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THE EFFECTS OF HYPOXIA ON CYCLOOXYGENASE-2 EXPRESSION AND EICOSANOID SYNTHESIS

by

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EFFECTS OF HYPOXIA ON MONOCYTE INFLAMMATORY MEDIATOR PRODUCTION: dissociation between changes in cyclooxygenase-2 expression and eicosanoid synthesis. Authors; **Demasi-M**, Cleland-LG, Caughey-GC, Cook-Johnson-RJ, James-MJ. Journal of Biological Chemistry (2003) Oct 3;278(40):38607-16.

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ABBREVIATIONS

α	alpha
AA	arachidonic acid
Ab	antibody
A	amp (s)
AD	actimomycin D
BSA	bovine serum albumin
Bq	bequerel
bp	base pairs
β	beta
°C	degrees Celsius
Ca^{2+}	calcium
CM	conditioned medium
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
CSF	colony stimulating factor
d	day (s)
Da	dalton
DPBS	dulbecco's PBS
ELISA	enzyme linked immunoassay
EP	prostaglandin E receptor
ERK	extracellular regulated kinase
FA	fatty acid

FCS	fetal calf serum
FLS	fibroblast-like synoviocytes
flt-1	fms-like tyrosine kinase-1
g	gram (s)
γ	gamma
h	hour (s)
HUVEC	human umbilical vein endothelial cells
HIF	hypoxia inducible factor
IL-1	interleukin-1
IL-1R	interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
IF	immunofluoresence
k	kilo
κ	kappa
L	litre
LPS	lipopolysaccharide
m	milli
M	molar
MAPK	mitogen activated protein kinase
MI	myocardial infarction
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MeOH	methanol
min	minute
n	nano

NF-κB	nuclear factor-kappa B
NSAID	non-steroidal anti-inflammatory drug
o/n	overnight
O_2	oxygen
p	pico
PBS	phosphate buffered saline
PG	prostaglandin
PGI_2	prostacyclin
PLA_2	phospholipase A_2
PMA	phorbol myristol acetate
PUFA	polyunsaturated fatty acid
RA	rheumatoid arthritis
RIA	radioimmunoassay
s	second (s)
SD	standard deviation
SEM	standard error of mean
STZ	serum treated zymosan
TNF	tumor necrosis factor
TIMP	tissue inhibitor metalloproteinase
TF	tissue factor
TX	thromboxane
UV	ultraviolet
μ	micro
3'-UTR	3'-untranslated region
VEGF	vascular endothelial cell growth factor

VSMC	vascular smooth muscle cell
Zn-PP	zinc protoporphyrin

SUMMARY

Low oxygen tension (hypoxia) often accompanies inflamed lesions such as those in rheumatoid joints, atherosclerotic plaques or solid tumors. Inflammatory mediators play a significant role in the perpetuation of these inflammatory conditions. Therefore, improving our understanding of the way in which hypoxia may influence their regulation, should be an aid to the design and implementation of treatments for unwanted inflammation.

To date, most in vitro studies of inflammatory mediator production are undertaken in room air, (20% O_2). However, these are not physiologically relevant conditions that inflammatory cells encounter in hypoxic (1% O_2) lesions. Therefore, the aim of this thesis was to examine the effects of hypoxia on the regulation of inflammatory mediator production in cell types that are relevant to inflamed lesions such as arthritic joints and atheromatous plaques. These include monocyte/macrophages, fibroblast-like synoviocytes and endothelial cells.

This thesis highlights the differences in the response of various cell types to hypoxic conditions and explores the potential mechanisms involved in regulating inflammatory gene expression. The results of these studies indicate that hypoxia is an important, but neglected determinant of inflammation and vascular homeostasis and demonstrates that cells respond uniquely to hypoxia, depending on their lineage. Lastly, this thesis provides a strong rationale for targeting factors which upregulate hypoxia-responsive genes, as a potential therapeutic tool in the treatment of inflammatory disease.

AUTHORS DECLARATION

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

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

Maryanne Demasi

25 / 03 / 2004

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This thesis is dedicated to my Mum and Dad

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Inflammatory lesions differ from healthy tissue in a number of ways, not least of which is a disruption of normal microvasculature with vasodilation, leucocyte transmigration, and extravasation of fluid. In an enclosed area such as a diarthrodial joint, fluid accumulation can increase intra-articular pressure. It has been demonstrated that rheumatoid joints (and non-rheumatoid joints) with effusions are hypoxic due to increased intra-articular pressure (James et al. 1990; James et al. 1992). A reciprocal relationship exists between the extent of synovial effusion and oxygen tension in the joint and it is possible that hypoxia may initiate physiological and biochemical alterations in the synovium which contribute to inflammatory synovitis (Richman et al. 1981). Similarly, the suggestion that hypoxia plays a key role in the pathological development of atherosclerotic plaques (Boxen 1985; Crawford and Blankenhorn 1991; Simanonok 1996) is derived from studies demonstrating decreased oxygen concentrations in the media of atherosclerotic plaques (Heughan et al. 1973; Bjornheden et al. 1999).

Among the key inflammatory mediators are the biologically active lipid eicosanoids, prostaglandin (PG) E_2, prostacyclin (PGI_2) and thromboxane (TX) A_2. Eicosanoids are derived from cyclooxygenase (COX) –1 and/or –2 activity, the key enzymes involved in

the oxidation of C20 fatty acids (predominantly AA) following their cleavage from membrane phospholipids by phospholipase A_2 (PLA_2) activation.

Eicosanoids have been shown to regulate the synthesis of the peptide cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-1β. These cytokines are present in high levels in synovial fluids of rheumatoid joints and in concert with matrix metalloproteinases (MMPs), have been implicated in the destructive processes of joint disease by regulating cartilage degradation and bone thinning.

The reduction of the hyperalgesia associated with PGE_2 through inhibition of COX activity is the primary therapeutic action of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) (Vane et al. 1998; Vane and Botting 1998). However, inhibition of both COX isoenzymes is associated with gastric irritation. These outcomes motivated the commercial development of selective COX-2 inhibitors, two of which, celecoxib and rofecoxib, are the market leaders (Chan et al. 1999; Fort 1999). Clinical trials have shown that these drugs achieve analgesic and anti-inflammatory effect in patients with arthritis to a similar extent to standard NSAIDs. As hoped, rofecoxib, the agent with the highest COX-2 selectivity, showed evidence of reduced gastric toxicity compared with conventional NSAIDs (Bombardier et al. 2000). However, this drug was associated with increased myocardial infarction in a long term safety study (Bombardier et al. 2000) and on a survey of events occurring during general prescribing (Ray et al. 2002).

Similarly, monoclonal antibodies targeting the action of TNFα and IL-1β have recently become available. However, whilst these latter agents have shown efficacy in reducing the destructive processes of joint disease, there are still patients who experience unsatisfactory

outcomes. Overall, much remains to be understood about the cellular biology of COX gene expression and eicosanoid synthesis, as well as the inter-relationships between eicosanoids and cytokines.

To date, much of our understanding about the regulation of eicosanoids and cytokines is derived from in vitro studies conducted in normoxic conditions (20% O_2). However, there is strong evidence demonstrating that normoxia does not reflect the conditions encountered in inflammatory lesions which are hypoxic. Thus hypoxia is a potential environmental modulator to be considered in studies characterising inflammatory mediator production. In view of this, the aim of this thesis was to examine the effects of hypoxia on COX-2 expression and activity in a highly relevant cell type involved in the inflammatory process, blood derived monocytes (Chapter 3-4). In addition, this thesis characterises the synthesis of the monocyte-derived inflammatory cytokines, TNFα and IL-1β in hypoxia and explores any inter-relationships between eicosanoids and cytokines (Chapter 5).

Following the recruitment of blood derived monocytes to a site of injury, they are capable of synthesising potent, soluble mediators to elicit an inflammatory response. Not only can these soluble mediators regulate autocrine responses in monocytes, but they may also regulate responses in adjacent cell types such as endothelial cells and synoviocytes (paracrine regulation). Therefore, this thesis characterises the effects of soluble monocyte-derived mediators on COX-2 expression and activity in synoviocytes (Chapter 6) and endothelial cells (Chapter 7-8) in hypoxic conditions.

This thesis focuses on inflammatory mediator production in hypoxia. This chapter reviews the current knowledge regarding the synthesis and regulation of eicosanoids and cytokines

and the receptors through which they exert their biological effects. Where appropriate, reference is made to the current knowledge of hypoxia as a regulatory influence over eicosanoid synthesis.

1.2 BIOSYNTHETIC PATHWAY OF EICOSANOIDS

Eicosanoids have diverse biological functions ranging from maintaining normal homeostatic states within tissues to being involved in the pathophysiology of inflammatory diseases. Some eicosanoids are highly labile due to chemical instability and others have short half-lives in vivo due to enzymatic degradation rendering them inactive. Once synthesised and secreted extracellularly, eicosanoids are thought to exert their biological effects on the cell from which they are derived (autocrine effect) or on neighbouring cells (paracrine effect). The synthetic pathways for the production of prostaglandins (PG) and thromboxane (TX) are complex. COX substrates are 20-carbon polyunsaturated fatty acids that are cleaved from membrane phospholipids following the activation of phospholipase A_2 (PLA_2), frequently by a noxious stimulus. The prime fatty acid released, arachidonic acid (AA), undergoes bisoxygenation to PGG_2 followed by a peroxidation to PGH_2. Both the reactions are catalysed by COX (DeWitt 1991; Smith et al. 1991; Smith et al. 1996; Marnett et al. 1999) - terminal synthases then convert PGH_2 to the respective eicosanoids (Fig 1.1).

1.2.1 Membrane phospholipid composition

Because the cellular phospholipid polyunsaturated fatty acid (PUFA) composition of humans can be dynamic and influenced by dietary PUFA intake, dietary PUFA can influence the production of eicosanoids (Spector and Yorek 1985).

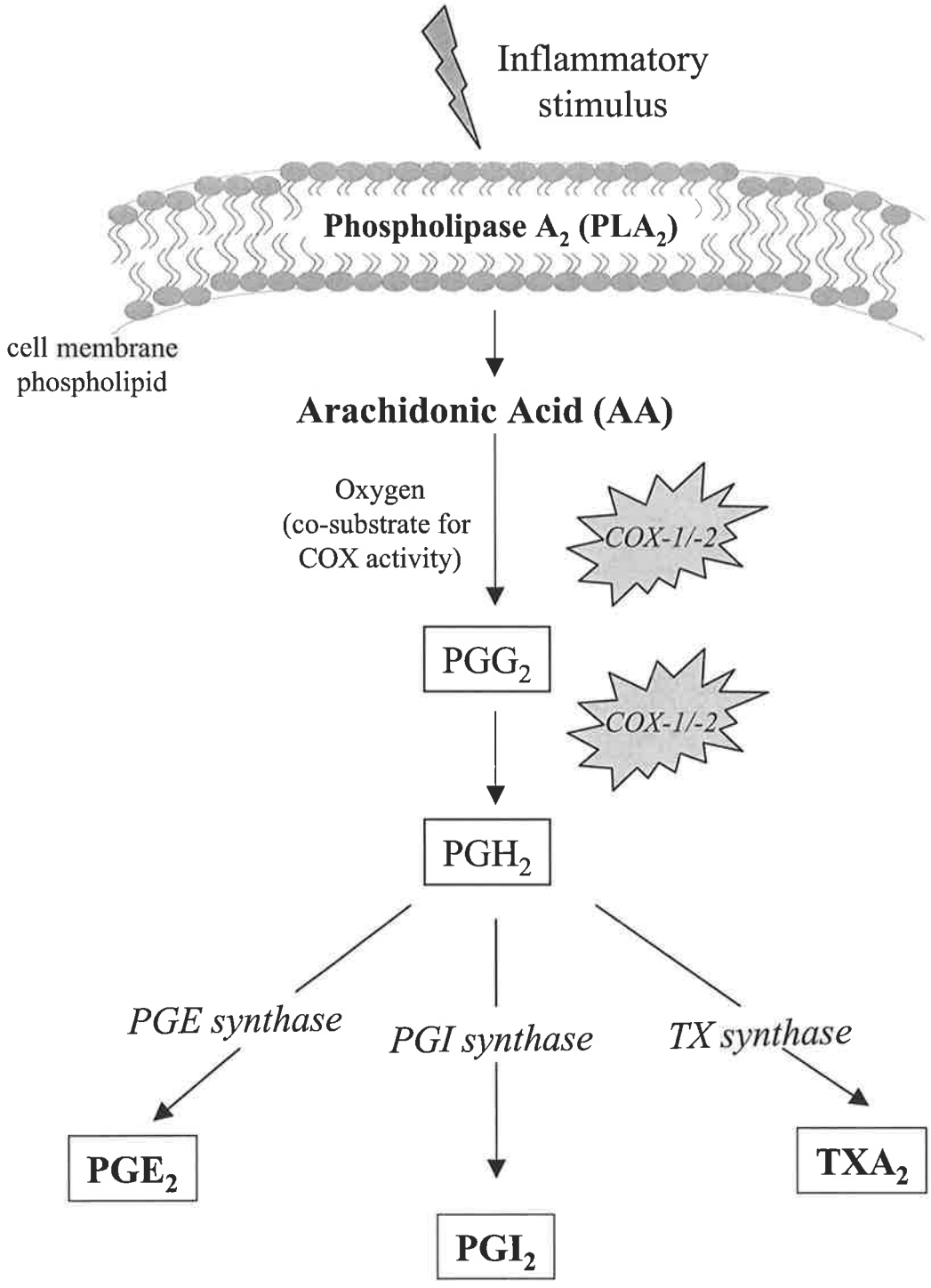


Figure 1.1
Pathway of eicosanoid biosynthesis

There are two classes of fatty acids in membrane phospholipids that may contribute to, or influence COX-mediated eicosanoid synthesis. The dietary 18-carbon n-3 fatty acid, α-linolenic acid (α-LNA) and the 18-carbon n-6 polyunsaturated fatty acid, linoleic acid (LA) can be metabolised by a series of elongase and desaturase enzymes to their respective 20 and 22 carbon members. The relative rates of conversion are determined by competition between the amount of substrate available and the relative affinity for the desaturase and elongase enzymes. Linoleic acid is the major dietary PUFA in the western diet and very little α-LNA is ingested. Due to the preponderance of n-6 PUFA in the modern Australian diet, LA is the predominant substrate for elongase and desaturase enzymes in human tissues (Fig 1.2). Therefore, the 20-carbon metabolite of LA, AA is the predominant 20-carbon PUFA in the membranes of people consuming the modern Western diet. As a consequence of this dietary n-6 fat preponderance, AA is the main COX substrate cleaved from membrane phospholipids by PLA_2.

1.2.1.1 Membrane phospholipids and hypoxia

Several reports indicate that the n-6:n-3 balance in cell membranes can alter the tissue responses to hypoxia or hypoxia followed by re-oxygenation. For example, increasing n-3 PUFA levels in cardiac membranes increases protection of cardiac tissue from hypoxic-reoxygenation injury (Hayashi et al. 1995; Oudot et al. 1995; Agnisola et al. 1996; Durot et al. 1997; O'Farrell and Jackson 1997) or ischaemia/reperfusion injury (Takeo et al. 1998). Vascular endothelial cell membranes enriched with n-3 PUFA displayed reduced eicosanoid synthesis following hypoxia-reoxygenation, compared with n-6 PUFA enriched

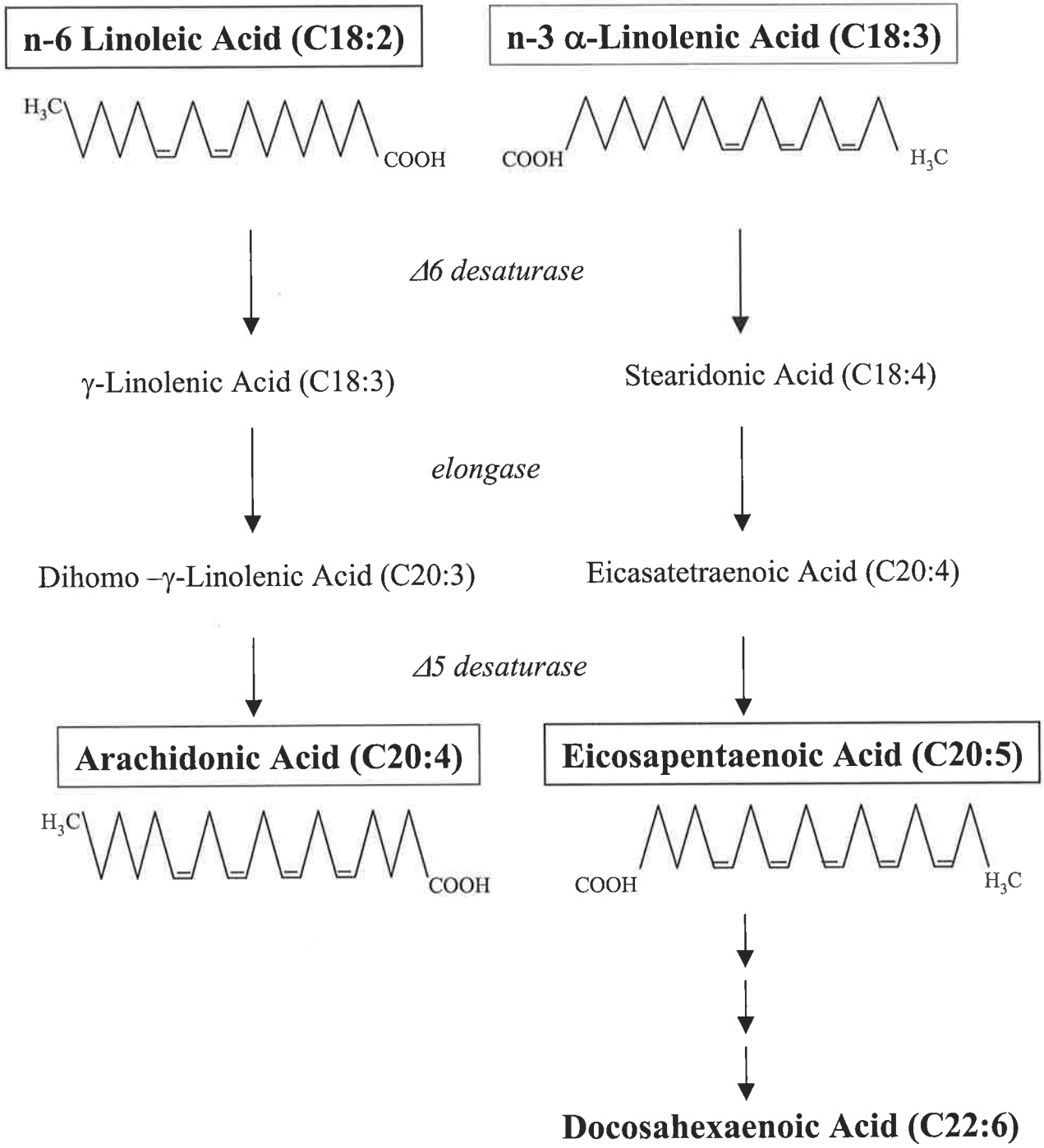


Figure 1.2
Membrane phospholipid fatty acids
The metabolism of dietary 18-carbon unsaturated fats to 20-carbon fatty acids which serve as precursors to eicosanoid synthesis.

cells (Oudot et al. 1998). This suggests that changes in dietary n-3 or n-6 PUFA may influence the response of tissues to hypoxic stress.

1.2.2 Phospholipase A_2 (PLA_2)

Following an inflammatory stimulus, activation of PLA_2 results in hydrolysis of the *sn*-2 fatty acyl bond in cellular phospholipids. As outlined in section 1.2.1, the preponderance of n-6 PUFA in the modern Western diet results in AA being the predominant fatty acid in the sn-2 position. Therefore, the main PLA_2 products are free AA and lysophospholipids (Mayer and Marshall 1993) (Fig 1.3). PLA_2 enzymes are found in most cell types, where they play a role in normal biosynthesis and remodelling of membrane phospholipids and cellular signalling. The cleavage of AA from membrane phospholipids has generally been considered the rate-limiting step in the generation of lipid-derived inflammatory mediators (Flower and Blackwell 1976; Irvine 1982).

1.2.2.1 Classes of PLA_2

PLA_2 enzymes are generally classified into three groups: secretory ($sPLA_2$), cytosolic ($cPLA_2$) and calcium independent ($iPLA_2$) (Six and Dennis 2000).

The $sPLA_2$ class is represented by a group of low molecular weight (~14kDa) enzymes that require millimolar amounts of Ca^{2+} for catalytic activity. They are selective for the *sn*-2 position but apparently indifferent to the PUFA located therein (Tischfield 1997). They contain a secretory signal peptide and their tertiary structure contains numerous disulfide bonds that render their enzymatic activities susceptible to reducing agents. Of the

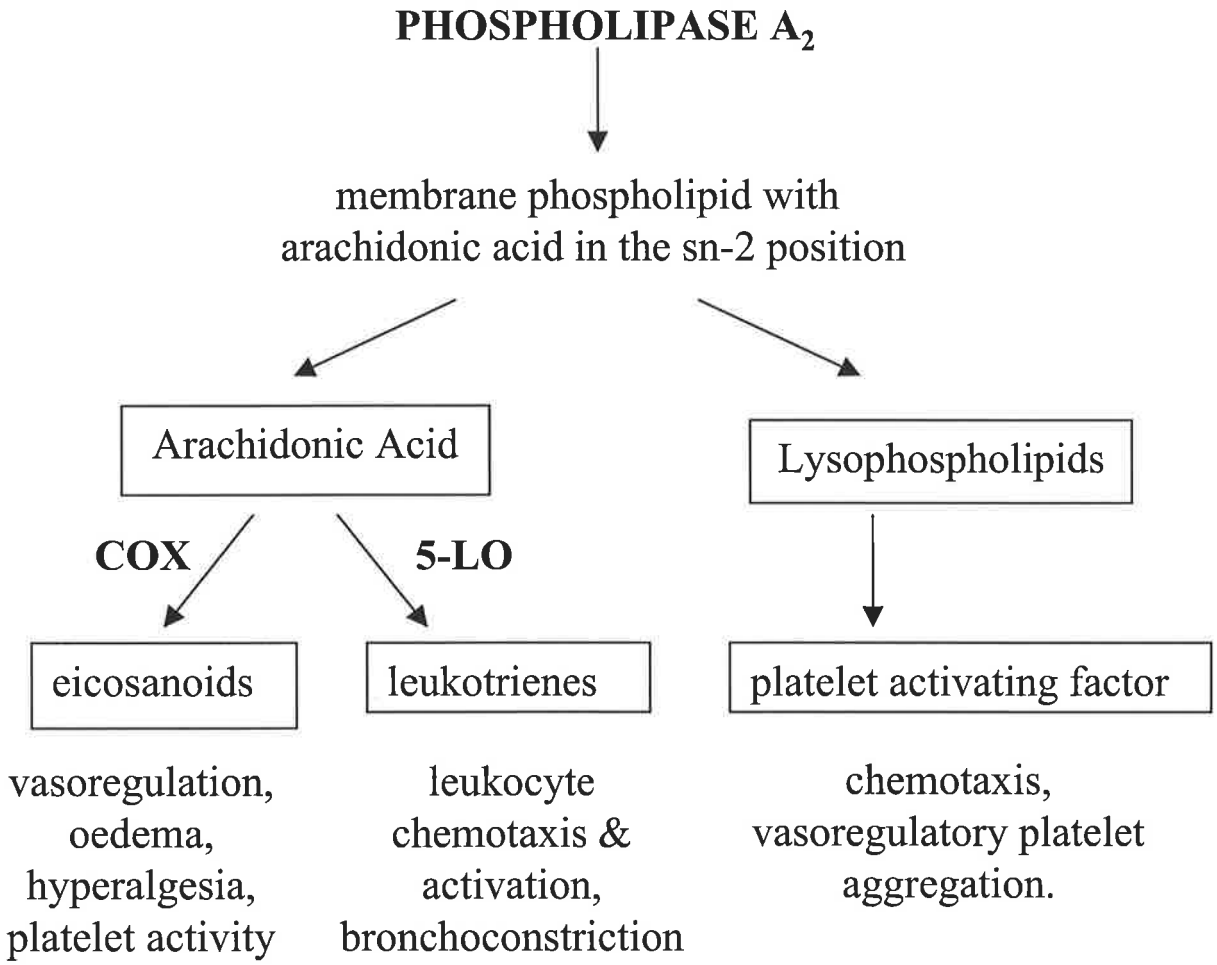


Figure 1.3
Phospholipase A_2 products

various types of $sPLA_2$ identified, type II is found in rheumatoid synovial fluid (Seilhamer et al. 1989; Bomalaski and Clark 1993).

In contrast to $sPLA_2$, $cPLA_2$ enzymes have a higher molecular weight (85-kDa) and their activity is not affected by reducing agents, reflecting the absence of disulfide bonds in their structure. In addition, these enzymes have selectivity for hydrolysis of AA and have little activity toward other fatty acids at the *sn*-2 position in phospholipids (Dennis 1994). The $cPLA_2$ enzymes require submicromolar Ca^{2+} concentrations for function. Unlike $sPLA_2$, $cPLA_2$ does not require Ca^{2+} for catalytic activity, but for translocation from its resting location in the cytosol to the perinuclear and endoplasmic reticulum membrane (Leslie 1997). Structural analysis has shown that $cPLA_2$ has been mapped to chromosome 1q25, the same region to which the gene encoding COX-2 has been mapped (Tay et al. 1994; Tay et al. 1995). Prostaglandins (Murakami et al. 1997) and cytokines, including IL-1β and TNFα (Clark et al. 1995) are able to induce $cPLA_2$ mRNA in a variety of cell types and the expression correlates with enhanced prostaglandin production.

The $iPLA_2$ class is an intracellular enzyme and does not require Ca^{2+} for catalysis. This group does not liberate AA from intact macrophages and its role is thought to be in membrane remodelling rather than directly in eicosanoid generation (Balsinde et al. 1995; Balsinde and Dennis 1997).

Both $sPLA_2$ (Barbour and Dennis 1993; Reddy et al. 1997; Kuwata et al. 1998; Bidgood et al. 2000) and $cPLA_2$ (Bonventre et al. 1997; Akiba et al. 1999; Fujishima et al. 1999; Gijon and Leslie 1999; Gijon et al. 2000) can be involved in prostaglandin production, with the

class of PLA_2 varying with cell types, the stimuli involved and the activation state of the cell.

1.2.2.2 PLA_2 and hypoxia

Several investigators have shown that exposure to low oxygen tensions causes a decrease in total intact phospholipids (Chien et al. 1978; Farber and Young 1981; Block et al. 1989) and an increase in the free fatty acids (AA) of cell membranes due to an increase in the activity of PLA_2 (Freyss-Beguin et al. 1989; Portilla et al. 1992; Kozlovsky et al. 1997). The increased AA release leads to the generation of inflammatory mediators. For example, hypoxic macrophages generate reactive oxygen species (ROS) and there is increased intracellular Ca^{2+} (Mishra and Delivoria-Papadopoulos 1999), both of which are prerequisites for $cPLA_2$ activation (Goldman et al. 1997; Woo et al. 2000; Martinez and Moreno 2001). This could ultimately lead to an increase in COX derived inflammatory eicosanoids. The experimental sections of this thesis examine the effect of hypoxia on $cPLA_2$ activation in various primary cell types.

1.2.3 Cyclooxygenase (COX) pathway

Cyclooxygenase catalyses the first two steps in the metabolism of AA to eicosanoids. There are two isoforms, COX-1 and COX-2 which are encoded by separate genes (Fletcher et al. 1992). However, both isoforms catalyse the same two reactions, i.e. the bisoxygenation of AA to PGG_2 followed by the peroxidation to PGH_2 (DeWitt 1991; Smith et al. 1991; Smith et al. 1996; Marnett et al. 1999).

1.2.3.1 Structure of COX

COX-1 (on chromosome 9) and COX-2 (on chromosome 1) have similar structures with molecular weights of approximately 66kDa and 72kDa, respectively. The two isoforms share ~61% homology (Shimokawa and Smith 1992; Herschman 1996). COX-1 and COX-2 exhibit similar enzymatic properties in that they both undergo suicide inactivation and have similar V_{max} and binding affinities (K_m) for AA (Meade et al. 1993; Barnett et al. 1994; Laneuville et al. 1994). Hydrophobic residues line the channelled active site of COX and this favours entry of the fatty acid substrate, AA. The bisoxygenation of COX adds oxygen atoms to AA to form PGG_2, and the peroxidisation activity of COX reduces the 15-hydroperoxide group of PGG_2 to form PGH_2. The heme prosthetic group of COX is essential for this 2-electron reduction.

Both isoenzymes are inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) (Meade et al. 1993; Smith et al. 1994). However, an important difference between the two isoforms is the substitution of isoleucine to valine at position 523 in COX-2 that exposes a "side-pocket" of the active site. This difference has been exploited to design drugs that are able to inhibit COX-2 selectively. These agents are discussed below in section 1.2.3.3.

1.2.3.2 Regulation and function of COX

Whilst COX-1 and COX-2 have similarities in structure, regulation of their expression is very different. COX-1 is constitutively expressed in most tissues and responsible for synthesising eicosanoids involved in general cellular homeostatic events like gastric mucosal protection. In contrast, COX-2 can be rapidly induced by inflammatory stimuli

such as cytokines, growth factors and lipopolysaccharide (LPS) and the best characterised consequence of COX-2 induction is an inflammatory response (Hempel et al. 1994; Smith et al. 2000). While COX-2 is generally not detected in most tissues under basal physiological conditions, it has been found in normal brain, kidney, testes and tracheal epithelial cells (Brooks et al. 1999). The COX-2 derived prostaglandins produced from these tissues may thus have normal physiological functions such as signalling in the brain (Cao et al. 1997), and regulation of renal perfusion (Komhoff et al. 1997). COX-2 also appears to be expressed in vascular endothelium in response to shear stress caused by laminar flow (Seibert et al. 1997; Gimbrone 1999).

Unlike COX-1 mRNA expression, COX-2 levels are reduced by glucocorticoids through mechanisms that involve reducing message stability and/or inhibition of transcription (O'Banion et al. 1991; Crofford et al. 1994). There are a variety of transcription factors that regulate the expression of the COX-2 gene. Putative transcription factor binding sites in the COX-2 promoter include those for NF-κB, NF-IL-6, AP-1, Sp1, SRE and CRE (Tanabe and Tohnai 2002).

Unlike COX-1, the mRNA of COX-2 has an extended 3' region containing multiple repeats of the sequence AUUUA. This sequence is often present in rapidly degraded and unstable mRNA species and may regulate the stability of the message (Appleby et al. 1994).

1.2.3.3 Pharmacological inhibition of COX

In the early 1970s, Vane recognized the therapeutic significance of aspirin and other NSAIDs in inhibiting COX activity and the subsequent synthesis of PGE_2 for treatment of inflammatory disease (Vane 1971). Aspirin acts by irreversibly acetylating serine 530 which is close to the active site tyrosine 385 of COX-1 which is necessary for "activation' of AA (Smith et al. 2000). This covalent binding leads to irreversible inhibition of the enzyme (DeWitt et al. 1990). Aspirin also inhibits COX-2 by a similar mechanism but with less potency (Mitchell et al. 1993) because the substrate channel of COX-2 is larger and more flexible than that of COX-1 (Kurumbail et al. 1996). For example in intact cells, aspirin is 166 times more active against COX-1 (IC_{50} = 0.3 μg/ml) than against COX-2 (IC_{50} = 50μg/ml) (Mitchell et al. 1993). I reported similar selectivity of aspirin for COX-2 in monocytes (Demasi et al. 2000). Aspirin completely inhibited bis-oxygenation of arachidonate by COX-1 whereas aspirin-treated COX-2 metabolises AA primarily to 15-hydroxyeicosatetraenoic acid (15-HETE) instead of PGH_2 (Meade et al. 1993).

