially during inflammation. At the transcription level, IDO is promoted by Forkhead box O3 (FOXO3) and interferon regulatory factor (IFR)-8 and suppressed by DANK-activation protein 12 (DAP12) (Orabona et al. 2006). IDO is also post-translationally controlled, for example via the supply and insertion of heme and changes in the intracellular redox status (Thomas et al. 2001) or via certain cytokine activities (Mellor & Munn 2004). Post-translationally, IDO can bind to SOCS3 leading to its ubiquitination and degradation (Orabona et al. 2008).

1.3.4 Biological functions

IDO is accepted as being an inducible negative regulator of T-cell viability, proliferation and activation during inflammation (Frumento et al. 2002; Terness et al. 2002; Munn & Mellor 2013), IDO contributes to immune regulation by catalysing the oxidative cleavage of the pyrrole ring of Trp to generate N-formyl-kynurenine that is metabolised to formic acid and the more stable-end product Kyn. IDO has a high affinity for Trp (Km ~0.02 mM) and therefore can rapidly catabolise Trp to create a local tissue microenvironment devoid of this essential amino acid (Munn & Mellor 2013). IDO is thought to suppress T-cell functions by two mechanisms: decreasing the local concentration of Trp (Hwu et al. 2000) and increasing the levels of the Trp metabolism by product Kyn, which is a natural ligand for the aryl hydrocarbon receptor (AhR) which in turn increases the number and activity of T-reg cells (Figure 1-3) (Dykens, Sullivan & Stern 1987; Frumento et al. 2002; Munn & Mellor 2013).

Cells can sense amino acid concentration via two pathways: mammalian target of rapamycin (mTOR) and general control nonderepressible 2 (GCN2) (McGaha et al. 2012; Munn & Mellor 2013). The depletion of Trp causes an increase in uncharged Trp tRNA, which activates GCN2 (Dong et al. 2000). Activation of GCN2 leads to T-cell cycle arrest (Munn et al. 2005; Munn & Mellor 2007). At the same time, increased Kyn metabolites activate AhR, activate GCN2 and suppress mTOR, which together lead to T-reg activation

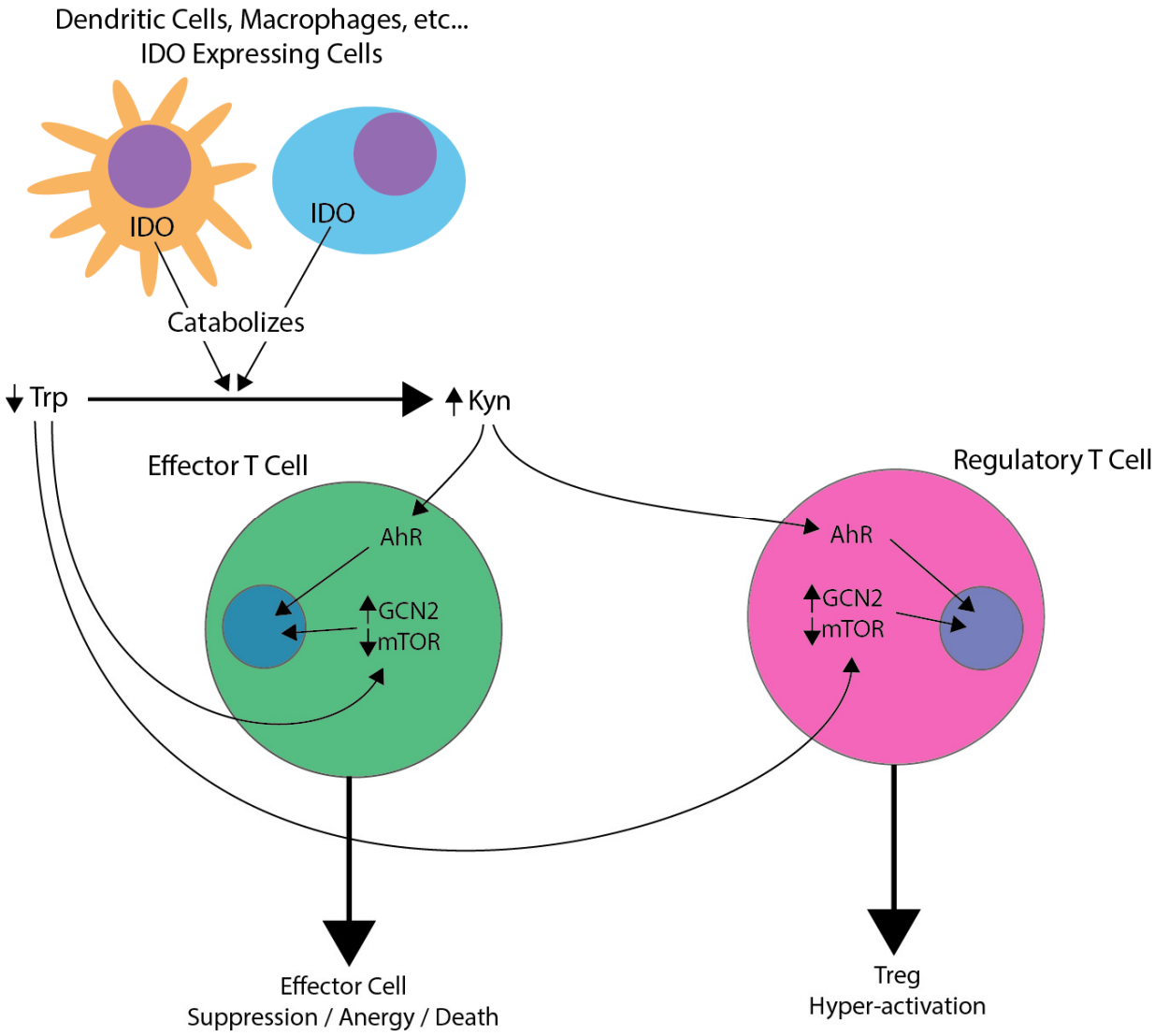


Figure 1-3 IDO controls T cells and T-reg cells

APCs which express IDO decrease local Trp and increase Kyn production. Kyn can activate AhR, GCN2 and suppress mTOR. These effects lead to activation of T-reg cells and suppression, anergy and death of T-effector cells. AhR, aryl hydrocarbon receptor; GCN2, general control nonderepressible 2; mTOR, mammalian rapamycin; Trp, tryptophan; T-reg, T regulatory cells; Kyn, kynurenine. (Adapted from Munn & Mellor 2013).

(Munn & Mellor 2013) and induction of T-cell apoptosis (Fallarino et al. 2002; Terness et al. 2002; Munn et al. 2005; Munn & Mellor 2007, 2013). Both T-cell cycle arrest and T-cell apoptosis lead to the depletion of T cells in the tumor microenvironment, leading to tumor evasion (Figure 1-4) (Munn et al. 2005; Munn & Mellor 2007). However, this cellular stress created by Trp insufficiency can be reversed by the addition of excess Trp *in vitro* (Hayashi et al. 2001; Munn et al. 2004; Munn, Sharma & Mellor 2004; Swanson et al. 2004). As it is difficult to lower the general body level of Trp, APCs that express IDO and the responding T cells have to function in close proximity to each other. Thus the concentration of Trp can be lowered sufficiently to activate the GCN2 signalling pathway. As such, it is now suggested that Trp depletion, the GCN2 pathway and Trp metabolites function synergistically to produce the biological effect of IDO (Fallarino et al. 2006).

1.3.5 Acquired tolerance

In vivo IDO is a molecule that contributes to immune acquired tolerance (Munn & Mellor, 2013). Acquired tolerance can be beneficial and necessary but can also be undesirable and harmful (Iweala & Nagler 2006). Both of these aspects of IDO acquired tolerance will now be discussed.

1.3.5.1 Benefits of IDO acquired tolerance

1.3.5.1.1 Infection control

The biological importance of IDO was first identified in studies examining its role in bacterial, viral and parasitic infections. Such pathogens elicit a strong IFN-γ response that in turn leads to an elevation in local IDO mRNA (*INDO1*) and IDO protein. IDO expression was found to increase in host Antigen-presenting cells (APCs). APCs that can express IDO include human-derived macrophages (Mellor & Munn 1999) and human monocyte-derived DCs (Hwu et al. 2000; Munn et al. 2002; Munn, Sharma & Mellor 2004; Orabona et al. 2006). The increased expression of IDO in these cells leads to a decrease in the local environment of free Trp. Lower Trp can inhibit pathogen proliferation aiding in the control of intracellular pathogens and their multiplication and growth, which explains the anti-microbial effects of IDO (Pfefferkorn 1984; Neurauter et al. 2003; Heseler et al. 2013; Schmidt et al. 2013).

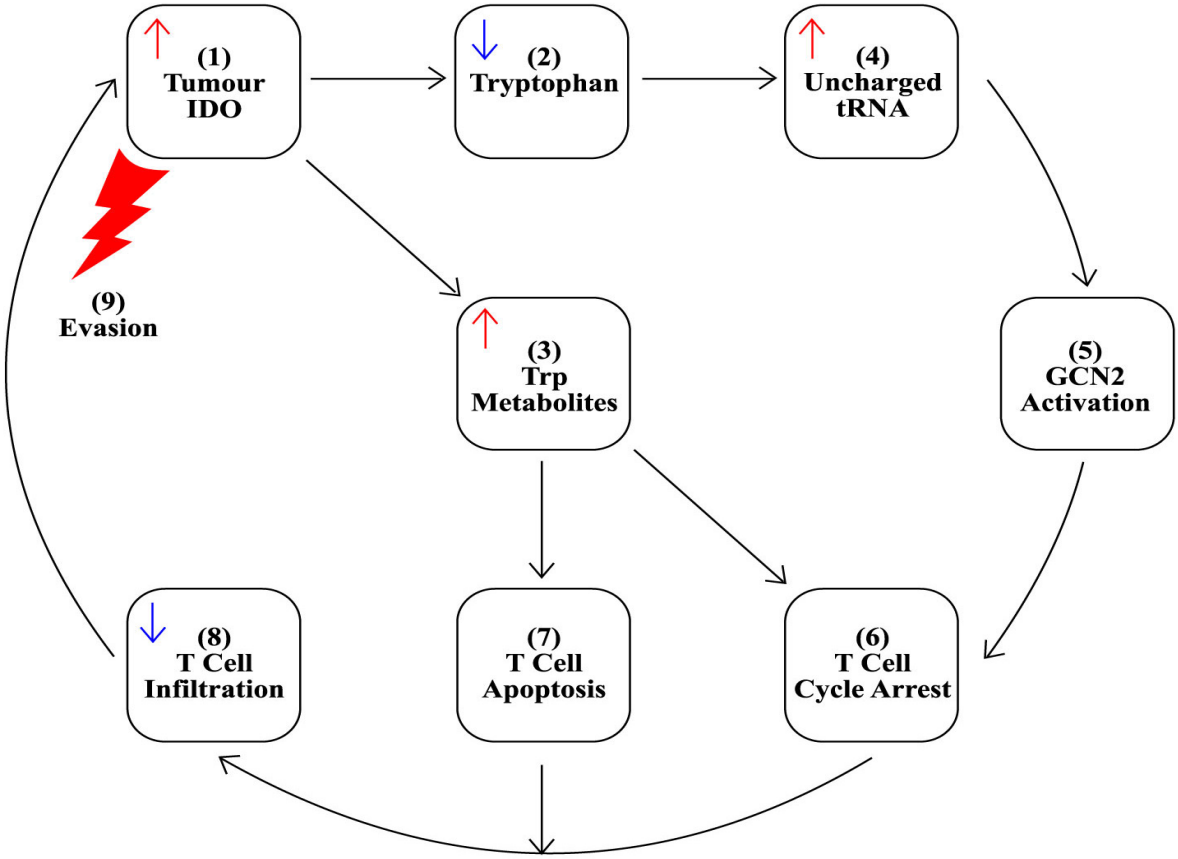


Figure 1-4 Key events in IDO induced immunosuppression and tumor evasion

Tumor cells express high levels of IDO (1), which leads to the catabolism of Trp, leading to Trp depletion (2) which causes an increase in Trp metabolites (3). Depletion of Trp (2) will also cause an increase in uncharged tRNA in surrounding T cells (4), which activates GCN2 kinase (5), leading to T-cell cycle arrest (6). At the same time, Trp metabolites (3) can cause T-cell cycle arrest (6) and T-cell apoptosis (7), which both lead to a decrease in T-cell infiltration into the tumor microenvironment (8) and enhance tumor evasion (9). (Adapted from Dong et al. 2000; Munn & Mellor 2007).

1.3.5.1.2 Pregnancy

Schroecksnadel et al. (1996) showed accelerated Trp breakdown by IDO during pregnancy however at that time there was no link between IDO and maternal immune tolerance (Schrocksnadel et al. 1996). Subsequent studies showed that IDO was important for immunosuppression of the maternal immune response providing a means of inducing immune tolerance (Munn et al. 1998; Neurauter et al. 2003; Mellor & Munn 2004; Muller et al. 2005). Historically, a major question in reproductive biology focused on why the maternal immune system does not reject the allogeneic foetus. Three mechanisms were proposed to explain how a foetus survives the potential rejection via the maternal immune system (Munn et al. 1998):

1. the anatomical separation between the maternal lymphocytes and the foetus by the maternal trophoblast (the placenta)
2. the antigenic immaturity of the foetus
3. maternal tolerance.

As the first two mechanisms failed to fully explain the foetal allogenic survival, attention was focused on the third mechanism (Munn et al. 1998). Maternal tolerance can be justified by two reasons: first, the high level of IDO expression by the placenta, and second, the use of the IDO inhibitor, 1-methyl trypotophan (1-MT), which reduced IDO activity, and hence reduced local immunosuppressive activity. This inhibitor was found to lead to rejection of semi-allogeneic, but not syngeneic, foetuses in mice via maternal T-cell activation (Munn et al. 1998).

1.3.5.1.3 Transplantation

Elevated levels of IDO have also been demonstrated to be beneficial in transplant acceptance. In a similar situation to maternal–foetal differences, transplantation of allogeneic tissue is rejected via innate, humoral and cellular immune responses. Immunosuppression in the local graft environment assists long-term acceptance of the transplanted tissue. In a model of lung transplantation, increased IDO expression or a Trp metabolite, 3-hydroxyanthranilic acid (3HAA), resulted in, after a slight acute rejection, near normal lung function (Alexander et al. 2002; Iken et al. 2012). Again over expressing IDO in lung transplants abrogated acute allograft rejection (Swanson et al. 2004).

The effects of IDO and 3HAA were due to modulation of the T-cell receptor, which leads to T-cell activation. The level of IDO activity correlated with transplant acceptance. Elevated levels of IDO are also associated with increased levels of T-regs, which in turn mediate local immunosuppressive effects. For example, Cytotoxic T lymphocyte-

associated antigen 4 immunoglobulin (CTLA-4-Ig) treatment leads to IDO induction and T-reg generation, which are essential for murine cardiac allograft survival (Sucher et al. 2012), and induce long-term survival of pancreatic islet allografts (Grohmann et al. 2002). IDO and Trp metabolites are also essential in the control of graft vs. host disease (G-vs.-HD). Furthermore, inhibition of IDO activity has been demonstrated recently to prevent long term allograft survival (Bauer et al. 2005; Wan et al. 2012). However, some controversy still exists about the role of IDO, as Landfried et al. demonstrated that elevated levels of Kyn were associated with a worsening of G-vs.-HD in human allogeneic stem cell transplantation (Landfried et al. 2011).

1.3.5.1.4 Autoimmunity

Given its immunosuppressive activity, it is not surprising that up-regulation of IDO facilitates peripheral tolerance in autoimmune diseases, such as non-obese diabetes (Grohmann et al. 2003b), allergic asthma and autoimmune diseases and experimental autoimmune encephalomyelitis (EAE) (Kwidzinski et al. 2005). As IDO leads to increased levels of Kyn, which has been identified to be an endogenous ligand and activator of the AhR (McGaha et al. 2012; Munn & Mellor 2013), activation of AhR leads to the generation of T-reg cells which have potent inhibitory activity, modulate effector T-cell activation, differentiation and survival and consequently amelioration of autoimmune symptoms (Grohmann et al. 2003b).

An important point is that IDO is not needed for self-tolerance. Its action is selective and is a specific form of acquired peripheral tolerance (Munn & Mellor 2007). Studies have shown that IDO knockout mice ($IDO^{-/-}$) do not develop lethal auto-immune diseases or lymphoproliferative disorders (Mellor et al. 2003) and treating mice for 28 days with a systemic IDO inhibitor did not cause the mice to develop spontaneous auto-immunity (Gurtner et al. 2003; Uyttenhove et al. 2003). These results suggest that using IDO inhibitors, for example as a goal in treating cancer, will not affect or cause severe auto-immunity as a major side effect (Munn & Mellor 2007).

1.3.5.1.5 Allergy

Allergy is defined as ‘a reaction of the body to foreign substances’, for example, hay fever, hives and asthma. Some allergies can be mild, like allergic asthma, and others can be life-threatening, like anaphylactic shock (Zaknun et al. 2011). Allergic immune responses rely on Th1 cytokines, Th2 cytokines, macrophages and B cells. During an allergic reaction, activation of Th1 cells leads to the release of IL-2 and IFN-γ, which is the

physiological inducer of IDO. Also, activation of Th2 cells leads to the release of IL-4, -5 , -6 and -13, which stimulate B cells to produce IgE which leads to histamine release from mast cells. Recently, IDO has been implicated in the control of allergic inflammation as it inhibits T-cell proliferation due to decreased levels of Trp (Zaknun et al. 2011). Elevated levels of IDO lead to a local anti-inflammatory response, minimising the allergic inflammatory reaction. Similarly, elevated levels of IDO-1 in immature DCs reduced Th2 cytokine production and allergic airway inflammation to ovalbumin (Zaknun et al. 2011).

1.3.5.2 Harmful effects of IDO acquired tolerance

1.3.5.2.1 IDO expression/activity in cancer cells

One of the earliest reports demonstrating that IDO expression/activity linked to cancer was by Yasui et al. (1986). Human lungs bearing cancer exhibited up to a 20-fold increase in IDO enzyme activity compared to lungs with benign lesions (Yasui et al. 1986). More recently, IDO has been demonstrated to be constitutively expressed in a large number of diverse human cancers including breast, prostate, colorectal, pancreatic, leukemia and cervical carcinomas, while normal cells of the same stroma are negative for IDO expression (Uyttenhove et al. 2003). Many cancer cell lines and primary tumors that express IDO also demonstrate reduced or abolished anti-tumor T-cell responses (Friberg et al. 2002). Furthermore, the level of IDO activity has been shown to correlate with disease progression in endometrial carcinoma (de Jong et al. 2012), colorectal cancer (Ferdinande et al. 2012), oesophageal squamous cell carcinoma (Zhang, G et al. 2011) and vulva squamous cell carcinoma (Sznurkowski et al. 2011).

Expression of IDO by immunogenic mouse tumor cells prevents their rejection by pre-immunised mice. This is in part due to a lack of antigen specific T cells accumulating at the tumor site (inhibition of infiltration) and the stimulation of T-cell apoptosis (Fallarino et al. 2002). Consistent with this, P815 cells (murine mastocytoma), which express IDO-1, form large tumors when introduced into pre-immunised hosts, while IDO-1 negative or low expressing cells are rejected (Muller et al. 2005; Gajewski et al. 2006). Inhibition of IDO-1 by the competitive inhibitor 1-MT also led to tumor rejection (Agaugue et al. 2006). Thus, clearly many cancer cells express IDO and this expression plays an important role in the mechanism of tumor evasion. Another important point is that cancer cells have more efficient transport of Trp than normal cells, allowing them to grow in low Trp levels (Uyttenhove et al. 2003). The evidence for this role will be presented in the next section.

1.3.5.2.2 Impact of IDO expression on cancer outcomes

Numerous studies have examined the relationship between IDO expression/activity and disease progression in various cancers. High IDO expression by cancer cells correlates with a poor clinical outcome. For example, oesophageal squamous cell carcinoma patients with high levels of IDO expression had lower survival rates than patients with low IDO expression (Sakurai et al. 2004). When endometrial cancer tissue was assessed, nearly 50% of all samples had high IDO expression (Ino et al. 2006). These patients had significantly impaired overall survival and significantly worse progression-free survival compared to patients with no or weak expression of IDO. High expression of IDO was also positively correlated with tumor involvement, including myometrial invasion, lymph-vascular space involvement, and lymph node metastases (Ino et al. 2006). In colorectal cancer, cells with high IDO expression had significantly reduced numbers of tumor infiltrating T cells compared to low IDO expressing tumors (Brandacher et al. 2006). Furthermore, high IDO expression correlated significantly with the frequency of liver metastases, and was an independent prognostic variable of disease outcome (Brandacher et al. 2006).

Using microarray analysis, Okamoto et al. (2005) showed that Paclitaxel resistant ovarian cancer cells from patients consistently demonstrated elevated levels of *INDO1* expression. A clear inverse relationship between IDO staining patterns and overall survival was found. All patients with negative IDO staining survived without relapse, while the 50% survival of patients classified as having sporadic, focal, and diffuse staining was 41, 17 and 11 months respectively (Okamoto et al. 2005). Thus, IDO expression was positively associated with Paclitaxel resistance and with impaired survival in patients with serous-type ovarian cancer (Okamoto et al. 2005). These results further demonstrate the importance of exploitation of the IDO enzyme by drug resistant cancer cells as part of their immune evasion strategy.

In cells whose expression of *INDO1* was blocked using small interfering RNA (siRNA), tumor formation was delayed and tumor size was significantly decreased, while T-cell apoptosis was inhibited. *In vivo*, IDO-siRNA treatment resulted in recovery of T-cell responses and enhancement of tumor-specific killing (Zheng et al. 2006).

Thus, in summary, IDO activation and acceleration of Trp catabolism correlates with disease progression, drug resistance and shortened survival rate in a number of cancers (Huang, A et al. 2002; Weinlich et al. 2007). The manipulation of IDO-1 expression shows promise as a target for breaking tumor-induced immune suppression. The blockade of IDO activity/expression also has been suggested as a possible mechanism for increasing the efficacy of tumor-specific T-cell therapies in cancer patients (Gajewski

et al. 2006). In support of this hypothesis, both immuno-pharmacological and genetic studies targeted at modulating *INDO1* levels and IDO-1 enzyme activity could break IDO tumor-derived immune suppression and enhance cancer treatment by the individuals own immune system (Zheng et al. 2006).

1.3.5.2.3 IDO sites of action in cancer

Several mechanisms can induce tumor tolerance both at the tumor microenvironment and at tumor associated draining lymph nodes (Staveley-O'Carroll et al. 1998; Cuenca et al. 2003; Munn & Mellor 2007). These mechanisms act together to block the initial immune response to tumor antigens (Staveley-O'Carroll et al. 1998; Cuenca et al. 2003; Munn & Mellor 2007), inhibit activated T cells that kill tumor cells (Gajewski et al. 2006) and enhance the suppressive activity of T-reg cells (Zou 2006).

In the tumor microenvironment, most primary tumor cells express IDO (Uyttenhove et al. 2003). As mentioned previously, IDO can be expressed by tumor cells constitutively (Muller et al. 2005), or may be induced by inflammatory cytokines such as IFN-γ (Taylor & Feng 1991). IDO also can be expressed by host APCs as a result of exposure to inflammatory cytokines e.g. IFN-γ (Munn et al. 1999). These inflammatory cytokines can be produced as an initial host response to the tumor cells themselves (Dunn, Koebel & Schreiber 2006). These host IDO^+ APCs have been identified in tumor associated draining lymph nodes, in patients with many cancers such as melanoma, breast (Munn et al. 2002; Munn et al. 2004; von Bergwelt-Baildon et al. 2006; Lee, HJ et al. 2007) and although small in number, it can cause immune tolerance and need to be interrupted in order for clinical anti-tumor immunotherapy to be successful (Munn & Mellor 2006, 2007).

1.3.5.2.4 IDO and cancer immunotherapy

Immunotherapy is a new promising strategy for cancer treatment (Munn & Mellor 2007; Avril et al. 2010). Immunotherapy is designed based on the molecular identification of human tumor Ags that are recognised by T cells and using these Ags as a vaccination against various tumors (Van den Eynde & van der Bruggen 1997; Boon & Van den Eynde 2003). However, until now there has been no record of desirable success (Garber 2012). The aim of immunotherapy is to induce inflammation and immune activation to produce antibodies against tumor antigens and make T cells attack the tumor (Garber 2012). During inflammation, IDO can be expressed by DCs, macrophages, epithelial cells (Hayashi et al. 2001; Barcelo-Batllori et al. 2002; Beutelspacher et al. 2006), eosinophils (Odemuyiwa et

al. 2004) and endothelial cells (Beutelspacher et al. 2006) and can act as a counter regulatory mechanism to protect the host from excessive immune activation, which can be damaging or even lethal to the host (Munn & Mellor 2007). Therefore, in cancer immunotherapy treatment, if IDO is produced as in the physiological case of inflammation, it could antagonise the desired effect of tumor immunotherapy (Munn & Mellor 2007). Thus, for cancer immunotherapy to achieve the desirable effect, it is important to use IDO inhibitors.

1.3.5.2.5 IDO and cancer chemotherapy

Chemotherapy is an essential line of treatment for many cancers due to its many effects. The main effect is the killing of tumor cells. Cell death leads to the release of tumor antigens, which are presented by local APCs in the tumor associated draining lymph nodes (Nowak, Robinson & Lake 2003; Broomfield et al. 2005). Chemotherapy can also deplete or inactivate tumor protective T-reg cells (Awwad & North 1988; Ghiringhelli et al. 2004; Beyer et al. 2005; Ercolini et al. 2005; Lutsiak et al. 2005). Finally, chemotherapy can enhance immunotherapy (Dudley et al. 2002; Morgan et al. 2006).

Unfortunately, until now, no protective immune response of chemotherapy has been fully established, which can be explained as follows. When tumors release their antigens, APCs in tumor associated draining lymph nodes present these antigens to T cells. If the APCs are able to induce IDO, will lead to re-suppression of the local immune response and re-establishment of immune tolerance (Munn & Mellor 2007). Thus, inhibition of IDO after chemotherapy could delay or disrupt immune suppression and the re-establishment of immune tolerance and subsequently improve the chemotherapeutic effect (Munn & Mellor 2007).

Pre-clinical studies in mice show synergistic effects when the IDO inhibitor 1-MT is combined with the chemotherapy drugs, cyclophosphamide, doxorubicin, paclitaxel or cisplatin, leading to a decrease in tumor size (Podsypanina, Li & Varmus 2004; Muller et al. 2005; Muller & Prendergast 2005; Hou et al. 2007). This synergistic effect was shown to be immune-mediated as it was lost in immune deficient hosts (Hou et al. 2007). This finding further indicates that inhibition of IDO activity in conjunction with chemotherapy may be able to break established tumor tolerance (Munn & Mellor 2007). However, this area needs further investigation.

In summary, IDO is constitutively expressed in various types of tumor cells, and can be induced by pro-inflammatory cytokines. IDO enhances tumor growth, escapes from the immune system, induces immunotolerance, and accelerates distal metastases and

worsens the prognosis for patients (Uyttenhove et al. 2003). Furthermore, it acts as an impediment of both immunotherapy and chemotherapy treatment in cancer (Okamoto et al. 2005; Chamuleau et al. 2008). All of the above effects suggest that IDO inhibition is an attractive therapeutic target (Uyttenhove et al. 2003).

1.3.6 IDO inhibitors

The most widely used IDO inhibitor is 1-Methyl tryptophan (1-MT) (Cady & Sono 1991). It is present in both D and L isomers, each with different levels of activity and toxicity. It is still unclear which of the two stereoisomers might be preferred in both clinical trials and as a treatment of diseases such as cancer. The L-isomer was shown to be more potent *in vitro* as it inhibits IDO-1 activity using the purified enzyme and in a HeLa cell–based assay, however similar results have not been seen *in vivo* (Hou et al. 2007). In contrast, D-MT has shown promising results *in vivo* reversing the suppression of T cells resulting from IDO-1 expression in DCs, however not *in vitro* (Munn, Sharma & Mellor 2004; Zhu et al. 2006; Hou et al. 2007). D-MT is more effective than L-MT when used in anti-tumor chemo-immunotherapy regimens in mouse models of melanoma and breast cancer. D-MT has been shown to specifically target IDO-1 as its anti-tumor effect is completely lost in IDO-1-knockout mice (Hou et al. 2007). However, Optiz et al., (2011) showed that proliferation of T-cells co-cultured with IDO-1-expressing tumors was unexpectedly inhibited by D-MT. They also showed that D-MT could increase Kyn production, and induced *INDO1* as well as IDO-1 protein expression. These findings potentially indicated that treatment of cancer patients with D-MT could promote anti-tumor immune escape by increasing IDO-1 production and care should be taken when using D-MT in clinical trials (Opitz et al. 2011). These findings raise an important question about the anti-tumor effect of D-MT and whether it's anti-tumor effect is due to inhibition of IDO-2 rather than IDO-1 should be considered. These findings further highlight the importance of finding more effective IDO inhibitors. Another important point is that 1-MT does not inhibit TDO (Suzuki et al. 2001). Due to the variation in the effect of both isomers, many clinical trials use a racemic mixture of both L and D isomers. Thus still more effective and specific inhibitors of IDO need to be developed.

In a comprehensive review by Dolusic & Frederick (2013), IDO inhibitors available from 2008 to 2012 were reviewed (Table 1-2). In their review, some of the challenges in discovering effective IDO inhibitors are mentioned. Firstly, the active site of IDO is relatively small and the highly lipophilic binding cleft obstructs binding of large

Table 1-2 Some IDO inhibitors and their sources

Inhibitor	Source	References
L-1MT and D-1MT	Tryptophan derivatives	(Cady & Sono 1991; Macchiarulo et al. 2009)
Antioxidants		(Thomas et al. 2001)
Tenatoprazole	Drug that inhibit proton pumps inhibit IDO-2	(Bakmiwewa et al. 2012)
B-carbolines	Mammalian sources	(Eguchi et al. 1984)
Potent nanomolar inhibitors	Marine invertebrate extracts	(Brastianos et al. 2006; Pereira et al. 2006)
Methylthiohydanatoin-DL-tryptophan	-	(Muller et al. 2005)
Brassinin and a synthetic derivative 5-bromo-brassinin (5-Br-brassinin)	Plants	(Takikawa et al. 1986; Gaspari et al. 2006; Banerjee et al. 2008)
3-Butyl-β-carboline	Tryptophan derivatives	(Ruddick et al. 2006)
Synthetic analogue derived from a marine sponge	-	(Munn et al. 1998)
4-amino-1,2,5-oxadiazole-3-carboximidamid	-	(Yue, EW et al. 2009)
tryptamine quinine	Exiguamine A	(Carr et al. 2008)
Naphthoquinone	Annulin B28	(Kumar et al. 2008)
Marine alkaloids Tsitsikammamine A derivatives	Pyrroloiminoquinone scaffold	(Dolusic et al. 2013)
3-indoleethanol (IET) (or tryptophol)	-	(Sono 1989)
4-phenylimidazole (PIM); Norhaarman		(Sono & Cady 1989; Sugimoto et al. 2006)
Indol-2-yl ethanones		(Dolusic et al. 2011; Dolusic & Frederick 2013; Dolusic et al. 2013)
Tryptoline derivatives	-	(Tanaka, M et al. 2013)

This table shows some of the proposed IDO inhibitors, sources and their references. 1-Methyl-L-tryptophan (L-1MT).

molecules. Secondly, there is a need to understand IDO inhibitory kinetics, as some IDOs inhibitors are competitive inhibitors (Hydroxyamidines), other are uncompetitive inhibitors (PIM derivatives) and a third group has redox activity (quinone-type derivatives). Thirdly, promising *in vitro* results need to show matching effective results *in vivo* with appropriate physiochemical and bioavailability without toxicity. Finally, the recent discovery of TDO expression in many cancer cells has further highlighted the need to discover combined IDO/TDO inhibitors. For example, when 104 human tumor lines were examined for IDO/TDO expression, 20 expressed TDO only, 17 expressed IDO only and 16 expressed both IDO and TDO, including colorectal carcinoma, glioblastoma, mesothelioma, head and neck carcinoma, pancreatic adenocarcinoma, non-small-cell lung carcinoma, bladder and renal carcinomas (Pilotte et al. 2012; Dolusic & Frederick 2013).

Thus inhibiting both TDO and IDO may result in improvement in cancer immunotherapy. The design and discovery of single or combined IDO/TDO inhibitors is thus important for better effective treatments of cancer and other diseases such as neurodegenerative (Miller et al. 2004; Wu et al. 2013), inflammatory, autoimmune and infectious diseases (Di Pucchio et al. 2010).

1.3.7 Post-translation modification of IDO

One important mechanism that affects the activity of any protein is its post-translational modifications (PTMs). PTMs can change a protein's physical or chemical properties, conformation, activity, cellular location or stability. Many proteins are changed by the addition or removal of a chemical group on either an amino acid or the N- or C-terminus. Some PTMs can be added and removed dynamically as a mechanism for reversibly controlling protein functions (Fujigaki, H, Seishima & Saito 2012). Thus, identifying the PTMs sites in IDO and fully understanding their biological roles is essential in developing or modifying any IDO inhibitors. The PTMs of IDO remain largely unknown. Only a few important PTMs of IDO have been found, for example nitration, N-terminal acetylation, and phosphorylation.

1.3.7.1 Nitration and inactivation of IDO

Activated macrophages can generate large amounts of NO and superoxide (Beckman & Koppenol 1996; Gobert et al. 1998). Both compounds combine to produce the more reactive peroxynitrite anion. Peroxynitrite is produced by inflammatory cells to defend against many pathological conditions including cancer, parasites, viruses and

bacterial infections (Ischiropoulos et al. 1992; Denicola et al. 1993; Brito et al. 1999). IDO activity can be regulated by its PTMs (Fujigaki, H, Seishima & Saito 2012). Peroxynitrite inhibits IDO activity via the nitration of tyrosine residues (Tyr15, Tyr345, and Tyr353) of IDO. This inhibition occurs at the post-translational level since peroxynitrite inhibits IDO enzyme activity without affecting the expression of IDO protein. Importantly, IDO-1 and IDO-2 proteins share identical nitration-targeted tyrosine residues. Thus IDO-2 activity may also be regulated by nitration of these tyrosine residues However, further experiments are needed to verify nitration of IDO2 (Fujigaki, H, Seishima & Saito 2012).

1.3.7.2 N-terminal modifications of IDO

To further analyse the PTMs of IDO, the enzyme was isolated from IFN-γ stimulated THP-1 cells by immune precipitation and the PTMs analysed using mass spectrometry (Fujigaki, H, Seishima & Saito 2012). The fragmentation spectrum showed that methionine had been removed from the N-terminus of IDO and the N-terminal alanine had been acetylated. The N-terminus of IDO was identified only in the cleaved methionine and acetylated forms (Fujigaki, H, Seishima & Saito 2012). During protein synthesis and maturation, the N-terminal protein sequences of both intracellular and extracellular proteins undergo a number of modifications (Driessen et al. 1985). The methionine at the N-terminus is cleaved from the growing chains of most eukaryotic proteins (Bradshaw, Brickey & Walker 1998) followed by acetylation of the N-terminal group which occurs in 80–90% of all soluble proteins (Polevoda & Sherman 2003). This acetylation of the N-terminal group occurs either at transcription or posttranslationally, after the removal of the N-terminal methionine. The widespread occurrence of N-acetylation suggests its important biological role, however, its exact function is still unknown (Fujigaki, H, Seishima & Saito 2012). Several hypotheses have been proposed to explain the biological function of this acetylation. One suggests that this N-acetylation exerts an influence on protein stability (Hwang, Shemorry & Varshavsky 2010; Scott et al. 2011) and protects IDO from proteolysis.

1.3.7.3 IDO Phosphorylation

Two tyrosine residues in mouse IDO, Tyr115 and Tyr253, can be phosphorylated (Orabona et al. 2008). The phosphorylated IDO subsequently can bind to SOCS3 forming an IDO/SOCS3 complex. This interaction leads to ubiquitination and subsequent

proteasomal degradation (Orabona et al. 2008). This is just one mechanism of modulating the IDO immunosuppressive effect (Figure 1-5) (Fujigaki, H, Seishima & Saito 2012).

1.3.8 Physiological inducers

One of the most potent *in vitro* and *in vivo* inducers of IDO is IFN-γ, which is produced following T-cell activation in both innate and adaptive immune responses (Ghiringhelli et al. 2004). IFN-γ mediates its effect through the Janus kinases (JAK1/2) and signal transducer and activator of transcription (STAT1) signalling pathway. Consistent with this, the *INDO1* promoter contains multiple sequence elements that confer responsiveness to IFN-γ (King & Thomas 2007). STAT1 and IFN-regulatory factor-1 (IRF1) function cooperatively to mediate the induction of IDO expression in response to IFN-γ (Chon et al. 1995). IFN-α and IFN-β also induce IDO although they are markedly less efficient, requiring 10–100 greater levels to elicit the same response (Darnell, Kerr & Stark 1994; Leonard & O'Shea 1998; Platanias 2005).

Another important inducer of IDO is LPS, which is found in the cell wall of gram negative bacteria (Fujigaki, S et al. 2001). The LPS mediated induction of IDO is through an IFN-γ independent pathway and is transduced through the activities of the p38 mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) pathways (Lu, Yeh & Ohashi 2008). LPS activates NF-κB via toll-like receptor 4 (TLR4). TLR4 depends on other co-receptors for full ligand sensitivity. In this case, TLR4 recognition of LPS requires MD-2. CD14 and LPS binding protein (LBP) are known to facilitate the presentation of LPS to MD-2 (Tobias, Soldau & Ulevitch 1986; Wright, SD et al. 1989; Gioannini & Weiss 2007). CD14, which is highly expressed by macrophages, neutrophils and monocytes, can only bind LPS in the presence of LBP (Wright, SD et al. 1989; Wright, SD et al. 1990). TLR4 signalling has been divided into MyD88-dependent and MyD88-independent (TRIF-dependent) pathways. The MyD88-dependent pathway was shown to be responsible for pro-inflammatory cytokine expression, while the MyD88-independent pathway mediates the induction of Type I interferon and interferon-inducible genes (Fitzgerald, Rowe & Golenbock 2004; Miyake 2007).

Another inducer of IDO is prostaglandin E_2 (PGE_2) (von Bergwelt-Baildon et al. 2006). PGE_2 is a metabolic derivative of the polyunsaturated fatty acid (PUFA) arachidonic acid (AA) through the cyclooxygenase pathway (COX1/2) (von Bergwelt-Baildon et al. 2006; Lanzinger et al. 2012). It is the most abundant PG and the most studied PUFA metabolite due to its many important biological and sometimes opposing roles in immune

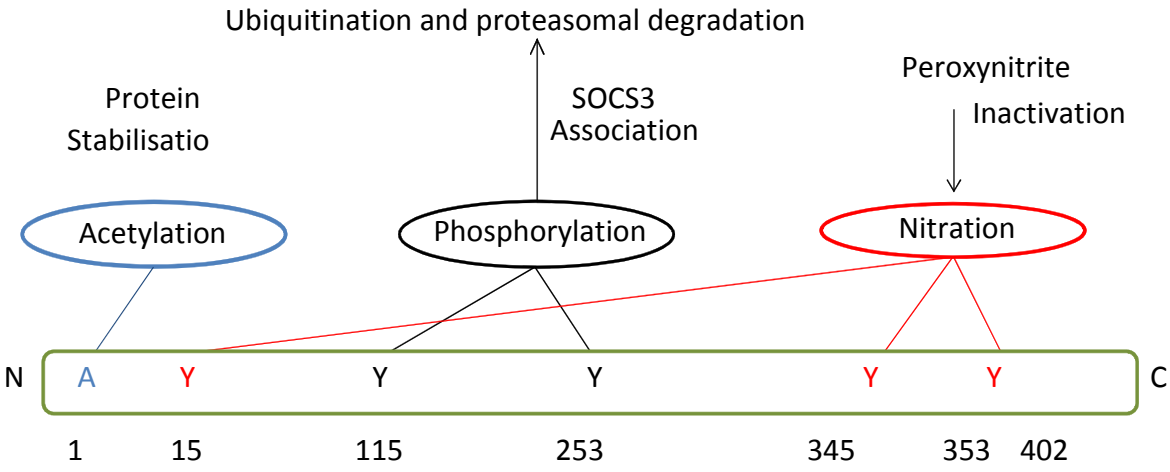


Figure 1-5 Post-translational modifications of IDO

The bar represents the IDO polypeptide. The position of modification sites is indicated. The N-terminal methionine is cleaved, and the resulting N-terminal alanine is acetylated (Fujigaki, H, Seishima & Saito 2012). Nitration of IDO at Tyr15, Tyr345 and Tyr353 by peroxynitrite inhibits IDO activity (Fujigaki, H, Seishima & Saito 2012). Phosphorylation of murine IDO at Tyr115 and Tyr253 leads to its association with suppressor of cytokine signalling 3 (SOCS3) and its subsequent ubiquitination and proteasomal degradation. (Adapted from Fujigaki, H, Seishima & Saito 2012).

modulation (Thurnher 2007; Legler et al. 2010). It is a lipid messenger released during inflammation and can activate four G-protein coupled receptors, EP1–EP4. Experimentally, PGE_2 is used in a cocktail to induce monocyte-derived DCs maturation, as part of cancer immunotherapy. PGE_2 can significantly increase *INDO1* expression, however, another signal through the TNF receptor (TNF-R) or toll-like receptor (TLR) is necessary for full IDO enzymatic activity. The effect of PGE_2 is mediated by activation of adenylate cyclase (AC) via the EP2 receptor (Braun, Longman & Albert 2005).

1.3.8.1 IFN-γ mediated induction of IDO

Since IFN-γ is the main physiological inducer of IDO, it is important to briefly discuss the main signal transduction pathway that is used to induce IDO. Binding of IFN-γ to its receptors (IFN-γ R1 & IFN-γ R2) induces receptor phosphorylation and dimerisation, which is followed by auto-phosphorylation of JAK1 and JAK2, which are associated with the two receptors respectively. Once activated, JAK1 and JAK2 phosphorylate STAT1 on tyrosine 701 (Tyr^{701}). Activation of STAT1 leads to their dimerisation and nuclear translocation and DNA binding to the ISRE and GAS, resulting in the transcriptional regulation of *INDO1* (Figure 1-6) (Darnell, Kerr & Stark 1994; Leonard & O'Shea 1998; Platanias 2005). IFN-γ is also able to mediate some activity independently of this signal transduction cascade, such as the p38 MAPK-dependent pathway on Ser^{727} in response to IFN-γ mediated signals (Wen, Zhong & Darnell 1995; Valledor et al. 2008). However, the action of IFN-γ through this pathway is less well characterised, and will not be discussed further.

1.4 Fatty acids (FAs)

The role of fatty acids (FAs) in health and disease has received the attention of researchers over many decades (Riediger et al. 2009). FAs are important constituents in all cells as they affect cell membrane stability, permeability and fluidity. FAs also affect protein function, energy provision, regulation of cell signalling and gene expression (Simopoulos 1999; Calder 2006b, 2008; Patterson et al. 2012). Many studies have shown that FAs have important roles in infant development (Dunstan et al. 2008), cardiovascular disease (Colussi et al. 2007; Kelley et al. 2007), mental illness (Hallahan et al. 2007), dementia and Alzheimer's disease (Boston et al. 2004), inflammation (Riediger et al. 2009; Patterson et al. 2012), platelet aggregation (Patterson et al. 2012), hypercholesterolemia (Riediger et al. 2009; Patterson et al. 2012), hypertension and both positive and negative

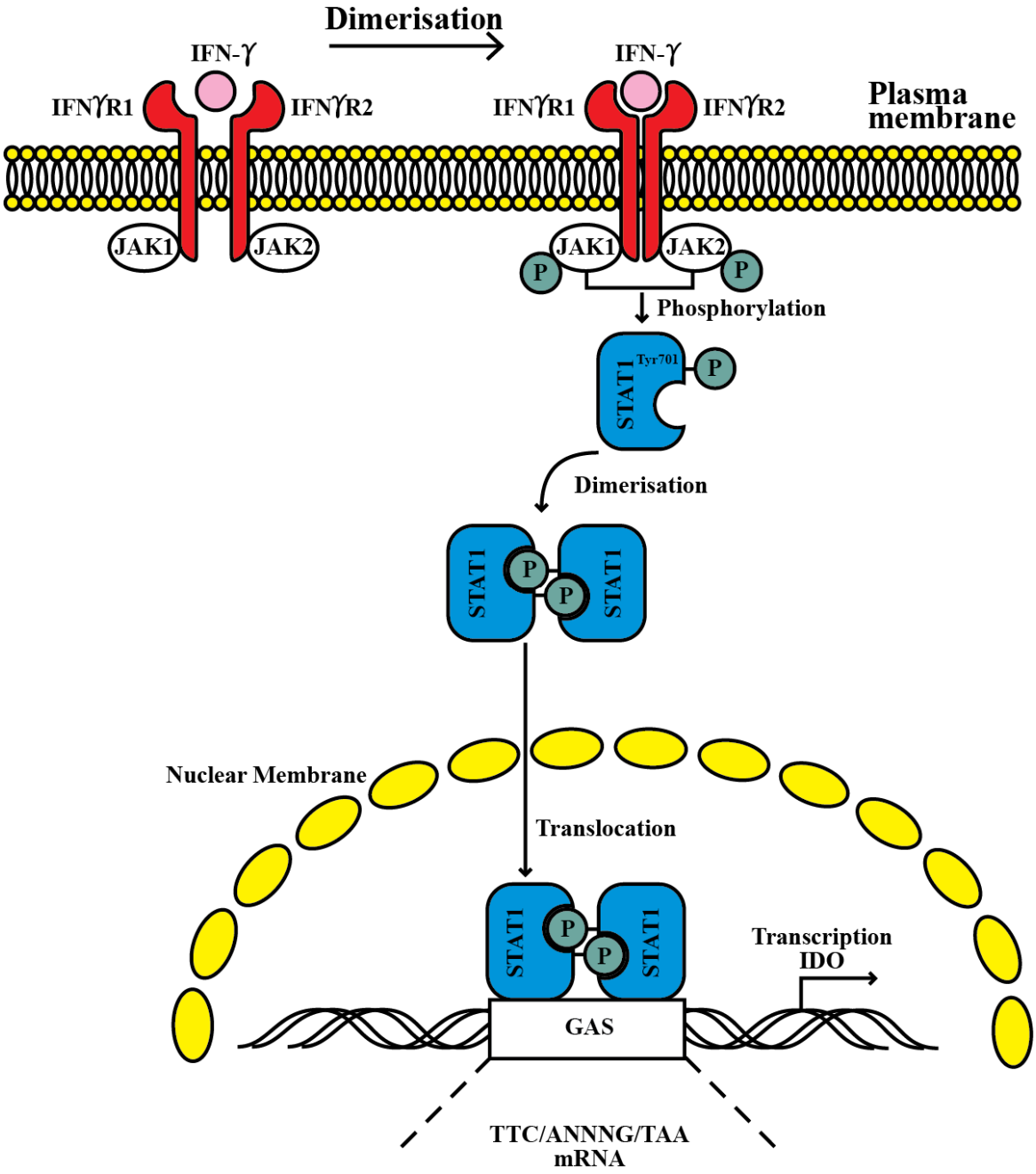


Figure 1-6 The classical signal transduction pathway of IFN-γ

Binding of IFN-γ to the IFN-γ R1 and IFN-γ R2 receptors induces receptor aggregation. This leads to auto-phosphorylation of JAK1 and JAK2 which in turn phosphorylate (P) STAT1 on tyrosine 701 (Tyr^{701}). Phosphorylated STAT1 dimerises and migrates to the nucleus where it binds to γ-interferon activated sequences (GAS, TTC/ANNNG/TTA) on DNA, resulting in the transcriptional regulation of genes. (Adapted from Aaronson & Horvath 2002; Platanias 2005).

effects on cancer progression (Riediger et al. 2009). Recently, additional effects of FAs on IDO have been identified. We demonstrated that AA can inhibit IDO induction in THP-1 cells (Bassal 2008; Bassal, Hughes & Costabile 2012). Braun et al. (2005) in their analysis of DCs maturation revealed a strong up-regulation of IDO in the presence of PGE_2, and TNF-α (Braun, Longman & Albert 2005; Jiang et al. 2010) Jiang et al. (2010) demonstrated that sodium butyrate (NaB) down-regulated IDO via both transcriptional and post-transcriptional mechanisms (Jiang et al. 2010). Jung et al. (2010) reported that curcumin inhibits IDO protein expression via the COX2/PGE_2-dependent pathway in LPS-stimulated murine bone marrow derived dendritic cells (BMDCs) (Jung et al. 2010).

1.4.1 Lipids and fatty acids

FAs, the basic units of lipids, are essential components of all cells. They are stored in adipose tissue and perform a number of important functions. A FA is a hydrocarbon chain with a carboxyl group at one end and a methyl group at the other (Calder 2008). The hydrocarbon chain is non-polar and hydrophobic, while the carboxyl group is polar, hydrophilic, and reactive and forms ester links with alcohol groups. The biological activities of FAs are affected by chain length, the number and the position of the double bonds as well as their orientation. When two or more double bonds are present, unsaturated fatty acids are called polyunsaturated fatty acids (PUFAs) (Calder 2008). FAs have major roles in any cell as follows:

1. are the building blocks of cell membrane phospholipids and glycolipids (Simopoulos 1999; Riediger et al. 2009; Patterson et al. 2012)
2. modify proteins to target them to the cell membrane (Simopoulos 1999).
3. are cellular fuels which when fully metabolized liberate 2.5 times as much ATP per gram compared to the metabolism of an equivalent amount of proteins or carbohydrates (Lehninger 1993).
4. metabolism into eicosanoids, which act as second messengers (Calder 2008; Patterson et al. 2012).
5. induce different types of cytokines that are both pro-inflammatory and anti-inflammatory (Calder 2008).

1.4.2 Nomenclature

The systematic name of a FA originates from its parent hydrocarbon by removing the final *e* and replacing it with *oic*. For example, C18, octadecan*e* is octadecan*oic* acid. Second, the name includes the number of double bonds the FA has, for example, C18 with one double bond is octade*cen*oic acid, C18 with two double bonds is octadeca*di*enoic acid and C18 with three double bonds is octadeca*tri*enoic acid. Third, the name also includes a notation to specify the number of carbon atoms, for example, 18:0 indicates a FA with 18 carbon atoms and 0 double bonds, while 18:2 indicates a FA with 18 carbon atoms and 2 double bonds (Calder 2008; Berg, JM, Tymoczko & Stryer 2012). There are two alternate forms of nomenclature for numbering the position of the double bonds in a FA. The first method denotes the locations of the double bonds which are present in the molecule, starting from the carboxyl end, for example arachidonic acid (AA) is 5,8,11,14-eicosatetraenoic acid. The second method uses three descriptive points to relay structural information which are: the number of carbon atoms present in the molecule, the number of double bonds and the carbon number from which the first double bond is located from the methyl or omega (ω) end. Thus AA is 20:4,n-6 (vas Dias 1984; Berg, JM, Tymoczko & Stryer 2012). At least three principal series of unsaturated fatty acids exist, depending on the position of the first double bond. Fatty acids with the first double bond on the third, sixth or ninth carbon atom from the methyl end are referred to as n-3, n-6 and n-9 fatty acids respectively (Figure 1-7).

1.4.3 Saturation and chain length

FAs usually contain an even number of carbon atoms and may be saturated fatty acids (SAFAs) where all carbon atoms are linked by a single bond (Table 1-3) or unsaturated fatty acids. Monounsaturated fatty acids (MUFAs) have a single double bond and polyunsaturated fatty acids (PUFAs) have more than one methylene-interrupted double bond present (Table 1-4). FAs can also be categorised as short, medium, long or very long according to the carbon chain length. Short chain FAs have less than 6 carbon atoms, medium chain FAs have 6–12 carbon atoms, long chain FAs have more than 12 carbon atoms and very long chain FAs have more than 22 carbon atoms (Sanders et al. 2006). The most common FAs have 16 or 18 carbon atoms. Of the FAs series found in nature, the most common are n-6 and n-3 (Wall et al. 2010). The human body can produce all but two essential FAs, namely, linoleic acid (LA, 18:2,n-6), the precursor to the n-6 series of FAs, as well as α-linolenic acid (ALA, 18:3,n-3), the precursor to the n-3 series of FAs.

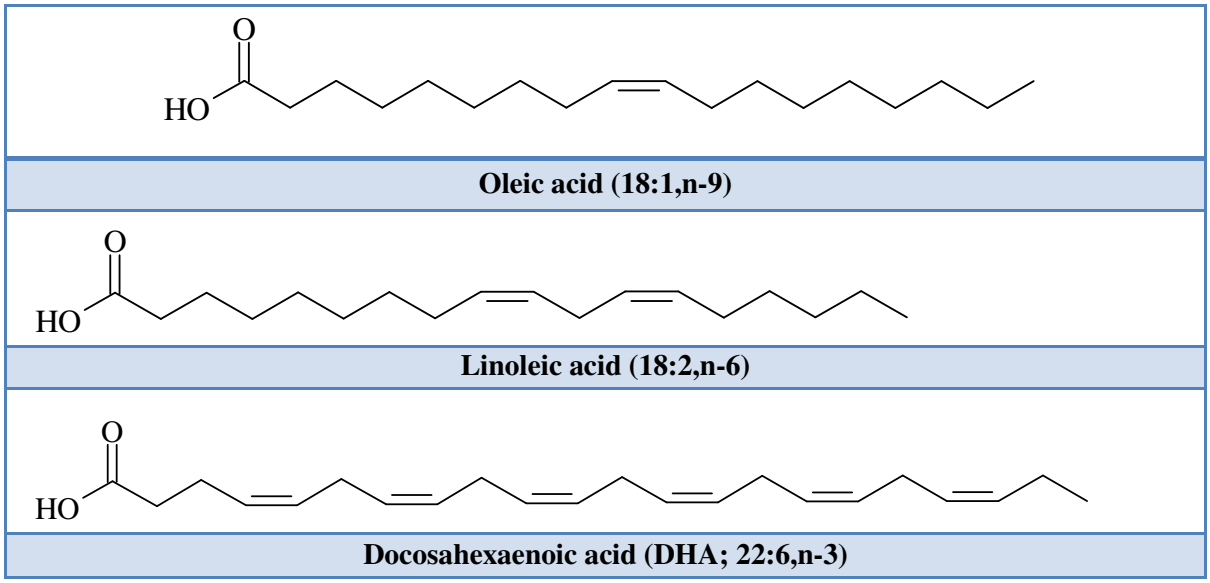


Figure 1-7 Examples of fatty acids with n-9, n-6 and n-3 series

The series of FAs is determined by the position of the first double bond from the omega or methyl end of the fatty acid. Fatty acids with the first double bond on the third, sixth or ninth carbon atom from the methyl end are referred to as n-3, n-6 and n-9 fatty acids respectively. (Adapted from Calder 2008).

Table 1-3 Examples of common saturated fatty acids

Common FAS name	Chemical structure	C:DB
Butyric	$CH_3(CH_2)_2COOH$	4:0
Hexanoic	$CH_3(CH_2)_4COOH$	6:0
Octanoic	$CH_3(CH_2)_6COOH$	8:0
Decanoic	$CH_3(CH_2)_8COOH$	10:0
Lauric	$CH_3(CH_2)_{10}COOH$	12:0
Myristic	$CH_3(CH_2)_{12}COOH$	14:0
Palmitic	$CH_3(CH_2)_{14}COOH$	16:0
Stearic	$CH_3(CH_2)_{16}COOH$	18:0
Arachidic	$CH_3(CH_2)_{18}COOH$	20:0
Behenic	$CH_3(CH_2)_{20}COOH$	22:0
Lignoceric	$CH_3(CH_2)_{22}COOH$	24:0

FAs, fatty acids; C, number of carbon atoms; DB, number of double bonds. (Adapted Calder 2008).

Table 1-4 Examples of common monosaturated and polyunsaturated fatty acids

Fatty acid name	Chemical structure	Configuration-Δ^x	C:DB	Series
Myristoleic	$CH_3(CH_2)_3CH=CH(CH_2)_7COOH$	*cis*-Δ^9	14:1	n-5
Palmitoleic	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	*cis*-Δ^9	16:1	n-7
Oleic	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	*cis*-Δ^9	18:1	n-9
Elaidic	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	trans-Δ^9	18:1	n-9
Vaccenic	$CH_3(CH_2)_5CH=CH(CH_2)_9COOH$	*cis*-Δ^{11}	18:1	n-7
Linoleic	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	*All cis*-Δ^9,Δ^{12}	18:2	n-6
Linoelaidic	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	All trans-Δ^9,Δ^{12}	18:2	n-6
α-Linolenic	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	*All cis*-$\Delta^9,\Delta^{12},\Delta^{15}$	18:3	n-3
Arachidonic	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	*All cis*-$\Delta^5\Delta^8,\Delta^{11},\Delta^{14}$	20:4	n-6
Eicosapentaenoic	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	*All cis*-$\Delta^5,\Delta^8,\Delta^{11},\Delta^{14},\Delta^{17}$	20:5	n-3
Erucic acid	$CH_3(CH_2)_7CH=CH(CH_2)_{11}COOH$	*cis*-Δ^{13}	22:1	n-9
Docosahexaenoic	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$	*All cis*-$\Delta^4,\Delta^7,\Delta^{10},\Delta^{13},\Delta^{16},\Delta^{19}$	22:6	n-3

Δ^x (delta x), location of the double bond; C, number of carbon atoms; DB, number of double bonds; n-x series, the location of the first double bond starting from the omega carbon atom. (Adapted from Calder 2008).

Thus, these FAs must be supplied in the diet (Patterson et al. 2012).

1.4.4 Configuration

The double bonds in MUFAs and PUFAs can exist in either the *cis* (Z) or *trans* (E) configuration. In the *Z* configuration, adjacent hydrogen atoms are on the *same* side of the double bond. The rigidity of the double bond freezes its conformation and causes the chain to bend and restricts the conformational freedom of the FAs. The more double bonds the chain has in the *Z* configuration, the less flexibility it has. When a chain has multiple *Z* bonds, it can achieve a curved configuration (Figure 1-8). For example, oleic acid with one double bond is bent, whereas LA with two double bonds has a more pronounced bend and ALA with three double bonds favors a hooked shape. In restricted environments, such as when FAs are components of membrane phospholipids, *Z* bonds limit the ability of FAs to be closely packed, affecting the FA melting temperature (Berg, JM, Tymoczko & Stryer 2012). In contrast, in the *E* configuration the two hydrogen atoms are bound to *opposite* sides of the double bond so they do not bend and their shape is similar to saturated FAs. *E* bonds allow FAs to be closely packed, lowering their melting temperatures compared to the Z bond configuration. In most naturally occurring unsaturated fatty acids, each double bond is followed by three carbon atoms, all of which are in the *Z* configuration. Most FAs in the E configuration are not found in nature, instead they are synthetically produced. The differences in configuration between the various types of FAs play an important role in their biological functions (Calder 2008).

1.4.5 Sources

The level of specific FAs in an organism is dependent on dietary intake and *de novo* synthesis by desaturases, elongases and other enzymes (Chen, Y, Perussia & Campbell 2007; Chen, YQ et al. 2007). The level of specific FAs is also affected by individual differences due to polymorphisms in the genes encoding the metabolic enzymes, which subsequently affect the resulting metabolites (Simopoulos 2010). FAs can be obtained from the diet or via *de novo* synthesis. Dietary FAs are obtained from many sources particularly animal meats, leafy green vegetables, nuts, oils and fish (Kinsella 1990). Certain dietary sources are particularly enriched in certain classes of FAs. Animal meats generally contain large amounts of saturated FAs. Oleic acid is abundant in olive oil

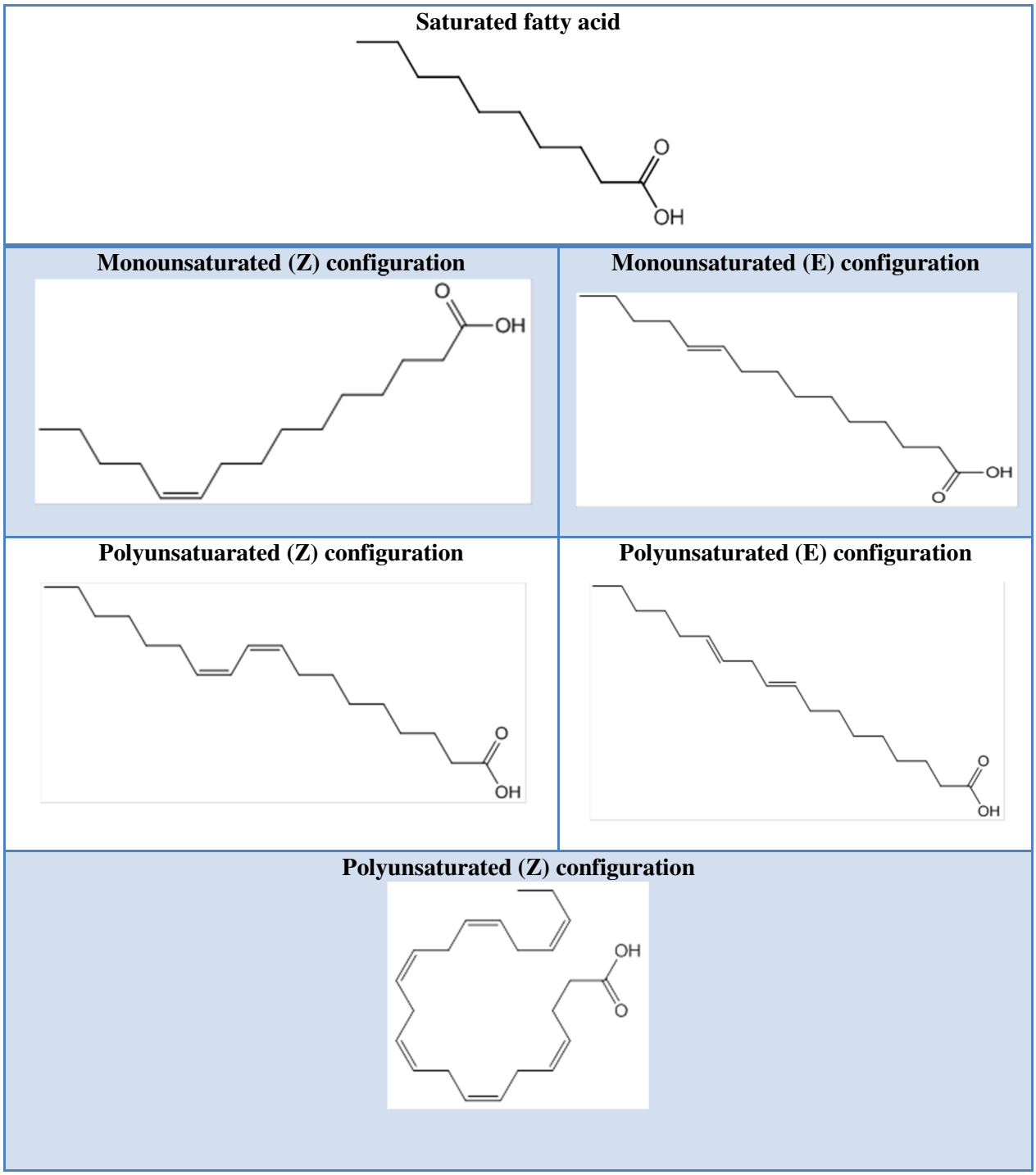


Figure 1-8 Examples of saturated, monosaturated and polyunsaturated fatty acids in the Z or E configuration

(Adapted from Enzo Life Sciences library).

(18:1,n-9), while corn, safflower and sunflower oil are rich in LA (18:2,n-6), linseed oil is rich in ALA (18:3,n-3), and evening primrose oil contains γ-LA (20:3,n-6) (Calder & Newsholme 1993). Fish oil is particularly rich in eicosapentaenoic acid (EPA) (20:5,n-3) and docosahexaenoic acid (DHA) (22:6,n-3) (Simopoulos 1999). The main dietary sources of LA include lean meat, cereals, and wholegrain bread and vegetable oils, for example sunflower, safflower, and corn oils. Rich dietary sources of ALA include green leafy vegetables, flaxseed, rapeseed oils and fatty fish (Sinclair 1991; Wall et al. 2010). Both LA and ALA can be metabolised to other fatty acids by addition of double bonds and elongation of the acyl chain to form the long chain PUFAs, AA (n-6) or EPA (n-3) and DHA (n-3) respectively (Figure 1-9) (Calder 2008).