COX-1 derived PGE_2 is involved in protection of the gastric mucosa (Meyer-Kirchrath and Schror 2000; Peskar 2001) and non-selective inhibition of COX activity has been associated with adverse gastric events (Meade et al. 1993; Barnett et al. 1994; Laneuville et al. 1994; Smith and DeWitt 1995). However, the distinct structural differences between the active sites of the two isoenzymes have allowed the pharmaceutical development of selective COX-2 inhibitors (Futaki et al. 1993a; Futaki et al. 1993b; Futaki et al. 1994). The more selective of these have anti-inflammatory effects with fewer upper gastrointestinal adverse events compared with traditional non-steroidal anti-inflammatory drugs (NSAIDs) (Bombardier et al. 2000). The consequences of inhibition of COX expression and activity with relevance to inflammatory disease is discussed in section 1.4.3.

1.2.3.4 Hypoxia and COX-2

Only a few studies have focused on the effects of hypoxia on COX-2 expression. In human endothelial cells, it was reported that hypoxia increased COX-2 transcription through increased binding of the transcription factor, NF-κB to one of the two binding sites in the COX-2 promoter region (Schmedtje et al. 1997). Subsequent research by the same group demonstrated that hypoxia also caused an increase in the expression of high-mobility-group protein I(Y) (HMG I(Y)) mRNA and protein (Ji et al. 1998). HMG I(Y) protein is associated with NF-κB transactivation and is thought to play a role in NF-κB mediated induction of COX-2 gene expression in hypoxia. An extension of this research showed that there is a binding site for the transcription factor, Sp1 just upstream of the NF-κB-3' element. Sp1 also plays a role in regulating COX-2 expression in hypoxia, demonstrated by the lack of COX-2 induction after mutation of the Sp1 binding site at the COX-2 promoter (Xu et al. 2000).

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that can regulate gene transcription in response to their ligands, which include prostaglandins (eg 15-deoxy-Δ-12, 14-prostaglandin J2) and NSAIDs (Forman et al. 1996; Willson and Wahli 1997). Recently, it has been reported that PPAR activation in hypoxia upregulates COX-2 expression in corneal epithelium (Bonazzi et al. 2000). Cobalt-simulation of hypoxia results in the sustained upregulation of COX-2 expression, prostaglandin synthesis and vascular endothelial cell growth factor (VEGF) expression in prostate cancer cells (Liu et al. 1999). Similar studies have shown that hypoxia or ischaemia causes upregulation of COX-2 expression in neurones (Vartiainen et al. 2001), lung (Chida and Voelkel 1996;

Matuschak et al. 1998) and cerebrum (Planas et al. 1995; Nogawa et al. 1997; Nogawa et al. 1998).

COX-2 is also found to be upregulated in rabbit models of cardiac ischemic preconditioning (IPC) (Shinmura et al. 2000). RNase protection assays were used to quantify COX-2 mRNA in myocardial tissue samples. Low but detectable COX-2 mRNA levels were present in control rabbits compared to increased levels detected at 1-3 h in IPC rabbits which returned to near control levels after 24 h (Shinmura et al. 2000). Similarly, low levels of COX-2 protein were detected in control rabbits but these were significantly increased in IPC rabbits. Increased COX-2 expression in IPC rabbits was completely prevented when animals were pretreated with a PKC inhibitor, a specific tyrosine kinase inhibitor, or a NF-κB inhibitor (Shinmura et al. 2002). These results indicate that induction of COX-2 expression in preconditioned myocardium requires PKC-, Src/Lck PTK-, and NF-κB- dependent signalling. Inhibitor studies in these animal models confirmed that COX-2 upregulation had a cytoprotective role in the myocardium and challenged the common paradigm that COX-2 activity is detrimental (Bolli et al. 2002). Analogous results to those in the rabbit were observed in mice (Guo et al. 2000), indicating that COX-2 upregulation in IPC is not species-specific. These studies demonstrate that ischemia or hypoxia associated with ischemia has the potential to influence the expression of COX-2.

1.2.4 Eicosanoids

The products of COX-1 or COX-2 activity are prostaglandins and thromboxane.

1.2.4.1 Prostaglandin (PG) E_2

PGE_2 has multiple functions and at times may have opposing effects on tissues. PGE_2 is a potent modulator of vascular and bronchial tone and stimulates bone and cartilage resorption. PGE_2 may also modulate responses by immune cells by promoting immunoglobulin (Ig) isotype switching and a type II immune response (Fedyk and Phipps 1996). PGE_2 suppresses proliferation of mature T cells (Minakuchi et al. 1990; Elliott et al. 1992) as well as inhibiting proliferation and function of B lymphocytes (DeWitt 1991) and macrophages (Ferreri et al. 1992). Exogenous PGE_2 inhibits TNFα (Knudsen et al. 1986; Kunkel et al. 1988) and IL-1[Haynes, 1992 #894; Ferreri et al. 1992; Haynes et al. 1992) production in monocytes/macrophages (Kunkel et al. 1988; Hart et al. 1989). This inhibitory effect is reported to be mediated by an increase in intracellular cAMP (Minakuchi et al. 1990; Anastassiou et al. 1992). The inhibitory effect of PGE_2 on TNFα synthesis has also been attributed to its action to inhibit signal transduction though tyrosine kinase pathways (Kolenko et al. 1999). Furthermore, PGE_2 increases soluble TNF receptors, which overall neutralises TNF activity (Choi et al. 1996). PGE_2 is produced by a variety of cell types. However, cells of the monocyte/macrophage lineage are the primary immune cells producing PGE_2.

1.2.4.1.1 PGE synthase

PGE synthase catalyses the conversion of PGH_2 to PGE_2. The activity of the enzyme is dependent on glutathione that appears to be a cofactor in the isomerization of PGH_2 (Ogino et al. 1977). Two isoforms exist; a cytosolic isoform that is a constitutively expressed 26kDa protein (Murakami et al. 2000; Tanioka et al. 2000) and an inducible isoform,

which was identified as a 15kDa protein in the membrane fraction of human microsomes of A549 cells and whose levels were increased by IL-1β (Jakobsson et al. 1999)

1.2.4.1.2 PGE receptors

PGE_2 binds to EP receptors, of which four subtypes, each encoded by distinct genes, have been identified. These receptors are designated EP_1, EP_2, EP_3 and EP_4 and are likely to account for the diverse effects of PGE_2. EP receptor activity is thought to be mediated by coupling to one of several classes of G proteins (Sugimoto et al. 1992; Nishigaki et al. 1996).

EP_1 expression is restricted to several organs including kidney, lung and stomach (Watabe et al. 1993). Selective EP_1 receptor antagonists appear to have analgesic activity (Hallinan et al. 1993), whereas EP_1 engagement causes smooth muscle constriction. By contrast, the EP_2 receptor has a functional role in smooth muscle relaxation (bronchioles) and it is suggested that EP_2 receptor agonists could be used to treat asthma and other pulmonary disorders (Gardiner 1986). Nuclease protection and northern analysis demonstrated relatively high levels of EP_3 receptor mRNA in several tissues including kidney, uterus, adrenal gland and stomach (Breyer et al. 2001). Targeted deletion of EP_3 receptors exhibit impaired febrile responses to PGE_2, suggesting that EP_3 receptor antagonists could be effective anti-pyretic agents (Ushikubi et al. 1998). EP_4 is the most recently described and is similar in function to EP_2 as it is linked to stimulation of adenylate cyclase via G_s (Nishigaki et al. 1996). However, EP_4 mRNA is more widely expressed compared with EP_2. In general, EP_2 and EP_4 receptor engagement leads to increases in cAMP whereas EP_3 receptor engagement leads to increases in intracellular Ca^{2+}.

1.2.4.2 Thromboxane A_2

Monocytes and platelets are the predominant sources of TXA_2 production in the vascular system. TXA_2 has a $t_{1/2}$ ~30 seconds under physiological conditions as it is readily hydrolysed to the stable but inactive metabolite, TXB_2. TXA_2 is important in vascular homeostasis through regulation of smooth muscle tone and its role on platelets in maintaining the integrity of the blood circulation. However, TXA_2 also plays a role in the pathology of disorders like thrombosis and asthma (Samuelsson et al. 1978; Oates et al. 1988b; Oates et al. 1988a) through its action as a platelet aggregator and bronchoconstrictor. Aside from its vascular effects, TXA_2 may facilitate monocyte TNFα and IL-1β production in an autocrine and paracrine manner (Caughey et al. 1997) and play a role in monocyte leukotaxis and adhesion to endothelial cells by increasing adhesion molecule expression (Campbell and Tolson 1988; Ishizuka et al. 1996).

1.2.4.2.1 Thromboxane synthase

Thromboxane (TX) synthase catalyses the conversion of PGH_2 to TXA_2. This 58kD enzyme is not-inducible by LPS and has a long half-life (>24h) (Orlandi et al. 1994). It is located at chromosome 7q33-34 (Miyata et al. 1994b) and shares 34-36% amino acid homology to the cytochrome P450 gene family (Haurand and Ullrich 1985; Nusing and Ullrich 1990; Yokoyama et al. 1991). In blood, TX synthase is most concentrated in platelets and monocytes (Nusing and Ullrich 1990). Organs with abundance of this enzyme include lung and liver (Nusing and Ullrich 1990).

Studies using isolated enzyme or intact platelets demonstrate that TX synthase undergoes 'suicide' inactivation (Jones and Fitzpatrick 1991). TX synthase loses activity following an association between PGH_2 substrate and the prosthetic heme group (Jones and Fitzpatrick 1991).

Although TX synthase inhibitors (i.e. imidazoles) suppress TXA_2 production, the accumulation of PGH_2 results in shunting of this substrate towards the synthesis of other eicosanoids (Gresele et al. 1991). Clinical trials with TX synthase inhibitors as anti-thrombotic agents have had little success (Fiddler and Lumley 1990). This is thought to be due to the accumulation of PGH_2, which is equally as potent as TXA_2 at inducing platelet aggregation and vasoconstriction of VSMC and has a higher affinity for the TX receptor than TXA_2 (Gresele et al. 1991).

1.2.4.2.2 TX receptor

TX receptors are highly expressed in vascular endothelium, monocytes, thymocytes and platelets (Namba et al. 1992; Ushikubi et al. 1993; Allan and Halushka 1994; Borg et al. 1994). The cellular response to TXA_2 is regulated by phosphorylation of the receptor (Borg et al. 1994) and both platelets and monocytes respond to TX receptor agonists with an increase in intracellular free Ca^{2+} (Allan and Halushka 1994).

Dual TX synthase inhibition and TX receptor blockade has provided a superior anti-thrombotic strategy and this combination was trialed in animals and humans and were effective and safe in reducing thrombosis and arrhythmias (De Clerck et al. 1989a; De Clerck et al. 1989b; Salvati et al. 1994).

1.2.4.3 Prostacyclin (PGI_2)

Prostacyclin (PGI_2) is predominantly synthesised by vascular endothelial cells (Zimmerman et al. 1990) and it has an important function in vascular homeostasis as a vasodilator and inhibitor of platelet aggregation. PGI_2 has a short half-life of 5 to 10 min (Sinzinger and Weber 1988) and the stable chemical hydrolysis product is 6-keto-prostaglandin $F_{1\alpha}$, which is inactive. PGI_2 elicits a strong vasodilating effect and platelet aggregation inhibitory effect through an increase of the cAMP levels mediated via its G protein-coupled receptor (Moncada and Vane 1979). PGI_2 actions oppose those of TXA_2 and the balance between these two eicosanoids contributes to the maintenance of normal vascular homeostasis. A disrupted ratio of PGI_2 and TXA_2 has been associated with the development of atherosclerosis (Sinzinger et al. 1991).

Intra-arterial and intravenous injection of PGI_2 or its stable analogues (iloprost, beraprost) are effective in vasodilatation and platelet aggregation inhibition. Therefore, these analogues have been used for the therapy of peripheral vascular occlusive disease (Staben and Albring 1996) and peripheral circulatory dysfunction due to heart failure (Califf et al. 1997).

1.2.4.3.1 Prostacyclin synthase

PGI synthase catalyses the conversion of PGH_2 to PGI_2 and is a member of the cytochrome P450 superfamily (Wang and Chen 1996). PGI synthase is widely expressed in tissues such as ovary, skeletal muscle, lung, prostate, and particularly in vascular endothelial and

smooth muscle cells (Hara et al. 1994; Miyata et al. 1994a; Tone et al. 1997). These results suggest a variety of physiological roles of PGI_2 in addition to those in the vascular system.

Studies have demonstrated that cytokines IL-1, IL-6 (Miyata et al. 1994a) and TNFα (Hara et al. 1994) can stimulate mRNA expression of PGI synthase. Several putative potential binding sites for transcription factors have been located, including shear stress responsive element, NFκB, Sp1 and AP2 binding sites (Frangos et al. 1985; Wang and Chen 1996).

1.2.4.3.2 Prostacyclin receptor

The IP receptor is present on smooth muscle cells and platelets (Coleman et al. 1994), indicating that PGI_2 acts to modulate the functions of these two cell types (Majerus 1983). Although IP receptors are present on arterial media, PGI_2 is not synthesised constitutively and PGI_2 does not appear to regulate basal vascular tone (FitzGerald et al. 1983). However, murine knockouts of the IP receptor demonstrate an anti-thrombotic as well as an anti inflammatory role for PGI_2 (Murata et al. 1997).

The IP receptor is coupled to adenylate cyclase to increase cyclic AMP levels in VSMCs (Kukovetz et al. 1979). Elevated cyclic AMP stimulates ATP-sensitive K^+ channels causing hyperpolarization of the cell membrane (Parkington et al. 1999) and extrusion of Ca^{2+} from the cytosol (Bukoski et al. 1989; Abe and Karaki 1992) which results in the inhibition of contractile machinery in VSMCs.

1.2.4.4 Eicosanoids and hypoxia

A central feature of tissue injury in hypoxia results from an exaggerated inflammatory response due in part to the production of pro-inflammatory eicosanoids. There are inconsistencies in the literature regarding the level of eicosanoids produced during hypoxic exposure. While there is a relative paucity of studies reporting the effects of hypoxia on eicosanoid synthesis in monocytes and fibroblast like synoviocytes, there are some relevant studies performed on endothelial cells and other cells types summarised below.

In endothelial cells, hypoxic exposure resulted in an increase in cytosolic Ca^{2+} concentration, PLA_2 activity and increased prostaglandin synthesis, which was sensitive to COX inhibitors (Michiels et al. 1993). Following reoxygenation, eicosanoid synthesis decreased to basal levels. Similarly, exposure to hypoxia in rat mesangial cells resulted in an increase in PGE_2 (Kurtz et al. 1985; Kurtz et al. 1986) and an increase in TXA_2 / PGI_2 production in human term villous trophoblasts (Blumenstein et al. 2001). Conversely, in rheumatoid synovial fibroblasts, PGE_2 production was unchanged during hypoxia (McGough et al. 1997). Rabbit corneal epithelial cells exposed to hypoxic condition resulted in a significant reduction in PGE_2 synthesis despite upregulated COX-2 protein (Bonazzi et al. 2000). The authors attributed this dissociation in COX-2 protein amount and enzymatic activity to a reduction in heme stores as a result of upregulated heme oxygenase-1 activity in hypoxia (Bonazzi et al. 2000). In rabbit myocardium subjected to hypoxia followed by reperfusion, there was a significant increase in PGI_2 and PGE_2, which was completely inhibited when rabbits were administered the selective COX-2 inhibitors NS-398 or celecoxib (Shinmura et al. 2000)). Interestingly, there was no significant

change in the level of TXA_2 with hypoxia or COX-2 inhibitors, suggesting that TXA_2 was a result of constitutive COX-1 activity (Shinmura et al. 2000).

Farber *et al*, investigated the effect of acute versus chronic hypoxia in endothelial cells (Farber and Barnett 1991). Cells that were exposed acutely to hypoxia, rapidly and transiently increased eicosanoid production. In comparison, cells that were exposed chronically to hypoxia displayed suppressed eicosanoid production (while demonstrating no evidence of cellular injury) (Farber and Barnett 1991).

Thus, the current understanding of eicosanoid synthesis in hypoxia remains incomplete. The conflicting results may be attributed to different cell types, methods of inducing hypoxia, the length of hypoxic exposure and the extent of hypoxia. This thesis attempts to improve the understanding of eicosanoid synthesis under hypoxia conditions in three cell types i.e. monocytes, endothelial cells and fibroblast like synoviocyte, which are relevant to inflammatory diseases such as RA and cardiovascular disease.

1.3 CYTOKINES

Cytokines are synthesised by most nucleated cells (Billingham 1987). They are secreted peptides involved in pivotal biological processes such as cell growth and activation, inflammation, immunity, cell differentiation and angiogenesis. The cytokines, tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) and vascular endothelial cell growth factor (VEGF) and the receptors through which they exert their effects are discussed in the following section.

1.3.1 Tumor Necrosis Factor-α (TNFα)

The TNF family of peptides includes TNFα that has a wide range of biological effects in inflammatory diseases including upregulation of endothelial and leukocyte adhesion molecules, stimulation of the inflammatory responses of leukocytes and other cells (Beekhuizen et al. 1990; Takahashi et al. 1994; Takahashi et al. 1996a) and cartilage degradation and bone resorption (Lam et al. 2002).

TNFα is synthesised initially as a large transmembrane precursor (26kDa) (Kriegler et al. 1988; Jue et al. 1990; Niehorster et al. 1990) and proteolytic cleavage of the hydrophobic extracellular domain initiates release of the 17kDa TNFα peptide (Kriegler et al. 1988). The biologically active form of TNFα is a trimeric complex of the 17kDa peptides (Smith and Baglioni 1987).

TNFα is secreted predominantly by activated cells of the monocyte/macrophage lineage in response to an inflammatory stimulus such as bacterial endotoxin, other cytokines or phagocytic stimuli (Collart et al. 1990). The expression of the TNFα gene is regulated by transcription factors which bind to regulatory elements in the promoter region of the gene including NF-κB (Collart et al. 1990), Egr-1 (Kramer et al. 1994), AP-1 (Rhoades et al. 1992) and NF-IL-6 (C/EBPβ) (Pope et al. 1994). For example, LPS initiates TNFα transcription by inducing binding of NF-κB to the TNF promoter with TNFα mRNA appearing within 15min of stimulation. TNF mRNA may be equally rapidly degraded (Dinarello 2000).

1.3.1.1 TNF receptors

The biological response to TNF is mediated through two distinct TNF receptors, TNF-RI (p55) and TNF-RII (p75) (Dembic et al. 1990; Loetscher et al. 1990). Receptor-ligand interaction is thought to lead to a trimeric cluster of TNF receptors per one TNFα trimer to initiate signal transduction (Banner et al. 1993). TNF receptors are present on most cell types, although not on erythrocytes. Expression of TNF-RI is constitutive whereas TNF-RII can be induced by mitogens, cAMP and LPS (Rothe et al. 1993). Both TNFα receptors bind TNFα with high affinity and even cells that possess only one of the two receptors are still fully responsive to TNFα.

Soluble forms of the receptors are released from cells after proteolytic cleavage of the receptors extracellular domain by a metalloproteinase (also known as TNFα converting enzyme). Soluble TNF receptors have been detected in biological fluids in both normal

and pathological conditions such as RA (Novick et al. 1989; Cope et al. 1992; Roux-Lombard et al. 1993). The effects of soluble TNFα receptors appear to depend on their concentration. For example, at low concentrations, soluble TNF receptors can stabilise the bioactivity of circulating TNFα (Aderka et al. 1992), compared to high concentrations that down regulate surface receptors and can inhibit bioactivity of TNFα by competitive ligand binding (Higuchi and Aggarwal 1992).

1.3.2 Interleukin-l (IL-1)

Like TNFα, IL-1α and IL-1β are both synthesised as high molecular weight precursor molecules (pro-IL, 30kDa) which require cleavage by specific proteases to achieve their mature forms (17kDa) (Dinarello 1996). Mature IL-1β appears to be a product of a protease called IL-1β converting enzyme (ICE) (Cerretti et al. 1992) which does not cleave IL-1α. As a result, IL-1α remains largely cell associated while IL-1β is the predominant circulating form and will be discussed further.

Similar to TNFα, IL-1β mediates inflammatory processes and is predominantly produced by cells of the monocyte/macrophage lineage (Dinarello 1996). LPS can trigger IL-1β transcription with an increase in IL-1β mRNA levels up to 4h, after which there is a rapid decrease following the synthesis of a transcriptional repressor (Fenton et al. 1987; Fenton et al. 1988). Transcription factors that regulate the expression of IL-1β include those that act via the NF-κB and AP-1 binding sites (Jung et al. 2002).

Another member of the IL-1 family that evolved as a single peptide is IL-1ra which is a naturally occurring receptor antagonist and shares ~26% amino acid homology to IL-1β. IL-1ra binds to cell receptors with similar affinity to IL-1β but does not transduce an intracellular signal, thereby acting as an antagonist (Hannum et al. 1990).

1.3.2.1 IL-1 Receptors

There are two receptors that bind IL-1. Firstly, there is IL-1RI (80kDa), which binds IL-1β with low affinity. However, following the binding of an accessory protein named IL-1R-AcP, this results in a high affinity binding of IL-1 to the IL-1 RI-IL-1R-AcP complex which then initiates an intracellular signal (Greenfeder et al. 1995). Secondly, IL-1 RII (68kDa), although it does not conduct an intracellular signal, binds IL-1β with high affinity. IL-1RII appears to act as a decoy receptor and by competitively inhibiting IL-1 RI binding, it antagonises IL-1β actions (Colotta et al. 1994).

Soluble forms of both IL-1 receptors, termed IL-1sRI and IL-1sRII, circulate in healthy and diseased tissue and function as natural buffers binding all members of the IL-1 family (Giri et al. 1990; Symons et al. 1991; Dinarello 1996). Elevated levels of these two receptors have been detected in inflammatory synovial fluids (Arend et al. 1994) and patients with sepsis (Giri et al. 1994). Soluble IL-1 receptors act as antagonists by competing for IL-1β binding with cell surface IL-1 receptors.

1.3.3 Vascular Endothelial Cell Growth Factor (VEGF)

VEGF was originally named and identified as vascular permeability factor (VPF) secreted by malignant cells with the ability to render microvasculature hyperpermeable and thereby increase fluid accumulation in tissues (Senger et al. 1983; Yeo et al. 1993). VPF, later named VEGF, was also reported to act as a potent endothelial cell mitogen involved in angiogenesis (Connolly et al. 1989a; Ferrara et al. 1992), wound healing (Frank et al. 1995; Elcin et al. 2001), cardiac vascularization (Giordano et al. 2001) and pannus formation in rheumatoid joints (Fava et al. 1994; Kasama et al. 2000; Kasama et al. 2001).

VEGF is encoded by a single gene but as a result of alternate mRNA splicing, there are four isoforms of 121, 165, 189 and 206 amino acids (Connolly et al. 1989b; Houck et al. 1991). By virtue of its amino acid sequence homology, VEGF is considered to be a member of the platelet-derived growth factor (PDGF) family (Conn et al. 1990). The bioactivities among VEGF isoforms may differ. For example, VEGF 165 has greater endothelial mitogenic activity compared with VEGF 121 (Neufeld et al. 1999). Most cell lines investigated express predominantly the 165 or the 189 amino acid isoforms of VEGF (Ferrara et al. 1991). VEGF has been shown to be induced by a variety of factors such as IL-6 (Cohen et al. 1996), IL-1β (Li et al. 1995), growth factors such as TGFβ (Dolecki and Connolly 1991; Pepper et al. 1993; Pertovaara et al. 1994), prostaglandins (Harada et al. 1994; Ben-Av et al. 1995; Hoper et al. 1997; Cheng et al. 1998; Pai et al. 2001), glucose deprivation (Satake et al. 1998) and hypoxia (Shweiki et al. 1992; Ladoux and Frelin 1993; Banai et al. 1994; Minchenko et al. 1994; Jackson et al. 1997).

1.3.3.1 VEGF Receptors

The biological action of VEGF is not only regulated at the level of VEGF production, but also at the level of VEGF receptor expression. VEGF exerts its biological effects by interacting with membrane receptors, VEGF receptor-1 (flt-1) and VEGF receptor-2 (KDR/flk-1) and the recently identified neurophilin-1 (Ikeda et al. 2000). These receptors are predominantly expressed in microvascular endothelial cells and synovial fibroblasts (de Vries et al. 1992; Terman et al. 1992; Fava et al. 1994; Lu et al. 2000). Resting monocytes express low levels of the flt-1 receptor gene. However, brief exposure to LPS led to a significant upregulation of the flt-1 mRNA (Barleon et al. 1996). In addition, these monocytes showed upregulated binding for VEGF 165, suggesting responses to VEGF are mediated via the flt-1 receptor (Barleon et al. 1996; Clauss et al. 1996; Sawano et al. 2001).

VEGF 165 and VEGF 121 can bind with high affinity to flt-1 and KDR receptors but neurophilin-1 is an isoform specific receptor for VEGF 165. Neurophilin-1 acts to enhance the bioactivity of VEGF by increasing the binding affinity of VEGF 165 to KDR (Soker et al. 1998). Hence, expression of VEGF 165 and its receptors KDR and neurophilin-1 may contribute to VEGF induced angiogenesis in tissue hypoxia. Receptor antagonists have potential to reduce VEGF-stimulated receptor autophosphorylation and proliferation of endothelial cells (Siemeister et al. 1998).

1.3.4 Cytokines in hypoxia

Numerous studies have examined the effects of hypoxia on the regulation of cytokine production. In human macrophages, it has been reported that mRNA, protein and/or release of TNFα (Scannell et al. 1993; Hempel et al. 1996; Scannell 1996; Guida and

Stewart 1998; Leeper-Woodford and Detmer 1999; Chandel et al. 2000) and soluble receptors (Scannell et al. 1993) are upregulated by hypoxia or by hypoxia-reoxygenation (Tamion et al. 1999). Recently, it was reported that the upregulation of TNFα gene expression is regulated transcriptionally due to enhanced nuclear binding of NF-κB in hypoxia (Leeper-Woodford and Detmer 1999; Chandel et al. 2000) and is dependent on ROS (Chandel et al. 2000). Conversely, hypoxic exposure combined with glucose deprivation in human monocytes resulted in a decreased mRNA and protein level of TNFα (Guida and Stewart 1998).

IL-1 mRNA and protein levels are upregulated in mononuclear phagocyte cultures following hypoxia and reoxygenation. When the culture supernatants were added to endothelial cells, there was increased expression of tissue factor and enhanced endothelial adhesiveness for neutrophils (Koga et al. 1992). In addition to monocytes, IL-1β (and/or TNFα) is reported to be induced by hypoxia in VSMCs (Cooper and Beasley 1999), synovial fibroblasts (Berse et al. 1999), neurones (del Zoppo et al. 2000; Liu et al. 2000), cardiac myocytes (Nakano et al. 1998) and epithelial cells (Taylor et al. 1998; Taylor et al. 1999). However, it was reported recently that IL-1β mRNA and protein synthesis was down-regulated following brief exposure to hypoxia in murine macrophages (Ndengele et al. 2000). Overall, hypoxia appears to up-regulate TNFα and IL-1β synthesis although different *in vitro* regimens of hypoxia may produce different effects on cytokine production.

Pathological angiogenesis is recognised as a fundamental component of the pannus development in RA (Koch et al. 1994; Koch 1998). In view of the fact that VEGF is a potent inducer of angiogenesis and that angiogenic responses are elicited under hypoxic

conditions, VEGF expression in hypoxia has been widely investigated. VEGF induction is very responsive to hypoxia (Shweiki et al. 1992; Goldberg and Schneider 1994) and low oxygen tension can induce its synthesis in a variety of cells types including endothelial cells (Namiki et al. 1995) fibroblasts (Jackson et al. 1997; Berse et al. 1999) and monocytes (Bottomley et al. 1999). The expression is mediated by a heterodimeric transcription factor, hypoxia inducible factor-1 (HIF-1), the level of which is increased when cells are exposed to hypoxia (Forsythe et al. 1996). The regulation of HIF-1 is discussed in more detail in section 1.6.2.1. Preclinical studies have employed different strategies to antagonize VEGF action, including the use of VEGF neutralising antibody, the use of soluble versions of VEGF receptors and the design of inhibitors of the VEGF receptor II tyrosine kinase. For example, treatment with a monoclonal antibody specific for VEGF inhibited the growth of three human tumor cell lines in nude mice (Kim et al. 1993). These findings suggested that inhibition of VEGF, which is spontaneously produced by tumor cells, can suppress tumor growth in vivo. Furthermore, systemic administration in mice of the potent and selective inhibitor of the Flk-1/KDR receptor tyrosine kinase, SU5416, was shown to inhibit subcutaneous tumor growth of cells derived from various tissue origins (Fong et al. 1999). These findings give impetus to pharmacological inhibition of the enzymatic activity of the VEGF receptor as a strategy for limiting the growth of a wide variety of tumor types.

This thesis examines the effects of pathologically relevant levels of hypoxia on cytokine production in monocytes, and investigates autocrine / paracrine roles they may have in the regulation of inflammatory eicosanoid production.

1.4 INFLAMMATORY DISORDERS

Inflammation consists of a series of normal biochemical and cellular responses to tissue injury. In disordered inflammation, the inflammatory reactions appear to have overcome normal regulation resulting in exaggerated inflammation and tissue destruction. Thus, in chronic inflammatory diseases like rheumatoid arthritis (RA), prolonged inflammation can lead to progressive joint damage, deformity and joint failure. Inflammation also appears to play a central role in the pathogenesis of atherosclerosis that is increasingly being described as an inflammatory disease. The following sections will focus on two inflammatory disorders, RA and atherosclerosis, and the relevance of a hypoxic environment in these inflammatory conditions, along with the inflammatory mediators likely to orchestrate clinically significant events.

1.4.1 Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease characterised by inflammation of diarthrodial joints that can lead to structural failure in multiple joints. Prevalence is estimated to be between 1-2% of Western populations, with the ratio of affected men to women being approximately 1:2. Mortality in patients with RA is increased due to cardiovascular events, infections and treatment-related illnesses (Callahan and Yelin 1995; Pincus and Callahan 1995).

Although RA is widely considered an autoimmune disease for which the initiating factors and putative self-targets have not been identified, the possibility of bacterial or viral infections initiating the disease has received considerable attention without positive

identification of a causal organism. Genetic studies have indicated that the severity of RA has an association with the presence of a conserved sequence of alleles of the HLA-DRβ1 gene and in particular in the HLA-DR4 and HLA-DR1 subtypes (Jawaheer et al. 1994; MacGregor et al. 1995). While the presence of this sequence does not assure development of the disease, the association suggests that presentation of processed antigen in the peptide binding groove of MHC class II molecules is likely to be involved in the pathogenesis.

1.4.1.1 Pathology of rheumatoid arthritis

The joint pathology of RA typically involves inflammation localised in the synovial lining. There are two cell types that constitute the synovial lining, which in its healthy state, is a monolayer. These are type A synoviocytes, which have phagocytic macrophage type features with microvilli and cytoplasmic vacuoles, and the mesenchymal type B cells, which are fibroblast-like and rich in endoplasmic reticulum (Mapp and Revell 1988; Stevens et al. 1990). In RA, the synovial lining becomes thickened due to synoviocyte proliferation and infiltration of inflammatory cells such as lymphocytes and blood-derived monocytes. Monocytes transform into macrophages and interactions with thickened synoviocyte lining are associated with increased collagenase and inflammatory mediator production (Dayer et al. 1979). The hyperplastic synoviocyte lining, called an inflammatory pannus, invades cartilage and peri-articular tissue, eventually resulting in irreversible joint damage and loss of function.

1.4.1.2 Synovial hypoxia

The hypoxic nature of the RA synovium was originally suggested on the basis of measurements of oxygen tensions within the inflamed joint cavity. Synovial fluids from patients with a range of arthritides were collected and measured for oxygen tension (Falchuk et al. 1970; Lund-Olesen 1970). The lowest pO_2 values were found in patients with RA, in whom the mean pO_2 concentration was ~26mm Hg (Lund-Olesen 1970). It was suggested that the measurement of oxygen tension in aspirated synovial fluid did not reflect the situation in the synovial tissue. However, fine polarographic needle electrodes have been used to measure O_2 tensions directly in the knee synovium and peri-articular tissue of patients with RA and OA as well as normal subjects. The lowest O_2 tensions in these tissues were found in the RA group (Ellis et al. 1994).

Synovial hypoxia is believed to be due to increases in intra-articular pressure (IAP). A healthy joint has slightly negative IAP and very little free fluid. However, associated with the typical joint effusion seen in patients with rheumatoid arthritis is an increase in IAP, particularly during everyday activities such as standing, walking, and even modest flexion (James et al. 1990). When IAP exceeds synovial capillary perfusion pressure, there is ischaemia, possibly episodic (Jayson and Dixon 1970a; Jayson and Dixon 1970b; Gaffney et al. 1995) and chronic (James et al. 1990) leading to hypoxia. Unsworth et al demonstrated that exercise of the RA joint is associated with large increased in IAP and correlated with significant falls in pO_2 levels (Unsworth et al. 1988). James et al also demonstrated that IAPs of greater that 45mmHg correlated with increased synovial fluid lactate concentrations and pCO_2 levels and decreased pH (James et al. 1990). A fall in pH and rise in pCO_2 and lactate concentration, correlated with histological changes of synovial proliferation, focal necrosis and focal obliterative microangiopathy (Falchuk et al. 1970).

In addition to a decrease in the supply of O_2 due to tamponade of blood vessels by high IAP, there is also a functional hypoxia due to increased metabolic demand in the presence of synovitis. In vitro studies have shown that rheumatoid synovium has a 10 to 20-fold increase in oxygen utilisation compared with that of normal synovium per mg of tissue (Dingle and Page-Thomas 1956). Similarly, the in vitro lactate production by rheumatoid synovium has been found to be between 3 to 6-fold that of normal synovium per mg of tissue (Dingle and Page-Thomas 1956; Roberts et al. 1967). The increased number of cells in the synovial fluid of patients with RA would also require greater quantities of oxygen. Thus, despite evidence from ^{133}Xe washouts for increased local circulation in the rheumatoid joint (Goetzl et al. 1971), it was concluded that the flow of blood does not change proportionately with the total increase in metabolic demand. The overall increase in blood flow is therefore insufficient to meet the demand of the synovial tissue.

This 'perfusion-demand mismatch' may alter the expression of inflammatory genes relevant to the synovial hyperplasia and leukocyte infiltration seen in RA joints. For example, Kourembanas et al showed that hypoxia induces the expression and secretion of endothelin-1 (ET-1) and platelet derived growth factor (PDGF) which act as potent mitogens for synovial fibroblasts (Kourembanas et al. 1991). Exposure of endothelial cells to hypoxia results in the upregulation of IL-8 gene expression (Karakurum et al. 1994) and VEGF (Schmedtje et al. 1997). Furthermore, hypoxic endothelial cells have an increased ability to synthesise platelet activating factor (PAF) and express ICAM-1 resulting in enhanced adhesiveness of hypoxic endothelium for leucocytes (Arnould et al. 1993; Arnould et al. 1994; Arnould et al. 1995). More recently, Arnould et al, reported that endothelial cells are stimulated by hypoxia to release prostaglandin $F_{2\alpha}$ which acts as a chemoattractant for neutrophils (Arnould et al. 2001). Mononuclear phagocytes exposed

to hypoxia have shown increases in tissue factor expression that resulted in fibrin formation (Lawson et al. 1997). In addition, hypoxia has been reported to increase IL-1 and TNF in peripheral mononuclear cells after exposure to endotoxin (Ghezzi et al. 1991). Melillo et al, recently demonstrated that hypoxia induces VEGF mRNA and protein levels in human monocytes (Melillo et al. 1999).

Overall, there is evidence that hypoxia is present and could play a role in the perpetuation of an inflammatory response in rheumatoid joints.

1.4.1.3 Eicosanoids and arthritis

PGE_2 (Robinson et al. 1975; Robinson et al. 1979; Blotman et al. 1980), 6-keto-$PGF_{1\alpha}$ and TXB_2 (Salmon et al. 1983) have been found in the synovial fluid of patients with RA (Henderson and Higgs 1987; Sano et al. 1992). IL-1β action on synovial fibroblasts in rheumatoid joints induces $cPLA_2$ and COX-2, resulting in an increase in PGE_2 production (Hulkower et al. 1994). PGE_2 contributes to pain and swelling during inflammation through induction of hyperalgesia and increased vascular permeability (Portanova et al. 1996) and modulates bone resorption through stimulation of osteoclast formation from precursor monocytic cells (Lader and Flanagan 1998). In rat models of arthritis, increased levels of PGE_2 were related to increased COX-2 mRNA and protein. Treatment with a selective COX-2 inhibitor reduced COX-2 mRNA expression and protein and reduced PGE_2 levels in rat paws to baseline levels (Anderson et al. 1996) and inhibited acute and chronic inflammation in rat adjuvant arthritis models (Simon 1999). This suggests that selective COX-2 inhibitors may interrupt an autocrine positive feedback loop, where PGE_2 upregulates COX-2 expression at sites of inflammation.

In addition, there has been considerable evidence demonstrating that eicosanoids can modulate cytokine synthesis. For example, exogenous PGE_2 was shown to inhibit monocyte TNFα production (Kunkel et al. 1988; Ferreri et al. 1992; Haynes et al. 1992) or IL-1 production in mouse peritoneal macrophages (Brandwein 1986). In contrast, TXA_2 synthesis is reported to be a facilitator of TNFα production (Caughey et al. 1997). The significance of several cytokines in RA is discussed in the following section.

1.4.1.4 Cytokines and arthritis

TNFα and IL-1β are considered to be important cytokines in RA. TNFα protein is readily detected in synovial fluids and increased levels of mRNA have been detected in the rheumatoid synovium (Di Giovine et al. 1988; Saxne et al. 1988). TNF receptor expression is also increased in rheumatoid tissues (Alsalameh et al. 1999). Furthermore, mononuclear cells isolated from rheumatoid arthritic joints showed increased expression of TNFα and receptor mRNA compared with normal or RA peripheral blood MNC (Brennan et al. 1992; Deleuran et al. 1992). The notion that TNFα has an important role in rheumatoid joints was supported initially by investigations in animal models of arthritis. Administration of exogenous TNFα exacerbated the disease (Cooper et al. 1992), while inhibiting TNFα activity prevented onset of the disease and acted to reduce the severity of the established disease (Thorbecke et al. 1992; Williams et al. 1992).

As previously described in section 1.3.1.1, soluble TNF receptors have been detected in biological fluids in both normal and pathological conditions such as RA (Novick et al. 1989; Cope et al. 1992; Roux-Lombard et al. 1993). At high concentrations, these soluble

TNF receptors appear to inhibit bioactivity of TNFα by competitive ligand binding (Higuchi and Aggarwal 1992). Recombinant soluble receptors have been evaluated as potential therapeutic agents with favourable results. Patients receiving a therapeutic dose of soluble TNF receptors exhibited good tolerance and a >20% improvement in the American College of Rheumatology clinical response criteria in 75% of patients (Moreland et al. 1997).

Similarly, studies have now shown that intravenous therapy with monoclonal anti-TNFα antibodies in patients with RA have resulted in biochemical and symptomatic improvements (Elliott et al. 1993; Elliott et al. 1994; Maini et al. 1998; Paleolog et al. 1998; Taylor 2001).

IL-1β is elevated in synovial fluid (Fontana et al. 1982; Hopkins et al. 1988), synovial tissue (Firestein et al. 1990), and plasma (Eastgate et al. 1988) of patients with RA. The level of IL-1β in the joint has a correlation with disease activity (Rooney et al. 1990). Synovial tissue and circulating monocytes obtained from patients with active RA secrete IL-1β in vitro (Shore et al. 1986; Miyasaka et al. 1988). In situ hybridisation and immunostaining revealed that CD14 + macrophages in the synovial lining and intermediate areas of the synovium, were responsible predominantly for expression of IL-1β (Firestein et al. 1992; Lipsky and Isakson 1997). The administration of intraarticular IL-1β into the joints of rabbits induced transient synovitis, leukocyte infiltration into the joint and loss of proteoglycan from cartilage (Pettipher et al. 1986; Ghivizzani et al. 1997). This is consistent with the actions of TNFα and IL-1β in inducing matrix metalloproteinases, such as MMP-1 (collagenase) and MMP-3 (stromelysin) that are involved in cartilage destruction (Dayer et al. 1985; Dayer et al. 1986). Antibodies against IL-1 have been

shown to suppress proteoglycan degradation and inflammation in collagen induced arthritis in mice (van den Berg et al. 1994).

Soluble forms of both IL-1 receptors (IL-1sRI and IL-1sRII) have been detected in human biological fluids and these can act as natural antagonists by preventing binding of IL-1β to its receptors on cells (Symons et al. 1991; Arend et al. 1994). It has been proposed that this effect could be exploited as an anti-IL-1β therapy. In several animal models of inflammation the soluble IL-1RI suppressed inflammation (Jacobs et al. 1991; Schorlemmer et al. 1993). However, in patients with active RA, there was marginal clinical benefit and treatment limiting toxicity was observed (Drevlow et al. 1996).

The IL-1 receptor antagonist (IL-1ra) is also present in RA joints. However, the concentration may be too low to inhibit the action of IL-1β (Firestein et al. 1990; Malyak et al. 1993). Clinical trials using a recombinant human IL-1ra suggest that this biological agent is effective in reducing joint erosion in RA (Bresnihan and Cunnane 1998; Schiff 2000)

VEGF has been detected in healthy knee joints (Pfander et al. 2001) but in RA, higher levels of VEGF have been detected in synovial fluid, macrophages lining the synovium, fibroblasts surrounding microvessels, VSMCs and synovial lining cells (Nagashima et al. 1995). VEGF mRNA and protein are expressed by RA synovial macrophages (Fava et al. 1994; Koch et al. 1994; Neufeld et al. 1999), suggesting VEGF is a key mediator in ncovascular changes in RA synovium. VEGF, which is induced by hypoxia (Shweiki et al. 1992; Ladoux and Frelin 1993; Banai et al. 1994; Minchenko et al. 1994; Jackson et al. 1997), has been shown to activate $cPLA_2$ resulting in an increase in prostacyclin synthesis

(Wheeler-Jones et al. 1997; He et al. 1999). Hence, in a hypoxic joint the production of PGI_2 may be a response to elevated VEGF levels.

Overall, these studies highlight the significance of cytokines as key regulators in the pathogenesis of inflammation and as potential therapeutic targets.

1.4.2 Atherosclerosis

Atherosclerosis is a disease of the arterial wall putatively arising as a result of persistent physical or environmental stresses (Munro and Cotran 1988). Atherosclerosis is the principle cause of death in Western countries (Ross 1993) despite a myriad of blood-pressure and lipid-lowering drug interventions. Under regular circumstances, it is thought to be a protective response to endothelial and smooth muscle cell insult of the artery wall. However, an excessive inflammatory, fibroproliferative response gives rise to an advanced lesion which may become unstable, resulting in plaque rupture and thrombotic occlusion of the vessel (Ross 1993). Risk factors for atherosclerosis include elevated LDL, diabetes, cigarette smoking, and impaired endothelial cell function. It is generally accepted that atherosclerosis is an inflammatory disease. Local inflammation occurs in the formation of a plaque, as macrophages and other immuno-competent cells are present in the lesions from an early stage. In addition, inflammation plays an important role in the weakening of the fibrous cap of the advanced plaque, eventually leading to plaque rupture and acute coronary syndrome and other ischaemic episodes.

1.4.2.1 Pathology of atherosclerosis

An atherosclerotic plaque essentially consists of three main cell types, vascular endothelial cells, VSMCs and monocytes/macrophages. The lesion is characterised by smooth muscle cell proliferation and infiltration of monocytes that can accumulate large amounts of oxidised low density lipoprotein (LDL) to become foam cells (Steinberg et al. 1989; Ross 1993). These foam cells are pivotal in the development of the atheromatous plaque (Klurfeld 1985; Jonasson et al. 1986; Hansson et al. 1988; Tipping et al. 1989).

1.4.2.2 Hypoxia in atherosclerosis

The anoxemia theory of atherosclerosis proposes that hypoxia is a key factor in the development of atherosclerotic lesions (Hueper 1944; Boxen 1985). When atherosclerotic plaques develop, the arterial wall thickness increases and diffusion capacity becomes limiting. The arterial wall depends on diffusion for its supply of oxygen and nutrients. However in atherosclerosis, both diffusion distance and oxygen consumption increase (Morrison et al. 1972; Bjornheden and Bondjers 1987). This results in a disparity of energy metabolism that is believed to support the development of the plaque with the formation of a necrotic core. Oxygen microelectrodes have been used in vitro and ex vivo to demonstrate decreased oxygen tension in the arterial media in atherosclerotic (Heughan et al. 1973) and hypertensive (Crawford and Kramsch 1988; Santilli et al. 1992) rabbits. A method was developed to enable measurement of hypoxia in arterial tissue in vitro, utilising a tissue bound marker of hypoxia which was a nitroimidazole derivative called NITP (Bjornheden et al. 1996). NITP undergoes nitroreduction intracellularly and reactive radicals are formed in the absence of oxygen. This method was later applied to in vivo studies where NITP was administered in animals, with the demonstration of hypoxic zones in atheromatous plaques (Bjornheden et al. 1999).

Overall, it is apparent that hypoxic areas exists within the arterial wall of a developing atherosclerotic plaque and may have potential to influence the expression of inflammatory genes of cells within the lesion.

Deleterious effects of hypoxia on atherosclerosis have been reported in rabbit models, where the development of aortic lesions increased following exposure to hypoxic conditions (Kjeldsen et al. 1968; Helin and Lorenzen 1969; Okamoto et al. 1983). Hypoxia may effect lipid metabolism in cultured aortic tissue (Howard 1972; Filipovic and Rutemoller 1976), myocardial cells (Hollenberg 1971), aortic smooth muscle cells (Albers and Bierman 1976; Tsukitani et al. 1984) and monocyte-derived macrophages (Matsumoto et al. 2000). In addition, hypoxia induces changes in lactate dehydrogenase synthesis (Lindy et al. 1974), glycosaminoglycans (Helin et al. 1970), connective tissue (Helin et al. 1974) and increases oxygen consumption (Bjornheden and Bondjers 1987) by aortic tissues or cells.

In addition to metabolic changes in response to hypoxia, some of the inflammatory-type reactions in atheromatous plaques could also be altered by hypoxia. This possibility is discussed in section 1.4.2.4.

1.4.2.3 Eicosanoids in atherosclerosis

A balance between PGI_2 synthesis (predominantly by endothelial cells) and TXA_2 synthesis (predominantly by platelets) maintains a healthy vascular system and prevents thrombotic events and spontaneous bleeding. PGI_2 dilates blood vessels and inhibits

aggregation of platelets, whereas TXA_2 causes vasoconstriction and induces platelet aggregation. A disruption in the balance of these two eicosanoids in favour of TXA_2 dominance has been associated with the development of atherosclerosis (Sinzinger et al. 1991).

PGI_2 is not only involved in vasodilatation and inhibition of platelet aggregation, but inhibits leukocyte activation and adhesion and VSMC proliferation, migration and contraction. PGI_2 is a product of COX activity and COX-2 expression has been demonstrated in endothelial cells, VSMC and monocyte/macrophages of human and animal atherosclerotic tissue (Baker et al. 1999; Schonbeck et al. 1999; Belton et al. 2000). The presence of COX-2 in cells of atheromatous lesions as well as its inducibility by mediators of atherogenesis (cytokines, hypoxia) are in keeping with data demonstrating that prostaglandin production is increased in atherosclerosis (FitzGerald et al. 1983; Belton et al. 2000). However, actions of PGI_2 described above suggest that increased COX-2 expression and eicosanoid production may be protective responses to the formation of atheromatous lesions, rather than drivers of pathogenesis.

1.4.2.4 Inflammatory proteins in atherosclerosis

Inflammatory proteins are altered in atherosclerosis and these may influence the recruitment of leukocytes to the developing atheromatous lesion. Migration of leukocytes into the plaque may predispose to catastrophic plaque complications.

1.4.2.4.1 Monocyte /Endothelial interactions

Tethering and rolling of leukocytes in post capillary venules of the systemic circulation is mediated by the selectins (L-, P- and E-selectins) and α4-integrins (α4β1 and α4β7) (Butcher 1991; Berlin et al. 1995). Endothelial cell E-selectin can be induced by a diverse array of mediators including cytokines and bacterial endotoxin (Bevilacqua and Nelson 1993; Nelson et al. 1993) and may be induced under hypoxic conditions with co-stimuli (Zund et al. 1996). Following this, firm adhesion and emigration of cells, including monocytes is mostly dependent on monocyte expression of LFA-1, a member of the CD18 (β2) integrin family (Luscinskas et al. 1991) which binds to the adhesion molecule, intercellular adhesion molecule-1 (ICAM-1) expressed on endothelial cells. Cognate interactions between the α4 integrins and vascular cell adhesion molecule-1 (VCAM-1) stabilise firm adhesion. Antibodies against CD18 inhibit monocyte adherence to endothelial cells (Arnaout et al. 1988; Beekhuizen et al. 1990; Kuijpers et al. 1990; Meerschaert and Furie 1995).

Hypoxia increased macrophage and neutrophil adhesiveness to rat epithelial cells by inducing expression of VCAM-1 and ICAM-1 mRNA and protein expression (Beck-Schimmer et al. 2001). $CoCl_2$, used as a hypoxia mimetic by stabilising HIF-1α, induced VCAM-1 expression in endothelial cells via the ERK1/2 MAPK pathway and caused a 2 to 3-fold increase in the rate of transendothelial migration of monocyte like HL-60 cells (Sultana et al. 1999).

1.4.2.4.2 Cytokines, VEGF and ORP

The production of and responses to TNFα and IL-1β by cells in atherosclerotic plaques (Barath et al. 1990; Rus et al. 1991) suggests these cytokines participate as autocrine or

paracrine mediators in atherosclerosis. Both cytokines are absent in normal vessels. Inumohistochemical analysis of post mortem and amputated specimens has revealed the presence of TNFα in foam cells, smooth muscle cells, mast cells and endothelial cells of atheromatous vessels (Barath et al. 1990; Kishikawa et al. 1993; Kaartinen et al. 1996). These cytokines would activate endothelial cells for increased leukocyte adherence and could activate plaque macrophages for inflammatory reactions that could lead to clinical complications (see 1.4.2.4.3).

Endothelin-1, a potent vasoconstrictor peptide and a mitogen for smooth muscle cells and fibroblasts, is increased in vascular cells exposed to TNFα and IL-1β suggesting that cytokines may play a significant role in the control of vascular tone (Kahaleh and Fan 1997). Furthermore, cytokines may indirectly contribute to the development of proliferative vascular lesions by stimulating smooth muscle and interstitial cell proliferation through their effects on endothelin release by vascular cells.

In view of the fact that VEGF can induce endothelial cell migration, growth, differentiation and regeneration, it was proposed that VEGF protects the artery from atherosclerosis (Van Belle et al. 1998; Baumgartner and Isner 2001). However, VEGF has the potential to induce migration and activation of infiltrating monocytes into atherosclerotic lesions via the Flt-1 receptor (Barleon et al. 1996). Activation events include expression of adhesion molecules (Kim et al. 2001) and monocyte chemoattractant protein-1 (MCP-1) (Marumo et al. 1999). Furthermore, inhibition of VEGF using a soluble form of the Flt-1 receptor (sFlt-1) in rats attenuated vascular inflammation and atherosclerosis suggesting that VEGF participates in plaque formation, possibly by increasing angiogenesis as well as having leukocyte effects. It has been reported that endothelial cells do not express VEGF in

significant levels under basal conditions, but that VEGF can be induced following hypoxic exposure (Namiki et al. 1995). Given that inflammatory atherosclerosis is accompanied by hypoxic conditions, it is not surprising that, whereas normal human arteries showed no substantial VEGF expression, atheromatous lesions showed distinct VEGF positivity (Inoue et al. 1998).

The importance of the presence of VEGF in cardiovascular disease is still under debate (Isner 2001). Intra-arterial administration of recombinant VEGF into ischemic limbs of experimental animals, induced angiogenesis and improved tissue perfusion (Takeshita et al. 1994). Furthermore, VEGF has the potential to induce endothelium-dependent relaxation of coronary arteries (Ku et al. 1993) and stimulate vascular endothelial cell proliferation after balloon-induced arterial injury, thereby militating against restenosis (Asahara et al. 1995). However, VEGF may also induce neovascularisation in atherosclerotic plaques leading to further plaque development, intraplaque haemorrhage, and plaque rupture (Kuzuya et al. 1995). The enhanced permeability of endothelial cells induced by VEGF may also render the plaque surface vulnerable to further damage. Overall, it appears as if the functions of vascular wall cells appear to be regulated by cytokines which can influence lesion initiation, progression, or complication.

The 150kDa- oxygen-regulated protein (ORP) was originally characterised in astrocytes exposed to oxygen deprivation (Kuwabara et al. 1996) and later found to be expressed in high levels in mononuclear phagocytes (MPs) derived from atherosclerotic plaques (Tsukamoto et al. 1996). In culture, ORP150 expression was potentiated by exposure to pathophysiologically relevant agents such as modified LDL (Tsukamoto et al. 1996). Furthermore, expression of ORP150 conferred resistance to cell death as indicated by

introduction of antisense oligonucleotide for ORP150 which selectively diminished hypoxia-mediated induction of ORP150 antigen and reduced viability of hypoxic MPs (Tsukamoto et al. 1996). These data indicate that expression of ORP150 in MPs of atherosclerotic vessel walls may provide a protective mechanism for survival, allowing MPs to carry out their role in tissue remodelling and scavenging under environmentally challenging conditions. Furthermore, the presence of autoantibodies against ORP in the sera of patients with severe atherosclerosis may be a marker of vascular pathology.

1.4.2.4.3 Plaque complications

The term plaque complication is used here to encompass vascular occlusive incidents that result from plaque rupture and consequent acute thrombosis and vascular occlusion. The propensity to rupture is know as plaque instability.

With regard to plaque instability, HUVEC can generate matrix metalloproteinase (MMP)-1 activity. This proteolytic enzyme may weaken the structural strength of plaques thereby predisposing to rupture. Direct contact of HUVEC in co-culture with human monocytic THP-1 cells for 48h induced increases in MMP-1 levels by 5-fold via the Src and mitogen activated protein kinase (MAPK) pathways. Furthermore, neutralising antibodies against IL-1β and TNFα significantly inhibited MMP-1 production ((Hojo et al. 2000). Indirect incubation of THP-1 monocytic cells with conditioned HUVEC medium for 18h induced MMP-9 mRNA and protein 4 to 8-fold and this was confirmed with fresh human monocytes (Amorino and Hoover 1998). Similar increases were detected following interactions between THP-1 cells and VSMC mediated by IL-6 and TNFα production (Zhu et al. 2000). The observation that MMP activity is increased in the coronary blood

circulation of patients with ischemic heart disease post angioplasty (Hojo et al. 2002) indicates that MMP activity may contribute to vascular remodelling. The effect of hypoxia on MMP-1 production is examined in this thesis.

Tissue factor (TF) plays a role as an initiator of the thrombotic complications of atherosclerosis, and in situ expression of TF activity by monocyte-derived macrophages and lesion-associated macrophage foam cells has been documented (Wilcox et al. 1989). A series of in vitro studies in the early to mid 90s, demonstrated that interaction between monocytes and endothelial cells induced significant amounts of TF expression (Wharram et al. 1991; Collins et al. 1995; Lewis et al. 1995; Lo et al. 1995; Herbert et al. 1996; Napoleone et al. 1997). Whilst some investigators reported that direct cell contact was necessary for TF induction (Wharram et al. 1991; Lewis et al. 1995; Lo et al. 1995; Herbert et al. 1996), others demonstrated that soluble mediators from conditioned medium were sufficient to induce TF (Collins et al. 1995; Napoleone et al. 1997). Hypoxia also potentiated TF production in monocyte-endothelial cell interactions (Herbert et al. 1996). TF in co-culture was diminished by neutralisation of IL-1β and TNFα activity (Napoleone et al. 1997), establishing a direct involvement of these cytokines in the induction of TF synthesis in monocyte-endothelial interactions.

1.4.3 Inhibition of COX and eicosanoids in inflammatory disease

The goal for treating inflammatory diseases should be to suppress not only the symptoms of inflammation but also the destruction of tissue structure and function. However, symptomatic relief is the only therapeutic outcome achieved by the NSAIDs including the selective COX-2 inhibitors. Since the discovery of the inducible COX-2 isoform of COX,

there has been significant interest in the development of selective COX-2 inhibitors for antiinflammatory and analgesic therapy of osteo- and rheumatoid arthritis as well as other pain syndromes. The rationale for this development is that these compounds offer the potential for inhibition of COX-2 derived inflammatory eicosanoids with sparing of COX-1 derived eicosanoids involved in gastro-protection and other homeostatic functions.

Animal studies suggested that administration of selective COX-2 inhibitors could achieve anti-inflammatory efficacy with reduced ulcerogenic and nephrotoxic effects (Anderson et al. 1996; Herschman 1996). In addition, double blind placebo controlled trials involving RA patients ingesting the moderately selective COX-2 inhibitor, celecoxib twice daily, resulted in significant improvement in the number of painful and tender joints by comparison to the placebo group (Lipsky and Isakson 1997). An equally favourable outcome for symptom relief was achieved in osteoarthritic patients taking celecoxib compared with the placebo groups (Zhao et al. 1999). The level of analgesia achieved is comparable to that with standard NSAIDs. However, in the CLASS study which compared the gastric safety of celecoxib against that of two other NSAIDs, diclofenac and ibuprofen, there was no statistically significant difference in gastric ulcer complications between celecoxib and the other NSAIDs (Juni et al. 2002). In retrospect, this is hardly surprising since the selectivity of celecoxib for COX-2 relative to COX-1 is no greater than that for diclofenac (Patrignani et al. 1994).

While COX-2 expression is frequently associated with inflammation and other pathophysiological states, there is increasing evidence that COX-2 plays a physiological role in renal, bone and vascular homeostasis. COX-2 knockout mice developed serious renal and bone disease (Morham et al. 1995). The finding that genetic disruption of COX-

2 results in cardiac fibrosis (Dinchuk et al. 1995) also suggests that COX-2 expression may be cardioprotective. In addition, COX-2 inhibitors in clinical use have a similar renal adverse event profile as traditional NSAIDs.

Platelet derived TXA_2 is COX-1 derived because this is the only COX isoform present in platelets. However, endothelial-derived PGI_2 depends on COX-2 induction to produce sufficient PGH_2 for PGI synthase activity (Caughey et al. 1997). Therefore, COX-2 inhibitors may encourage a 'pro-thrombotic state' or a 'pro-atherogenic state' (Cheng et al. 2002). Also, myocardial expression of COX-2 may result in the production of eicosanoids that are protective for ischaemic and oxidant induced damage. Protection from oxidant damage as well as protection from ischemic damage by ischaemic pre-conditioning was abolished by selective COX-2 inhibitors in cardiomyocytes and in a rabbit model (Adderley and Fitzgerald 1999; Bolli et al. 2002).

These results provide possible mechanistic explanations for the increased risk of myocardial infarction (MI) seen in the VIGOR study. The VIGOR study was a long-term double blind safety study, in which the highly selective COX-2 inhibitor, rofecoxib (10x more selective than celecoxib) was given to patients with RA (Bombardier et al. 2000). The trial included over 8000 patients with RA who received either naproxen or rofecoxib for 12 months (excluding aspirin therapy for all patients since mucosa protection in the upper GI tract was being evaluated). Despite rofecoxib demonstrating a 50% reduction in complicated GI effects, there was a 4-fold increase in incidence of MI. A large percentage of these adverse events occurred in a subset of patients, identified retrospectively as being at risk for vascular events. These findings suggest that highly selective COX-2 inhibitors can perturb the homeostatic balance between COX-1 and COX-2 derived eicosanoids in

the vascular space in ways that may increase risk for cardiovascular events, especially but not exclusively in those with prior high risk (FDA analysis of primary data from VIGOR study – http://www.fda.gov/ohms/dockets/ac/01briefing/377b2.html). Clearly, imbalance between COX-1 and COX-2 derived eicosanoids could also have undesirable effects in other extravascular tissues, especially under hypoxic conditions.

1.5 HYPOXIA AND GENE REGULATION

Mammalian cells adapt to hypoxia by increasing the expression of genes coding for proteins which facilitate cell survival. For example, hypoxia induces expression of glycolytic enzymes for energy production and VEGF for neovascularization (Semenza 2000). However, tissue injury in hypoxia may result in an exaggerated inflammatory response due to the upregulation of many inflammatory genes by hypoxia. This has been reviewed in the previous sections and this next section will review the mechanisms for the effects of hypoxia on gene expression and the transcription factors involved in their regulation.

1.5.1 Hypoxia and message stability

The wide range of mRNA decay rates in higher eukaryotes contributes significantly to regulation of the expression of gene products and several decay pathways have been characterised (Guhaniyogi and Brewer 2001). The half-life of mRNA is determined by cis-elements that associate with binding proteins which localise to the 3' untranslated region (3'-UTR) on the message. AU-rich elements (ARE) in the 3'UTR confer post-transcriptional control of mRNA expression by influencing mRNA stability (Xu et al. 1997) and translational efficiency (Rajagopalan and Malter 1996). HuR is a ubiquitously expressed nucleo-cytoplasmic shuttling protein that regulates the stability of mRNA by selectively binding to ARE-containing mRNA thereby increasing their half-life (Ma et al. 1996; Fan and Steitz 1998a; Fan and Steitz 1998b; Nabors et al. 2001).

VEGF is an example of a protein regulated in this manner. Hypoxia not only upregulates VEGF gene transcription, but also increases the half-life of VEGF mRNA (Shima et al. 1995; Stein et al. 1995; Levy et al. 1996; Claffey et al. 1998; Dibbens et al. 1999). A recent study demonstrated that hypoxia-induced stabilisation of VEGF mRNA is mediated through the activation of stress activated protein kinases like p38 and JNK (Pages et al. 2000). The regulation is generally achieved through the interaction of specific proteins such as HuR, with various regions of the VEGF 3'-UTR. HuR, which binds to the VEGF ARE with high affinity and specificity mediates hypoxia-induced stabilisation (Levy et al. 1998). Deletion of the AREs of the VEGF 3'-UTR resulted in significant stabilisation of VEGF mRNA in normoxia (Levy et al. 1996), thereby indicating the unbound AREs are associated with mRNA instability.