1.4.6 Metabolism

The metabolism of 20 carbon atom PUFAs can occur via cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 pathways. As cells contain a high portion of n-6 PUFAs, AA (20:4,n-6) will be the focus of this research. The metabolism of AA produces prostaglandins (PGs), prostacyclins, thromboxanes (TXs) and leukotrienes (LTs), which are collectively called eicosanoids. They have a short life and are considered as hormones as they alter the activities of cells they are synthesised by as well as their neighbouring cells. They can modulate the intensity and duration of inflammation (Lewis, Austen & Soberman 1990; Tilley, Coffman & Koller 2001) and most frequently they have opposing effects, for example anti-inflammatory and pro-inflammatory (Calder 2008). Their action can vary depending on the cells they are produced in, the stimuli that lead to their production, the timing of their production, their concentration, the type and expression of the receptors they activate, their affinity to bind to those receptors and the sensitivity of the target cells and tissues they affect (Calder 2008).

PGs contain a 5-carbon atom ring and are produced by reductase and isomerase enzymes. Prostacyclins and TXs are produced from further metabolism of PGs and are generated by prostacyclin synthase and thromboxane synthase respectively. LTs are produced from AA by the action of 5, 12 or 15- lipoxygenase enzymes (Figure 1-10) (Calder 2008; Berg, JM, Tymoczko & Stryer 2012).

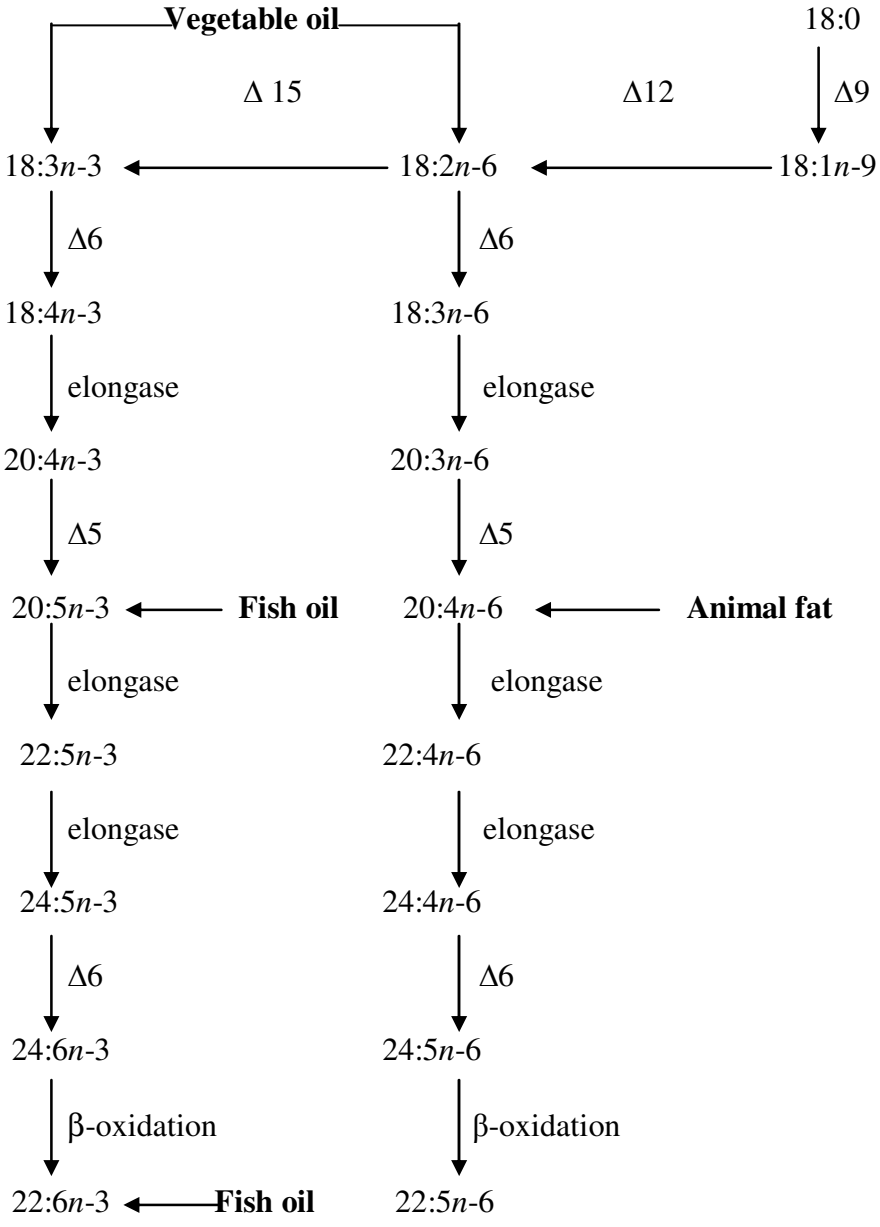


Figure 1-9 Pathway involved in the synthesis of n-3, n-6 and n-9 fatty acids

The symbol Δ and the following number indicate the position of the first double bond. (Adapted from Calder 1997).

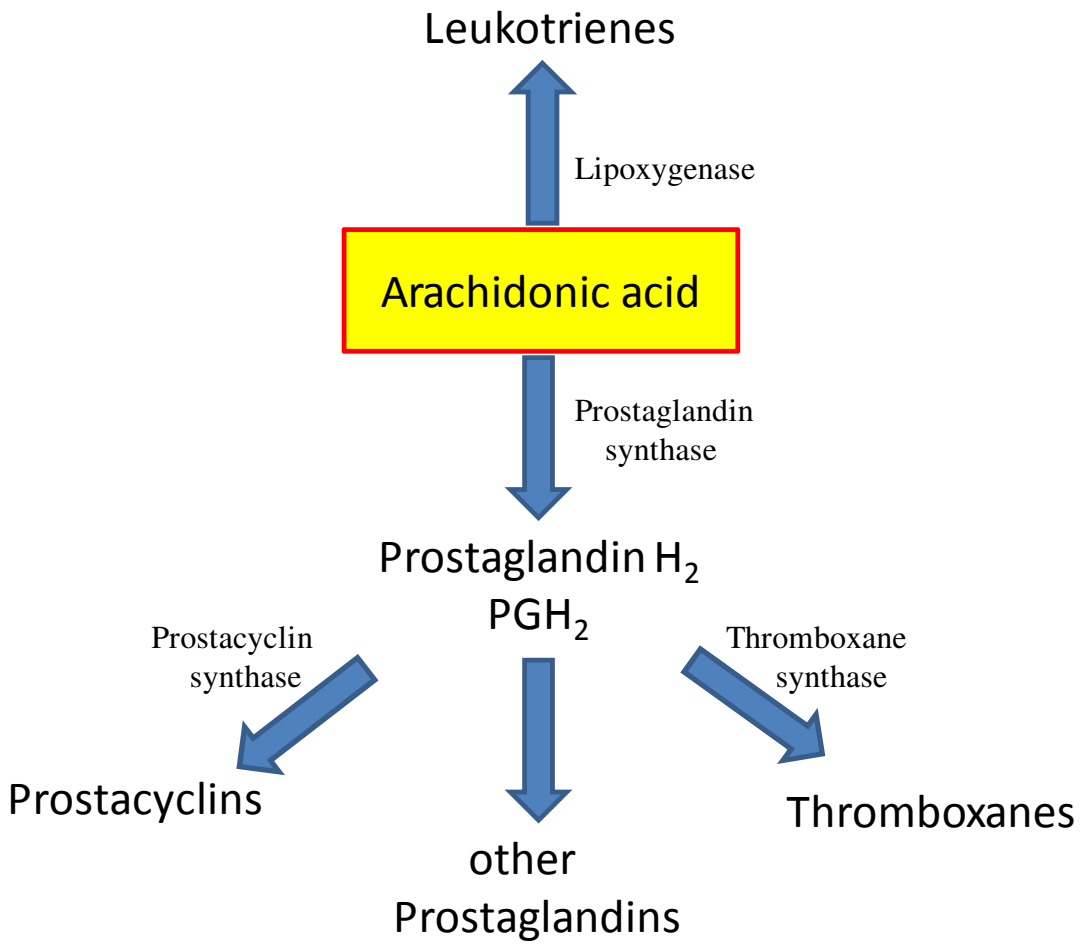


Figure 1-10 Arachidonic acid (AA) is the major precursor of eicosanoid hormones

Prostaglandin synthase catalyses the first step in a pathway leading to prostaglandins (PGs); prostacyclins by prostacyclin synthase and thromboxanes (TXs) by thromboxane synthases while lipoxygenase catalyzes the initial step in a pathway leading to leukotrienes (LTs). (Adapted from Berg, JM, Tymoczko & Stryer 2012).

1.4.6.1 Cyclooxygenase pathway (COX)

The general pathway for biosynthesis of PGs starts with the release of esterified AA from plasma membrane phospholipids via the action of phospholipase A_2. AA is then converted to PGG_2 and PGH_2 by the cyclooxygenase (COX) and peroxidase activity of PGH synthase. The two forms of PGH synthase are PGH synthase 1 (COX1) and PGH synthase 2 (COX2) (Straus & Glass 2001). COX1 is constitutively expressed in many tissues and is considered a house keeping enzyme. The PGs that are formed via COX1 primarily act in normal physiological conditions, for example homeostasis, renal water retention and gastric acid secretion (Straus & Glass 2001). In contrast, COX2 can be expressed constitutively in the brain and kidney, and can be induced by inflammatory cytokines causing pain, fever and inflammation, or induced during ovulation and implantation (Kang et al. 2007). Prostaglandin synthase catalyses the first step in a pathway leading to PGs, prostacyclin and TXs (Serhan & Sheppard 1990). PGH_2 is then converted to PGD_2, PGE_2, PGI_2, TXA_2 or $PGF_{2\alpha}$ by specific synthase enzymes (Figure 1-11) (Straus & glass 2001). The cyclopentane PGs, PGE_2 and PGD_2, can be further dehydrated to the cyclopentenone PGA_2 and PGJ_2 respectively. PGD_2 also can be transformed to 15-deoxy $\Delta^{12,14}$-PGJ_2 in the presence of albumin *in vitro*, while PGJ_2 isomerizes to yield Δ^{12}-PGJ_2 (Straus & Glass 2001).

1.4.6.2 Lipoxygenase pathway (LOX)

The second pathway of FA metabolism is the lipoxygenase (LOX) pathway. Lipoxygenase enzymes catalyse the initial step in a pathway leading to LTs (Calder 2008; Berg, JM, Tymoczko & Stryer 2012). The products of the LOX pathway include hydroperoxy, hydroxy and dihydroxy FAs and LTs including LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4. The letter following the abbreviation LT denotes the nature and position of the oxygen containing substituent and the numerical subscript indicates the number of double bonds present. Three LOX enzymes exist, which can introduce oxygen atoms into AA at positions 5, 12 and 15 (Ford-Hutchinson 1994) to give 5-lipoxygenase (5-LOX) (Jakobsson et al. 1995), 12-LOX (Reynaud & Pace-Asciak 1997) and 15-LOX (Borgeat & Samuelsson 1979) respectively. 5-LOX is present in neutrophils, eosinophils, basophils, monocytes, macrophages, mast cells and B-lymphocytes (Lewis & Austen 1984; Jakobsson et al. 1995). 12-LOX is present in platelets (Serhan & Sheppard 1990), neutrophils (Serhan & Brezinski 1991) and macrophages (Sun & Funk 1996),while

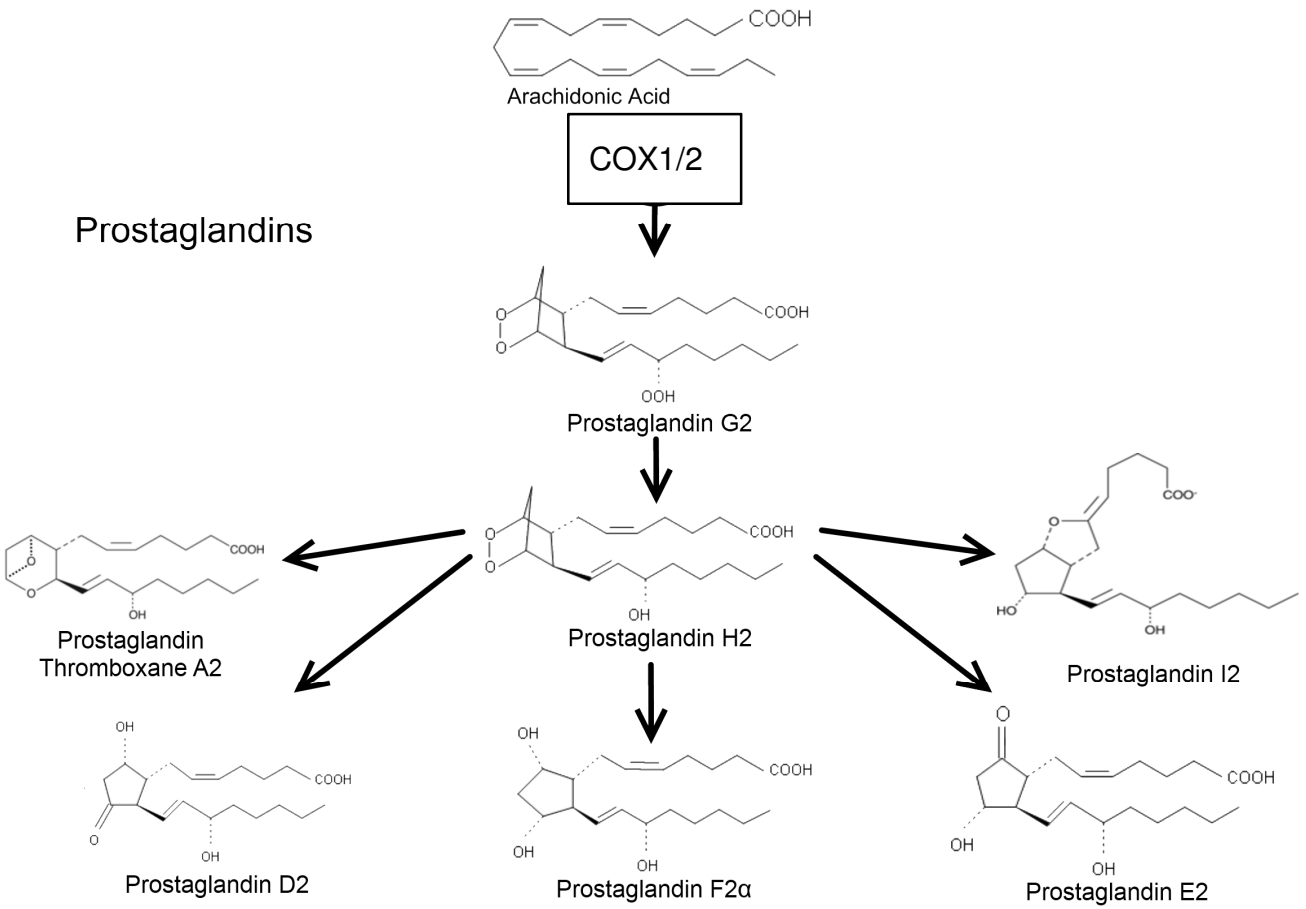


Figure 1-11 AA Cyclooxygenase pathway

AA is released from plasma membrane phospholipids via the phospholipase A_2. AA is then converted to Prostaglandin G_2 and Prostaglandin H_2 by PGH synthases (COX1 and COX2). PGH_2 is further metabolised to Prostaglandin Thromboxane A_2, Prostaglandin D_2, Prostaglandin $F_{2\alpha}$, Prostaglandin E_2 and prostaglandin I_2. (Adapted from Straus & Glass 2001).

15-LOX is present in neutrophils (Chabannes et al. 2003), eosinophils (Turk et al. 1982), macrophages (Pawlowski et al. 1982) and platelets (Wong et al. 1985).

The first metabolic product of AA metabolism via the LOX pathway is hydroperoxy eicosatetraenoic acid (HPETE). Due to the presence of the OOH group, HPETE is rapidly converted to the more stable hydroxy fatty acid (HETE) either enzymatically or non-enzymatically (Figure 1-12) (Ford-Hutchinson 1994). Further addition of oxygen leads to the production of dihydroxy HETE, while dehydration of HPETE can lead to the formation of epoxy fatty acids. LTA_4 is the first leukotriene formed from the metabolism of AA and once formed it can undergo non-enzymatic hydrolysis to di-HETEs. In addition, LTA_4 is enzymatically converted to LTB_4 by a LTA_4 hydrolase (Ford-Hutchinson 1994). LTA_4 can also be converted to LTC_4 by LTC_4 synthase, which in turn can be converted to LTD_4 and finally to LTE_4 by a γ-glutamyltranspeptidase (Ford-Hutchinson 1994). 5, 12 and 15-LOX enzymes can also use hydroperoxy, hydroxy and epoxide containing compounds as substrates to produce lipoxins (LXs) (Serhan & Sheppard 1990; Serhan 1991; Calder 2006a, 2008).

1.4.6.3 Cytochrome P450

AA can also be metabolised by cytochrome P450 enzymes to produce a number of hydroxy and carboxy products, including 19-hydroxy, 19-oxo, 20-hydroxy and 20-carboxy metabolites (Capdevila et al. 1982). The main types of P450 products are epoxy acids, hydroxy and dihydroxy acid derivatives of AA (Oliw 1994). The formation of the cytochrome P450 derived products are probably of minimal importance in some tissues due to their extremely low levels, for example in the skin (Ford-Hutchinson 1990; Reiners et al. 1992; Ford-Hutchinson 1994). However, they have many important roles in the physiology of other tissues, for example kidney and cornea (Ford-Hutchinson 1990). Of the three pathways of FAs metabolism, COX, LOX and P450, the focus of this study will be on the AA COX pathway and its metabolites as our preliminary results and (Bassal 2008; Bassal, Hughes & Costabile 2012) showed that AA and its COX1/2 metabolites significantly inhibited IDO in THP-1 cells.

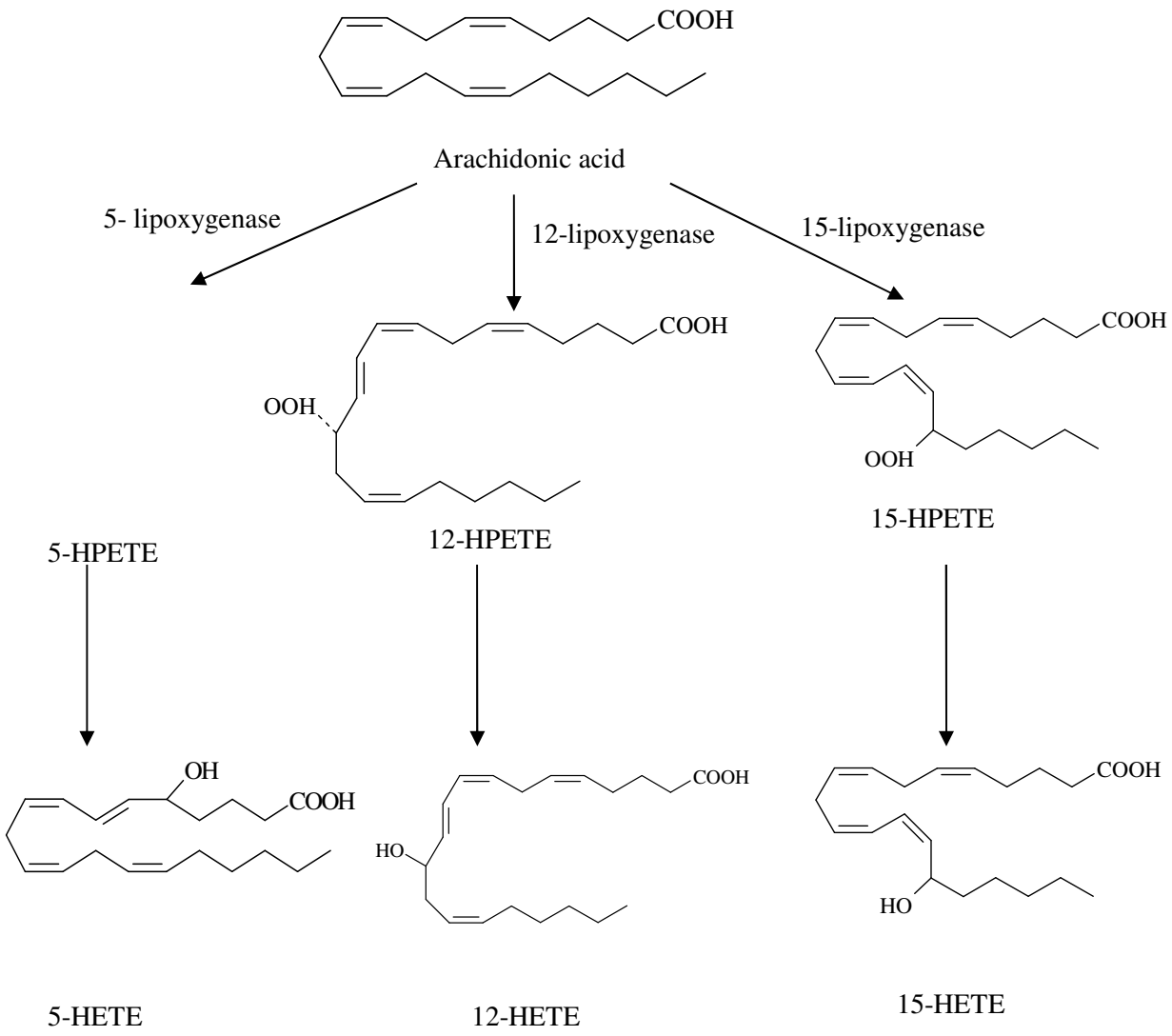


Figure 1-12 Metabolism of AA via the lipoxygenase pathway (LOX) pathway produces 5-HETE, 12-HETE and 15-HETE

AA metabolism via the LOX pathway produces: hydroperoxy eicosatetraenoic acid (HPETE) and more stable hydroxy fatty acids (HETE). (Adapted from Ford-Hutchinson 1994; Calder 1997, 2008).

1.4.6.4 Prostaglandins

PGs are defined as molecules that possess a cyclopentane ring, a *trans* double bond between carbons 13 and 14 and a hydroxyl group on carbon 15. PGs include PGD, PGE, PGF, PGJ and PGI series (Bergstrom & Samuelsson 1968; Lands 1979). The letter following PG indicates the nature and location of the oxygen containing substituent in the cyclopentane ring (Willis 1981). Numerical subscripts are used to indicate the number of double bonds present in the molecule. *In vivo*, PGE_1 is derived from Dihomo-gamma-linolenic acid (DGLA 20:3n-6), PGE_2 from AA and PGE_3 from EPA (Willis 1981). Both DGLA and AA are equally good substrates for the COX pathway, however, the level of DGLA is about a third to a fifth that of AA (Willis 1981). Thus PGE_2 is the main PG produced. Both EPA and AA compete for the common COX and LOX enzymes (Riediger et al. 2009) and determine the eicosanoids produced. Thus the n-6: n-3 ratio is an important determining factor for the enzymatic pathways.

1.4.6.4.1 PGs mechanism of action

PGs are known to mediate their effect mainly via seven transmembrane G-protein coupled receptors (GPCRs). These receptors transduce signals from many kinds of stimuli, for example light, lipids and hormones. Activation of these receptors leads to activation of a specific G-protein, which in turn alters specific enzymatic signalling pathways resulting in different effects.

1.4.6.4.2 G-protein coupled prostanoid receptors

The mode by which PGs exert their biological actions depends on binding to their respective GPCRs. Although most prostaglandin GPCRs are localised in the plasma membrane of platelets, vascular smooth muscle cells and mast cells, some are situated at the nuclear envelope (Ide et al. 2003). Specific GPCRs have been identified for all prostanoids, with at least nine known prostanoid receptor forms in mice and humans (Narumiya & FitzGerald 2001; Hata & Breyer 2004). There are 4 PGE receptors, EP1–4, one PGF receptor, one PGI receptor and two PGD receptors. To activate these receptors, the appropriate ligand binds to a specific ligand binding domain. This domain can be extracellular or fixed in the plasma membrane. Now the GPCR can activate a trimeric guanosine-triphosphate (GTP)-binding protein. The G-protein consists of three subunits: Gα, Gβ and Gγ. Both Gα and Gγ are post-translationally modified to have a lipid tail that allows them to anchor to the plasma membrane. In the inactive state, Gα is bound to guanosine-diphosphate (GDP). Upon activation, GDP is exchanged for GTP leading to

activation of the Gα and the Gβ/γ complex. The Gα can either separate from Gβ/γ or remain attached to it. In both cases, the activated subunits can now regulate the target protein in the plasma membrane, for example they can activate adenylate cyclase (AC) or potassium and calcium ion channels. Then the GTP is hydrolysed to GDP (Sjogren, Blazer & Neubig 2010). The inactive Gα then reunites with the Gβ/γ complex and makes them inactive.

1.4.6.4.3 GPCRs signalling pathways

There are two types of Gα subunits leading to two signalling pathways: Gαs (stimulatory) and Gαi (inhibitory). Gαs stimulates the activity of certain forms of AC, a family of proteins that synthesise cyclic adenosine monophosphate (cAMP) from ATP. cAMP is degraded by phosphodiesterases (PDE) or it can activate two pathways: protein kinase A (PKA) or exchange protein activated by cAMP (Epac). PKA consists of a tetramer: two regulatory subunits and two catalytic subunits. If cAMP activates PKA, it binds to the regulatory subunit of PKA leading to dissociation of the two catalytic subunits. These catalytic subunits then phosphorylate downstream target proteins (Shabb 2001). PKA mainly phosphorylates cAMP responsive element binding protein (CREB), which binds the cAMP response element (CRE) and alters gene transcription. Alternatively, if cAMP activates Epac, GTP is replaced by GDP in Ras-related proteins (RAP), and Epac can modulate kinase signalling, mitogenesis and exocytosis (Patten et al. 2007). In humans, the Gαs consists of 17 subunits and all can be activated by cholera toxin.

The other signalling pathway of GPCR is through the Gαi inhibitory subunit. In this case, Gαi suppresses the generation of cAMP, blocking AC through Gαi subunits. The Gβ/γ dimers of Gαi dissociate and activate other cascades, for example phospholipase C (PLC) or K^+ channels (Figure 1-13). The Gαi consists of 8 subunits and all except one can be inhibited by pertussis toxin.

1.4.7 Biological effects of FAs

The biological effects of FAs depend on many factors including degree of unsaturation and series. Saturated FAs are believed to have no or less activity than MUFAs, which also have less activity than PUFAs. This section discusses the general biological effects of FAs and pays particular attention to PUFAs.

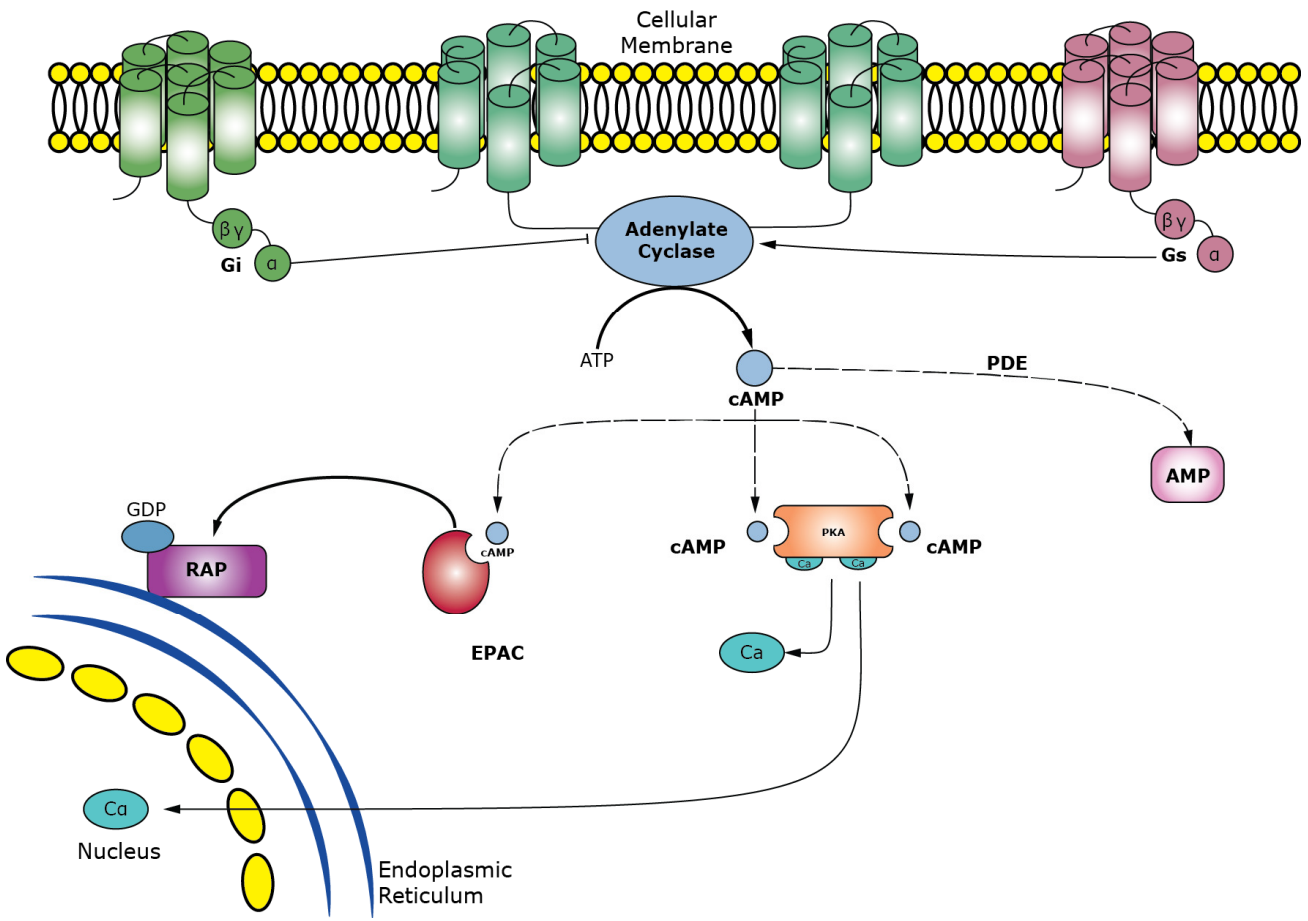


Figure 1-13 Signalling through Gαs and Gαi G-protein coupled receptors

AC, adenylate cyclase; PDE, phosphodiesterases; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; AMP, adenosine monophosphate; PKA, protein kinase A; Epac, exchange protein activated by cAMP; Ca, catalytic unite; GDP, guanosine-diphosphate; RAP, Ras-related protein. (Adapted from Cayman Chemicals Company Ann Arbor, MI, USA).

The roles of PUFAs, in particular of the n-3 and n-6 series in human health and disease, have been the focus of attention for many years (Riediger et al. 2009; de Lorgeril & Salen 2012; Patterson et al. 2012). A multitude of studies have shown many benefits ofn-3 PUFA intake, especially in infant development, cardiovascular diseases, mental illnesses, auto-immune diseases and cancer. The n-3 PUFAs also have multiple beneficial effects as anti-inflammatory mediators, inhibitors of platelet aggregation and reducing hypertension and hyperlipidemia (Riediger et al. 2009). In contrast, n-6 PUFAs have harmful effects as pro-inflammatory mediators, increasing the risk of many cancers, cardiovascular disease, obstructive lung disease and inflammatory bowel diseases (de Lorgeril & Salen 2012; Patterson et al. 2012). In Western countries, a trend to increase n-6 PUFA consumption has led to the production of AA-derived eicosanoids, which in most cases have pro-inflammatory properties.

These metabolites contribute to the pathology associated with numerous diseases, for example metabolic fatty liver diseases, cardiovascular diseases, obesity, inflammatory bowel disease, rheumatoid arthritis and Alzheimer's disease (Patterson et al. 2012). Although the effects of n-3 derived eicosanoids are also important, there have been fewer studies about their effects than the n-6 eicosanoids (Calder 2009).

Since both n-6 (mainly AA) and n-3 (mainly EPA and DHA) can be metabolised by COX1 and COX2 enzymes, the ratio of n-6:n-3 PUFAs in the diet directly affects the profile of eicosanoids produced, which have different or even opposing biological effects. As a result of these mediators, the biological effects of FAs in physiological and pathological conditions will vary. This section will focus on the roles of FAs and their eicosanoids in inflammation and cancer as they are related to this study.

1.4.7.1 Inflammation

Inflammation is a normal defence mechanism that occurs in response to infection, injury or abnormal stimuli. Signs of inflammation include pain, redness, heat, oedema and loss of function. Localised acute inflammation facilitates the elimination of pathogens and leads to tissue repair and the restoration of homeostasis. The inflammatory response is highly complex, induced by multiple mediators acting in concert. These mediators are produced by different cell types. Normally, the inflammatory response is well regulated, self-limited by negative feedback mechanisms and resolves rapidly. When self-regulating mechanisms fail to resolve acute inflammation, chronic inflammation ensues and can lead to pathological disease and consequent damage to host tissues, for example Rheumatoid

arthritis, Crohn's disease, Ulcerative colitis, Type-1 and Type-2 diabetes and cancer cachexia (Calder 2009).

1.4.7.1.1 FAs and inflammation

FAs, especially the n-6 PUFA, AA and the n-3 PUFAs, EPA and DHA, are precursors of eicosanoids. These molecules play important roles in inflammation. In any inflammatory condition, both n-3 and n-6 PUFAs interact together and the result determines the outcome of these inflammatory conditions. Generally, n-6 PUFA derived eicosanoids and leukotrienes have pro-inflammatory effects, while those derived from n-3 PUFAs have anti-inflammatory effects (Harizi, Corcuff & Gualde 2008; Patterson et al. 2012). A mechanism of this interaction has been proposed where inflammation occurs as a result of an infection or tissue injury (Richard, AG et al. 2003). During the first 6 h, neutrophils infiltrate the affected area directed by IL-1, TNFα and IL-8. These activated neutrophils increase their surface expression of Fc receptors and complement receptors to facilitate antibody binding and phagocytosis. They stimulate reactive nitrogen species (RNS) and a respiratory burst producing ROS, both of which are responsible for killing of pathogens and associated tissue damage during inflammation. They also promote the switch of AA-derived eicosanoids to lipoxins at the same time as the biosynthesis of n-3 derived resolvins and protectins and initiate neutrophil apoptosis. These cells then undergo phagocytosis by macrophages, leading to neutrophil clearance and the release of anti-inflammatory cytokines (Serhan & Savill 2005). While the initial response of the n-6 PUFAs derived eicosanoids to promote inflammation is beneficial, the increase in the ratio of n-6:n-3 PUFAs leads to an overall increase in the production of pro-inflammatory cytokines and an unnecessary over-reactive inflammatory response and damage to tissues (Richard, AG et al. 2003; Calder 2006a, 2006b; Funahashi et al. 2008; Patterson et al. 2012). FAs in cell membrane phospholipids principally contain saturated FAs esterified in the *sn*-1 position, while both n-3 and n-6 PUFAs are present in the *sn*-2 position. PUFAs in the *sn*-2 position are released by the action of phospholipase A_2 and are a major substrate for eicosanoid synthesis (Figure 1-14). The effects of different eicosanoids are dependent on a number of factors, for example cell specificity, stimuli, respective prostanoid receptors expression, receptor binding affinity, the result of activating those receptors and the cellular response to such effects. Of these eicosanoids, some have pro-inflammatory effects, some have anti-inflammatory effects and some have both pro-inflammatory and anti-inflammatory effects. A summary of the many roles of eicosanoids in physiological

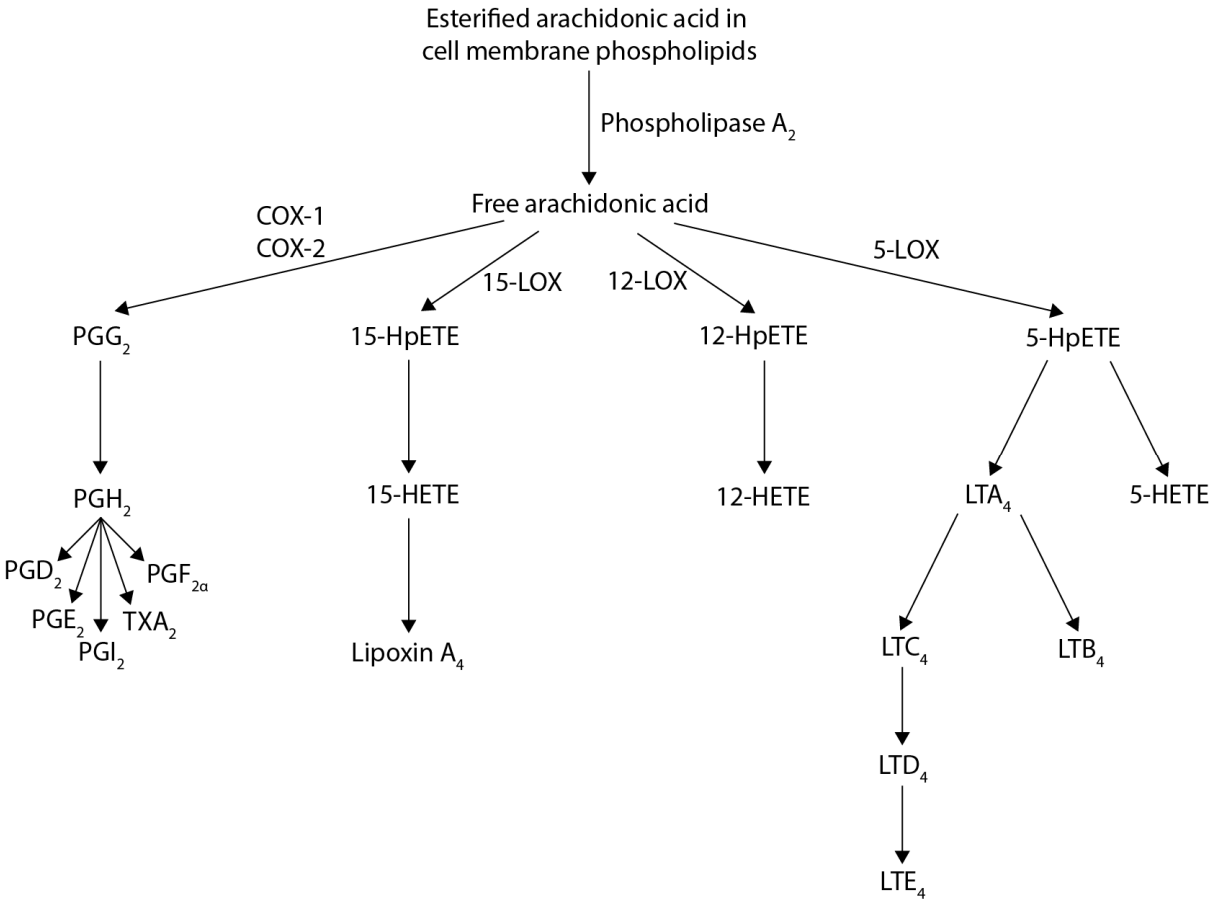


Figure 1-14 Pathway of eicosanoid synthesis from AA

COX1/2, cyclooxygenase 1/2; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxy-eicosatetraenoic acid; LOX, lipoxygenase; LTs, leukotrienes; PG, prostaglandin; TX, thromboxanes. (Adapted from Calder 2008).

and pathological conditions is shown in Table 1-5 and Table 1-6 (Lewis, Austen & Soberman 1990; Tilley, Coffman & Koller 2001; Calder 2009).

AA-derived eicosanoids are considered pro-inflammatory in many immune responses. The n-6 prostaglandin PGD_2 can promote inflammation via ligation of the CRTH2 receptor that activates eosinophils (Hata & Breyer 2004; Pettipher, Hansel & Armer 2007). LTB_4 is pro-inflammatory as it induces leukocyte infiltration and leads to the release of pro-inflammatory cytokines, for example IL-1β and TNF-α (He et al. 2002) via its interaction with the LT receptors BTL1 and BTL2 (Lundeen et al. 2006). PGE_2 and PGI_2, are pro-inflammatory via their activation of the EP2 and IP receptors, respectively. They increase vascular permeability and leukocyte infiltration.

Interestingly, other evidence indicates that AA-derived eicosanoids can also display anti-inflammatory effects in certain settings (Calder 2009; Ricciotti & FitzGerald 2011). For example, PGE_2 was found to inhibit the production of two classic pro-inflammatory cytokines, TNF-α and IL-1, by monocytes and macrophages (Miles, Allen & Calder 2002) and inhibit 5-LOX leading to decreased production of 4-series LTs (Levy et al. 2001), and induction of 15-LOX promoting the formation of lipoxins (Levy et al. 2001) which all have anti-inflammatory effects (Gewirtz et al. 2002; Serhan et al. 2003). So, PGE_2 has both pro-inflammatory and anti-inflammatory effects. Anti-inflammatory effects can be caused by LTs, resolvins and neuroprotectin (Calder 2009). These anti-inflammatory effects have been linked to fish oil as it is a rich source of EPA and DHA. Fish oil has been shown to decrease colonic inflammation, weight loss and mortality in animal models of colitis (Varnalidis et al. 2011). Fish oil supplementation in patients with inflammatory bowel disease results in the incorporation n-3 PUFAs into gut mucosal tissue and modification of inflammatory mediator profiles. Some trials showed that n-3 dietary supplementation improved gut histology, inhibited disease activity, decreased use of corticosteroids and reduced disease relapse. Other studies showed variable effects of fish oil, indicating that more studies are needed (Calder 2008).

1.4.7.1.2 FAs mechanism of action in inflammation

There are many proposed mechanisms that explain the anti-inflammatory effects of EPA and DHA. Firstly, their incorporation and replacement of AA in membrane phospholipids (Calder 2001b) results in inhibition of the production of AA-derived pro-inflammatory eicosanoids, for example PGE_2, TXB_2, LTB_4, 5-HETE and LTE_4 (Straus & Glass 2001; Calder 2009; Jones, Giembycz & Woodward 2009). Secondly, they are precursors of anti-inflammatory eicosanoids, for example LTB_5 and LTE_5

Table 1-5 n-6 PUFA derived eicosanoids and some of their effects

Metabolite class	AA-derived eicosanoids	Physiological effects	Target
Prostaglandins	PGD_2	Bronchoconstriction Pro-inflammatory	Bronchi Activation of eosinophils
	PGE_2	Pro-arrhythmic Induces fever	Blood vessels Nociceptor sensory
	PGF_2	Causes pain	neurons
	PGI_2	Increases production of IL-6 Bronchoconstriction Pro-arrhythmic Causes pain	Bronchi Vessels Nociceptor sensory neurons
Thromboxanes	TXA_2 TXB_2	Pro-aggregation Vasoconstriction Bronchoconstriction Pro-aggregation Vasoconstriction Bronchoconstriction	Platelets Vessels Bronchi Platelets Vessels Bronchi
Leukotrienes	LTA_4 LTB_4 LTC_4 LTD_4 LTE_4	Pro-inflammatory Chemotaxis Release of reactive oxygen species	Leukocytes Leukocytes Granulocytes

n-6 PUFA, AA derived eicosanoids, and some of their physiological and pathological effects and target cells/organs affected. Prostaglandin, PG; Leukotriene,LT and Thromboxane, TX. (Adapted from Patterson et al. 2012).

Table 1-6 n-3 PUFA derived eicosanoids and some of their effects

Metabolite	EPA and DHA derived eicosanoids	Physiological effects	Target
Prostaglandins	PGD_3 PGE3 PGF3 PGI3	Anti-arrhythmic	Blood vessels
Thromboxanes	TXA3 TXB3	Anti-aggregation	Platelets
Leukotrienes	LTA_5 LTB_5 LTC_5 LTD_5 LTE_5	Anti-inflammatory	Leukocytes
Resolvins	RVE1 RVD	Anti-aggregation Inflammatory Anti-inflammatory	Platelets Dendritic cells
Neuroprotectin	NPD1	Anti-inflammatory Anti-apoptotic Decrease oxidative stress	Retina Brain

n-3 PUFA, EPA and DHA, derived eicosanoids and some of their physiological and pathological effects and target cells/organs affected. Prostaglandin,PG; Leukotriene, LT, Thromboxane,Tx; Resolvin,RV. (Adapted from Patterson et al. 2012).

(Endres et al. 1989; Sperling et al. 1993; Lee, SY et al. 2009). Thirdly, they exert effects on other aspects of inflammation, such as decreasing leukocyte chemotaxis and adhesion molecule expression (Calder 2001a; Bagga et al. 2003). Fourthly, they can decrease gene expression (Berquin et al. 2011). Lastly, both EPA and DHA give rise to a new family of anti-inflammatory mediators, resolvins and related mediators. These mediators are produced from EPA and DHA via COX2 and 5-LOX to produce trihydroxy-eicosapentaenoic acid mediators (E-series resolvins), and trihydroxydocosahexanoic acid mediators (D-series resolvins) respectively. DHA also generates a dihydroxydocosatriene termed neuroprotectin D1 via 5-LOX (Figure 1-15) (Serhan et al. 2000a; Serhan et al. 2000b; Serhan, Chiang & Van Dyke 2008).

In any inflammatory condition both n-3 and n-6 derived eicosanoids interact together and the result determines the outcome of the inflammatory conditions. The pro-inflammatory effects of AA-derived PGs and LTs and the anti-inflammatory effects of those produced from n-3 PUFAs have been described by Harizi and colleagues (Harizi, Corcuff & Gualde 2008). A mechanism of this interaction has been proposed for resolution that initiates in the first few hours after the inflammatory stimuli. A switch occurs when AA-derived PGs and LTs, which have initiated inflammation, undergo further metabolism to become second generation eicosanoids termed lipoxins and hence terminate inflammation at the local sites (Serhan & Savill 2005). Since these lipoxins are involved in the resolution of acute inflammation, they are said to have “pro-resolving” and anti-inflammatory functions. These events happen together with the biosynthesis of resolvins and protectins from n-3 PUFAs (Serhan & Savill 2005). However, while the initial response of the AA-derived eicosanoids to promote inflammation is beneficial, for example in the control of blood flow and vessel dilation, the increase in the ratio of n-6:n-3 PUFAs leads to an overall increase in the production of pro-inflammatory cytokines and an unnecessary over-reactive inflammatory response. The decrease in consumption of n-3 PUFAs leads to an overall decrease in production of resolvins and protectins, which is detrimental to the host as the inflammatory response cannot be terminated effectively (Figure 1-15) (Calder 2006a, 2006b; Funahashi et al. 2008; Patterson et al. 2012).

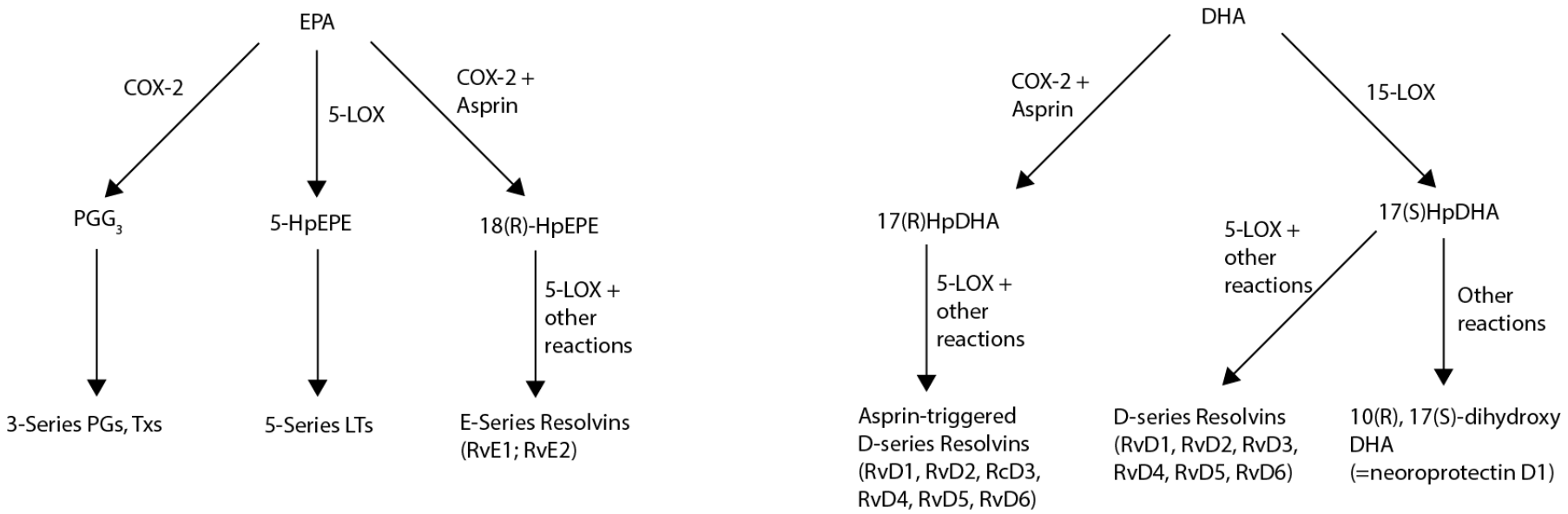


Figure 1-15 Synthesis of resolvins and other related mediators from EPA and DHA cyclooxygenase pathway

HPDHA, hydroperoxy docosahexaenoic acid; HPEPE, hydroperoxy eicosapentaenoic acid; LOX; lipoxygenase; LT, Leukotriene; PG, prostaglandin; Rv, resolvin; TX, thromboxane. (Adapted from Serhan et al. 2000a; Serhan et al. 2000b; Mukherjee et al. 2004; Calder 2009).

1.4.7.2 Cancer

Many studies have examined the relationship between FAs and the development of different types of cancer. In general, a diet rich in saturated FAs and n-6 PUFAs is most commonly associated with increased risk of cancer (Cohen, LA 1992; Cohen, ND et al. 2008), while monounsaturated FAs and n-3 PUFAs have protective effects (Berquin et al. 2007; Calviello, Serini & Piccioni 2007; Chen, YQ et al. 2007; Berquin, Edwards & Chen 2008; Chen, X et al. 2008; Cui et al. 2012).For example, some studies have shown that a diet rich in SAFAs or n-6 PUFAs can increase the risk of colorectal cancer and breast cancer *in vivo* (Giovannucci & Willett 1994; Ames, Gold & Willett 1995; Cui et al. 2012) and stimulate pancreatic cancer *in vitro* (Funahashi et al. 2008). Other studies have shown that a higher intake of monounsaturated FAs or n-3 PUFAs is associated with a lower incidence of colorectal cancer and breast cancer *in vivo* (Kuriki et al. 2006; Kimura et al. 2007; Kuriki et al. 2007; Theodoratou et al. 2007) and suppression of pancreatic cancer *in vitro* (Table 1-7) (Funahashi et al. 2008). The daily consumption of EPA by patients with colorectal adenomas led to a suppression of crypt cell proliferation and increased apoptosis in colonic mucosa (Courtney et al. 2007). In a recent study, FAs were measured in the colorectal mucosa in both normal and adjacent cancerous tissues (Zhang, J et al. 2013). The results showed that, palmitoleic acid, oleic acid and EPA and DHA were significantly lower in colorectal cancerous tissues than the adjacent normal tissues.

Furthermore AA, eicosadienoic acid, eicosatrienoic acid and adrenic acid were higher in cancerous colorectal tissue. According to Dukes classification (1932), colorectal cancers are classified to A, B, C and D stages. Stage A is where there is invasion into but not through the bowel wall, stage B invasion through the whole bowel wall but not involving any lymph nodes, stage C there is involvement of local lymph nodes and stage D there are metastases (Dukes 1932). A higher level of LA and AA was detected in the C cancer stage than in the B cancer stage, but a lower level of oleic acid and DHA was detected in the C cancer stage (Zhang, J et al. 2013).

However, the results of many other studies have shown conflicting results about the association between dietary FAs intake and the risk of breast cancer (Sieri et al. 2008). Breast cancer, one of the most common worldwide cancers, can be affected by life style, environmental factors, age, hormonal treatment and the diet consumed (Key, Verkasalo & Banks 2001; Sieri et al. 2008).

Strong associations between high fat intake and greater breast cancer incidence have been found in international correlation studies (Armstrong & Doll 1975) and animal

Table 1-7 Effect of MUFAs and PUFAs in colorectal, pancreatic and breast cancer

FAs	Type of cancer	References
↑MUFAs	↓ colorectal cancer	(Kuriki et al. 2006; Kuriki et al. 2007; Theodoratou et al. 2007)
↑MUFAs ↑ n-3 PUFAs	↓ pancreatic cancer ↓ risk of latest stages	(Astorg 2005; Funahashi et al. 2008)
↑SAFAs ↑n-6 PUFAs	↑ colorectal cancer	(Giovannucci & Willett 1994; Ames, Gold & Willett 1995)
↑SAFAs ↑n-6 PUFAs	↑ pancreatic cancer	(Giovannucci & Willett 1994; Ames, Gold & Willett 1995; Funahashi et al. 2008)

Summary of some *in vitro* studies showing the effect of FAs and types of cancer. MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SAFAs, saturated fatty acids.

studies (Aylsworth et al. 1984; Welsch 1992; Rogers, Zeisel & Groopman 1993). However, most case-control studies indicate only a weak association (Howe et al. 1990) and prospective cohort studies have shown little or no association (Velie et al. 2000; Byrne, Rockett & Holmes 2002; Kim, EH et al. 2006; Lof et al. 2007). A US study on post-menopausal women found that dietary fat intake was weakly but significantly associated with the risk of invasive breast cancer, whereas intakes of saturated FAs, monounsaturated FAs or PUFAs significantly increase the risk of breast cancer (Thiebaut et al. 2007). A pooled analysis of data from cohort studies found a weak increase in breast cancer risk associated with dietary saturated FAs and an iso-caloric diet (Smith-Warner et al. 2001a; Smith-Warner et al. 2001b). Another analysis of 45 epidemiologic studies (14 cohort studies and 31 case-control studies) found a weak but statistically significant relation between high fat intake and increased risk of breast cancer (Boyd et al. 2003). An interesting point is that fat intake was significantly associated with breast cancer risk in European but not in Canadian or US studies, which most probably is due to variation in dietary fat intake (Boyd et al. 2003). Published results of the randomised US Women's Health Initiative found that low fat diets were associated with a 9% reduction in the risk of invasive breast cancer (Prentice et al. 2006). Conflicting results with respect to monounsaturated FAs also have been reported. Some cohort studies identified a protective effect of monounsaturated FAs intake on breast cancer risk (Wolk et al. 1998; Holmes et al. 1999; Voorrips et al. 2002; Prentice et al. 2006), whereas others identified no effect or even a positive association with breast cancer (Table 1-8) (Velie et al. 2000; Smith-Warner et al. 2001a; Cho et al. 2003; Kim, EH et al. 2006; Thiebaut et al. 2007).

Similar conflicting results have been reported on the effect of FAs on prostate cancer (Suburu & Chen 2012), which is the second most common cancer in men worldwide (Baade, Youlden & Krnjacki 2009). In general, n-6 PUFAs increase prostatic cancer risk and progression while n-3 PUFAs show protective effects (Chen, YQ et al. 2007; Baade, Youlden & Krnjacki 2009; Berquin et al. 2011). Prostate cancer appears to require both dietary FAs (SAFAS and MUFAS and PUFAS) and *de novo* synthesis of FAs (SAFAs and MUFAs only) (Table 1-9).

Many factors can explain these controversial results. In epidemiological studies, factors include: the data rely on patient questionnaires which may not be accurate regarding the quantity of diet consumed; the quantity of specific types of saturated FAs, monounsaturated FAs and PUFAs; and, the type of diet since different types of food contain different amounts of the same FAs. These discrepancies cannot be avoided even in preclinical studies where specific formulae would be given to participants, as there are

Table 1-8 Summary of some studies showing the effect of different FAs on breast cancer

FAs	Breast cancer risk	References
↑MUFAs	↓ tumor growth and metastasis	(Wolk et al. 1998; Kuriki et al. 2006; Prentice et al. 2006; Kimura et al. 2007; Theodoratou et al. 2007; Cui et al. 2012).
↑ n-3PUFAs	↓	(Kuriki et al. 2007)
↑SAFAs ↑n-6 PUFAs	↑	(Giovannucci & Willett 1994; Ames, Gold & Willett 1995; Funahashi et al. 2008)
SFAS	↑	(Smith-Warner et al. 2001a; Smith-Warner et al. 2001b)
High fat intake	↑	(Armstrong & Doll 1975; Boyd et al. 2003)
High fat intake	↑	(Aylsworth et al. 1984; Welsch 1992; Rogers, Zeisel & Groopman 1993)
High fat intake	Weak association	(Howe et al. 1990)
High fat intake	Little association	(Knekt et al. 1990)
High fat intake	No association	(Velie et al. 2000; Byrne, Rockett & Holmes 2002; Kim, EH et al. 2006; Lof et al. 2007);
Low fat diet	9% ↓ in invasive type	(Prentice et al. 2006)
↑SAFAs, ↑MUSAs ↑PUFAs	↑ risk of invasive type	(Thiebaut et al. 2007)

Some studies reporting different effects of FAs in breast cancer. MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids and SAFAs, saturated fatty acids.

Table 1-9 Summary of some studies indicating the relation between SAFAs and prostate cancer

FAs	Name of FAs	Prostate cancer	Race	References
↑ SAFAs	Palmitic, Stearic, Lauric, Myristic	↑ risk ↓ progression free survival	Human	(Whittemore et al. 1995; Strom et al. 2008; Park, SY et al. 2009)
↑ SAFAs		No significant effects	Human	(Crowe et al. 2008; Jackson et al. 2012)
↑ SAFAs		No significant effects	Mice	(Lloyd et al. 2010)
↑ SAFAs	soybean, pork fat, linseed	↑ in prostatic weight and epithelial hyperplasia and significantly lighter organ	Rat	(Escobar, Gomes-Marcondes & Carvalho 2009)
↑ SAFAs ↑MUFAs	Palmitic, Oleic	↑ prostate weight ↑ testosterone ↑ cell proliferation	Rat	(Escobar, Gomes-Marcondes & Carvalho 2009)
Very long SAFAs	VLSFAs	↑tumor growth ↑ testosterone	Mouse	(Tamura et al. 2009)
MUFAs Olive oil	Oleic	Protective effects		(Lopez-Miranda et al. 2010)
MUFAs Olive oil		No effect		(Crowe et al. 2008; Park, SY et al. 2009)
MUFAs		Promoting effect	Human Mice	(Bidoli et al. 2005; Narita et al. 2008)

Different effect of FAs in prostate cancer. MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids and SAFAs, saturated fatty acids.

many variations in these formulated diets between different studies. Therefore it is difficult to connect the different results in different studies to obtain conclusive results (Sieri et al. 2008; Suburu & Chen 2012).

Although the issue is still unclear, multiple mechanisms by which FAs exert their effects in many cancers have been suggested. Many studies have focused on the effect of different FA metabolites from different pathways including COX, LOX and P450.

1.4.7.2.1 FAs metabolites and cancer

The resulting FAs metabolites have been implicated in various types of cancer (Panigrahy et al. 2010; Wang & Dubois 2010). For example, to assess the interaction between metabolites of dietary PUFAs in a single *in vivo* model and prostate cancer, Berquin et al. (2011) demonstrated that both n-3 and n-6 PUFAs have differential effects in prostate-specific null mice. They used COX1, COX2, 5-LOX, 12-LOX, or 15-LOX gene knock out mice and concluded that the interaction between FAs and their metabolites can play an important role in determining cancer risk (Table 1-10). Substantial evidence from human studies supports the important role of COX1 and COX2 in PUFAs metabolism and cancer (Wang & Dubois 2010). 5-LOX also has been implicated in the progression of several types of human cancers, for example, lung (Avis et al. 1996), colorectal (Soumaoro et al. 2006), colon (Ye et al. 2005), renal (Faronato et al. 2007), bladder (Avis et al. 1996; Ye et al. 2005; Hayashi, Nishiyama & Shirahama 2006; Soumaoro et al. 2006; Faronato et al. 2007) and prostate cancer (Ghosh & Myers 1998; Ghosh 2003; Sundaram & Ghosh 2006; Sharma, Pilania & Singh 2009). In summary, results indicate an importance in the relationship between COX1, COX2, n-6 PUFAs and 5-LOX PUFAs and cancer.

1.5 Evidence for a relationship between fatty acids and IDO

The ability of PUFAs to regulate IDO activity has received limited attention to date. Many molecules modulate IDO induction in different cell types. For example, inhibition of COX2 activity in vivo was associated with an increase in cytotoxic T lymphocytes (CTLs), which is attributed to a significant decrease in levels of tumor-associated IDO protein (Basu et al. 2006). Many cancers, for example breast, are characterised by high expression of COX2 and its metabolite PGE2. COX2 can have many effects in tumorigenesis. It can cause angiogenesis, tumor invasion and immune suppression (Dannenberg & Subbaramaiah 2003; Zha et al. 2004), many of which are through PGE2 (Okuno et al. 1995; Takayama et al. 2002; Harizi, Grosset & Gualde 2003; Chang et al. 2004; Pockaj et al. 2004). PGE2, when used as part of a DCs maturation cocktail, induced INDO1 and

Table 1-10 The involvement of different pathways on FAs effects on prostate cancer.

PUFAs	Enzymes inhibited	Effect on prostate cancer
n-3	COX1	↓
n-6	COX1	↑
n-3	COX2	↑
n-6	COX2	↑
n-3	5-LOX	No effect
n-6	5-LOX	↑
n-3 and n-6	12-LOX and 15-LOX	No effect

The effect of different FAs on prostate cancer as a result of blocking different metabolic pathways. (Adapted from Berquin et al. 2011, Chen et al. unpublished).

resulted in production of inactive IDO protein; however, when used with TNFα this resulted in IDO activation (von Bergwelt-Baildon et al. 2006). In another study, curcumin inhibited IDO expression through the inhibition of both COX2 and PGE_2 in DCs (Jung et al. 2010).

IDO activity has also been demonstrated to be affected by the short chain FA sodium butyrate (NaB, 4:0). Jiang et al. (2010) investigated the ability of NaB to modulate IDOactivity in human nasopharyngeal cell carcinoma. IDO protein expression was significantly reduced at 0.2 mM NaB and almost completely inhibited at 0.5 mM. NaB was found to modulate its effect by significantly inhibiting STAT1 phosphorylation at Tyr^{701}, its nuclear translocation and transcriptional activity. NaB had no effect on *INDO1* expression when added post-IFN-γ treatment, yet IDO protein was undetectable. Thus, the effect of NaB was mediated at both the transcriptional and post-transcriptional levels. Treatment of cells with NaB resulted in a significant increase in total content of ubiquitinated proteins, including IDO. Using the specific 26S proteasome inhibitor, Bortezomib, there was significant suppression of NaB-induced down regulation of IDO protein. Thus, NaB caused the ubiquitin-mediated proteasome degradation of IDO and this mechanism of action could be one way other FAs use to down regulate IDO.

We recently demonstrated that AA and its COX1/2 metabolites inhibited IFN-γ mediated induction of IDO at the transcriptional and post-transcriptional levels in THP-1 cells and human monocytes. These effects were via inhibition of STAT1 phosphorylation on Tyr701 (Bassal 2008; Bassal, Hughes & Costabile 2012). Surprisingly, Holmes (1998) did not find any effect of other modifiers, including phorbol 12-myristate 13-acetate (PMA), AA, lipopolysaccharide (LPS), analogs of cAMP and cGMP, and sphingosine, on the induction of IDO in the absence of IFN-γ. Nor did they modulate IFN-γ induction of IDO expression in cultured human foreskin fibroblasts. However, apart from these studies, the regulation of IDO activity by FAs and their eicosanoid derivatives has received very little attention.

These studies indicated a relationship between FAs and their metabolites and IDO which raised many questions about the effect of other naturally occurring FAs and their eicosanoids on IDO.