The 3' UTR of TNFα and COX-2 genes also contain multiple repeats of ARE regions which cause mRNA instability (Chen and Shyu 1995; Dixon et al. 2000; Cok and Morrison 2001). Disruption of the 3' UTR of TNFα mRNA impaired binding of HuR in murine macrophages (Di Marco et al. 2001) and this impaired binding of HuR resulted in hindered transport of TNFα mRNA from the nucleus to the cytoplasm (Dean et al. 2001; Di Marco et al. 2001). Similarly, deletion of regions in the 3'-UTR using transfected chimeric luciferase-COX-2 mRNA 3'-UTR reporter constructs, increased reporter gene mRNA stability and translation (Dixon et al. 2000; Cok and Morrison 2001; Faour et al. 2001).

Overall, there is potential for hypoxia to stabilise COX-2 mRNA by regulating elements in the 3'-UTR, but this has not been examined. This is a possible mode of upregulated COX-2 protein expression in hypoxia because LPS (Barrios-Rodiles et al. 1999) and a combination of IL-1β and TNFα (Huang et al. 2000) have been shown to stabilise COX-2

mRNA in human macrophages in normoxia. This thesis will characterise the stability of COX-2 mRNA in normoxic and hypoxic conditions in three cell types relevant to an inflammatory lesion. As well as affecting the stability of mRNA transcripts, hypoxia has the potential also to induce gene transcription by influencing the activity of transcription factors.

1.5.2 Transcription factors in hypoxia

Transcription factors bind to promoter regions of their target genes and regulate gene expression by interacting with the basal transcription machinery. The properties of these transcription factors are commonly regulated on distinct levels such as expression and stability of the transcription factor protein as well as modulation of its binding properties by post-translational modification. There are numerous transcription factors that are affected by hypoxia. The following section will describe three factors that may play an important role in inflammation; namely, hypoxia-inducible factor-1 (HIF-1), early growth response-1 (Egr-1) and nuclear factor kappa-B (NF-κB).

1.5.2.1 Hypoxia inducible factor (HIF)

HIF-1 is a transcription factor expressed in response to physiologically relevant levels of hypoxia. However, it may also be upregulated by certain transition metals, (Co^{2+}, Ni^{2+}, Mn^{2+}) and by iron chelation. HIF-1α is oxygen-responsive whereas HIF-1β (also know as aryl hydrocarbon receptor nuclear translocator (ARNT)), is constitutively expressed. The heterodimer of HIF-1α and HIF-1β/ARNT binds to hypoxia responsive elements (HRE) (Iyer et al. 1998a; Iyer et al. 1998b) containing the sequence 5'-CGTG-3' in the promoter

region of genes regulated by HIF-1. HIF-1α is continually synthesised and degraded by the ubiquitin-proteosome pathway under normoxic conditions but rapidly accumulates following exposure to low oxygen tension (Huang et al. 1996; Wiener et al. 1996; Shih and Claffey 1998). A central component regulating HIF-1α turnover is the product of the tumor suppressor gene *vhl*, encoding the von Hippel-Lindau protein (pVHL) (Maxwell et al. 1999). pVHL forms the recognition component of an E3 ubiquitin ligase complex leading to ubiquitinylation of HIF-1α in the presence of oxygen (Ivan and Kaelin 2001). Loss of function of pVHL leads to an accumulation of HIF which in turn, leads to excessive transcription of HIF target genes such as VEGF. Hence, tumors associated with VHL disease (germline mutations in the *vhl* tumor suppressor gene) are known to be hypervascular primarily due to their inability to suppress VEGF (Ivan and Kaelin 2001). In support of this, high levels of HIF-1α are found in tumor cells with mutations of the *vhl* gene (Maxwell et al. 1999).

During hypoxia, HIF-1α dissociates from the chaperone protein, heat shock protein 90 (Hsp 90) (Minet et al. 1999) and translocates to the nucleus where it dimerises with HIF-1β. Studies have demonstrated that HIF-1β deficient cells showed reduced induction of hypoxia responsive genes (Wood et al. 1996).

HIF-1 activation by hypoxia plays an important role in the adaptive responses to oxygen deprivation and is capable of upregulating genes encoding for erythropoietin, VEGF, glucose transporters and glycolytic enzymes as well as genes involved in iron metabolism and cell survival (Semenza 1999b; Semenza 1999a; Wenger 2002). In the case of VEGF, hypoxia leads to HIF-1 binding to an enhancer element in the 5'-flanking region of the VEGF gene (Ikeda et al. 1995; Forsythe et al. 1996; Damert et al. 1997; Levy et al. 1997).

VEGF is one of the best characterised hypoxia-sensitive angiogenic factors and not only is VEGF involved in hypoxia-dependent remodelling, but is also involved in aberrant angiogenesis. Consequently, HIF-dependent gene regulation is emerging as a target for anti- or pro-angiogenic treatments (Iyer et al. 1998a; Iyer et al. 1998b; Kung et al. 2000; Vincent et al. 2000).

The HIF-α family contains two other members, HIF-2α and HIF-3α, both of which have limited tissue expression (Wenger 2002). However, they contain domains similar to those in HIF-1α and exhibit a similar heterodimerisation with HIF-1β subunits followed by binding to the same DNA sequence in vitro (Semenza 1999b). The relative contribution of HIF-1α and HIF-2α to the regulation of gene expression in hypoxic macrophages is not well understood and may vary between tissues. It has been demonstrated that the main HIF upregulated in hypoxic macrophages is HIF-2α rather than HIF-1α (Talks et al. 2000). Conversely, immunoblotting studies have shown that murine alveolar macrophages exhibit upregulated HIF-1α protein under hypoxia (Yu et al. 1998) and immunoreactive HIF-1α has been detected in human macrophages in the hypoxic synovia of arthritic human joints. HIF-3α has recently been characterised (Gu et al. 1998) indicating that a family of ARNT-binding factors may be present in cells under hypoxia. However, there is no evidence in the literature for binding of HIF-3α in hypoxic macrophages. To date, there has been no examination of the effects of HIF activity as a mediator of the effects of hypoxia on COX-1 or COX-2 expression.

1.5.2.2 Early growth response-1 (EGR-1)

The Early Growth Response gene product, Egr-1 is reported to cause *de novo* expression of tissue factor in mononuclear phagocytes along with consequent fibrin deposition in response to hypoxia (Yan et al. 1999). Hypoxia activates the expression of Egr-1 via protein kinase pathways in monocyte-derived macrophages (Yan et al. 2000b) and endothelial cells (Lo et al. 2001). The Egr-1 product activates high affinity binding to GC-rich elements in the promoter region of many genes including TNFα, IL-1β, ICAM-1 and tissue factor (TF) (Kramer et al. 1994; Yan et al. 2000a). Therefore, it is possible that hypoxia may regulate genes in macrophages and endothelial cells via the Egr-1 pathway. Egr-1 also mediates TF production by endothelial cells in response to stimulation by VEGF (Mechtcheriakova et al. 1999). Hence, elevated levels of circulating VEGF in hypoxic rheumatoid synovium may be responsible for Egr-1 mediated activation of macrophages and endothelial cells. In a murine model of lung ischaemia/reperfusion, there was an 11-fold induction of VEGF mRNA in wild-type mice lung tissue, compared to a 2.6-fold increase in Egr-1-null mice lung tissue. This suggests that Egr-1 plays a role in the regulation of VEGF in hypoxia (Yan et al. 2000a). Overall, these results suggest that there is an Egr-1/VEGF regulatory inter-relationship, which plays a role in the regulation of angiogenesis and cytokine expression in the rheumatoid synovium.

1.5.2.3 Nuclear factor-κB (NF-κB)

NF-κB is a heterodimer of two DNA-binding subunits, p50 and p65, which share structural homology with the Rel family of proteins (Ghosh et al. 1998). NF-κB exists in the cytoplasm of cells in an inactive form bound to the inhibitor, I-κBα. Upon receiving an activation signal, possibly via Src and Ras kinase activation, I-κBα becomes phosphorylated (Koong et al. 1994b). This leads to degradation via an ubiquitin-

proteosome pathway and NF-κB is released, translocating to the nucleus (Ghosh et al. 1998). It was established that NF-κB binds with high affinity to a κB decameric recognition sequence 5'-GGGPuNNPyPyCC-3' (Wang et al. 2000). With regard to COX-2 induction, there are two NF-κB consensus binding sites in the promoter region of the COX-2 gene, NF-κB-5' and NF-κB-3' (Appleby et al. 1994). The NF-κB-5' binding site has been shown to play a role in the mechanism of COX-2 induction by TNFα in murine osteoblasts (Yamamoto et al. 1995; Yamamoto et al. 1997). The NF-κB-3' binding site may play a role in facilitating the induction of COX-2 by LPS and by hypoxia in endothelial cells (Inoue et al. 1995; Schmedtje et al. 1997). The activation of NF-κB is rapid as it does not require *de novo* synthesis and among the factors known to activate NF-κB are cytokines, LPS and oxidants such as reactive oxygen species (ROS) (Barnes and Karin 1997). The genes that NF-κB is known to regulate include TNFα, IL-1β, MCP-1, iNOS, COX-2 and $cPLA_2$ genes (Grimm and Baeuerle 1993; Barnes and Karin 1997; Ghosh et al. 1998).

It was demonstrated that hypoxia resulted in I-κBα degradation, increased NF-κB DNA binding activity, and transactivation of a reporter gene construct containing two NF-κB DNA binding sites (Koong et al. 1994a). Inhibition of tyrosine phosphorylation of I-κBα prevented I-κBα degradation and NF-κB binding in response to hypoxia, suggesting that tyrosine phosphorylation of Iκ-Bα during hypoxia is an important proximal step which precedes its dissociation from NF-κB and degradation (Koong et al. 1994a). Hypoxia is reported to increase the coordinate activation of HIF-1α and NF-κB DNA binding by 3 to 4 fold which is essential for the upregulation of COX-2 and VEGF mRNA and protein (Lukiw et al. 2003). In addition, hypoxia followed by reoxygenation increased NF-κB

activation in synovial fibroblasts, and also increased lymphocyte adhesiveness to synovial fibroblasts and increased synovial ICAM-1 expression, both of which were completely blocked by an NF-κB antagonist (Han et al. 2003).

Overall, hypoxia-induced activation of NF-κB appears to be involved in the upregulation of inflammatory and angiogenic genes as well as being involved in the recruitment of leukocytes to hypoxic sites of inflammation.

1.6 SUMMARY

Blood derived monocytes that are attracted to sites of inflammation, exert their biological effects by secreting pro-inflammatory eicosanoids (PGE_2, TXA_2) and cytokines (TNFα, IL-1β and VEGF). These soluble mediators are capable of regulating an inflammatory response in an autocrine manner or by inducing changes in heterotypic cells that lie within close proximity. For example in a rheumatoid joint, fibroblast-like synoviocytes (FLS) line the synovial cavity and lie in close proximity to infiltrating blood derived monocytes. Similarly, endothelial cells are closely associated with monocytes not only in inflamed joint disease, but also in atheromatous vascular pathology. The expression and activity of inflammatory mediators may be altered by hypoxia, and since hypoxia is a feature of rheumatoid joints with effusions and of atheromatous plaques, it is important to consider oxygen tension when conducting studies of inflammatory mediator production. Studies of inflammation that take into account the effects of hypoxia may reveal additional molecular and cellular mechanisms involved in inflammation and disordered inflammation, such as occurs in a rheumatoid pannus or atherosclerotic plaque. Novel therapeutic targets may thereby emerge.

1.7 AIMS

The aims of this thesis were:

(1) In human monocytes,

- To characterise the effects of hypoxia on induction of human monocyte COX-2 mRNA and protein expression with various co-stimulators likely to be found in rheumatoid joints (Chapter 3).
- To characterise the production of the eicosanoids, PGE_2 and TXA_2 arising from COX-2 activity in the presence of hypoxia (Chapter 4).
- To characterise the production of the cytokines, TNFα and IL-1β in response to hypoxia and any autocrine eicosanoid/cytokine inter-relationships (Chapter 5).

(2) In human fibroblast-like synoviocytes (FLS),

- To characterise the effect of hypoxia on induction of human FLS COX-2 mRNA and protein (Chapter 6).
- To characterise the production of the eicosanoids, PGI_2 and PGE_2 arising from COX-2 activity in the presence of hypoxia (Chapter 6).
- To characterise the response of FLS to monocyte-derived mediators (Chapter 6).

(3) In Human Umbilical Vein Endothelial Cells (HUVEC).

- To characterise the effect of hypoxia on induction of HUVEC COX-2 mRNA and protein (Chapter 7).
- To characterise the production of the eicosanoids, PGE_2, PGI_2 and TXA_2 arising from COX-2 activity in the presence of hypoxia (Chapter 7).
- To characterise the response of HUVEC on monocyte-derived mediators (Chapter 8).

1.8 HYPOTHESES

The general hypotheses of the studies were that:

(1) hypoxia alters COX-2 expression and eicosanoid synthesis by all cell types studied i.e. monocytes, fibroblast-like synoviocytes and endothelial cells

(2) hypoxia-induced changes in eicosanoid production can influence cytokine synthesis by monocytes

(3) interactions between various cell types in hypoxia alters COX-2 expression and eicosanoid synthesis

CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1 BUFFERS AND SOLUTIONS

All recipes for buffers and solutions are described in appendix A.

2.2 METHODS/MATERIALS

2.2.1 Counter current elutriation

Counter current elutriation is a method used to separate cells on the basis of size and density. This separation occurs in a v-shaped chamber built into the base of a centrifuge rotor (Fig 2.1). A preparation of cells in buffer are injected into the chamber whilst the centrifuge is at a specific rotor speed. The flow of buffer provides resistance in the opposite direction to the centrifugal force and can be adjusted to elute sub-populations of cells according to their densities. Cells with low sedimentation properties are washed out first (eg. platelets). Smaller cells (lymphocytes and erythrocytes) are subsequently eluted as the flow rate increases and the larger population of cells (monocytes) remain inside the centrifuge chamber. The final population of larger cells may be collected once the centrifuge is turned off and the flow rate is turned up to maximum.

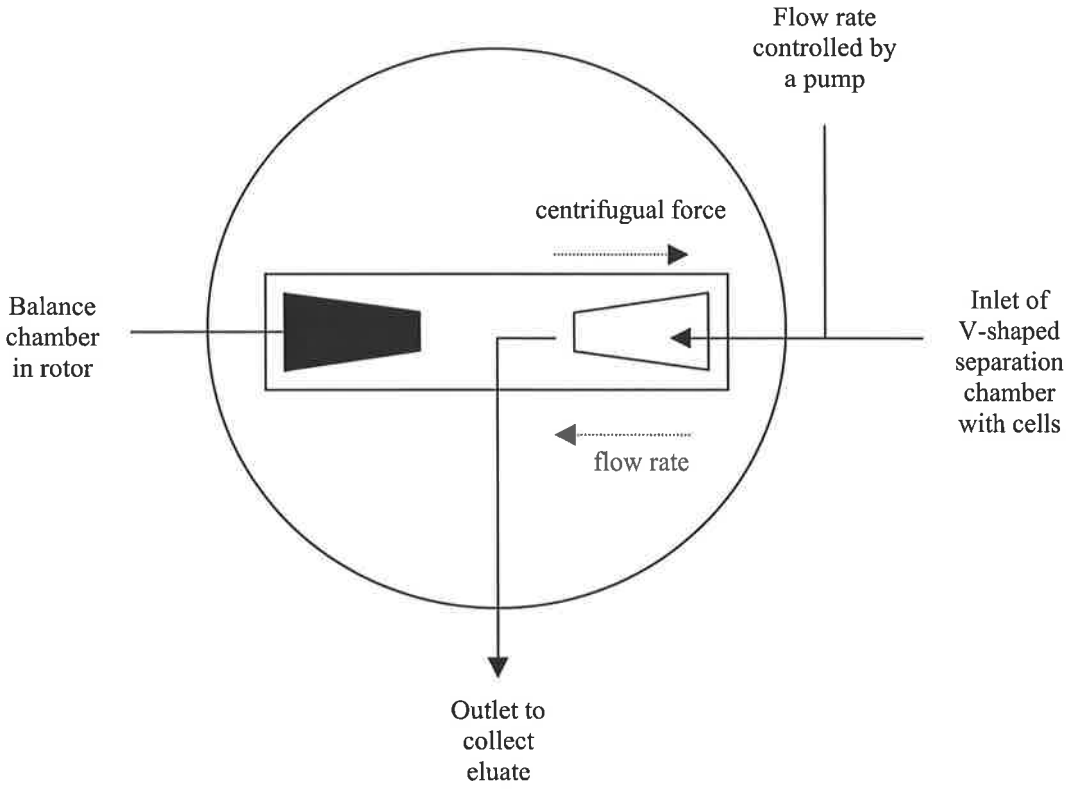


Figure 2.1

Overview of counter current elutriator chamber

The V-shaped chamber is embedded into the rotor of the elutriator. Running buffer flows through the apparatus whilst the cells in suspension are injected into the chamber. The rate at which they travel through the chamber is due to the net effect of flow rate and centrifugal force (2000rpm). These can be adjusted so that smaller cells (platelets, erythrocytes) are eluted early in the elutriation and larger cells (e.g. monocytes) remain in the chamber.

2.2.2 Isolation of monocytes by counter current elutriation

Buffy coats (leukocyte rich plasma) were obtained fresh from the Red Cross Blood Centre, Adelaide, South Australia. A total of 50ml buffy coat was diluted 1:2 with sterile PBS and 20ml was overlayed on 7ml of pyrogen-free Lymphoprep™ (density 1.077g/ml) (Nycomed Pharma, Olso, Norway). Following centrifugation (800 x *g*, 30min) the mononuclear cell (MNC) layer was removed from the interface with a sterile plastic pipette, pooled in a 50ml centrifuge tube and washed 2 x with sterile PBS. The MNC pellet was resuspended in 10ml of running buffer (HBSS, 0.21% tri-sodium citrate) and injected into the elutriator (J-6 M/E Elutriation System, Beckman, Palo Alto, CA). The rotor speed was constant at 2000rpm and the flow rate was gradually increased (over 10min) to reach a final flow rate of 12ml/min and run for a further for 20min. After a total of approx 30min, the rotor was stopped and the flow rate was increased to its maximum to collect the final eluate of monocytes. Purity of the obtained monocyte fraction (>85%) was assessed by FACS analysis. Contaminant cells were essentially all lymphocytes. For the maintenance of minimal LPS contamination, the mononuclear cell isolation procedure was performed under sterile conditions and elutriator tubing was flushed with 600ml of E-Toxa-Clean, 70% ethanol, Milli Q water and finally running buffer prior to each elutriation.

2.2.3 Cell count and stimulation

Cells were pelleted by centrifugation at 200 x*g* then resuspended in 1ml of RPMI-1640 tissue culture medium supplemented with 10% (v/v) heat-inactivated low-LPS fetal calf serum (FCS), L-glutamine, HEPES, penicillin (100U/ml) and gentamycin (100µg/ml). 10µl of cell suspension was diluted 1/100 and a haemocytometer (Improved Neubauer)

was used to count cells. An average of 4 counts was used to calculate the final cell concentration (mean cell count x magnification (10^4) x dilution factor (10^2)). The monocyte suspension was adjusted to a final volume of $2x10^6$cells/ml or $5x10^6$ / 2.5ml for each experiment.

Monocytes were incubated in single, duplicate or triplicate in Minisorp non-adherent teflon tubes (Nunc, Copenhagen, Denmark) in a total of 1ml (or 2.5ml for Western and Northern Analysis) at 37°C, 5% CO_2 unless otherwise stated. When pharmacologic agents were added, cells were pre-incubated for 15-30min with the respective agents before stimulation. Cells incubated short term with arachidonic acid (AA) or calcium ionophore A23187 were resuspended in RPMI (FCS free). Incubations with LPS or cytokines were performed in complete medium (10% FCS) at 37°C. Cell suspensions were centrifuged and cell-free supernatants were stored at –20°C until eicosanoid or cytokine determination.

2.2.4 Preparation of monocyte-derived macrophages

Monocytes were prepared by elutriation and resuspended at $1x10^6$/ml in RPMI 1640 containing 10% FCS. Cells were incubated with M-CSF (50ng/ml) or GM-CSF (100ng/ml) for 6 days in teflon pots (Savillex Corporation, Minnetonka, Minnesota, USA) to prevent cells from adhering. Following this treatment, monocytes had differentiated into macrophages (Young et al. 1990). After long term culture, monocyte derived macrophages were washed and incubated with fresh culture medium prior to an experimental procedure.

2.2.5 Preparation of U937 monocytic cell line and differentiation by PMA

U937 cells were cultured in RPMI 1640 containing 10% FCS in T75 flasks (Corning Laboratory Sciences Co., Corning, NY), at 37°C, 5%CO_2. U937 cells ($5x10^6$/2.5ml) were differentiated in Minisorp teflon tubes with 50ng/ml of phorbyl 12-myristate 13-acetate (PMA) (Sigma Chem Co) for 5–7 days to allow for differentiation (Pedrinaci et al. 1990). Following this incubation period, the cells were pooled, recounted and resuspended ($5x10^6$ /2.5ml) ready for use. Cells were stimulated with serum treated zymosan (STZ at 100μg/ml) for all experiments.

2.2.6 Preparation of serum treated zymosan (STZ)

Typically, 100mg of Zymosan A (*Saccharomyces cerevisiae*, Sigma Chem Co., St Louis MO) was suspended in 20ml of sterile saline. The preparation was boiled for 30min then cooled and centrifuged at 800g for 10min. The sediment was washed 2x in Dulbecco's PBS (DPBS) then resuspended in 10mls of fresh human serum in a T25 flask (Corning Laboratory Sciences Co., Corning, NY) and incubated overnight at 37°C. Following this incubation the zymosan was washed twice in DPBS and resuspended in 5ml of DPBS to achieve a final concentration of 20mg/ml. Aliquots of STZ (1ml) were stored at –20°C until further use and typically used at a concentration of 100μg/ml).

2.2.7 Fibroblast-like synoviocytes (FLS) from Synovial Fluid

2.2.7.1 Ethics Approval

Ethics approval was obtained from the Royal Adelaide Hospital (RAH) Human Research Ethics Committee. All participants were given written information regarding the nature of the study and signed consent was obtained.

2.2.7.2 Preparation of fibroblast-like synoviocytes (FLS)

This method of FLS isolation and culture has been previously described (Neidhart et al. 2003). Synovial fluid from knees of arthritis patients with effusions was collected in a non-clotting tube and cells were pelleted by centrifugation at 800g for 10min. The fluid was discarded and the cell pellet was resuspended in RPMI-1640 tissue culture medium supplemented with 20% (v/v) heat-inactivated, low-LPS foetal calf serum (FCS), L-glutamine, HEPES, penicillin (100U/ml) and gentamycin (100µg/ml), fungizone (2µg/ml). FLS were cultured in T75 flasks (Corning Laboratory Sciences Co., Corning, NY). Experiments with FLS were typically used between 2 and 4 passages and discarded after 5 passages. FLS ($5x10^5$ / 2ml) were plated in 6-well dishes and allowed to reach confluency within 24h. FLS experiments were performed in RPMI 1640 containing 10% FCS and generally stimulated with IL-1β (2ng/ml). Other stimuli were used and described in experiments.

2.2.8 Human Umbilical Vein Endothelial Cell (HUVEC) culture

Human Umbilical Vein Endothelial Cell (HUVEC) were kindly supplied by Assoc Prof Jennifer R Gamble (Immunology, The Hanson Institute, Adelaide, South Australia) after isolation by collagenase digestion of umbilical cords as described (Gamble et al. 1989). The cells were cultured on gelatin-coated culture flasks in medium M199 with Earle's salts (Cytosystems, Sydney, Aust) supplemented with 20% FCS, 50μg/ml endothelial growth factor (Collaborative Research) and 50μg/ml heparin (Sigma Chem Co, St Louis, MO). For experiments, cells were typically used at passage 4, cultured in 6-well plates ($5x10^5$/well) and allowed to reach confluency within 24h. HUVEC experiments were performed in RPMI 1640 containing 10% FCS and generally cells were stimulated with IL-1β (2ng/ml). Other stimuli were used also and described in the results.

2.2.8.1 Hypoxia

Hypoxic conditions at ambient oxygen concentrations of 1% were maintained using a controlled incubator with CO_2/O_2 monitoring and CO_2/N_2 gas sources (Edwards Instrument Co., Wilmington, MA). CO_2 was maintained at 5%. Periodic analysis of the oxygen conditions with CO_2 and O_2 electrodes assured a controlled environment. Culture medium was pre-equilibrated overnight in the hypoxia chamber prior to cell exposure. The pO_2 of the medium was 33mmHg in hypoxia and 154mmHg in normoxia. A pH indicator in the culture medium demonstrated that the medium was maintained at pH of 7.4. Reoxygenation effects at cell harvest were prevented by immediate replacement of hypoxic medium with lysis buffers while the cells were on ice. The physical appearance by light microscopy of cells in hypoxia was indistinguishable from those maintained in normoxic conditions.

2.2.10 Eicosanoid measurement by radioimmunoassay (RIA)

PGE_2, TXA_2 and PGI_2 levels were measured by radioimmunoassay (RIA) by means of competitive binding to antibodies against a specific eicosanoid. Rabbit anti-human PGE_2 antisera (Sigma Chem Co., St Louis, MO) was diluted in 10ml RIA buffer (0.1% gelatin, 0.9% NaCl, 0.01M Trisbase, pH 7.3) and stored as a stock solution at –20°C. Working dilutions of the antisera were made by a further 1:10 dilution of the stock. PGI_2 is unstable, therefore the stable hydrolysis product 6-KetoPGF$_{1\alpha}$ was measured and the antiserum was diluted as described for PGE_2. TXA_2 has a $t_{1/2}$ of ~30s under physiological conditions and is hydrolysed to the stable metabolite TXB_2, which was measured. TXB_2 antisera was prepared from rabbits immunised with thyroglobulin-conjugated TXB_2 (James and Walsh 1988) and was diluted 1:8000 in RIA buffer to achieve the working dilution. For both PGE_2, 6-keto PGF$_{1\alpha}$ or TXB_2 determinations, 100µl of each of the following (1) RIA buffer (2) PGE_2, 6-keto PGF$_{1\alpha}$ or TXB_2 antisera (3) standards or sample supernatants and (4) [^{3}H] PGE_2 (183 Ci/mmol), [^{3}H] 6-keto PGF$_{1\alpha}$ (190 Ci/mmol) or [^{3}H] TXB_2 (219 Ci/mmol) in a total volume of 400µl for each eicosanoid were incubated for 2h at 37°C followed by 1h at 4°C. Free PGE_2, 6-keto PGF$_{1\alpha}$ or TXB_2 were removed by addition of dextran-coated charcoal (1% Dextran T70, 1% Charcoal, 0.05%Na Azide). After centrifugation at 1500g for 20min, the supernatant (500µl) containing the antibody/antigen complex was placed into scintillation fluid (1.5ml) and the radioactivity was determined in a liquid scintillation counter (Wallac 1409, Wallac Oy, Turku, Finland). The limit of detection of each assay was 10pg/100µl. All determinations were carried out in triplicate

or duplicate of duplicate incubations and the means of the counts were used to calculate eicosanoid levels from standard curves of known concentrations.

2.2.11 Cytokine measurement by Enzyme Linked Immunosorbent Assay (ELISA)

IL-1β and TNFα ELISAs were developed using commercially available monoclonal matched pair antibodies and recombinant proteins (Endogen, Boston MA). Nunc 96-well plates were coated with mouse monoclonal coating antibody against human IL-1β or TNFα (5μg/ml in 0.2mol/L Na_2CO_3, pH, 9.4) overnight at 4°C. The plate was then blocked by addition of 200μL of 0.5% BSA for 1hr at 37°C. Serial dilutions of human recombinant IL-1β or TNFα (ranging from 20ng/ml – 0.312ng/ml) or samples (1:16 dilution for IL-1β, 1:3 dilution for TNFα) in a volume of 50μl were added together with 50μl of mouse monoclonal (second matched pair antibody) against human Il-1β or TNFα (0.05μg/ml) for 2h at room temperature. Plates were washed between steps with PBS containing 0.05% Tween 20. 100μl of Extravidin ® peroxidase (1:4000 dilution in 0.5% BSA) was added for 15min at 37°C. Finally, 100μL of the peroxidase substrate, tetramethylbenzidine (TMB) (Sigma Chem Co.) in 0.5M phosphate-citrate buffer (according to manufacturers protocol) was added. The reaction was stopped by addition of 100μl of 2M H_2SO_4. Absorbance was measured at 450nM in a microplate reader (Model 450, Bio-Rad Laboratories, NSW, Aust).

2.2.12 Gelatin zymography for matrix metalloproteinase activity

Gelatin zymography was the method used for detection of MMP activity in conditioned medium. Supernatants were treated with p-aminophenyl mercuric acetate (APMA) (Sigma Chemical Co.) at 1mM for 1h at 37°C (to dissociate MMPs from tissue inhibitor metalloproteinases (TIMPs)). 20µl of sample was mixed with 20µl of 2x non-reducing buffer (30mM Tris HCl (pH 6.8), 10% glycerol, 4% SDS. 0.005% bromophenol blue)) and 25µl was loaded onto a 10% polyacrylamide gel containing gelatin (2mg/ml). The gelatin was a gift from Dr Steve Bozinovski, Dept of Pharmacology, University of Melbourne). Proteins were electrophoresed on a Mighty Small apparatus (Hoeffer Scientific Instruments, San Fancisco, CA) at 120V for 2h. The gel was then washed in 2.5% Triton-X 100 for 30min to renature proteins and then incubated in substrate buffer (50mM Tris/HCl pH 6.8, 5mM $CaCl_2$, 1mM $ZnCl_2$, 0.5% Triton-X 100, 0.01% NaN_3) overnight at 37°C with agitation. The following day, the gel was stained with Coomassie blue staining solution for 30min then de-stained with Destain solution until digested bands were visible. A broad range of molecular weight markers was used to provide putative identification of the type of MMP according to the molecular weight of the digested band.

2.2.13 Western Blot Analysis

This is a semi-quantitative technique that begins with separation of proteins on a SDS-polyacrylamide gel according to their molecular weight, followed by transfer onto a membrane and immunoblotting with an enzyme labelled antibody directed against the protein of interest.

2.2.13.1 Protein Extraction

Monocyte pellets ($5x10^6$) were washed twice in PBS to remove serum proteins before addition of 60µl ice-cold lysis buffer (HEPES-buffered HBSS, pH 7.4, 0.5% TritonX-100, 10µg/ml leupeptin, 10µg/ml aprotinin) and 60µl of 2x sample buffer (0.125M Trizma base, pH 6.8, 20% glycerol, 4% sodium dodecyl sulphate (SDS), 10% 2-mercaptoethanol). Samples were heated at 95°C for 7min before storing at –20°C. FLS or HUVEC, which grow as attached cells, were washed twice in PBS before the addition of 50µl of ice-cold lysis buffer. Using a rubber policeman, the wells were scraped and another 50µl of lysis buffer was added before collecting into an Eppendorf tube and combining with 25µl of 5x sample buffer. Samples were heated at 95°C for 7min before storing at –20°C.

2.2.13.2 Protein separation and transfer

Proteins (20-50µg/lane) were separated on 9% SDS-polyacrylamide gel in the presence of SDS on a 15-well (0.75mm) vertical slab gel unit (Model SE 400, Hoeffer Scientific Instruments, San Fancisco, CA). Prestained broad range SDS-PAGE molecular weight markers were run with every experiment to verify size of proteins of interest. Proteins and molecular weight markers (BioRad) were allowed to migrate through the initial stacking gel for 1.5h at 20mA, after which 170V constant voltage was used until the dye front reached the base of the gel. Separated proteins on the gel were then transferred onto either a Sequi-Blot™ PVDF membrane or a nitrocellulose membrane (BioRad) at -4°C for 16h at 300mA in transfer solution (25mM Tris base, pH8.3, 0.19M Glycine, 20% methanol (v/v)).

2.2.13.3 Protein Immunoblot

Equivalence between samples with regard to protein loading and transfer efficiency was

monitored by Ponceau Red staining. The membrane was blocked for 1h at RT in tris-buffered saline (TBS: 25mM Tris-HCl, 0.2M NaCl, 0.15%Tween-20, pH 7.6) containing 5% (w/v) dried milk to minimise non-specific binding. Subsequently, membranes were treated with the relevant antibodies at the following dilutions: polyclonal COX-2 (1:1,000), polyclonal phospho-p38 MAPK (1:500), phospho-p44/42 MAPK (1:500), phospho-$cPLA_2$ (1:500), and monoclonal β-actin (1:2,500) antibody for 1h at room temperature or overnight at 4°C. All antibodies were diluted in TBS. The membranes were washed twice in TBS (over 0.5h) and incubated with the secondary antibodies, either horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse antibodies. Bound antibodies were revealed with the Supersignal WestPico chemiluminescent system following the manufacturer's protocol (Pierce, Rockford, IL).

2.2.14 Northern Blot Analysis

This semi-quantitative technique begins with separation of messenger ribonucleic acids (mRNA) on a 1% agarose gel according to their molecular weight. Following transfer onto a nylon membrane, hybridisation with a radioactive labelled probe indicates the presence or absence of expression of a particular mRNA of interest.

2.2.14.1 RNA isolation

For RNA isolation, monocytes ($5x10^6$) were washed twice in PBS to remove serum proteins and pellets were resuspended in 1ml of Trizol (Invitrogen) and stored at 4°C for no more than two days. Chloroform (200µl) was added and vortexed for 10s only to minimise shearing of RNA. The sample was left to stand at room temperature for 2min.

The tube was then centrifuged for 15min at 12,000g at 4°C. After centrifugation, the preparation forms two phases, the lower chloroform phase and the colourless aqueous phase above. The RNA was collected from the aqueous phase, ensuring that the DNA and proteins at the interface of the two phases was not collected. The aqueous phase (~500µl) was then mixed with an equal volume of isopropanol, inverted 3x and allowed to stand for 10min at RT. The RNA precipitate was pelleted by centrifugation (12,000g, 4°C, 30min), and washed 1x with ice-cold ethanol (75%) and centrifuged again (12,000g, 4°C for 5min). The ethanol was discarded by gentle aspiration and the pellet was dissolved in sterile DEPC-H_20 (20µl) and heated for 5min at 65°C, then stored at –80°C until use.

2.2.14.2 RNA integrity and separation

Integrity of RNA and equal loading of the samples was checked by running a portion of the RNA on a check gel. RNA (2µl) was made up to a volume of 10µl with DEPC-H_20 and mixed with an equal volume of 2x RNA loading buffer (containing ethidium bromide). The samples were heated (65°C, 3min) and placed on ice before loading. The 1% agarose gel with formaldehyde allowed the RNA to migrate through at 100V for 30min. Separation of the 28S and 18S bands were visualised using an UV light box and photographed for future reference. An identical gel was made along with the check gel, the latter gel used to adjust the volumes of RNA in each sample to ensure equal loading. RNA is separated on the gel at 100V for 2h or until the dye front reached the end of the gel.

2.2.14.3 RNA Transfer

A 1% agarose gel with formaldehyde containing the RNA samples, was briefly rinsed in DEPC-H_2O before mRNA was transferred onto a positively charged nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Piscataway, NJ) by capillary action. Tissue towelling was placed at the base of the bench and wet with 10x SSC. Three layers of soaked filter paper were cut to the size of the gel and soaked in 10x SSC followed by the same size soaked nylon membrane. A pipette was used to roll out all the air bubbles that could impede transfer. The gel was carefully placed on the membrane, followed by 3 layers of soaked filter paper and topped with 10cm highly absorbent towels. 10x SSC was intermittently added for 3-4hr before transfer was complete. The nylon membrane was rinsed briefly in 2x SSC and the RNA was fixed to the membrane by cross-linking under UV light.

2.2.14.4 RNA hybridisation

Membranes were prepared for hybridisation by incubation in hybridisation buffer for 3h at 43°C with rotation. The cDNA probe (COX-2) was labelled with α^{32}P-labelled CTP using a GIGAPrime DNA labelling Kit (Bresatec, Adelaide, Aust) according to the manufacturers protocol. The reaction was stopped by heating to 65°C for 10min before purification of the probe by passage through a wool column and addition directly to the hybridisation mix (43°C, overnight). Following incubation, the membrane was washed in 2x SSC, 0.1% SDS for 10min at room temperature. The temperature and stringency of the wash buffer was increased gradually to minimise background radiation. The membrane was sealed in cling wrap and placed in a cassette with film and exposed overnight at –80°C.

2.2.14.5 Making the COX-2 probe

The COX-2 primers used to produce the probe were: hCOX-2f, 5'-GCT GAC TAT GGC TAC AAA AGC TGG-3'; and hCOX-2r, 5'-ATG CTC AGG GAC TTG AGG AGG GTA-3'. These have been used in previous studies (Pouliot et al. 1997). Total RNA (1μg) was converted to cDNA with Superscript II (Invitrogen Life Sciences) using random primers, as per manufacturer's instructions. cDNA was then subjected to 'hot start' PCR amplification using 1.0U for each reaction, Amplitaq Gold (Perkin Elmer, CT), 1.5mM $MgCl_2$, 1x (10x) Buffer (100mM Tris-HCl, pH 8.3, 500nM KCl, 15mM $MgCl_2$, 0.01% (w/v) glycerin), 0.2mM dNTPs (New England Biolabs), 100ng of each primer and HPLC grade water (Sigma) to a total volume of 20μL. COX-2 specific oligonucleotide primers were designed from published genomic sequences such that the recognition sites of the upstream and downstream primers resided in separate exons (GeneWorks, Adel, Aust) (Table 2.1). PCR was performed for 35 cycles for COX-2 and the product could be assayed in the exponential phase of the amplification curve in a thermal cycler (Corbett Research, Vic, Aust). The PCR conditions were 95°C for 9min to activate the polymerase, and 35 cycles at 1min at 94°C, 1min at 62°C and 1min at 72°C. This was followed by an additional extension step for 1min at 72°C. A control reaction included a preparation in which the target RNA was omitted. Amplification products were resolved by electrophoresis on a 2% agarose gel. Oligonucleotide primer sequences and the expected PCR product size is listed (Table 2.1). The COX-2 PCR product was sequenced using the QIAGEN Dye terminator protocol (Qiagen Pty Ltd, Vic, Aust). The obtained sequences were aligned with the published COX-2 sequence using the Clustal X Multiple sequence alignment program.

Table 2.1 RT-PCR analysis for primers.
The synthetic oligonucleotides used in this study were primer pairs spanning the intron-exon boundaries of COX-2.

Target	Accession No.	Forward primer	Reverse primer	Product
hCOX-2	L15326	5'-GCT GAC TAT GGC TAC AAA AGC AGC TGG- 3'	5'-ATG CTC AGG GAC TTG AGG AGG GTA -3'	~ 450bp

2.2.15 COX-2 promoter-reporter construct

These were engineered and provided by Rebecca Cook-Johnson, Rheumatology Unit, Royal Adelaide Hospital. A vector containing a 7kb fragment (Accession No. AF044206) of the COX-2 promoter region was the kind gift of Dr Steven Prescott of the Huntsman Cancer Institute (University of Utah) (Meade et al. 1999). This was used as the template for amplification by PCR of a fragment containing bases –531 or –922 through to +65 relative to the COX-2 transcriptional start site. A reporter construct driven by this segment was responsive to hypoxia in endothelial cells (Schmedtje et al. 1997). Briefly, the conditions for the PCR were, 0.2U AmpliTaq Gold ® (Applied Biosystems), 1.5mM $MgCl_2$, 1x (10x) Buffer (100mM Tris-HCl, pH 8.3, 500nM KCl, 15mM $MgCl_2$, 0.01% (w/v) glycerin) 0.2mM dNTPs (New England Biolabs), 100ng of each primer and HPLC grade water (Sigma) to a total volume of 20µL. Conditions were 95°C for 10min, 30 cycles of (94°C 30sec, 50°C 30sec, 72°C 1min) then 72°C for 10min. The primers used had specific restriction sites built into the 5' most ends to facilitate ligation in a specific orientation into pGL3-Basic (Promega). This vector contains the gene coding the firefly luciferase gene, though lacks a promoter to drive its expression. The specific primers with the restriction sites in bold italics were, fpro-531COX-2 5' GC***GGTACC***GTTACTCGCCCCAGTCTGTC 3' and rpro+65COX-2 5' GG***CTCGAG***CGAGGCGCTGCTGAGGAG 3'.

The PCR product was purified using the MinElute™ PCR Purification Kit (Qiagen), restricted in separate reactions with *Kpn*I and *Xho*I (NEB) according to manufacturers instructions and then further purified for transformation using the MinElute™ Reaction

Cleanup Kit (Qiagen). The isolated restricted PCR products were then ligated at the *Kpn*I and *Xho*I sites located in the multiple cloning site of the pGL3-Basic vector using T4 DNA Ligase (Promega). The vectors pGL3-COX-2-531 or pGL3-COX-2-922 were then transformed into MAX Efficiency ® DH5α^{TM} competent cells according to manufacturers instructions. Sequencing (ABI Prism® Model 3700) confirmed the orientation and sequence of COX-2 –531 or COX-2-922. Overnight cultures were then grown and plasmids isolated using the Endofree® Plasmid Maxi Kit (Qiagen) to ensure minimal LPS contamination.

2.2.16 Transient Transfections

These were established and performed by Rebecca Cook-Johnson, Rheumatology Unit, Royal Adelaide Hospital. U937 monocytic cells were plated in 12 well plates ($2x10^6$cells/2ml) in RPMI with 10% FCS and PMA (50ng/ml) which promotes differentiation after 3-5 days of treatment (Pedrinaci et al. 1990). After differentiation, cells were transfected using Jet PEI (PolyTransfection), according to the manufacturers instructions. Briefly, 4μg of the pGL3-COX-2-531 construct and 1μg pGL-3-*Renilla* was suspended in 75μl of sterilised NaCl solution (150mM). 4μl of Jet PEI solution was also suspended in 75μl of sterilised NaCl solution (150mM). The Jet PEI/NaCl solution was then added to the DNA/NaCl solution and incubated at RT for 30min. The medium in the wells was replaced, and 150μl of the DNA/Jet PEI was added to each well. The transfection was allowed to proceed for 5h after which the medium was replaced again with hypoxic or normoxic medium. The cells were then stimulated with serum treated zymosan (STZ) (100μg/ml) for specified times and normoxic or hypoxic conditions respectively. Following the transfection period, the medium was removed and discarded

and the cells lysed with 100µL of Passive Lysis Buffer (PLB) supplied in the Dual-Luciferase™ Reporter Assay kit. The lysate was then assayed for luciferase activity.

Transfection of the COX-2 promoter-luciferase construct into FLS was performed using 4µg of the pGL3-COX-2-922 and Jet PEI (PolyTransfection) (as described above). Following the 5h transfection period, FLS were immersed in either hypoxic or normoxic medium and stimulated with IL-1β (2ng/nl) for 8h in normoxic or hypoxic conditions, respectively. Following this, the medium was removed, 100µl of PLB was added and then assayed for luciferase activity.

HUVEC were transfected using Amaxa HUVEC (Vs.2) Nucleofector™ kit (Amaxa, Maryland, USA) according to the manufacturer's instructions. Briefly, cells were plated at 80% confluency and the next day the medium was changed to EGM-2 medium (Clonetics) overnight. Cells were then trypsinised and resuspended at $1x10^6$ cells / 100µl of HUVEC Nucleofector Solution with a total concentration of 5µg plasmid DNA with a 4:1 ratio of COX-2-531 promoter reporter-construct:control *Renilla* plasmid. This solution was then transferred to an Amaxa cuvette and subjected to electroporation. Cells were then resuspended in an appropriate volume of EGM-2 medium and transferred to 24-well plates. Cells were allowed to recover overnight, after which the medium was changed to normoxic or hypoxic medium and incubated in the presence of IL-1β (2ng/ml) at 37°C for 18h under normoxic or hypoxic conditions respectively. Cells were then assayed using the Dual-Luciferase™ Reporter Assay kit (Promega, New South Wales, Aust).

2.2.17 Fluorescence activated flow cytometry

Flow cytometry was used to assess monocyte purity using forward and side scatter to assess cell size and intracellular complexity, respectively. Immunostaining for COX-2 was performed on adherent HUVEC or non-adherent monocytes. HUVEC ($2x10^5$) in each well of a 12-well plate or monocytes ($1x10^6$ / tube) were used.

Non-specific binding through Fc receptors on cells was blocked by incubation with 10% normal human serum (NHS), which had been heat inactivated for 40min at 56°C prior to the addition of the staining antibody. Control incubations containing isotype matched Abs against irrelevant specificities were run concurrently with each analysis. Intracellular staining (for COX-2) involved incubating cells with 0.1% saponin (Sigma Chem Co) to permeabilise cells, together with primary COX-2 PAb for 30min at 4°C.

This was followed by 3x washes in FACS wash buffer (PBS, 2% FCS, 0.01M azide) then incubated with secondary anti-rabbit PAb for 40min at 4°C. Adherent cells were trypsinised with Trypsin EDTA (0.05% Trypsin, 0.53mM EDTA) (Gibco) for 30s followed by neutralisation with 3ml of FACS wash buffer. After 3x washing with FACS wash buffer, cells were resuspended in 300µl of FACS Fix (PBS, 1% formalin (v/v), 2% glucose (w/v), 0.02% azide). Cells were stored at 4°C in the dark until flow cytometric analysis could be performed (not longer that 2 days). A minimum of 10,000 cells were analysed using a COULTER® EPICS®XL-MCL flow cytometer and SYSTEM II™ v.3 software. A table of the antibodies used are summarised (Table 2.2).

2.2.18 Immunohistochemistry

Table 2.2 Antibodies used for flow cytometric analysis.

Antibody	Specificity	Class	Form	Conc^{n}	Source
COX-2	COX-2	Polyclonal	Purified	20μg/ml	Cayman Chem
Rabbit polyclonal	Giardia	Polyclonal Control	Purified	20μg/ml	Prof G Mayrhofer
FITC rabbit 2^{o} Ab	rabbit	Polyclonal	Purified	Neat	Dr P. Hurtardo

Immunohistochemistry was performed on HUVEC ($2x10^4$/well) plated on a fibronectin coated 8-well chamber slide (Nalge Nunc, Naperville, IL). Following incubation with IL-1β in the presence or absence of hypoxia, cells were fixed with 96% ice-cold ethanol for 30sec, before washing 2x with FACS wash. Rabbit anti-human COX-2 polyclonal Ab at 20μg/ml was added to cells (or isotype control) for 30min at 4°C. Cells were washed 2x gently with FACS wash to avoid cells detaching. Anti-rabbit-FITC was added for 40min at 4°C followed by 2x washing, the walls of the chamber were removed and a coverslip on the slide allowed the fluorescence to be detected by microscopy. Five fields/ treatment were photographed and analysed for intensity of fluorescence using V++ Precision Digital Imaging System (Auckland, NV).

2.2.19 Statistical Analysis

Results are expressed as the mean ± S.E.M of triplicate incubations. Analysis of variance followed by the Neuman-Keuls multiple comparisons test was used to identify the statistically significant changes in eicosanoid production between treatments using WINKS (Texasoft, Cedar Hill, TX).

CHAPTER 3

EFFECT OF HYPOXIA ON HUMAN MONOCYTE COX-2 EXPRESSION AND ACTIVITY

3.1 INTRODUCTION

Blood derived monocytes are found at sites of inflammation as well as in solid tumors and atheromatous plaques. They have important roles in the pathology of inflammation which in part, involves their production of inflammatory eicosanoids such as PGE_2 and TXA_2 (Zhang et al. 1997; James et al. 2001).

Cyclooxygenase-2 (COX-2) is an immediate early response gene and commonly associated with inflammatory responses due its augmentation of PGE_2 and TXA_2 biosynthesis (Dubois et al. 1998; Turini and DuBois 2002). Studies of inflammatory mediator production are performed invariably in medium equilibrated under normoxic conditions, i.e. 20% O_2. However, many monocyte-containing lesions such as solid tumors (Vaupel et al. 1991; Runkel et al. 1994; Hockel et al. 1996; Lartigau et al. 1997; Knocke et al. 1999; Movsas et al. 1999; Rofstad and Maseide 1999), rheumatoid joints (Falchuk et al. 1970; Lund-Olesen 1970; Treuhaft and McCarty 1971) and atherosclerotic lesions (Heughan et al. 1973; Jurrus and Weiss 1977; Crawford and Blankenhorn 1991; Bjornheden et al. 1999) are reported to be hypoxic. Consequently, it is important to consider the effects of hypoxia

on monocyte inflammatory eicosanoid production and the key enzyme responsible for their synthesis, COX-2.

It has been reported that hypoxia resulted in upregulation of COX-2 in endothelial cells and a rabbit corneal epithelial cell line (Schmedtje et al. 1997; Bonazzi et al. 2000). However, the effects of hypoxia on monocyte COX-2 and eicosanoid production have not been examined and the mechanisms responsible for COX-2 upregulation by hypoxia remain poorly explored. Chapter 3 will characterise the effects of hypoxia on COX-2 expression and eicosanoid synthesis in fresh human monocytes, monocyte-derived macrophages and U937 monocytic cells.

3.2 MATERIALS and METHODS

3.2.1 Materials

Materials were obtained from the following sources: COX-2 rabbit polyclonal Ab, β-actin mouse monoclonal antibody (Cayman Chemicals (Ann Arbor, MI), PGE_2 antiserum (Sigma), rabbit TXB_2 antiserum was prepared from rabbits immunised with TXA_2 conjugated to human thyroglobulin as used in previous studies (James and Walsh 1988), pyrogen free Lymphoprep, (Nycomed, Oslo, Norway) LPS, PMA, gliotoxin, zymosan A, Actinomycin D (Sigma Chem Co, St Louis, MO), protein trans-blot transfer membranes (Bio-Rad, North Ryde, AUS), RNA nylon transfer membrane (Hybond N+, Amersham Pharmacia Biotech, Pisacataway, NJ), peroxidase labelled donkey anti-rabbit Ab, peroxidase labelled goat anti-mouse Ab, ^{3}[H].PGE_2, ^{3}[H].TXB_2 (Amersham International, Little Chalfont, ENG), Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) IL-1β, TNFα (Endogen, Boston, MA), α^{32}[P]-dCTP (Perkin Elmer, CT), GIGA Prime probe labelling kit, (Bresatec, Adelaide, AUS), GAPDH and COX-2 mRNA probe (prepared as per method in Chapter 2, section 2.2.15.5) phosphorylated p38 polyclonal Ab (New England Biolabs, Beverly, MA), GM-CSF (gift from Dr Andrew Zannetino, Hanson Institute, Adelaide), M-CSF (gift from Dr Prue Hart, Flinders Medical Centre, Adelaide) anti-rabbit FITC (gift from Dr Plinio Hurtardo).

3.2.2 Methods

Cell incubations

Fresh human monocytes were isolated by counter current centrifugal elutriation as described in section 2.2.1. The purity of all preparations was assessed by FACS analysis and typically >85% monocytes. Lymphocytes were the major cell contaminant. However, they are not responsive to LPS stimulation and therefore did not contribute significantly to eicosanoid or cytokine synthesis.

Where it is indicated that LPS treatment was 'transient', LPS was added to cells for 15min in normoxia or hypoxia at 37°C. After 15min, monocytes were washed 2x in their respective medium and the incubation period was allowed to continue with fresh normoxic or hypoxic medium.

Where indicated, inhibitors were incubated for 15min prior to LPS stimulation. Inhibitors were dissolved in DMSO or ethanol and all controls contained the vehicle for the duration of the incubation period.

Monocyte-derived macrophages and U937 cells were prepared as described in sections 2.2.4 and 2.2.5, respectively.

Flow cytometry

For flow cytometry of COX-2, cells were permeablised with saponin (0.1%v/v) and 50µl of COX-2 primary antibody or isotype control (rabbit polyclonal Ab) (20µg/ml). Following 3x washing, 50µl of secondary FITC anti-rabbit Ab was used at a 1/200

dilution. FACS Fix was added after another series of washing and stored at –20°C until analysis.

Northern analysis

Northern membranes were processed by hybridising membranes with a COX-2 cDNA or GAPDH cDNA probe overnight at 43°C on a rotator. Initial washes began at low temperatures (RT) with low stringency wash buffer (2x SSC, 0.1% SDS) for 30min. A Geiger counter was used to determine the extent of background radioactivity on the membrane. If the membrane required further washing, a higher temperature (43°C) and more stringent wash buffer (0.1x SSC, 0.1% SDS) was used. Radiation sensitive film was exposed to the membranes for 24h at –80°C.

Transcription Studies

A COX-2 promoter-reporter construct was designed as indicated in section 2.2.15 in the methods section. Briefly, this comprised a region –531bp upstream from the transcription start site of the COX-2 gene ligated into the pGL3-Basic vector (Promega), which contains a luciferase reporter gene downstream from the site of ligation. Transfection of the reporter construction into U937 monocytic cells was performed following seeding 12 well plates with $2x10^6$ cells / 2ml in RPMI with 10% FCS and phorbol 12-myristate 13-acetate (PMA) (50ng/ml) which promotes differentiation after 3-5 days of treatment (Pedrinaci et al. 1990). After differentiation, cells were transfected using 4µg of the pGL3-COX-2-531 construct and 4µl of Jet PEI were each suspended in 75µl of sterile NaCl solution (150mM). The Jet PEI / NaCl solution was then added to the DNA/NaCl solution and

incubated at RT for 30min. The medium in the wells was then changed to fresh medium, and 150µl of the DNA/Jet PEI was added to each well. The transfection was allowed to proceed for 5h and the medium replaced again with either hypoxic or normoxic medium and stimulated with STZ (100µg/ml). Following this, the medium was removed and cell lysates were then assayed for luciferase activity.

Western analysis

Transfer membranes were treated with rabbit polyclonal COX-2 Ab at 1/1,000 dilution for 1h at RT or phospho-p38 MAPK Ab at a 1/500 dilution in TBS-Tween overnight at 4°C. β-actin protein was stained with mouse monoclonal β-actin at 1/2,500 dilution in TBS-Tween for 60min at RT. Following washing, the membrane was treated with donkey anti-rabbit horseradish peroxidase (HRP) Ab (for COX-2) or goat anti-mouse HRP Ab (for β-actin) at 1/10 000 dilution in TBS-Tween for 45min. The chemiluminescent substrate solution was added to the membrane (5min) prior to exposure to light sensitive film for 2-5min.

3.3 RESULTS

3.3.1 Effect of hypoxia on monocyte COX-2 message and protein.

LPS (200ng/ml) added to fresh human monocytes induced COX-2 mRNA and protein over 18h. COX-2 protein bands have a characteristic doublet due to glycosylation heterogeneity. The up-regulation of COX-2 mRNA and protein was greatly augmented by hypoxia (1% O_2) (Fig 3.1). This augmentation of COX-2 expression by hypoxia was observed with a variety of co-stimuli (Fig 3.2). Flow cytometry was also used to confirm the effect of hypoxia on monocyte COX-2 induction. Again, the induction of COX-2 protein in monocytes stimulated with LPS was upregulated in hypoxia compared to normoxia (Fig 3.3).

Following these observations, two potential mechanisms for the upregulation of COX-2 mRNA in hypoxia were investigated.

3.3.2 Effect of hypoxia on transcription of COX-2 mRNA

It has been reported that hypoxia can increase transcription of COX-2 in endothelial cells (Schmedtje et al. 1997), and therefore this mode of regulation was examined. Many attempts to transfect fresh human monocytes transiently with a COX-2 promoter/luciferase reporter construct were unsuccessful. However, the human monocytic cell line, U937, was

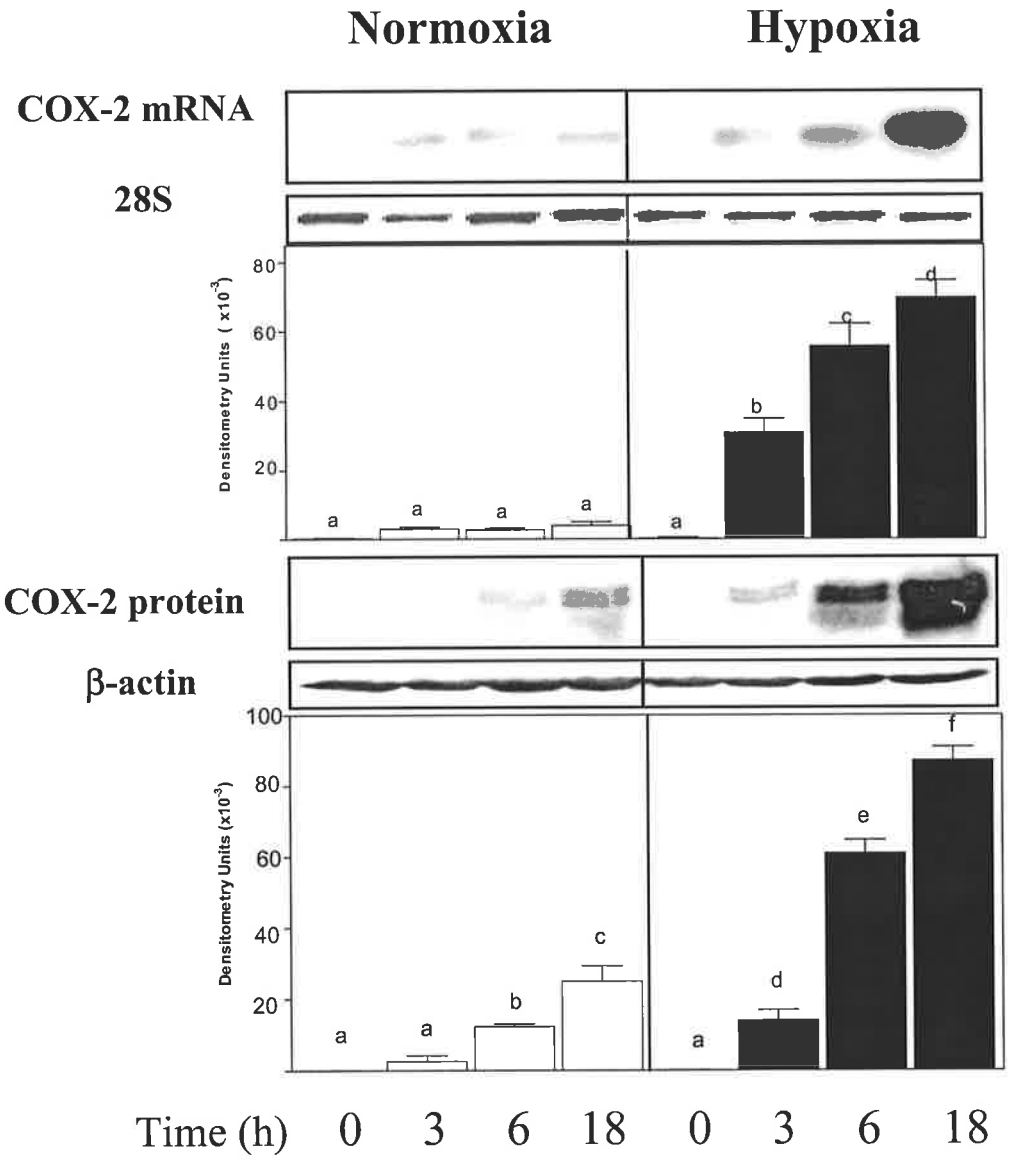


Figure 3.1
Effect of hypoxia on COX-2 mRNA and protein
Monocytes ($5x10^6$) were stimulated with LPS (200ng/ml) in normoxia or hypoxia. Cells were processed for Northern blot and Western blot analysis as described in the Methods section. Blots are representative of 3 separate experiments, the mean values of which are shown in the graph. Within the Northern or Western Blot series, bars with different letters are significantly different from each other ($p<0.05$).

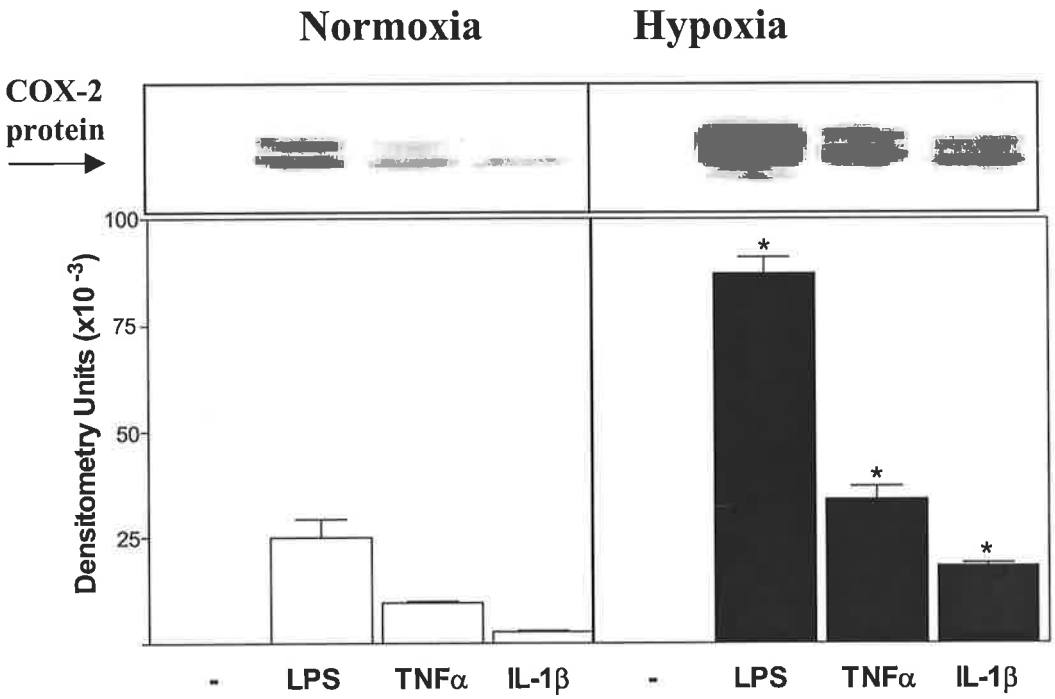


Figure 3.2

Effect of hypoxia on COX-2 protein induction in monocytes with various stimuli

Monocytes ($5x10^6$) were stimulated with LPS (200ng/ml), TNFα (1ng/ml), IL-1β (2ng/ml) for 24h in normoxia or hypoxia. Cells were processed for western blot analysis as described in the Methods section. Western blot is representative of 3 separate experiments, the mean values of which are shown in the graph. *$p<0.05$, by comparison to the same stimuli in normoxic monocytes.