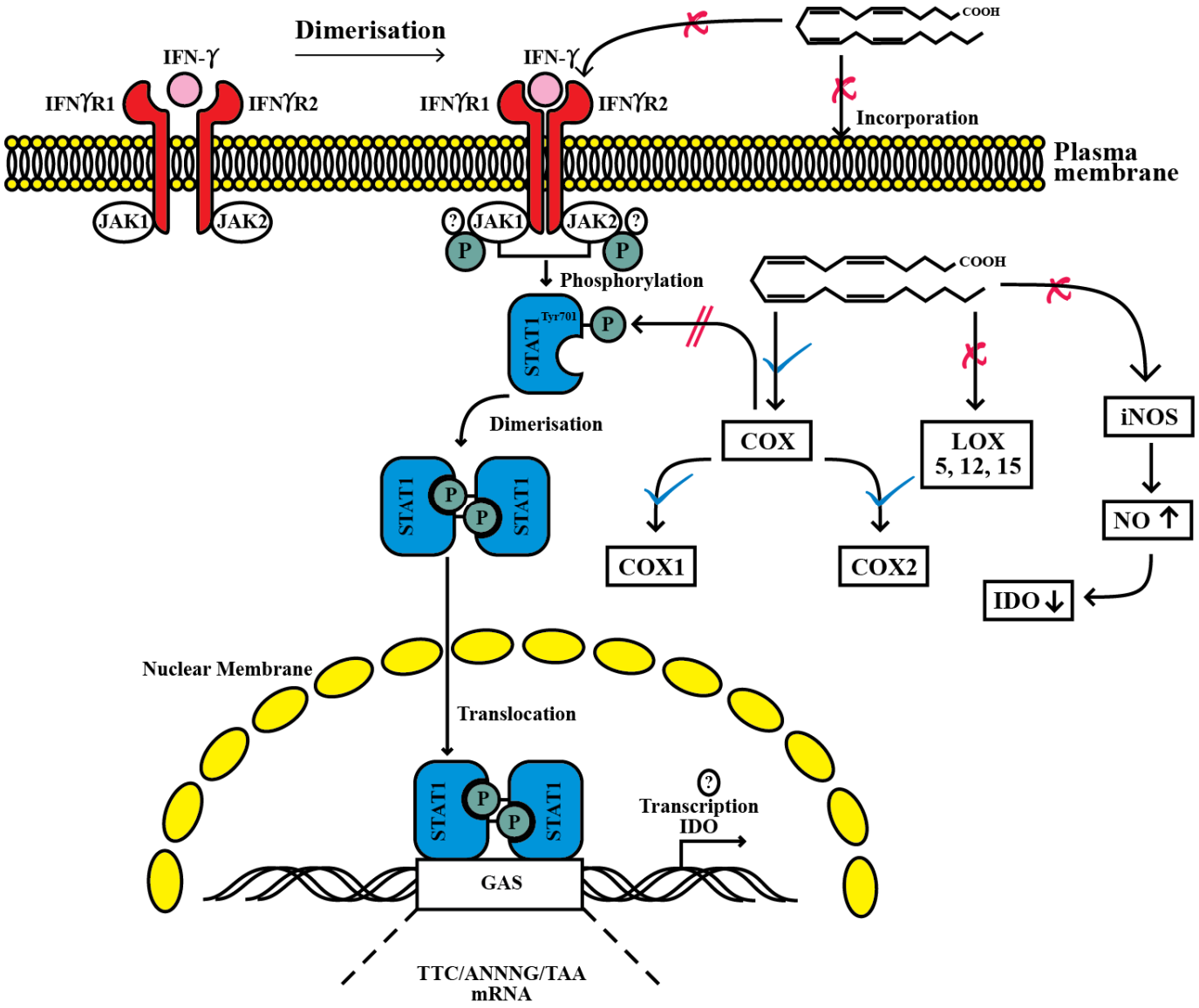
1.6 Preliminary data

The effect of AA on IFN-γ mediated induction of IDO was studied using THP-1 cells, which are an acute monocytic cell line model of leukemia. These cells are well

characterised and have been widely used to study IDO. THP-1 cells were stimulated with 1000 IU/mL of IFN-γ for 72 h to achieve maximal IDO activity, as assessed indirectly by Kyn production. The quantification of Kyn was optimised using two independent assays: a colorimetric assay, where Kyn was reacted with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) and the product was quantitated spectrophotometrically against a Kyn standard curve, and HPLC assay based on measuring both Kyn and Trp in culture supernatants. The colorimetric assay was used for routine analysis as it has both good throughput and sensitivity, while the HPLC assay allows for changes in both Kyn and Trp levels to be quantitated. Both assays demonstrated excellent linearity over the normal range of Kyn produced in tissue culture (up to 100 μmol/L). Resting THP-1 cells consistently produced low levels of Kyn (<10 μmol/L) while IFN-γ stimulated a significant increase in Kyn. Using a standard pre-treatment time of 30 min with AA (20 μM) prior to IFN-γ stimulation, a significant decrease in Kyn production was detected. These effects were dose dependent and AA had a calculated IC_{50} of 20 μmol/L in THP-1 cells. The inhibitory effects were not due to changes in cell viability (% cell viability; IFN-γ 90.25 ± 1.1; AA + IFN-γ; 86.9 ± 2.8, n=4) or changes in interferon gamma receptors (IFN-γR) expression as determined by flow cytometry. The presence of double bonds was essential for the inhibitory activity, as the saturated derivative, arachidic acid (Ar, 20:0), failed to inhibit Kyn levels (% inhibition; Ar + IFN-γ, 4.83 ± 1.03, n=5) in THP-1 cells. The inhibitory activity of AA was found to be dependent on its metabolism via the COX, but not LOX pathway. After pre-treating cells with indomethacin (IM), a COX inhibitor, AA was unable to inhibit Kyn production in response to IFN-γ. In contrast, AA retained its ability to inhibit Kyn production after pre-treatment with nordihydroguaiaretic acid (NDGA), a LOX inhibitor. To examine the mechanism of inhibition, and since IFN-γ was used as the agonist, the kinetics of STAT1 phosphorylation in response to IFN-γ treatment was determined. Under these conditions, AA significantly inhibited the phosphorylation of STAT1, at Tyr701, in response to IFN-γ alone. Also when cells were pre-treated with IM, AA inhibition was completely abolished, indicating that a metabolite(s) of AA rather than the parent FAs AA was/were responsible for the inhibitory activity (Figure 1-16). These results may explain another mechanism by which FAs can modulate cancer proliferation, metastasis and prognosis. These preliminary data add background and rationale for this project.

Figure 1-16 Action of AA on IFN-γ mediated induction of IDO in THP-1 cells

AA has no effect on IFN-γ cell surface receptor expression and is unlikely to mediate its effects through incorporation into membrane phospholipids. The inhibitory activity of AA is mediated via COX1/2 and not LOX metabolites. The undetermined COX metabolite of AA blocks STAT1 phosphorylation. AA has no effect on nitric oxide mediated inhibition of IDO activity. COX1/2, cyclooxygenase metabolites1&2; COX, cyclooxygenase pathway; GAS, TTC/ANNNG/TAA gamma interferon activated sequence; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric oxide synthase; IFN-γR1/2, interferon gamma receptors; JAK1/2, Janus kinases; LOX, lipoxygenase; NO, nitric oxide; P, phosphorylation; STAT1, signal transducer activator of transcription; Y701, tyrosine 701; ×, not involved; **?**, not determined; ✓, involved; // blocked. (Adapted from Aaronson & Horvath 2002; Platanias 2005; Bassal 2008).


Dimerisation
IFN-γ
IFNγR1
IFNγR2
IFN-γ
IFNγR1
IFNγR2
COOH
Incorporation
Plasma membrane
JAK1
JAK2
JAK1
JAK2
P
P
Phosphorylation
STAT1 Tyr701
P
Dimerisation
STAT1
P
P
STAT1
COOH
COX
LOX 5, 12, 15
iNOS
COX1
COX2
NO ↑
IDO ↓
Nuclear Membrane
Translocation
STAT1
P
P
STAT1
Transcription IDO
GAS
TTC/ANNNG/TAA
mRNA

1.7 Research focus

IDO plays an important role in modulating the immune response in both physiological and pathological conditions. IDO is accepted as an immuno suppressive molecule and an inducer of immune tolerance thereby helping cancer cells evade the immune response. IDO also has detrimental effects in both chemotherapy and immunotherapy. It is known that FAs, especially PUFAs, play many roles in immune modulation mainly in inflammation and cancer with little attention being given to the relationship between FAs and IDO. There are a number of reports in the literature which highlight the potential for regulation of IDO by FA, e.g. the inhibition of IDO expression by inhibiting COX2 enzyme (Jung et al. 2010), the modulation of IDO by NaB (Jiang et al. 2010) , the induction of IDO by PGE_2 (Thurnher 2007; Legler et al. 2010; Sakata, Yao & Narumiya 2010) and the inhibition of IFN-γ mediated induction of IDO by AA. A detailed understanding of the regulation of AA and its derived eicosanoids on IDO activity is important. The analysis of the structure–function of both FAs and their derived eicosanoids can be significant as it can be used successfully to develop more effective compounds to modulate IDO activity. IDO inhibitors are potentially a major target in cancer treatment as well as in combination with chemotherapy and immunotherapy for cancer patients.

1.8 Hypothesis

Fatty acids and their eicosanoid derivatives can modulate the IFN-γ mediated induction of indoleamine 2,3-dioxygenase in THP-1 cells and human monocytes.

1.9 Aims

The aims of this thesis were to determine in both THP-1 cells and human monocytes:

- the effect of naturally occurring fatty acids on IFN-γ mediated induction of IDO
- the modulation of IFN-γ mediated induction of IDO in by AA and the mechanism of action
- the effect of biologically active AA's COX1/2 metabolites on IFN-γ mediated induction of IDO, and their structure–function relationships
- the characterisation of the activity of AA's active metabolite, PGD_2, on IFN-γ mediated induction of IDO and the mechanism of its action.

1.10 Significance

IDO contributes to different immunological conditions: maternal tolerance to semi allogeneic foetal tissues and transplanted organs, inhibits local tissue inflammation and autoimmunity, and suppresses immunity to chronic infections and cancer. IDO is constitutively expressed in various types of tumor cells, but importantly can also be induced by cytokines. It enhances tumor growth, escapes from the immune system, induces immuno tolerance, and accelerates distal metastasis and worsens prognosis for the patients (Uyttenhove et al. 2003). IDO expression has been positively associated with tumor drug resistance, Paclitaxel resistance as part of immune evasion strategy (Okamoto et al. 2005). Furthermore, IDO acts as an impediment of both immunotherapy and chemotherapy treatment in cancer (Okamoto et al. 2005; Chamuleau et al. 2008). All the above reasons suggest that IDO inhibition is an attractive therapeutic target for many diseases, cancer therapy, chemotherapy and immunotherapy (Uyttenhove et al. 2003; Munn & Mellor 2007; Yoshida et al. 2008). The manipulation of IDO expression shows promise as a target for breaking tumor-induced immune suppression. Both immuno-pharmacological and genetic studies targeted at modulating the IDO enzyme and/or mRNA levels provide direct evidence that suppression of IDO may break tumor-derived immune suppression and enhance cancer treatment by the individual's own immune system (Zheng et al. 2006).

COX activity *in vivo* may regulate IDO expression within the tumor microenvironment as well as PGE_2 production, and lead to an induction of IDO after DC maturation. IDO protein expression was significantly reduced and almost completely inhibited by NaB (Jiang et al. 2010). NaB caused the ubiquitin-mediated proteasome degradation of IDO and this mechanism of action could be one way that other FA use to down regulate IDO. These studies indicated a relationship between FAs and their metabolites and IDO which raised many questions about the effect of other naturally occurring FAs and their eicosanoids on IDO. Further evidence for the relation between IDO and FAs has been shown in a study on the effect of the PUFA AA and its COX metabolites (Bassal 2008; Bassal, Hughes & Costabile 2012). Thus, it is feasible to assume that AA, or other naturally occurring fatty acids, may affect IDO activity.

Chapter 2

Materials and Methods

2.1 Reagents

Ammonium acetate, β-mercaptoethanol (β-ME), copper sulphate, dithiothreitol (DTT), ethylenediaminotetraacetic acid (EDTA), ethylene glycol-bis (β-aminoethyl ether) tetraacetic acid (EGTA), Folin and Ciocalteu's phenol reagent, N-(2-hydroxyethyl) piperazine-N`-(2-ethanesulfonic acid) (HEPES), L-kynurenine, L-3-nitro-L-tyrosine, L-tryptophan, Nonidet P-40, *p*-dimethylaminobenzaldehyde (Ehrlich's reagent), phenylmethylsulfonyl fluoride (PMSF), sodium acetate, sodium bicarbonate, sodium dodecyl sulphate (SDS), sodium hydroxide, sodium orthovanadate, sodium tartrate, sucrose, trichloroacetic acid (TCA), Tris (hydroxy methyl) amino methane hydrochloride; Tris hydrochloride, Trizma base; 2-amino-2-(hydroxymethyl)-1,3-propanediol were all purchased from Sigma Chemical Company (St. Louis, MO, USA). Diethyl ether, glacial acetic acid and hexane were from Ajax Finechem (NSW, Australia). HPLC-grade methanol was from BDH (Kilsyth, VIC, Australia). Polvoxyethylene (20) sorbitan monolaurate (Tween 20) was from Asia Pacific specialty chemicals (APS) (Auckland, New Zealand). Acetonitrile was from Malinckrodt Baker (NJ, USA). Western blot recycling kit was from Alpha Diagnostic International (TX, USA). Chemiluminescence Reagent Plus was from Perkin Elmer Life Science (Boston, USA). MilliQ water was used to prepare all reagent solutions.

2.2 Fatty acids and prostaglandins

Arachidic acid (Ar; 20:0), arachidonic acid (AA; 20:4, n-6), arachidonic acid methyl ester (AA-ME; 20:4, n-6 ME), eicosapentaenoic acid (EPA; 20:5, n-3), docosahexaenoic acid (DHA; 22:6, n-3) and all prostaglandins (see Chapter 5 for full listing) were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). The purity of all fatty acids was routinely monitored by thin layer chromatography (TLC). All fatty acids were dissolved in absolute ethanol and kept in the dark at –20 °C for routine use or –80 °C for long-term storage. The fatty acid library (see Chapter 4 for full listing) was from Enzo Life Sciences (Ann Arbor, MI, USA).

2.3 IDO agonist

Recombinant human IFN-γ was from PeproTech (Princeton Business Park, Rocky Hill, NJ, USA).

2.4 Inhibitors, agonists and antagonists

Aprotinin, benzamidine hydrochloride, indomethacin (IM), leupeptin, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide (NS-398), nordihydroguaiaretic acid (NDGA), pepstatin A and 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC-560) were all from Sigma Chemical Company (St Louis, MO, USA). BAY-u3405 (Ramatroban), 3-(3-cyclohexyl-3-hydroxypropyl)-2, 5-dioxo-(R*, S*)-(±)-4-imidazolineheptanioc acid (BW 245C), BWA 868C, CAY 10595 and MK 0524 (Laropiprant) were from Cayman Chemical (Ann Arbor, MI, USA).

2.5 Antibodies

Mouse monoclonal IgG anti-STAT1 p91 (C-111), goat polyclonal IgG phospho-STAT1 (Tyr701), goat polyclonal IgG phospho-STAT1 (Ser727), rabbit polyclonal IgG IDO (H-110), donkey anti-goat IgG-HRP, goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP were all from Santa Cruz Biotechnology (California, USA). Rabbit anti-GAPDH was from Sigma-Aldrich (St. Louis, MO, USA).

2.6 THP-1 cells

THP-1 cells were purchased from Sigma-Aldrich (NSW, Australia). Cells were cultured in RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (100 mmol/L) from Sigma-Aldrich (NSW, Australia) and β-mercaptoethanol (20 µmol/L) from SAFC Bioscience (Kansas, TX, USA) at 37 °C in a humid atmosphere of 5% CO_2. Cells were sub-cultured every 2–3 days and were maintained between 3–8 x 10^5/mL. THP-1 cells (ATCC code: TIB-202) are an acute monocytic (AML) cell line derived from the peripheral blood of a 1-year-old male suffer of acute monocytic leukemia. THP-1 cells express Fc and C3b receptors and lack surface and cytoplasmic immunoglobulin. These cells can be differentiated into macrophage-like cells by prolonged culture with dimethyl sulfoxide (DMSO). THP-1 cells have the following mutant genes: CDKN2A (homozygous), KDM6A (homozygous) and NRAS (heterozygous) (Tsuchiya et al. 1982).

2.7 Primary human monocytes

Primary human monocytes were isolated from the peripheral blood of healthy volunteers as described previously (Ferrante, JV et al. 1997). Briefly, venous blood from

healthy volunteers was collected into tubes containing 25 IU/mL heparin. The blood was then layered onto a density gradient consisting of 8% Ficoll 400 (v/v) (Pharmacia Biotech, MN, USA) and the density of 1.114 achieved by the addition of sodium diatrizoate from Sigma Chemical Company (St. Louis, MO, USA) and Angiografin (Schering, NSW, Australia). The blood was centrifuged at 600 g x 45 min. Following centrifugation, the leukocytes had separated into two layers. The top layer contained the peripheral blood mononuclear cells (PBMCs), the lower layer contained neutrophils, while red blood cells were at the bottom of the tube. The upper layer was gently aspirated and washed with RPMI-1640 medium by repeated centrifugation (3 x 5 min at 600 g) and suspension of the cells and adjusted to 4 x 10^6/mL. Ethical approval was granted by the University of South Australia Human Research Ethics Committee and informed consent was obtained from all participants (Ethics approval number: 0000022047). All healthy volunteers were students and employees of the University of South Australia.

2.8 Colorimetric assay for kynurenine quantification

Kynurenine (Kyn) was quantified as described previously (Takikawa et al. 1988) with minor modifications. Briefly, Kyn standards (0–100 µmol/L) were prepared in tissue culture medium (Section 2.6). To 100 µL of each sample was added 40 µl of 30% (w/v) trichloroacetic acid (TCA). The samples were vortexed and centrifuged (16,000 g x 5 min) at room temperature. A 100 µL aliquot of the supernatant was transferred to a 96-well flat bottom plate (Iwaki, Japan) and to each was added 100 µL of 4% (w/v) *p*-dimethylaminobenzaldehyde (Ehrlich's reagent ER), dissolved in 100% (v/v) glacial acetic acid. The samples were incubated for 10 min in the dark at room temperature. The absorbance of the samples was measured at 492 nm using a Multiskan Ascent plate reader, Labsystem (model 354) (Helsinki, Finland) using the supplied Ascent software (version 2.4.1). The results were analysed using Graphpad Prism version 5, Graphpad Software Inc., (San Diego, CA, USA).

To exclude the possibility that the FAs can directly interfere with the colorimetric assay, an experiment was performed where AA was added directly to the Kyn standard curve assay. Even at the highest concentration used (20 µmol/L), which would account for 100% carry over, which is unlikely to happen after treatment of cells for 72 h, a non-significant 5% reduction was observed, confirming that the AA does not directly interfere with the colorimetric assay.

2.9 High performance liquid chromatography (HPLC) for kynurenine and tryptophan quantification

Kyn and tryptophan (Trp) were quantified via HPLC as described previously (Laich et al. 2002) with minor modifications. Briefly, both Kyn and Trp standards (0–100 µmol/L) were prepared in tissue culture medium (Section 2.6). To 100 µl of each sample were added 100 µl of 50 µmol/L of L-3-nitro-L-tyrosine (NT) and 25 µL of 2 mol/L TCA. The samples were mixed and centrifuged (16,000 g x 5 min) at room temperature. Ten microlitres of the supernatant from each sample was injected into a C_{18} column (100 x 4.6 mm) from Phenomenex (NSW, Australia), with an elution buffer flow rate of 0.5 mL/min. The elution buffer consisted of 15 mmol/L acetic acid–sodium acetate (pH 4.0) supplemented with 2.5% (v/v) acetonitrile. The concentration of components was calculated according to peak heights and was corrected using NT as the internal standard. HPLC analysis was performed at 360 nm on a Shimadzu system controlled by CLASS-LCIO software with a photodiode array detector SPD-10A (Shimadzu, Kyoto, Japan).

2.10 Treatment of cells with recombinant human interferon gamma (IFN-γ)

THP-1 cells (4 x 10^5/100 µL) or human monocytes (4 x 10^6/100 µL) were suspended in tissue culture medium (Section 2.6) supplemented with 100 µmol/L of L-Trp, and treated with an equal volume of recombinant human interferon gamma (IFN-γ; 1000 IU/mL) for 0–72 h in a 24-well flat bottom plate (Iwaki, Japan) at 37 °C/5% CO_2 in a humidified atmosphere. After this time, the supernatant was collected and centrifuged (600 g x 5 min). Kyn levels were quantitated using the colorimetric assay or Trp levels quantitated via HPLC as described above.

2.11 Assessment of fatty acid purity by thin layer chromatography (TLC)

Fatty acids (100 µg) were spotted onto a 20 x 20 cm thin layer chromatography plate (Merck, Whitehouse Station, NJ, USA) and dried under a stream of nitrogen. The plate was developed using a diethyl ether: hexane: glacial acetic acid (60:40:1, v/v) solvent system. Once the solvent front had travelled 16 cm, the plate was allowed to air dry and developed by exposure to iodine vapour for 30 min. No material was found to migrate with a lower R_f value than the fatty acids, indicating the absence of contaminating or degraded non-polar molecules (Figure 2-1).

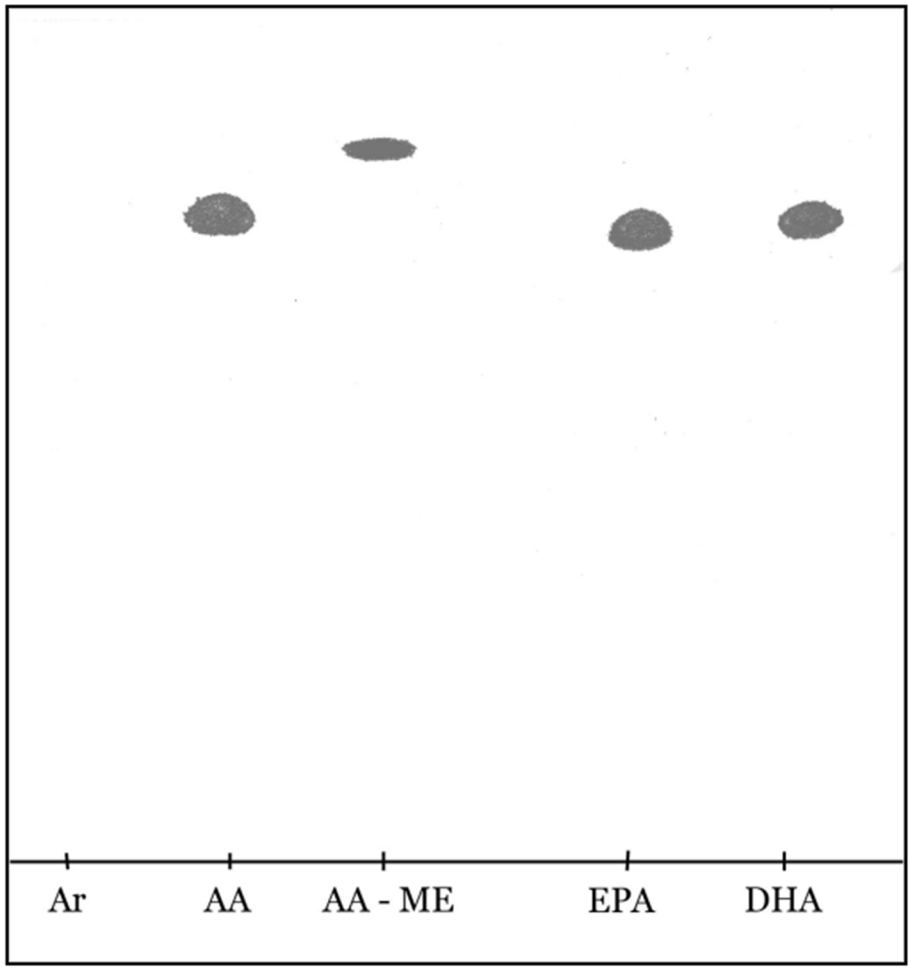


Figure 2-1 A typical thin layer chromatogram of arachidic (Ar), arachidonic (AA), arachidonic acid methyl ester (AA-ME), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA)

A total of 100 µg of fatty acid was spotted onto a TLC plate and developed as described in Section 2.11. No spot was seen for Ar as it is a saturated fatty acid and fails to react with the iodine vapour.

2.12 Presentation of fatty acids and prostaglandins to THP-1 cells

Treatment of THP-1 cells with fatty acids or prostaglandins were performed as described previously (Bassal, Hughes & Costabile 2012). Briefly, THP-1 cells were first washed in tissue culture medium lacking serum and suspended in the same media supplemented with L-Trp (100 µmol/L) at 4 x 10^5/100 µL. One hundred microliter of THP-1 cells was placed into a flat bottom well. An aliquot of fatty acid or prostaglandin, dissolved in 100% (v/v) ethanol, was prepared in tissue culture medium lacking serum and 100 µL of fatty acid (0–30 µmol/L), prostaglandin (0–10 µmol/L) or an equivalent amount of ethanol (100%, v/v) (concentration never > 0.03%) was added to the cells for (0–30 min) at 37 °C/5% CO_2 in a humidified atmosphere. The cells were then stimulated with 200 µL of IFN-γ (1000 IU/mL) prepared in tissue culture medium (Section 2.6) supplemented with L-Trp (100 µmol/L) or an equivalent amount of diluent and incubated at 37 °C/5% CO_2 in a humidified atmosphere for 0.5–72 h.

2.13 Presentation of fatty acids and prostaglandins to monocytes

Treatment of human monocytes with fatty acids or prostaglandins was performed as for THP-1 cells with some modifications as how to prepare the cells for treatment. Human monocytes were purified as described previously and then washed in tissue culture medium and suspended in the same medium as for THP-1 cells. Two hundred microliters of plasma was placed into a flat bottom well and incubated for 20 min at 37 °C/5% CO_2 in a humidified atmosphere. The plasma was removed and monocytes (4 x 10^6/mL/well) were added and incubated for 1 h at 37 °C/5% CO_2 in a humidified atmosphere. The wells were washed 3 times with tissue culture medium lacking serum, to remove non-adherent cells, and then 100 µL of medium lacking serum and supplemented with L-Trp (100 µmol/L) was added to each well. Cells were then treated as for the THP-1 cells (Section 2.12).

2.14 Treatment of cells with COX, COX1/2 and LOX inhibitors

THP-1 cells and human monocytes were prepared as described previously (Sections 2.12 and 2.13). Fifty microlitres of cells were pre-treated with 50 µl of indomethacin (IM; 100 µM), nordihydroguaiaretic acid (NDGA; 10 µmol/L), SC-560 (1 or 10 µmol/L) or NS-398 (1 or 25 µmol/L) all prepared in tissue culture medium lacking serum and supplemented with L-Trp (100 µmol/L) or equivalent amount of ethanol, for 30 min at 37 °C/5% CO_2 in a humidified atmosphere. The cells were then treated with 100 µL of AA (20 µmol/L), prepared in tissue culture medium lacking serum or equivalent amount of

ethanol (100%, v/v) for 30 min at 37 °C/5% CO_2 in a humidified atmosphere. The cells were then stimulated with 200 µL of IFN-γ (1000 IU/mL) and incubated at 37 °C/5% CO_2 in a humidified atmosphere for 72 h. Samples were collected and assessed for Kyn and Trp quantification using either the colorimetric (Section 2.8) or HPLC assay (Section 2.9).

2.15 Treatment of cells with DP1/CRTH2 agonists

THP-1 cells and human monocytes were prepared as described previously (Sections 2.12 and 2.13). One hundred microlitres of THP-1 cells or human monocytes were pre-treated with 100 µl of BW 245C (0.1–10 µmol/L) or 13,14 dihydro 15-keto PGD_2 (10 µmol/L) all prepared in tissue culture medium supplemented with L-Trp (100 µmol/L) or diluent for 30 min at 37 °C/5% CO_2 in a humidified atmosphere. The cells were stimulated with 200 µL of IFN-γ (1000 IU/L) and incubated at 37 °C/5% CO_2 in a humidified atmosphere for 72 h. Samples were collected and assessed for Kyn quantification using the colorimetric assay (Section 2.8).

2.16 Treatment of cells with DP1/CRTH2 antagonists

THP-1 cells and human monocytes were prepared as described previously (Sections 2.12 and 2.13). Fifty microlitres of THP-1 cells or human monocytes were pre-treated with 50 µl of BAY-u3405 (0.1–1 µmol/L), BW A868C (2.5-10 µmol/L), CAY10595 (0.1–1 µmol/L) or MK 0524 (2.5–10 µmol/L) all prepared in tissue culture media supplemented with L-Trp (100 µmol/L) or diluent, for 30 min at 37 °C/5% CO_2 in a humidified atmosphere. The cells were treated with 100 µL of PGD_2 (10 µmol/L), prepared in tissue culture medium lacking serum or diluent for 30 min at 37 °C/5% CO_2 in a humidified atmosphere. The cells were stimulated with 200 µL of IFN-γ (1000 IU/mL) and incubated at 37 °C/5% CO_2 in a humidified atmosphere for 72 h. Samples were collected and assessed for Kyn quantification using the colorimetric assay (Section 2.8).

2.17 Protein quantification

Protein content was determined according to the method of Lowry et al. (1951). Briefly, serial dilutions of bovine serum albumin (0, 3.125, 6.25, 12.5, 25 and 50 µg/50 µL) were prepared in MilliQ water in duplicate. The experimental samples (5 µl sample + 45 µL H_2O) were also prepared in duplicate. To each tube was added 150 µLof solution 1; 2% (w/v) sodium carbonate, 1% (w/v) sodium dodecyl sulphate, 0.4% (w/v) sodium

hydroxide and 0.16% (w/v) sodium/potassium tartrate in MilliQ water: copper sulphate (0.1 mol/L = 100:1). The samples were vortexed and incubated for 20 min at room temperature. To each tube was added 15 µL of solution 2; (Folin Ciocalteu reagent: H_2O 1:1). The samples were vortexed and incubated for 20 min at room temperature. One hundred and eighty microlitres from each sample was transferred into a 96-well flat bottom plate and the absorbance read at 540 nm on a Dynatech plate reader (Dynatech, Chantilly, VA, USA) using the supplied Dynatech software (version 3.5). The results were analysed using Graphpad Prism version 5 (Graphpad Software Inc., CA, USA).

2.18 Western blot

Western blot was performed as described previously (Bassal, Hughes & Costabile 2012). Briefly, THP-1 cells or human monocytes (4 x 10^5/100 µL; 1 x 10^7/total) were treated with fatty acids, prostaglandins or inhibitors (Sections 2.12–2.14), prepared in serum free tissue culture medium (Section 2.6) for 30 min at 37 °C/5% CO_2 in a humidified atmosphere. The cells were stimulated with IFN-γ (1000 IU/mL) for (0–72 h) and collected by centrifugation (2000 g x 5 min, 4 °C). The supernatant was discarded and the cells suspended in 200 µl of lysis buffer (20 mM HEPES pH 7.4, 0.5% (v/v) Nonidet P-40, 100 mmol/L NaCl, 1 mmol/L EDTA, 2 mmol/L Na_3VO_4, 2 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, 10 µg/L pepstatin A, 10 µg/L benzamidine and 100 KIU aprotinin). The cells were sonicated (3 x 10 sec, setting 2) (Misonix, NY, USA), centrifuged (16,000 g x 20 min, 4 °C) and the supernatant collected. The protein concentration was determined as described in Section 2.17. The samples (100 µg of protein) plus reducing buffer (20 mM Tris-HCl, pH 6.8, 40% sucrose, 6% sodium dodecyl sulphate (SDS) and 10 mmol/L β-mercaptoethanol, 3:1) were heated at 100 °C for 5 min and then loaded and separated by 10% polyacrylamide gels (Bio-Rad) using a BioRad mini gel apparatus from BioRad (Gladesville, NSW, Australia), run at 170 V for 45 min. The gel was then soaked in transfer buffer (25 mM Tris base, 20% (v/v) methanol, 125 mM Glycine and MilliQ water) for 20 min and the nitrocellulose membrane (0.2 mm) from Amersham International (Amersham, Bucks, UK) equilibrated by being briefly soaked in deionised water and then in transfer buffer for 10 min. The separated proteins were electrophoretically transferred to the nitrocellulose membrane at 300 mA for 1.5 h. Following transfer, the blots were stained with Ponceau S (0.1% (w/v) in 5% acetic acid) to confirm even transfer. The membrane was blocked for 1 h with blocking buffer (20 mM Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5% (w/v) skim milk and 0.1% (v/v) Tween 20). This

was followed by overnight incubation with anti-IDO (H-110) rabbit polyclonal IgG (1:100), phospho-STAT1 (Tyr701) (1:400), STAT1α p91 mouse monoclonal IgG (1:400), and anti-GAPDH rabbit polyclonal IgG (1:10,000) at 4 °C. The membrane was washed (3 x 10 min) with blocking buffer and incubated for 1 h with donkey anti-goat IgG-HRP conjugated (1:5000) or goat anti-rabbit IgG-HRP conjugated (1:2000) or goat anti-mouse IgG-HRP conjugated (1:2000) at room temperature. The membrane was washed (3 x 10 min) with blocking buffer. The immune reactive complex was visualised using a Western Lighting™ Chemiluminescence Reagent Plus Kit from Perkin Elmer Life Sciences (Boston, USA). The membrane was covered for 1 min with a 1:1 mixture of enhancer luminol and oxidising reagent. The membrane was visualised using an Image quant LAS 4000 and Image Quant TL software. To ensure that equal amounts of protein had been loaded, the membrane was incubated for 2 x 15 min in stripping solution, followed by 1 h incubation in blocking solution from Alpha Diagnostic (TX, USA). After stripping, the membrane was re-probed with an anti-GAPDH rabbit polyclonal IgG (1:10,000) followed by incubation with a polyclonal goat anti-rabbit-HRP conjugated (1:2000) and visualised as above. Volume integration was used to evaluate the relative density of bands after correction for GAPDH loading using Image Quant TL software.

2.19 Total RNA isolation

Total RNA was isolated using a commercial ISOLATE RNA mini kit (Bioline, Alexandria, Australia) according to the manufacturer's protocol. Briefly, treated THP-1 cells (5 x 10^6/total) or human monocytes (6 x 10^6/total) were harvested by centrifugation (300 g x 5 min) and the supernatant discarded. The pellet was suspended by pipetting in 400 µl of lysis buffer R and incubated at RT for 3 min. The supernatant was transferred to a spin column and centrifuged at 10,000 g x 2 min. The spin column was discarded and 400 µL of 70% ethanol (v/v) added to the filtrate, mixed well and transferred to a new spin column. The column was centrifuged at 10,000 g x 2 min, and the spin column was washed with 500 µl of wash buffer AR (10,000 g x 1 min). The spin column was washed with 700 µl of wash buffer BR (10,000 g x 1 min). The eluate was discarded and the spin column centrifuged once more (10,000 g x 3 min) to remove any residual buffer. A total of 35 µL of RNase-free water was added to the spin column membrane and incubated at RT for 5 min and then centrifuged (6000 g x 1 min). Total RNA was quantitated using a Nano drop 2000 spectrophotometer (260/280 = 1.8–2) from Thermo Fisher Scientific (New Zealand). Samples were stored at –20 °C until used.

2.20 cDNA synthesis

Single strand cDNA was synthesised from total RNA using a Tetro cDNA synthesis kit from Bioline (Alexandria, Australia) according to the manufacturer's protocol. Briefly, to 2 µg of RNA was added 1 µL of random hexamer, 1 µL of 10 mM dNTP mix and DEPC-treated water up to 10 µL. The tube was mixed gently and then incubated at 65 °C for 10 min. The tubes were then placed on ice for 2 min. The reaction mix was prepared as follows: 4 µL of 5 x RT-buffer, 1 µL of RNase inhibitor, 1 µL of reverse transcriptase (200 U/µL) and DEPC-treated water up to 10 µL. Ten microlitres of the reaction mix was added to each tube containing the primed RNA and incubated at 42 °C for 30 min. The reaction was terminated by incubation at 70 °C for 15 min. Samples were stored at –20 °C until required.

2.21 Reverse transcription PCR (RT-PCR)

Reverse transcription PCR (RT-PCR) was used to detect mRNA levels for PGH synthase 1 (COX1), PGH synthase 2 (COX2) and for the PGD_2 receptor, CRTH2, in THP-1 cells and human monocytes. In order to optimise the conditions for the detection, the annealing temperature (55–70 °C), magnesium ion (Mg^{2+}) concentration (1.5–3.5 mmol/L) and cycle number (29–37) were verified. Reactions contained cDNA (1 µL), 0.5 µmol/L of each forward and reverse primer (GeneWorks, Adelaide, Australia) (Table 2-1), 1 µL of Phusion DNA polymerase, 10 mmol/L of each dNTP and 5 µL of Phusion HF buffer in a total volume of 25 µL. β-actin was used as an internal standard. A no template control was included in each amplification reaction. DNA amplifications were performed using a Bio-Rad Cycler (Bio-Rad Laboratories, CA, USA). The optimal conditions for COX1, COX2, β-Actin and CRTH2 are summarised in Table 2-2. The cycling conditions for RT-PCR amplification are listed in Table 2-3 and Table 2-4. Following amplification, one volume of DNA loading buffer was added to 5 volumes of sample and then loaded onto a 1.5% agarose gel (Amresco, Ohio, USA) containing 1 µg/ml ethidium bromide. The amplified DNA was electrophoresed at 125 V for 45 min in Tris-acetate-EDTA (TAE) buffer, with a 100 bp DNA ladder (Invitrogen, Victoria, Australia). The gel was subsequently viewed under a Bio-Rad UV transilluminator connected to a desktop computer, and the software program Quantity One version 4.2.1from Bio-Rad Laboratories (CA, USA) was used to view and capture an image of the gel.

Table 2-1 Primers sequences and product sizes for COX1, COX2, β-actin and CRTH2 mRNA

Target mRNA	Primers	Product size
COX1	F (5'- CCG GAT GCC AGT CAG GAT GAT G- 3')	529 bp
COX1	R (5'- CTA GAC AGC CAG ATG CTG ACA G- 3')	
COX2	F (5'- ATC TAC CCT CCT CAA GTC CC- 3')	708 bp
COX2	R (5'- TAC CAG AAG GGC AGG ATA CAG- 3')	
β-actin	F (5'-AGC ACT GTG TTG GCG TAC AG-3')	240 bp
β-actin	R (5'-GGA CTT CGA GCA AGA GAT GG-3')	
CRTH2	F (5'-AAC ACC AGC ATC CGC TAC AT-3')	281 bp
CRTH2	R (5'-CTG GCG AAC ATG TTG AGA AA-3')	

COX1, PGH synthase 1, COX2; PGH synthase 2, CRTH2; PGD_2 CRTH2 receptor, F, forward; R, reverse.

Table 2-2 Optimal conditions for the detection of β-actin, COX1 and COX2 mRNA by RT-PCR in THP-1 cells

	Annealing Temperature (°C)	Mg^{2+} (mM)	Optimal cycle number.
β-actin	60	1.5	33
COX1	67	1.5	33
COX2	60	2	33
CRTH2	49.5	2	35

The above table shows the optimal conditions for detecting β-actin, COX1, COX2, and CRTH2 mRNA. Mg^{2+} (mM), magnesium concentration; and optimal cycle number.

Table 2-3 Cyclic conditions for COX1, COX2 and β-actin RT-PCR

Step		Temperature (°C)	Time
Denaturation		98	30 sec
33 cycles	denaturation	98	10 sec
	annealing	55–70	30 sec
	extensions	72	20 sec
Final extension		72	10 min
Hold		4	∞

Table 2-4 Cyclic conditions for CRTH2 and β-actin RT-PCR

Step		Temperature (°C)	Time
Denaturation		95	5 min
35 cycles	denaturation	98	10 sec
	annealing	49.5	30 sec
	extensions	72	30 sec
Final extension		72	10 min
Hold		4	∞

2.22 Quantitative Real-time PCR (qRT-PCR)

Quantitative real time-PCR (qRT-PCR) of *INDO1* (IDO-1), *INDOL* (IDO-2) or β-actin was carried out using a TaqMan protocol and a 7300 quantitative RT-PCR instrument from Life Technology (Melbourne, Australia). For quantification of *INDO1*, *INDOL* and β-actin mRNA, we used primers and a fluorogenic oligonucleotide probe designed to hybridise to the specific target sequence (Assay on-demand: *INDO1* (IDO-1); Hs-00158027, *INDOL* (IDO-2); Hs 0158975, β-actin mRNA, Hs-99999903, from Applied Biosystems (Melbourne, Australia). The following instrument settings were used: initial denaturation, 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 sec, and 60 °C for 1 min. Relative quantification of *INDO1, INDOL* and β-actin mRNA expression in stimulated and fatty acid/prostaglandin treated cells was compared to control samples using the $2^{-\Delta\Delta CT}$ method and the relative *INDO1* and *INDOL* fold changes determined by comparison to the control, which was set as 1. It should be noted that basal levels of mRNA in resting cells could be detected by this technique, but all levels were set to 1.

2.23 Prostaglandin and leukotriene quantification

Prostaglandin and leukotriene production by THP-1 cells was quantified using a prostaglandin screening EIA kit (Cat No. 514012) and Cysteinyl Leukotriene EIA kit (Cat No. 500390) from Cayman Chemical Company (Ann Arbor, MI, USA). THP-1 cells (4 x 10^5/50 µL) were pre-treated with indomethacin (IM, 100 µmol/L), nordihydroguaiaretic acid (NDGA, 10 µmol/L), SC-560 (10 µmol/L), NS-398 (25 µmol/L) or diluent for 30 min. Cells were then treated with an equal volume of AA (20 µmol/L) for 30 min and then stimulated with IFN-γ (1000 IU/L) for 24 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was removed and a 4-fold excess of ethanol (100%) added. The samples were vortexed, incubated at 4 °C for 5 min and centrifuged at 3000 g x 10 min at 4 °C. The supernatant was removed and the ethanol evaporated under a stream of nitrogen. The pH of the samples was adjusted to pH 4.0 using citrate buffer. Five hundred microlitres of EIA buffer was added to each sample. The samples were added to the EIA plate according to the manufacturers protocol (Table 2-5), covered and incubated overnight at 4 °C. The plate was then washed five times with wash buffer. To each well was added 200 µL of Ellman's Reagent and 5 µL of tracer added to the total activity well. The plate was covered, shaken at 160 rpm x 60–120 min to develop. The absorbance was read at 405 nm and a result used when the absorbance of the B_0 wells was between 0.3–1.0 (blank subtracted). The prostaglandin and leukotriene cross reactivity of the two kits is shown in Table 2-6 and Table 2-7 respectively.

Table 2-5 Pipetting summary for the Prostaglandin and Leukotriene assay kits

Well	EIA Buffer	Standard/sample	Tracer	Antiserum
BLK	-	-	-	-
TA	-	-	5 µL	-
NSB	100 µL	-	50 µL	-
B_0	50 µL	-	50 µL	50 µL
Standard/sample	-	50 µL	50 µL	50 µL

A total of 100 µL of EIA buffer was added to the non-specific binding (NSB) wells and 50 µL of EIA buffer was added to the maximum binding (B_0) wells. Fifty microlitres of each sample were assayed in duplicate. Fifty microlitres of prostaglandin or leukotriene screening acetylcholine esterase tracer were added to each well except the total activity (TA) and blank (BLK) wells. Fifty microlitres of prostaglandin or leukotriene screening EIA antiserum was added to each well except the TA, NSB and BLK wells.

Table 2-6 Specificity of the Prostaglandin EIA screening kit

Compound	Cross reactivity (%)	Compound	Cross reactivity (%)
PGE_1	100	TXB_2	5.0
PGE_2	100	LTD_4	0.2
$PGF_{1\alpha}$	100	AA	< 0.01
$PGF_{2\alpha}$	100	LTB_4	< 0.01
$PGF_{3\alpha}$	51.3	LTC_4	< 0.01
PGE_2 ethanolamide	44.0	LTE_4	< 0.01
6-keto $PGF_{1\alpha}$	43.6	PGA_1	< 0.01
8-iso $PGF_{2\alpha}$	38.4	PGA_2	< 0.01
8-iso PGE_2	28.5	PGA_3	< 0.01
PGD_2	26.6	PGB_1	< 0.01
8-iso-2,3 dinor $PGF_{1\alpha}$	20.0	15-keto PGE_2	< 0.01
PGE_3	9.5	13,14-dihydro-15-keto $PGF_{2\alpha}$	< 0.01

The table shows the different COX metabolites that can be detected by the prostaglandin EIA screening kit. Arachidonic acid, AA; Leukotriene, LT; Prostaglandin, PG. Data from manufacturer, Cayman Chemical Company (Ann Arbor, MI, USA).

Table 2-7 Specificity of the Cysteinyl Leukotriene EIA Kit

Compound	Cross reactivity (%)
LTC_4	100
LTD_4	100
LTE_4	79
5,6-DiHETE	3.7
LTB_4	1.3
5(S)-HETE	0.04
AA	< 0.01

The table shows the LOX metabolites that can be detected by the cysteinyl leukotriene EIA Kit screening kit. Arachidonic acid, AA; Leukotriene, LT. Data from manufacturer, Cayman Chemical Company (Ann Arbor, MI, USA).

2.24 PGD_2 quantification

PGD_2 in tissue culture medium was quantified using a prostaglandin D_2-MOX EIA kit (Cat. No. 512011) from Cayman Chemical Company (Ann Arbor, MI, USA). The degradation of PGD_2 in the presence of serum proteins such as albumin, give rise to a variety of decomposition products. Similarly, antigenic protein conjugates of PGD_2, synthesised for the production of antiserum, show considerable decomposition. Thus, the resulting antibody response is heterogeneous with poor specificity. This makes PGD_2 assay systems based on the parent compound unreliable and difficult to interpret. A PGD_2-MOX EIA was chosen as it is based on the conversion of PGD_2 to a stable MOX derivative. Treatment of the sample with methoxylamine hydrochloride (MOX HCl) converts PGD_2 into PGD_2-MOX, preventing its further chemical degradation. The antiserum used in the assay was developed using conjugates of this derivative and is specific for PGD_2-MOX. The assay has been validated against stable isotope dilution GC/MS. Measurements using both techniques on identical samples showed a correlation coefficient of 0.97 Cayman Chemical Company (Ann Arbor, MI, USA). THP-1 cells (4 x 10^5/50 µL) were pre-treated with indomethacin (IM, 100 µmol/L), nordihydroguaiaretic acid (NDGA, 10 µM), SC-560 (10 µmol/L), NS-398 (25 µmol/L) or diluent for 30 min. Cells were then treated with an equal volume of AA (20 µmol/L) for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 24 h at 37 °C/5% CO_2 in a humidified atmosphere. Five hundred microlitres of the supernatant was collected, diluted 1:1 with cold acetone (100%) and incubated on ice for 5 min. Samples were then centrifuged at 3000 g x 10 min. The supernatant was collected and evaporated to dryness under a stream of nitrogen. Samples were re-suspended in 100 µL of supplied EIA buffer and the plate was prepared according to the manufacturer protocol (Table 2-5), covered and incubated overnight at 4 °C. The plate was washed five times with wash buffer and to each well were added 200 µL of Ellman's Reagent and 5 µLof tracer added to the total activity well. The plate was covered, shaken at 160 rpm x 60–120 min for development. The plate was read at 405 nm and a result used when the absorbance of the B_0 wells was in the range of 0.3–1.0 (blank subtracted). The cross reactivity of the kit is shown in Table 2-8.

2.25 cAMP quantification

Changes in intracellular cAMP level in THP-1 cells were quantitated using a cyclic AMP EIA Kit (Cat no 581001) from Cayman Chemical (Ann Arbor, MI, USA). THP-1

Table 2-8 Specificity of the Prostaglandin D_2-MOX EIA kit

Compound	Cross reactivity (%)	Compound	Cross reactivity (%)
PGD_2	100	6-keto $PGF_{1\alpha}$	0.05
$PGF_{2\alpha}$	92.4	13,14-dihydro-15-keto PGD_2	0.02
PGJ_2	21.6	AA	< 0.01
PGE_2	2.86	LTD_4	< 0.01
TXB_2	2.54	Tetranor-PGDM	< 0.01
11β-$PGF_{2\alpha}$	1.99	Tetranor-PGEM	< 0.01
8-iso $PGF_{2\alpha}$	1.90	Tetranor-PGFM	< 0.01
PGA_2	0.72	Tetranor-PGJM	< 0.01

The table shows the PGD_2 metabolites that can be detected by the Prostaglandin D_2-MOX EIA kit. Arachidonic acid, AA; Leukotriene, LT; Prostaglandin; PG; Thromoxane, TX. Data from manufacturer, Cayman Chemical Company (Ann Arbor, MI, USA).

cells (4 x 10^6/10 mL) were pre-treated with 3-isobutyl-1-methylxanthine (IBMX) (0.25 µmol/L) for 5 min. Cells were then treated with PGD_2 or 11β-$PGF_{2\alpha}$ (10 µmol/L) for 10 min at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was discarded and 400 µL of 0.1 mol/L HCl added to each tube, the cells suspended and incubated at room temperature for 20 min. The samples were centrifuged at 1000 g x 10 min, and the supernatant diluted 1:2 with supplied EIA buffer. The plate was prepared according to the manufacturers protocol (Table 2-5), covered with plastic film and incubated for 18 h at 4 °C. The plate was washed five times with wash buffer. To each well was added 200 µL of Ellman's Reagent and 5 µL of tracer added to the total activity well. The plate was covered, shaken at 160 rpm x 60–120 min to develop. The absorbance was read at 405 nm and a result used when the absorbance of the B_0 wells was in the range of 0.3–1.0 (blank subtracted). The cross reactivity of the kit is shown in Table 2-9.

2.26 Measurement of changes in STAT1 (Tyr^{701}), total CREB and pCREB (Ser^{133}) via ELISA one.

STAT1 phosphorylation (Tyr701), total CREB and pCREB (Ser^{133}) was assessed using an Alpha Screen Surefire kit from TGR BioSciences (Adelaide, Australia) according to the manufacturer's instructions. Briefly, THP-1 cells (4 x 10^5/mL) were collected by centrifugation (600 g x 5 min) and washed (2 x) in serum free HBSS by centrifugation (600 g x 5 min). Cells were then incubated at 37 °C/5% CO_2 for 2 hours to reduce basal cell activation. A total of 4 x 10^5/100 µL cells were then placed into glass tubes, to which was added an equal volume of PGD_2 (10 µmol/L), Forskolin (100 µmol/L) or 11β-$PGF_{2\alpha}$ (10 µmol/L) for 30 min. Cells were then treated with 200 µL of IFN-γ (1000 IU/mL) prepared in HBSS containing 5% fetal calf serum for 30 min at 37 °C/5% CO_2. Cells were collected by centrifugation (3000 g x 3 min, 4 °C) and suspended in 200 µL of supplied lysis buffer. After 10 min, 50 µL of supernatant in duplicate was transferred into a flat bottom ELISA plate to which was added 50 µL of supplied capture and detection Abs. The plate was covered and incubated at room temperature for 60 min with shaking at 300 rpm. After 60 min, the liquid was discarded and the wells washed 3 times with 200 µL of supplied wash buffer. To each well was then added 100 µL of substrate and the plate incubated at room temperature in the dark for 10 min with shaking at 300 rpm. A total of 10 µL of supplied stop solution was then added to each well and the fluorescence measured with an excitation wavelength of 530–540 nm and detection wavelength of 590–600 nm using Wallac 1420 multilabel counter from Perkin Elmer Life and Analytical Sciences (Turku, Finland).

Table 2-9 Specificity of the Cyclic AMP EIA Kit

Compound	Cross reactivity (%)
cAMP	100
cGMP	1.5
AMP	< 0.001
ATP	< 0.001
Adenoside	< 0.001

cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate. Data from manufacturer, Cayman Chemical Company (Ann Arbor, MI, USA).

2.27 Statistical analysis

Data were statistically analysed using an ANOVA. Statistical comparisons between groups were made using the Kruskal-Wallis test. Post-hoc comparisons between specific pairs were performed using an unpaired exact Mann Whitney. The results are expressed as mean ± standard error of the mean (SEM), unless stated otherwise. Statistics were calculated using Prism Graphpad software version 5. A p value of < 0.04 was considered statistically significant.

Chapter 3

Effect of naturally occurring fatty acids on IFN-γ mediated induction of IDO in THP-1 cells

3.1 Introduction

Fatty acids (FAs) are esterified in the cell membrane and are released by phospholipases as a result of environmental or cellular signals. The main characteristic of FAs is the presence of both a hydrophilic and hydrophobic moiety. FAs and their metabolites are known to play many important roles in both physiological and pathological conditions. Physiologically, they have important roles in both the structure and function of the cell membrane. They are concentrated stores of energy. FAs metabolites can act as signalling molecules and can be released intercellularly, acting as hormones or intracellularly, acting as second messengers. They can act at the receptor level and activate different cellular signalling cascades affecting gene expression (Diggle 2002). On the other hand, FAs, particularly polyunsaturated FAs (PUFAs), are known to modulate the immune response (Cohen, LA 1992; Chon et al. 1995) and affect many inflammatory diseases. In general, n-6 PUFAs are considered to have pro-inflammatory activities, while n-3 PUFAs have anti-inflammatory activities (Calder 2009). While many studies have shown a strong correlation between different types of dietary FAs and colon (Weisburger 1997; Bartsch, Nair & Owen 1999; Kuriki et al. 2007), breast (Rose 1997; Weisburger 1997; Bartsch, Nair & Owen 1999; Funahashi et al. 2008) and prostate cancer (Rose 1997; Weisburger 1997; Park, SY et al. 2009), there are also reports with contradictory findings about their effect on cancer (Table 1-7, Table 1-8, Table 1-9). Again it has been suggested that n-6 PUFAs and saturated FAs (SAFAs) have a stronger correlation with cancer progression, while n-3 PUFAs and monosaturated FAs (MUFAs) have a lower correlation with cancer progression or are even protective against cancer development (Hursting, Thornquist & Henderson 1990; Caygill, Charlett & Hill 1996; Diggle 2002).

In our study (Bassal, Hughes & Costabile 2012), THP-1 cells, an acute monocytic leukaemic cell line were used to study the effect of arachidonic acid (AA) on the activation and regulation of IDO. THP-1 cells have previously been demonstrated to express IDO at basal levels (Taylor & Feng 1991), which can be increased following treatment with agonists, such as interferon gamma (IFN-γ) (Werner-Felmayer et al. 1989a; Taylor & Feng 1991), and lipopolysaccharide (LPS) (Werner-Felmayer et al. 1989a; Fujigaki, S et al. 2001; Travers et al. 2004). In this current study, IFN-γ was used as the main inducer of IDO. The effect of IFN-γ on IDO induction/activity was determined by indirectly measuring the level of kynurenine (Kyn) and/or the corresponding decrease in tryptophan (Trp) levels. It was previously determined that a cell density of (4×10^5/well) for THP-1 cells and (4×10^6/well) for human monocytes, treatment time of 72 h and IFN-γ concentration of 1000 IU/mL were optimal for stimulation of Kyn production in these

cells. Having demonstrated that AA inhibited IDO activity, it was important to examine the effect of other FAs on IDO activity. As the activities of FAs are affected by chain length, saturation and series (Bates et al. 1995; Ferrante, JV et al. 1997), a commercially available library consisting of 68 naturally occurring FAs with saturated, monounsaturated and PUFAs varying in chain length, degree of unsaturation, configuration and series was purchased. These FAs were screened for their ability to modulate the IFN-γ mediated increase in Kyn production in THP-1 cells. Based on the obtained results, several structural features of FAs were shown to play a role in their biological inhibitory activity towards IDO. To the best of our knowledge no study has examined, in detail, the regulation of IDO activity by different FAs.

3.2 Effect of different FAs on IFN-γ induced IDO activity in THP-1 cells

THP-1 cells (4 x 10^5/well) were pre-treated with the individual FA at 20 µmol/L (a concentration that is routinely used in the literature) or diluent (maximum 0.02% v/v) for 30 min in medium lacking serum. Cells were then stimulated with IFN-γ (1000 IU/mL) prepared in medium containing serum for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. Unstimulated cells produced no to very low levels of Kyn, while treatment with IFN-γ caused a significant increase in Kyn production. In most cases, cells that were pre-treated with FAs alone, showed no significant effect on basal Kyn production. In cells that had been pre-treated with different FAs and then stimulated with IFN-γ, there was a spectrum of biological activities. The results were analysed and presented in tables in the following sections.

3.3 Structure–function analysis

From the results it was clear that there was a spectrum of inhibitory activity across the different FAs. To determine why a FA gave a particular response, we examined the relationship between FA structure and inhibitory activity of these FAs towards IFN-γ induced Kyn production.

3.3.1 Saturated fatty acids (SAFAs)

Of the 16 SAFAs available in the library, none displayed any significant inhibitory activity toward IFN-γ stimulated Kyn production in THP-1 cells. There was no relationship between chain length and biological activity (Table 3-1). For example, the shortest FA

Table 3-1 Ability of SAFAs to modulate the IFN-γ mediated induction/avtivity of IDO in THP-1 cells

Fatty acid name + IFN-γ	*C*:*DB*	Kyn (umol/mL)	% inhibition ± SEM
Control	-	4 ± 4	
IFN-γ	-	52 ± 13***	
Decanoic	10:0	41 ± 12	20 ± 3
Undecanoic	11:0	52 ± 12	1.49 ± 11
Dodecanoic	12:0	53 ±1 3	–3.64 ± 9
Tridecanoic	13:0	48 ± 12	6.30 ± 15
12-Methoxydodecanoic	13:0	49 ± 13	5.66 ± 3
Tetradecanoic	14:0	39 ± 14	24.35 ± 25
Pentadecanoic	15:0	44 ± 10	14.68 ± 4
Hexadecanoic	16:0	48 ± 13	6.6 ± 4
Heptadecanoic	17:0	55 ± 12	–7.37 ± 15
Octadecanoic	18:0	37 ± 13	28.17 ± 22
Nonadecanoic	19:0	48 ± 15	6.15 ± 28
Eicosanoic	20:0	56 ± 15	-7.66 ± 3
Heneicosanoic	21:0	61 ± 13	–18.58 ± 13
Docosanoic	22:0	37 ± 14	28.32 ± 22
Tricosanoic	23:0	57 ± 14	-10.63 ± 7
Tetracosanoic	24:0	57 ± 14	–9.7 ± 2

THP-1 cells (4 x 10^5/well) were treated with the individual saturated fatty acid (20 µmol/L) for 30 min, and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. The culture supernatant was removed and Kyn measured using the colorimetric assay. The table shows: fatty acid name; C, number of carbon atoms; DB, number of double bonds. Results are presented as mean ± SEM of Kyn production, n=4 separate experiments. Kruskal-Wallis test was not significant, p=0.2649.

tested, decanoic acid (10:0), had no inhibitory activity, yet Jiang et al, (2010) showed that the very short chain FA, sodium butyrate (NaB; 4:0), could modulate IDO activity in GNE2 (nasopharyngeal carcinoma) cells (Jiang et al. 2010). In this study, NaB was used at 0.5 mmol/L, a concentration much higher than that used in this study.

3.3.2 Monounsaturated fatty acids (MUFAs)

A total of 31 MUFAs were available for screening. None of the MUFAs modulated the basal levels of Kyn produced in unstimulated cells. Of these 31 FAs, only three had significant inhibitory effects towards Kyn produced by IFN-γ stimulated cells, 10(Z)-Nonadecenoic (19:1;n-9), 14(Z)-Tricosenoic (23:1;n-9) and 15(Z)-Tetracosenoic (24:1;n-9). Three fatty acids stimulated Kyn production following IFN-γ treatment, 10-Undecenoic acid (11:1; n-1), 11-Dodecenoic acid (12:1;n-1) and 11(E) Eicosenoic acid (20:1;n-9) yet there were not statistically significant.(Table 3-2). A structure–function analysis of the results demonstrated that there was a large amount of variation between chain length and the biological activity of the MUFAs. Interestingly, FAs with 20 carbon atoms did not have any inhibitory activity. Also, varying MUFAs with the Z configuration were in general more biologically active than the corresponding MUFAs in the E configuration. When comparing the biological effect of the Z vs. E configuration of the same FAs (12 pairs tested in the library), we found that 58% of FAs in the Z configuration were more active than the same FAs in the E configuration. For example, 10(Z)-pentadecenoic acid (15:1;n-5) had more inhibitory activity than 10(E)-pentadecenoic acid (15:1;n-5). The same was seen with 9(Z)-hexadecenoic acid (16:1;n-7) being active while 9(E)-hexadecenoic acid (16:1;n-7) was not. When the inhibitory activity of the different FAs series of MUFAs was examined, only three of the n-9 were inhibitory. Thus, there does seem to be some relationship between series and configuration and ability of the monounsaturated fatty acids to inhibit IDO activity.

3.3.3 Polyunsaturated fatty acids (PUFAs)

A total of 21 PUFAs were available for screening. None of the PUFAs modulated the basal levels of Kyn produced in unstimulated cells. Of the 21 PUFAs tested, AA, DHA and Dihomo-γ-Linolenic acid had significant inhibitory activity. A total of 14 (66.7%) displayed inhibitory activity on Kyn production in IFN-γ induced stimulated THP-1 cells, however this was not statistically significant. Only 6(Z),9(Z),12(Z),15(Z)-octadecatetraenoic acid caused an increase in Kyn production in these cells (Table 3-3).

Table 3-2 Ability of MUFAs to modulate IFN-γ mediated induction/activity of IDO in THP-1 cells

Fatty acid name + IFN-γ	C:DB;n-	Kyn (umol/L)	% inhibition ± SEM
Control	-	1 ± 0.56	
IFN-γ	-	31 ± 7***	
10-Undecenoic	11:1;n-1	53 ± 13	–8 ± 7
11-Dodecenoic	12:1;n-1	33 ± 6	–12 ± 9
12-Tridecenoic	13:1;n-1	31 ± 7	2 ± 8
9(Z)-Tetradecenoic	14:1;n-5	20 ± 4	21 ± 14
9(E)-Tetradecenoic	14:1;n-5	19 ± 4	21 ± 14
10(Z)-Pentadecenoic	15:1;n-5	26 ± 5	16 ± 5
10(E)-Pentadecenoic	15:1;n-5	27 ± 5	14 ± 7
9(Z)-Hexadecenoic	16:1;n-7	24 ± 5	18 ± 9
9(E)-Hexadecenoic	16:1;n-7	27 ± 5	5 ± 14
10(Z)-Heptadecenoic	17:1;n-7	27 ± 5	11 ± 4
10(E)-Heptadecenoic	17:1;n-7	28 ± 6	12 ± 6
6(Z)-Octadecenoic	18:1;n-12	15 ± 3	30 ± 11
6(E)-Octadecenoic	18:1;n-12	22 ± 5	16 ± 11
9(Z)-Octadecenoic	18:1;n-9	14 ± 3	40 ± 7
9(E)-Octadecenoic	18:1;n-9	28 ± 6	11 ± 5
11(Z)-Octadecenoic	18:1;n-7	21 ± 4	32 ± 5
11(E)-Octadecenoic	18:1;n-7	31 ± 6	–4 ± 7
7(Z)-Nonadecenoic	19:1;n-12	17 ± 4	26 ± 13
7(E)-Nonadecenoic	19:1;n-12	24 ± 6	–3 ± 12
10(Z)-Nonadecenoic	19:1;n-9	11± 2*	54 ± 5*
10(E)-Nonadecenoic	19:1;n-9	27 ± 6	13 ± 3
11(E)-Eicosenoic	20:1;n-9	36 ± 6	–22 ± 9
8(Z)-Eicosenoic	20:1;n-12	21 ± 8	43 ± 12
11(Z)-Eicosenoic	20:1;n-9	22 ± 4	18 ± 8
5(Z)-Eicosenoic	20:1;n-15	25 ± 5	8 ± 12
12(Z)-Heneicosenoic	21:1;n-9	17 ± 4	47 ± 7
13(Z)-Docosenoic	22:1;n-9	19 ± 2	23 ± 11
13(E)-Docosenoic	22:1;n-9	16 ± 3	29 ± 10
14(Z)-Tricosenoic	23:1;n-9	9 ± 2**	59 ± 7**
14(E)-Tricosenoic	23:1;n-9	28 ± 6	11 ± 8
15(Z)-Tetracosenoic	24:1;n-9	12 ± 3*	45 ± 13*

THP-1 cells (4 x 10^5/well) were treated with the individual polyunsaturated fatty acid (20 µM) for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. The culture supernatant was removed and Kyn measured using the colorimetric assay. The table shows: fatty acid name; C, number of carbon atoms; DB, number of double bonds and n-, series. Results are presented as mean ± SEM of Kyn production, n=12 separate experiments. Comparisons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; **, $p < 0.001$, ***, $P < 0.000$. A negative value of the % inhibition means there is an increase production of Kyn after pre-treatment of cells with FA and then treated with IFN-γ.

Table 3-3 Ability of PUFAs to modulate IFN-γ mediated induction /activity of IDO in THP-1 cells

Fatty acid name + IFN-γ	C:DB;n-	Kyn (μmol/L)	% inhibition ± SEM
Control		1 ± 0.56	-
IFN-γ		31 ± 7***	-
9(Z),12(Z)-Octadecadienoic	18:2;n-6	26 ± 5	6±12
9(E),11(Z)-Octadecadienoic	18:2;n-7	23 ± 4	26 ± 8
9(E),12(E)-Octadecadienoic	18:2;n-6	24 ± 6	23 ± 5
9(Z),12(Z),15(Z)-Octadecatrienoic	18:3;n-3	24 ± 6	23 ± 7
6(Z),9(Z),12(Z)-Octadecatrienoic	18:3;n-6	32 ± 6	-3 ± 8
6(Z),9(Z),12(Z),15(Z)-Octadecatetraenoic	18:4;n-3	35 ±6	–13 ± 10
10(Z),13(Z)-Nonadecadienoic	19:2;n-6	21 ± 4	32 ± 8
7(Z),7(Z)-Dimethyleicosadienoic	19:2;n-12	22 ± 2	29 ± 18
5(Z),8(Z)-Eicosadienoic	20:2;n-12	18 ± 3	42 ± 10
11(Z),14(Z)-Eicosadienoic	20:2;n-6	18 ± 5	42 ± 8
5(Z),8(Z),11(Z)-Eicosatrienoic	20:3;n-9	17 ±6	45 ± 7
8(Z),11(Z),14(Z)-Eicosatrienoic	20:3;n-6	10 ±1**	68 ± 10**
11(Z),14(Z),17(Z)-Eicosatrienoic	20:3;n-3	25 ±5	19 ± 10
5(Z),8(Z),11(Z),14(Z)-Eicosatetraenoic	20:4;n-6	14 ± 2**	55 ± 11**
5(Z),8(Z),11(Z),14(Z),17(Z)-Eicosapentaenoic	20:5;n-3	25 ±4	719 ± 18
12(Z),15(Z)-Heneicosadienoic	21:2;n-6	19 ± 4	39 ± 13
13(Z),16(Z)-Docosadienoic	22:2;n-6	19 ± 4	39 ± 8
13(Z),16(Z),19(Z)-Docosatrienoic	22:3;n-3	15 ± 2**	52 ± 5**
7(Z),10(Z),13(Z),16(Z)-Docosatetraenoic	22:4;n-6	18 ± 3	42 ± 12
4(Z),7(Z),10(Z),13(Z),16(Z)-Docosapentaenoic	22:5;n-6	16 ± 3	48 ± 13
4(Z),7(Z),10(Z),13(Z),16(Z),19(Z)-Docosahexaenoic	22:6;n-3	20 ± 4	35± 14

THP-1 cells (4 x 10^5/well) were treated with the individual polyunsaturated fatty acid (20 μM) for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. The culture supernatant was removed and Kyn measured using the colorimetric assay. The table shows: fatty acid name; C, number of carbon atoms; DB, number of double bonds and n-, series. Results are presented as mean ± SEM of Kyn production, n=12 separate experiments. Comparisons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; **, $p < 0.001$, ***, $P < 0.000$. A negative value of the % inhibition means there is an increase production of Kyn after pre-treatment of cells with FA and then treated with IFN-γ.

There was a general trend of increasing activity associated with increasing chain length. The most noticeable relationship between structure and function was again that the Z configuration in general had more inhibitory activity than the E configuration. Of the 14 biologically active FAs, 12 were in the Z configuration, one in the E configuration and one FA has both Z and E configurations that were active. The only exception was 9(Z),12(Z)-octadecadienoic acid (18:2;n-6) which did not have any significant activity while 9(E),12(E)-octadecadienoic acid (18:2;n-6) was found to have an inhibitory activity. When the inhibitory activity of different series of PUFAs was examined, it was found that there were no significant differences between the n-6 PUFAs and n-3 PUFAs. However, it should be noted that the number of FAs in each series was not equal and thus testing of additional PUFAs may alter these results.

3.4 Summary

After screening a library of 68 saturated, monounsaturated and polyunsaturated FAs, 6 (9%) were identified that significantly inhibited IFN-γ stimulated Kyn production in THP-1 cells. Of the 16 saturated FAs tested, none had any significant effect. The introduction of one or more double bond to the FAs resulted in an increase in the inhibitory activity of the FAs. Of the 31 monounsaturated FAs tested, 3 had significant inhibitory activity. Of 21 polyunsaturated FAs tested, 3 were significantly inhibitory. The inhibitory activity of FAs increased with increasing chain length as well as increasing the number of double bonds. FAs with the Z configuration were found to have more inhibitory activity than FAs with the E configuration. The activity of the same FAs with both Z and E configuration showed that the Z configuration was more active than the E configuration. We also identified no significant differences in activity between different series; the n-6 PUFAs appeared to have the same activity as n-3 PUFAs although in some cases the number of FAs available for testing was limited.

Chapter 4

Modulation of IFN-γ mediated induction of IDO in THP-1 cells and human monocytes by arachidonic acid and its mechanism of action

4.1 Introduction

We recently demonstrated that arachidonic acid (AA) inhibited the interferon gamma (IFN-γ) mediated induction of IDO in THP-1 cells (Bassal 2008; Bassal, Hughes & Costabile 2012). This inhibitory effect was dose (IC_{50}~20μM) and time dependent (significant inhibition appearing as early as 20 min post pre-treatment) and was not due to apparent cell toxicity. A structural analysis identified that both the terminal carboxyl and methyl groups of AA were important for its inhibitory activity. The metabolism of AA through the cyclooxygenase pathway (COX) but not lipoxygenase pathway (LOX) was required for the inhibitory activity. AA did not affect IFN-γ cell surface receptor expression; however, it caused an 80% decrease in STAT1 phosphorylation on Tyr701.

In this chapter, the effect of AA on the IFN-γ mediated induction of IDO in human monocytes is examined. Since THP-1 cells were used as a model cell line of monocytes, it was important to confirm the activity of AA in primary human monocytes. Further, the mechanism of action of AA on the IFN-γ mediated induction of IDO in both THP-1 cells and human monocytes was characterised. Previously the optimal conditions for measuring maximal Kyn levels were determined to be a cell density of 4 x 10^5/100 μL, an IFN-γ concentration of 1000 IU/mL, an AA pre-treatment time of 30 min and an IFN-γ treatment time of 72 h (Bassal 2008). After performing a dose curve analysis of IFN-γ on Kyn production, maximal stimulation was seen at 1000 IU/mL (Bassal 2008). Since this project aimed to identify inhibitory compounds, we chose to continue with 1000 IU/mL of IFN-γ. It is possible that at lower levels of IFN-γ, any stimulatory effects of the compounds may also be seen, however this is beyond the scope of this project. Kyn was used as an indirect measure of IDO activity and was quantified using a colorimetric assay. The colorimetric method using ER should be used with caution when measuring Kyn concentrations, especially in monocyte culture medium, as it is not specific for Kyn, but also reacts with other Kyn pathway metabolites (Alegre, Lopez & Gonzalez 2005). However we also used HPLC to examine the specificity and accuracy of the colorimetric method. Our results indicated that both methods could be used and they gave the same results and given the specificity of HPLC, one assumes that the colorimetric assay has acceptable specificity (Bassal 2008). In some cases, we also repeated experiments stimulating cells with IFN-γ before the addition of AA, AA was still able to inhibit Kyn production, although the level of activity was less than when used prior to IFN-γ stimulation (data not shown). These same conditions were used when assessing the effect of AA on IFN-γ mediated induction of IDO in human monocytes, the only modification being that monocytes were used at a density of 4 x 10^6/100 μL.

4.2 Effect of AA on the IFN-γ mediated induction of IDO in human monocytes

Human monocytes (4 x 10^6/100 µL) were pre-treated with AA (20 µmol/L) or diluent for 30 min in medium lacking serum, supplemented with L-Trp (100 µmol/L) at 37 °C/5% CO_2 in a humidified atmosphere. The cells were then treated with IFN-γ (1000 IU/ml) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere in medium containing serum and supplemented with L-Trp (100 µmol/L). The supernatant was collected and Kyn levels measured using a colorimetric assay. Consistent with the results obtained with THP-1 cells (Bassal, Hughes & Costabile 2012), IFN-γ caused a significant increase in Kyn production compared to unstimulated cells, while AA alone had no significant effect on basal Kyn production. AA pre-treatment of cells followed by IFN-γ stimulation caused a significant inhibition in Kyn production (86.3% ± 2) compared to IFN-γ alone (Figure 4-1). The viability of the human monocytes was not significantly affected after this incubation period (% viability of cells treated with AA+IFN-γ compared to cells treated with IFN-γ was 94.6% ± 7).

4.3 Effect of AA concentration on inhibitory activity towards monocytes

Having confirmed that AA was also able to inhibit Kyn production by human monocytes, we next examined the effect of AA concentration on this inhibitory effect. Cells were pretreated with AA (0–30 µmol/mL) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h. The supernatant was collected and Kyn levels measured using the colorimetric assay. Consistent with previous results IFN-γ caused a significant increase in Kyn production compared to unstimulated cells. AA alone had no significant effect on basal Kyn production. AA pre-treatment of cells followed by IFN-γ stimulation caused a significant dose dependent inhibition of Kyn production compared to IFN-γ stimulated cells alone with significant inhibition seen with as little as 5 µM AA. The IC_{50} was calculated to be 12.8 ± 2.6 µmol, which was lower than that observed in THP-1 cells (Figure 4-2).

4.4 Modulation of INDO1 transcription by AA

It is well-known that IFN-γ mediates the induction of IDO at both the transcriptional and translational levels (Munn et al. 2004). Since AA inhibited the IFN-γ mediated induction/activity of IDO in THP-1 cells, as well as human monocytes, it was important to examine the effect of AA at the level of transcription. The effect of AA on IFN-γ mediated

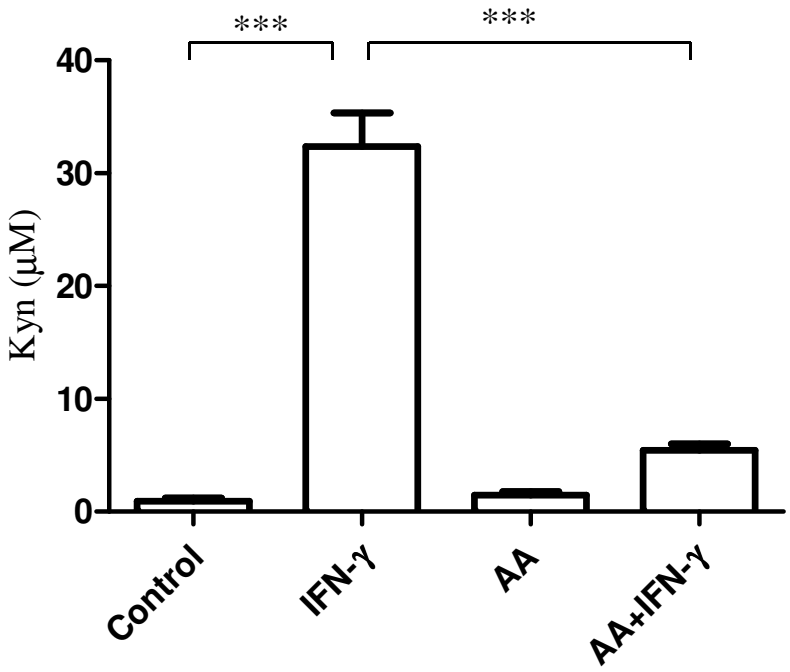


Figure 4-1 Effect of AA on IFN-γ mediated Kyn production in human monocytes
Monocytes (4 x 10^6/100 μL) were pre-treated with AA (20 μmol/L) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are presented as mean ± SEM, n=11 separate experiments. Comparisons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; *** indicates $p < 0.0001$.

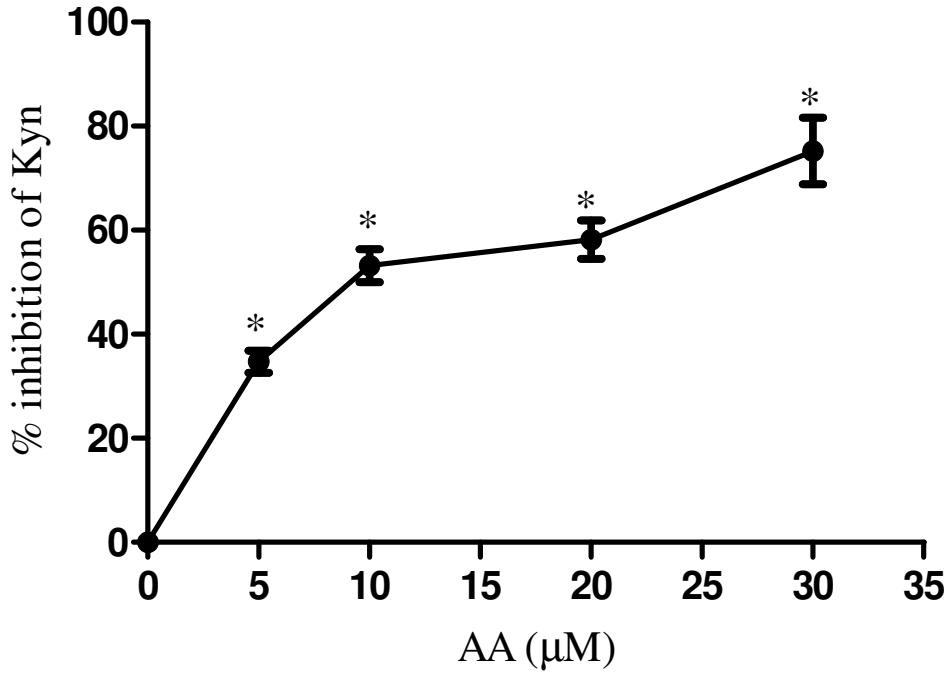


Figure 4-2 Effect of AA concentration on IFN-γ mediated induction of IDO in human monocytes

Monocytes (4 x 10^6/well) were treated with AA (0–30 μmol/mL) for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are presented as mean ± SEM of % inhibition of Kyn production, n=4 separate experiments. Comparisons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$. Kyn produced in resting cells was 0.09 ± 0.3 μmol/mL while in response to IFN-γ it was 32.3 ± 0.3 μmol/mL.

induction of *INDO1* and *INDOL* in THP-1 cells and human monocytes was examined using a TaqMan gene expression assay. However, prior to analysis, the conditions for measuring *INDO1* were optimised.

4.4.1 Real- time quantitative PCR (qRT-PCR) efficiency

The efficiency of a qRT-PCR is evaluated by performing 10-fold serial dilutions of a target sample. After correctly setting the baseline and threshold, the slope of the standard curve can be used to calculate the efficiency of amplification, using the following equation: efficiency = $10^{(-1/\text{slope})}$-1. Thus, if the slope is –3.32, the efficiency is 100%. The 7300 Real-Time PCR system (software version 2.0) calculates the efficiency of the qRT-PCR of a standard curve as shown in Table 4-1. Ideally, the efficiency of the qRT-PCR should be between 90–100% (–3.6 ≥ slope ≥ –3.3). If the efficiency is 100%, the C_T (the cycle number at which the fluorescence generated within a reaction crosses the threshold line) value of the 10-fold dilution will occur every 3.3 cycles (i.e. a 2-fold change will occur for each change in C_T). Due to their ready availability, we chose to use THP-1 cells for the optimisation process. Cells (4 x 10^6/total) were treated with IFN-γ (1000 IU/mL) or diluent for 24 h. Total RNA was extracted and 1, 2 or 3 µg of RNA used to prepare the corresponding cDNA. A series of 10-fold dilutions of cDNA were prepared and *INDO1* and β-actin used to determine the efficiency of the qRT-PCR. Using 1, 2 and 3 µg of total RNA, the slope for β-actin mRNA was calculated to be –3.7, –3.6 and –3.9 respectively which corresponded to 86%, 90% and 80% efficiency respectively. In IFN-γ stimulated cells, the slope for β-actin mRNA was –3.3, –4 and –4 with 100%, 78% and 78% efficiency respectively (Figure 4-3). Based on these results, a total of 2 µg of RNA was used to prepare cDNA as it gave the best result considering both β-actin mRNA slope for control and IFN-γ stimulated cells. This amount is also consistent with the manufacturer's recommendation.

4.4.2 Determining the optimal concentration of cDNA for measuring *INDO1*

Having determined the starting amount of total RNA to use, we next determined the optimal concentration of cDNA to be used. THP-1 cells (4 x 10^6/total) were treated with IFN-γ (1000 IU/mL) or diluent for 24 h. Total RNA was extracted and 2 µg of total RNA was used to prepare the corresponding cDNA. Using a range of cDNA concentrations (25–400 ng), the qRT-PCR was performed to measure *INDO1* and β-actin mRNA as the endogenous control. The results showed that there were no significant differences between

Table 4-1 Relationship between slope and percentage efficiency of a qRT-PCR

Slope	% Efficiency
–3.32	100
–3.5	93
–3.6	90
–3.8	83
–4	78

The efficiency of any RT-PCR is evaluated by performing 10-fold serial dilutions of a target sample. After correctly setting the baseline and threshold, the efficiency can be calculated from the slope of the standard curve using the following equation: efficiency = 10(-1/slope) – 1. (Data from manufacturer Life Technology (Melbourne, Australia).

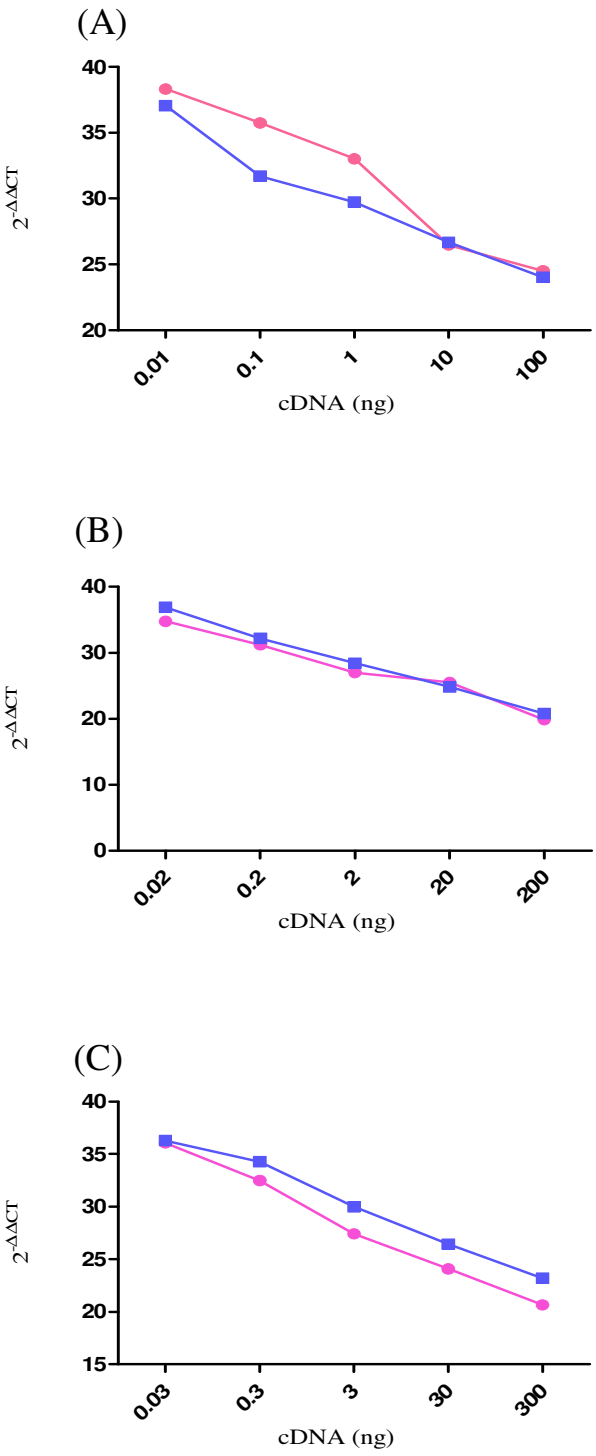


Figure 4-3 Determination of real-time quantitative PCR (qRT-PCR) linearity at varying cDNA levels

THP-1 cells (4 x 10^6/total) were treated with IFN-γ (1000 IU/mL) or diluent for 24 h. Total RNA was extracted and cDNA prepared from 1, 2 or 3 µg of total RNA. qRT-PCR was performed using Taq-man gene expression assay. Results are representative of one experiment performed in triplicate. A=1 µg, B=2 µg and C=3 µg. Relative quantification ($2^{-\Delta\Delta CT}$). β-actin in control cells (●) and β-actin in IFN-γ treated cells (■).

25, 50 and 100 ng of cDNA in detecting *INDO1*. At higher levels, there were significant differences between 100 ng and 200 ng and 400 ng respectively but no significant difference between 200 ng and 400 ng concentrations (Figure 4-4). From these results, a concentration of 100 ng was chosen for future experiments.

4.4.3 Kinetics of IFN-γ mediated induction of *INDO1* in THP-1 cells

In order to fully assess the impact of fatty acid treatment on changes in *INDO1* levels, we next determined the kinetics of *INDO1* expression in THP-1 cells. To do so, THP-1 cells (4 x 10^6/total) were treated with IFN-γ for 24, 48 or 72 h. Cells were collected total RNA extracted, and 2 µg of total RNA was converted to cDNA. Using 100 ng of cDNA, changes in *INDO1* were measured and corrected against β-actin mRNA. The results showed that maximal *INDO1* could be detected after 24 h (116.5 ± 9 fold increase over basal levels) compared to (81.29 ± 15.42 fold increase) and (41.76 ± 4.94 fold increase) after 48 and 72 h respectively (Figure 4-5). Although there was a trend of decreasing *INDO1* over time, there was no significant difference between the 24 and 48 h time points and the only significant difference was between the 24 and 72 h time points. As a result, the 24 h treatment time point was chosen for all future experiments.

4.5 Effect of AA on IFN-γ mediated induction of INDO1 and INDOL

Having optimised the conditions for measuring changes in *INDO1*, we next assessed the effect of AA on the IFN-γ mediated induction of *INDO1* and *INDOL* in THP-1 cells and human monocytes. Cells were pre-treated with AA (20 µmol/L) or diluent for 30 min followed by stimulation with IFN-γ (1000 IU/mL) or diluent for 24 h. The cells were collected, total RNA extracted and 2 µg of total RNA converted to cDNA. Using 100 ng of cDNA, *INDO1, INDOL* and β-actin mRNA were measured by qRT-PCR. Consistent with changes in Kyn levels, IFN-γ caused a significant increase in *INDO1* in both cell types compared to unstimulated cells. AA treatment alone caused no significant change in basal *INDO1* levels, however, when cells were pre-treated with AA before treatment with IFN-γ although not significant, there was a decrease in *INDO1* levels. AA caused a 33% inhibition of *INDO1* in THP-1 cells and a 50% inhibition in *INDO1* in human monocytes compared to IFN-γ alone (Figure 4-6. These results further confirmed that the inhibitory effect of AA on IFN-γ mediated induction of IDO is at the level of transcription. Unfortunately, *INDOL* could not be detected in resting or IFN-γ stimulated cells (data not shown).

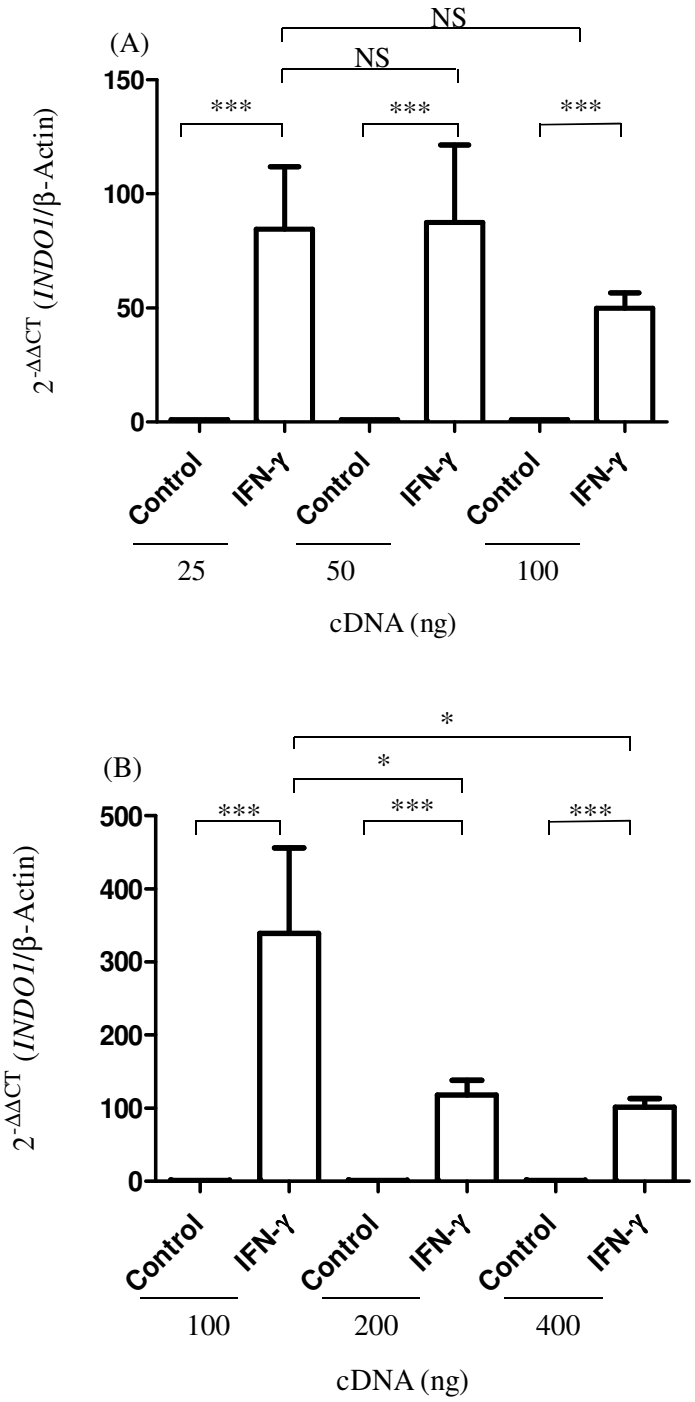


Figure 4-4 Determination of the optimal concentration of cDNA for measuring *INDO1*

THP-1 cells (4 x 10^6/total) were treated with IFN-γ (1000 IU/mL) or media for 24 h. Total RNA was extracted and 2 µg of total RNA was converted to cDNA. (A) Using 25, 50 or 100 ng of cDNA or (B) 100, 200 or 400 ng of cDNA, qRT-PCR was performed using a Taq-man gene expression assay. Results are presented as mean ± SEM of 4 separate experiments. The control set to 1 and *INDO1* normalised against β-actin mRNAComparisons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$. *** indicates $p < 0.0001$.

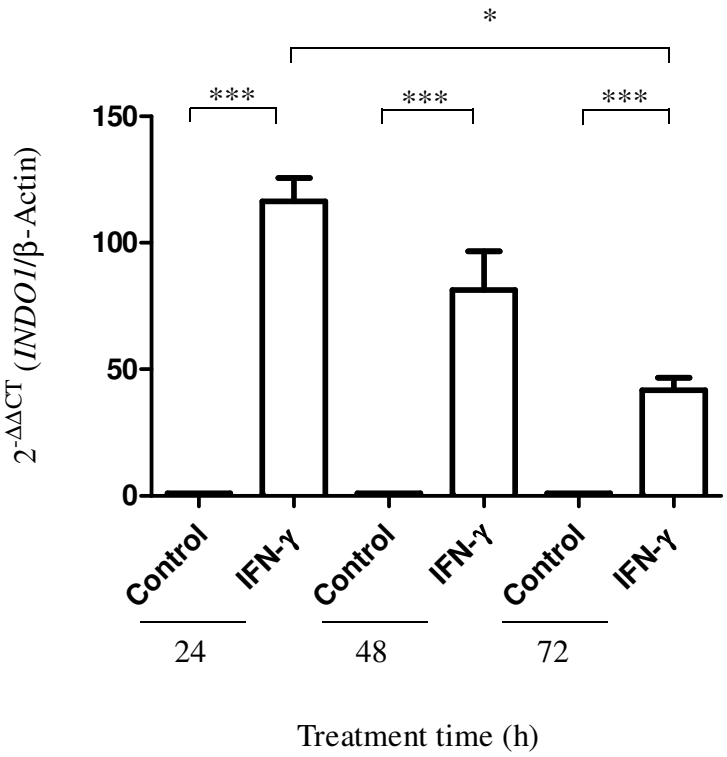


Figure 4-5 Kinetics of *INDO1* in IFN-γ stimulated THP-1 cells

THP-1 cells (4 x 10^6/total) were treated with medium or IFN-γ (1000 IU/mL) for 24, 48 or 72 h. Total RNA was extracted and 2 µg was converted to cDNA. Using 100 ng of cDNA, qRT-PCR was performed using Taq-man gene expression assay. Results are presented as mean ± SEM 4 separate experiments with control set to 1 and *INDO1* normalised against β-actin mRNA. Compareson were performed using Kruskal-Wallis, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$, *** indicates $p < 0.0001$.

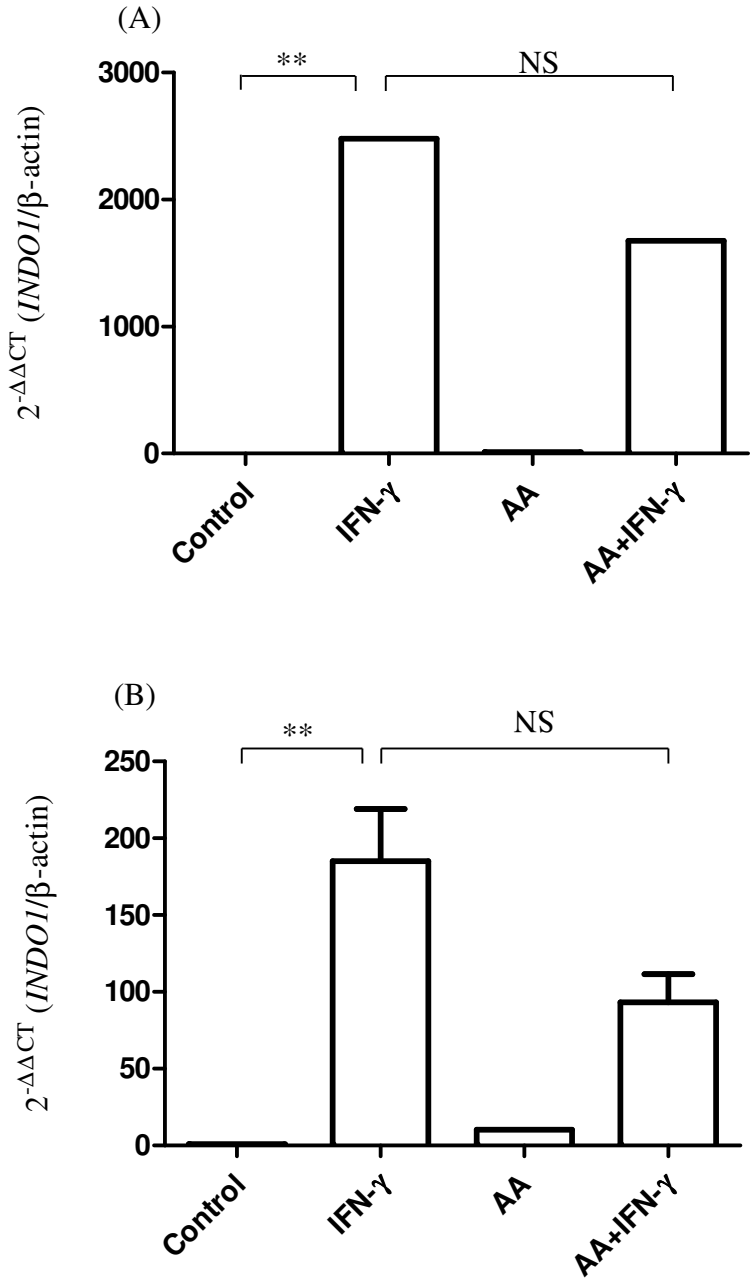


Figure 4-6 Effect of AA on IFN-γ mediated induction of *INDO1* in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^6/total) or (B) human monocytes (5 x 10^6/Total) were pre-treated with AA (20 µmol/mL) or diluent for 30 min followed by stimulation with IFN-γ (1000 IU/mL) for 24 h at 37 °C/5% CO_2 in a humidified atmosphere. RNA was extracted and 2 µg converted to cDNA. Using 100 ng of cDNA, qRT-PCR was performed using Taq-man gene expression assay. Results are expressed as mean ± SEM of 4 separate experiments each in triplicate, with *INDO1* normalised against β-actin mRNA. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired ecaxt Mann Whitney test; ** indicates $p < 0.001$. For the THP-1 cells, error bars are plotted but are too small to be seen.

4.6 Effect of AA on IFN-γ mediated induction of IDO protein

From the literature, it is known that changes in *INDO1* levels do not necessarily correlate with changes in IDO protein expression (Fujigaki, H, Seishima & Saito 2012). As a result, it was important to determine if the reduction in IDO activity, as seen by reduced Kyn levels and a reduction in *INDO1*, also led to a reduction in IDO protein expression. THP-1 cells or monocytes (1 x 10^7/total) were treated with AA (20 µmol/mL) or diluent for 30 min. The cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. Cells were collected and protein was extracted and assessed for IDO protein via Western blot. Resting cells showed no detectable IDO protein, while in IFN-γ treated cells, a strong immunoreactive band was readily observed. Consistent with the Kyn results, AA alone did not stimulate IDO protein production. In contrast, AA pre-treatment followed by IFN-γ stimulation caused a significant inhibition of IFN-γ mediated induction of IDO-1 protein (31.7%) in THP-1 cells and (86.5%) in monocytes compared to protein production from IFN-γ stimulated cells (Figure 4-7).

4.7 Effect of indomethacin and nordihydroguaiaretic acid on the AA inhibitory effect

Previously we examined if metabolism of AA via the cyclooxygenase (COX) or lipoxygenase (LOX) enzymes was required for its inhibitory activity (Bassal, Hughes & Costabile 2012). THP-1 cells were pre-treated with indomethacin (IM, 100 µmol/L), nordihydroguaiaretic acid (NDGA, 10 µmol/L) or diluent for 30 min. Cells were then treated with AA (20 µmol/L) or diluent for an additional 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was harvested and Kyn levels quantitated. Consistent with previous results, IFN-γ caused a significant increase in Kyn production compared to unstimulated cells and AA alone had no significant effect on basal Kyn production. AA pre-treatment of cells followed by IFN-γ stimulation caused a significant inhibition of Kyn production. IM had no significant effect on Kyn production compared to baseline, and no significant effect on IFN-γ mediated production of Kyn or any significant effect on AA induced basal Kyn levels. However, when cells were pre-treated with IM followed by AA and then IFN-γ, the inhibitory activity of AA was significantly reduced compared to cells treated with AA and then IFN-γ alone. In contrast, NDGA, a LOX inhibitor, failed to cause any correction of the AA mediated inhibition of Kyn. These results confirmed that metabolism of AA via COX was required for the inhibitory activity observed (Figure 4-8).

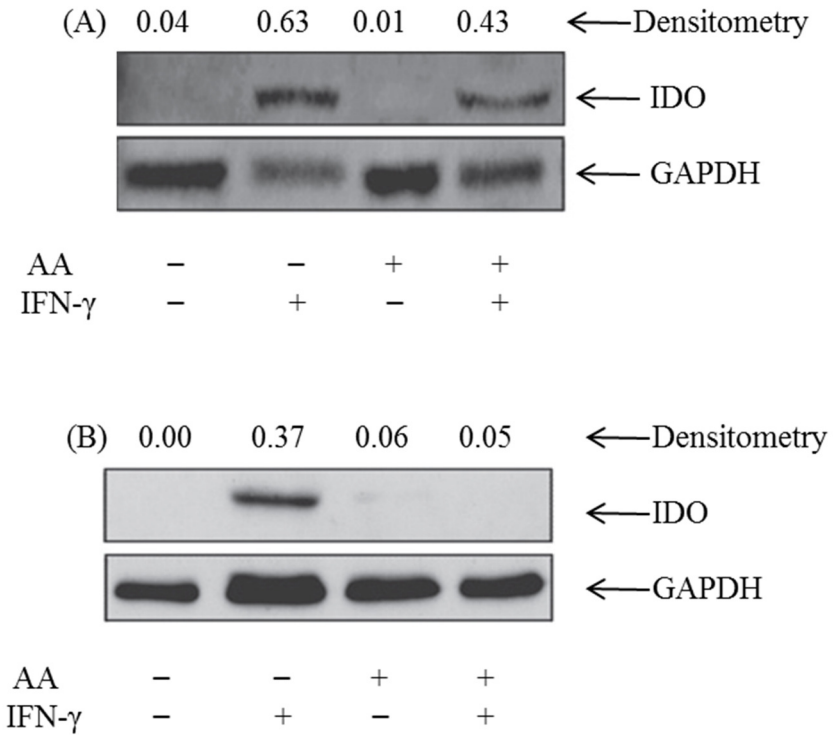


Figure 4-7 Effect of AA on IFN-γ mediated induction of IDO-1 protein in THP-1 cells and human monocytes

(A) THP-1 cells (1 x 10^7/total) or (B) monocytes (1 x 10^7/total) were treated with 20 µmol/L AA or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. Protein was extracted, quantified and 100 µg was used to detect IDO protein and GAPDH by Western blot. Densitometry of the immunoreactive bands was calculated using image Quant software version 1.2 and total protein loading was corrected using GAPDH as the endogenous control. A representative result from one of four separate experiments is shown.

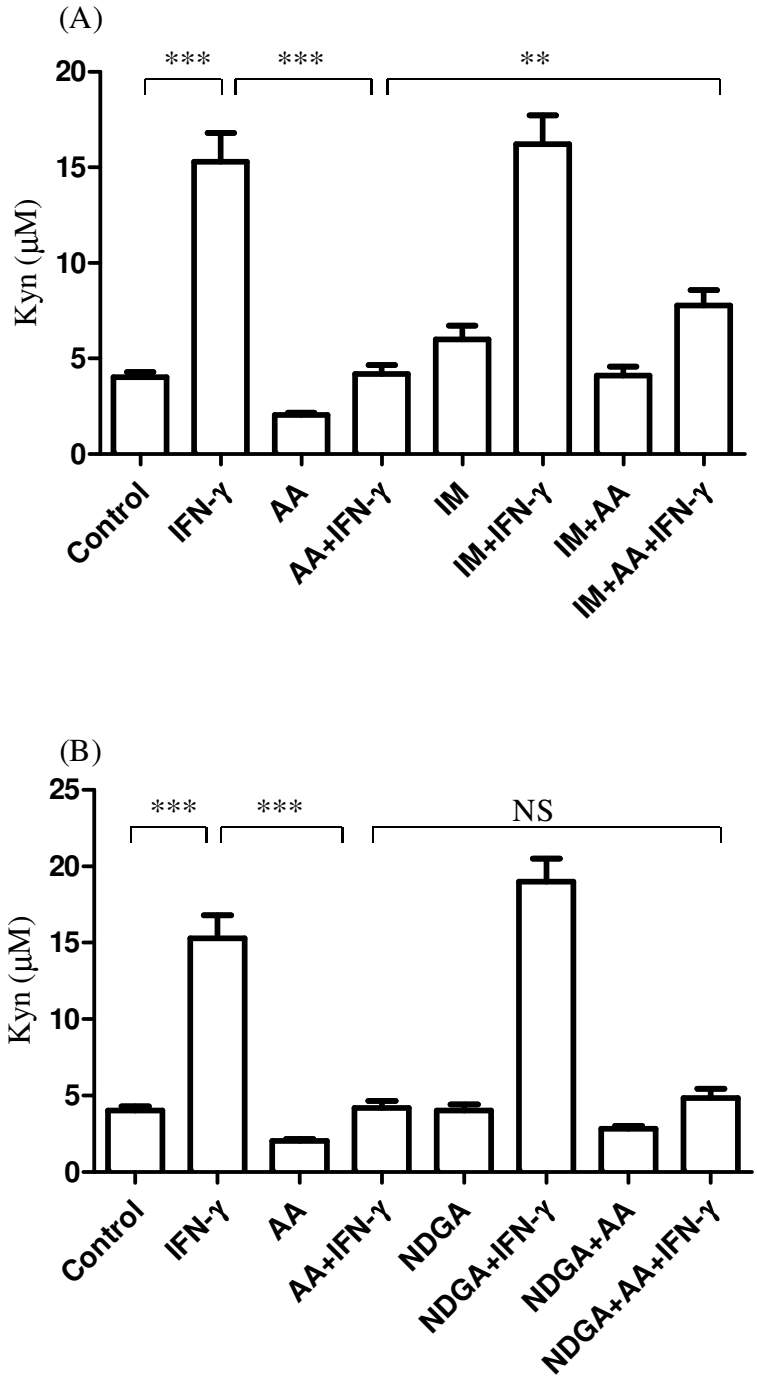


Figure 4-8 Effect of IM and NDGA on AA mediated inhibition of IFN-γ induced IDO-1 activity

THP-1 cells (4 x 10^5/well) were pre-treated with (A) IM (100 μmol/L), or (B) NDGA (10 μmol/L) or diluent for 30 min and then treated with AA (20 μmol/L) or diluent for a further 30 min in media lacking serum. The cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Data are presented as the mean ± SEM of Kyn in culture supernatants, n=12 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; ** indicates $p < 0.001$, *** indicates $p < 0.0001$. This result has previously been reported (Bassal 2008).

4.8 Effect of IM and NDGA on the production of AA metabolites

Our previous result suggested that the COX, but not LOX, derived metabolites of AA played a role in inhibiting IDO activity. In order to confirm these results and that the inhibitors tested were functional, we quantified the production of AA-derived COX and LOX metabolites in the presence/absence of IM and NDGA. THP-1 cells (4 x 10^5/50 µL) were pre-treated with IM (100 µmol/L), NDGA (10 µmol/L) or diluent for 30 min. Cells were then treated with an equal volume of AA (20 µM) or diluent for an additional 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 24 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was harvested and metabolites quantified using a commercial prostaglandin EIA screening kit. The inhibitory activity of IM was confirmed by its ability to completely block the production of AA COX metabolites in THP-1 cells (Figure 4-9). The level of these PGs in unstimulated cells and IFN-γ stimulated cells were very low, AA treated cells showed an increase in total PGs produced, while AA plus IFN-γ stimulated cells also showed an increase of PGs over basal levels. In cells treated with IM, IM plus IFN-γ, IM plus AA or IM plus AA plus IFN-γ, all stimulated cells showed 95% inhibition of AA-derived PGs, indicating that IM was functionally active. The inhibitory activity of NDGA was confirmed by its ability to completely block the production of AA-derived LOX metabolites in THP-1 cells, using a cysteinyl leukotriene (LT) EIA ki+-t (Figure 4-10). The level of LTs in unstimulated cells and IFN-γ stimulated cells were very low, AA stimulated cells and AA plus IFN-γ stimulated cells showed an increase in LTs over basal levels. In cells treated with NDGA, NDGA plus IFN-γ, NDGA plus AA, or NDGA plus AA plus IFN-γ all showed a 95% inhibition of AA-derived LTs, confirming that NDGA was functionally active and that these cells could produce these metabolites.

4.9 Determination of COX1 and COX2 mRNA expression in THP-1 cells

The previous results showed that the inhibitory effect of AA was dependent on its metabolism via the COX but not the LOX pathway. Since the COX pathway consists of prostaglandin-endoperoxidase synthase-1 (COX1) and prostaglandin-endoperoxidase synthase-2 (COX2) enzymes, which are encoded by PGHS1 (COX1) and PGHS2 (COX2) respectively, we needed to determine the contribution of the individual COX1 and COX2 enzymes to the AA inhibitory effect. To determine the role of these individual enzymes, we first examined the mRNA expression of both COX1 and COX2 in THP-1 cells. We first optimised the conditions for detecting both COX1 and COX2 mRNA (Section 2.21). The optimal conditions for detecting β-actin, COX1 and COX2 mRNA are shown in Table 2-2 and Table 2-3. The results showed that mRNA for both COX1 and COX2 was

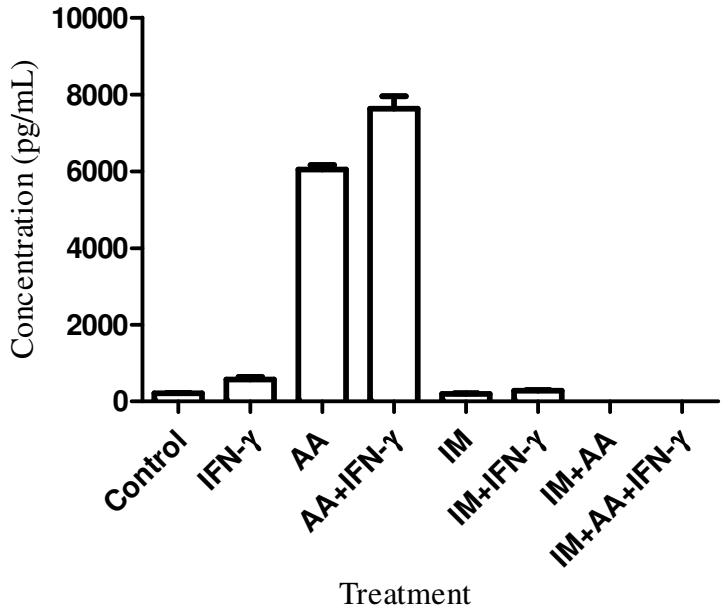


Figure 4-9 Effect of IM on AA-derived PG metabolite production

THP-1 cells (4 x 10^5/well) were pre-treated with IM (100 µmol/L) or diluent for 30 min and then treated with AA (20 µmolm/L) or diluent for a further 30 min. The cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 24 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was collected and total PGs were quantified according to the manufacturer's protocol (Section 2.23). Data of two experiments each performed in triplicate are presented as the mean ± SEM of total metabolites in culture supernatants. Comparesons were performed using the Kruskal-Wallis test; ** indicates $p < 0.0021$.

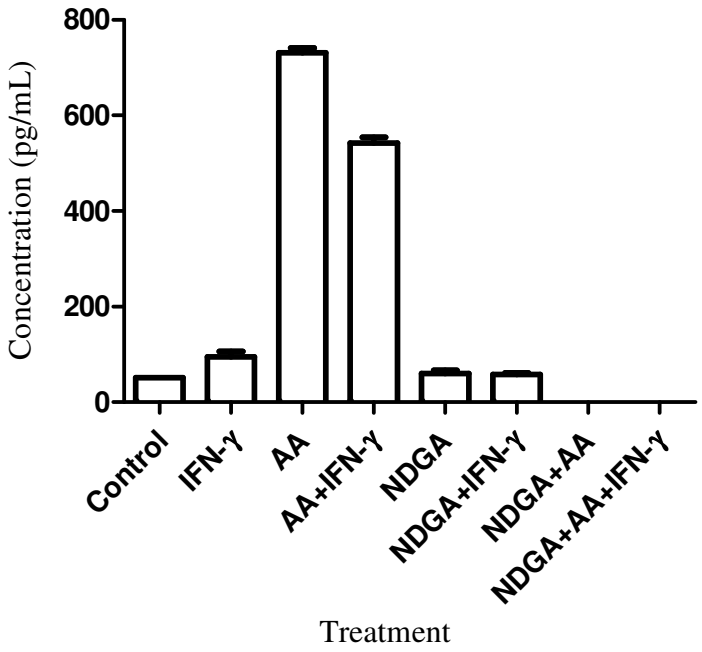


Figure 4-10 Effect of NDGA on AA-derived LT metabolite production

THP-1 cells (4 x 10^5/well) were pre-treated with NDGA (10 µmol/L) or diluent for 30 min and then treated with AA (20 µmol/L) or diluent for a further 30 min. The cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 24 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatants were collected and total LTs were quantified according to the manufacturer's protocol (Section 2.23). Data of two separate experiments each performed in triplicates are presented as the mean ± SEM of different metabolites in culture supernatants. Comparesons were performed using the Kruskal-Wallis test; ** indicates $p < 0.0029$.

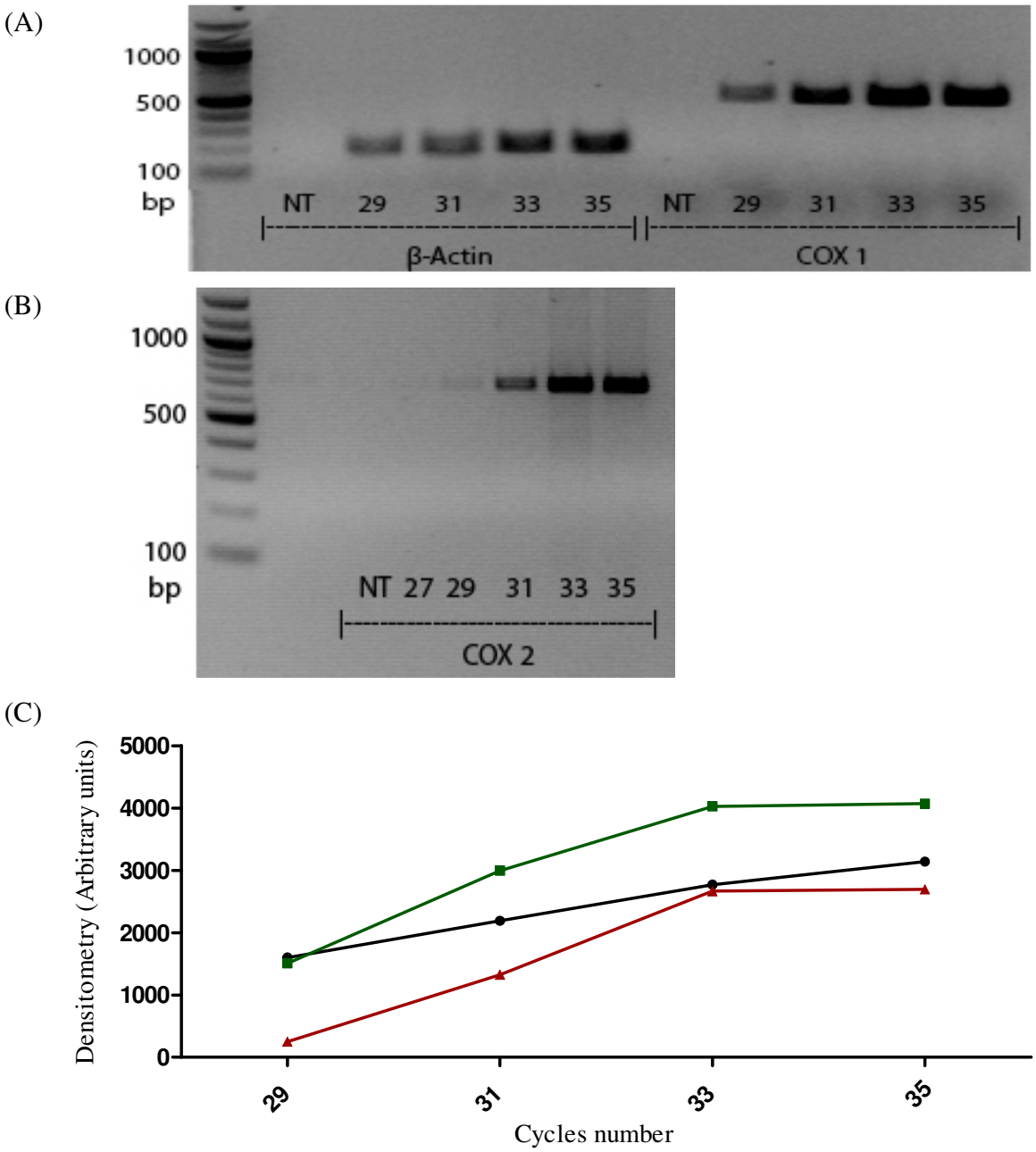


Figure 4-11 Determination of optimal cycle number for detection of COX1, COX2 and β-actin mRNA

Total RNA was extracted from THP-1 cells (4 x 10^6/total), converted to cDNA and analysed via RT-PCR for 29, 31, 33 and 35 cycles, then separated by 1.5% agarose gel. (A) β-actin mRNA and PGHS1 mRNA (B) PGHS2 mRNA (C) densitometry was used to quantify changes in product formed. The results show that using 33 cycles was suitable to detect β-actin mRNA, COX1 mRNA and COX2 mRNA before the amplification is no longer exponential. Representative data of one experiment repeated 3 times is shown. (●) β-Actin, (■) COX1 and (▲) COX2. NT, no template control.

expressed in THP-1 cells and there was a trend of increasing COX1 and COX2 and β-actin mRNA amplification from cycles 29–33. After these numbers of cycles, both COX1 and COX2 amplification began to plateau (Figure 4-11).

4.10 Effect of IFN-γ and AA on the expression of PGHS1 (COX1) and PGHS2 (COX2) mRNA in THP-1 cells

Since both COX1 and COX2 mRNA were expressed by THP-1 cells, we next examined the effect of treatment with IFN-γ and AA on mRNA expression. THP-1 cells (4 x 10^5/well) were treated with AA (20 μmol/L) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 24 h at 37 °C/5% CO_2 in a humidified atmosphere. Cells were then collected, total RNA extracted, cDNA prepared and RT-PCR performed. The data demonstrated that THP-1 cells constitutively expressed COX1 mRNA and that IFN-γ caused an increase in COX1 mRNA expression. AA alone had no effect on COX1 mRNA levels while AA+IFN-γ showed a decrease in COX1 mRNA level compared to IFN-γ only stimulated cells. COX2 mRNA could also be detected in resting cells, while both IFN-γ alone and AA alone had no effect on COX2 mRNA levels. AA+IFN-γ caused an increase in COX2 mRNA expression, compared to IFN-γ only stimulated cells (Figure 4-12).

4.11 Ability of COX1/COX2 inhibitors to modulate the AA inhibitory effect

Having demonstrated that both COX1 and COX2 mRNA were expressed in THP-1 cells, the contribution of the individual enzymes towards the AA inhibitory effect was examined. To do so, we used selective pharmacological inhibitors of COX1, SC-560, and COX2, NS-398. THP-1 cells (4 x 10^5/well) were pre-treated with SC-560 (1 and 10 μmol/L) (Figure 4-13A) or NS-398 (1 and 25 μmol/L) (Figure 4-13B) or both each at 1 μmol/L (Figure 4-13C) or diluent for 30 min. Cells were then treated with AA (20 μmol/L) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h. The supernatant was collected and Kyn levels measured. Consistent with previous results, IFN-γ caused a significant increase in Kyn production over basal, while AA had no significant effect on basal Kyn levels. Cells pre-treated with AA followed by IFN-γ stimulation demonstrated significant inhibition of Kyn production compared to IFN-γ stimulated cells. Neither SC-560 nor NS-398 had any significant effect on Kyn production compared to basal levels. Cells pre-treated with the inhibitors and then AA or inhibitors followed by IFN-γ showed no significant change of Kyn level compared to Kyn levels produced by cells stimulated with AA alone or IFN-γ alone respectively. Treating cells with each of the

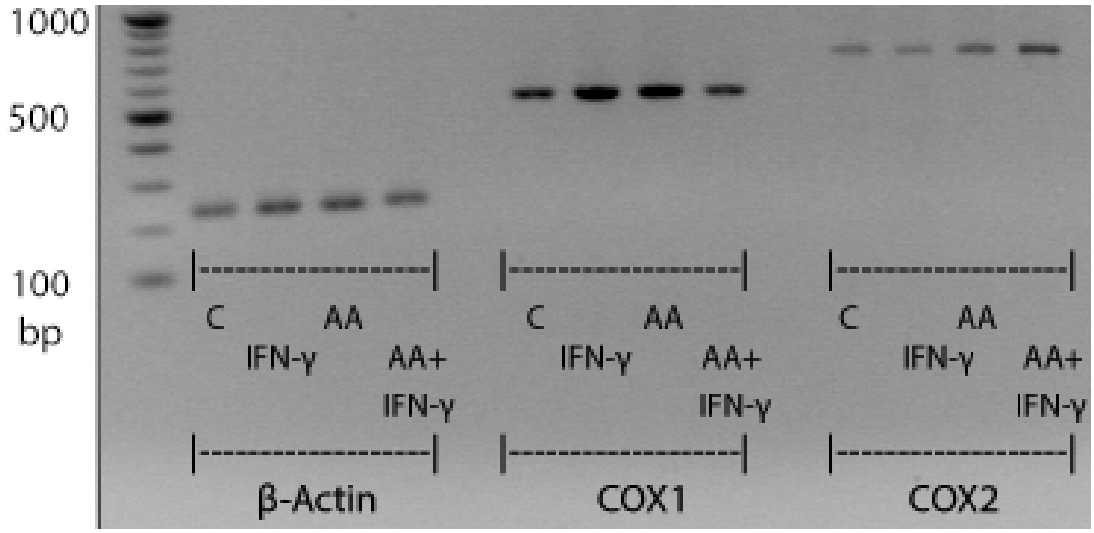
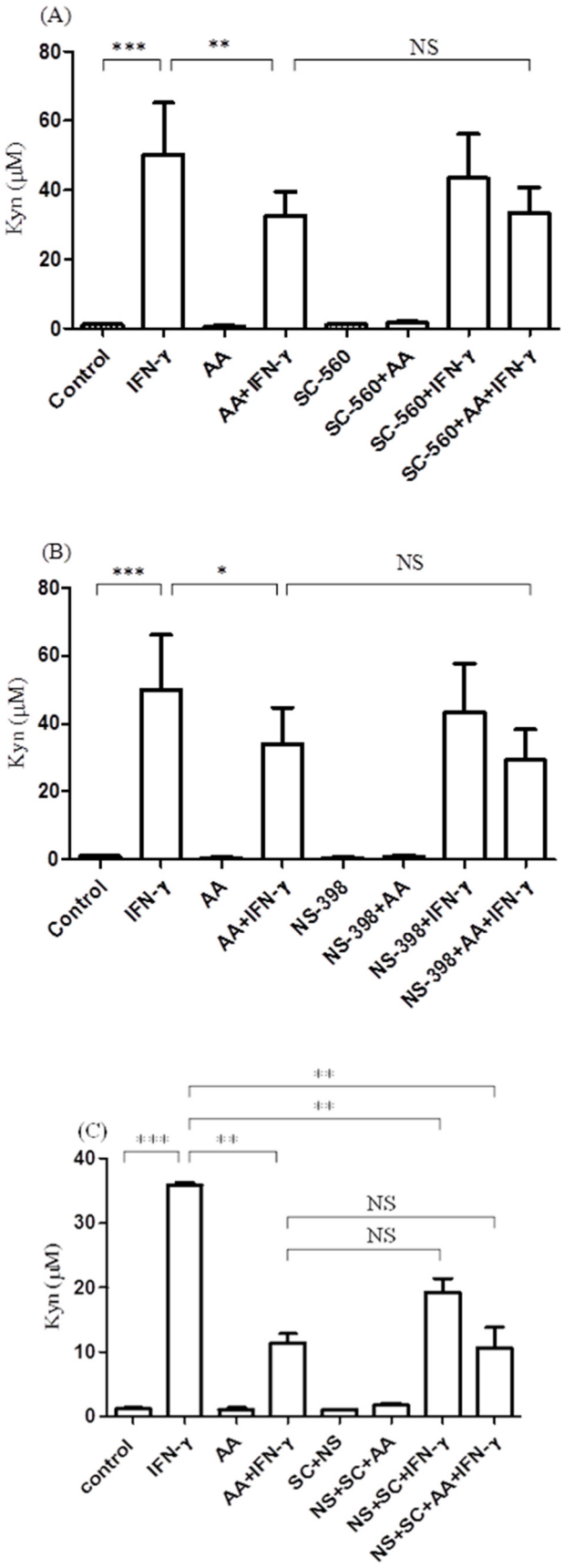


Figure 4-12 Effect of IFN-γ and AA on the expression of both COX1 and COX2 mRNA in THP-1 cells

THP-1 cells (4 x 10^6/well) were treated with AA (20 µmol/L) or diluent for 30 min then stimulated with IFN-γ (1000 IU/mL) or diluent for 24 h at 37 °C/5% CO_2 in a humidified atmosphere. Cells were collected, RNA was extracted, used to prepare cDNA and RT-PCR was performed. Representative data of one of 3 separate experiments is shown.

Figure 4-13 Effect of COX1 (SC-560) and COX2 (NS-398) inhibitor on the AA mediated inhibition of Kyn production

THP-1 cells (4 x 10^5/well) were treated with (A) SC (SC-560) (10 µmol/L) or (B) NS (NS-398) (25 µmol/L) or (C) SC-560 and NS-398 together (1 µmol/L each) or diluent for 30 min and then treated with AA (20 µmol/L) or diluent for 30 min and then stimulated with 1000 IU/ml of IFN-γ or diluent for 72 h. Kyn was measured using the spectrophotometric assay. Data are presented as the mean ± SEM of Kyn of 3 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$, ** indicates $p < 0.001$, *** indicates $p < 0.0001$.


(A)
***
**
NS
Kyn (μM)
80
60
40
20
0
Control
IFN-γ
AA
AA+IFN-γ
SC-560
SC-560+AA
SC-560+IFN-γ
SC-560+AA+IFN-γ
(B)
***
*
NS
Kyn (μM)
80
60
40
20
0
Control
IFN-γ
AA
AA+IFN-γ
NS-398
NS-398+AA
NS-398+IFN-γ
NS-398+AA+IFN-γ
(C)
**
**
***
**
NS
NS
Kyn (μM)
40
30
20
10
0
control
IFN-γ
AA
AA+IFN-γ
SC+NS
NS+SC+AA
NS+SC+IFN-γ
NS+SC+AA+IFN-γ

inhibitors then AA followed by IFN-γ showed no correction of the AA inhibitory effect. We hypothesised that the absence of any correction was due to the presence of the other enzyme produced biologically active metabolites. When cells were treated with both inhibitors together and then IFN-γ, total Kyn production in response to IFN-γ treatment alone was significantly inhibited. Thismade any effect of both inhibitors plus AA on IFN-γ production of Kyn very difficult to detect. Although not conclusively demonstrated, these results are consistent with the hypothesis that both enzymes were responsible for the AA inhibitory effect.

4.12 Effect of SC-560 and NS-398 on the induction of AA metabolites

Our previous results suggested that both COX1 and COX2 were involved in the generation of AA derived metabolites that were biologically inhibitory. In order to confirm that the inhibitors we tested were functionally active, we confirmed their effects on the production of AA COX derived metabolites by EIA. THP-1 cells (4 x 10^5/50 µL) were pre-treated with SC-560 (10 µmol/L), NS-398 (25 µmol/L) or both for 30 min. Cells were then treated with an equal volume of AA (20 µmol/L) for an additional 30 min and then stimulated with IFN-γ (1000 IU/mL) for 24 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was harvested and AA derived COX metabolites quantified using a commercial PG screening EIA kit (Figure 4-14). The total level of PGs in unstimulated cells and IFN-γ stimulated cells were very low, while AA treatment and AA plus IFN-γ treated cells showed a significant increase in total PGs produced over basal levels. In cells treated with SC-560 or SC-560 plus IFN-γ the level of PGs were very low as in the control and IFN-γ only treated cells. In cells treated with SC-560 plus AA or SC-560 plus AA plus IFN-γ, there was still a significant increase in total PGs produced over basal levels. Similarly, in cells treated with NS-398 or NS-398 plus IFN-γ the level of PGs was very low. In cells treated with NS-398 plus AA or NS-398 plus AA plus IFN-γ, there was a significant level of total PGs produced over basal levels. These results further supported our hypothesis that by inhibiting one COX enzyme, the other COX enzyme was still metabolically active and was responsible for producing some of the inhibitory PGs. Another possibility is that many PGs can be metabolised both enzymatically and non-enzymatically, so by inhibiting both COX1 and COX2 enzymes, we could still detect some AA non-enzymatically derived metabolites. It would be interesting to test the effect of both COX1 and COX2 inhibitors together on the production of AA COX metabolites by EIA. However, it was shown previously that when cells were treated with both inhibitors

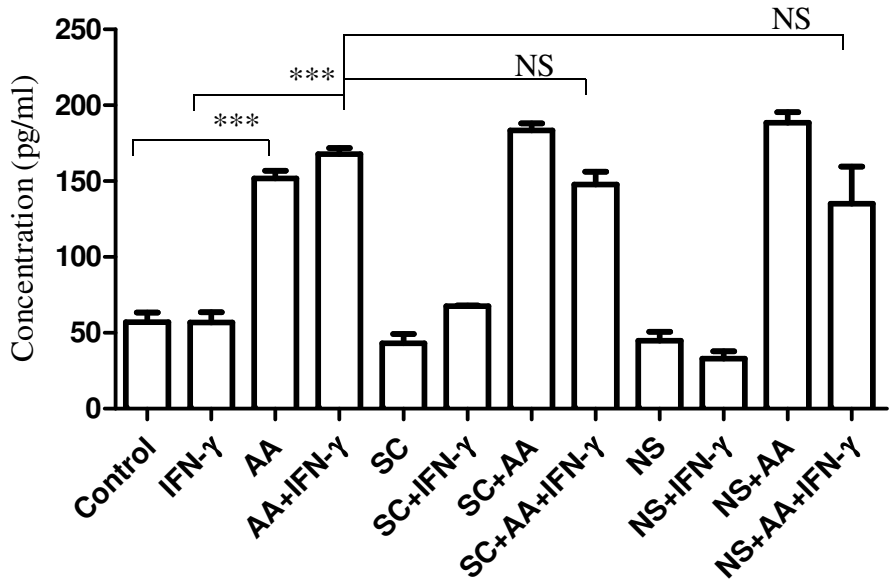


Figure 4-14 Effect of SC-560 and NS-398 on induction of AA-derived PG metabolites
THP-1 cells (4 x 10^5/well) were pre-treated with SC (SC-560) (10 µmol/L) or NS (NS-389) (25 µmol/L) or diluent for 30 min and then treated with AA (20 µmol/L) or diluent for a further 30 min. The cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 24 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatants were collected and total PGs metabolites were measured according to the manufacturer's protocol. Data of three separate experiments each performed in triplicate are presented as the mean ± SEM of total metabolites detected in culture supernatants. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; *** indicates $p < 0.0001$.

together and then IFN-γ, total Kyn production in response to IFN-γ treatment alone was significantly inhibited. This made any effect of both inhibitors plus AA on IFN-γ production of Kyn difficult to detect.