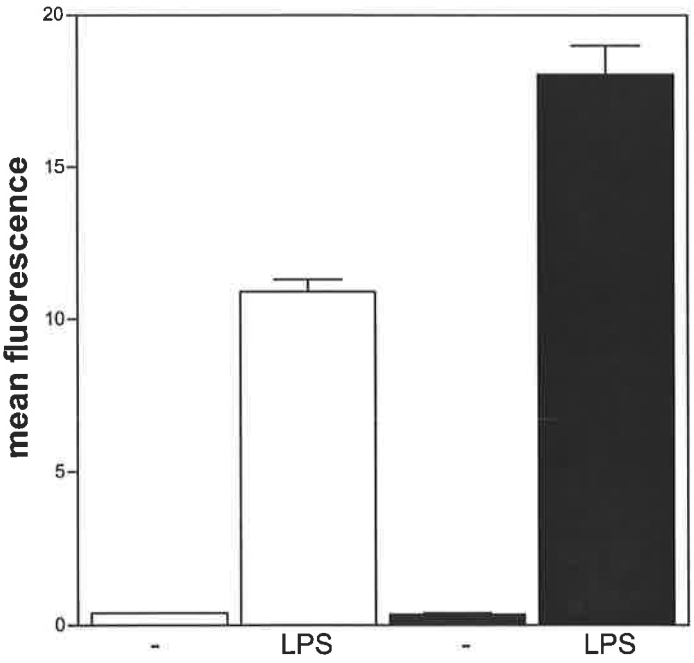


Figure 3.3
Effect of hypoxia on COX-2 protein by flow cytometry
Monocytes ($1x10^6$) were stimulated with LPS (200ng/ml) in normoxia (open bars) or hypoxia (solid bars). Cells were processed for FACS analysis (mean ± SEM, n=4).

transfectable and these cells were used. Hypoxia augmented activity of the -531bp segment of the COX-2 promoter in U937 cells (Fig 3.4).

Post transcriptional regulation of COX-2 mRNA can occur and has been shown to occur with LPS treatment of monocytes (Barrios-Rodiles et al. 1999). However, this had not been examined in hypoxia and therefore, the effect of hypoxia on COX-2 mRNA stability in monocytes was examined.

3.3.3 Effect of hypoxia on monocyte COX-2 mRNA stability

Monocytes were treated with LPS in two ways. In one case, cells were treated with LPS for 15min after which cells were washed and allowed to incubate for another 3h (transient treatment). In the other case, LPS was left in contact with the cells for a full 3h of incubation (sustained treatment). Incubations were undertaken under normoxic or hypoxic conditions. Actinomycin D (AD) was added to inhibit further transcription and then the level of COX-2 mRNA was measured for a further 3h incubation. In monocytes where LPS was left in (sustained treatment), COX-2 mRNA did not show any signs of decreased mRNA levels over time in normoxia or hypoxia (Fig 3.5a). By comparison, monocytes which had transient treatment with LPS, showed over 90% decrease in the level of COX-2 mRNA in normoxia within 3h following AD addition (Fig 3.5a). However, in hypoxia, COX-2 mRNA levels decreased by less than 20% in the 3h following AD addition (Fig 3.5a). A graphical representation of COX-2 mRNA normalised against GAPDH is shown (Fig 3.5b).

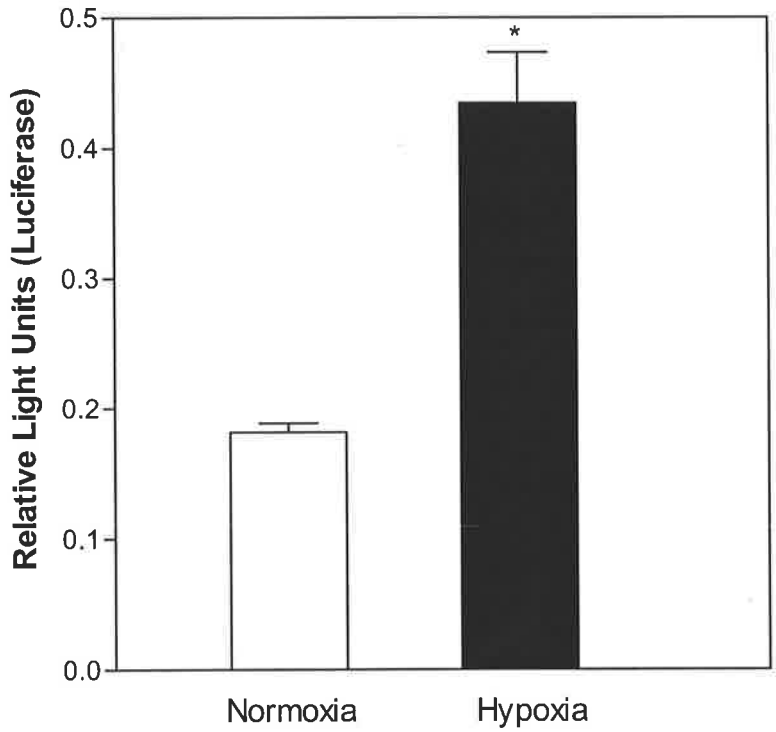


Figure 3.4

Effect of hypoxia on COX-2 transcription in U937 monocytic cells

U937 monocytic cells ($2x10^6$ cells/2mL) were transfected with COX2 -531 construct (4μg) using Jet PEI and incubated for 5h. The media was changed, and stimulated with STZ (100μg/ml) for 8h in normoxia (open bar) or hypoxia (solid bar). Cells were then lysed and assayed for luciferase activity (n=3). *$p<0.05$, by comparison to normoxic cells. Results are representative of 4 separate experiments assayed in triplicate.

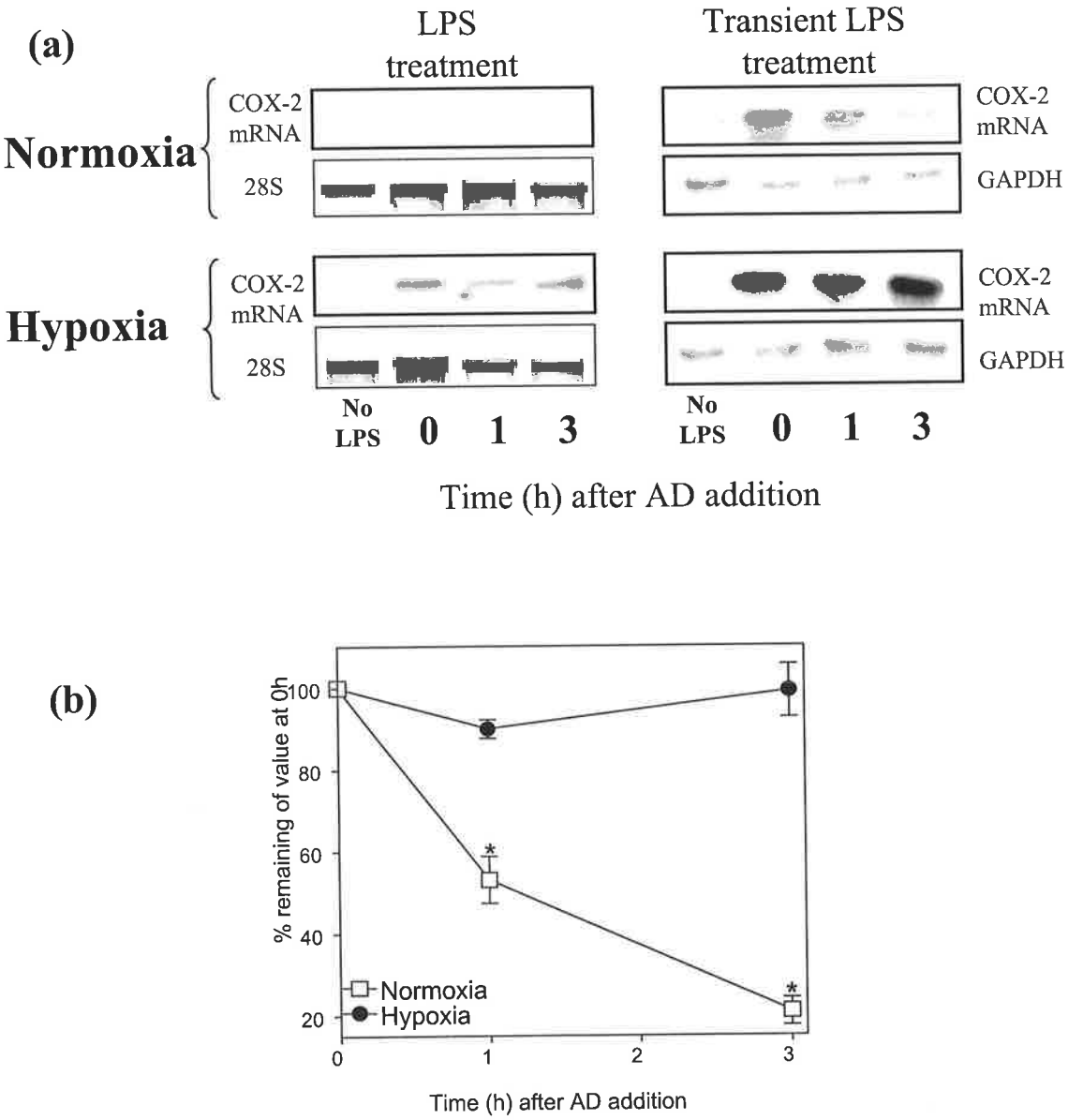


Figure 3.5

Effect of hypoxia on COX-2 mRNA stability

(a) Monocytes ($5x10^6$) were stimulated with LPS (200ng/ml) or transiently with LPS (15min) for 3h at 37°C in normoxia or hypoxia. AD (5μg/ml) was then added and the level of COX-2 mRNA was assessed for a further 3h (37°C) by Northern analysis. Blots are representative of 3 separate experiments (b) In the graphic depiction of densitometry results from transient LPS stimulation, COX-2 mRNA is normalised against GAPDH and the rate of decay is shown with values at 1 and 3h expressed as a % of value at 0h (mean ±SEM, n=3); normoxia (□) and hypoxia (●). *$p<0.05$, by comparison to the equivalent times in hypoxic monocytes.

To determine whether the increased COX-2 expression in hypoxia led to an increase overall COX activity, we determined the effect of hypoxia on synthesis of the major eicosanoids PGE_2 and TXA_2, (measured as TXB_2), produced by monocytes.

3.3.4 Effect of hypoxia on monocyte eicosanoid activity

Fresh monocytes were stimulated transiently or throughout with LPS under normoxic or hypoxic conditions and the accumulation of PGE_2 and TXB_2 in the cell supernatants was measured. There was a time dependent increase in PGE_2 and TXB_2 accumulation in monocytes over 18h, when LPS was present transiently or throughout (Fig 3.6). However, in hypoxia, there was a marked reduction in the rate of synthesis of PGE_2 and TXB_2 compared to the rate of synthesis in normoxia with both modes of stimulation (Fig 3.6). The reduced synthesis of these eicosanoids in hypoxia did not equate with the increased expression of COX-2 protein in hypoxia, described above in Figure 3.1.

Possible explanations for the disparate hypoxia induced changes in COX-2 expression and eicosanoid synthesis are examined in Chapter 4.

3.3.5 Effect of hypoxia on p38 mitogen activated protein kinase (MAPK)

The activation of the p38 MAPK is essential for the induction of COX-2 in fresh human monocytes in normoxia (Pouliot et al. 1997). Therefore, it was important to determine whether an increase in the phosphorylation of p38 MAPK also contributed to the augmented expression of COX-2 in hypoxia.

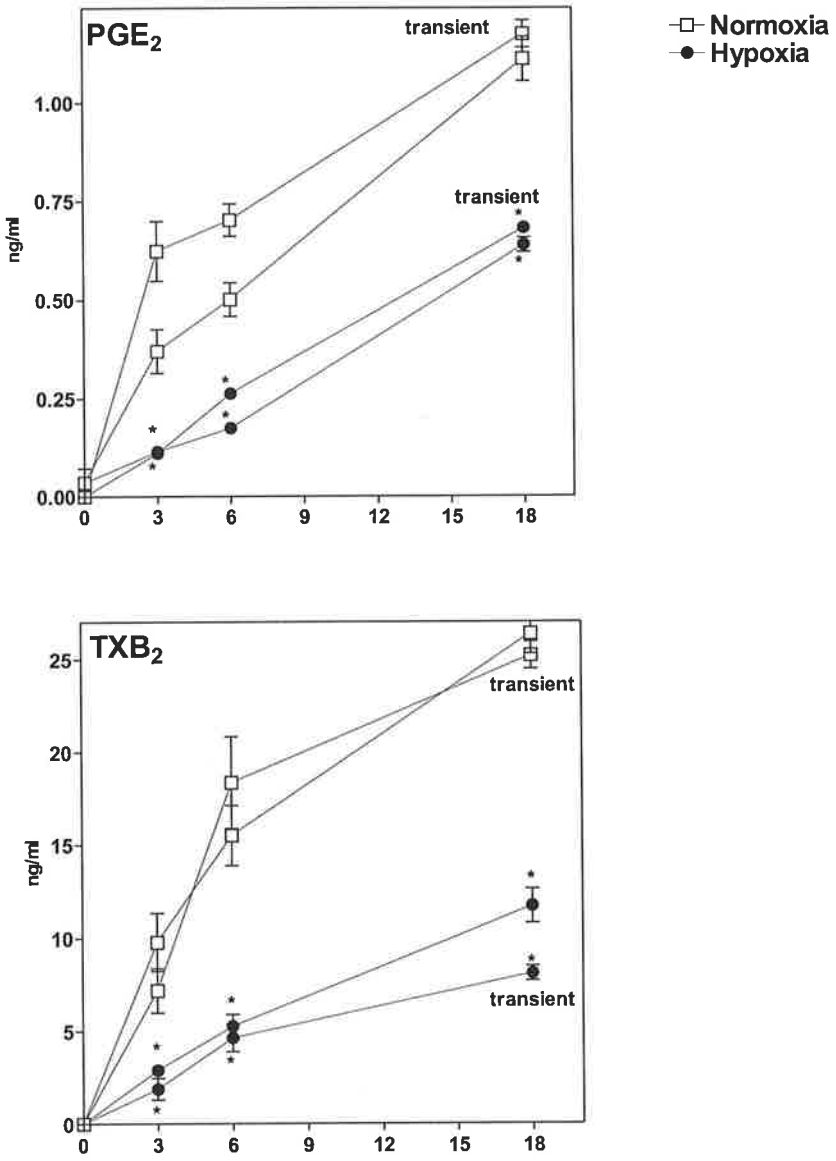


Figure 3.6

Effect of hypoxia on COX-2 activity

Monocytes ($5x10^6$) were stimulated with LPS (200ng/ml) or transiently with LPS (15min) for the times shown in normoxia (□) or hypoxia (●). Supernatants were collected and assayed for PGE_2 and TXB_2 by RIA (mean ± SEM, n=3). *$p<0.05$, by comparison to normoxic monocytes.

Fresh monocytes were stimulated with LPS for up to 120min. There was no change in the extent of phosphorylation of p38 MAPK or time course of decay in the amount of phosphorylated enzyme (Fig 3.7).

3.3.6 Effect of gliotoxin, a NFκB inhibitor, on COX-2 mRNA, protein and activity in hypoxia

The induction of COX-2 in endothelial and colonic epithelial cells is dependent on transcription factors including NFκB activation (Schmedtje et al. 1997; Jobin et al. 1998). To determine whether the induction of COX-2 in hypoxia was dependent on the activation of NFκB, monocytes were treated with gliotoxin (100ng/ml), prior to stimulation with LPS. For examination of mRNA, LPS treatment was transient (15min) and followed by washout and incubation for a further 3h. For examination of protein, LPS treatment was sustained (18h). Gliotoxin inhibits IκBα degradation and thereby inhibits NF-κB DNA binding activity (Liu et al. 2003).

COX-2 mRNA and protein was significantly inhibited with the addition of gliotoxin (Fig 3.8a). This was reflected in the activity of COX-2, since eicosanoid synthesis was also significantly decreased (Fig 3.8b).

3.3.7 Effect of hypoxia on M-CSF and GM-CSF differentiated monocyte-derived macrophages

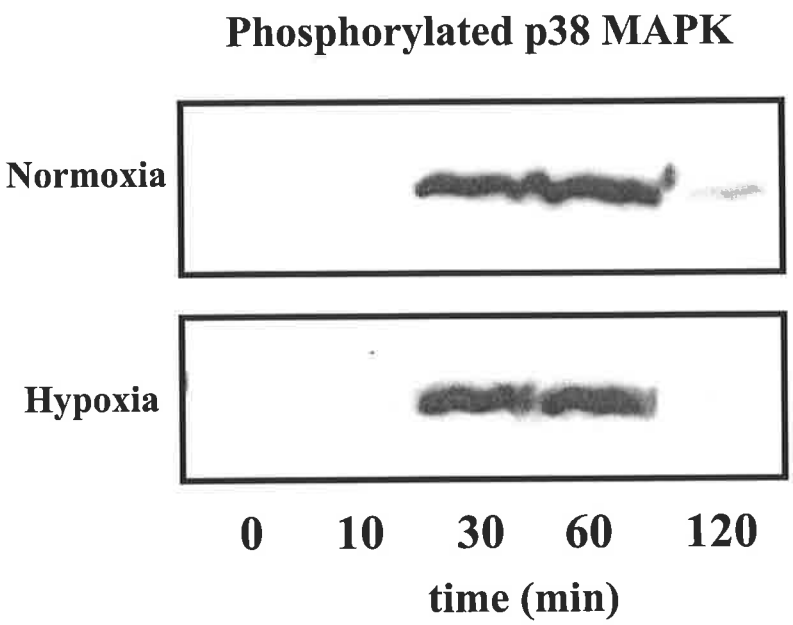


Figure 3.7
Effect of hypoxia on phosphorylated p38 MAPK
Monocytes ($5x10^6$) were stimulated with LPS (200ng/ml) under normoxic or hypoxic conditions for up to 120min. At each time point, cells were processed for western blot analysis as described in the Methods section. Results are representative of 3 experiments.

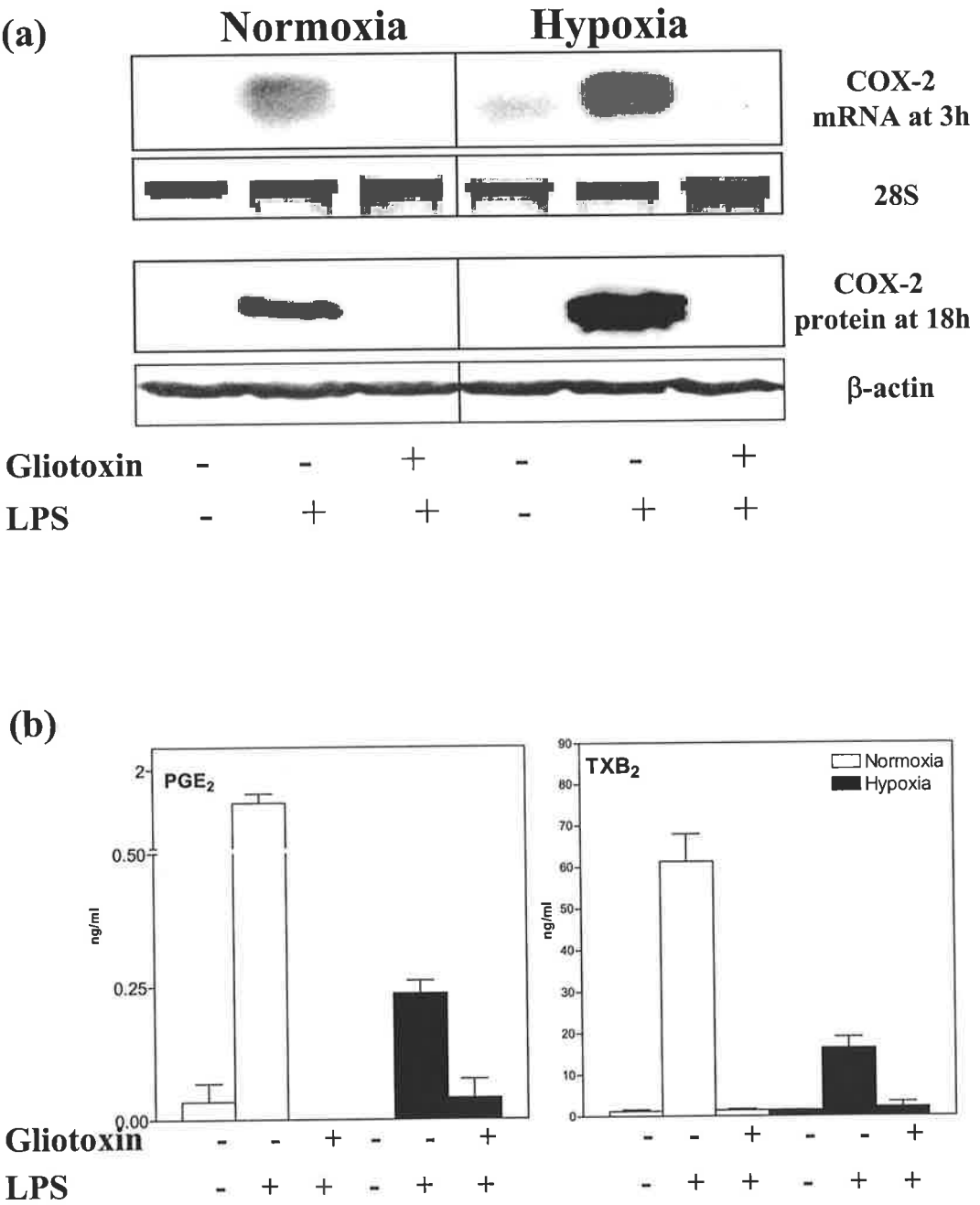


Figure 3.8

Effect of gliotoxin, a NFκB inhibitor, on COX-2 mRNA, protein and activity

(a) Monocytes ($5x10^6$) were stimulated with LPS (200ng/ml) for 3h (for mRNA) or 18h (for protein) in normoxia or hypoxia . (b) Supernatants were collected after 18h and assayed for PGE_2 and TXB_2 by RIA (mean ± SEM, n=3). Normoxic supernatants (open bars) were compared to hypoxic supernatants (solid bars).

All of the studies described above have been conducted with fresh human monocytes. To examine whether cells with a macrophage phenotype had similar responsiveness to hypoxia, monocytes were pre-treated with M-CSF or GM-CSF (as described in section 2.2.4) before experiments were commenced. Thus, monocyte derived macrophages ($5x10^6$) were incubated in the absence or presence of LPS for 18h following 6 days differentiation with M-CSF or GM-CSF.

LPS induced COX-2 protein after 18h in M-CSF and GM-CSF treated macrophages under normoxic conditions (Fig 3.9a). As observed in fresh monocytes, COX-2 induction was augmented in hypoxia (Fig 3.9a). Despite the upregulated COX-2 expression in hypoxia, the accumulation of PGE_2 and TXB_2 in macrophages was reduced (Fig 3.9b). Thus the macrophages were similar to monocytes in their COX-2 and eicosanoid responses to hypoxia.

3.3.8 Effect of hypoxia on PMA differentiated U937 monocytic cells

Following differentiation of U937 cells with PMA (50ng/ml) for 3 days, cells ($5x10^6$) were incubated with STZ (100ng/ml) for up to 18h. STZ induced COX-2 time dependently in normoxia over this period (Fig 3.10a). Again, COX-2 induction was augmented in hypoxia (Fig 3.10a) and the accumulation of PGE_2 and TXB_2 in the supernatants was reduced in hypoxia compared to normoxia (Fig 3.10b)

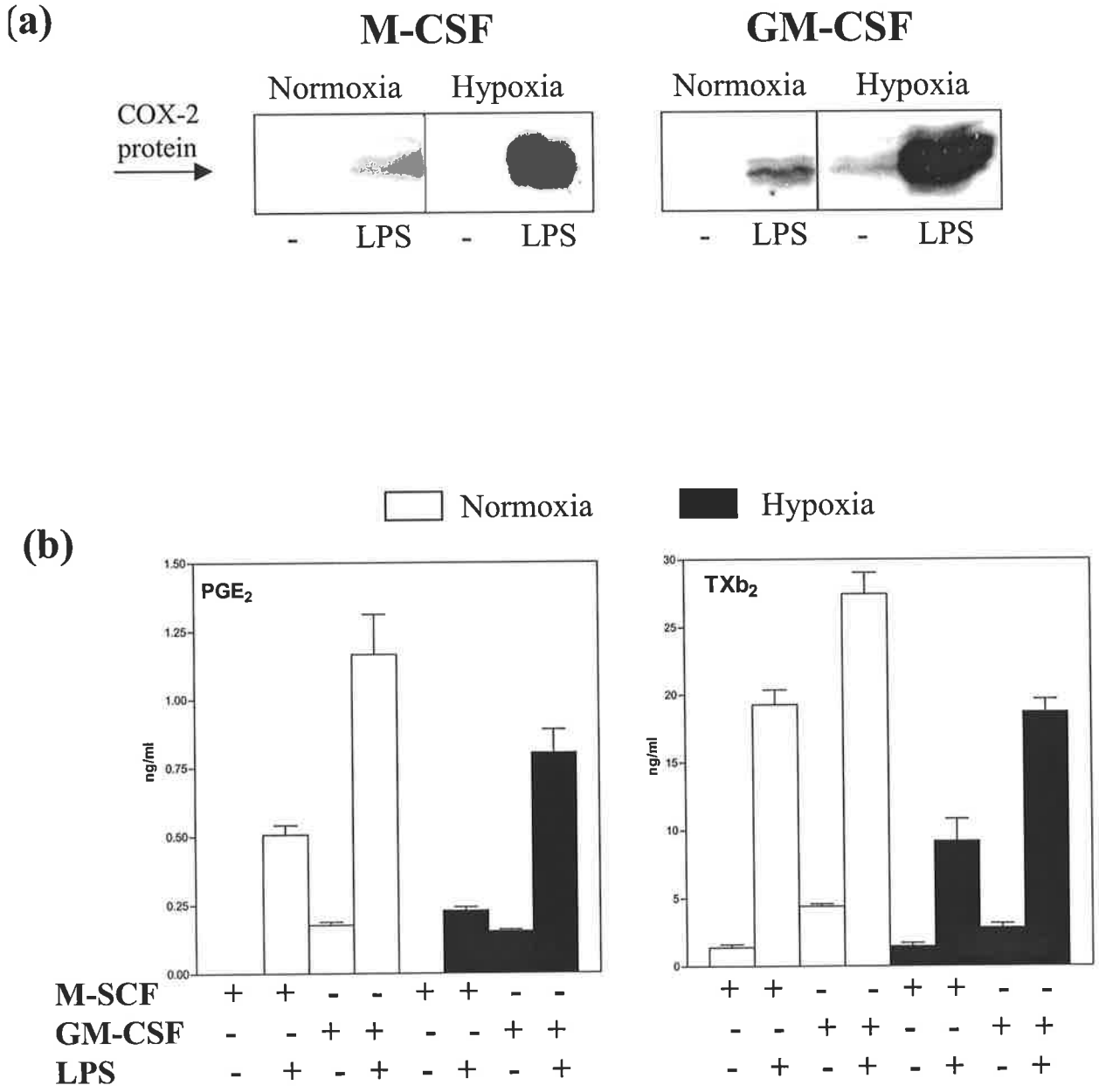
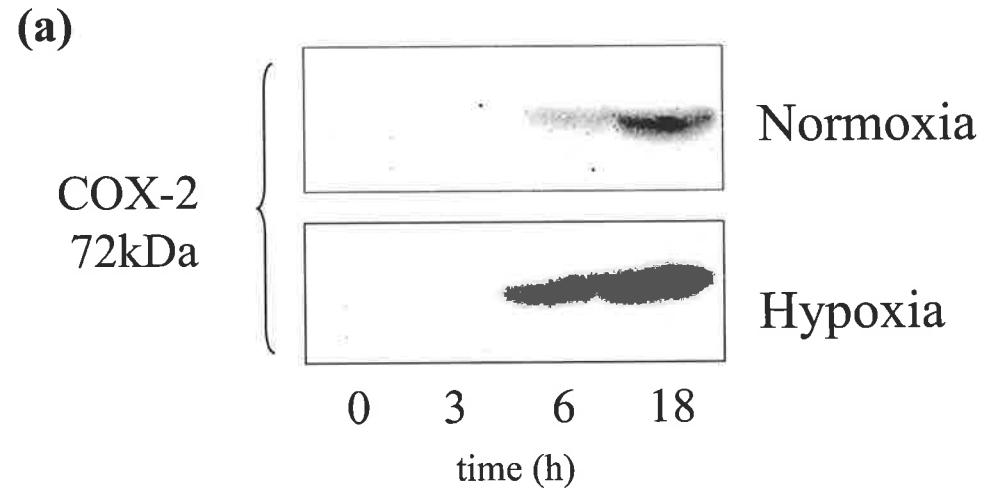


Figure 3.9

Effect of hypoxia on M-CSF and GM-CSF monocyte derived macrophage COX-2 expression and activity

(a) Monocytes ($5x10^6$) treated 6 days with M-CSF (50ng/ml) or GM-CSF (100ng/ml) for 6 days, then washed and stimulated with LPS (200ng/ml) for 18h in normoxia or hypoxia. Cell pellets were processed for western blots as per methods. (b) Supernatants were collected after 18h in normoxia (open bars) or hypoxia (solid bars) and assayed for PGE_2 and TXb_2 by RIA (mean ± SEM, n=3).



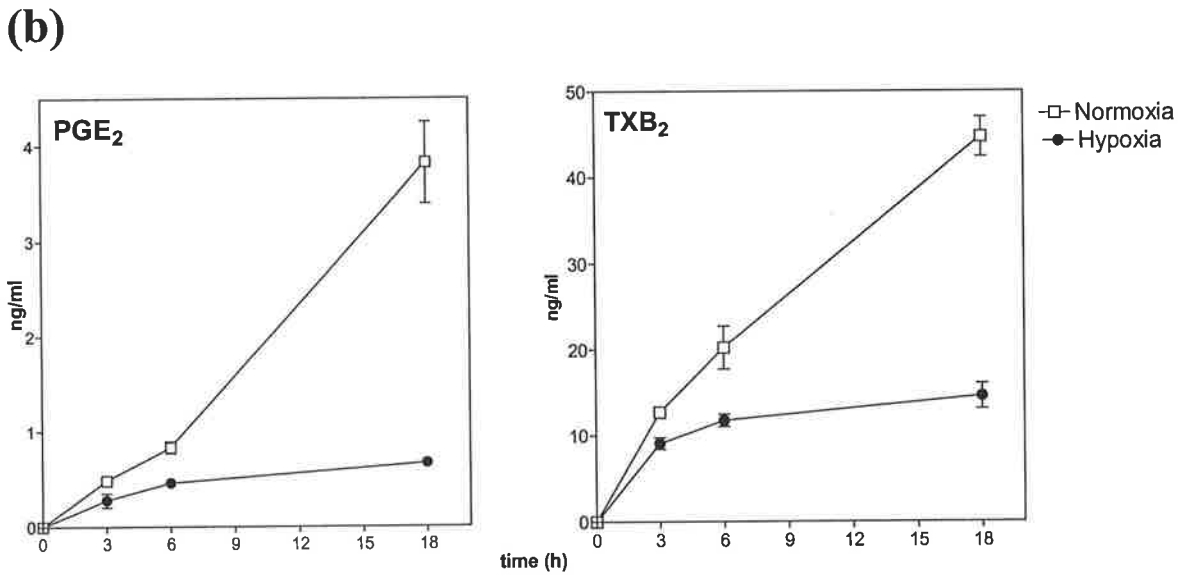


Figure 3.10

Effect of hypoxia on PMA differentiated U937 monocytic cell COX-2 protein and activity

(a) PMA-treated U937s ($5x10^6$) were stimulated with STZ (100μg/ml) for 3, 6 and 18h normoxia or hypoxia. Cell pellets were processed for westerns as per methods (b) Supernatants were collected at 3, 6 and 18h and assayed for PGE_2 and TXB_2 by RIA (mean ± SEM, n=3). Normoxic supernatants (□) were compared to hypoxia supernatants (●).