4.13 Summary

AA significantly inhibited the IFN-γ mediated induction of IDO in THP-1 cells and human monocytes. The inhibitory effect was time dependent with significant inhibition occuring in the first 10 min of pre-treatment in THP-1 cells. The inhibitory effect was dose dependent with a calculated IC_{50} of 20 μmol/L for THP-1 cells and 12.8 ± 2.6 μmol/L in human monocytes. The inhibitory effect was not due to toxicity. The AA inhibitory effect on both THP-1 and human monocytes was mediated at the transcriptional level with AA causing a 33% inhibition of *INDO1* expression in THP-1 and 50% inhibition in *INDO1* expression in human monocytes compared to IFN-γ. The AA inhibitory effect was also exerted at the level of translation in human monocytes as AA caused a 87% inhibition in human monocytes compared to IFN-γ. AA metabolites via the COX but not LOX enzymes were involved in the inhibitory effect.

Both PGHS1 and PGHS2 were expressed in THP-1 cells however the level of PGHS1 was higher than PGHS2. AA-derived metabolites were produced by THP-1 cells and their production could be blocked by IM and NDGA. However, the inhibitory effects of SC-560 and NS-890 were different. When used individually, significantly high levels of AA derived metabolites could still be detected. Thus both AA COX enzymes, COX1 and COX2, could be responsible for the production of inhibitory metabolites.

Chapter 5

Determination of biologically active COX1/2 metabolites and analysis of their structure–function relationships

5.1 Introduction

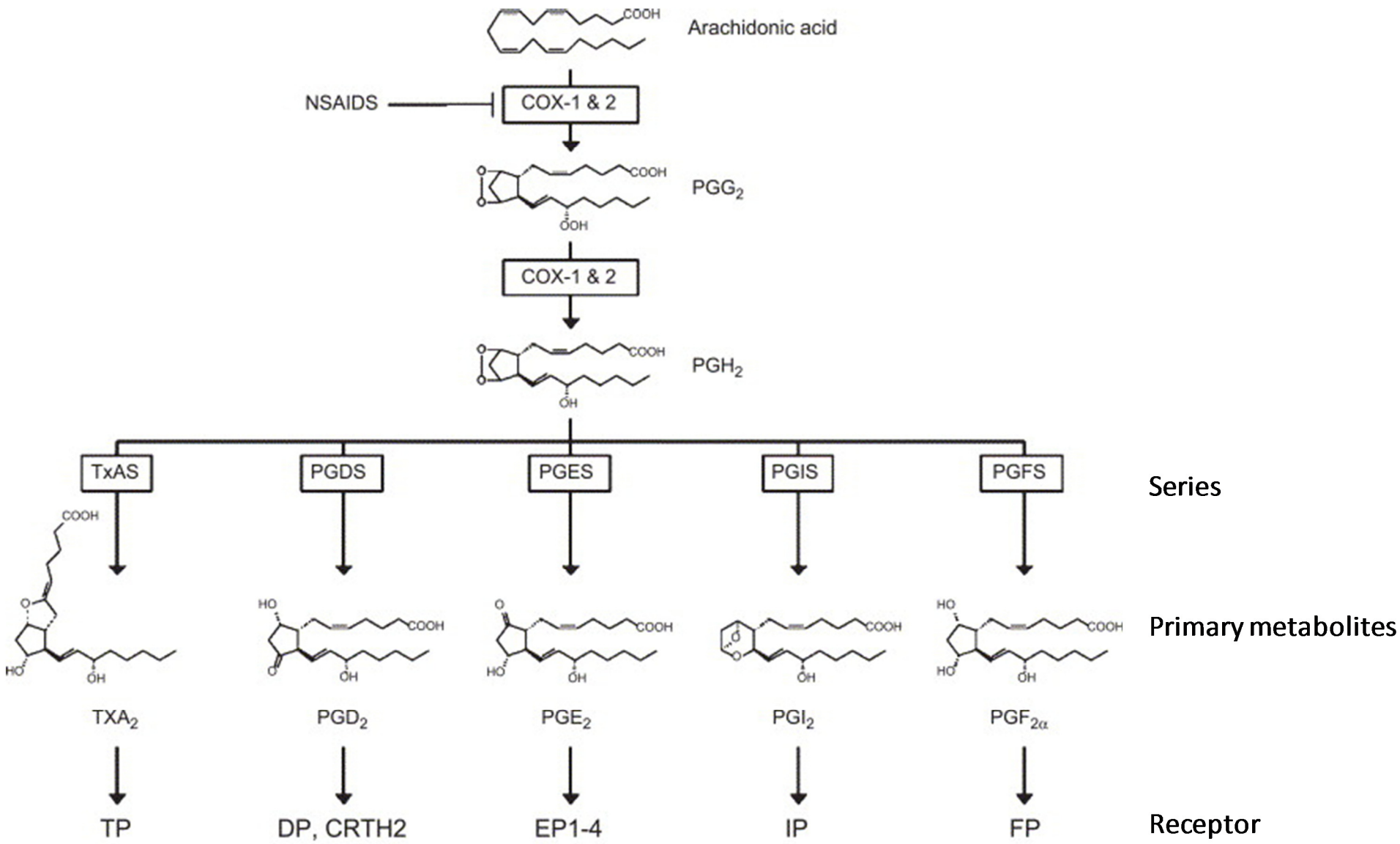
Having established that the cyclooxygenase metabolism of AA was required for inhibition of IDO-1 activity, we next focused on identifying the molecule(s) with this inhibitory activity. We screened a range of available COX1/2 derivatives of AA for inhibitory activity. To better place these into a biological context, we have arranged our results based on the order of the metabolite's synthesis. The general pathway for the biosynthesis of PGs involves the release of esterified AA from phospholipids via the action of phospholipase A_2. AA is then converted to PGG_2 and PGH_2 by the cyclooxygenase and peroxidase activity of PGH synthase. PGH synthase has been shown to exist in at least two isoforms, named PGH synthase 1 (COX1) and PGH synthase 2 (COX2). COX1 is constitutively expressed in nearly all mammalian tissues (O'Neill & Ford-Hutchinson 1993). The PGs formed via COX1 primarily act in regulating normal physiological conditions, for example homeostasis, renal water retention and gastric acid secretion (Kang et al. 2007). In contrast, COX2 is constitutively expressed in many tissues, for example prostate, lung brain, kidney, and during ovulation and implantation (O'Neill & Ford-Hutchinson 1993; Kang et al. 2007). COX2 is inducible in other cells and organs during inflammation and thus can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin and indomethacin (IM) (O'Neill & Ford-Hutchinson 1993; Herschman 1999) (Figure 5-1). Our results to date have suggested that both COX1 and COX2 derived metabolites are responsible for inhibiting the IFN-γ induced IDO activity. To identify the biologically active metabolite(s) responsible for this activity, we screened a library of AA-derived PGs for their ability to inhibit IDO activity in THP-1 cells. The PGs that had an inhibitory effect on THP-1 cells were then examined for their effect on human monocytes. Although human research ethics approval from the University of South Australia was obtained to use human monocytes, there was a limitation to the number of participants that ethics approval allowed for blood donation, which precluded testing of all AA COX metabolites.

5.2 Effect of PGG_2 and PGH_2 on IFN-γ mediated induction of IDO-1

The first two PGs produced by the metabolism of AA via COX1 and COX2 are PGG_2 and PGH_2. Consequently, these were examined for their ability to inhibit Kyn production. THP-1 cells (4 x 10^5/well) or human monocytes (4 x 10^6/well) were treated with PGG_2 or PGH_2 at 10 µmol/L or diluent for 30 min, and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was collected and Kyn levels quantified using the modified spectrophotometric assay. Consistent

Figure 5-1 Biosynthesis of AA-derived prostaglandins via COX1/COX2 enzymes

AA is metabolised by COX1/2 enzymes to the unstable endoperoxide PGG_2 that is then converted to PGH_2, the common precursor for the five principal prostaglandins. Thromboxane A_2 (TXA_2), prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin I_2 (PGI_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are generated by individual prostaglandin synthase enzymes (TXAs, PGDs, PGEs, PGIs, and PGFs respectively) and elicit their biological effects by activating their cell surface G-protein-coupled receptors TP, DP and Chemoattractant receptor homologous molecule expressed on T-helper-2 cells (CRTH2), EP1-4, IP and FP respectively. (Adapted from Hata & Breyer 2004).


Arachidonic acid
NSAIDS
COX-1 & 2
PGG_2
COX-1 & 2
PGH_2
TxAS
PGDS
PGES
PGIS
PGFS
Series
TXA_2
PGD_2
PGE_2
PGI_2
$PGF_{2\alpha}$
Primary metabolites
TP
DP, CRTH2
EP1-4
IP
FP
Receptor

with our previous results, control cells produced very low levels of Kyn, while IFN-γ stimulated cells showed a significant increase in Kyn over baseline. Neither PG significantly affected basal Kyn production, yet both PGs caused significant inhibition in Kyn production following IFN-γ stimulation in THP-1 cells (Figure 5-2A), yet surprisingly, no significant effect on human monocytes, which may have been due to the small sample size, although there was a trend of inhibitory activity moving between PGG_2 and PGH_2 (Figure 5-2B).

5.3 PGD_2 inhibits the IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

Since the previous results suggested that both PGG_2 and PGH_2 inhibited IDO-1 activity (at least in THP-1 cells) we next examined the activity of the first five principal PGs produced from PGH_2 metabolism, namely thromboxane A_2 (TXA_2), PGD_2, PGE_2 PGI_2 and $PGF_{2\alpha}$. These 5 principal metabolites work via different receptors (Figure 5-1). It should be noted that although TXA_2 is the first TX produced, it is unstable and is rapidly transformed to TXB_2 and as such it was not available for testing. As a result the inhibitory activity of TXB_2 was assessed instead. THP-1 cells (4 x 10^5/well) or human monocytes (4 x 10^6/well) were treated with TXB_2, PGD_2, PGE_2, PGI_2, $PGF_{2\alpha}$ (10 µmol/L) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was collected and Kyn levels quantified using the colorimetric assay. Consistent with our previous results, control cells produced very low levels of Kyn, while IFN-γ stimulated cells showed a significant increase in Kyn over basal levels. Cells treated with the PGs alone showed no significant change in Kyn production over basal levels. Of the five major PGs tested only PGD_2 caused significant inhibition in Kyn production in both THP-1 cells (Figure 5-3A) and human monocytes (Figure 5-3B).

5.4 Effect of PGD_2 metabolites on the IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

PGD_2 is a major precursor of a range of D_2 derived metabolites (Figure 5-4). Thus, to determine whether the parent compound and/or its metabolites were responsible for the inhibitory activity, each of these molecules was assessed for its ability to inhibit Kyn production in THP-1 cells. Active compounds were then tested using human monocytes. THP-1 cells (4 x 10^5/total) were treated with 10 µmol/L of PGJ_2, Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2, 13,14-dihydro-15-keto PGD_2, Tetranor PGDM, 11β $PGF_{2\alpha}$, 2,3-dinor-11β

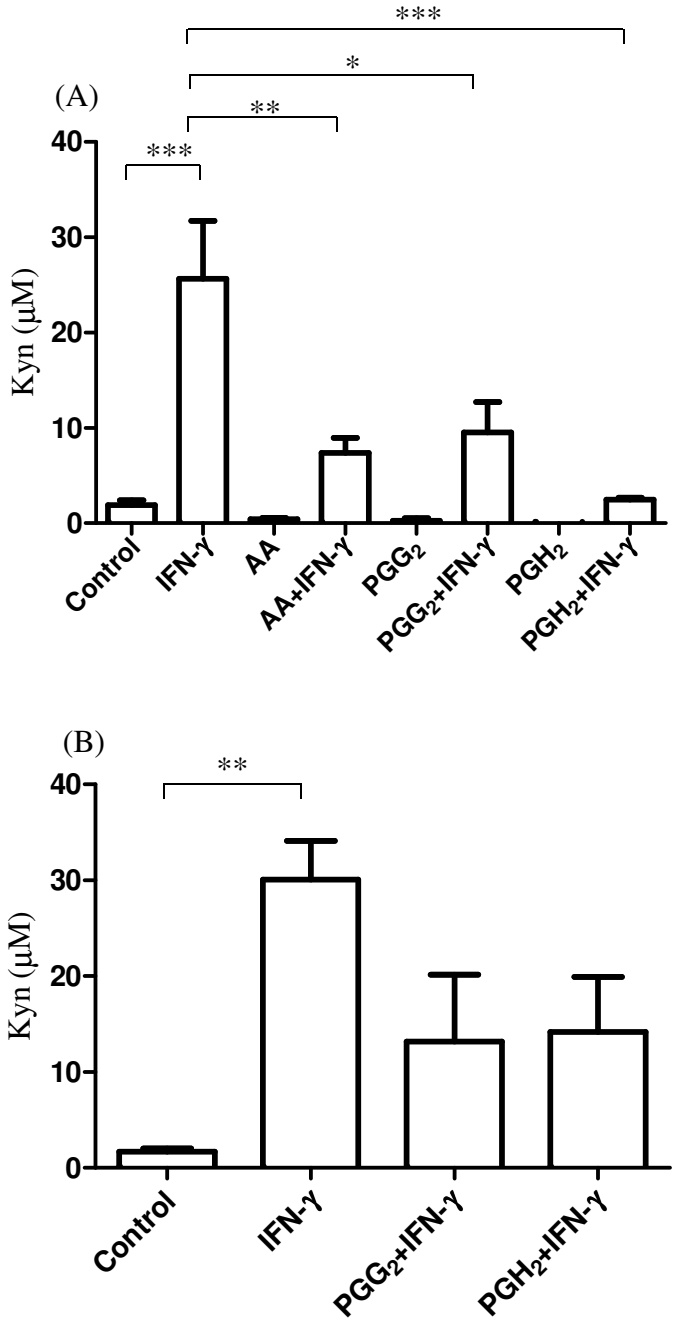


Figure 5-2 Effect of PGG_2 and PGH_2 on Kyn production in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/total) or (B) human monocytes (4 x 10^6/tatal) were pre-treated with PGG_2, PGH_2 (10 µmol/L) or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM Kyn produced. THP-1 n=10 separate experiments, human monocytes n=3 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$, ** indicates $p < 0.01$, *** indicates $p < 0.0001$.

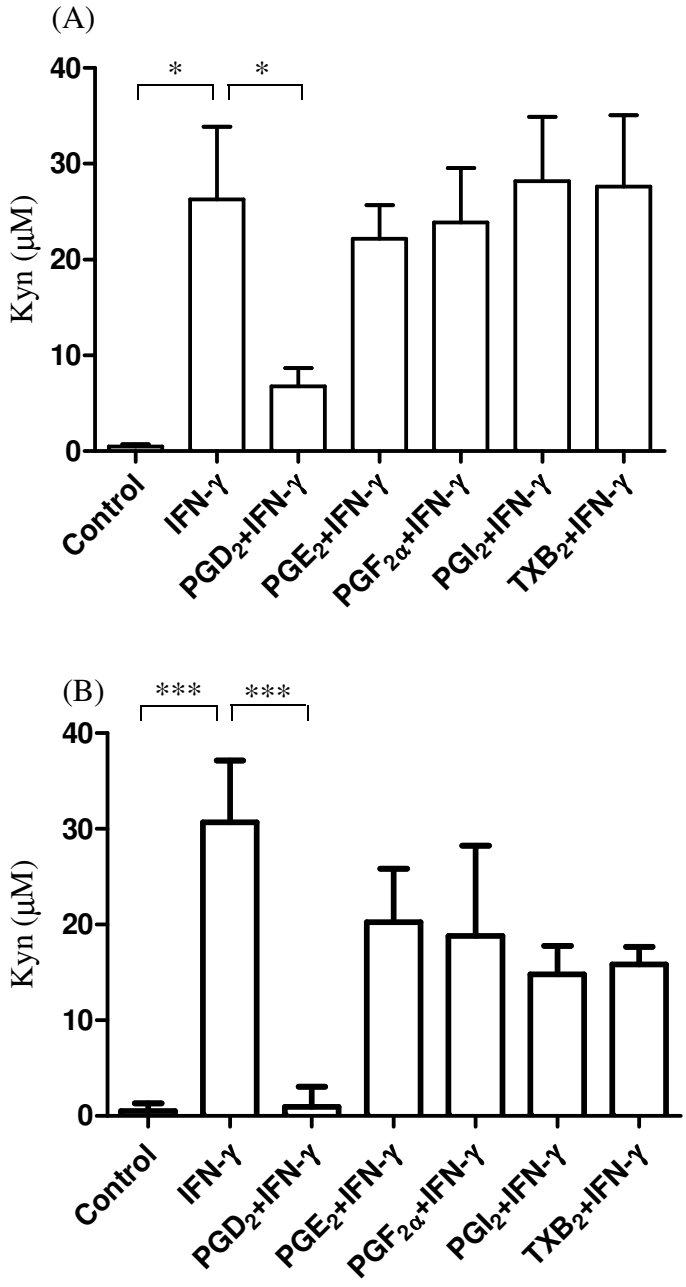


Figure 5-3 PGD_2 inhibits the IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/total) or (B) human monocytes (4 x 10^6/total) were pretreated with 10 μmol/L of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, TXB_2 or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 ^oC/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM. THP-1 n=10 separate experiments, human monocytes n=5 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$, *** indicates $p < 0.0001$.

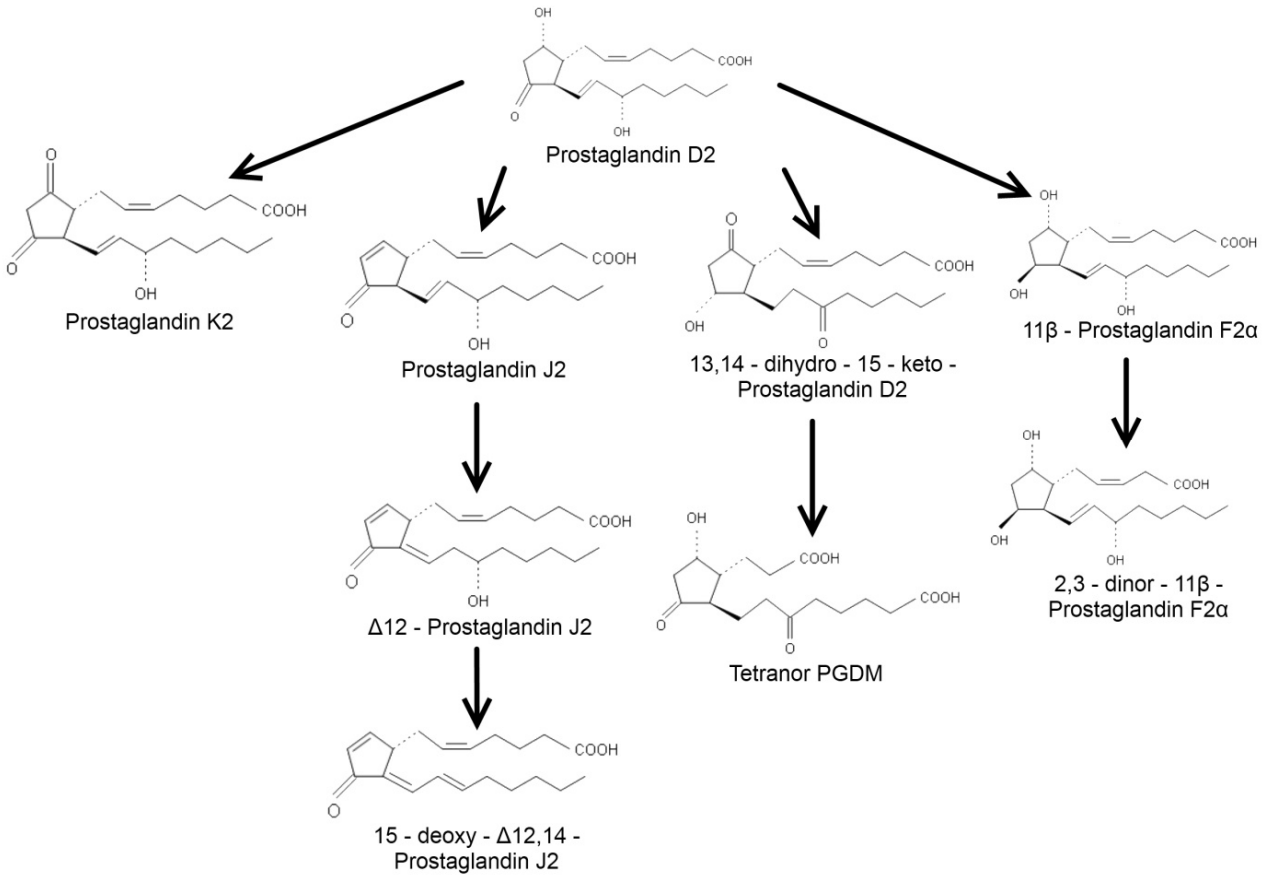


Figure 5-4 Metabolic pathway of PGD_2 and its derivatives

PGD_2 is metabolised enzymatically to 13, 14 dihydro-15-keto-PGD_2 which is metabolised to tetranor PGDM and 11β $PGF_{2\alpha}$ which in turn is metabolised to 2, 3-dinor-11β $PGF_{2\alpha}$. The remaining PGD_2 is metabolised non-enzymatically to PGK_2 and PGJ_2. PGJ_2 further dimerise to Δ^{12}-PGJ_2 and then to 15-deoxy $\Delta^{12,14}$-PGJ_2. (Adapted from Cayman chemicals, Ann Arbor, MI, USA).

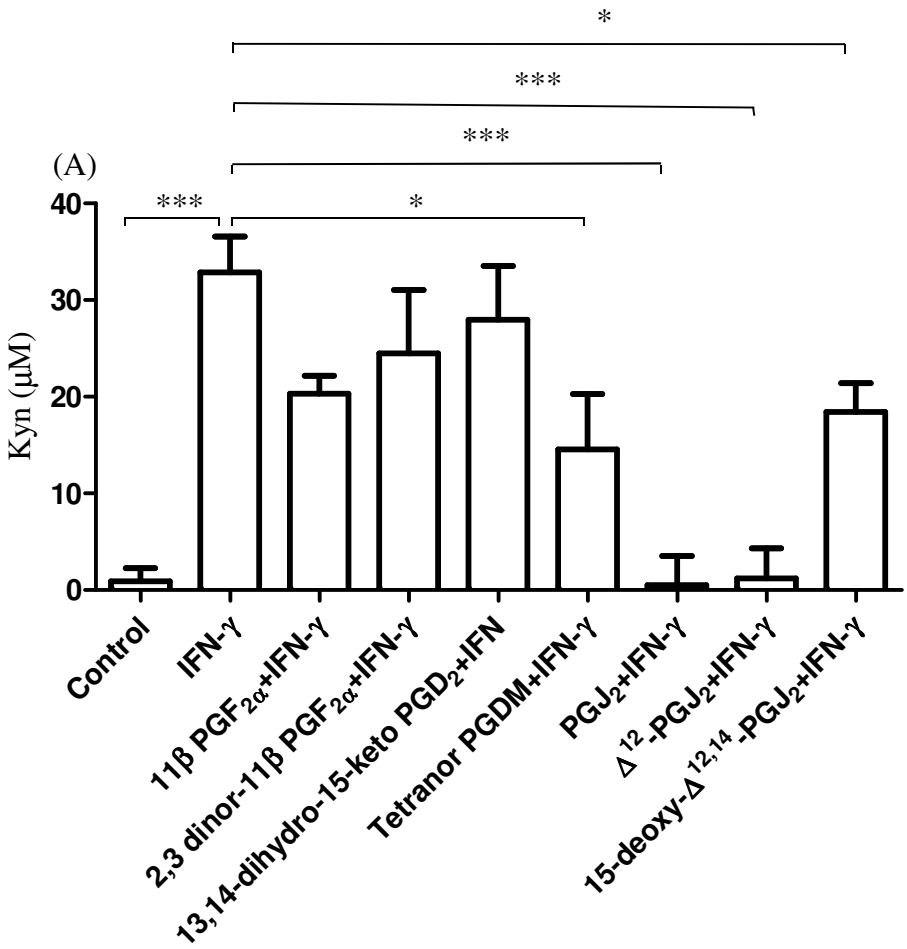
$PGF_{2\alpha}$ or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was collected and Kyn levels quantified using the modified spectrophotometric assay. Consistent with previous results, control cells produced very low levels of Kyn, while IFN-γ stimulated a significant increase in Kyn levels over basal levels. Cells treated with PGs alone showed no significant change in Kyn production over basal levels. A significant inhibition of Kyn production was seen in THP-1 cells pre-treated with PGJ_2, Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 or Tetranor PGDM and then stimulated with IFN-γ compared to IFN-γ alone. 11β $PGF_{2\alpha}$, 2, 3-dinor-11β $PGF_{2\alpha}$ and 13, 14-dihydro-15-keto PGD_2 failed to significantly inhibit Kyn production (Figure 5-5A). Due to the limitation in access to primary human monocytes, we only screened those derivatives with significant inhibitory activity in THP-1 cells on human monocytes. Human monocytes (4 x 10^6/total) were treated with 10 µmol/L of PGJ_2, Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 ºC/5% CO_2 in a humidified atmosphere. The supernatant was collected and Kyn levels quantified using the colorimetric assay. Consistent with previous results, control cells produced very low levels of Kyn, while IFN-γ stimulated a significant increase in Kyn over basal levels. Cells treated with PGs alone showed no significant change in Kyn production over basal levels. A significant inhibition in Kyn production was seen in human monocytes pre-treated with PGJ_2, Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2, and then stimulated with IFN-γ compared to IFN-γ alone (Figure 5-5B).

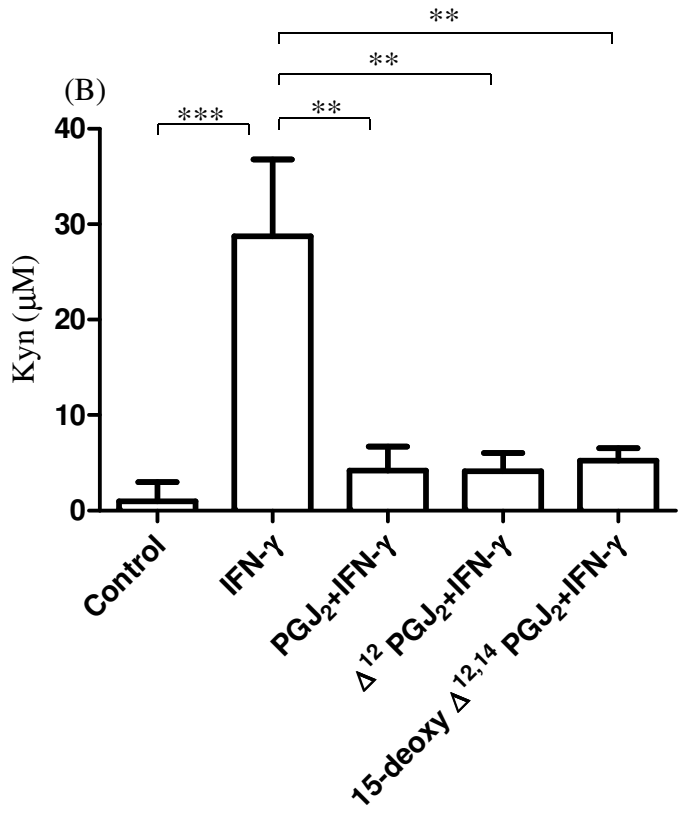
5.5 Effect of PGE_2 metabolites on the IFN-γ mediated induction of IDO in THP-1 cells and human monocytes

Although PGE_2 did not significantly inhibit Kyn production in either cell, it has been extensively reported as regulating IDO activity when used as part of a maturation cocktail leading to the generation of *in vitro* derived DCs (Braun, Longman & Albert 2005; Lanzinger et al. 2012). We thus examined PGE_2 and its metabolites in further detail (Figure 5-6). THP-1 cells (4 x 10^5/total) were treated with 10 µmol/L of PGA_2, PGB_2, PGK_2, Tetranor PGEM, 15-keto PGE_2, 13,14-dihydro-15 keto PGE_2, 13,14-dihydro-15-keto PGA_2, Bicyclo PGE_2, $PGF_{2\beta}$, 19(R)-hydroxy PGE_2 or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was collected and Kyn levels quantified using the modified spectrophotometric assay. Consistent with previous results, control cells produced very low levels of Kyn, while IFN-γ stimulated a significant increase in Kyn production over basal

Figure 5-5 Effect of PGD_2 metabolites on IFN-γ induced Kyn production in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/total) were pre-treated with 10 µmol/L of PGJ^2, Δ^{12}-PGJ^2, 15-deoxy-$\Delta^{12,14}$-PGJ_2, 13,14-dihydro-15 keto PGD_2, Tetranor PGDM, 11β $PGF_{2\alpha}$, 2,3-dinor-11β $PGF_{2\alpha}$ or diluent for 30 min. (B) Human monocytes (4 x 10^6/well) were pre-treated with 10 µM of PGJ_2, Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37°C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are presented as mean ± SEM Kyn produced. THP-1 n=10 separate experiments, human monocytes n=5 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$, ** indicates $p < 0.001$, *** indicates $p < 0.001$.


(A)
Kyn (μM)
40
30
20
10
0
*
***
***
***
*
Control
IFN-γ
11β PGF2α+IFN-γ
2,3 dinor-11β PGF2α+IFN-γ
13,14-dihydro-15-keto PGD2+IFN
Tetranor PGDM+IFN-γ
PGJ2+IFN-γ
Δ12 -PGJ2+IFN-γ
15-deoxy-Δ12,14 -PGJ2+IFN-γ


(B)
Kyn (μM)
40
30
20
10
0
**
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**
Control
IFN-γ
PGJ2+IFN-γ
Δ12 PGJ2+IFN-γ
15-deoxy Δ12,14 PGJ2+IFN-γ

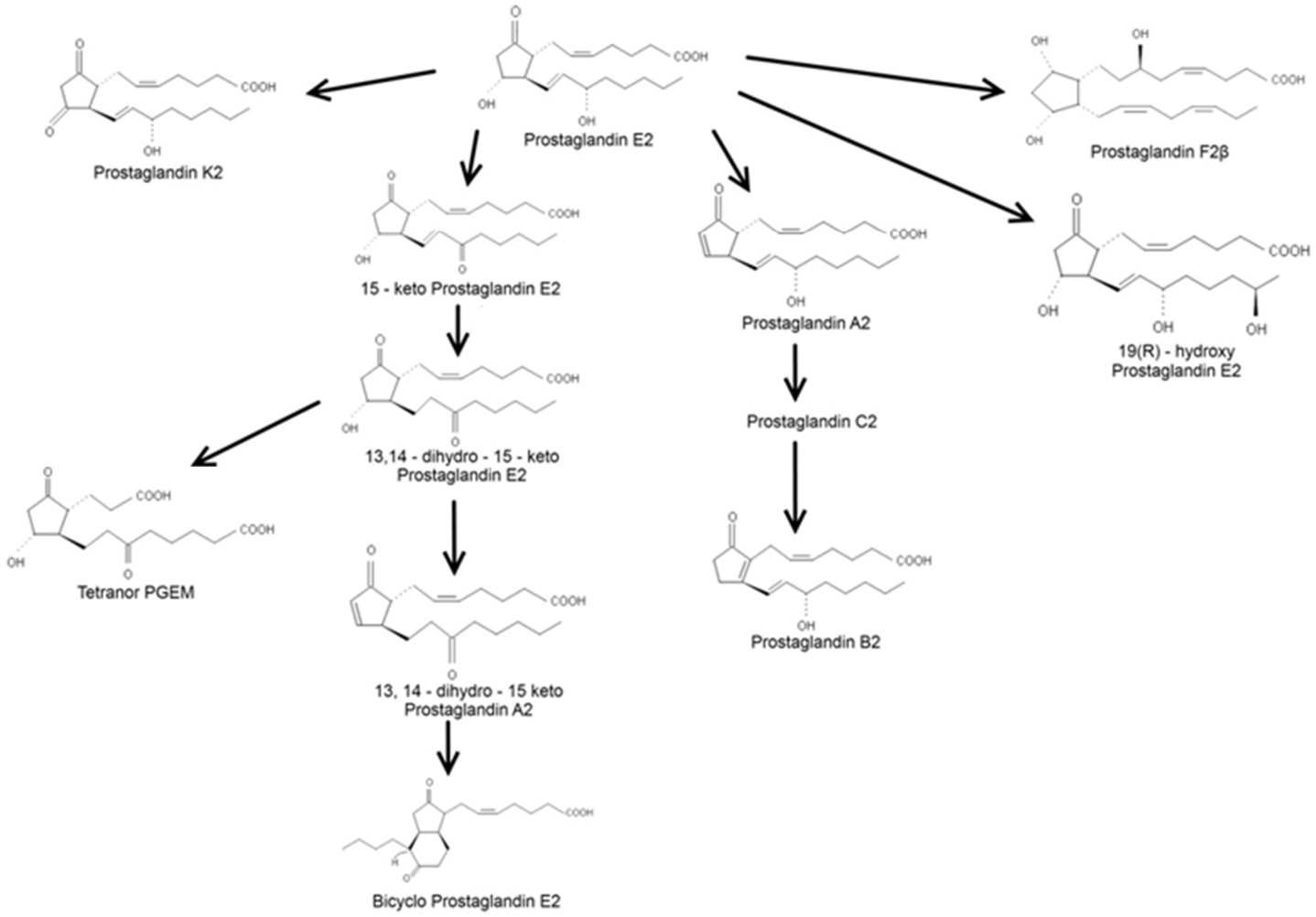


Figure 5-6 Metabolic pathway of PGE_2 and its derivatives

This diagram illustrates the metabolism of PGE_2 to different metabolites through the COX1 pathway. (Adapted from Cayman chemicals) (Ann Arbor, MI, USA).

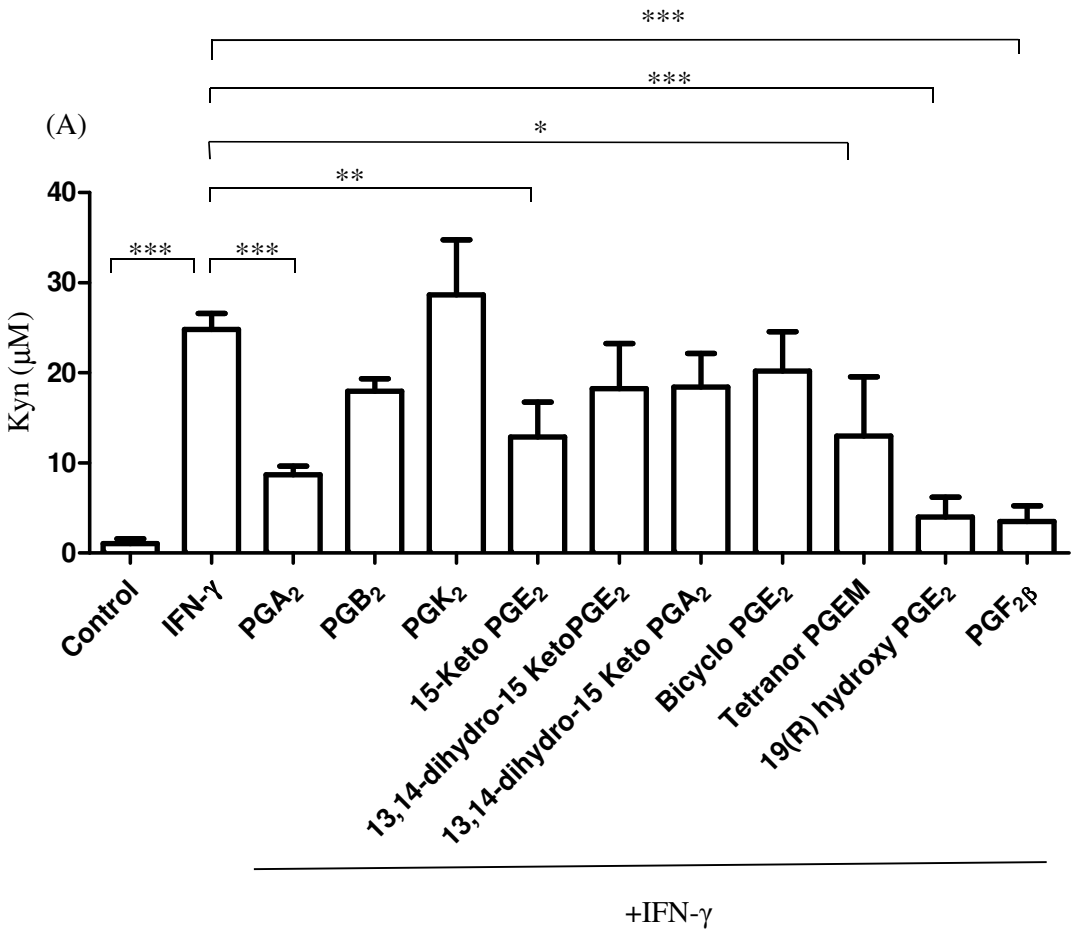
levels. Cells treated with PGs alone showed no significant change in Kyn production over basal levels. PGA_2, Tetranor PGEM, 15-keto PGE_2, $PGF_{2\beta}$ and 19(R)-hydroxy PGE_2 all significantly inhibited IFN-γ induced Kyn production in THP-1 cells (Figure 5-7A). None of the other PGs tested caused significant inhibition in Kyn levels compared to IFN-γ alone. Human monocytes (4 x 10^6/Total) were only treated with 10 μM of PGA_2, 15-keto PGE_2, $PGF_{2\beta}$ or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. The supernatant was collected and Kyn levels quantified using the modified spectrophotometric assay. Consistent with our previous results, control cells produced very low levels of Kyn, while IFN-γ stimulated cells showed a significant increase in Kyn production over basal levels. Cells treated with PGs alone showed no significant change in Kyn production over basal levels. In monocytes, only PGA_2 and 15-keto PGE_2 significantly inhibited Kyn production compared to IFN-γ alone. The lack of a significant effect of 19(R)-hydroxy PGE_2 and $PGF_{2\beta}$ in human monocytes may have been due to the small sample size (Figure 5-7B).

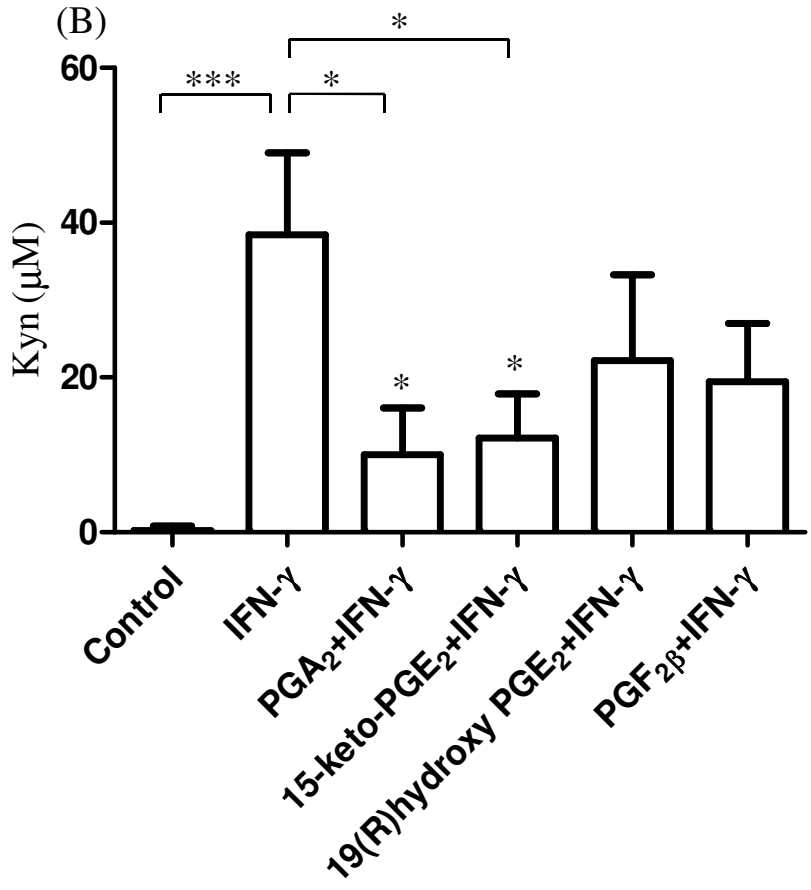
5.6 Effect of $PGF_{2\alpha}$ metabolites on the IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

We next examined the biological activity of metabolites of $PGF_{2\alpha}$ (Figure 5-8). THP-1 cells (4 x 10^5/Total) were treated with 10 μM 15-keto $PGF_{2\alpha}$, 20-hydroxy $PGF_{2\alpha}$, 13, 14-dihydro-15 keto $PGF_{2\alpha}$, Tetranor PGFM, 19(R)-hydroxy $PGF_{2\alpha}$ or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was collected and Kyn levels quantified using the modified spectrophotometric assay. Consistent with previous results, unstimulated cells produced very low levels of Kyn, while IFN-γ stimulated a significant increase in Kyn levels. Cells treated with PGs alone showed no significant change in Kyn production over basal. THP-1 cells that were treated with 15-keto $PGF_{2\alpha}$ and then stimulated with IFN-γ showed significant inhibition in Kyn production compared to cells treated with IFN-γ alone (Figure 5-9A). None of the other PGs tested significantly inhibited Kyn production compared to IFN-γ alone. We also confirmed the THP-1 cell results with 15-keto $PGF_{2\alpha}$ using human monocytes. In contrast to our THP-1 results, 15-keto $PGF_{2\alpha}$ failed to significantly inhibit IFN-γ induced Kyn production compared to IFN-γ alone (Figure 5-9B).

Figure 5-7 Effect of PGE_2 metabolites on IFN-γ mediated induction of IDO in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/Total) were pretreated with 10 µmol/L of PGA_2, PGB_2, PGK_2, Tetranor PGEM, 15- keto PGE_2, 13,14-dihydro-15-keto PGE_2, 13,14-dihydro-15 keto PGA_2, Bicyclo PGE_2, $PGF_{2\beta}$, 19(R)-hydroxy PGE_2 or diluent for 30 min. (B) Human monocytes (4 x 10^6/Total) were pre-treated with 10 µmol/L of PGA_2, 15-keto PGE2, $PGF_{2\beta}$ or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are presented as mean ± SEM Kyn produced. THP-1 n=10 separate experiments, human monocytes n=5 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.


(A)
***
***
*
**
***
***
40
30
20
10
0
Kyn (μM)
Control
IFN-γ
PGA2
PGB2
PGK2
15-Keto PGE2
13,14-dihydro-15 KetoPGE2
13,14-dihydro-15 Keto PGA2
Bicyclo PGE2
Tetranor PGEM
19(R) hydroxy PGE2
PGF2β
+IFN-γ


(B)
*
***
*
60
40
20
0
Kyn (μM)
*
*
Control
IFN-γ
PGA2+IFN-γ
15-keto-PGE2+IFN-γ
19(R)hydroxy PGE2+IFN-γ
PGF2β+IFN-γ

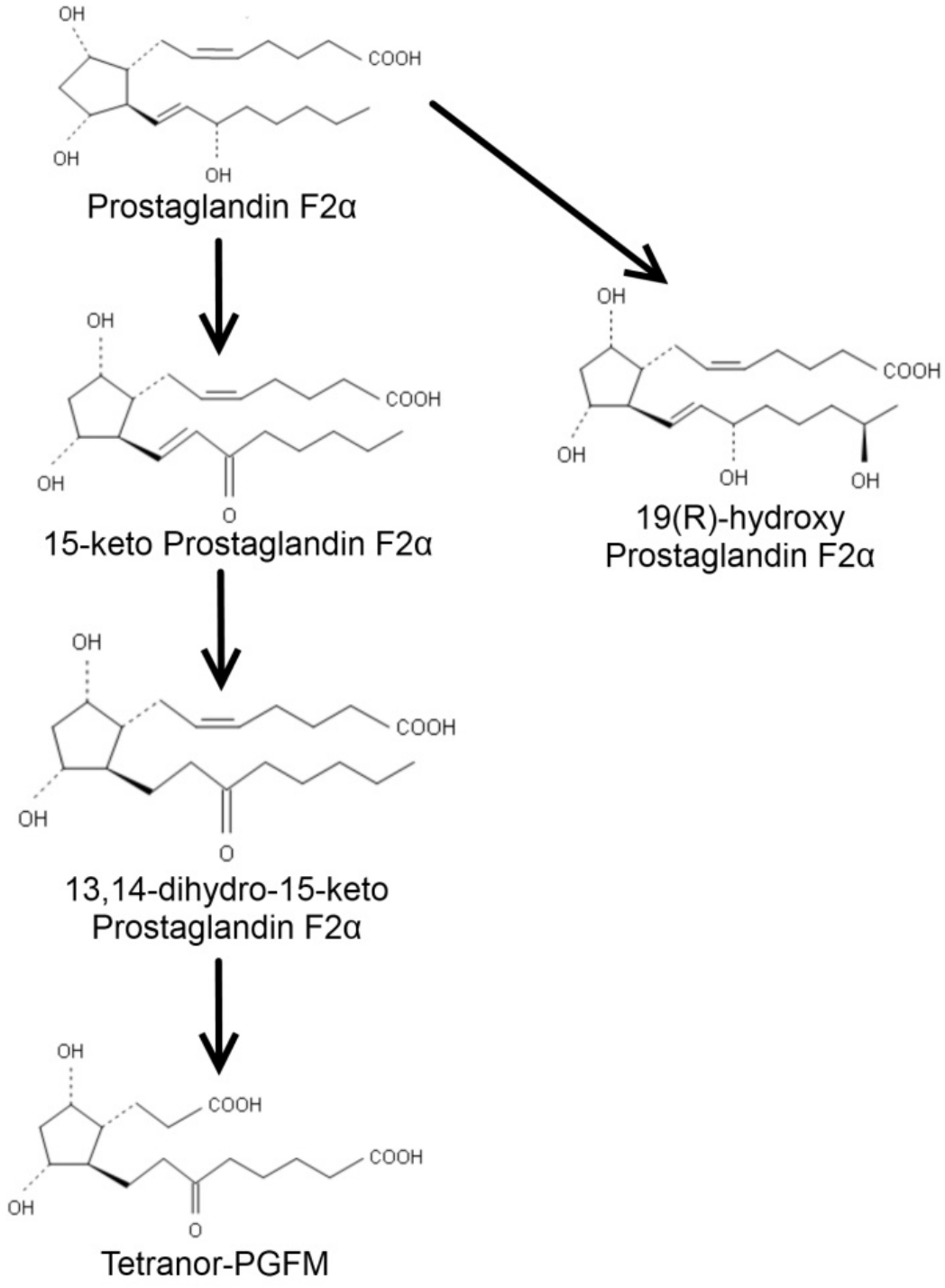


Figure 5-8 Metabolic pathway of $PGF_{2\alpha}$ and its derivatives

This diagram illustrates the metabolism of PGF2α to different metabolites through the COX1 pathway. (Adapted from Cayman chemicals, Ann Arbor, MI, USA).

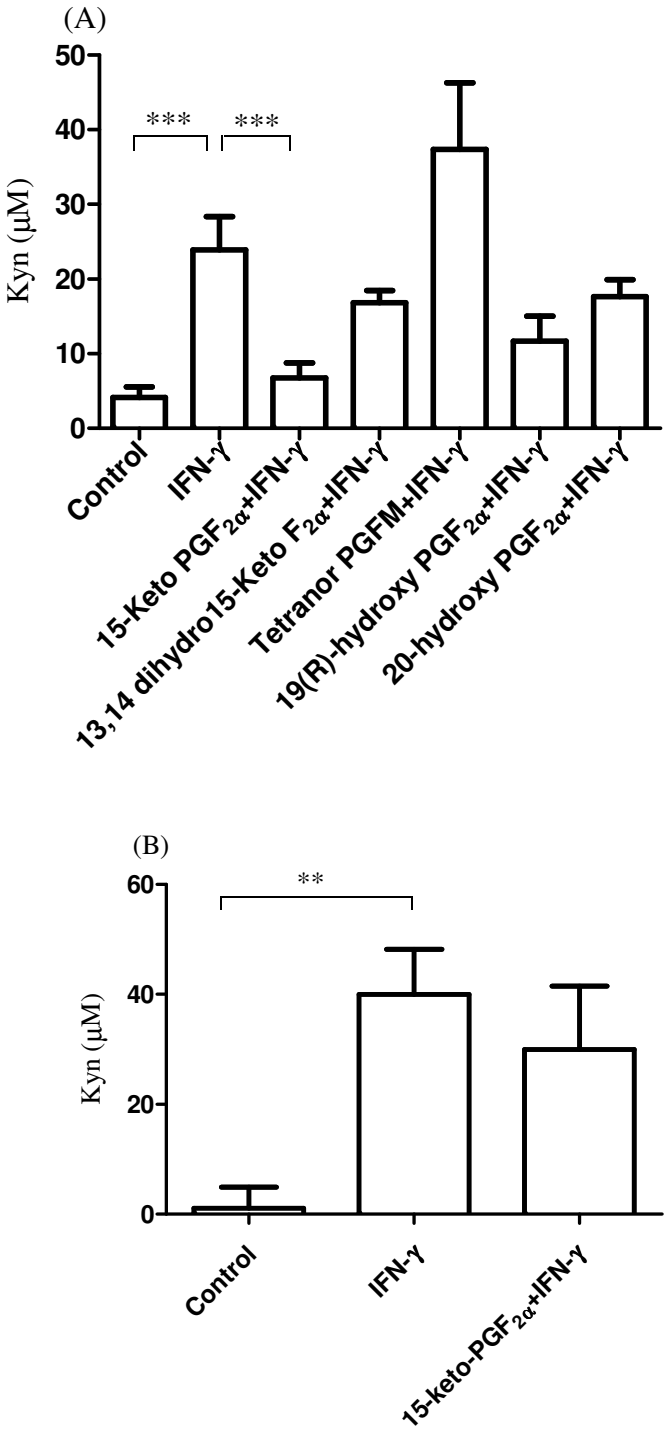


Figure 5-9 Effect of $PGF_{2\alpha}$ metabolites on Kyn production in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/Total) were pre-treated with 10 μmol/L of 15-keto $PGF_{2\alpha}$, 20 hydroxy $PGF_{2\alpha}$, 13,14-dihydro-15 keto $PGF_{2\alpha}$, Tetranor PGFM, 19(R)-hydroxy $PGF_{2\alpha}$ or diluent for 30 min. (B) human monocytes (4 x 10^6/Total) were treated with 15-keto-PGF2α or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM Kyn produced. THP-1 n=10 separate experiments, human monocytes n=5 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

5.7 Effect of COX2 metabolites on the IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

We next examined the biological activity of PGs produced by the COX2 enzyme (Figure 5-10). THP-1 cells (4 x 10^5/Total) were treated with arachidonyl ethanolamide, 20 HETE, $PGF_{2\alpha}$ ethanolamide, PGE_2 ethanolamide, PGD_2 ethanolamide, 20-hydroxy $PGF_{2\alpha}$, 20-hydroxy PGE_2 (10 µmol/L) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was collected and Kyn levels quantified using the modified spectrophotometric assay. Consistent with previous results, control cells produced very low levels of Kyn, while IFN-γ stimulated a significant increase in Kyn. Cells treated with the PGs alone showed no significant change in Kyn production over basal levels. PGE_2 ethanolamide, PGD_2 ethanolamide and 20-hydroxy PGE_2 all significantly inhibited Kyn production compared to IFN-γ alone. All other PGs failed to significantly inhibit Kyn production compared to IFN-γ alone (Figure 5-11A). Human monocytes (4 x 10^6/Total) were also treated with PGE_2 ethanolamide, PGD_2 ethanolamide, 20-hydroxy PGE_2 (10 µmol/L) or diluent as described for THP-1 cells. Consistent with our previous results, control cells produced very low levels of Kyn, while IFN-γ stimulated a significant increase in Kyn levels over baseline. Cells treated with PGs alone showed no significant change in Kyn production over basal levels. Consistent with the THP-1 results, PGE_2 ethanolamide and PGD_2 ethanolamide significantly inhibited IFNγ induced Kyn production in human monocytes while 20-hydroxy PGE_2 plus IFN-γ had no significant effect on Kyn production compared to IFN-γ stimulated cells alone (Figure 5-11B)

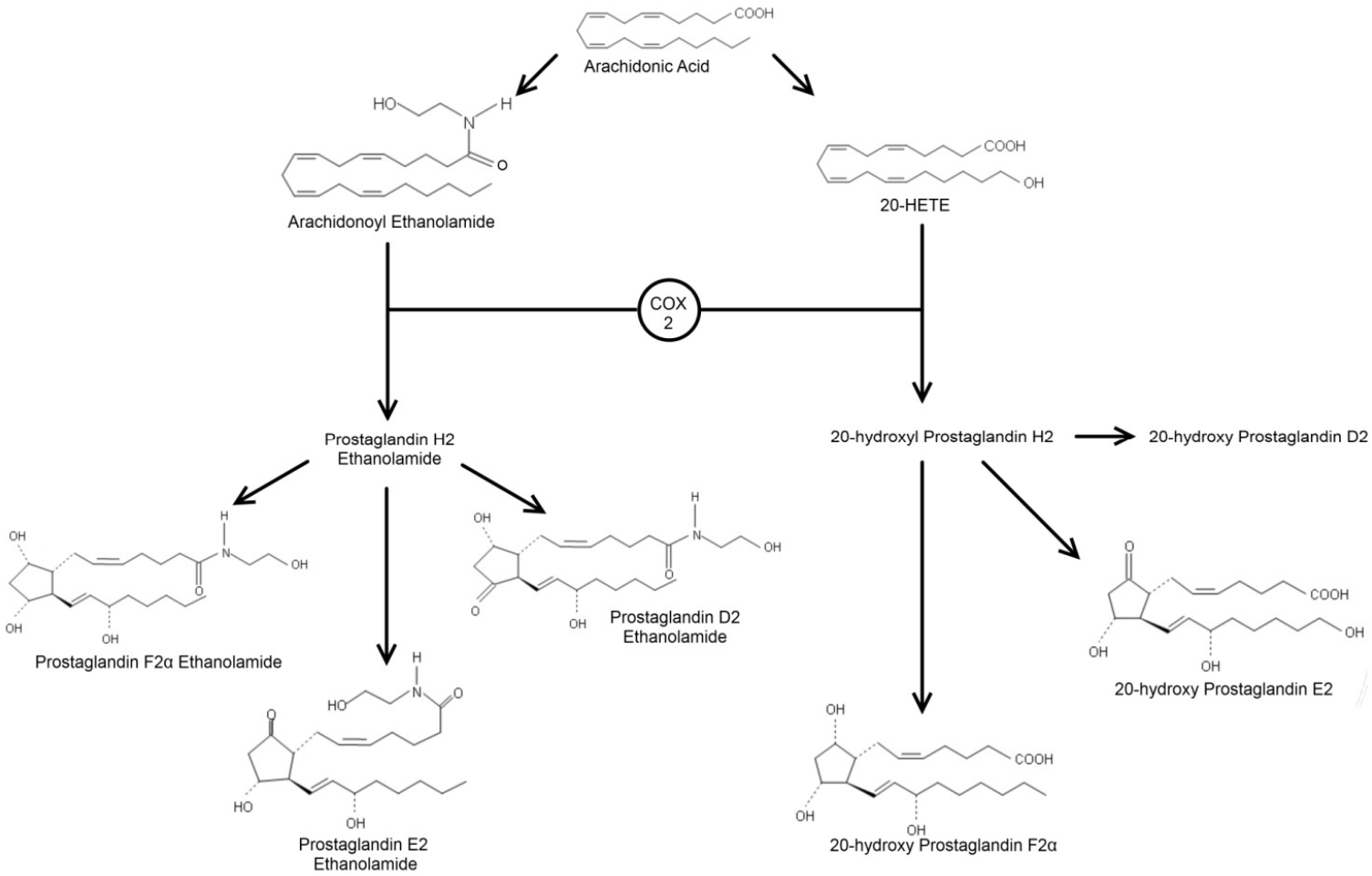


Figure 5-10 Metabolic pathway COX1/2 metabolites

This diagram illustrates the metabolism of AA to different metabolites through the COX2 pathway. (Adapted from Cayman chemicals, Ann Arbor, MI, USA).

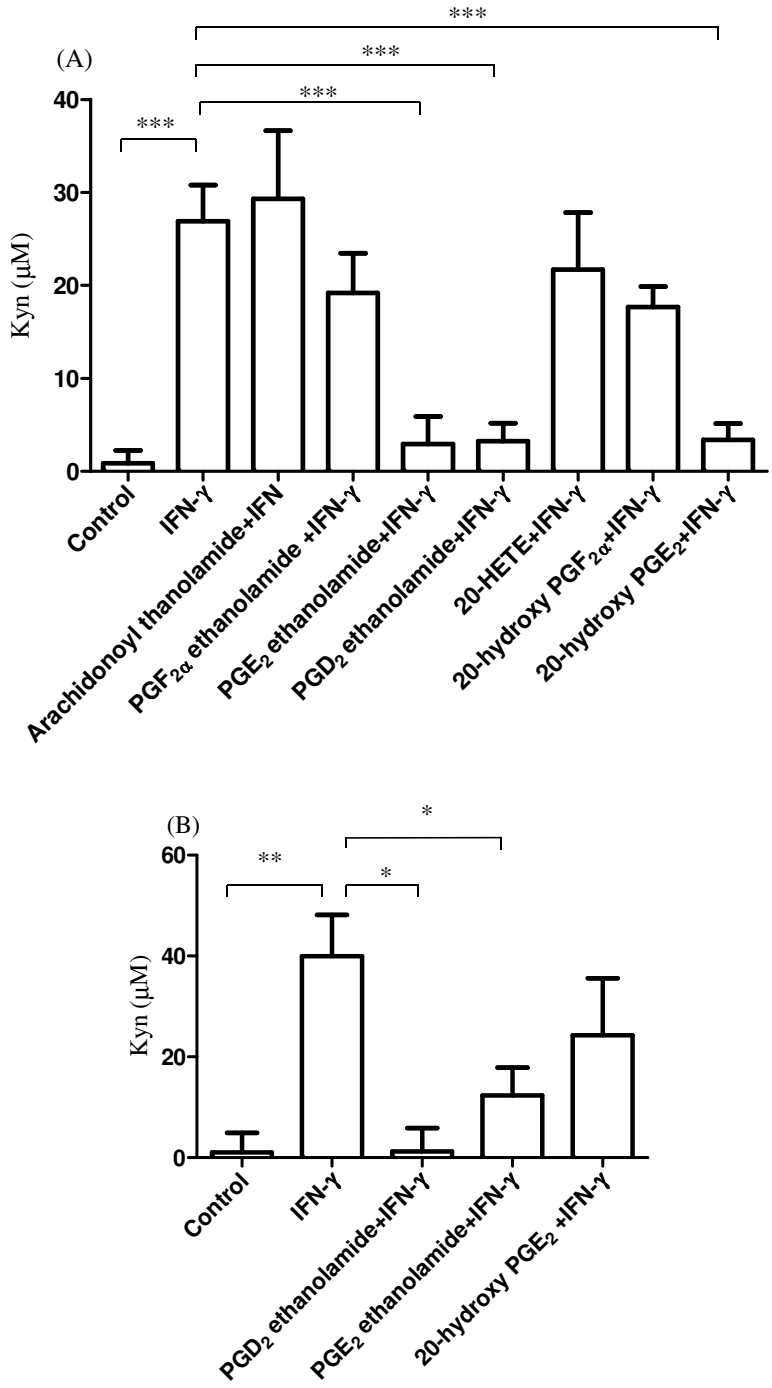


Figure 5-11 Effect of COX2 metabolites on Kyn production in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/Total) were pre-treated with arachidonoyl ethanolamide, 20 HETE, PGF2α ethanolamide, PGE2 ethanolamide, PGD_2 ethanolamide, 20-hydroxy $PGF_{2\alpha}$, 20-hydroxy PGE_2 (10 µmol/L) or diluent for 30 min. (B) Human monocytes (4 x 10^6/Total) were pre-treated with PGE_2 ethanolamide, PGD_2 ethanolamide, 20-hydroxy PGE_2 (10 µmol/L) or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM Kyn produced. THP-1 n=10 separate experiments, human monocytes n=5 separate experiments. Compareson were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

5.8 Determining the IC_{50} for the biologically active COX1/2 metabolites

The initial examination of the AA-derived metabolites identified a total of 16 COX1/COX2 metabolites with significant inhibitory activity in both THP-1 cells and human monocytes. Since these compounds were all tested at one concentration (10 μM), to further characterise their biological activity, we next determined the IC_{50} for each of the metabolites, also examining their effect on cell viability. THP-1 cells (4×10^5) or human monocytes (4×10^6) were pre-treated with each metabolite (0.1–10 μM) or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/ml) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was removed and Kyn was measured using the colorimetric assay. Using the computer program Prism Graphpad (version 5), the IC_{50} for each compound was calculated. To calculate viability, THP-1 cells were collected and viability determined using trypan blue and the results expressed compared to control cells (Table 5-1). For monocytes, since these cells are adherent, trypan blue was not appropriate, so monocyte viability was assessed using a commercial MTS assay. In brief, to 100 μl of stimulated monocytes, was added 20 μl of MTS reagent and incubated for 2 h at 37 °C /5% CO_2 in a humidified atmosphere and the absorbance was read at 492 nm (Table 5-1).

There was a wide range of activity in both THP-1 cells and human monocytes and most were consistent in both cells. Only 15-deoxy-$\Delta^{12,\ 14}$-PGJ_2 was found to be toxic to THP-1 cells, but not to human monocytes. Similarly, 13, 14-dihydro-15-keto PGE_2 was biologically active in human monocytes but not in THP-1 cells. In general, the IC_{50} for all compounds was lower for monocytes than for THP-1 cells. These levels are higher than found physiologically, however they are well within the pharmacological levels reported by others (Fukushima et al. 1982b; Kikuchi et al. 1994).

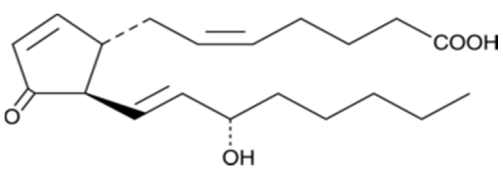
Table 5-1 Percentage inhibition, IC_{50} and percentage viability of biologically active COX1/2 PGs in THP-1 cells and human monocytes

Name	THP-1 cells			Human monocytes		
	% inhibition (Mean ± SEM)	IC_{50} (µM)	% Viability	% inhibition (Mean ± SEM)	IC_{50} (µM)	% Viability
PGD_2	67.6 ± 2.8**	7.4 ± 0.2	77.8 ± 3.4	100.0 ± 4.8***	4.1 ± 0.1	106.5± 2.6
PGJ_2	76.6± 13.0***	6.7 ± 0.7	67.3 ± 2.8	100.0 ± 2.9**	4.2 ± 0.1	93.5 ± 1.0
15-deoxy-$\Delta^{12, 14}$-Prostaglandin J_2	41.5 ± 9.9*	12.5 ± 1.8	35.7 ± 7.0	100.0 ± 3.7**	4.4 ± 0.0	255.8 ± 10.5
Δ^{12}-PGJ_2	72.8 ± 15.2***	7.1 ± 0.8	64.4 ± 5.1	100.0 ± 4.8**	4.6 ± 0.1	143.2 ± 21.3
PGD_2 Ethanolamide	88.6 ± 1.6***	5.7 ± 0.1	34.4 ± 4.3	100.0 ± 6.4*	4.7± 0.2	ND
PGA_2	64.5 ± 3.1***	7.8 ± 0.2	64.0 ± 4.6	100.0 ± 17.0*	4.9 ± 0.5	172.1 ± 26.7
PGE_2 Ethanolamide	100.0 ± 16.6***	4.6 ± 0.4	70.6 ± 4.7	81.1 ± 14.0*	6.3 ± 0.6	ND
13,14-dihydro 15-keto PGE_2	18.2 ± 5.3	30.1 ± 5.1	42.0 ± 2.6	76.5 ± 13.1	6.7 ± 0.7	267.8 ± 20.0
15-Keto PGE_2	55.3 ± 4.3***	9.0 ± 0.5	55.5 ± 7.1	74.9 ± 12.5*	6.8 ± 0.7	222.8 ± 28.8
$PGF_{2\beta}$	68.3 ± 6.0***	7.4 ± 0.4	71.5 ± 4.1	73.1 ± 16.4	7.1 ± 0.9	211.7 ± 10.4
19 (R) hydroxy PGE_2	84.4 ± 4.8***	5.9 ± 0.2	60.1 ± 5.6	55.5 ± 14.9	9.5± 1.5	180.3 ± 21.0
PGH_2	70.6 ± 11.0***	7.2 ± 0.7	ND	57.0 ± 11.6	9.6± 1.4	ND
PGG_2	55.1 ± 8.6*	9.2 ± 0.8	ND	51.7 ± 23.6	11.2 ± 3.3	ND
20-hydroxy PGE_2	71.7± 21.4***	7.4 ± 1.3	82.4 ± 4.9	49.6 ± 17.9	11.2 ± 2.5	188.4 ± 17.5
15-Keto $PGF_{2\alpha}$	65.7 ± 5.0***	7.6 ± 0.3	61.5 ± 3.9	33.2 ± 22.2	23.1 ± 11.2	ND
PGE_2	48.6 ± 4.8	10.4 ± 0.6	ND	26.5 ± 12.1	22.1 ± 6.5	113.0 ± 2.1

THP-1 cells and human monocytes were treated with 0.1–10 µmol/L of the individual PG or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as Mean ± SEM % inhibition of Kyn production, IC_{50} or cell viability, THP-1, n=12 separate experiments; human monocytes, n=6 separate experiments; unpaired exact Mann Whitney test; * indicates $p < 0.04$, *** indicates $p < 0.0001$. ND: not determined. Percentage viability over 100% indicates cellular proliferation. Viability of control cells was 91.9% ± 3.4 and viability of IFN-γ stimulated cells was 83.1% ± 6.7.

5.9 Structure–function analysis of inhibitory COX1/2 metabolites

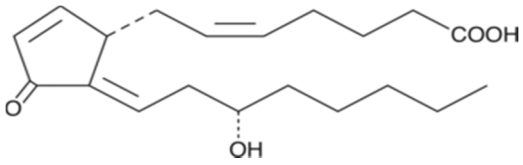
Having screened a large number of metabolites, we questioned why some displayed potent activity and others were inactive. To determine which chemical features of the molecule(s) were important for activity, we performed a structure–function analysis using the significance of the inhibitory effect of individual PG compared to IFN-γ treatment alone * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. When comparing PGJ_2 and Δ^{12}-PGJ_2, there was no significant difference in activity, IC_{50} or effect on cell viability. The only difference was that the carbon between C13–14 is projected backwards in PGJ_2 and in the same direction as the other carbons in Δ^{12}-PGJ_2. Therefore, we concluded that this difference had no effect on the function of the two compounds, especially as the arms are free to move and rotate around the remainder of the structure.



PGJ_2

76.6 ± 13.0***

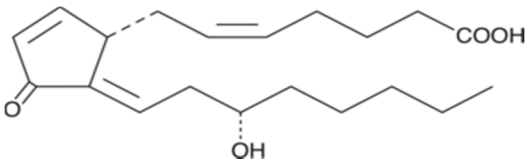
vs.



Δ^{12}-PGJ_2

72.8 ± 15.2***

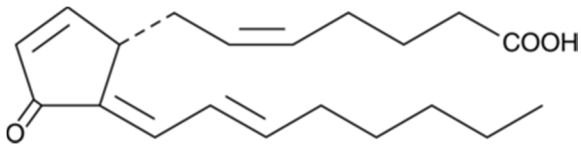
- When comparing Δ^{12}-PGJ_2 to 15-deoxy-$\Delta^{12,14}$-PJ_2, removal of the OH group from C15 caused a significant decrease in activity and increased the toxicity towards the THP-1 cells.



Δ^{12}-PGJ_2

72.8 ± 15.2***

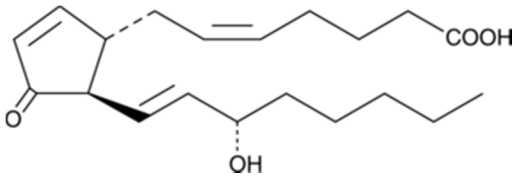
vs.



15-deoxy-$\Delta^{12, 14}$-Prostaglandin J_2

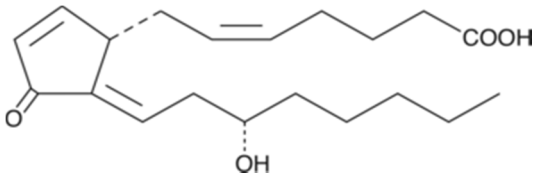
35.7 ± 7.0*

- When PGJ_2 and Δ^{12}-PGJ_2 are compared with PGD_2, the removal of the OH group from the α position in the cyclopentenone ring and the presence of a double bond in the α-β position resulted in an increase in inhibitory activity while cell viability was not affected.



PGJ_2

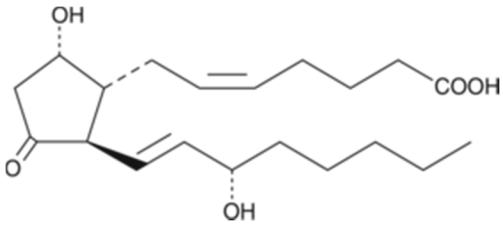
76.6 ± 13.1***



Δ^{12}-PGJ_2

72.8 ± 15.2***

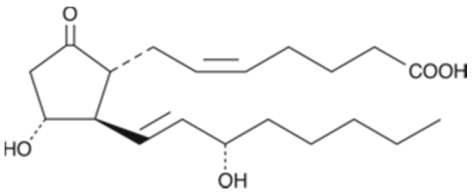
vs.



PGD_2

67.6 ± 2.8**

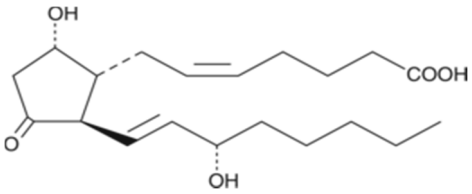
- Changing the orientation of the O atom from the α position in the cyclopentanone ring as in PGE_2 to the γ position as in PGD_2 led to a significant increase in inhibitory activity. This effect may be due to reduced congestion under the ring and/or the O atom playing a greater role in the γ position than in the α position.



PGE_2

48.6 ± 4.8

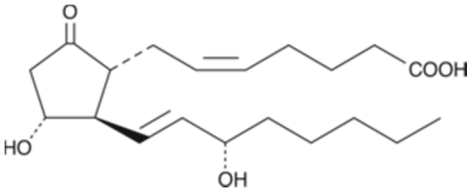
vs.



PGD_2

67.6 ± 2.8**

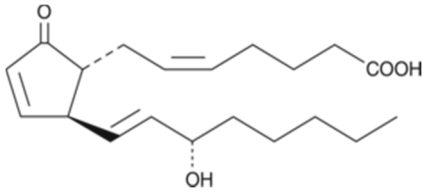
- In comparing PGE_2 and PGA_2, the removal of the OH group from the γ position in the cyclopentanone ring and addition of double bonds to become a cyclopentenone ring caused a significant increase in the inhibitory activity without affecting cell viability.



PGE_2

48.6 ± 4.8

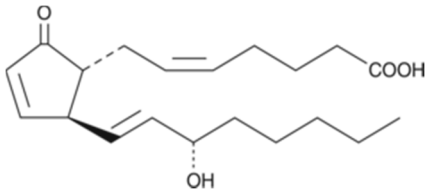
vs.



PGA_2

64.5 ± 3.0***

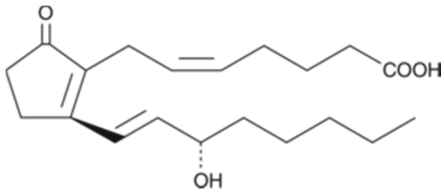
- When comparing PGA_2 and PGB_2, which have the same basic structure but in a different configuration, changing the position of the double bonds in the cyclopentenone ring caused a significant decrease in inhibitory activity.



PGA_2

64.5 ± 3.0***

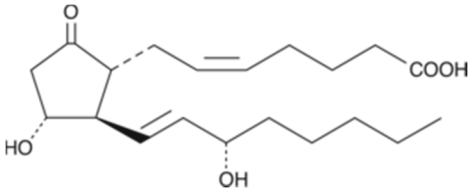
vs.



PGB_2

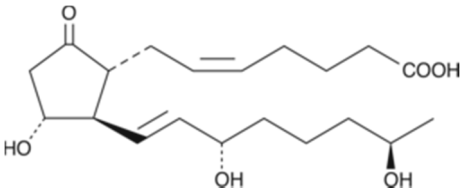
16.7 ± 4.5

- When PGE_2 was compared with 19(R)-hydroxy PGE_2 and 20-hydroxy PGE_2, the addition of an OH group in the side arm significantly increased inhibitory activity without affecting cell viability.



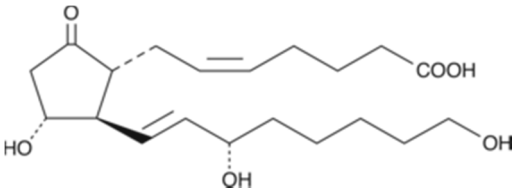
PGE_2

48.6 ± 4.8



19 (R) hydroxy PGE_2

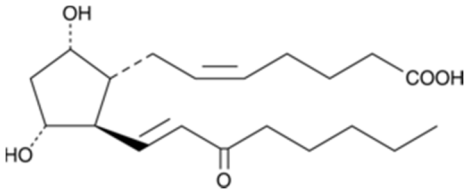
84 ± 5***



20-hydroxy PGE_2

72 ± 21***

- Based on our previous observations, we expected 15-keto PGE_2 to be more active than 15-keto $PGF_{2\alpha}$, as it has an O group in the α position of the cyclopentenone ring, and this result was indeed observed with human monocytes but not in THP-1 cells.

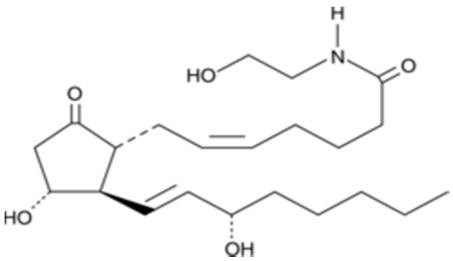


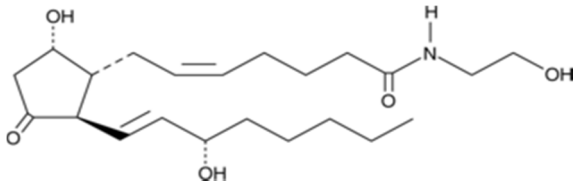
15-Keto $PGF_{2\alpha}$ vs. 15-Keto PGE_2

65.7 ± 5.0*** (THP-1) 55.3 ± 4.3** (THP-1)

33.2 ± 22.2 (monocytes) 74.9 ± 12.5* (monocytes)

- We also observed that all PGs that contained an ethanolamide group showed significant inhibitory activity on Kyn production.





PGE_2 Ethanolamide vs. PGD_2 Ethanolamide

100.0 ± 16.6*** 88.6 ± 1.6***

5.10 Summary of the structure–function analysis

The following structural features were found to affect the activity of the PGs towards IDO activity.

1. The γ position in the cyclopentenone ring with a double bond in the α-β position was important for inhibitory activity.
2. An OH group in the side arm was important for inhibitory activity.
3. The presence of more than one OH group in the side arm increased inhibitory activity.
4. The orientation of the OH group in the α position in the cyclopentanone ring appeared to be important, leading to a flatter structure.
5. Any steric interactions or congestion of the groups under the ring seemed to interfere with the inhibitory activity of the PGs.
6. The addition of an ethanolamide group significantly increased the inhibitory activity of the different metabolites.

5.11 Summary

A wide range of AA derived COX1 and COX2 metabolites (38) were screened for their ability to inhibit the IFN-γ mediated induction/ activity of IDO-1 in THP-1 cells. The inhibitory effects of the biologically active compounds were confirmed in human monocytes. Of the first five principal PGs produced from PGH_2 metabolism, only PGD_2 showed an inhibitory effect in both THP-1 cells and human monocytes. Of the 38 metabolites tested, 13 PGs in THP-1 cells and 11 in human monocytes modulated IFN-γ mediated induction/activity of IDO-1 without apparent toxicity. It should be noted that some compounds were toxic at the concentration tested, e.g. 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2. Analysis of the structure–function relationship revealed that the γ position in the cyclopentenone ring with a double bond in the α-β position, the presence of more than one OH group in the side arm and the addition of an ethanolamide group to the side arm significantly increased the inhibitory activity of the metabolite.

Chapter 6

Characterisation of the activity of PGD_2 on IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

6.1 Introduction

We previously demonstrated that COX1 and COX2 metabolites of AA inhibited the IFN-γ mediated induction of IDO in THP-1 cells and human monocytes (Bassal, Hughes & Costabile 2012). In the previous chapter, we screened a wide range of AA-derived COX1 and COX2 metabolites to identify which metabolite(s) was/were responsible for the inhibitory activity. A total of 13 prostaglandins (PGs) significantly inhibited IFN-γ mediated induction/activity of IDO-1in THP-1 cells and 11 PGs in human monocytes. While it is important to further characterise each of these PGs, such an undertaking was beyond the limit of this research. As a result, we have focused our attention on the PGs that displayed the greatest inhibitory activity, namely PGA_2 in monocytes and PGD_2 in THP-1 cells. Of these two, only PGD_2 had commercially available agonists and antagonists that would allow us to study its mechanism of action in detail. As a result, the mechanism of action of PGD_2 and its derivatives are elucidated in this chapter.

6.2 Prostaglandin D_2 (PGD_2)

AA undergoes oxidation to PGG_2, which is further reduced to PGH_2. PGH_2 is then metabolised to PGD_2 by two enzymes: the hematopoietic-type of PGD synthase (H-PGDS), which is expressed by immune and inflammatory cells and localized in APCs, and the lipocalin-type PGD synthase (L-PGDS) which is expressed by neurons, oligodendrocytes, Leydig cells, endocardial and myocardial cells of the heart (Urade & Hayaishi 2000b, 2000a; Trivedi et al. 2006; Rajakariar et al. 2007). PGD_2 is a prostanoid produced predominantly by mast cells (Tanaka, K et al. 2004). However, it can also be produced by Th2 lymphocytes and dendritic cells (Pettipher 2008). PGD_2 has many roles in immune responses (Urade et al. 1989; Tanaka, K et al. 2004). In an *in vitro* study using murine mast cells, the early phase of PGD_2 production was found to be COX1 dependent, while the more prolonged PGD_2 production was COX2 dependent (Reddy et al. 1999). PGD_2 undergoes spontaneous dehydration and isomerisation *in vitro* and is enzymatically catalysed *in vivo* to a wide range of metabolites (Figure 6-1) (Sandig, Pease & Sabroe 2007).

PGD_2 is involved in many pathological conditions, principally allergy and inflammation. PGD_2 has been detected in high concentrations (micromolar) at the site of allergic inflammation and initiates IgE-mediated type 1 acute allergic responses (Roberts et al. 1980; Lewis et al. 1982). After an allergen challenge, PGD_2 is released into the airways causing bronchoconstriction (Hardy et al. 1984) and eosinophil infiltration

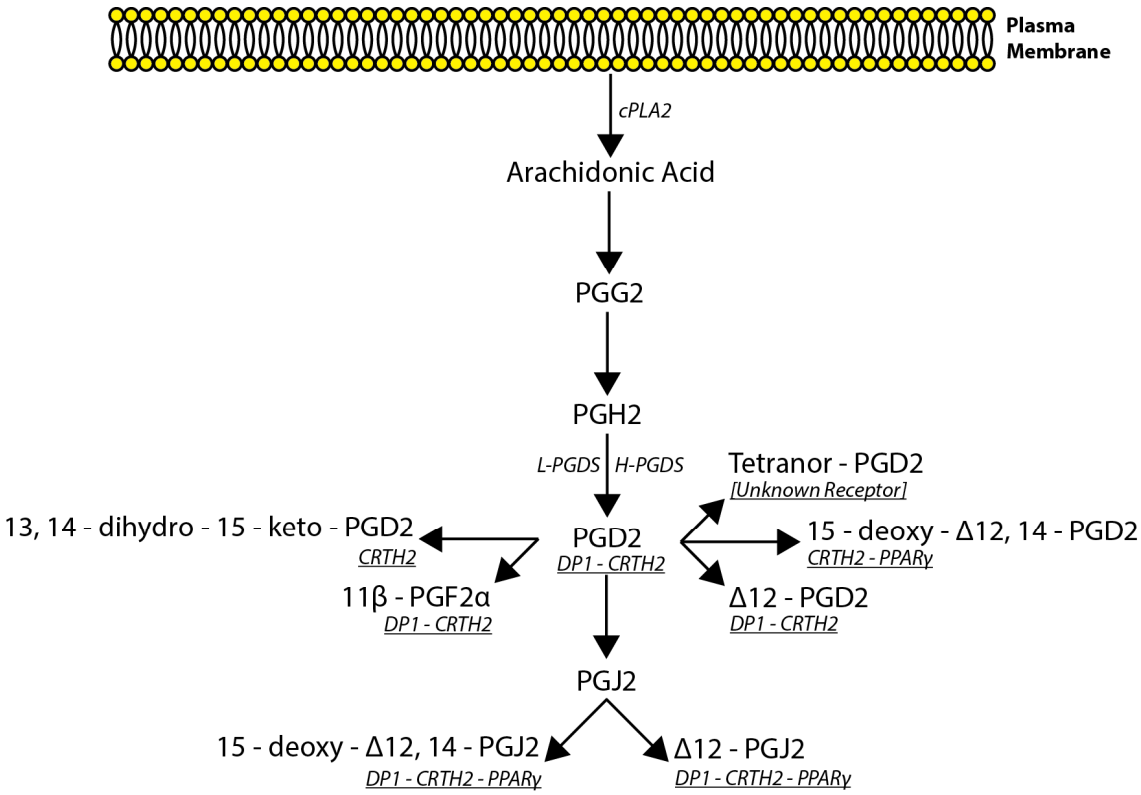


Figure 6-1 PGD_2, its metabolites and the receptors they activate

AA undergoes oxidation to PGG_2 that is then reduced to PGH_2 and then metabolised to PGD_2 (Chantal et al., 2007) and ultimately to a range of metabolites through the action of 2 specific synthases. AA, arachidonic acid; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGD_2, prostaglandin D_2; H-PGDS, hematopoietic-type of PGD and L-PGDS, synthase and lipocalin. DP1, prostaglandin D receptor; CRTH2, chemoattractant receptor-homologous molecule expressed on T-helper cells. (Adapted from Sandig, Pease & Sabroe 2007, and Cayman Chemical, used with permission).

(Emery et al. 1989). During acute allergic responses; PGD_2 is released into the skin causing atopic dermatitis (Murray, JJ et al. 1986; Barr et al. 1988). PGD_2 has also been shown to display anti-inflammatory activity (Hata & Breyer 2004), inhibit platelet aggregation (Whittle et al. 1985), smooth muscle relaxation and contraction (Narumiya & Toda 1985), vasodilatation and vasoconstriction (Giles & Leff 1988), mucus secretion (Wright, JK et al. 2000) and promotes sleep induction (Mizoguchi et al. 2001). PGD_2 also has been shown to inhibit human ovarian tumor cell proliferation at a pharmacological IC_{50} of 6.8 μM (Kikuchi et al. 1994).

6.3 PGD_2 signal transduction

PGD_2 and its metabolites mediate their effects through three different mechanisms: via G-protein coupled receptors, nuclear receptors and direct modification of proteins.

6.3.1 PGD_2 signalling via G-protein coupled receptors (GPCRs)

PGD_2 mediates its effect mainly via a pair of seven transmembrane, G-protein coupled receptors: the D-prostanoid receptor (DP1) and chemoattractant receptor-homologous molecule expressed on T-helper cells (CRTH2) (Nagata et al. 1999b; Hirai et al. 2001). PGD_2 has low affinity to bind and signal via the thromboxane T receptor (TP receptor) causing bronchoconstriction (Hamid-Bloomfield et al. 1990; Johnston et al. 1992).

DP1 is a member of the prostanoid receptor family: EP 1–4, IP and TP. DP1 is expressed by vascular smooth muscle, platelets (Pettipher 2008), basophils, eosinophils (Gervais et al. 2001), monocytes, dendritic cells (Gosset et al. 2003), NK cells (Chen, Y, Perussia & Campbell 2007) and Th1 and Th2 cells although at low levels (Tanaka, K et al. 2004). CRTH2 is expressed on Th2 lymphocytes, eosinophils and basophils (Nagata et al. 1999b). It is closer in structure to other chemotactic receptors such as the LTB4 receptor, BLT1 and BLT2, complement receptor C5a and formyl peptide receptors (Pettipher, Hansel & Armer 2007).

The two receptors also differ in their signal transduction pathways. DP1 is coupled to Gαs, which is directly linked to adenylate cyclase (AC), and its activation leads to the elevation of intracellular levels of cAMP and suppresses Ca^{2+} mobilization (Chen, Y, Perussia & Campbell 2007). In contrast, CRTH2 is a Gαi-coupled receptor and its activation leads to a reduction in cAMP levels (Sawyer et al. 2002), stimulation of

phosphatidylinositol-3-kinase (PI3K), p38 mitogen-activated protein kinase (MAPK) and release of Gβγ-subunits, which cause an elevation in intracellular calcium (Hata & Breyer 2004; Murray, AJ 2008) (Figure 6-2).

DP1 binds to both PGD_2 and PGJ_2 and the selective DP1 agonists BW 245C and L-644,698 with high affinity and to the partial agonist BW 868C (Nagata et al. 1999b; Hirai et al. 2001), CRTH2 binds PGD_2 and also PGD_2 metabolites: 13,14-dihydro-15-keto PGD_2, 11β $PGF_{2\alpha}$, 15-R-methyl-PGD_2, Δ^{12}-PGD2, Δ^{12}-PGJ2, 15-deoxy-$\Delta^{12,14}$-PGD_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 but not BW 245C (Nagata et al. 1999b; Hirai et al. 2001). PGD_2 binding via DP1 promotes Th2 cytokine production, especially IL-12 and Th2 polarisation during allergen sensitisation (Kabashima & Narumiya 2003; Pettipher 2008). Furthermore, PGD_2 binding to CRTH2 has been shown to promote recruitment and activation of Th2 lymphocytes, eosinophils and chemotaxis of leukocytes *in vivo* that leads to atopic dermatitis (Nagata et al. 1999b; Hirai et al. 2001; Iwasaki et al. 2002). Also through CRTH2, PGD_2 can promote the production of Th2 cytokines especially IL-4, IL-5 and IL-13 which promote the induction of IgE (Pettipher 2008) (Table 6-1).

It is clear that PGD_2 has a wide range of activities, some of which are opposing. This can be explained by the ability of PGD_2 to activate multiple signalling pathways leading to different effects depending on whether DP1 and/or CRTH2 are expressed on specific cells (Breyer et al. 2001; Tilley, Coffman & Koller 2001; Hata & Breyer 2004; Luster & Tager 2004). For example, apart from its pro-inflammatory effects, PGD_2 also has anti-inflammatory effects in other conditions. In humans, intradermal injection of PGD_2 causes erythema (Flower, Harvey & Kingston 1976) and, in dogs (Emery et al. 1989) and rats (Almishri et al. 2005), intra-tracheal PGD_2 administration induces lung eosinophilia. Also mice that over-express lipocalin-type PGD_2 synthetase display increased eosinophil and T-lymphocyte recruitment to the lung (Fujitani et al. 2002). PGD_2 is further metabolised to a number of metabolites that are known to be bioactive, especially PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2, which are involved in many of the anti-inflammatory effects of PGD_2 (Gilroy et al. 1999; Gilroy et al. 2003; Sandig, Pease & Sabroe 2007). PGD_2 and 15-$\Delta^{12,14}$-PGJ_2 production is observed during the resolution of induced pleural inflammation and when the production of these PGs is blocked, inflammation worsens (Gilroy et al. 1999; Gilroy et al. 2003). It also was observed that 15-deoxy-$\Delta^{12,14}$-PGJ_2 induces apoptosis of infiltrating neutrophils and stimulates macrophage recruitment to clear apoptotic cells, thus aiding in the clearance of the inflammatory infiltrate (Gilroy et al. 2003). Evidence for an anti-inflammatory role for PGD_2 and its metabolites was also generated in studies using mice deficient in H-PGDS$^{-/-}$ (Rajakariar et al. 2007).

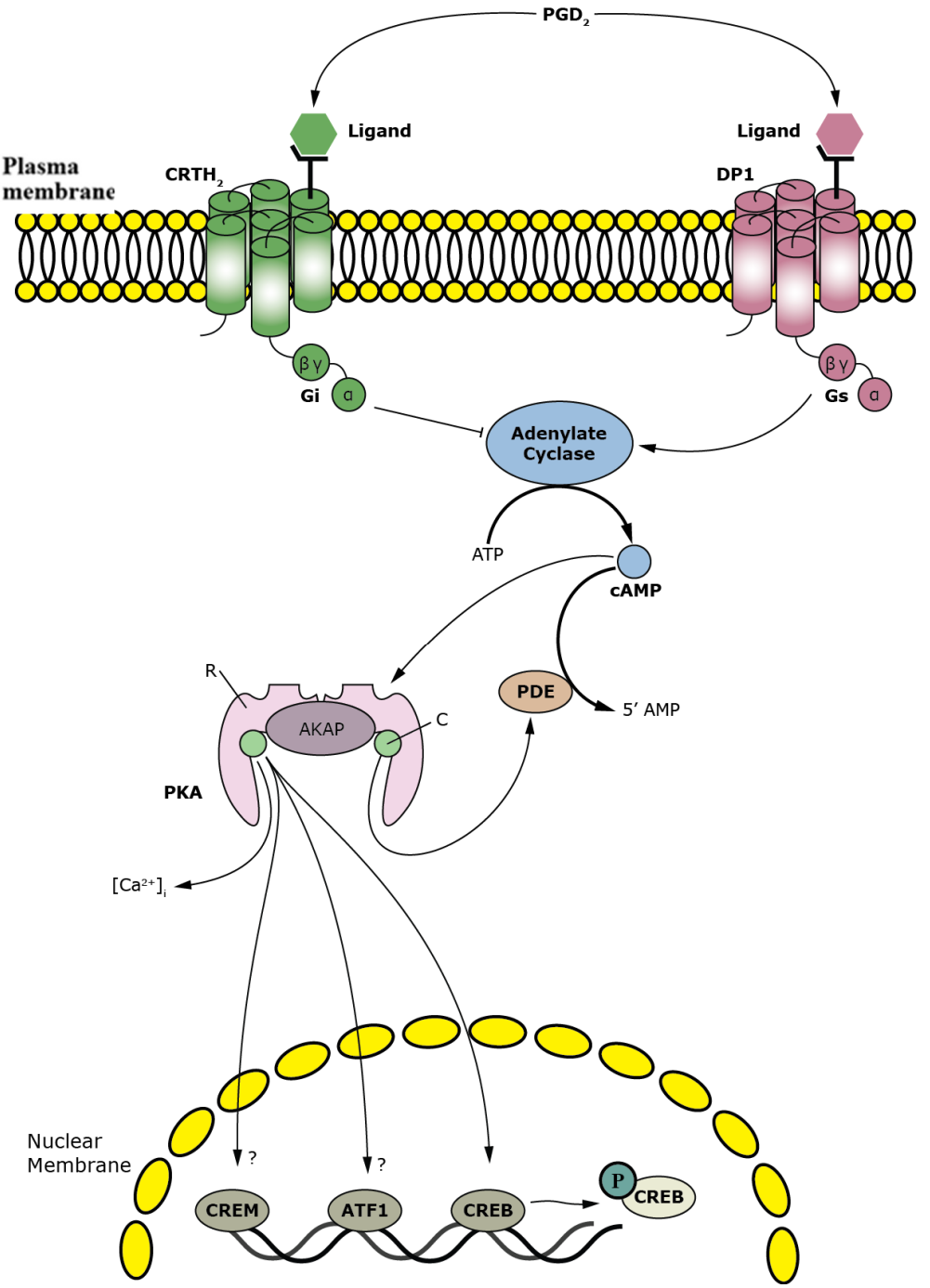


Figure 6-2 Summary of the PGD_2 mediated signalling cascade

DP1 is a Gαs-coupled receptor which, following binding of PGD_2, leads to dissociation of the α subunit and activation of adenylate cyclase (AC), which converts ATP into cAMP and decreases or increases intracellular Ca^{+2} (Hirata et al. 1994; Hirata & Murad 1994; Boie et al. 1995). CRTH2 is a Gαi-coupled receptor and binding of PGD_2 leads to the α subunit inhibiting AC activation (Hirai et al. 2001; Sawyer et al. 2002; Kostenis & Ulven 2006). cAMP activates protein kinase A (PKA) which phosphorylates the transcription factor CREM, ATF and CREB that are normally upstream of target genes. cAMP is degraded to 5' AMP via the action of a specific phosphodiesterase (PDE) enzyme. AKAP, A-kinase anchoring protein; ATF1, activating transcription factor 1; ca, catalytic unit; CREB, cAMP response element-binding protein; CREM, cAMP response element modulator; R, regulatory unit. (Adapted from Murray, AJ 2008).

Table 6-1 Comparison of the biological characteristics of DP1 and CRTH2 receptors

	DP1	CRTH2	References
Ligand	PGD_2 PGJ_2	PGD_2 13,14-dihydro-15-keto PGD_2 15-deoxy-$\Delta^{12,14}$-PGJ_2	(Nagata et al. 1999a; Hirai et al. 2001; Kostenis & Ulven 2006)
Agonist	BW 245C ZK110841	Indomethacin, L-644,698 13,14-dihydro-15-keto PGD_2 15(R)-15-methyl PGD_2	(Hirai et al. 2001)
Antagonist	BW 868C MK 0524 S-5751	BAY u4305 Ramatroban	(Hirai et al. 2001)
Signalling mechanism	Gαs ↑ intracellular cAMP	Gαi ↓cAMP ↑intracellular mobilization of calcium	(Boie et al. 1995; Hirai et al. 2001; Sawyer et al. 2002; Kostenis & Ulven 2006; Chen, Y, Perussia & Campbell 2007)
Cellular expression	Bronchial smooth muscle Vascular smooth muscle DCs, Platelets, CNS	Brain, heart, thymus, spleen Th2 lymphocytes; Eosinophils Basophils, CNS	(Hirai et al. 2001; Sawyer et al. 2002; Kostenis & Ulven 2006)
Biological effects	Anti-inflammatory, Bronchodilatation and Vasodilatation, Increase inflammatory cell survival, Increase inflammatory cell extravasation into allergic sites, Decrease IL-12, IFN-γ and IL-2, Inhibition of platelet aggregation, Induce sleep and pain cognition	Pro-inflammatory Chemotaxis in Th2 cells Activation of Th2 lymphocytes, Migration of eosinophils and basophils	(Hirai et al. 2001; Hata & Breyer 2004; Kostenis & Ulven 2006)

CNS, central nervous system; DCs, dendritic cells; DP1, D-prostanoid receptor; CRTH2, chemoattractant receptor-homologous moleculeexpressed on T-helper cells; PGD_2, prostaglandin D_2; Th2 cells, T-helper type 2 cells; ↑, increase; ↓, decrease. (Adapted from Hirai et al. 2001; Sawyer et al. 2002; Kostenis & Ulven 2006).

animals show impaired resolution of inflammation, and animals transgenic for H-PGDS show a reduced inflammatory response (Rajakariar et al. 2007).

6.3.2 PGD2 and nuclear receptors

A third type of receptor involved in PGD_2 metabolite activity is the peroxisome proliferator-activated receptor (PPAR). PPARs are nuclear receptors that modify gene expression in a ligand-dependent manner. PPARs include PPARα, PPARβ and PPARγ and they play many important roles in the immune system (Rossi et al. 2000; Woerly et al. 2003; Scher & Pillinger 2005). PPARs are expressed by monocytes and macrophages (Ricote et al. 1998a; Ricote et al. 1998b), eosinophils (Ueki et al. 2003; Woerly et al. 2003) and T lymphocytes (Clark et al. 2000). PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGD_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 have been shown to activate PPARα and PPARγ (Forman et al. 1995; Kliewer et al. 1995) although only at micromolar concentrations and with less affinity than for CRTH2 (Nosjean & Boutin 2002).

6.4 The effective inhibitory concentration of PGD_2

Having identified PGD_2 as being a potent inhibitor of IDO-1 activity, we examined its biological activity in further detail. We first determined its IC_{50} by treating both THP-1 cells and human monocytes with PGD_2 (10^{-9}–10^{-5} mol/L). Results are shown as the percentage inhibition of Kyn production compared to treatment with IFN-γ alone. PGD_2 had no effect on basal Kyn production at any of the concentrations tested in either cell type, yet it caused a dose dependent decrease in Kyn production in both cell types (Figure 6-3). The IC_{50} was calculated to be 7.2±0.3 µmol/L in THP-1 cells and 5.2±0.9 µmol/L in human monocytes (data not shown). These values are consistent with the pharmacological *in vitro* activity of PGD_2 in inhibiting ovarian tumor cell proliferation with an IC_{50} of 6.8 µmol/L (Kikuchi et al. 1994).

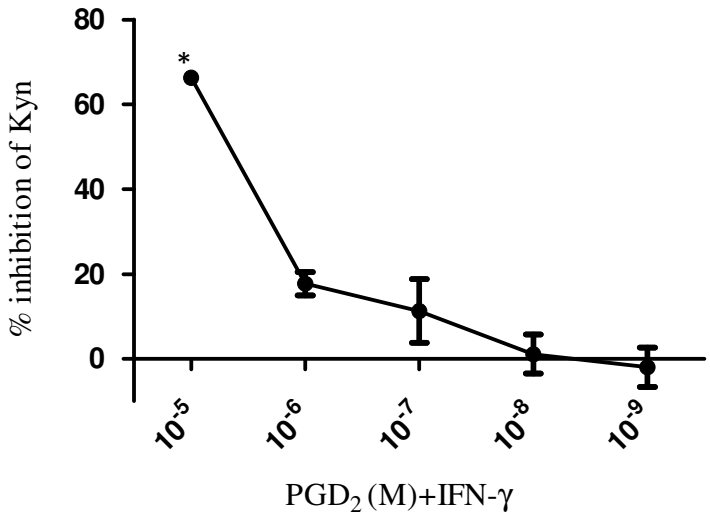


Figure 6-3 Determination of the IC_{50} for PGD_2 in THP-1 cells and human monocytes

THP-1 cells (4 x 10^5/total) were treated with PGD_2 (10^{-9}–10^{-5} M) for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are presented as mean ± SEM of % inhibition of Kyn production compared to IFN-γ alone. Results are shown as mean ± SEM % inhibition of Kyn produced. n = 8 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicates $p < 0.04$.

6.5 Effect of PGD_2 on cell viability

We next examined whether the PGD_2 inhibitory activity was due to cell toxicity rather than true inhibitory effect. THP-1 cells were treated with the highest dose of PGD_2 (10 µmol/L) for 30 min, then stimulated with IFN-γ (1000 IU/mL) for 24–72 h and viability was then assessed by their ability to exclude trypan blue. There were no significant differences in cell viability between all treatments after 24 and 48 h. After 72 h, there were no differences in cell viability between the control and PGD_2 treated cells, however there was a significant decrease in the viability of the cells treated with IFN-γ and PGD_2+IFN-γ but, importantly, no difference between the two treatments. In cells pre-treated with PGD_2 and then treated with IFN-γ, the cell viability was lower than the control cells but higher than cells treated with IFN-γ alone. We therefore concluded that the inhibitory effect of PGD_2 was not due to toxicity (Figure 6-4).

6.6 Effect of Kyn on THP-1 cell viability

Having demonstrated that IFN-γ alone decreased THP-1 cell viability compared to control cells, we next aimed to determine whether the reduced viability was due to the increased levels of Kyn in the culture media or was due to a direct effect of IFN-γ. Cells were thus treated with 100 µmol/L of Kyn, a level that can be achieved *in vitro,* or IFN-γ (1000 IU/mL) for 24–72 h. Cells were collected at different times and treated with trypan blue. Consistent with our previous results, IFN-γ decreased cell viability compared to control cells. We found no differences in cell viability between cells treated with Kyn compared to untreated cells. We therefore concluded that the decrease in viability was independent of Kyn levels and was a direct effect of the IFN-γ (Figure 6-5).

6.7 Effect of PGD_2 on IFN-γ mediated induction of *INDO1*

IFN-γ is well-known to induce IDO at the transcriptional level (Zhang, YJ et al. 2010). Previously, we demonstrated that *INDO1* levels were reduced by AA pre-treatment (Figure 4-6). Consequently, it was important to determine if PGD_2 could transcriptionally regulate *INDO1*. Cells were pre-treated with PGD_2 (10 µmol/L) for 30 min followed by stimulation with IFN-γ (1000 IU/mL) for 24 h. *INDO1* was then measured as previously described (Bassal, Hughes & Costabile 2012). With control *INDO1* levels set to 1, IFN-γ significantly induced *INDO1* in both THP-1 cells and human monocytes, while PGD_2 alone had no significant effect on basal expression. When cells were pre-treated with PGD_2 and then stimulated with IFN-γ, there was a significant decrease in *INDO1* levels compared to

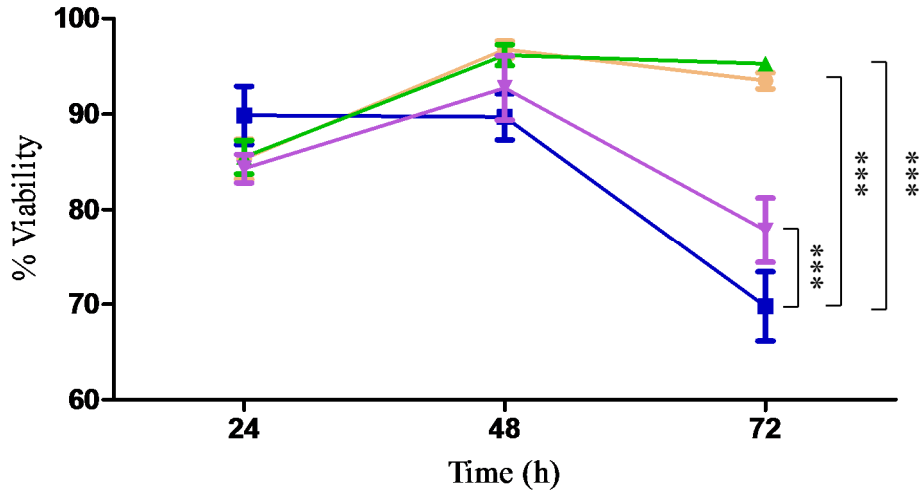


Figure 6-4 Effect of PGD_2 on THP-1 cell viability

THP-1 cells (4 x 10^5/Total) were pre-treated with PGD_2 (10 µmol/L) for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 24–72 h at 37 °C/5% CO_2 in a humidified atmosphere. At each time point, cells were collected and treated with trypan blue. Data are presented as the mean ± SEM of % viability compared to control cells as assessed by trypan blue exclusion, n=6 separate experiments. (●) Control, (■) IFN-γ, (▲) PGD_2, (▼) PGD_2+IFN-γ. Comparesons were performed using the Kruskal-Wallis test, followed by an upaired exact Mann Whitney test; *** indicates $p <$ 0.0001.

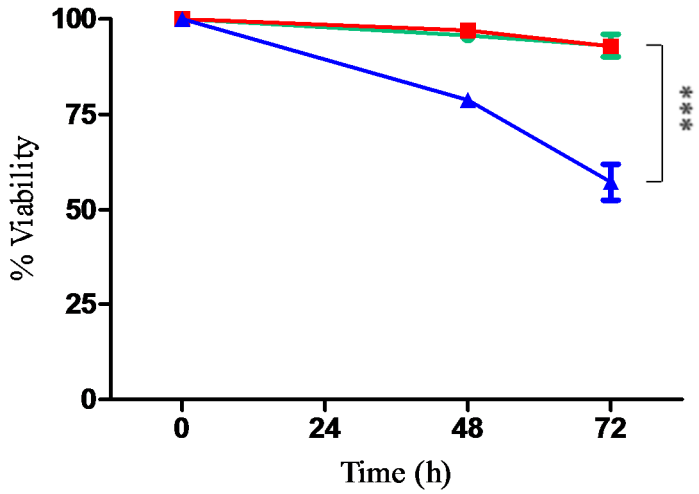


Figure 6-5 Effect of Kyn on THP-1 cell viability

THP-1 cells (4 x 10^5/Total) were treated with Kyn (100 µmol/L) or IFN-γ (1000 IU/mL) for 24–72 h at 37 °C/5% CO_2 in a humidified atmosphere. The cells were collected and treated with trypan blue Data are presented as the mean ± SEM of % viability compared to control cells as assessed by trypan blue exclusion, n=6 separate experiments. (●) Control (■) Kyn (▲) IFN-γ. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; *** indicate $p < 0.0001$.

IFN-γ stimulated cells. THP-1 cells showed a 45% decrease (Figure 6-6) and human monocytes a 72% decrease over IFN-γ stimulated cells. These results confirmed that the inhibitory effect of PGD_2 on IFN-γ mediated induction of IDO-1 was mediated at the level of transcription.

6.8 Effect of PGD_2 on IFN-γ mediated induction of IDO-1 protein

IDO-1 is also well-known to undergo post-translational modification (Takikawa et al. 1988; Du, Sotero-Esteva & Taylor 2000). Hence, changes in mRNA levels may not directly correlate to a change in IDO-1 protein expression. To determine if this was the case, we examined the effect of PGD_2 on the IFN-γ mediated induction of IDO-1 protein. THP-1 cells or monocytes (1 x 10^7/total) were treated with PGD_2 (10 µmol/L) for 30 min and then stimulated with IFN-γ (1000 IU/L) or diluent for 72 h. Cells were collected, protein was extracted and Western blot performed as previously described (Bassal, Hughes & Costabile 2012). In unstimulated THP-1 cells, a low level of IDO-1 expression was observed while IFN-γ caused a 325% increase in IDO protein level in THP-1 cells and a 251% increase in human monocytes. PGD_2 had no effect on basal IDO-1 protein expression, however, PGD_2 followed by IFN-γ stimulation caused a 32% inhibition of IFN-γ mediated induction of IDO-1 protein in both THP-1 cells and monocytes (Figure 6-7).

6.9 PGD_2 and its metabolites

PGD_2 is readily degraded by a series of spontaneous dehydration and isomerisation reactions *in vitro* and either enzymatically or non-enzymatically *in vivo* to a wide range of metabolites (Fitzpatrick & Wynalda 1983; Shibata et al. 2002). PGD_2 yields PGJ_2 in aqueous solutions (Fukushima et al. 1982a; Fukushima 1990) and, in the presence of serum albumin, PGJ_2 isomerises to yield Δ^{12}-PGJ_2 (Fitzpatrick & Wynalda 1983). *In vitro*, PGD_2 also can be transformed into 15-deoxy-$\Delta^{12,14}$-PGJ_2 via an albumin-catalyzed reaction (Fitzpatrick & Wynalda 1983)

Enzymatically, PGD_2 is metabolised to 13, 14-dihydro-15-keto PGD_2 by 15 prostaglandin dehydrogenase, 11β $PGF_{2\alpha}$ by prostaglandin 11-ketoreductase and tetranor-PGDM (Song et al. 2008). Alternatively, PGD_2 is non-enzymatically converted to 15-deoxy-$\Delta^{12,\ 14}$-PGD_2 and Δ^{12}-PGD_2 (Sandig, Pease & Sabroe 2007). Interestingly, some of these metabolites can activate more than one receptor, depending on the level of expression of these receptors on different cell types and the affinity of these metabolites to the different receptors. Consequently, these metabolites (Figure 6-1) can produce different

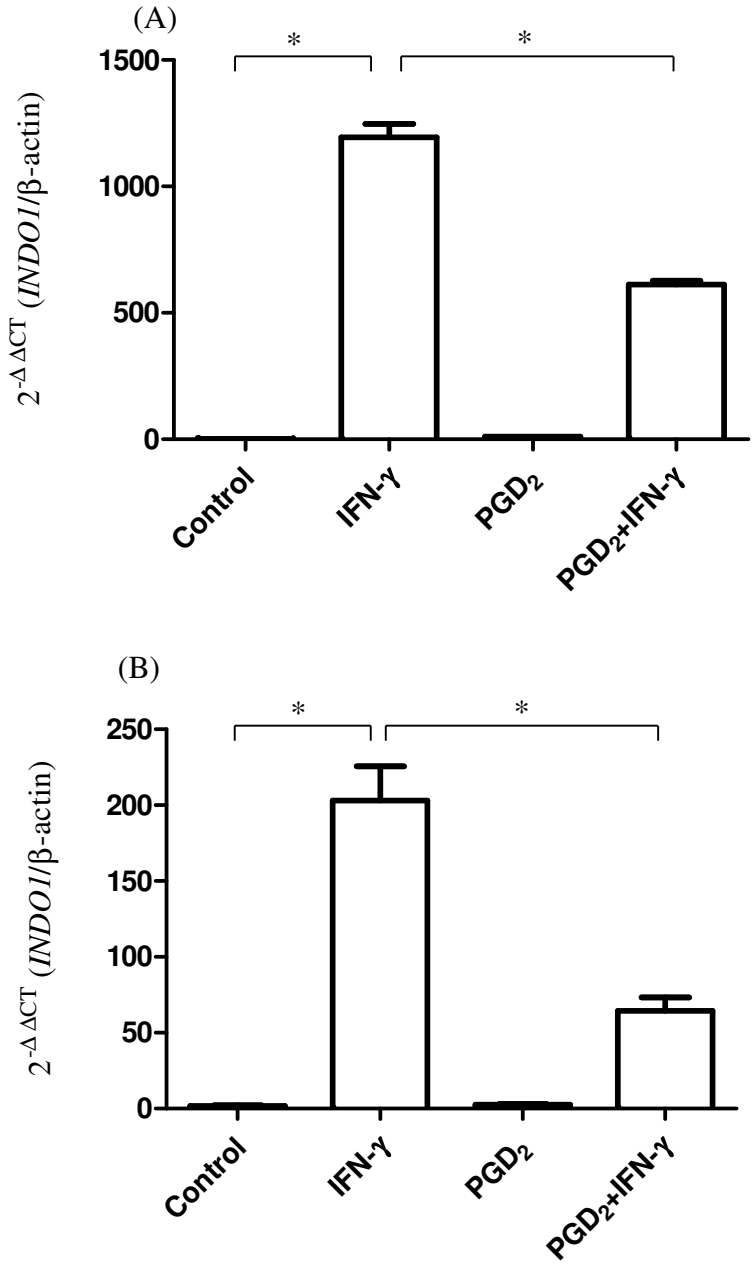


Figure 6-6 Effect of PGD2 on IFN-γ mediated induction of *INDO1*

(A) THP-1 cells (5 x 10^6/total) or (B) human monocytes (5 x 10^6/total) were pre-treated with PGD_2 (10 µmol/L) or diluent for 30 min followed by stimulation with IFN-γ (1000 IU/mL) for 24 h. RNA was extracted and 2 µg used to synthesise cDNA. Using 100 ng of cDNA, qRT-PCR was performed. Results are expressed as mean ± SEM of the *INDO*1 ratio corrected for β-actin mRNA. n=4 separated experiment. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$.

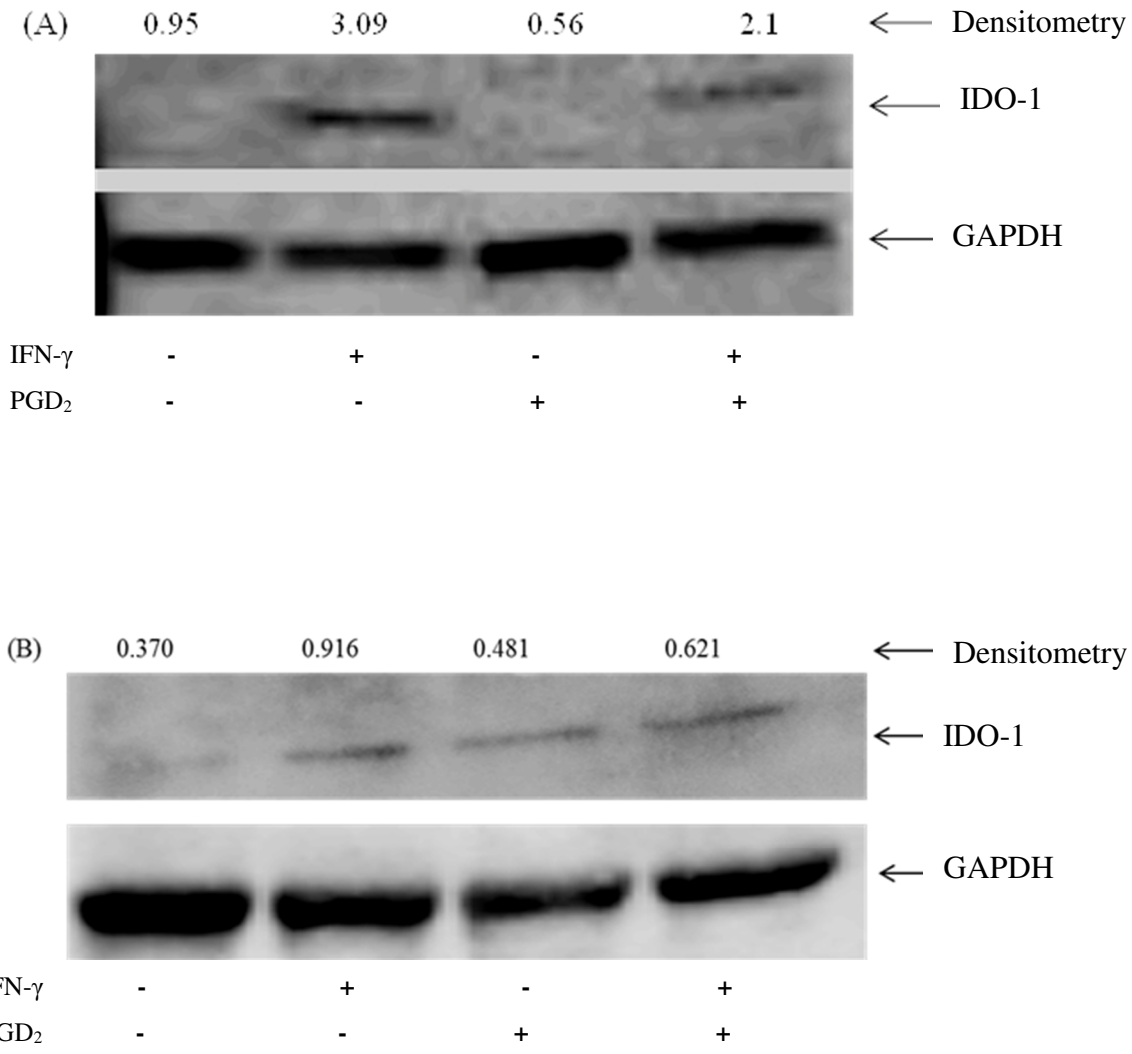


Figure 6-7 Effect of PGD_2 on IFN-γ mediated induction of IDO-1 protein

(A) THP-1 cells (1 x 10^7/total) or (B) human monocytes (1 x 10^7/total) were treated with PGD_2 (10 µmol/L) for 30 min and then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. Protein was extracted, 50 µg separated by 10% SDS PAGE and probed for IDO-1 protein and GAPDH by Western blot. The membrane was visualised using an Image Quant LAS 4000 and densitometry for both IDO-1 and GAPDH calculated using Image Quant LAS 4000 and Image Quant TL software. Densitometry values represent level of IDO-1 protein corrected for the total GAPDH protein loaded. The result of one representative experiment of three independent experiments is shown.

effects in different cells. Since PGD_2 significantly inhibited IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes, we next determined whether the effect was due to the parent PG or to one of its metabolites.

6.10 Enzymatically produced metabolites of PGD_2 fail to inhibit Kyn production

We examined the biological activity of the enzymatically produced PGD_2 metabolites 13,14-dihydro-15 keto PGD_2 (DK-PGD_2) and 11β $PGF_{2\alpha}$ on THP-1 cells and human monocytes. Cells were treated with PGD_2 (positive control), metabolite (10 μmol/L) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h. As demonstrated previously, IFN-γ caused a significant increase in Kyn production over basal levels while PGD_2 significantly inhibited its production. Neither DK-PGD_2 nor 11β $PGF_{2\alpha}$ alone had any effect on basal Kyn production. Similarly, in cells that had been treated with 13,14-dihydro-15 keto PGD_2 or 11β $PGF_{2\alpha}$, and then stimulated with IFN-γ, neither metabolite caused significant inhibition of Kyn production (Figure 6-8).

6.11 Non-enzymatically produced metabolites of PGD_2 display inhibitory activity

PGD_2 can be also transformed spontaneously to different metabolites (Figure 6-1). To determine if PGD_2 or any of the non-enzymatic products of PGD_2 were responsible for the observed inhibitory activity, we examined the ability of each metabolite to inhibit Kyn production in both cell types. Cells were pre-treated with PGD_2 (positive control), PGJ_2, Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2, Δ^{12}-PGD_2, 15-deoxy-$\Delta^{12,14}$-PGD_2 (10 μmol/L) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/ml) or diluent for 72 h. Treatment with IFN-γ caused a significant increase in Kyn production over baseline while PGD_2 caused a decrease in IFN-γ stimulated Kyn production. None of the metabolites alone had any effect on basal Kyn production. In both THP-1 cells and human monocytes, all metabolites significantly inhibited the IFN-γ induced production of Kyn compared to IFN-γ stimulated cells (Figure 6-9). Of these metabolites, only 15-deoxy-$\Delta^{12,14}$-PGJ_2 was found to be toxic to cells at the concentration used (Table 6-2).

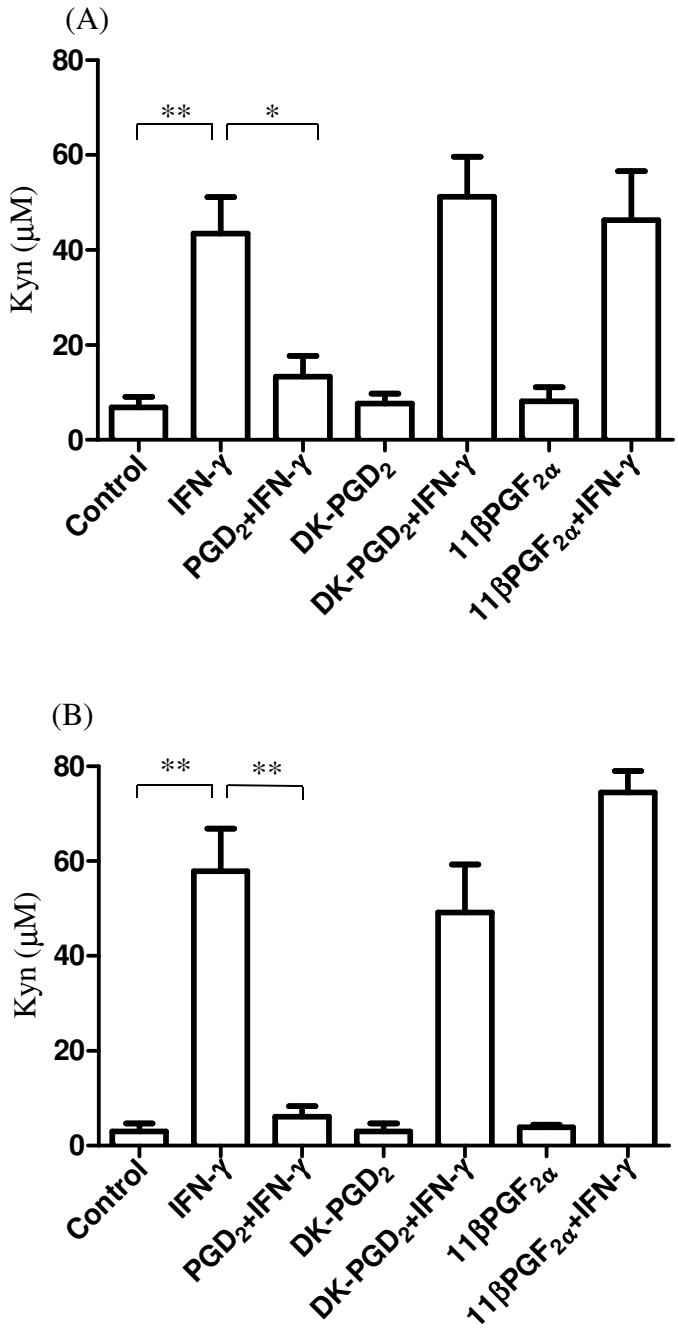


Figure 6-8 Effect of enzymatically produced metabolites of PGD_2 on IFN-γ induced Kyn production by THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/total) or (B) human monocytes (4 x 10^6/total) were pre-treated with PGD_2, 13,14 dihydro-15-keto PGD_2 (DK-PGD_2) or 11β $PGF_{2\alpha}$ (10 μmol/L) or diluent for 30 min in media lacking serum. Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent (control) prepared in media containing serum for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM Kyn produced. THP-1 cells, n=10 separate experiments; human monocytes, n=4 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$, ** indicate $p < 0.001$.

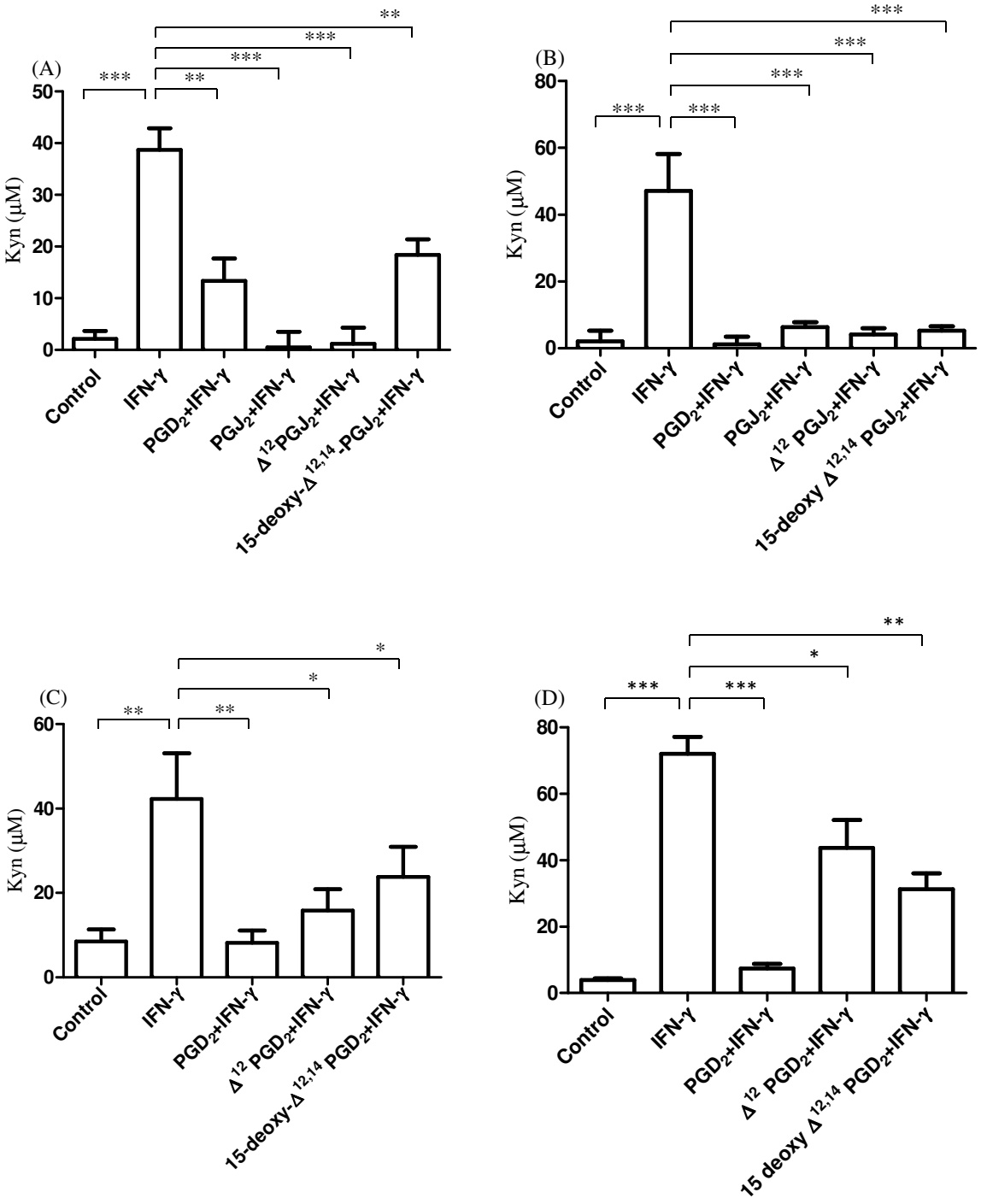


Figure 6-9 Effect of non-enzymatically produced PGD_2 metabolites on IFN-γ induced Kyn production by THP-1 cells and human monocytes.

(A & C) THP-1 cells (4 x 10^5/total) or (B & D) monocytes (4 x 10^6/total) were pre-treated with PGD_2, PGJ_2, Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 PGD_2, Δ^{12}-PGD_2 or 15-deoxy-$\Delta^{12,14}$-PGD_2 (10 µmol/L) for 30 min in medium lacking serum. Cells were then stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM Kyn produced.THP-1 cells, n=9 separate experiments; human monocytes, n=3 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$, ** indicate $p < 0.001$, *** indicate $p < 0.0001$.

Table 6-2 Summary of PGD_2 metabolites, their effect on IFN-γ stimulated Kyn production in THP-1 cells and human monocytes and the receptors to which they bind

Metabolite	Enzymatic production	Non-enzymatic production	Inhibition		Toxicity	Receptor
			THP-1	Monocytes		
13,14 dihydro-15-keto PGD_2	✓		×	×	No	CRTH2
11β $PGF_{2}\alpha$	✓		×	×	No	DP1 CRTH2
PGJ_2		✓	✓	✓	No	DP1
Δ^{12}-PGJ_2		✓	✓	✓	No	DP1 CRTH2 PPARγ
15-deoxy-$\Delta^{12,14}$-PGJ_2,		✓	✓	✓	Yes	DP1 CRTH2 PPARγ
Δ^{12}-PGD_2		✓	✓	✓	No	CRTH2
15-deoxy-$\Delta^{12,14}$-PGD_2		✓	✓	✓	No	CRTH2 PPARγ

PGD_2 enzymatic metabolites, 13,14-dihydro-15-keto PGD_2 and 11β $PGF_{2\alpha}$, failed to inhibit IFN-γ stimulated Kyn production in both THP-1 cells and human monocytes. PGD_2 non-enzymatic metabolites significantly inhibited Kyn production in THP-1 cells. PGD_2 non-enzymatic metabolites PGJ_2, Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 significantly inhibited IFN-γ induction of Kyn in monocytes. Only 15-deoxy-$\Delta^{12,14}$-PGJ_2 was toxic to both cell types. DP1, PGD_2 G-protein coupled receptor 1; CRTH2, chemoattractant receptor-homologous molecule; PPARγ, peroxisome proliferator-activated receptor gamma. ✓, inhibition; ×, No effect.

6.12 CRTH2 mRNA expression by THP-1 cells and human monocytes

From the previous results, it was clear that PGD_2 and its non-enzymatically produced metabolites inhibited Kyn production in THP-1 cells and human monocytes. These activities could be mediated through DP1, CRTH2 and/or PPARγ. Since PGD_2 mediates its effect only via binding to DP1 and CRTH2 and not PPARγ, we investigated the role of these two receptors in the inhibitory activity. DP1 is known to be expressed on both THP-1 cells (Hirano et al. 2007) and human monocytes (Gosset et al. 2003). While CRTH2 is known to be expressed on human monocytes (Gosset et al. 2003), its expression on THP-1 cells was unknown. To determine if CRTH2 was involved in the PGD_2 mediated effects, it was important to first determine its expression by examining CRTH2 mRNA levels in both cell types. THP-1 cells and human monocytes were stimulated with IFN-γ (1000 IU/mL) or diluent for 24 h. Cells were then collected, total RNA isolated and cDNA prepared. Using β-actin to correct for loading, RT-PCR was performed. The results showed that both cell types constitutively expressed CRTH2 mRNA, while CRTH2 mRNA expression could be further increased by IFN-γ stimulation in monocytes but not in THP-1 cells (Figure 6-10).

6.13 Identification of the receptor responsible for PGD_2 biological activity

PGD_2 can mediate its effects via both DP1 and CRTH2 (Straus & Glass 2001; Hata & Breyer 2004) and having demonstrated mRNA expression of CRTH2 in THP-1 cells and human monocytes, it was thus important to determine which receptor was involved in the PGD_2 mediated inhibitory activity. To do so, we used pharmacological agonists and antagonists of DP1 and CRTH2 to determine their contribution to the inhibitory activity in both cell types.