3.4 DISCUSSION

There is abundant evidence demonstrating that hypoxia exists in a variety of inflamed lesions including rheumatoid joints (Falchuk et al. 1970; Lund-Olesen 1970; Treuhaft and McCarty 1971), solid tumors (Vaupel et al. 1991; Runkel et al. 1994; Hockel et al. 1996; Lartigau et al. 1997; Knocke et al. 1999; Movsas et al. 1999; Rofstad and Maseide 1999) and arterial walls of atheromatous plaques (Heughan et al. 1973; Jurrus and Weiss 1977; Crawford and Blankenhorn 1991; Bjornheden et al. 1999). This provides a strong rationale for determining the effects of reduced oxygen tensions on inflammatory mediator production.

To date, *in vitro* studies of inflammatory mediator production by human monocytes/macrophages have been well characterised in normoxic conditions (20% O_2). However, this is unlikely to reflect conditions of oxygenation that monocytes encounter in many monocyte-containing lesions such as inflamed joints, atheromatous lesions and solid tumors. In joints with effusions, dissolved O_2 levels in the range of 8-80mmHg have been measured (Falchuk et al. 1970; Lund-Olesen 1970; Treuhaft and McCarty 1971). The presence of an effusion can readily increase intra-articular pressure to levels above capillary closure pressure, particularly during everyday activities such as standing, walking, and even modest flexion (James et al. 1990). Similarly, several studies have demonstrated a decreased oxygen concentration in the media of atherosclerotic arteries, ranging between 2 and 50mmHg (Heughan et al. 1973; Jurrus and Weiss 1977; Crawford and Blankenhorn 1991; Bjornheden et al. 1999). This led to the hypothesis that hypoxia is a component of the pathology of atherosclerotic plaques (Boxen 1985; Simanonok 1996). In addition, regions of reduced oxygen tensions have been reported in breast (Vaupel et al.

1991; Runkel et al. 1994), prostate (Movsas et al. 1999), melanoma (Lartigau et al. 1997; Rofstad and Maseide 1999) and cervical cancers (Hockel et al. 1996; Knocke et al. 1999) although the oxygen levels are very heterogenous within individual tumors. Thus, examination of the effects of hypoxia on monocyte inflammatory mediator production has relevance to many pathological situations in which monocytes are present.

In this study, we incubated monocytes in 1% O_2 (v/v) which provided dissolved O_2 levels of 33 mmHg, which is in the range observed in hypoxic joints (Falchuk et al. 1970; Lund-Olesen 1970; Treuhaft and McCarty 1971), solid tumors (Vaupel et al. 1991; Runkel et al. 1994; Hockel et al. 1996; Lartigau et al. 1997; Knocke et al. 1999; Movsas et al. 1999; Rofstad and Maseide 1999) and arterial walls of atheromatous plaques (Heughan et al. 1973; Jurrus and Weiss 1977; Crawford and Blankenhorn 1991; Bjornheden et al. 1999).

It has been demonstrated in human umbilical vein endothelial cells (HUVEC) that hypoxia increased COX-2 expression and that an increase in transcription was involved (Schmedtje et al. 1997). However, the effect on prostaglandin production of this COX-2 response to hypoxia was not measured (Schmedtje et al. 1997). We observed that hypoxia caused a marked upregulation of COX-2 mRNA and protein in fresh human monocytes. In addition, flow cytometry confirmed upregulated COX-2 protein in hypoxia and these results may be explained by an increase in COX-2 transcription and in COX-2 mRNA stability. Many RNAs coding for immediate-early response genes such as COX-2, are unstable and have short half-lives (Kruys et al. 1989; Cok and Morrison 2001). This is related to the presence of repeated AUUUA motifs in the 3'-untranslated region (3'-UTR) of the gene (Akashi et al. 1994). The 3'-UTR of the COX-2 gene contains 22 copies of the AUUUA motif. The response to hypoxia of COX-2 mRNA observed in this study may be a

more general phenomenon because the gene for vascular endothelial growth factor (VEGF) also contains instability motifs in its 3'-UTR and the mRNA is stabilised following hypoxia (Shima et al. 1995; Levy et al. 1998).

Another candidate mechanism for upregulation of COX-2 expression in hypoxia was prolonged phosphorylation of the p38 MAPK pathway. However, our results demonstrate no significant changes in activation of this enzyme in hypoxia compared to normoxia.

NF-κB is a transcription factor involved in the regulation of many genes. It is activated by LPS and appears to regulate COX-2 expression and an array of other inflammatory genes including TNFα, IL-1β, MCP-1, iNOS, and $cPLA_2$ (Grimm and Baeuerle 1993; Barnes and Karin 1997). Inhibition of NF-κB by gliotoxin, proved to be essential for the induction of COX-2 in monocytes and other reports have supported our findings (Pouliot et al. 1997; Schmedtje et al. 1997). However, these results do not allow determination of whether increased NF-κB activation in hypoxia can explain the augmentation of COX-2 expression in hypoxia.

Despite the finding that hypoxia caused upregulated COX-2 expression, the reduction in the accumulation of eicosanoids was a surprising observation. A similar outcome of increased COX-2 expression and reduced eicosanoid synthesis was observed in monocyte-derived macrophages and the U937 monocytic cell line. At face value, this appears to be a paradoxical response. In the following chapter, some of the possible explanations for this dissociation between COX-2 induction and activity were examined.

3.5 CONCLUSION

Hypoxia resulted in a significant, time dependent upregulation of COX-2 mRNA and protein in monocytes. This increased expression of COX-2 is likely to be due to an increase in the transcription rate of the gene in addition to an increase in the stability of the message. The upregulation of COX-2 did not appear to be influenced by any changes in the phosphorylation of the p38 MAPK pathway, and the induction of COX-2 in hypoxia is dependent on the activation of transcription factor NF-κB. Despite upregulated COX-2, there was a reduced accumulation of PGE_2 and TXA_2 in hypoxic monocytes. An investigation into the possible mechanisms for this dissociation was studied next.

CHAPTER 4

THE DISSOCIATION BETWEEN MONOCYTE COX-2 EXPRESSION AND EICOASANOID SYNTHESIS IN HYPOXIA

4.1 INTRODUCTION

In the preceding chapter, it was concluded that hypoxia augments the expression of monocyte COX-2 mRNA and translates this into increased COX-2 protein at times as early as 3h following co-incubation with a stimulus such as LPS. However, despite the increased COX-2 protein expression, the accumulation of eicosanoids derived from COX-2 activity, is significantly reduced. Similar results in monocyte-derived macrophages and the U937 monocytic cell line, corroborated these findings.

This chapter examines possible mechanisms for this paradoxical finding. For example, limited oxygen availability in hypoxia will be considered as a possible explanation for reduced eicosanoid synthesis, since oxygen is a co-substrate of the COX enzyme (Smith and Song 2002). In addition, co-factors such as heme are important to the activity of COX-2 (Smith and Song 2002) and were considered as well as the availability of COX-2 substrate (i.e. AA) as potential mechanisms for reduced eicosanoid synthesis in hypoxic monocytes despite upregulated COX-2 protein expression.

Finally, the effect of hypoxia on signalling pathways that may regulate the availability of substrate for COX-2 activity, were examined.

4.2 MATERIALS and METHODS

4.1.1 Materials

Materials were obtained from the following sources: arachidonic acid (AA), COX-2 rabbit polyclonal Ab, β-actin mouse monoclonal antibody, PGE_2, NS-398 (Cayman Chemicals (Ann Arbor, MI), PGE_2 rabbit antiserum (Sigma Chem), rabbit TXB_2 antiserum was prepared from rabbits immunised with TXA_2 conjugated to human thyroglobulin as used in previous studies (James and Walsh 1988), pyrogen free Lymphoprep, (Nycomed, Oslo, Norway) LPS, Heme, Zn-protoporphyrin-IX (Sigma Chem Co, St Louis, MO), protein trans-blot transfer membranes (Bio-Rad, North Ryde, AUS), peroxidase labelled donkey anti-rabbit Ab, peroxidase labelled goat anti-mouse Ab, [^{3}H]PGE_2, [^{3}H]TXB_2, [^{3}H].AA (Amersham International, Little Chalfont, ENG), Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) phosphorylated p44/42 polyclonal Ab, phosphorylated $cPLA_2$ polyclonal Ab, PD985059 (New England Biolabs, Beverly, MA)

4.1.2 Methods

Incubations

Fresh human monocytes were isolated by counter current centrifugal elutriation as described in section 2.2.1. Where indicated, inhibitors were incubated for 15min prior to LPS stimulation. Inhibitors were dissolved in DMSO or ethanol and all controls contained

the vehicle for the duration of the incubation period. The purity of all preparations was assessed by FACS analysis and typically >85% of cells were monocytes, with the remainder lymphocytes.

AA release studies were performed by incubating monocytes with [^{3}H]AA (2μCi/ml; 2x10^{-6}Ci/mmol) in normoxia overnight at 37°C to incorporate labelled AA into cell membranes. After 18h, cells were washed (3x) in pre-warmed medium, then stimulated with LPS in normoxia or hypoxia for 30h. In addition, some pre-labelled cells that had been hypoxic for 9h were returned to oxygenated conditions for a further 21h.

Western Analysis

Phosphorylated proteins p44/42 MAPK and cPLA$_2$ were stained by treating transfer membranes with the respective Abs at 1/500 dilution in TBS-Tween overnight at 4°C. Following washing, the membrane was treated with donkey anti-rabbit horseradish peroxidase (HRP) Ab at 1/10,000 dilution in TBS-Tween for 45min. The chemiluminescent substrate solution was added to the membrane (5min) prior to light sensitive film for 2-5min.

4.3 RESULTS

4.3.1 Effect of heme on COX-2 activity in hypoxia

COX-2 is a heme containing enzyme and cellular heme levels can be reduced by heme oxygenase (HO), including the inducible isoform, HO-1 which may be upregulated during hypoxia (Lee et al. 1997; Bonazzi et al. 2000; Haider et al. 2002). Therefore, in hypoxia, the cellular levels of heme may become limiting for adequate COX-2 constitution, leading to a functionally deficient enzyme. The addition of heme or a HO inhibitor, zinc-protoporphyrin (Zn-PP), under conditions previously reported to alter COX-2 activity in a hypoxic rabbit corneal epithelial cell line (Bonazzi et al. 2000), did not affect the production of PGE_2 and TXB_2 or the amount of COX-2 protein in normoxia or hypoxia under these experimental conditions in monocytes. This suggests that HO activity or reduced heme levels are not responsible for reduced COX-2 activity seen in monocytes in hypoxia (Fig 4.1).

4.3.2 Effect of O_2 levels in hypoxia on COX-2 activity

COX-2 utilises oxygen as a co-substrate during the conversion of arachidonic acid (AA) to PGH_2, the common precursor of PGE_2 and TXA_2 (Fig 1.1, Chapter 1). In this study, O_2 in the incubation chamber was set at 1% (v/v); *cf* approximately 20% for air at sea level. This level of hypoxia reduced dissolved oxygen in the incubation medium to 33 mmHg compared with 154mmHg in normoxic medium. To determine whether these levels of O_2

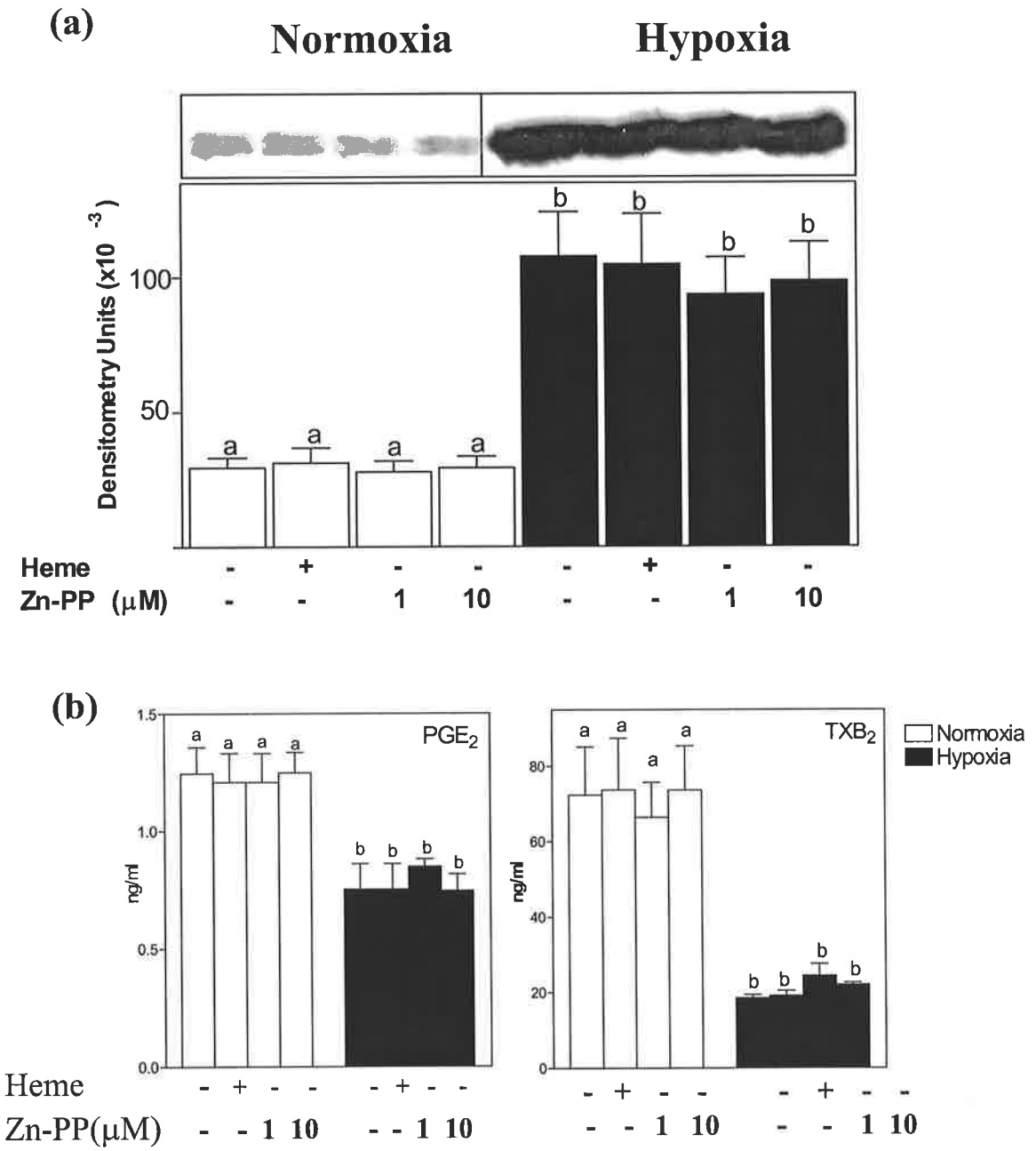


Figure 4.1

Effect of heme or Zn-protoporphyrin on COX-2 activity in LPS stimulated monocytes

Monocytes ($5x10^6$) were incubated with heme (5μM) or Zn-PP (1 or 10μM) for 30min then stimulated with LPS (200ng/ml) (18h, 37°C) in normoxia or hypoxia. (a) Cells were processed for western blot for COX-2 protein determination. Blot is representative of 3 separate experiments, the means of which were averaged to provide the mean (with SEM). (b) Supernatants were collected and assayed for PGE_2 and TXB_2 by RIA (mean ± SEM; n=3). Bars with different letters are significantly different from each other ($p<0.05$).

were rate-limiting for eicosanoid synthesis, monocytes were first incubated in hypoxia with LPS to induce COX-2. After 18h, cells were washed twice with hypoxic medium, then incubated in fresh hypoxic or normoxic medium with exogenous AA (10μM, 15min).

The levels of oxygenation of the medium had no effect on the production of PGE_2 and TXB_2 (Fig 4.2). These results indicated that dissolved O_2 at the levels of hypoxia used in this study were not rate-limiting for COX activity.

4.3.3 Effect of hypoxia on the metabolism of PGE_2 in hypoxia

Next, I determined whether the reduced accumulation of eicosanoids in hypoxia was due to increased metabolism of the eicosanoids ~~was examined~~. Monocytes were treated with the COX-2 inhibitor, NS-398 (1μM, 15min) to inhibit endogenous eicosanoid synthesis, then exogenous PGE_2 (0-1000ng/ml) was added prior to LPS stimulation for 18h in normoxia or hypoxia. PGE_2 concentrations in the supernatant after 18h were equivalent in normoxia and hypoxia (Fig 4.3). Thus, hypoxia did not cause an increase in metabolism of PGE_2 by monocytes.

4.3.4 Effect of exogenous arachidonic acid (AA) on COX-2 activity in hypoxia

Monocytes were incubated with LPS for 18h in the absence or presence of hypoxia to induce COX-2. On the following day cells were washed twice and incubated with fresh normoxic or hypoxic medium and exogenous AA (10μM) for 15min. In hypoxia, there was an increase in PGE_2 and TXB_2 synthesis (Fig 4.4). This contrasted with results above

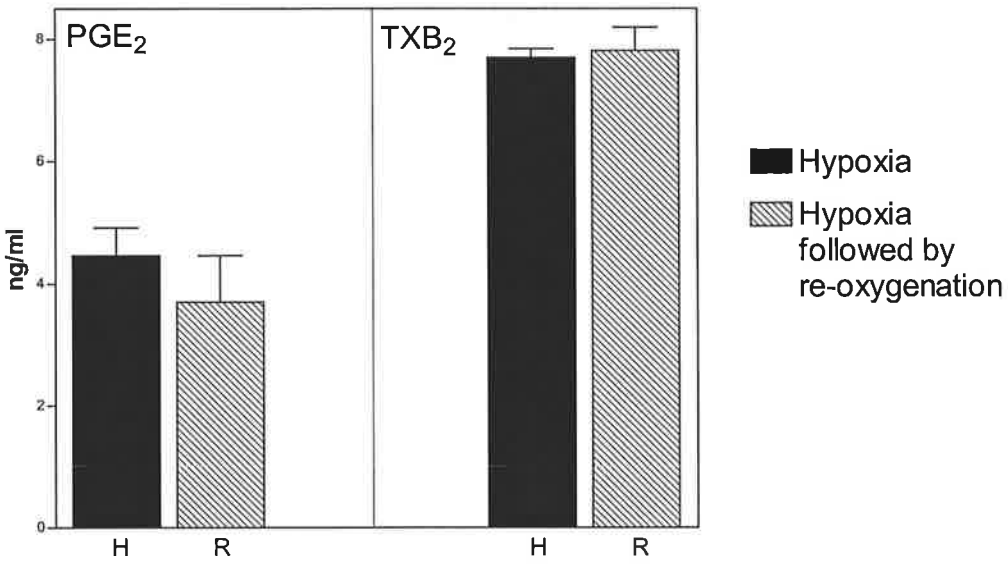


Figure 4.2

Effect of O_2 co-substrate on COX-2 activity in the presence of exogenous AA

Monocytes ($2x10^6$) were stimulated with LPS (200ng/ml, 18h, 37°C) in hypoxia to induce COX-2. Cells were washed and resuspended in fresh hypoxic or normoxic (reoxygenated cells) medium with exogenous AA (10μM, 15min). Supernatants were collected and assayed for PGE_2 and TXB_2 by RIA (mean ± SEM;n=3).

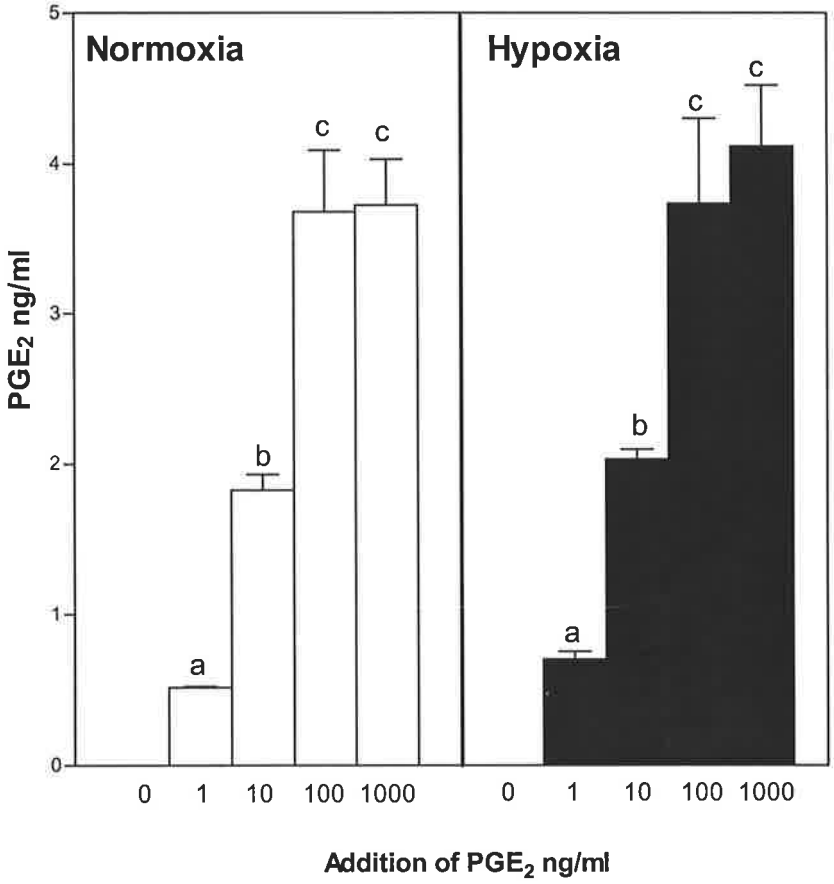


Figure 4.3

Effect of hypoxia on the metabolism of exogenous PGE_2

Monocytes ($2x10^6$) were treated with NS-398 (1μM, 15min) prior to addition of exogenous PGE_2 (0-1000ng/ml) and LPS (200ng/ml, 18h, 37°C) in normoxia (open bars) or hypoxia (solid bars). After 18h, supernatants were collected and assayed for PGE_2 by RIA (mean ± SEM; n=4). Bars with different letters are significantly different from each other ($p<0.05$).

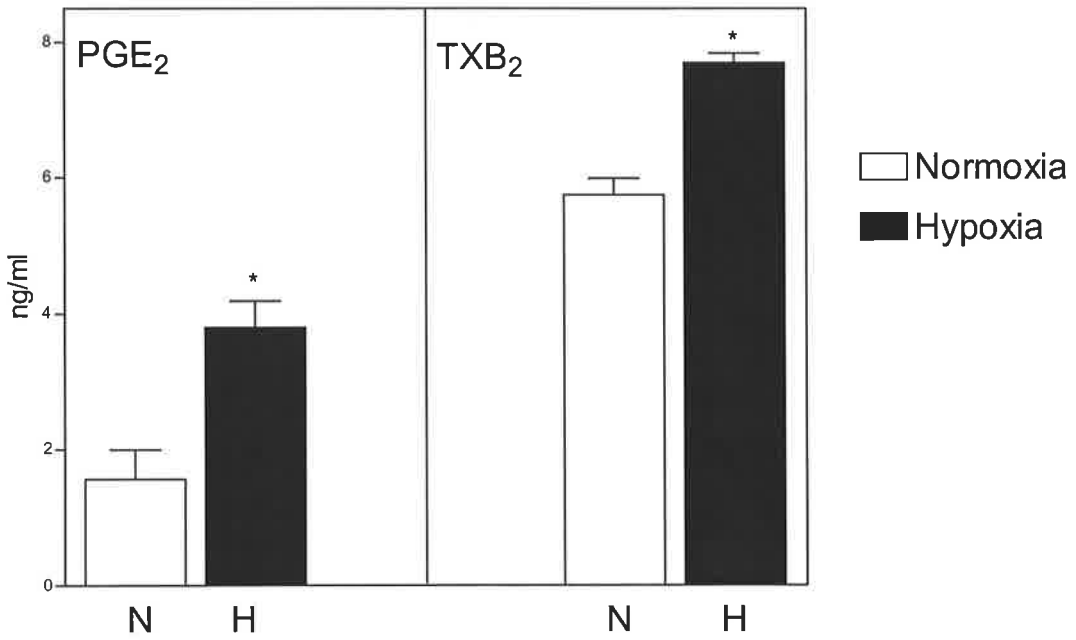


Figure 4.4

Effect of exogenous arachidonic acid on COX-2 activity in hypoxia

Monocytes ($2x10^6$) were stimulated with LPS (200ng/ml, 18h, 37°C) in normoxia or hypoxia to induce COX-2. Cells were washed and resuspended in fresh normoxic or hypoxic medium with exogenous AA (10μM). After 15min, supernatants were collected and assayed for PGE_2 and TXB_2 by RIA (mean ± SEM;n=3). *$p<0.05$, by comparison to normoxic monocytes.

(Fig 3.5) where eicosanoid synthesis from endogenous AA was reduced in hypoxia. This suggested that the reduced accumulation of eicosanoids observed in hypoxia, despite the up-regulated levels of COX, was due to a decreased availability of endogenous AA substrate.

4.3.5 Effect of hypoxia on endogenous arachidonic acid (AA) release

Monocytes were pre-incubated with [^{3}H]AA overnight at 37°C in normoxia. Cells were washed 3x and these [^{3}H]AA pre-labelled monocytes were stimulated with LPS in normoxia or hypoxia for 30h. Concurrently, some samples that had been hypoxic for 9h were returned to oxygenated conditions for the following 21h.

In normoxia, there was a time dependent increase in the release of labelled AA from monocytes when stimulated with LPS (Fig 4.5). By comparison, in monocytes stimulated with LPS in hypoxia, negligible release of AA was seen beyond 3h, the first time point examined (Fig 4.5). Re-oxygenation after 9h of hypoxia resulted in a restoration of AA release from cells to rates that were similar to those observed in normoxic cells (Fig 4.5).

Because $cPLA_2$ is predominantly involved in the release of AA from membrane phospholipids, the effect of hypoxia on $cPLA_2$ phosphorylation, a measure of $cPLA_2$ activation, was examined.

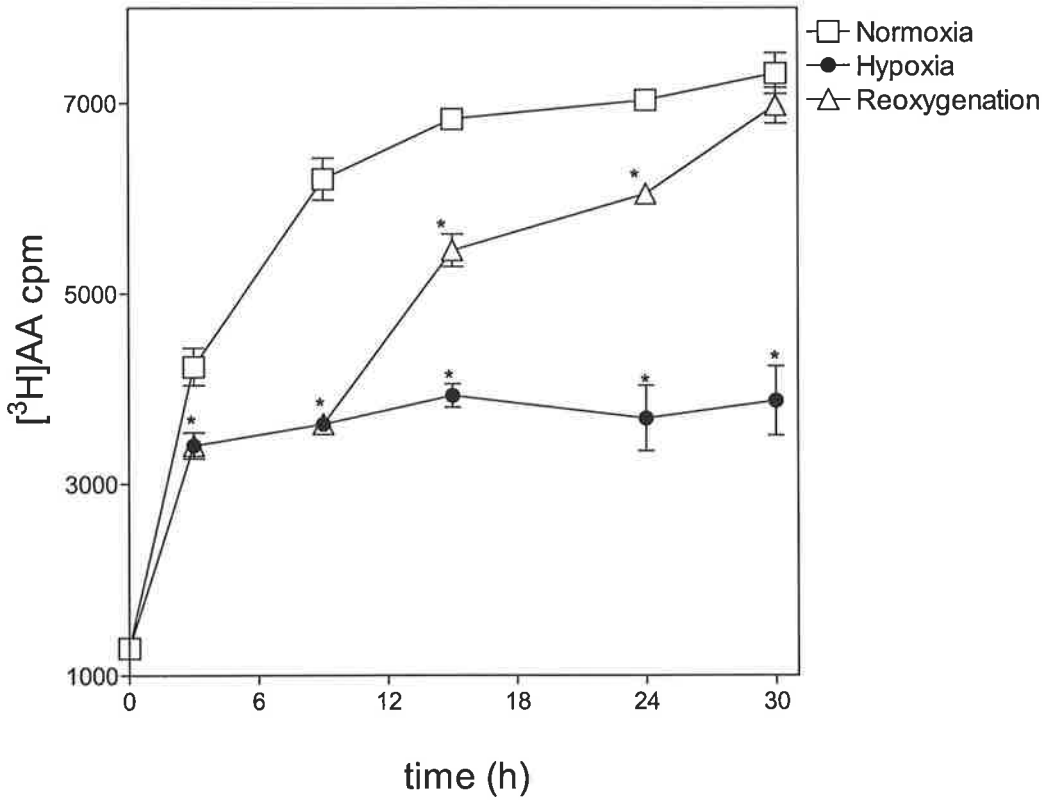


Figure 4.5

Effect of hypoxia on arachidonic acid release

Monocytes ($5x10^6$) were incubated with [^{3}H]AA (2μCi/ml) overnight. The following day the cells were washed 3x and stimulated with LPS (200ng/ml, 30h, 37°C) in normoxia (□) or hypoxia (●). In addition, cells were hypoxic for 9h and reoxygenated (Δ) for a further 21h. Supernatants were collected and [^{3}H]AA release was determined using a scintillation counter (mean ± SEM; n=3). *$p<0.05$, by comparison to normoxic monocytes

4.3.6 Effect of hypoxia on cytosolic phospholipase A_2 ($cPLA_2$) phosphorylation

Following preparation by elutriation of fresh human monocytes, $cPLA_2$ appeared in the phosphorylated form in resting cells, prior to stimulation. Following stimulation with the Ca^{2+} ionophore, A23187 (1μM), the amount of phosphorylated $cPLA_2$ decreased (Fig 4.6). In hypoxia, there was an accelerated dephosphorylation of the enzyme at later times (Fig 4.6).

The mitogen activated protein kinase (MAPK) of the extracellular responsive kinase (ERK) pathway can regulate the phosphorylation and activation of $cPLA_2$ (Fouda et al. 1995; Hazan et al. 1997; Hiller and Sundler 1999; Miura et al. 1999; Syrbu et al. 1999; Gijon et al. 2000). Therefore, the effects of hypoxia on the phosphorylation of p44/42 (ERK1/2), were examined.

4.3.7 Effect of hypoxia on the phosphorylation of p44/42 MAPK

Following LPS stimulation in normoxia, the phosphorylation of p44/42 was maximal at 10min followed by dephosphorylation up to 120min (Fig 4.7). In hypoxia, there was a reduction in the phosphorylation at 10min in addition to an accelerated dephosphorylation at later times (Fig 4.7).