6.13.1 Effect of a DP1 agonist on IFN-γ stimulated Kyn production

To confirm the involvement of DP1, a DP1 agonist should mimic the effect of PGD_2. For this, BW 245C was chosen as a DP1 agonist (Ayabe et al. 2013). Cells were treated with PGD_2 (10 μmol/L) or BW 245C (0.01–1 μmol/L) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h. IFN-γ caused a significant increase in Kyn production over baseline, while cells pre-treated with PGD_2 or BW 245C alone showed no effect on basal Kyn production. In both cell types, cells that had been pre-treated with PGD_2 or BW 245C (1 μmol/L) and then stimulated with IFN-γ, showed

(A)

(B)

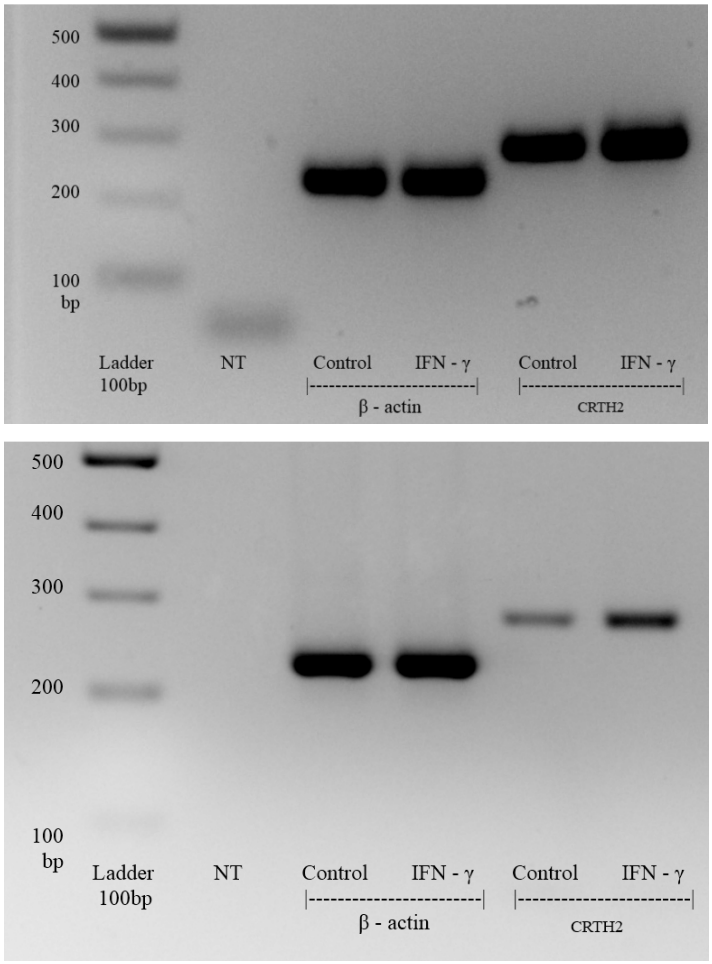


Figure 6-10 Expression of CRTH2 mRNA in THP-1 cells and human monocytes

(A) THP-1 cells (5 x 10^5/total) or (B) human monocytes (5 x 10^6/total) were treated with IFN-γ (1000 IU/mL) or diluent for 24 h. Cells were collected, total RNA extracted and cDNA prepared. RT-PCR was performed as described previously (Section 2.21). Amplified products were separated using a 2% agarose gel and subsequently viewed under a Bio-Rad UV trans-illuminator linked to a desktop computer and software program Quantity One (Version 4.2.1, Bio-Rad Laboratories, California USA) was used to view and capture an image of the gel. NT, No template; bp, base pair; β-actin size=240 bp, CRTH2 =281bp.

significant inhibition of Kyn production compared to cells stimulated with IFN-γ alone (Figure 6-11). Thus, binding of a ligand to DP1 was able to mimic the inhibitory effect of PGD_2 in THP-1cells and human monocytes, indicating that DP1 could modulate IDO activity in both THP-1 cells and human monocytes.

6.13.2 Effect of PGD_2 and BW 245C on IFN-γ mediated induction of *INDO1*

To further support the involvement of DP1, we determined the effect of BW 245C on IFN-γ mediated induction of *INDO1*. Cells were pre-treated with PGD_2 (10 µmol/L) or BW 245C (1 µmol/L) for 30 min followed by stimulation with IFN-γ (1000U/mL) for 24 h. The cells were collected, total RNA was extracted and the corresponding cDNA prepared and changes in *INDO1* measured by qRT-PCR. Consistent with previous experiments, IFN-γ significantly induced *INDO1* levels in THP-1 cells, while PGD_2 and BW 245C alone had no effect on basal levels of *INDO1*. Cells that were pre-treated with PGD_2 or BW 245C and then stimulated with IFN-γ showed significant inhibition of *INDO1* levels. These results further supported our finding that BW 245C mimicked the inhibitory effect of PGD_2 on IFN-γ mediated induction of IDO-1 at the level of transcription and that the effect of PGD_2 was via DP1 (Figure 6-12).

6.13.3 Effect of a CRTH2 agonist on THP-1 cells and human monocytes

Our previous result suggested that DP1 was involved in the PGD_2 inhibitory activity. Given the opposing signalling pathway of DP1 vs. CRTH2, we predicted that a CRTH2 agonist should display no inhibitory activity. THP-1 cells and human monocytes were pre-treated with PGD_2 (10 µmol/L) or the CRTH2 agonists 13,14-dihydro-15-keto PGD_2 or 15(R)-15-methyl PGD_2 (Monneret et al. 2003) (10 µmol/L) or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h. IFN-γ caused a significant increase in Kyn production over baseline, while PGD_2 or either agonist alone did not alter basal Kyn production. In cells that had been pre-treated with PGD_2 and then stimulated with IFN-γ, there was a significant inhibition of Kyn production, while cells pre-treated with either 13, 14-dihydro-15-keto PGD_2, (Figure 6-7) or 15(R)-15-methyl PGD_2 (Figure 6-13) and then stimulated with IFN-γ showed no significant inhibition of Kyn production. This lack of inhibitory activity supported our hypothesis that PGD_2 did not mediate its effects via CRTH2.

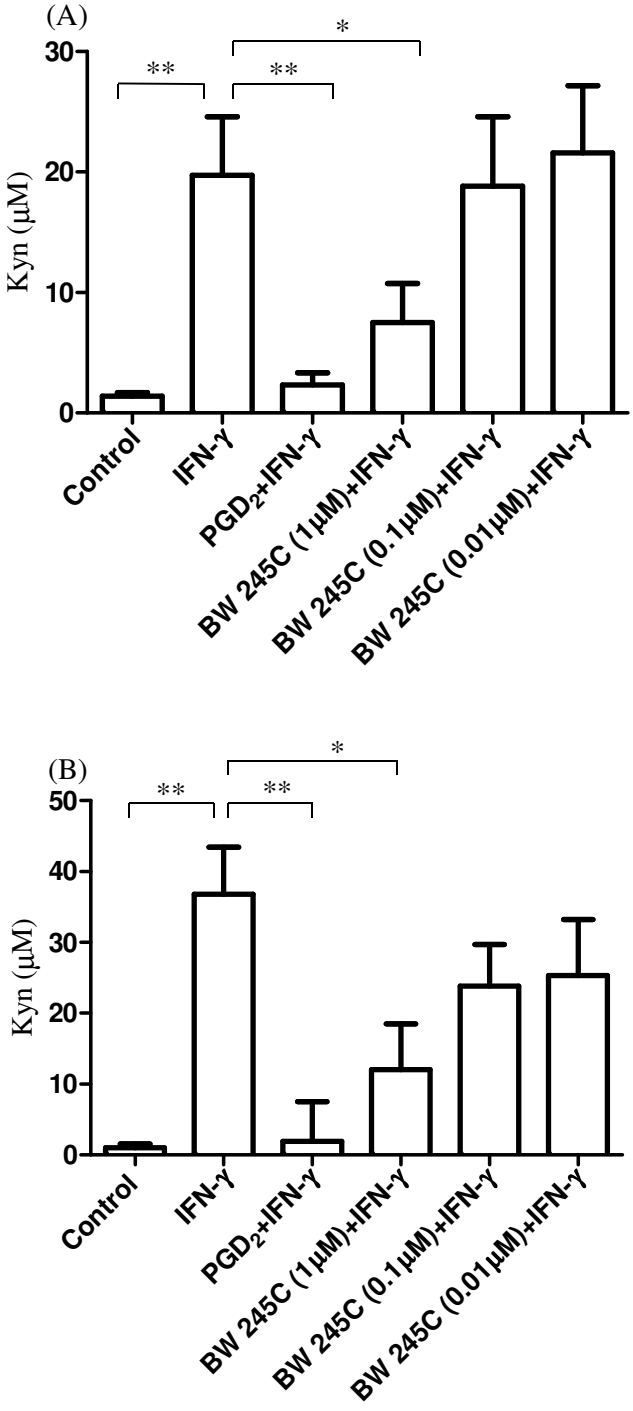


Figure 6-11 Effect of DP1 agonist BW 245C on IFN-γ mediated Kyn production by THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/total) or (B) human monocytes (4 x 10^6/total) were pre-treated with PGD_2 (10 μmol/L) or BW 245C (0.01, 0.1 and 1 μmol/L). Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent prepared in media containing serum for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM Kyn produced. THP-1 cells, n=8 separate experiments; human monocytes, n=3 separate experiments. Compareson were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$, ** indicate $p < 0.001$.

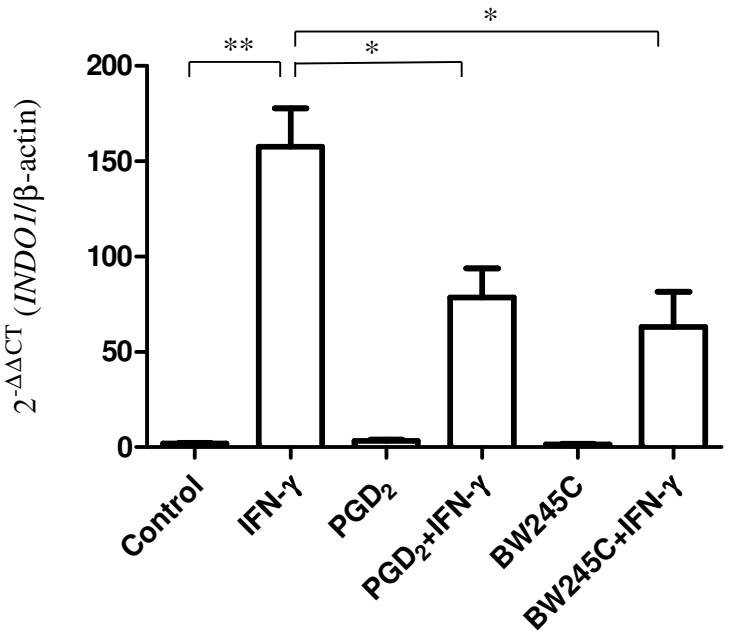


Figure 6-12 Effect of PGD_2 and BW 245C on IFN-γ mediated induction of *INDO1* in THP-1 cells

THP-1 cells (4 x 10^6/total) were pre-treated with PGD_2 (10 µmol/L) or BW 245C (1 µmol/L) or diluent for 30 min followed by stimulation with IFN-γ (1000 IU/mL) for 24 h. RNA was extracted and 2 µg used to synthesise cDNA. Using 100 ng cDNA, qRT-PCR was performed using Taq-man gene expression assay. Results are expressed as mean ± SEM of *INDO1* ratio corrected for β-actin. n=6 separet experiments each in triplicate. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$, ** indicate $p < 0.001$.

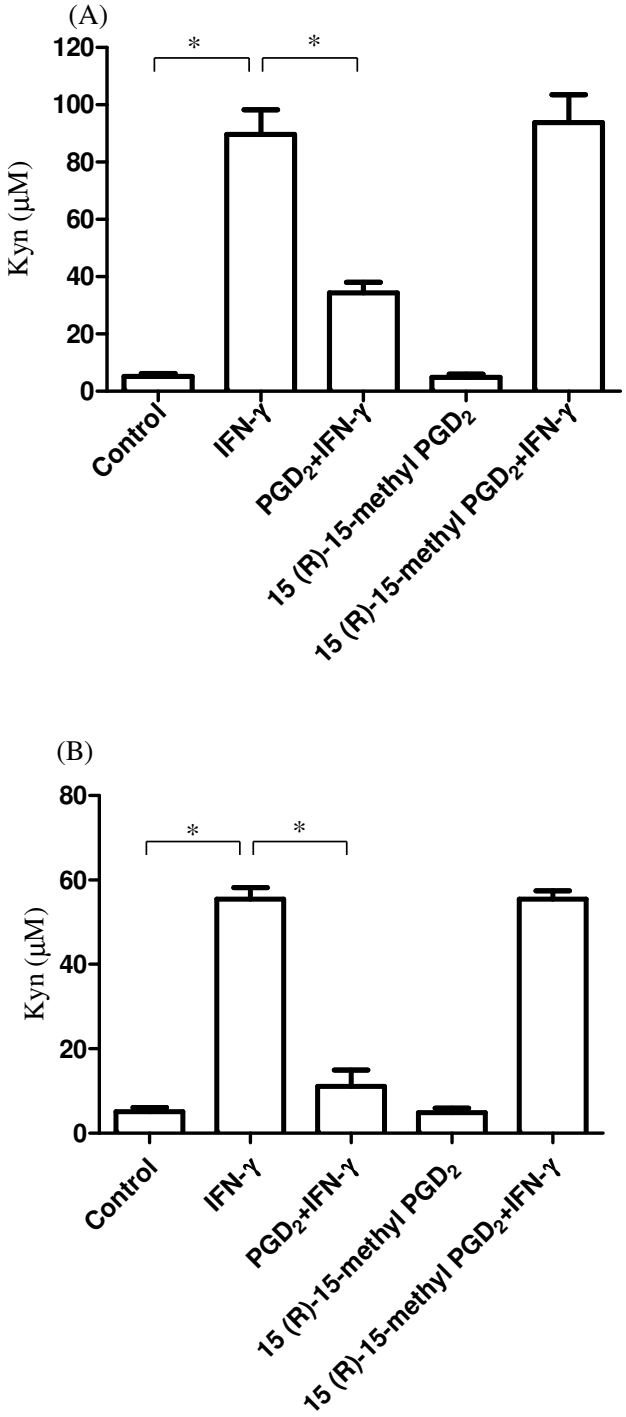


Figure 6-13 Effect of CRTH2 agonist 15(R)-15-methyl PGD_2 on IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/total) or (B) human monocytes (4 x 10^6/total) were pre-treated with PGD_2 15(R)-15-methyl PGD_2 (10 μmol/L), or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM Kyn produced, n=4 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$. .

6.13.4 Effect of DP1 and CRTH2 antagonists on the PGD_2 inhibitory activity

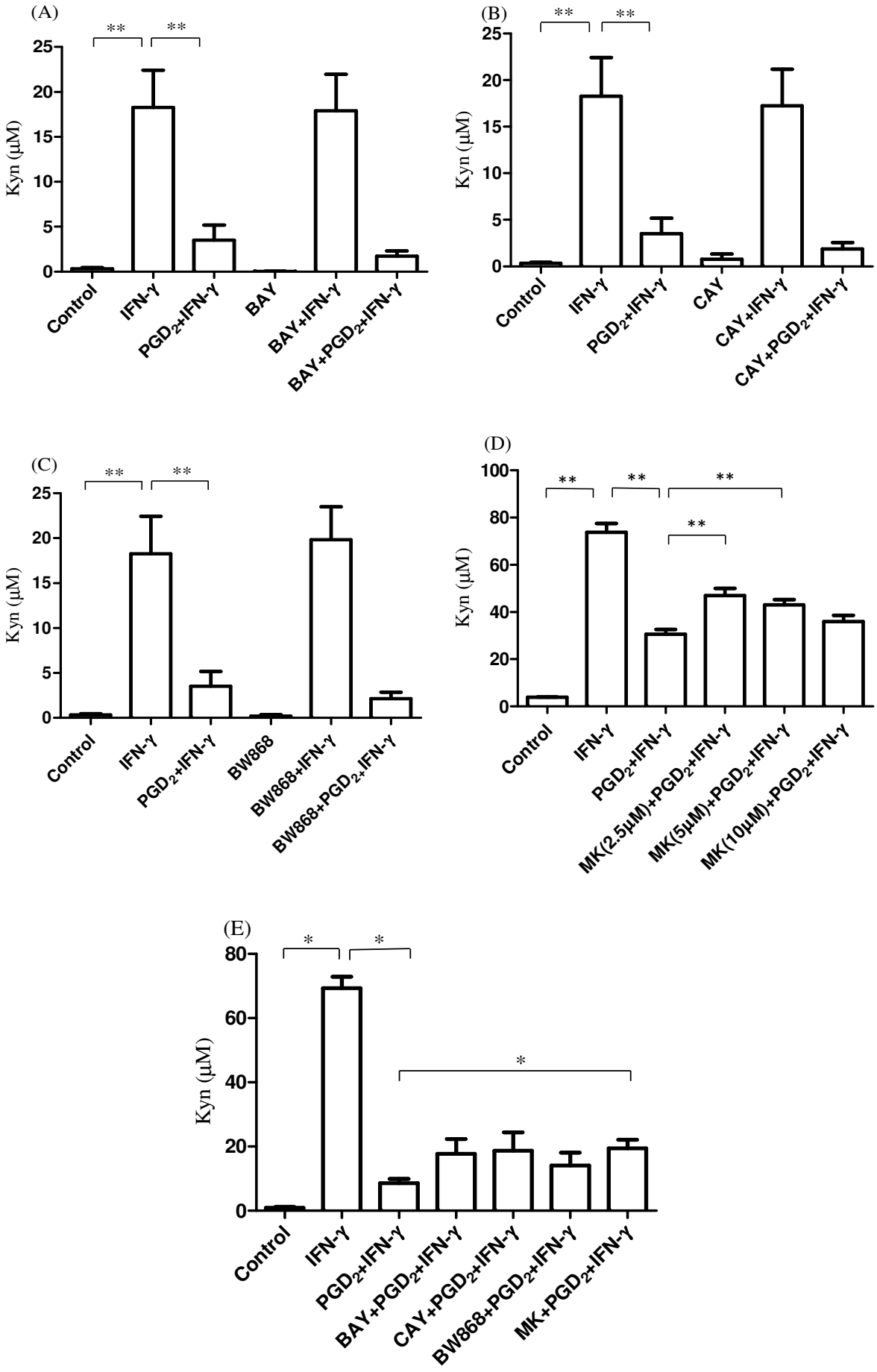
To further confirm that DP1 was responsible for the inhibitory activity, we tested the ability of DP1 and CRTH2 antagonists to block this activity in both cell types. THP-1 cells or human monocytes were treated with PGD_2 (5 µmol/L) or DP1 antagonists (BW 868C or MK 0524) (2.5–10 µmol/L) or CRTH2 antagonists (BAY u3405 or CAY 10595) (0.1–1 µmol/L) for 30–120 min with or without stimulation with IFN-γ (1000 IU/mL). In both cell types, treatment with IFN-γ caused a significant increase in Kyn production over basal levels, while cells pre-treated with PGD_2, DP1 or CRTH2 antagonists alone showed no significant effect on basal Kyn production. Cells that had been pre-treated with PGD_2 plus BAY u3405, CAY 10595 or BW 868C and then stimulated with IFN-γ, showed no significant correction of the inhibitory effect of PGD_2, while MK 0524 showed a small, yet significant correction of the inhibitory effect of PGD_2 in both cell types (Figure 6-14). This further suggested that the PGD_2 inhibitory effect was mediated via DP1 and not CRTH2.

6.13.5 Effect of MK 0524 on PGD_2 and its inhibitory metabolites

As summarised previously (Table 6-2), some PGD_2 metabolites that inhibited Kyn production in both cell types can mediate their effects via DP1, CRTH2 or even PPARγ. To test the involvement of DP1 in the inhibitory effect of these metabolites, we tested all of the inhibitory PGD_2 metabolites with the DP1 antagonist MK 0524. THP-1 cells were treated with MK 0524 or diluent for 30 min, then treated with PGD_2 or inhibitory metabolites or diluent for 30 min and then stimulated with IFN-γ or diluent for 72 h. The supernatant was collected and Kyn levels quantified using the colorimetric assay. In agreement with our previous results, IFN-γ significantly increased Kyn production compared to the baseline. All the metabolites tested plus IFN-γ showed significant inhibition of Kyn production compared to IFN-γ alone. Treatment of cells with MK 0524 before PGD_2 or its metabolites and then IFN-γ caused significant correction of the inhibitory effect of the following metabolites: PGD_2, PGJ_2, Δ^{12}-PGJ_2 and Δ^{12}-PGD_2, which all mediate their activity via DP1. In contrast, treatment of cells with MK 0524 before PGD_2 or its metabolites and then with IFN-γ caused no significant correction of the inhibitory effect of 15(R) PGD_2 and 15-deoxy-$\Delta^{12,14}$-PGD_2, which do not mediate their effect via DP1 but CRTH2. These results further support the involvement of DP1 in the inhibitory effect of PGD_2 and its metabolites (Figure 6-15).

Figure 6-14 Ability of DP1 and CRTH2 antagonists to correct the PGD_2 inhibitory activity in THP-1 cells and human monocytes

(A-D) THP-1 cells (4 x 10^5/total) or (E) human monocytes (4 x 10^6/total) were pre-treated with PGD_2 (5 µmol/L) or PGD_2 plus BAY u3405 (BAY; 1 µmol/L) or CAY 10595 (CAY; 1 µmol/L) or BWA 868C (BW686; 1 µmol/L) or MK 0524 (MK; 2.5-10 µmol/L) for 0.5–1 h and stimulated with IFN-γ (1000 IU/mL) for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM Kyn produced. THP-1 cells, n=7 separate experiments; human monocytes, n=4 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact test Mann Whitney test; * indicate $p < 0.04$, ** indicate $p < 0.001$.


(A)
Kyn (μM)
25
20
15
10
5
0
**
**
Control
IFN-γ
PGD2+IFN-γ
BAY
BAY+IFN-γ
BAY+PGD2+IFN-γ
(B)
Kyn (μM)
25
20
15
10
5
0
**
**
Control
IFN-γ
PGD2+IFN-γ
CAY
CAY+IFN-γ
CAY+PGD2+IFN-γ
(C)
Kyn (μM)
25
20
15
10
5
0
**
**
Control
IFN-γ
PGD2+IFN-γ
BW868
BW868+IFN-γ
BW868+PGD2+IFN-γ
(D)
Kyn (μM)
100
80
60
40
20
0
**
**
**
**
Control
IFN-γ
PGD2+IFN-γ
MK(2.5μM)+PGD2+IFN-γ
MK(5μM)+PGD2+IFN-γ
MK(10μM)+PGD2+IFN-γ
(E)
Kyn (μM)
80
60
40
20
0
*
*
*
Control
IFN-γ
PGD2+IFN-γ
BAY+PGD2+IFN-γ
CAY+PGD2+IFN-γ
BW868+PGD2+IFN-γ
MK+PGD2+IFN-γ

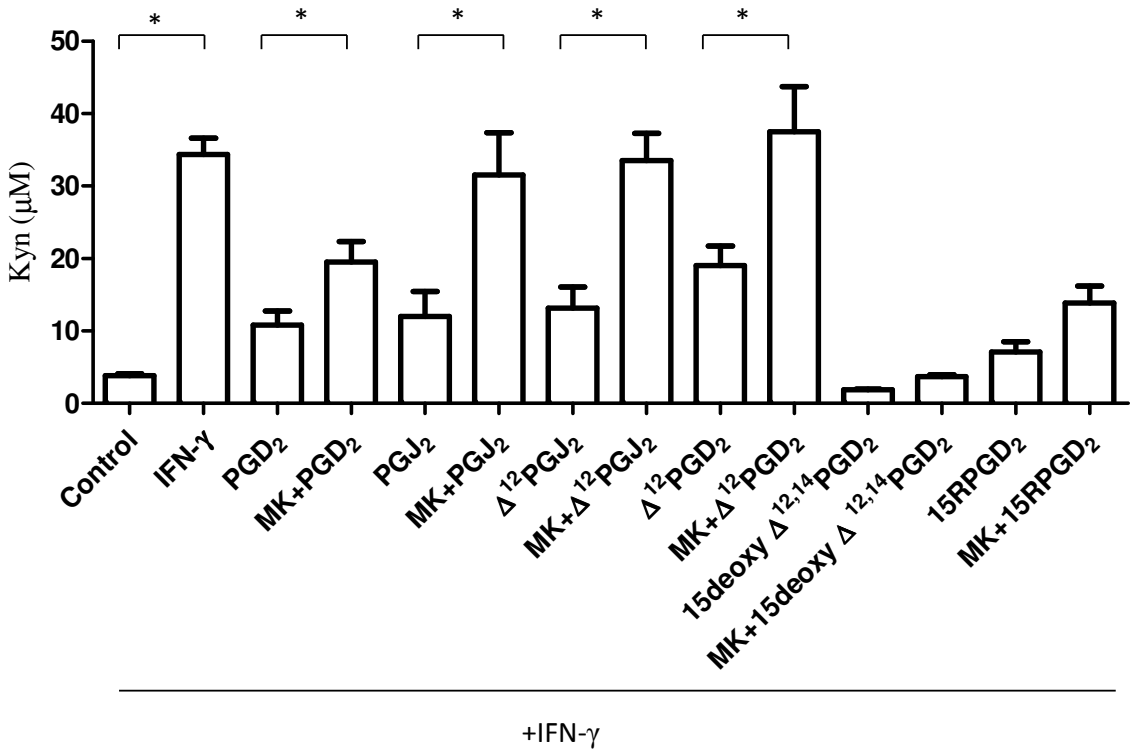


Figure 6-15 Ability of DP1 antagonist MK 0524 to correct PGD_2 and its metabolites inhibitory activity in THP-1 cells

THP-1 cells (4 x 10^5/total) were pre-treated with MK 0524 (2.5 μmol/L) or diluent for 30 min, then treated with PGD_2 (5 μmol/L), PGD_2 inhibitory metabolites PGJ_2, Δ12-PGJ_2, Δ^{12}-PGD_2, 15-deoxy $\Delta^{12,14}$-PGD_2 and 15(R) PGD_2 (5 μmol/L) or diluent for another 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay, THP-1 cells, n=6 separate experiments. Results are shown as mean ± SEM Kyn produced. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$.

6.14 Effect of Forskolin on IFN-γ mediated induction of IDO in THP-1 cells

To this point, the data strongly suggested that the effects of PGD_2 and its metabolites were mediated via DP1. DP1 is a Gαs linked receptor, which stimulates the activity of adenylate cyclase (AC), increasing intracellular cAMP. To confirm involvement of this signalling pathway, THP-1 cells were pre-treated with Forskolin (100 μmol/L), a direct activator of AC or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h. IFN-γ caused a significant increase in Kyn production over baseline, while cells pre-treated with Forskolin alone showed a significant reduction in basal Kyn production. In cells pre-treated with Forskolin and then stimulated with IFN-γ, there was a significant inhibition in Kyn production without any apparent toxicity (% viability of cells treated with Forskolin+IFN-γ compared to cells treated with IFN-γ alone was 85.6% ± 5) (Figure 6-16). These results demonstrated that direct activation of AC and increased levels of cAMP mimic the inhibitory effect of PGD_2.

6.15 PGD_2 stimulates production of intracellular cAMP in THP-1 cells

The Forskolin data confirmed that AC could modulate IDO-1 activity in THP-1 cells. To confirm this was true for PGD_2, changes in intracellular levels of cAMP in THP-1 cells following PGD_2 treatment were assessed by EIA (Section 2.25). To ensure any effects were specific for PGD_2, we also tested 11β $PGF_{2\alpha}$, a CRTH2 ligand that did not inhibit Kyn production (Figure 6-8). THP-1 cells were first pre-treated with the phosphodiesterase inhibitor IBMX (0.25 μmol/L) for 10 min, to prevent degradation of cAMP to 5' AMP, and then stimulated with PGD_2, 11β $PGF_{2\alpha}$ (10 μmol/L) or diluent for 10 min in medium lacking serum. A basal level of cAMP was detectable in resting cells. While PGD_2 caused a significant increase in cAMP levels (Figure 6-17), 11β $PGF_{2\alpha}$ did not significantly increase cAMP levels compared to baseline. This result further supported our hypothesis that the inhibitory effect of PGD_2 was mediated via DP1, increasing intracellular levels of cAMP.

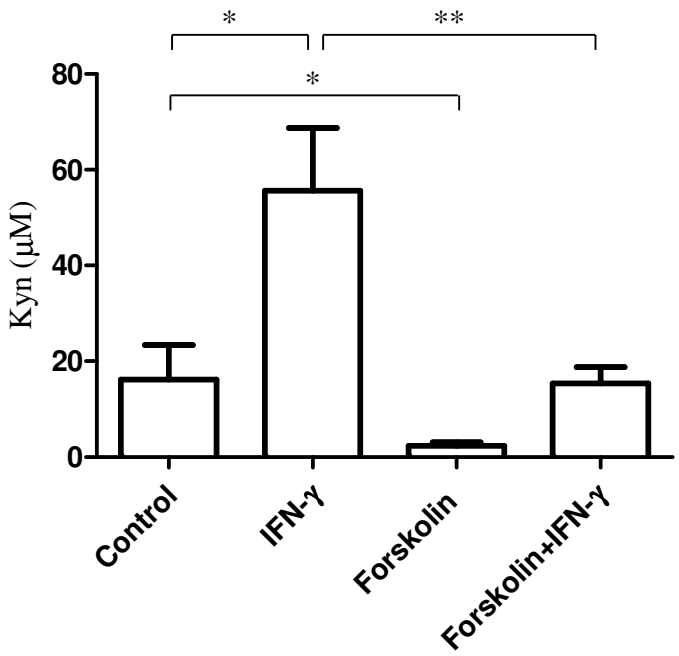


Figure 6-16 Effect of Forskolin on IFN-γ mediated induction of IDO in THP-1 cells

THP-1 cells (4 x 10^5/total) were treated with Forskolin (100 μmol/L) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are presented as mean ± SEM Kyn produced. n=6 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$, ** indicate $p < 0.001$.

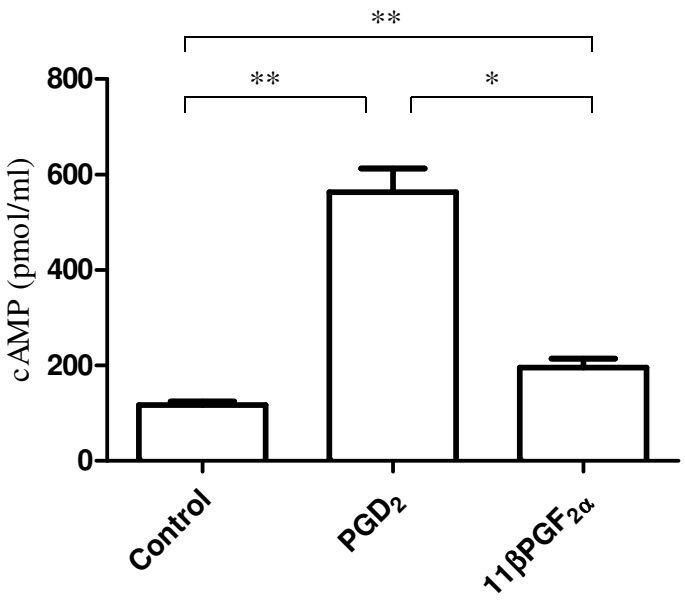


Figure 6-17 Effect of PGD_2 and 11β $PGF_{2\alpha}$ on intracellular cAMP levels in THP-1 cells

THP-1 (4 x 10^5/total) cells were pre-treated with IBMX (0.25 µmol/L) for 10 min and then stimulated with PGD_2, 11β $PGF_{2\alpha}$ (both at 10 µmol/L) or diluent for 10 min in medium lacking serum at 37 °C/5% CO_2 in a humidified atmosphere. The supernatant was removed and cAMP measured using a cAMP EIA kit according the manufacturer instructions. Data are shown as mean ± SEM cAMP. THP-1, n=6 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$, ** indicate $p < 0.001$.

6.16 Effect of N^6-cAMP on IFN-γ induced IDO-1

Having confirmed that PGD_2 increased intracellular cAMP levels in THP-1 cells, we further examined the role of the cAMP–PKA signalling pathway in modulating IDO-1 activity. We first tested the effect of a cell permeable cAMP analog, N^6-phenyl-cyclic AMP (N^6-cAMP), on the IFN-γ mediated induction of IDO-1. THP-1 cells and monocytes were pre-treated with N^6-cAMP (0.5 mmol/L) or diluent for 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h. In cells treated with IFN-γ, Kyn levels were significantly increased over basal levels. N^6-cAMP alone had no effect on basal Kyn levels, but when cells were subsequently stimulated with IFN-γ, there was a significant inhibition of Kyn production in both THP-1 and human monocytes compared to IFN-γ alone (Figure 6-18). Thus, a cAMP analog was able to inhibit IDO-1 activity, further supporting our hypothesis that cAMP plays a role in modulating IFN-γ mediated induction/activity of IDO-1 in both cell types. Consistant with the above results, N6-cAMP reduced *INDO1* expression in both THP-1 cells and human monocytes, with similar results for IDO-1 protein (data not shown)

6.17 Effect of phosphodiesterase inhibitor, IBMX on the inhibitory effect of PGD_2 and BW245C

The levels of intracellular cAMP are modulated by two direct mechanisms, increased production by AC and degradation to 5' AMP by a specific phosphodiesterase. Thus, the phosphodiesterase inhibitor, IBMX, will artificially maintain the level of intracellular cAMP. THP-1 cells and human monocytes were treated with IBMX (0.25 μmol/L) for 30 min, then PGD_2 (5 μmol/L) or BW 245C (1 μmol/L) for a further 30 min. Cells were then stimulated with IFN-γ for 72 h. IFN-γ caused a significant increase in Kyn production in both cell types. PGD_2, BW 245C and IBMX alone had no effect on basal Kyn levels. Consistent with previous results, cells pre-treated with PGD_2 or BW 245C and then stimulated with IFN-γ showed significant inhibition in Kyn production in both cell types. Cells that had been pre-treated with IBMX and then stimulated with IFN-γ also showed a significant decrease in Kyn levels compared to cells stimulated with IFN-γ alone. The combination of IBMX and PGD_2 or BW 245C prior to IFN-γ stimulation resulted in a significant increase in inhibition in Kyn levels than cells pre-treated with PGD_2 or BW 245C and IFN-γ alone. These results further confirmed that modulation of the level of cAMP could inhibit IDO in both cell types (Figure 6-19).

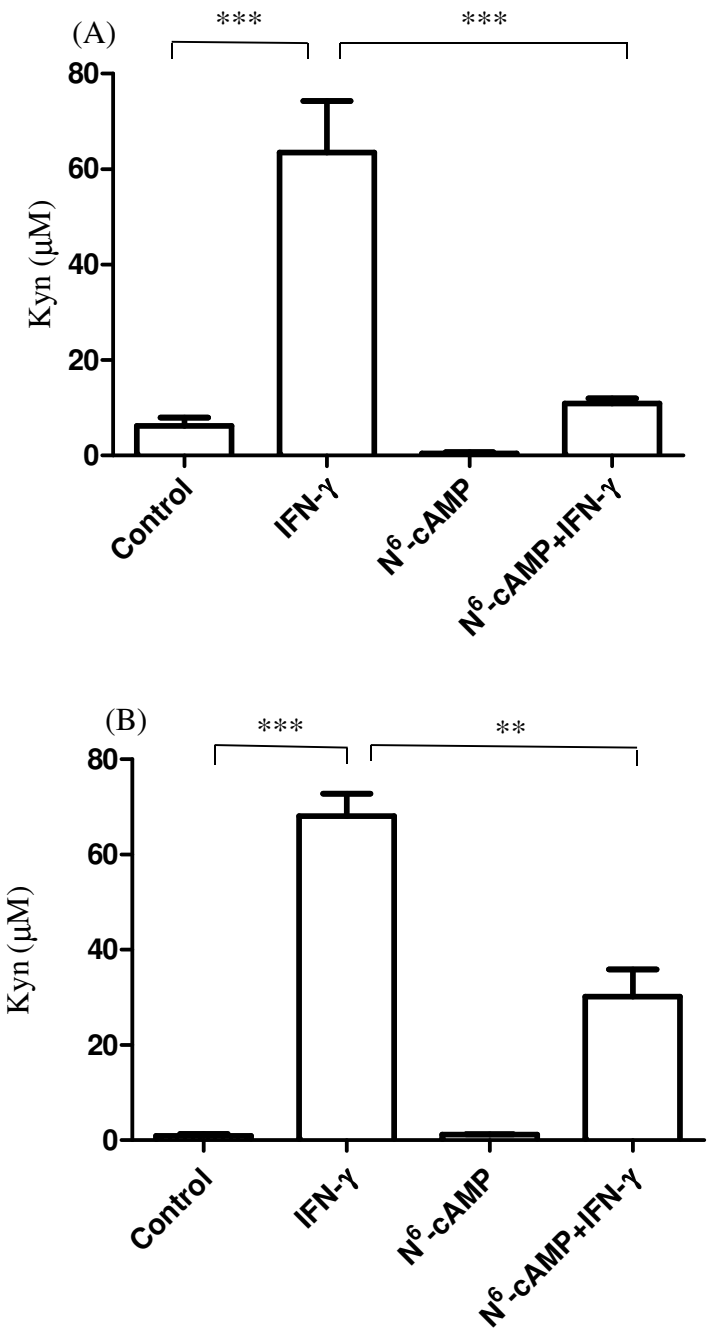


Figure 6-18 Effect of N^6-cAMP on IFN-γ mediated induction of IDO in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/total) or (B) human monocytes (4 x 10^6/total) were pre-treated with N^6-cAMP (0.5 mmol/L) or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM Kyn produced. THP-1, n=10 separate experiments; human monocytes, n=4 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; ** indicate $p < 0.001$, *** indicate $p < 0.0001$.

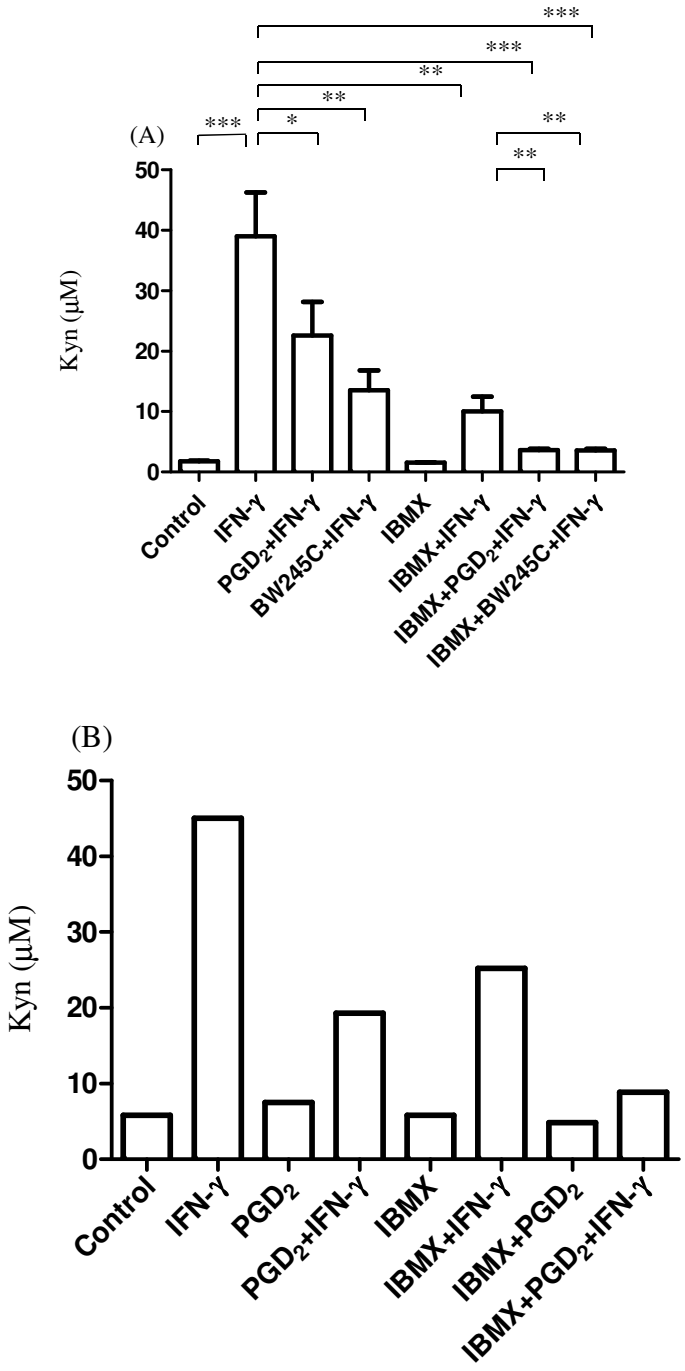


Figure 6-19 Effect of the phosphodiesterase inhibitor, IBMX, on the inhibitory activity of PGD_2 and BW245C on IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/total) and (B) human monocytes (4 x 10^6/total) were pre-treated with PGD_2 (5 μmol/L) or BW 245C (1 μmol/L) with or without IBMX (0.25 μmol/L) or diluent for 30 min in medium lacking serum. Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent prepared in media containing serum for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean±SEM Kyn produced.THP-1, n=6 separate experiments; human monocytes, n=1(confirmatory result). Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$, ** indicate $p < 0.001$, *** indicate $p < 0.0001$ compared to IFN-γ.

6.18 Effect of IBMX on IFN-γ mediated induction of INDO1

We next examined the effect of IBMX on the IFN-γ mediated induction of IDO at the transcriptional level. Cells were pre-treated with IBMX (0.25 μmol/L) or with diluent for 30 min followed by stimulation with PGD_2 or diluent for another 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 24 h. The cells were collected, total RNA extracted, cDNA prepared and *INDO1* measured by qRT-PCR. Consistent with previous results, IFN-γ significantly induced *INDO1* levels, PGD_2 and IBMX alone had no significant effect on basal *INDO1* levels and when cells were pre-treated with PGD_2 and then stimulated with IFN-γ, there was significant inhibition of *INDO1* levels. Again when cells were stimulated with PGD_2 and IBMX and then IFN-γ, there was a greater inhibition of *INDO1* levels compared to cells treated with PGD_2, plus IFN-γ. These results further supported the hypothesis that the inhibitory effect of PGD_2 at the transcription level involves cAMP (Figure 6-20).

6.19 Effect of the PKA inhibitor (Rp-cAMP) on the inhibitory effect of PGD_2

cAMP classically mediates its activity via activation of protein kinase A (PKA) (Andrew et al. 2008). To confirm the involvement of PKA in the PGD_2 effect, we examined the ability of the PKA inhibitor, Rp-cAMP (Andrew et al. 2008) to block the inhibitory effect of PGD_2. THP-1 cells or human monocytes were treated with Rp-cAMP (50 μmol/L) or diluent for 60 min and then stimulated with PGD_2 (10 μmol/L) or diluent for 30 min. Cells were then stimulated with IFN-γ (1000 IU/mL) or diluent for 72 h. IFN-γ caused a significant increase in Kyn over basal levels in both cell types. While PGD_2 alone had no effect on baseline Kyn levels, it caused a significant reduction when cells were stimulated with IFN-γ. Consistent with the JAK1/JAK2/STAT1 mediated induction of IDO via IFN-γ, pre-treatment of cells with Rp-cAMP followed by IFN-γ caused no significant effect on Kyn production compared to cells treated with IFN-γ alone. Pre-treatment of cells with Rp-cAMP and then PGD_2 caused no effect on Kyn production over basal levels. Pre-treatment of cells with Rp-cAMP, then PGD_2 and then IFN-γ led to a complete correction of the PGD_2 mediated inhibition of Kyn production in THP-1 cells, and less correction, but still significant, in human monocytes (Figure 6-21). This result confirmed that the action of PGD_2 was via PKA.

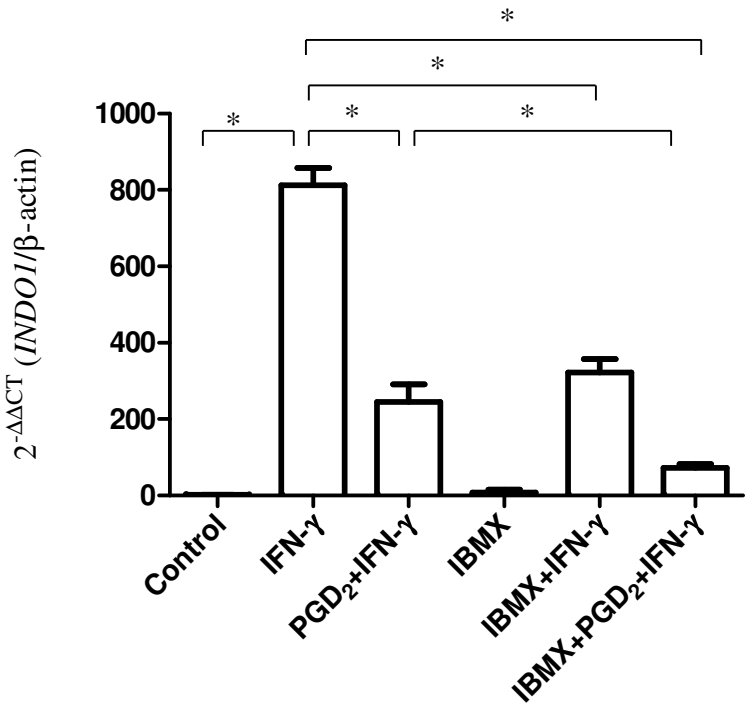


Figure 6-20 Effect of IBMX on PGD_2 mediated inhibition of IFN-γ induced *INDO1* in THP-1 cells

THP-1 cells (4 x 10^6/total) were pre-treated with PGD2 (10 µmol/L), IBMX (0.25 µmol/L) or diluent for 30 min followed by stimulation with IFN-γ (1000 IU/mL) for 24 h. RNA was extracted and 2 µg was used to synthesise cDNA. Using 100 ng cDNA, RT-PCR was performed using Taq-man gene expression assay. Results are expressed as mean ± SEM of *INDO1* ratio compared to β-actin mRNA. n=3 separete experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$.

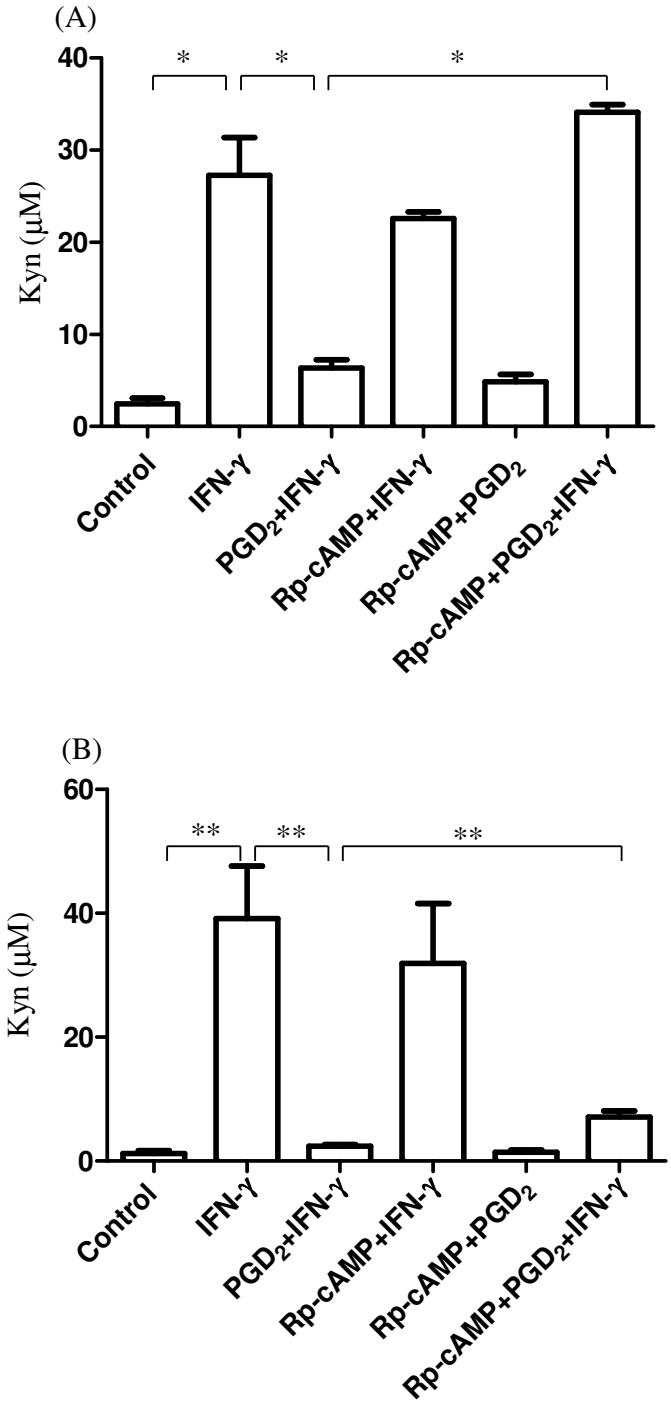


Figure 6-21 Effect of Rp-cAMP on the inhibitory effect of PGD_2 on IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

(A) THP-1 cells (4 x 10^5/total) or (B) human monocytes (4 x 10^6/total) were pre-treated with Rp-cAMP (50 μmol/L) for 60 min in media lacking serum. Cells were then treated with PGD_2 (10 μmol/L) for 30 min in media lacking serum and then stimulated with IFN-γ (1000 IU/mL) or diluent prepared in media containing serum for 72 h at 37 °C/5% CO_2 in a humidified atmosphere. The culture supernatant was removed and Kyn measured using the colorimetric assay. Results are shown as mean ± SEM Kyn produced. THP-1, n=4 separate experiments; human monocytes, n=3 separate experiments. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$, ** indicate $p < 0.001$, *** indicate $p < 0.0001$.

6.20 Effect of Rp-cAMP on PGD_2 mediated inhibition of IFN-γ induced INDO1

We next examined the effect of the PKA inhibitor, Rp-cAMP, on IFN-γ induced *INDO1* expression. Cells were pre-treated with Rp-cAMP (50 µmol/L) or with diluent for 1 h followed by stimulation with PGD_2 or diluent for another 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 24 h. The cells were collected, RNA extracted, cDNA prepared and *INDO1* measured by qRT-PCR. Consistent with the results from the previous experiments, IFN-γ significantly induced *INDO1* mRNA production and PGD_2 and Rp-cAMP alone had no significant effect on basal *INDO1* mRNA levels. When cells were pre-treated with PGD_2 and then stimulated with IFN-γ, there was 74% inhibition of *INDO1* mRNA levels. When cells were pre-treated with Rp-cAMP then PGD_2 and then IFN-γ, the inhibitory effect of PGD_2 was blocked compared to PGD_2 and IFN-γ treatment alone with an 81% correction, further confirming that the decrease in *INDO1* was mediated via PKA. These results further supported our hypothesis that the inhibitory effect of PGD_2 at the transcription level was through DP1, cAMP and PKA (Figure 6-22).

6.21 Kinetics of CREB phosphorylation in response to Forskolin

To date our results strongly supported the hypothesis that PGD_2 mediated its inhibitory effect through DP1/cAMP/PKA. PKA is known to mediate its effects through the transcription factor, cAMP response element binding protein (CREB). We thus examined the effect of PGD_2 on CREB activity by assessing its phosphorylation on Ser133. We first determined the kinetics of CREB phosphorylation in response to Forskolin, a direct AC activator. THP-1 cells (4 x 10^5/total) were treated with 100 µmol/L Forskolin for 0–60 min at 37 °C/5% CO_2 in a humidified atmosphere, cells collected, lysed and the lysates analysed using a pCREB ELISA-one assay kit. Increased pCREB was detectable within 5 min and reached a maximum after 20 min and was then maintained over a 60 min period (Figure 6-23). As a result a 20 min stimulation time with Forskolin was used in subsequent experiments.

6.22 Effect of PGD_2 and 11β $PGF_{2\alpha}$ on pCREB (Ser133)

To assess if CREB was involved in the inhibitory effect of PGD_2, we examined the ability of PGD_2 and Forskolin to modulate CREB phosphorylation on Ser133. THP-1 cells (4 x 10^5/total) were treated with PGD_2 (10 µmol/L), 11β $PGF_{2\alpha}$ (10 µmol/L) (CRTH2 ligand), Forskolin (100 µmol/L) (positive control) or diluent for 20 min. Cells were collected, lysed and the lysates assessed for total CREB and pCREB measurements using

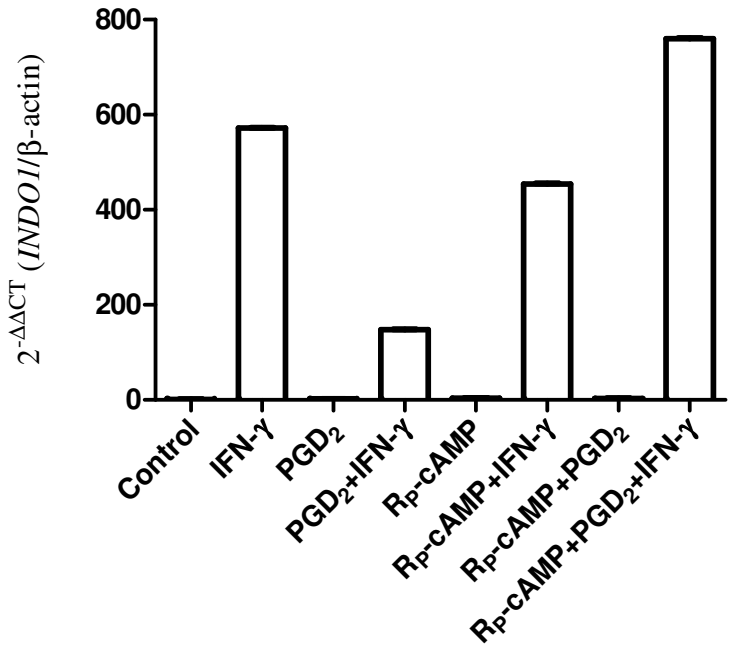


Figure 6-22 Effect of Rp-cAMP on PGD_2 mediated inhibition of IFN-γ induced *INDO1* mRNA in THP-1 cells

THP-1 cells (4 x 10^6/total) were pre-treated with Rp-cAMP (50 µmol/L) or diluent for 1 h, then with PGD_2 (10 µmol/L) or diluent for 30 min followed by stimulation with IFN-γ (1000 IU/mL) for 24 h. RNA was extracted and 2 µg was used to synthesise cDNA. Using 100 ng cDNA, RT-PCR was performed using Taq-man gene expression assay. Results are expressed as mean ± SEM of *INDO1* ratio compared to β-actin mRNA. Results of one experiment which was repeated three times each in triplicate. Comparesons were performed using the Kruskal-Wallis test and was significant, ** indicate $p < 0.0034$.

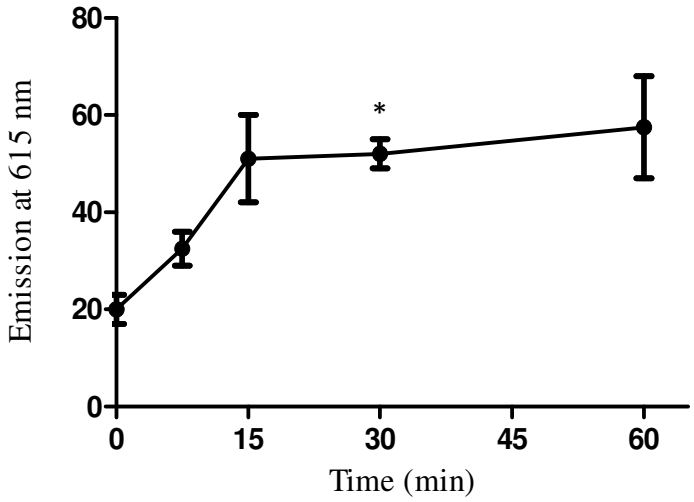


Figure 6-23 Kinetics of CREB phosphorylation at Ser^{133}

THP-1 cells (4 x 10^5/total) were treated with Forskolin (100 µmol/L) for 0–60 min at 37 °C/5% CO_2 in a humidified atmosphere. Cells were collected and lysed. Lysates were probed for pCREB according to the manufacturer's instruction (Section 2.26), and emission read at 615 nm. Results are expressed as mean ± SEM emission of 3 experiments each in duplicate. Unpaired exact Mann Whitney test; *indicate $p < 0.04$.

an ELISA-one assay kit. Control cells showed low levels of pCREB while IFN-γ also did not change pCREB levels. PGD_2 and Forskolin caused a significant increase in pCREB compared to control cells (Figure 6-24). Also PGD_2 and Forskolin plus IFN-γ caused significant increases in pCREB compared to IFNγ alone. As predicted, 11β $PGF_{2\alpha}$ alone and 11β $PGF_{2\alpha}$ plus IFN-γ caused no increase in pCREB levels compared to control and IFN-γ treated cells respectively. These results further support the hypothesis that PGD_2 mediated its inhibitory effect via DP1/AC/cAMP/PKA/pCREB.

6.23 Effect of PGD_2 on STAT1 (Tyr^{701})

We previously demonstrated that FAs could modulate STAT1 phosphorylation. In the previous series of experiments with PGD_2, we demonstrated that the cAMP pathway can also modulate IDO-1 activity. In an attempt to elucidate how these two pathways are connected, we examined the ability of PGD_2 to modulate STAT1 phosphorylation. To assess the effect of PGD_2 on STAT1 phosphorylation at Tyr^{701}, THP-1 cells (4×10^5/total) were treated with PGD_2 (10 µmol/L) for 30 min and then stimulated with IFN-γ (1000 IU/mL) or diluent for 30 min. Cells were collected, lysed and the lysates assessed for total CREB as a loading control since total STAT1 was not available in ELISA-one assay kit. Control cells showed low levels of STAT1 phosphorylation while IFN-γ caused a 1.5 fold increase in STAT1 phosphorylation. PGD_2 alone caused no significant effect on STAT1 phosphorylation compared to control cells. PGD_2 plus IFN-γ showed 0.7 fold inhibition in STAT1 phosphorylation compared to IFN-γ stimulated cells (Figure 6-25). This result further supported the hypothesis that PGD_2 mediates its inhibitory effect via DP1 and significantly inhibits the IFN-γ JAK/STAT signalling pathway at Tyr^{701}. Due to insufficient time we were unable to investigate this mechanism further, however the proposed experimentation is discussed in the final chapter of this thesis.

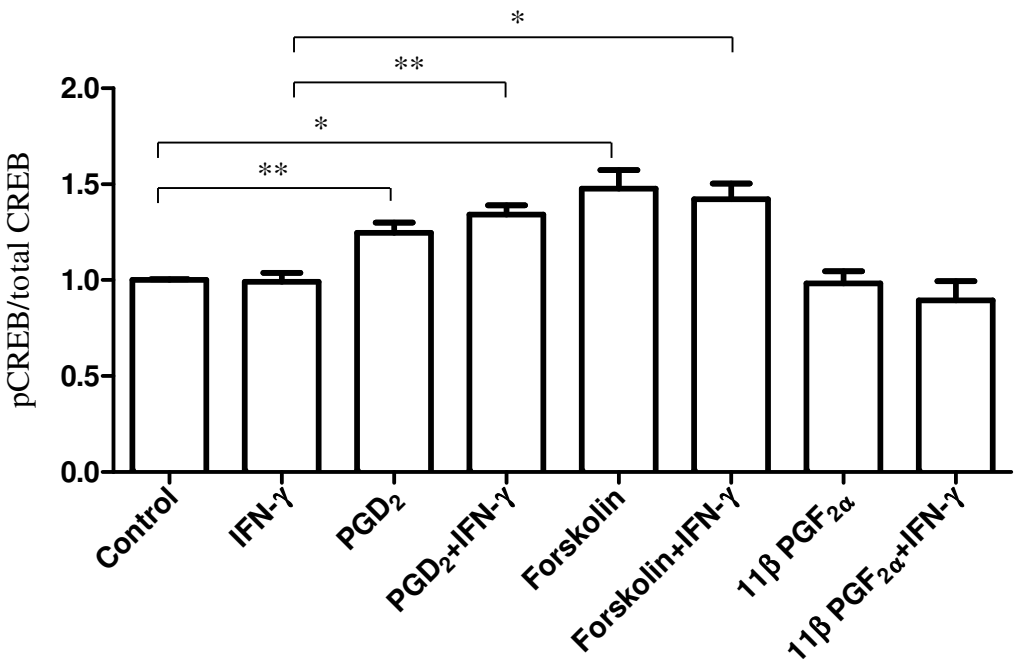


Figure 6-24 Effect of PGD_2, Forskolin and 11β $PGF_{2\alpha}$ on pCREB (Ser^{133}) in THP-1 cells

THP-1 cells (4 x 10^5/total) were treated with PGD_2 (10 µmol/L), 11β $PGF_{2\alpha}$ (10 µmol/L), Forskolin (100 µmol/L) or diluent for 30 min then were stimulated with (1000 IU/mL) IFN-γ or diluent at 37 °C/5% CO_2 in a humidified atmosphere for 20 min. Cells were collected, lysed and the lysates was used for CREB and pCREB using an ELISA-one assay kit (TGR BioSciences). Results are expressed as mean ± SEM of fold change of pCREB corrected for total CREB levels. n=4 separate experiments each in duplicate. Comparesons were performed using the Kruskal-Wallis test, followed by an unpaired exact Mann Whitney test; * indicate $p < 0.04$, ** indicate $p < 0.001$.

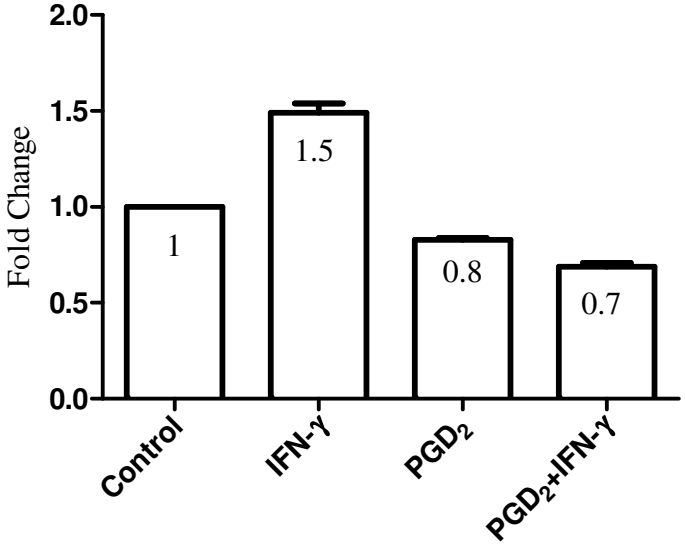


Figure 6-25 Effect of PGD_2, on pSTAT1 (Tyr^{701}) in THP-1 cells

THP-1 cells (4 x 10^5/total) were treated with PGD_2 (10 µmol/L) or diluent for 30 min then were stimulated with (1000 IU/mL) IFN-γ or diluent at 37 °C/5% CO_2 in a humidified atmosphere. Cells were collected, lysed and the lysates was used for CREB and pSTAT1 using an ELISA-one assay kit (TGR BioSciences). Results are expressed as fold change in pSTAT1 (Tyr^{701}). n=2 separate experiments each in duplicate. Comparesons were performed using the Kruskal-Wallis test and was not significant.

6.24 Summary

PGD_2 inhibited the IFN-γ mediated production of Kyn in THP-1 cells and human monocytes in a dose dependent manner with an IC_{50} of 7.2 ± 0.3 μmol/L and 5.2 ± 0.9 μmol/L respectively. The inhibitory effect was not due to toxicity in either cell type. PGD_2 inhibited both *INDO1* and IDO-1 protein in both cell types. Furthermore, PGD_2 was found to mediate its effects via the G-protein coupled receptor, DP1, but not CRTH2. This was shown using pharmacological agonists/antagonists of DP1 and CRTH2. The DP1 agonist BW 245C mimicked the PGD_2 inhibitory effects at the level of Kyn production and *INDO1* levels, while neither CRTH2 agonists, 13, 14-dihydro PGD_2 nor 11β $PGF_{2\alpha}$ affected IDO-1 at the level of activity. Furthermore, the DP1 antagonist MK 0524 corrected the PGD_2 inhibitory effect while CRTH2 antagonists, CAY 10595 and BAY u3405, failed to elicit the same response. PGD_2 was found to significantly increase cAMP levels in THP-1 cells. Forskolin, a direct AC activator, inhibited Kyn production. N^6-cAMP, a cell soluble cAMP derivative, also mimicked the inhibitory effect of PGD_2 on Kyn production, *INDO1* levels and IDO-1 protein expression. IBMX, a phosphodiesterase inhibitor, produced a greater inhibitory effect and decreased IFN-γ mediated induction of Kyn and *INDO1* levels. The PKA inhibitor, Rp-cAMP, corrected the PGD_2 inhibitory effect on Kyn levels and *INDO1* expression. PGD_2 and forskolin caused an increase in the phosphorylation of the PKA dependent transcription factor, CREB. Lastly, PGD_2 caused a significant inhibition in STAT1 phosphorylation at Tyr701.

Chapter 7

Discussion

7.1 Introduction

This research investigated the regulation of the enzyme indoleamine 2,3 dioxygenase (IDO-1) by fatty acids (FAs) and their eicosanoid derivatives in THP-1 cells and human monocytes. The data presented in this thesis showed evidence that 6 FAs modulated the IFN-γ mediated induction of IDO-1 in THP-1 cells. The activity of the FAs increased with an increase in chain length and number of double bonds. We also identified that fatty acids in the Z configuration were more inhibitory of IDO-1 induction/activity in THP-1 cells than the E configuration.

Extending our previous study (Bassal 2008), this research identified 13 AA derived COX1/2 metabolites that inhibited the IFN-γ mediated induction of IDO-1 in both THP-1 cells and human monocytes, with prostaglandins (PGs) A_2 and D_2 being the most active. Of these two PGs, we examined the mechanism of action of PGD_2 and its metabolites in detail determining that PGD_2 mediated its inhibitory effect via the DP1 receptor, adenylate cyclase, cAMP and PKA pathway. We also identified cross talk with the IFN-γ JAK/STAT signalling pathway. These findings may allow us to examine the potential of FAs and PGs as the basis of modulators of IDO-1.

7.2 Effect of naturally occurring fatty acids on IFN-γ mediated induction of IDO-1 in THP-1 cells

The biological activities of FAs are known to depend on their chain length, degree of unsaturation, and series (Ferrante, A et al. 1994; Bates et al. 1995; Ferrante, JV et al. 1997; Costabile et al. 2001; Zhang, YJ et al. 2010). Using a library of naturally available FAs (Chapter 3), a total of 6 of 68 FAs significantly inhibited IFN-γ mediated IDO-1 activity in THP-1 cells. An analysis of structure vs. inhibitory activity revealed some patterns consistent with the literature and others that had not been identified previously.

We found that none of the saturated FAs tested showed any significant effect on IDO-1 activity. Interestingly in a study by Jiang et al. (2010) the short chain unsaturated FA, NaB (4:0), down-regulated IDO at the transcriptional and post transcriptional levels via ubiquitination and degradation mechanisms in nasopharyngeal carcinoma cells. Apart from its effects on IDO, NaB also has been demonstrated to inhibit proliferation of cultured mammalian cells, induce differentiation and repression of gene expression through histone hyper-acetylation and inhibit histone deacetylase activity (HDAC) (Candido, Reeves & Davie 1978; Reeves & Candido 1978).

NaB is produced in the colon of mammals as a result of bacterial anaerobic fermentation of dietary fiber, undigested starch and protein (McIntyre, Gibson & Young

1993; Emenaker et al. 2001). In nasopharyngeal carcinoma cells, IDO protein expression was significantly reduced by 0.2 mmol/L NaB and almost completely inhibited by 0.5 mmol/L. NaB was found to modulate its effect partly by inhibiting STAT1 phosphorylation at Tyr^{701}, nuclear translocation of STAT1 and transcriptional activity of STAT1. NaB had no effect on *INDO1* expression when added post IFN-γ treatment, yet IDO protein was undetectable. Thus the effect of NaB was mediated at both the transcriptional and post-transcriptional levels. Treatment of cells with NaB resulted in a significant increase in total content of ubiquitinated proteins, including IDO. Using the specific 26S proteasome inhibitor, Bortezomib, there was significant suppression of NaB mediated inhibition of IDO protein expression. Thus NaB caused the ubiquitin-mediated proteasomal degradation of IDO (Jiang et al. 2010). One important point is that the activity of NaB was found when used at concentrations 0.2–0.5 mmol/L, significantly higher than the concentration of FAs used in our study (20 μmol/L maximum). Thus it is possible that other FAs may also be able to inhibit IFN-γ mediated induction of IDO-1 activity at higher concentrations. While not physiologically relevant, they may be pharmacologically appropriate.

The introduction of one or more double bonds into the FAs resulted in a significant increase in inhibitory activity. A total of 3 of 31 monounsaturated FAs displayed significant inhibitory activity towards IDO activity as assessed by Kyn production. When PUFAs were investigated, 3 of 21 PUFAs tested showed significant inhibitory activity. The inhibition of IDO-1 activity could be a possible mechanism by which monounsaturated FAs and PUFAs mediate their anti-tumor effects as shown in many cancers, some of which produce IDO-1.

From the literature, the longer chain C19–C22 n-3 monounsaturated FAs have been shown to inhibit proliferation, induce apoptosis, decrease PGE_2 formation and decrease cell invasion, while C16–C18 analogues were less active when tested in MDA-MB-468 breast cancer cells that overexpressed COX2 (MDA-COX2 cells). Thus, longer chain n-3 monounsaturated FAs may be potential anti-cancer agents that decrease the formation of PGE_2 in tumor cells that overexpress COX2 (Basu et al. 2006; von Bergwelt-Baildon et al. 2006; Cui et al. 2012). In our study, we found that in general the longer the chain length, the more effective the FA at inhibiting IFN-γ induced IDO activity. The inhibition of IDO activity/induction may be a possible new mechanism by which these long chain unsaturated FAs mediate their anti-cancer effects.

The configuration of FAs plays important roles in their biological functions (Calder 2008). In most naturally occurring unsaturated FAs, each double bond is followed by three

carbon atoms all in the Z configuration, while most FAs in the E configuration are not found naturally. This explains why our library contained FAs predominantly in the Z rather than E configuration. In general, our results indicate that FAs in the Z configuration were more effective than FAs in the E configuration at inhibiting the induction of IDO-1 activity. Furthermore, when the activity of both the Z and E configuration of the same FA were compared, the Z configuration was consistently more active. In the Z configuration, a double bond causes the chain to bend and restricts the conformational freedom of the FA. The more double bonds the chain has in the Z configuration, the less flexibility the FA has. When a chain has multiple Z bonds it can achieve a curved configuration and, in restricted environments, Z bonds limit the ability of FAs to move freely and change shape (Berg, JM , Tymoczko & Stryer 2003). It is possible that FAs, in the Z configuration, may directly interact with the active site of IDO-1 interfering with its activity and with restricted movement it will sustain its inhibitory effect. In contrast, FAs in the E configuration do not bend and they are free to change their shape and that would affect their ability to bind efficiently to the IDO-1 active site. To test this possibility directly, it would be necessary to test the effect of individual FAs on purified IDO-1 enzyme activity. An alternative approach we investigated made use of the *in silico* assessment of fatty acids interaction with the IDO-1 active site. While we were able to demonstrate that fatty acids could indeed interact with the active site, their degree of rotational flexibility made it impossible to definitively determine any direct relationship between binding and *in vitro* inhibitory activity (data not shown). Due to the predicted results and time constraints, examining this hypothesis in more detail was beyond the scope of this thesis.

It was predicted that n-6 PUFAs, which have pro-inflammatory activity, might display greater inhibitory effect towards IDO-1 activity than n-3 PUFAs which are generally considered to be anti-inflammatory (Ferrante, A et al. 1994; Ferrante, JV et al. 1997; Costabile et al. 2001; Costabile et al. 2005; Zhang, YJ et al. 2010). Although our results showed that the n-6 PUFA, AA, had more inhibitory effect than both n-3 PUFAs, DHA and EPA (Bassal 2008; Bassal, Hughes & Costabile 2012), surprisingly, no significant difference was found in the inhibitory effects between the n-6 vs. n-3 series.

7.3 Modulation of IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes by AA and the mechanism of action

AA is an important component of cell membranes. It is the precursor of pro-inflammatory eicosanoids and prolonged, elevated intake of AA is strongly associated with many types of cancer (Calder 2009; Patterson et al. 2012). Our study showed for the first

time that AA and its COX1/2 metabolites inhibit IFN-γ mediated induction/activity of IDO-1 in THP-1 cells (Bassal 2008) as well as in primary human monocytes (Chapter 4) (Bassal, Hughes & Costabile 2012).

In our previous study, the significant biological effect of AA was noted at concentrations as low as 2.5 µmol/L. However, even at the highest concentration tested (30 µmol/L) the level of AA is within normal physiological levels (Bassal 2008). Significant inhibition was seen after 20 min pre-treatment, while no significant increase in activity was seen when the pre-treatment time was extended to 24 h. Preliminary results indicated no effect of AA on IFN-γ receptor expression (Bassal 2008). The inhibitory activity of AA was mediated via its metabolism through COX1/2 enzymes (Bassal 2008; Bassal, Hughes & Costabile 2012). Consistent with a role for COX1/2 enzymes, THP-1 cells constitutively expressed COX1 mRNA, while lower levels of COX2 mRNA could also be detected, consistent with our hypothesis that the activity of both enzymes was responsible for the effects seen.

Although not directly tested, the rapid inhibitory effect of AA suggested that incorporation of the AA into membrane phospholipids was unlikely to be required for its inhibitory effect. While AA is known to be rapidly and preferentially incorporated into the *sn*-2 postion of membrane phospholipids and can be readily metabolised via COX and LOX enzymes, the incorporation is unlikely to be critical for the inhibitory effect. Firstly, the inhibitory effect of AA is mediated extremely rapidly with inhibitory effects seen after just 10 min. After this period of time, the amount of AA incorporated would be limited. Also when cells were pre-treated for longer periods of time, 24 h, there was no significant increase in inhibition. Over this longer period, a substantial amount of incorporation would be expected and a greater level of inhibitory activity should have been observed if this was a critical mode of action. So the rapid effect seen suggest that both cells metabolised the exogenouse FA directly via COX pathway (Bassal 2008). It is thus likely that the added AA is rapidly metabolised via COX1/2 enzymes. We were able to detect the expression of both COX1 and COX2 mRNA in THP-1 cells, which is consistent with the literature (Smith et al. 1993). Further evidence of their basal activity comes from the observation that in some experiments AA inhibited basal Kyn production.

Our findings showed that unsaturation was essential for activity as the saturated AA analog, arachidic acid, did not show any inhibitory effect. In addition, the carboxyl group of AA was found to play some role in the AA inhibitory effect with the methyl ester derivative displaying less inhibitory activity than AA (Bassal 2008; Bassal, Hughes &

Costabile 2012). We have previously show no effect of AA on iNOS in THP-1 cells (Bassal 2008).

IFN-γ affects IDO-1 at the transcriptional and translational levels (Travers et al. 2004) and this was supported by our results, which demonstrated that the AA inhibitory effect was at the level of transcription as it caused a 67.6% inhibition of *INDO1* expression in THP-1 and 50.4% inhibition of *INDO1* expression in human monocytes. Furthermore, AA affected IDO-1 at the level of translation as it caused a significant inhibition of IDO-1 protein levels, 31.7% in THP-1 cells and 86.5% in monocytes.

All of our experiments were performed using IFN-γ, the most potent physiological inducer of IDO-1. Through IFN-γ, the JAK1/2/STAT1 signalling pathway and phosphorylation of Tyr^{701} on STAT1 is essential for dimerisation, nuclear translocation and DNA binding (Wen, Zhong & Darnell 1995; King & Thomas 2007). Our data demonstrated that post-ligand binding events were affected, leading to the inhibitory effect of AA. We firstly demonstrated that AA inhibited STAT1 phosphorylation on Tyr^{701} (80% inhibition in THP-1 cells based on densitometry), while indomethacin (IM), a major COX inhibitor, corrected these inhibitory effects, indicating that the AA inhibitory effect was dependent on its metabolism via the COX pathway (Bassal 2008). The detection of AA-derived COX metabolites in control cells, and their complete inhibition in IM treated cells, confirmed the activity of IM as a COX inhibitor. Nordihydroguaiaretic acid (NDGA), a major lipoxygenase (LOX) inhibitor, failed to correct the AA inhibitory effect indicating that the LOX pathway was not involved. Similarly, the detection of AA-derived LOX metabolites by control cells, and their complete inhibition in NDGA treated cells, confirmed the activity of NDGA as a LOX inhibitor.

Surprisingly, Holmes (1998) did not find any effect of other modifiers, including phorbol 12-myristate 13-acetate (PMA), AA, LPS, analogs of cAMP and cGMP, and sphingosine, on induction of IDO in the absence of IFN-γ, nor did they modulate IFN-γ induction of IDO expression in cultured human foreskin fibroblasts. The differences between our findings and those of Holmes may be due the differences in the cells tested (THP-1 cells and human monocytes vs. human foreskin fibroblasts).

As mentioned previously, PGE_2 has received a great deal of attention for its regulatory effects towards *INDO1* induction and enzyme activity. PGE_2 is the most abundant PG produced from the metabolism of AA via COX2 (Lanzinger et al. 2012). PGE_2, as well as other PGs, can show opposing functions depending on cell type specificity and the experimental settings used (Thurnher 2007; Legler et al. 2010; Sakata, Yao & Narumiya 2010). Interestingly, in our experiments, PGE_2 did not display significant

inhibitory activity but, rather, PGA_2 and PGD_2 were the most potent inhibitors of Kyn production (Bassal, Hughes & Costabile 2012). To the best of our knowledge, there are no reports in the literature of either PGA_2 or PGD_2 modulating IDO activity/induction.

The first report examining the effect of PGE_2 on IDO demonstrated that it increased *INDO1* levels in monocyte-derived dendritic cells, but this was not accompanied by an increase in activity (Braun, Longman & Albert 2005). Only when TNFα was added could IDO enzymatic activity be detected. The differences between our findings and the above research are the species used (human vs. murine), cell type (THP-1 and human monocytes vs. monocyte-derived DCs) as well as the agonists used (IFN-γ vs. PGE_2 and TNFα). TNFα and IFN-γ have discrete signalling pathways and thus the immune modulatory properties of PGE_2 may be due to its actions in different points of the respective signalling cascade. Indeed, our results showed an inhibition of *INDO1* expression, inhibition of IDO protein expression and inhibition of STAT1 phosphorylation on Tyr701 by AA (Bassal 2008; Bassal, Hughes & Costabile 2012). Importantly, Braun et al. (2005) did not examine the effect of PGE_2 and IFN-γ on *INDO1* expression or IDO-1 protein, thus it is unclear if they would also observe an inhibitory effect.

Consistent with the diverse biological activity of PGE_2, Jung et al. (2010) demonstrated that curcumin inhibited IDO expression in DCs via increased expression of COX2 and PGE_2. Curcumin is a natural phenolic compound that can be isolated from the rhizome of *Curcuma longa* (turmeric) (Jung et al. 2010). Curcumin has many important effects: anti-oxidant (Stoner & Mukhtar 1995), anti-proliferative (Khar et al. 1999) and cancer chemo-preventative (Huang, MT et al. 1994). It inhibits pro-inflammatory cytokine production in mouse bone marrow-derived DCs (Kim, GY et al. 2005). In the Jung et al. (2010) study, PGE_2 was inhibitory towards IDO, but again the differences between our result and theirs may be due to variations in cell type (THP-1 and human monocytes vs. bone marrow DCs) and experimental agonists (IFN-γ vs. LPS).

AA is widely considered to be pro-inflammatory, which is why reducing the level of n-6 PUFAs in favor of n-3 PUFAs is a major approach in the treatment of a wide number of inflammatory diseases (Calder 2009). Our results suggest that the ability of AA and its COX1/2 metabolites to inhibit IDO-1 induction represents a pro-inflammatory mechanism of action. Excess AA may inhibit the immunosuppressive and anti-inflammatory activity of IDO-1 *in vivo* and thus lead to the presence of a pro-inflammatory state. This is supported by the findings that n-3 anti-inflammatory PUFAs, DHA and EPA, displayed significantly less inhibitory activity than an n-6 pro-inflammatory PUFA, AA, towards IDO-1 activity (Bassal 2008; Bassal, Hughes & Costabile 2012) (Figure 7-1).

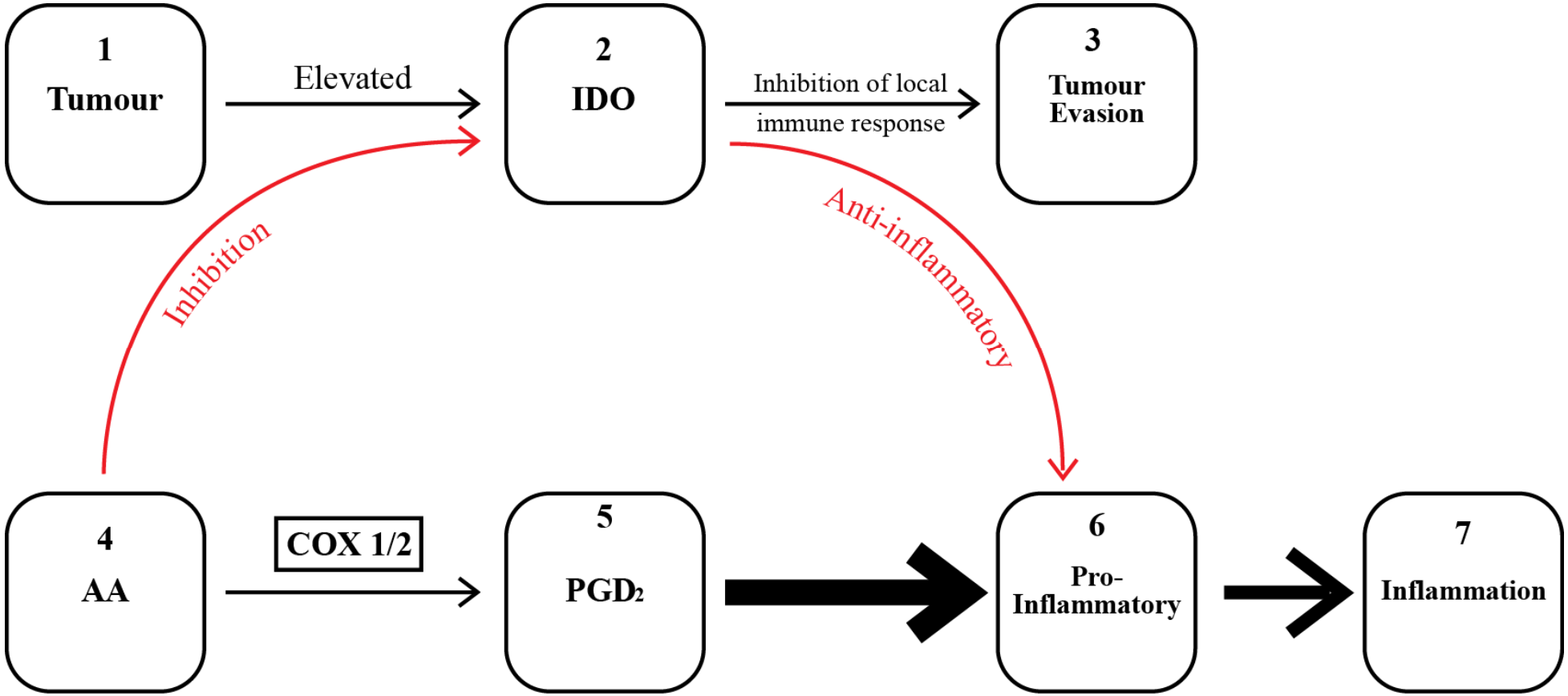


Figure 7-1 Summary of the inflammatory effects of AA and its derived eicosanoids

Tumors (1) that express high levels of IDO (2) inhibit the local immune response enhancing tumor evasion of the immune response (3). AA (4), through its COX-derived eicosanoids produces many active molecules such as PGG_2, PGH_2, or PGD_2 (5). These molecules are pro-inflammatory (6). We now show that AA inhibits the anti-inflammatory activity of IDO in cancer cells. Thus the pro-inflammatory fatty acid, AA, can interfere with IDO mediated anti-inflammatory activity with the net result still being an inflammatory response. (Adapted from Bassal 2008).

7.4 Determination of biologically active COX1/2 metabolites and analysis of their structure/function relationships

PGs play significant roles in many physiological and pathological conditions including inflammation, allergy and cancer (Lewis, Austen & Soberman 1990; Ferrante, JV et al. 1997; Costabile et al. 2001; Costabile et al. 2005; Calder 2009). PGs are principally produced from AA released from the plasma membrane forming PGG_2 and then PGH_2, which is further metabolised to different PGs by different synthase enzymes depending on the cell type.

As mentioned previously, no reports in the literature have assessed a broad range of PGs for their ability to modulate the induction/activity of IDO. Knowing that the inhibitory effect of AA was via its COX1/2 metabolism, it was important to identify the biologically active metabolite(s) behind this effect. Based on the hypothesis that both COX1/2 metabolites were involved in the AA inhibitory effect, the ability of a library of AA derived COX1/2 metabolites toward IFN-γ mediated induction of IDO in both cell types was studied in detail.

Of 38 commercially available AA derived COX1/2 metabolites, a total of 13 PGs in THP-1 cells and 11 PGs in human monocytes significantly inhibited IFN-γ mediated induction/activity of IDO-1 in both cell types without apparent toxicity (Chapter 5). Only 15-deoxy-$\Delta^{12,14}$-PGJ_2 showed toxicity towards THP-1 cells. Supporting the hypothesis that both COX1 and COX2 metabolites were responsible for the AA inhibitory effect, both COX1 and COX2 metabolites were found to be inhibitory. Both PGA_2 and PGD_2 were found to be the most effective at inhibiting Kyn production, as an indirect marker of IDO-1 activity, while PGE_2 was found to have no significant effect on IFN-γ meditated induction of IDO-1.

Since IFN-γ was used as the agonist, it was possible that the PGs interfered with the IFN-γ signalling cascade. Consistent with this hypothesis, 15-deoxy-$\Delta^{12,14}$-PGJ_2 has been shown to inhibit tyrosine phosphorylation of JAK1, JAK2 and STAT1 and STAT1 nuclear translocation (Panzer et al. 2008) as well as inducing SOCS1 and SOCS3 transcription (Park, EJ et al. 2003). However, neither of these manuscripts examined the effect of 15-deoxy-$\Delta^{12,14}$-PGJ_2 on the IFN-γ mediated induction of IDO.

As the structure of PGs affects their biological activity (Straus & Glass 2001; Cossette et al. 2007), we studied the relationship between the structure and activity of the inhibitory metabolites. We identified the following structural features as being important for biological activity. Some of these features are supported from the literature while some have not previously been reported.

- The α-β unsaturated carbonyl group is important for inhibitory activity (Atsmon et al. 1990; Parker & Ankel 1992; Straus & Glass 2001). The α-β unsaturated carbonyl group contains an electrophilic centre which makes these PGs susceptible to Michael addition reactions, with groups such as free sulfhydryl groups of cysteine residues located in reduced glutathione (GSH) or cellular proteins (Cagen et al. 1975; Honn & Marnett 1985; Parker & Ankel 1992). Several reports support this hypothesis. Firstly, PGs that do not have a cyclopentenone ring do not have a similar range of biological activities (Fukushima 1990). Secondly, conjugation of the reactive centre with GSH eliminates the activity of the cyclopentenone PGs (Honn & Marnett 1985; Bui & Straus 1998). Finally, many, if not all, of the biological activities of cyclopentenone PGs are mimicked by cyclopentenone (2-cyclopenten-1-one) itself, however, at 30–100 fold higher concentrations (Bui & Straus 1998; Straus et al. 2000). However, the related compounds of cyclopentenone which is the cyclopentanone are devoid of such or similar activity (Honn & Marnett 1985; Bui & Straus 1998; Straus et al. 2000; Straus & Glass 2001). These results are consistent with our finding, since both PGJ_2 and Δ^{12}-PGJ_2, which differ from PGD_2 by lacking a OH group in the α position in the cyclopentanone ring and the presence of a double bond in the α-β position respectively, resulted in an increase in inhibitory activity while cell viability was not affected.
- Schneider et al. (2002) indicated that the addition of an oxygen atom at positions 9, 11 or 15 could contribute to and affect the different biological activities of the PGs as different receptors distinguish different PGs on the basis of the nature of the hydroxy or keto groups at these positions. This was also found in our results as the removal of the OH group from C15 in Δ^{12}-PGJ_2 caused a significant decrease in activity and reduced the viability of THP-1 cells in relation to 15-deoxy-$\Delta^{12,14}$-PGJ_2. Thus the addition of other groups could further modulate the structure of different PGs to produce compounds more effective at inhibiting IFN-γ stimulated IDO-1 induction/activity.
- Another important structural feature was the presence of a hydroxyl group in the natural (S) configuration at C15, and not the (R) configuration, being critical for optimal activation of receptors (Main & Whittle 1975; Powell 2003). Oxidation of the above group can abolish the biological activity of the PG (Tai et al. 2002). These findings support our results as 15(S)-15-methyl PGD_2, via the DP1 receptor, had significant inhibitory activity while 15(R)-15-methyl PGD_2, via CRTH2, failed to do so. However, none of the above structural features of PGs that are important for their biologically activity have been validated in the context of IDO.

Based on the structure of the biologically active PGs we identified the following structural features that were important for biological activity:

- The presence of one or more OH groups in the side arm increased inhibitory activity.
- The orientation of the OH group in the α position in the cyclopentanone ring was important as it leads to a flatter structure.
- Any steric interactions or congestion of the groups under the ring interfered with the inhibitory activity of the PGs.
- The ethanolamide group had a significant effect in increasing the inhibitory activity of different metabolites.

In applying the above features, an ideal structure has been predicted (Figure 7-2). This PG can be further modified by removing an OH group in the side arm and replacing it with an ethanolamide group. This structure, if prepared in the laboratory and its activity tested, may become a basis for developing novel PG based compounds that can modulate IFN-γ stimulated IDO activity.

7.5 Characterization of the activity of PGD_2 on IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

Of the five major AA-derived COX metabolites (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2), PGD_2 was the only PG that inhibited the IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes. PGD_2 and its non-enzymatic metabolites significantly inhibited the IFN-γ stimulated IDO-1 induction/activity in both cells (Chapter 6). This inhibitory effect was seen with our standard 30 min pre-treatment with PGD_2 or its metabolites prior to IFN-γ treatment. Significant effects were seen at 10 µmol/L with PGD_2 and its metabolites with no apparent toxicity to both cells. This inhibitory effect of PGD_2 and its metabolites was at the level of transcription and translation. Since both IDO-1 and IDO-2 can metabolise Trp to produce Kyn, it was important to examine the expression of *INDO1* and *INDOL* in these cells. However, we could not detect *INDOL* in either THP-1 cells or human monocytes. It may be that these cells do not express *INDOL*. It should also be noted that historically TDO, which can also metabolise Trp, was considered to be limited to hepatic expression. However, recently it was also shown to be expressed at elevated levels in glioma cancer cells. This paper was released recently (Platten, Wick & Van den Eynde 2012) and, due to time limitations, we were unable to investigate whether FAs were also able to regulate TDO activity.

Many PGs with a cyclopentenone ring induce apoptosis, cause cell cycle arrest and

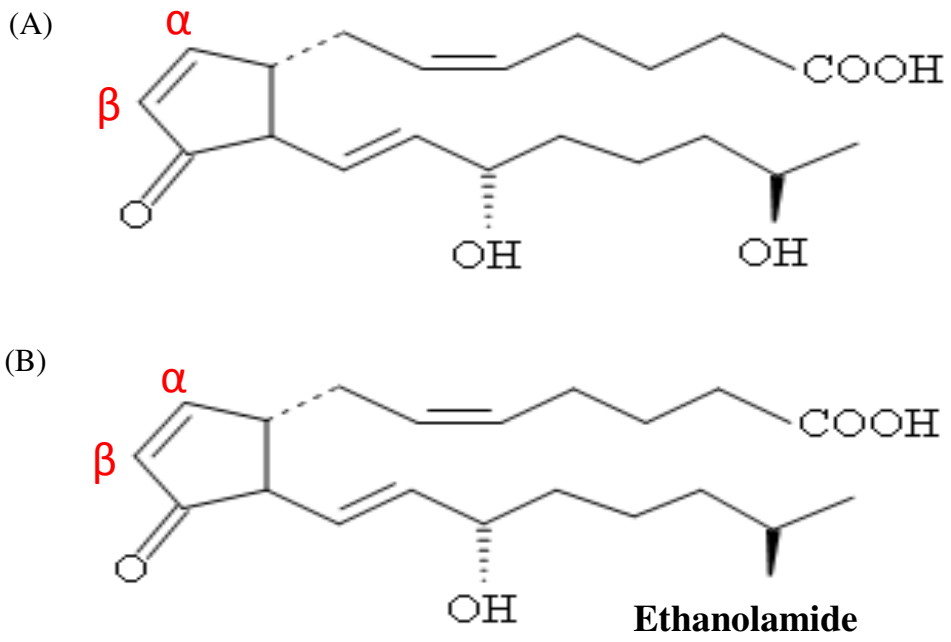


Figure 7-2 Proposed optimal structure of an inhibitory PG

The structure and features of the proposed biologically active compound characterised with the following: PG with 20 carbon atoms, cyclopentenone ring with double bond in the α-β position, O group under the ring, OH group in the side arm in the (R) position, and (A) another OH group or (B) ethanolamide group in the side arm.

have anti-tumor activity (Straus & Glass 2001). PGD_2 is known to have multiple roles in many pathological conditions. PGD_2 has been shown to be pro-apoptotic in human osteoclasts (Yue, L et al. 2012), PC12 neuronal cells (Ragolia et al. 2001; Yue, L et al. 2012), eosinophils and granulocytes (Ward et al. 2002). PGD_2 and PGJ_2 induce apoptosis in the human leukaemic cell HL-60 through ROS and caspase 3 cascades (Chen, YC, Shen & Tsai 2005). Interestingly, in our study PGD_2 and all inhibitory metabolites tested did not show any toxicity to either THP-1 cells or human monocytes, the only exception being 15 deoxy $\Delta^{12,14}$-PGJ_2, which was toxic towards THP-1 cells. PGD_2 also has been shown to inhibit growth of the murine leukaemic cell, L1210, with an IC_{50} of 6.9 µmol/L and human ovarian tumor cell proliferation with an IC_{50} of 6.8 µmol/L (Kikuchi et al. 1994). PGD_2 and PGA_2 also have been shown to inhibit NALL-1, RPMI-8226, RPMI-8402 and SK-Ly-16 growth with similar IC_{50} results (Fukushima et al. 1982b). However, none of the above studies examined the relationship between any of these PGs and IDO, which may explain some of the PGs anti-neoplastic effects.

PGD_2 acts via the classical G-protein coupled receptors, PGD_2 prostanoid receptor DP1 and chemoattractant receptor-homologous coupled receptor (CRTH2) (Oguma, Asano & Ishizaka 2008). PGD_2 is degraded *in vitro* and *in vivo* to a variety of metabolites, the majority of which were thought, until recently, to be physiologically inactive. However, many PGD_2 metabolites, particularly 15-deoxy $\Delta^{12,14}$-PGJ_2, have been shown to have a wide-range of effects, mainly anti-inflammatory, which inhibit NF-κB mediated transcription by PPARγ dependent and independent actions (Straus & Glass 2001) and eosinophil activation in asthma via CRTH2 (Powell 2003).

We hypothesised that the inhibitory effect detected could be due to PGD_2 alone or PGD_2 plus its metabolites. PGD_2 is degraded *in vitro* and *in vivo* to a variety of metabolites (Sandig, Pease & Sabroe 2007). In our research, both THP-1 cells and human monocytes were pre-treated with PGD_2 for 30 min followed by IFN-γ stimulation for up to 72 h, thus the inhibitory effect could be due to PGD_2, its metabolites or a combination of the two. This possibility was supported by our EIA data where both PGD_2 and its metabolites could be detected in culture supernatants.

PGD_2 is metabolised both enzymatically and non-enzymatically. Both enzymatic and non-enzymatic metabolites of PGD_2 can be detected *in vivo*, however, physiologically the concentration of the non-enzymatic products is lower than micromolar (Powell 2003). This is important for several reasons. First, being present at very low concentrations, they can be further used as a therapeutic treatment without concern about higher dosage side effects, which occur during many pathological conditions (Shibata et al. 2002). Second, it

is important to note that although the physiological concentration of many of these PGs *in vivo* is picomolar–to-nanomolar concentration (Fukushima 1990), their levels can be increased to micromolar during inflammation (Offenbacher, Odle & Van Dyke 1986). Third, although the IC_{50} is outside the normal physiological concentration range, pharmacologically it is well accepted as shown by many studies. For example, when PGD_2 was examined for its potential cytotoxic effects on L1210 mouse leukaemic cells, it inhibited cell growth at 3.6 µmol/L with complete inhibition seen at 14.3 µmol/L and a calculated IC_{50} of 6.9 µmol/L. PGD_2 also has been demonstrated to inhibit ovarian tumor cell proliferation with an IC_{50} of 6.8 µmol/L (Kikuchi et al. 1994). In our study, the PGD_2 IC_{50} was 7.2 µmol/L in THP-1 cells and 5.2 µmol/L in human monocytes, which is consistent with the literature and well accepted as a safe pharmacological concentration.

PGD_2 and its metabolites are biologically active over a broad concentration range and, interestingly, it appears that there are marked concentration-dependent variations in the consequences of signalling by these PGs, which have the potential to exert opposing effects. For example, PGD_2 can influence multiple stages of the mature eosinophil, from causing their release from the bone marrow to inducing their recruitment and activation and, ultimately regulating their apoptosis (Sandig, Pease & Sabroe 2007). Also, 15 deoxy $\Delta^{12,14}$-PGJ_2 at low concentrations activates PPAR-γ and in high doses activates CRTH2 (Kobayashi et al. 2005; Ueki et al. 2007; Oguma, Asano & Ishizaka 2008), each being linked to a different signalling pathway and, ultimately, different biological effects. It would be interesting to measure the level of PGD_2 and its different metabolites in our setting, however this is both expensive and time consuming and could not be performed in our study. To identify which PGD_2 derivatives were biologically active, both its enzymatic and non-enzymatic metabolites were studied in detail. Our results indicated that only the non-enzymatic metabolites were biologically active and inhibited IDO-1 activity in both cell types without any apparent toxicity.

As described previously, the chemically reactive cyclopentenone centre suggests that these compounds might alkylate exposed cysteine residues on key cellular target proteins. According to this hypothesis, alkylation of these cysteine residues would result in loss of function of the targets. Because the α-β unsaturated carbonyl group is a soft electrophile, alkylation of weaker nucleophilic sites located in other macromolecules such as DNA is less likely. Early studies indicated that these compounds reacted with cellular proteins rather than DNA (Fukushima 1990). The hypothesis that specific cysteine residues in cellular proteins are the key targets has been confirmed with the elucidation of the molecular mechanism for repression of NF-κB activity by 15-deoxy-$\Delta^{12,14}$-PGJ_2 (Rossi et

al. 2000; Straus et al. 2000). In this case, two target protein cysteine residues are involved. One of these is located in the activation loop of NF-κB Kinase, which is required for NF-κB activation, and the other is located in the DNA binding domain of NF-κB (Rossi et al. 2000; Straus et al. 2000). The question of whether IDO-1 is a target for binding to this group of PGs needs to be investigated.

The idea of chemically modifying PUFAs to modulate their immunological effects is known to be effective and can generate selectivity for different immunological parameters (Costabile et al. 2001; Costabile et al. 2005). The presence of another reactive electrophilic carbon located on one of the side chains can increase the inhibitory effect of the PGs as in the case of Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (Noyori & Suzuki 1993). The addition of an extra group, such as a methyl group can affect the biological activity (Cossette et al. 2007). Our results showed that the addition of the ethanolamide group significantly increased the inhibitory effect.

PGs can act through at least three mechanisms: activation of G-protein coupled receptors, activation of nuclear receptors and direct modification of proteins (Sandig, Pease & Sabroe 2007). PGs can have different effects or even opposite effects depending on the cell and the type of receptor they activate (Zhang, YJ et al. 2010). For example, the PGE series of PGs can stimulate the proliferation of some cell types (Taub et al. 1979; Taub et al. 1983) and inhibit the growth of others (Johnson & Pastan 1971; Huttner et al. 1977) both *in vitro* and *in vivo*. PGE_2 can significantly inhibit DNA synthase at 1 nM in IMR-90 human diploid fibroblasts (Straus & Pang 1984), while it did not inhibit DNA synthase in the NIL hamster fibroblast cell line (Straus & Pang 1984). These results indicate that the effect of PGE series PGs on cell growth is very much dependent on the cell specificity and the expression and affinity to the different EP-prostanoid receptors (Narumiya 1995).

In order to determine the mechanism of PGD_2 and its inhibitory metabolites, it was important to determine which receptor was involved, DP1 or CRTH2. PGD_2 and almost all of its metabolites can activate both receptors, while some can also activate PPAR-γ. However, as PGD_2 has been shown to activate DP1 or CRTH2 only, they were the focus of this research study.

DP1 is expressed on both THP-1 cells (Hirano et al. 2007) and human monocytes (Gosset et al. 2003). While CRTH2 was known to be expressed on human monocytes (Gosset et al. 2003), its expression on THP-1 cells was unknown. Our results confirmed the expression of CRTH2 mRNA in THP-1 cells and in human monocytes and that it could be further increased by IFN-γ stimulation in human monocytes. Thus it was possible that the inhibitory effect seen with PGD_2 and its metabolites was mediated through either

receptor. Activation of DP1 led to activation of adenylate cyclase (AC) and increased cAMP levels while activation of CRTH2 led to an increase in calcium and decrease in intracellular cAMP levels (Boie et al. 1995).

The effects of PGD_2 and its metabolites can depend on the expression of these two receptors in any given cell type and the PG's affinity to bind to them. For example, in monocytes, PGD_2 ligation and activation of CRTH2 induces monocyte migration *in vitro* while binding to DP1 inhibits monocyte migration (Gosset et al. 2003).

Thus, firstly, it was important to identify the receptor involved in the PGD_2 inhibitory activity. PGD_2 binds to DP1 with eight times greater affinity than to CRTH2 (Sawyer et al. 2002), thus it may be expected that the effect of PGD_2 is most likely mediated via DP1 and not CRTH2. Kyn production was significantly inhibited as a result of treatment with PGD_2 or the DP1 receptor agonists BW245C or BW868, which both bind to DP1 with the same affinity (Yue, L et al. 2012; Ayabe et al. 2013), before IFN-γ stimulation. Both CRTH2 agonists 13, 14-dihydro-15-keto PGD_2 (Yue, L et al. 2012) or 15(R)-15-methyl PGD_2 (Monneret et al. 2003) failed to mimic the PGD_2 inhibitory effect. Strongly supporting our findings, the DP1 antagonist MK 0524 (Pettipher & Hansel 2008) successfully corrected the inhibitory effect of PGD_2, PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy^{13,14}-PGD_2, which all act via DP1, while BAY u3405, Cay 10595 or Cay 10471 (Yue, L et al. 2012), CRTH2 antagonists, failed to cause any correction. Our mechanistic study revealed that the inhibitory effect of PGD_2 and its biologically active metabolites was via activating DP1 and not CRTH2.

DP1 is linked to AC, thus the significant inhibition of Kyn production by forskolin, a direct activator of AC (Zhang, YJ et al. 2010; Ayabe et al. 2013), in both monocytes and THP-1 cells further supported our data that DP1 and not CRTH2 was important for the inhibitory activity. Further evidence of the involvement of DP1 came from the increase in intracellular cAMP after PGD_2 treatment while the CRTH2 agonist, 11β $PGF_{2\alpha}$, caused no changes in the level of intracellular cAMP. Further supporting evidence for a role of cAMP was the demonstration that an exogenous analogue of cAMP, N^6-cAMP, inhibited the IFN-γ Kyn production in both cell types and inhibited both *INDO1* and IDO-1 protein levels. The phosphodiesterase inhibitor IBMX also led to an enhancement of the inhibitory effect of both PGD_2 and N^6-cAMP.

Intracellular cAMP can activate Epac or PKA signalling pathways depending on the cell type (Ayabe et al. 2013). Epac is activated directly by cAMP. Epac1 and Epac2 are cAMP-dependent guanine-nucleotide-exchange factors for the small GTPases that are important mediators for the cAMP signalling pathway. Both Epac and PKA are involved in

many important cellular functions as they can activate many domain proteins, implicated in various cellular processes such as integrin-mediated cell adhesion and cell–cell junction formation, the control of insulin secretion and neurotransmitter release (Holz et al. 2006; Ni et al. 2011). However, in most of these processes, cAMP signalling involves PKA, emphasising the interconnectivity between Epac- and PKA-mediated signalling.

Since PKA is classically associated with cAMP, we confirmed its role using its inhibitor H89 (Hirano et al. 2007), however this was found to be toxic towards THP-1 cells. As a result we used an alternative well-characterised PKA inhibitor, Rp-cAMP (Liu et al. 2004; Murray, AJ 2008), which significantly prevented the PGD_2 mediated inhibition in Kyn production without any toxicity in either cell type. Activation of PKA can lead to phosphorylation of the transcription factor, cAMP response element binding protein (CREB) (Murray, AJ 2008). Gosset et al. (2003) showed that PGD_2 affected the function of human monocyte derived DCs via activating DP1/PKA and CREB phosphorylation. However, it is important to note that this study did not examine the effect of that pathway on IDO-1 activity.

Consistent with the literature, both PGD_2 and forskolin (as a positive control) significantly induced CREB phosphorylation on Ser133 in THP-1 cells. Our results suggested that PGD_2 could modulate IDO expression at multiple levels and perhaps there is cross talk between PGD_2 signalling via DP1/cAMP/PKA and pCREB and IFN-γ JAK/STAT1 signalling pathways. Preliminary data indicated that PGD_2 significantly inhibited STAT1 phosphorylation on Tyr701, however additional experiments are required to continue and confirm this result. Even so this result is consistent with other reports in the literature. David et al. (1996) showed that treatment of the myeloma cell line, U266, with forskolin negatively regulated the JAK-STAT signalling pathway. Another study indicated that the cAMP/PKA signalling pathway could inhibit the IFN-γ JAK/STAT signalling pathway (Kalvakolanu 2003). As a result of their work, they concluded that cAMP/PKA/CREB can be a negative regulatory mechanism to the IFN-γ JAK/STAT signalling pathway. However, and most importantly, none of these studies examined the effect of their finding on IDO activity or the effect of PGD_2 through this mechanism on IFN-γ mediated induction of IDO.

In conclusion, our results demonstrated that PGD_2 activation of cAMP/PKA/CREB may be a negative regulatory mechanism to the IFN-γ JAK/STAT signalling pathway involved in modulating IDO induction in THP-1 cells and human monocytes.

7.6 Future Work

The demonstration that a wide range of FAs could inhibit the IFN-γ mediated induction/activity of IDO-1 in THP-1 cells is encouraging; these results pave the way for future study on the regulation of IDO-1 by FAs. Our testing was performed using a library of 68 FA. It would be beneficial to repeat the testing using human monocytes as well as other cancer cells which constitutively express elevated levels of IDO. Testing all of the FAs on human monocytes will provide additional evidence that the effect of the FAs is a true biological effect and is not an artifact for cancer cells. It would also be important to test the effect of AA and its metabolites on other cells e.g.T-cells as other compounds e.g. colchicine derivatives and colcemide are able to inhibit T cell activation within the cellular immune response leading to reduced production of IFN-γ which could lead to inhibition of IDO (Altindag et al. 1997).

Also, by testing different types of cancer cells, the applicability of this approach in a broader setting will be established.

It is also important to test as many FAs as possible for their modulatory effects. Increasing the testing, in particular of less commonly available FAs, will allow us to better appreciate the broad spectrum of activity. For example, while the activity of unsaturated FAs increased with increasing chain length, we only tested one monounsaturated FA with 11, 12, 13, 21 or 24 carbon atoms. In addition, more monounsaturated FAs with 18 and 21 carbon atoms were available for testing. Similarly for PUFAs, we tested 6 FAs that had 18 carbon atoms, 7 with 20 carbon atoms and 1 with 21 carbon atoms. A larger number of FAs with equal numbers of carbon atoms in each group of different FAs would allow us to better determine the relationship between structure and function. By applying the important feature of FAs that improve their inhibitory effects and using engineered FAs that exist (Costabile et al. 2001; Costabile et al. 2005), there can be further tailoring to achieve a significant effect of these biological compounds.

In the field of FAs, multiple studies report opposing effects for a single, or group of FAs. Part of the reason for such variability in biological activity is the variation in methodology used. Thus in order for our results to be internally consistent, several factors need to be addressed before studying the effect of a wider range of FAs on IDO-1 in different cancer cell types. Firstly, both cancer and normal cells must be tested under the same conditions, and since the growth rate of cancer cells is faster than that of normal cells of the same stroma, the density of the cells or the incubation period may need to be adjusted. Secondly, testing a wide range of FAs concentrations will be beneficial as many of the FAs and their eicosanoid derivatives can have different effects or even opposing

effects depending on the concentration and cell type being investigated (Sandig, Pease & Sabroe 2007). Thirdly, in our *in vitro* assays we used medium lacking serum, as it is known that serum can decrease the activity of PUFAs and their metabolites (Costabile et al. 2005). This setting is not available *in vivo,* so whether the effect of the inhibitory FAs will be the same or different will need more study. Finally, although albumin is important for binding and transporting different PUFAs in the blood, free PUFAs may more readily enter cells than when bound to albumin.

Numerous studies also have examined the regulation of IDO activity in immature DC particularly in response to PGE_2. Thus we could also examine the effect of FAs and their derived eicosanoids on monocyte-derived DCs. However as with any *in vitro* manipulation, one can argue that these cells may not be completely representative of the *in vivo* population.

Our results also showed that AA and its COX metabolites inhibited both *INDO1* and IDO protein expression in THP-1 cells and human monocytes. Recently, however, another mechanism, down-regulation of IDO at the transcriptional and post-transcriptional levels via ubiquitination and degradation (Jiang et al. 2010), has been reported. This could be another mechanism by which AA and its metabolites down-regulate IDO activity and could be investigated.

From the data presented, it is clear that all of our studies have been undertaken in an *in vitro* setting. It would be important to assess the activity of the FAs and eicosanoid derivatives *in vivo* also. Multiple studies have shown that PUFA and their eicosanoids derivatives inhibit the formation of ROS and have antioxidant activity. Anti-oxidants have been shown to inhibit Th1 cytokine production, of which IFN-γ is the prototypical example (Sies 1997; Richard, D et al. 2008). As outlined in the introduction, IFN-γ is a potent inducer of IDO-1 leading to degradation of Trp but it is also a powerful trigger of ROS (Nathan et al. 1983). Thus *in vivo*, it is possible that PUFA administration could lead to a reduction in IFN-γ levels from T cells. This reduced level of IFN-γ would then lead to a reduction in IDO-1 activation, which would be seen as reduced conversion of Trp to Kyn and its by-products (Jenny et al. 2011; Zaknun et al. 2011).

Similarly PGD_2 has been shown to suppress the Th1 response in favour of a Th2 profile (Tanaka, K et al. 2004), which is consistent with the above mechanism of action. Thus *in vivo*, it is possible that the PGD_2 effect may be due to a reduction in IFN-γ levels and a corresponding increase in IL-4 levels, hence giving rise to the lower level of IDO-1 activity. However, our *in vitro* results clearly show a direct effect of PGD_2 in modulating

the IFN-γ mediated signal transduction pathway leading to a reduction in IDO-1 message and protein expression.

To assess this possible mechanism of action, mice could be fed diets enriched with varying combinations of FAs. The PBMCs could then be isolated and either unstimulated or treated with Phytohaemagglutinin (PHA) or Concanavalin A (ConA) to induce cellular activation, proliferation and cytokine production. Differences in cellular activation could then be assessed by quantifying the level of neopterin produced, Trp/Kyn ratio and IFN-γ produced via ELISA (Nathan 1987).

At the commencement of this research, it was known that the metabolism of Trp was via three enzymes: the hepatic TDO and non-hepatic IDO-1 and IDO-2. As *INDOL* could not be detected, the study concentrated only on IDO-1. Recently TDO was found in sites outside the liver (Platten, Wick & Van den Eynde 2012), so it would be beneficial to study the effect of FAs on TDO induction/activity in our experimental setting in more detail.

PGs are able to modulate their effects in three ways: G-protein coupled receptors, PPAR-γ and direct binding and modification of other proteins. This thesis focused on DP1 and CRTH2 receptors, as they are known to be involved in PGD_2 activity. However, some PGs with biological activity mediate their effects via PPAR-γ. There are no reports of the involvement of PPAR-γ in the modulation of IDO-1 activity. Thus it would be useful to examine the involvement of PPAR-γ receptor in the PG modulation of IDO activity.

Many of these active PGs can activate more than one receptor and while it is useful to use chemically available agonists and antagonists, it would also be important to use other protocols to confirm our findings. For example, siRNA targeting DP1 and CRTH2 could be used to confirm the involvement of individual receptors in the PGD_2 mediated activity. It is important to investigate if the inhibitory effect is due to PGD_2 alone or PGD_2 plus some of its biologically active metabolites, mainly PGJ_2 and/or Δ^{12}-PGJ_2. Our preliminary experiments found that PGJ_2 did inhibit *INDO1* and IDO-1 protein in IFN-γ stimulated THP-1 cells (data not shown).

Our findings indicated that PGD_2 inhibited IDO-1 activity via DP1/AC/cAMP/PKA and pCREB and that PGD_2 also inhibited STAT1 phosphorylation at Tyr701. This cross talk between the two signalling pathways can be further investigated, testing the action of Forskolin and N^6cAMP on IFN-γ stimulated STAT1 phosphorylation and the result of IBMX and Rp-cAMP on PGD_2 and IFN-γ effect on STAT1 phosphorylation and confirming the key results using human monocytes.

Our research also revealed new important features in PG structure that affect the activity of these compounds. Based on these findings, we propose to chemically modify PGD_2 at selected positions to confirm the importance of these features and functional groups. In addition, we would selectively add reactive groups (Figure 7-2) to increase the inhibitory activity of the parent compounds. Our experimental approach, given that the ethanolamide group enhanced the PG's inhibitory activity, would begin by adding this group to various positions on the PGD_2 molecule and assess the inhibitory activity. The next approach would be the esterification and acetylation of available hydroxyl groups either individually or on multiple positions. Lastly, we would esterify the carboxyl group and determine the effect on the inhibitory activity. Esterification will enhance the lipid solubility of the compounds and thus may enhance the activity of the molecules. Based on these modifications, we will be able to identify which part of the molecule is/are required for inhibitory activity. Based on the result, a second round of synthesis reactions would be performed to combine important structural features to generate even more inhibitory derivatives. Each derivative would be assayed *in vitro* using the established IDO activity assay. The activity of modified compounds would be compared to the activity of the parent compound to assess their relative activity. Compounds that display inhibitory activity would also have their IC_{50} determined, while their effects on cell viability would be assessed using a commercial MTS assay.

Our results showed that the most biologically inhibitory PGs were PGD_2 and PGA_2. It would be of great interest to study PGA_2 in detail too. PGA_2 activity is of great interest as many studies have shown anti-neoplastic and anti-proliferative effects of PGA_2 towards cancer cells (Fukushima et al. 1982b; Straus & Glass 2001).

Our research will continue by testing the effect of FAs and their biologically active metabolites in more cancer cell lines that are known to express inducible levels of IDO, for example MRc-5 (normal foetal lung), HEp-2 (larynx carcinoma), KB (oral carcinoma), 1407 (normal embryonic intestine) and WiDr (colon carcinoma) (Ozaki, Edelstein & Duch 1988). It will also be important to test tumor cells that express IDO constitutively, for example prostatic, colorectal, pancreatic, cervical carcinomas, LB1610-MEL (melanoma), MZ-PC-1 (pancreatic carcinoma) and NCI-H596 (non-small-cell lung carcinoma) (Uyttenhove et al. 2003).

Although it is accepted to use measurement of Kyn levels as an indirect measure of IDO activity, it would be important to test the effect of FAs and their biologically active metabolites on other Trp metabolites e.g. serotonin and also directly on purified IDO enzyme activity.

Studying the effect of FAs and their biologically active metabolites on IDO2 and TDO activity will be of interest to continue this study.

7.7 Limitations of this study

A limitation of this study was that we were unable to test the effects of all FAs or all PGs on the IFN-γ mediated activity of IDO-1 in human monocytes. Since we collected blood from local donors, we were limited in the number of samples available as well as conforming to the requirements of the local institutional ethical committee. Given some minor differences in the activity between THP-1 cells and monocytes, it may be useful to assess these other compounds in the future, however, it should be noted that, in most cases, the results seen in THP-1 cells were representative of human monocytes.

Our results showed that the most biologically inhibitory PGs were PGD_2 and PGA_2. However, at the present time there is little information on the receptor involved in PGA_2 activity, therefore its mechanism of action could not be studied further. There is a possibility that PGA_2 may be acting via PPAR-γ, and it would be interesting to study the involvement of PPAR-γ in the PGs effect on IDO-1 activity.

Our results showed that PGD_2 and some of its metabolites can inhibit IDO-1 induction/activity in both cell types, so it will be important to measure the concentration of these compounds in the cells before and after treatment with IFN-γ.

Although it is accepted to use measurement of Kyn levels as an indirect measure of IDO-1 activity, it would be valuable to test the effect of our hypothesised compounds on direct IDO-1 activity. However this assay requires a comparatively greater number of cells, thus limiting the total number of questions that could have been asked.

Unfortunately we could not detect *INDOL* in either THP-1 cells or human monocytes. This limited our study on the effect of FAs and their biologically active metabolites on IDO-2 activity, which is why testing other cell lines that are known to express IDO-2 may indicate further effects of FAs and their metabolites on that enzyme.

Studying the effect of FAs and their biologically active metabolites on TDO activity will be of interest to continue this study. Unfortunately, the discovery of TDO was first published in late 2012 (Platten, Wick & Van den Eynde 2012), which due to time limitations was not investigated.

7.8 Summary

We have demonstrated that AA and its COX1/2 metabolites inhibited the IFN-γ mediated induction/activity of IDO-1 in a time and dose dependent manner in both THP-1 cells and human monocytes. We partially elucidated the mechanism of action, demonstrating that AA had no effect on the expression of IFN-γ receptors. COX1/2 metabolites of AA blocked the phosphorylation of STAT1 at Tyr^{701} with a concomitant decrease in *INDO1* and IDO-1 protein expression in both THP-1 cells and primary human monocytes (Figure 7-3).

A series of FAs were found to inhibit IFN-γ mediated induction/activity of IDO in THP-1 cells. The activities of these FAs were affected by chain length, degree of saturation and the Z configuration being more active than the E configuration. Although the n-6 PUFA, AA, showed more inhibitory activity than the n-3 PUFAs, EPA and DHA, no significant differences between the two series were found. We now suggest that inhibiting the anti-inflammatory effect of IDO could be another mechanism by which the pro-inflammatory n-6 PUFAs mediate their pro-inflammatory effect, leading to enhanced inflammation.

In all, 13 PGs were found to inhibit the IFN-γ mediated induction/activity of IDO-1 in THP-1 cells and in human monocytes, without apparent toxicity. The concentration used demonstrated the pharmacological potential for using PGs to modulate IDO-1 activity in inflammation and cancer. The structure/function analysis of the biologically active PGs may lead to the design of proposed biologically active compounds. This finding demonstrates that by alteration of the structure of PGs, unique compounds can be obtained that exhibit significant effects on IDO-1.

Modulating IDO-1 immunosuppressive activity may be a new mechanism by which PGs induce their anti-neoplastic effects in cancer cells. PGD_2 and its non-enzymatic metabolites are novel inhibitors of IFN-γ mediated induction/activity of IDO-1 in THP-1 cells and human monocytes. This inhibition was at both transcriptional and translational levels with no apparent toxicity. PGD_2 mediated its effect via DP1/AC/cAMP/PKA and pCREB at Ser133 (Figure 7-4). There is cross talk between this pathway and the IFN-γ JAK/STAT signalling pathway for IDO-1 induction, which has not been reported in the literature.

While FAs and their eicosanoids are known to have many effects in both physiological and pathological conditions, this research has highlighted the importance of more active molecules by the discovery of their activity towards inhibition of IFN-γ mediated induction/activity of IDO-1 in THP-1 cells and human monocytes. This research adds further evidence to the argument that novel FAs and their derivative eicosanoids may have the potential to become effective clinical therapeutic IDO-1 modulators.

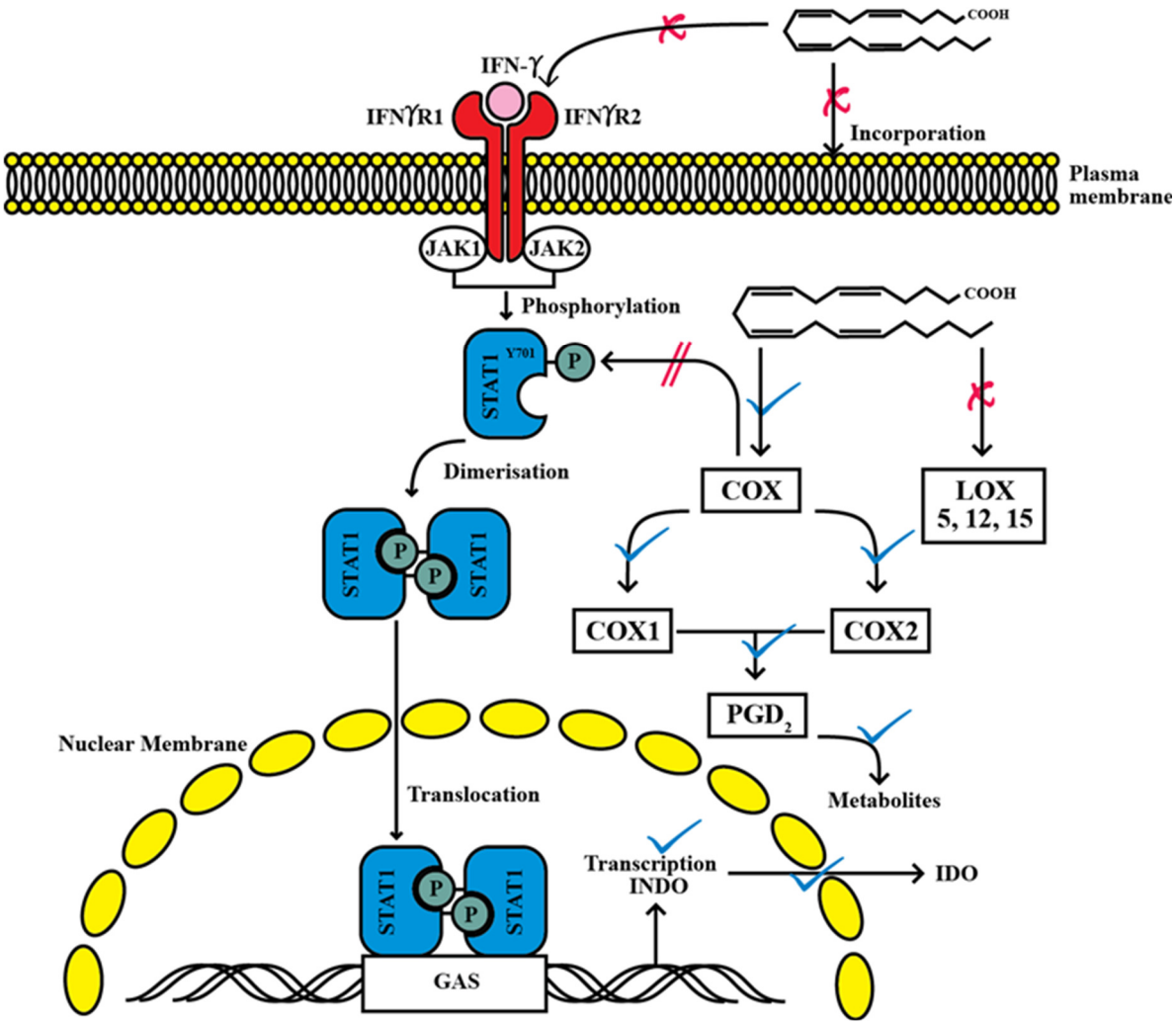


Figure 7-3 The action of AA on IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

AA has no effect on IFN-γ cell surface receptor expression and is unlikely to mediate its effects through incorporation into membrane phospholipids. The inhibitory activity of AA is mediated via COX1/2 and not LOX metabolites. The undetermined COX metabolite of AA blocks STAT1 phosphorylation. COX1/2, Cyclooxygenase metabolites1&2; COX, Cyclooxygenase pathway; GAS, TTC/ANNNG/TAA); gamma interferon activated sequence; IDO, Indoleamine 2,3-dioxygenase; IFN-γR1/2), Interferon gamma receptors 1 and 2; AK1/2, Janus kinases; LOX, lipoxygenase; P, phosphorylation; STAT1, Signal transducer activator of transcription; Y701, Tyrosine 701; ×, = not involved; ?, = not determined; ✓, = involved; //, = blocked. (Adapted from Aaronson & Horvath 2002; Platanias 2005; Bassal 2008).

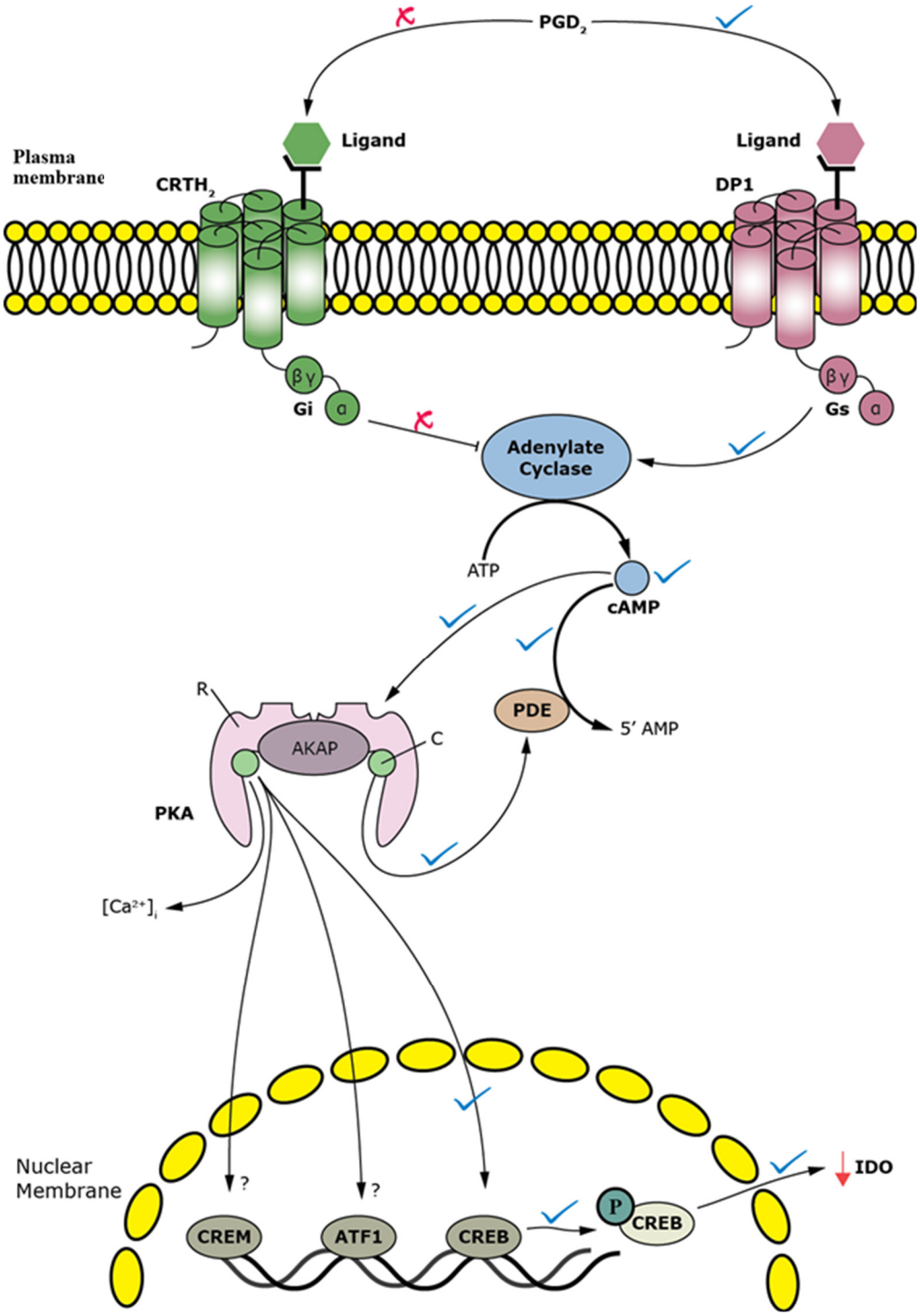


Figure 7-4 Proposed signalling pathway of the effect of PGD_2 on IFN-γ mediated induction of IDO-1 in THP-1 cells and human monocytes

PGD_2 binds to DP1 not CRTH2 receptor, activates adenylate cyclase, increases levels of cAMP, activates PKA and phosphorylate CREB. AKAP, A-kinase anchoring protein; ATF1, activating transcription factor 1; ATP, adenosine triphosphate; $Ca^{2+,}$ calcium; cAMP, cyclic adenosine monophosphate; CREB, cAMP responsive elements binding proteins; CREM, cAMP responsive elements modulator; CRTH2, chemoattractant receptor homologous molecule; DP1, D-prostanoid receptor; PDE, phosphodiesterase enzyme; 5' AMP, 5' adenosine monophosphate; PKA, protein kinase A; ✗, = not involved; ✓, = involved and ↓, = decrease. (Adapted from Murray, 2008).

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Appendices