4.3.8 Effect of inhibition of p44/42 MAPK activation on arachidonic acid (AA) release in hypoxia

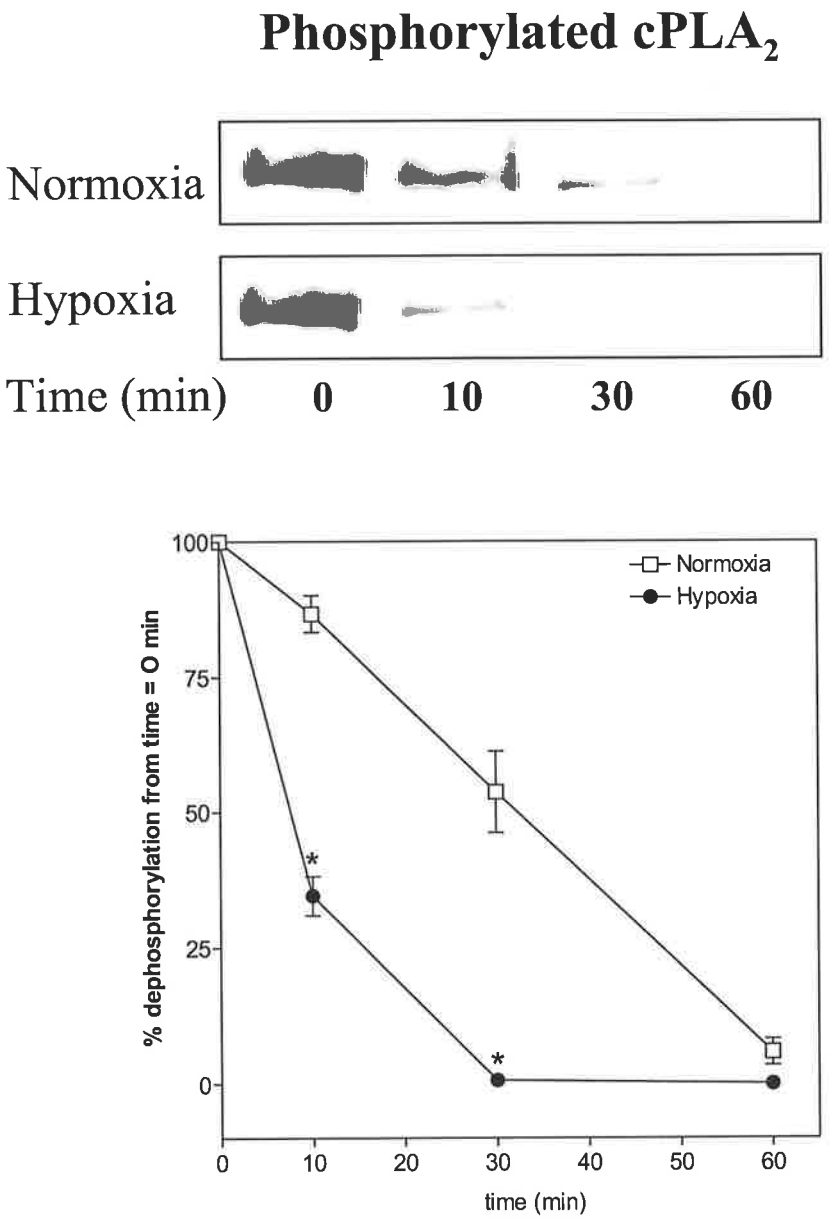


Figure 4.6

Effect of hypoxia on phosphorylated $cPLA_2$

Monocytes ($5x10^6$) were stimulated with A23187 (1μM) under normoxic or hypoxic conditions for up to 60min. At each time point, cells were processed for western blot analysis. Blot is representative of 3 separate experiments, the mean values of which are shown in the graph as % dephosphorylation from time 0h (mean ± SEM; n=3). *$p<0.05$, by comparison to normoxic monocytes.

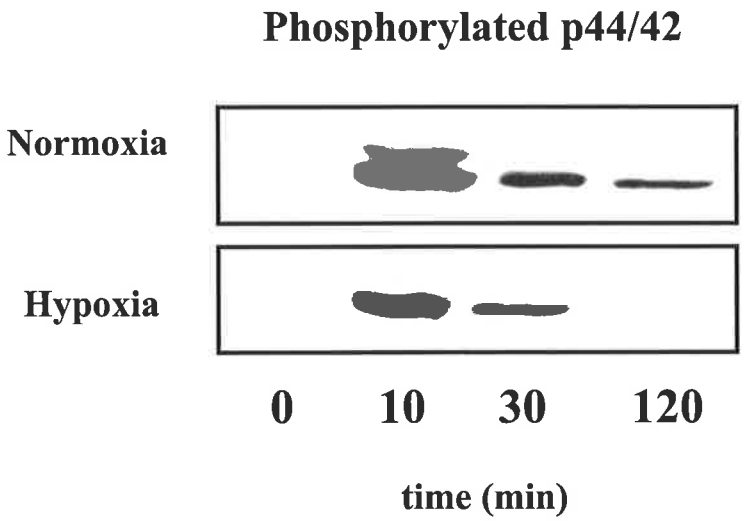


Figure 4.7
Effect of hypoxia on phosphorylated p44/42 MAPK
Monocytes ($5x10^6$) were stimulated with LPS (200ng/ml) under normoxic or hypoxic conditions for up to 120min. At each time point, cells were processed for western blot analysis. Results are representative of 3 experiments.

Monocytes were incubated with [^{3}H]AA in normoxia (18h, 37°C) to incorporate labelled AA into cell membranes. After 18h, cells were washed (3x) and incubated with LPS in normoxia or hypoxia for 30h. In addition, some pre-labelled cells that had been hypoxic for 9h were returned to oxygenated conditions with or without PD 98059, an inhibitor of p44/42 MAPK phosphorylation and thus, p44/42 activation.

PD 98059 inhibited the restoration of AA release from reoxygenated cells to levels similar to those observed in cells maintained in hypoxia (Fig 4.8).

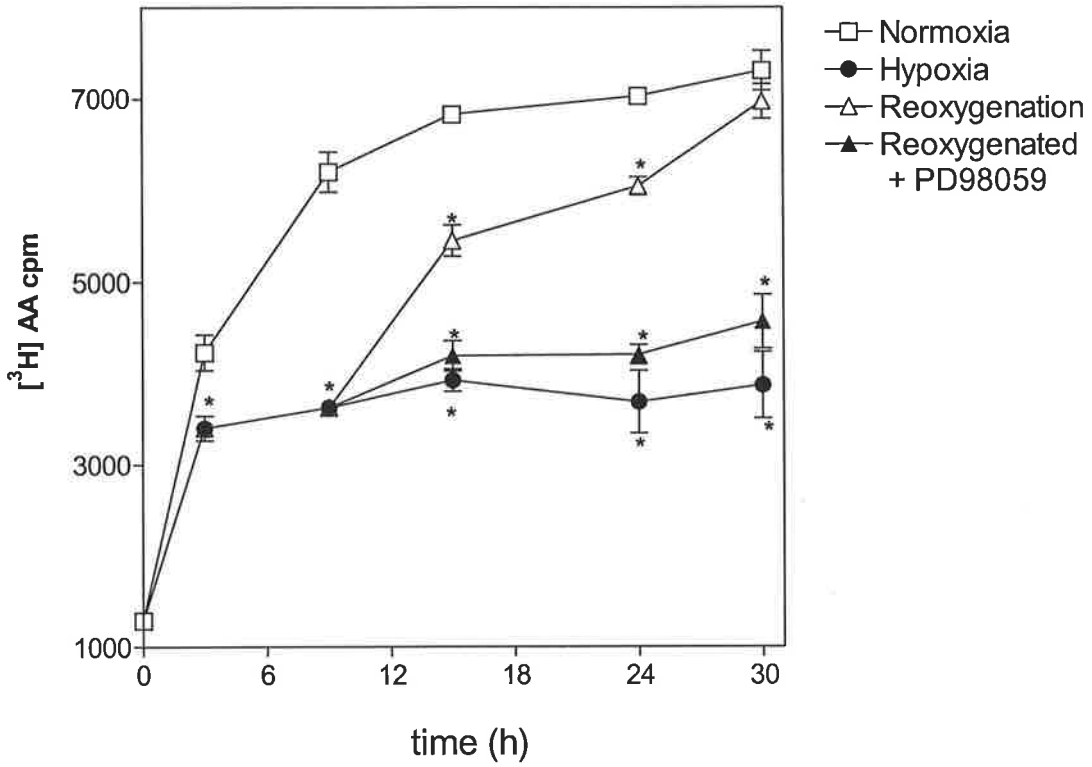


Figure 4.8

Effect of PD 98059 on arachidonic acid release during reoxygenation

Monocytes ($5x10^6$) were incubated with [^{3}H]AA (2μCi/ml) overnight. The following day, cells were washed 3x and stimulated with LPS (200ng/ml, 30h, 37°C) in normoxia (□) or hypoxia (●). In addition, cells were hypoxic for 9h and reoxygenated (Δ) or reoxygenated with PD 98059 (25μM) (▲) for a further 21h. Supernatants were collected and radioactivity was determined using a scintillation counter (mean ± SEM; n=3). *$p<0.05$, by comparison with normoxic monocytes.

4.4 DISCUSSION

Although there was an augmentation of monocyte COX-2 expression in hypoxia, this was accompanied by a decrease in PGE_2 and TXA_2 production (Chapter 3). This appears to be a paradoxical response. Rat lung exposed to hypoxia had increased COX-2 levels and increased prostaglandin production (Chida and Voelkel 1996; Matuschak et al. 1998). Similarly, ischaemia induced an increase in COX-2 mRNA and an increase in PGE_2 synthesis in mouse cerebrum (Nogawa et al. 1997). Nevertheless, there is a report of increased COX-2 expression but decreased PGE_2 synthesis in hypoxia in a rabbit corneal epithelial cell line (Bonazzi et al. 2000).

In the latter case, the authors attributed this dissociation to increased activity of heme oxygenase in hypoxia with resultant decreased heme for COX-2 activity (Bonazzi et al. 2000). COX-2 is a heme protein and the amount of available heme can be influenced by the activity of inducible heme oxygenase (HO) -1. HO-1 not only controls the amount of heme for synthesis of heme-binding proteins but generates carbon monoxide (CO) which binds to the heme moiety of heme proteins, thereby affecting their enzymatic activity (Haider et al. 2002). HO-1 is induced in hypoxia and may be responsible for heme depletion in hypoxic cells (Bonazzi et al. 2000; Haider et al. 2002). However, when we repeated the procedures of Bonnazi et al of adding heme or an inhibitor of heme oxygenase, there was no restoration of prostaglandin synthesis like that reported in the rabbit corneal epithelial cell line (Bonazzi et al. 2000). It is possible that this was an intrinsic difference between systems in monocytes and rabbit corneal epithelium, or it is possible that the rabbit corneal epithelial cells, which were a transformed cell line, were

depleted of heme stores due to long-term culture where as fresh human monocytes probably have adequate heme stores.

The possibility that reduced PGE_2 and TXA_2 accumulation in hypoxia was due to increased metabolism by monocytes or re-incorporation into their membrane phospholipids, was examined. Exogenous PGE_2 was added to monocytes following inhibition of synthesis of all endogenous COX-2 eicosanoids, and the fate of PGE_2 was determined by the level remaining in the supernatant after 18h in hypoxia. The results indicated that PGE_2 was not metabolised into another product or incorporated into the membrane phospholipids, as there was no significant difference between PGE_2 levels in normoxia or hypoxia. This could not be performed with the active TXA_2 analogue ($cTXA_2$) as it was not possible to measure $cTXA_2$ in our system.

Another possible explanation for decreased eicosanoid synthesis in hypoxia at 1% O_2 is that the co-substrate for COX, O_2, is rate-limiting. However, this was an unlikely explanation given that eicosanoid synthesis was similar in hypoxia when compared to re-oxygenated monocytes in the presence of an abundance of the other co-substrate for COX, AA, which had been added exogenously.

Next, I added exogenous AA to monocytes containing COX-2 protein induced following LPS stimulation in normoxia or hypoxia. This resulted in a significant increase in eicosanoid synthesis in hypoxic cells compared to normoxic cells. The results indicated that the presence of active COX-2 enzyme had increased more under hypoxic than normoxic conditions and suggested that the supply of AA substrate for COX activity was rate limiting in hypoxia. Therefore, I investigated AA release from monocytes.

Interestingly, AA release in hypoxia had a brief duration with failure of accumulation of released AA beyond the 3h time point. Later, re-oxygenation restored the release of AA to levels equivalent to those observed in normoxic cells. The stimulus-induced release of AA is regulated by cytosolic PLA_2, the major intracellular form of PLA_2 in monocytes (Bonventre et al. 1997; Leslie 1997). Phosphorylation of $cPLA_2$, which equates to activation of this enzyme, was shown to decline rapidly under hypoxic conditions. Hence, the observation of lack of sustained AA release in hypoxic monocytes, correlated with a more rapid dephosphorylation of $cPLA_2$ in hypoxia.

The pathway, in which p44/42 MAPK acts, is reported to regulate the phosphorylation and activation of $cPLA_2$ in macrophages (Hiller and Sundler 1999; Gijon et al. 2000), neutrophils (Fouda et al. 1995; Hazan et al. 1997; Syrbu et al. 1999) and basophils (Miura et al. 1999). The involvement of the p44/42 MAPK in AA release in monocytes was corroborated by the observation that inhibition of p44/42 MAPK activity by the MEK-1 inhibitor, PD 98059, inhibited 'catch up' AA release following re-oxygenation.

4.5 CONCLUSION

This chapter has investigated the possible explanations for the disparate hypoxia induced changes in COX-2 expression and eicosanoid synthesis.

A reduction in AA substrate available for COX-2 activity in hypoxia, relative to normoxia, is a significant finding. This limitation in AA availability is associated with more rapid dephosyphorylation and deactivation of $cPLA_2$, of which the phosphorylation appears to be regulated by p44/42 MAPK. An inhibitor of the phosphorylation of p44/42 MAPK resulted in restoration of AA accumulation, to levels seen in normoxia and was dependent on activity of this kinase pathway.

Despite these findings, it has been well documented that eicosanoids may regulate the synthesis of pro-inflammatory cytokines. More specifically, PGE_2 has potential to suppress the production of TNFα (Kunkel et al. 1988; Spatafora et al. 1991). Therefore, it is important to determine whether hypoxia, which results in the reduction of important pro-inflammatory eicosanoids, has the potential to regulate cytokine synthesis in monocytes. This was the major objective in Chapter 5.

CHAPTER 5

EFFECT OF HYPOXIA ON CYTOKINE SYNTHESIS IN MONOCYTES: AUTOCRINE RELATIONSHIPS BETWEEN EICOSANOID AND CYTOKINE SYNTHESIS IN MONOCYTES

5.1 INTRODUCTION

In the two preceding chapters, it is apparent that, despite upregulated COX-2 expression in hypoxia, there was a reduction in the synthesis of eicosanoids due to a decrease in the availability of AA substrate in hypoxic monocytes.

It was hypothesised that this reduction in eicosanoids may play a role in altering TNFα synthesis in hypoxia. It has been well documented that eicosanoids can regulate the synthesis of pro-inflammatory cytokines. For example, PGE_2 can suppress the production of TNFα (Knudsen et al. 1986; Kunkel et al. 1986; Kunkel et al. 1988; Spatafora et al. 1991; Choi et al. 1996). This is mainly due to the interaction of PGE_2 with its EP2 and EP4 receptors which elevates intracellular cAMP (Peters et al. 1990; Reinstein et al. 1994; Fennekohl et al. 2002). This in turn may alter the activation and phosphorylation of p38 MAPK, since cAMP is reported to regulate this pathway (Hansen et al. 2000). As previously observed in this thesis (Chapter 3), signalling through p38 MAPK is a key event in the response of monocytes for the production inflammatory mediators of which TNFα synthesis is no exception. In addition, the inhibitory effect of PGE_2 on TNFα production

has been attributed to inhibition of signal transduction through the janus kinase, JAK3 protein in T lymphocytes (Kolenko et al. 1999). Furthermore, it has been reported that PGE_2 can decrease TNFα activity by increasing the release of soluble TNF receptors in THP-1 monocytic cells (Choi et al. 1996). In this experimental system, the PGE_2 mediated release of 55- and 75kDa TNFα receptors exceeded spontaneous TNFα release and resulted in neutralisation of biologically active TNFα (Choi et al. 1996). Overall, PGE_2 may decrease TNFα total activity by co-ordinated inhibition of TNFα synthesis and an increase in soluble TNFα receptor release.

Since IL-1β, like TNFα, mediates inflammatory processes and is predominantly produced by cells of the monocyte/macrophage lineage (Dinarello 1996), this chapter will determine whether hypoxia alters IL-1β and TNFα synthesis by fresh human monocytes and whether hypoxia-induced changes in eicosanoid synthesis affects their production.

5.2 MATERIALS and METHODS

5.1.1 Materials

Materials were obtained from the following sources: COX-2 rabbit polyclonal Ab, β-actin mouse monoclonal antibody, PGE_2, NS-398 (Cayman Chemicals, Ann Arbor, MI), rabbit TXB_2 antiserum was prepared from rabbits immunised with TXA_2 conjugated to human thyroglobulin as used in previous studies (James and Walsh 1988), pyrogen free Lymphoprep, (Nycomed, Oslo, Norway), LPS, PGE_2 rabbit antiserum (Sigma Chem Co, St Louis, MO), protein trans-blot transfer membranes (Bio-Rad, North Ryde, Aust), peroxidase labelled donkey anti-rabbit Ab, peroxidase labelled goat anti-mouse Ab, [^{3}H]PGE_2, [^{3}H]TXB_2 (Amersham International, Little Chalfont, UK), Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL), phosphorylated p38 MAPK polyclonal Ab (New England Biolabs, Beverly, MA), matched pair monoclonal IL-1β Abs, matched pair monoclonal TNFα Ab, recombinant Il-1β and TNFα (Endogen, Boston MA,) neutralising anti-TNFα Ab (R&D Systems, Minneapolis, MN), 1B5 isotype control Ab (gift from GMayrhofer, Arthritis Lab, Hanson Institute, Adelaide, Aust))

5.1.2 Methods

Incubations

Fresh human monocytes were isolated by counter current centrifugal elutriation as described in section 2.2.1. Where indicated, inhibitors were incubated for 15min prior to

LPS stimulation. Inhibitors were dissolved in DMSO or ethanol and all controls contained the vehicle for the duration of the incubation period. The purity of all preparations was assessed by FACS analysis and typically >85% of cells were monocytes, with the remainder lymphocytes.

'Transient LPS stimulation' involved incubating monocytes with LPS in normoxia or hypoxia at 37°C. After 15min, cells were washed twice in their respective medium and the incubation period was allowed to continue with fresh normoxic or hypoxic medium.

Western Analysis

Transfer membranes were treated with rabbit polyclonal COX-2 Ab at 1/1,000 dilution for 1h at RT or phospho-p38 MAPK Ab at a 1/500 dilution in TBS-Tween overnight at 4°C. β-actin protein was revealed by mouse monoclonal β-actin at 1/2,500 dilution in TBS-Tween for 60min at RT. Following washing, the membrane was treated with donkey anti-rabbit horseradish peroxidase (HRP) Ab (for COX-2) or goat anti-mouse HRP Ab (for β-actin) at 1/10,000 dilution in TBS-Tween for 45min. The chemiluminescent substrate solution was added to the membrane (5min) prior to light sensitive film for 2-5min.

5.2 RESULTS

5.3.1 Effect of hypoxia on cytokine synthesis in monocytes

As expected, in normoxia, LPS induced TNFα and IL-1β synthesis in fresh human monocytes, over 18h. This was greatly potentiated by hypoxia (Fig 5.1).

To examine the possibility that the reduction in PGE_2 synthesis observed under hypoxic conditions may contribute to the augmentation of cytokine production in hypoxia, the effects of hypoxia on TNFα and IL-1β synthesis were examined in the presence of COX inhibitors.

5.3.2 Effect of COX inhibitors on cytokine synthesis in monocytes

Monocytes were pre-incubated (15min, 37°C) with a selective COX-2 inhibitor, NS-398 (1μM), or a non-selective COX inhibitor, indomethacin (10μM), prior to LPS stimulation in normoxia and hypoxia.

In normoxia, both NS-398 and indomethacin resulted in a marked reduction in PGE_2 production and a significant increase in TNFα synthesis (Fig 5.2). Furthermore, there was no additional effect of hypoxia on TNFα synthesis in the presence of NS-398 and indomethacin (Fig 5.2). COX inhibition did not alter the level of IL-1β in normoxia or hypoxia (Fig 5.2).

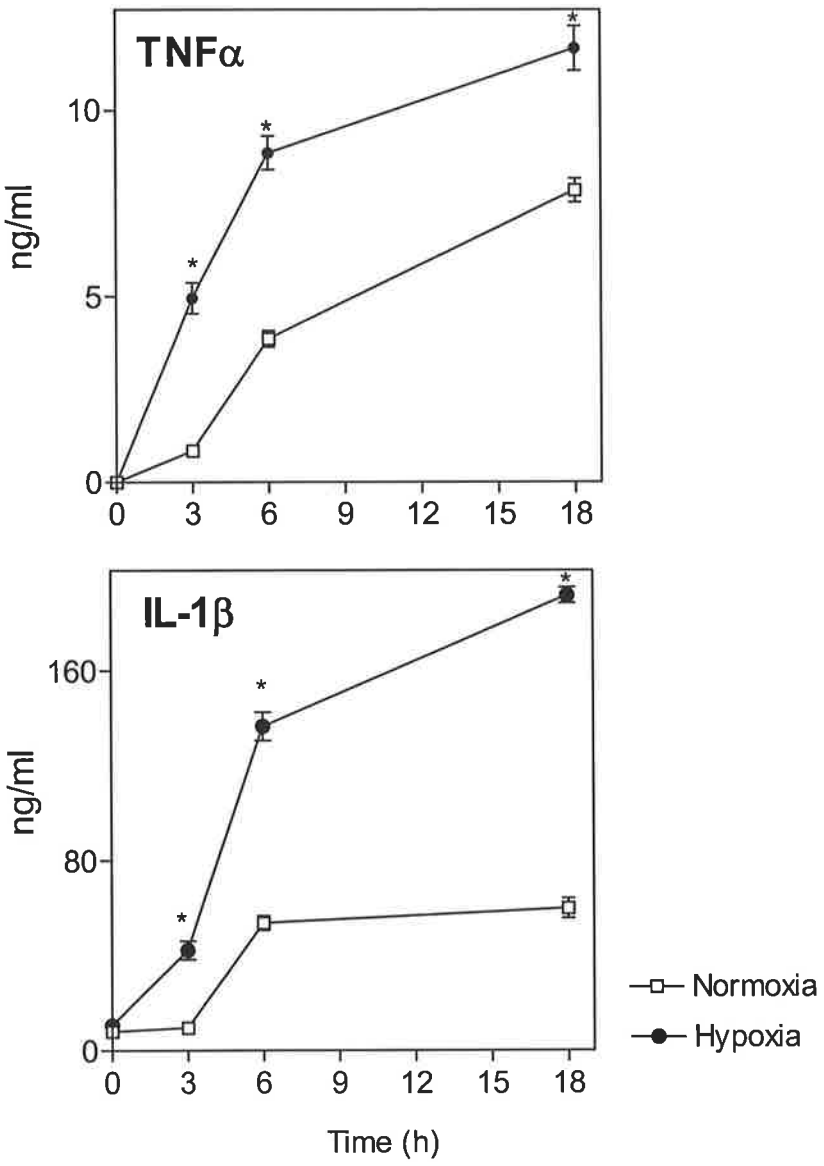


Figure 5.1

Effect of hypoxia on cytokine synthesis in LPS stimulated monocytes

Monocytes ($5x10^6$) were stimulated with LPS (200ng/ml) for 3, 6 & 18h in normoxia (□) or hypoxia (●). Supernatants were collected and assayed for TNFα and IL-1β by ELISA (mean ± SEM, n=3). *$p<0.05$, by comparison to normoxic monocytes.

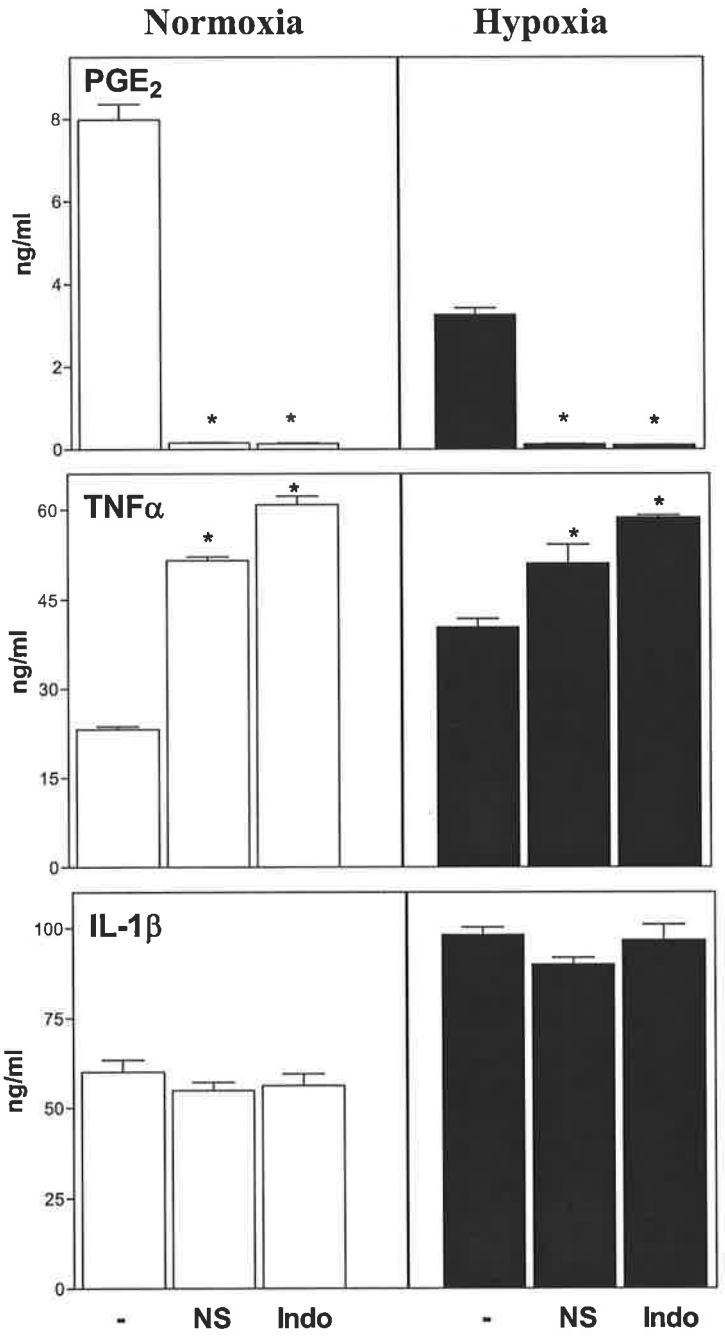


Figure 5.2

Effect of COX inhibitors on TNFα and IL-1β synthesis

Monocytes ($5x10^6$) were pre-incubated (15min, 37°C) with NS-398 (NS, 1μM) or indomethacin (Indo, 10μM) prior to LPS stimulation (18h, 37°C) in normoxia and hypoxia. Supernatants were collected and assayed for PGE_2 by RIA. TNFα and IL-1β was measured by ELISA. *$p<0.05$, by comparison to no inhibitor (mean ± SEM, n=3).

Since TNFα (but not IL-1β) synthesis increased with COX inhibition in this system, the capacity for exogenous PGE_2 to inhibit TNFα synthesis, was examined.

5.3.3 Effect of exogenous PGE_2 on TNFα synthesis in monocytes

Monocytes were pre-treated with NS-398 (1μM, 15min) to inhibit endogenous COX-2 activity in normoxia or hypoxia. Cells were then incubated with increasing concentrations of PGE_2 (0-1000ng/ml) prior to LPS stimulation.

PGE_2 dose dependently suppressed the production of TNFα synthesis in normoxia and hypoxia (Fig 5.3). Therefore, in hypoxic monocytes, reduced PGE_2 synthesis due to decreased availability of COX substrate (Chapter 4), could play a role in enhancing TNFα synthesis.

In light of this possibility, the effect of chemical inhibition of endogenous PGE_2 synthesis on p38 MAPK phosphorylation was examined.

5.3.4 Effect of inhibition of endogenous eicosanoid synthesis on the phosphorylation of p38 MAPK

Fresh monocytes were preincubated in the absence or presence of NS-398 (COX-2 inhibitor, 1μM) or indomethacin (non-selective COX inhibitor, 10μM) for 30min prior to stimulation with LPS for up to 120min.

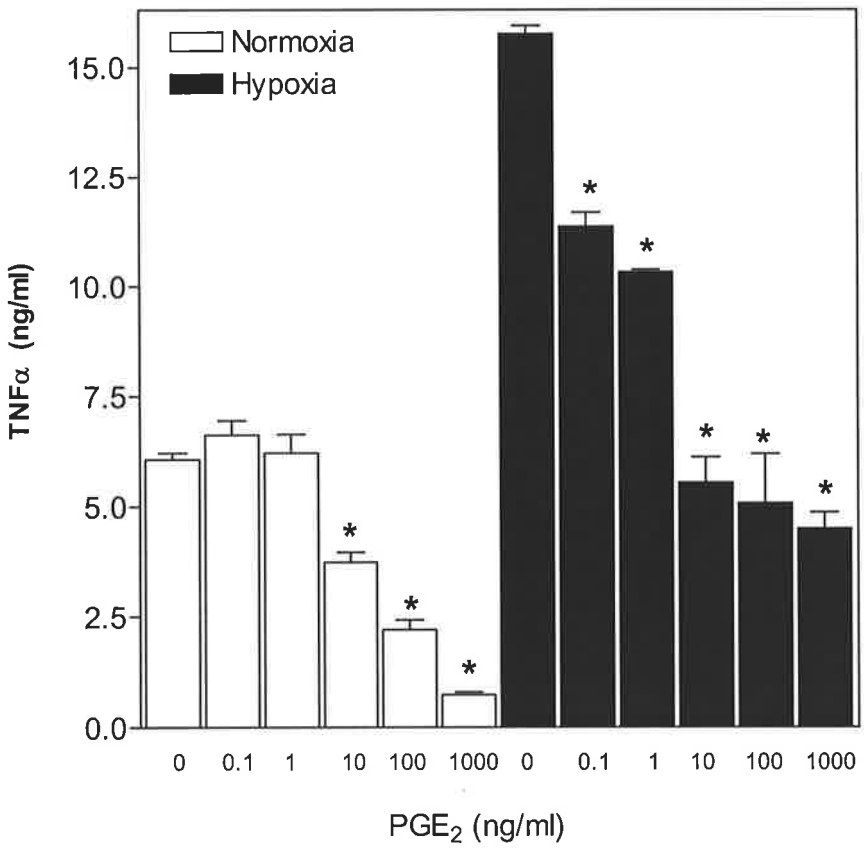


Fig 5.3

Effect of exogenous PGE_2 on TNFα synthesis

Monocytes ($5x10^6$) were pre-incubated (15min, 37°C) with NS-398 (1μM), then PGE_2 at the doses indicated prior to LPS stimulation (18h, 37°C) in normoxia or hypoxia. Supernatants were collected and TNFα was measured by ELISA (mean ± SEM, n=4). *$p<0.05$, by comparison to nil PGE_2 addition.

In normoxia, phosphorylation of p38 MAPK in LPS stimulated monocytes was maximal at 30min and dephosphorylation occurred at following times (Fig 5.4). Addition of NS-398 or indomethacin resulted in no significant changes in p38 MAPK phosphorylation (Fig 5.4).

A further consideration is that the synthesis of endogenous eicosanoids following LPS stimulation may have been insufficient to influence changes in p38 MAPK phosphorylation, which occurs within minutes of cell stimulation. Therefore, the effect of prior addition of exogenous eicosanoids on p38 MAPK phosphorylation was investigated. This treatment potentially mimics the effect of released eicosanoids within the ambient milieu of an inflammatory focus at the time a monocyte receives a stimulatory signal. In other words, the treatment explores the potential paracrine effects of the test eicosanoids on MAPK phosphorylation.

5.3.5 Effect of exogenous eicosanoids on the phosphorylation of p38 MAPK

Fresh monocytes were pre-incubated in the absence or presence of the stable TXA_2 analogue, carbocyclic TXA_2 ($cTXA_2$, 10μM) or PGE_2 (100ng/ml) for 10min prior to stimulation of LPS for up to 120min.

In normoxia, phosphorylation of p38 MAPK in LPS stimulated monocytes was maximal at 30min and dephosphorylation occurred at following times (Fig 5.5). The addition of exogenous $cTXA_2$ or PGE_2 did not alter the phosphorylation of p38 MAPK (Fig 5.5).

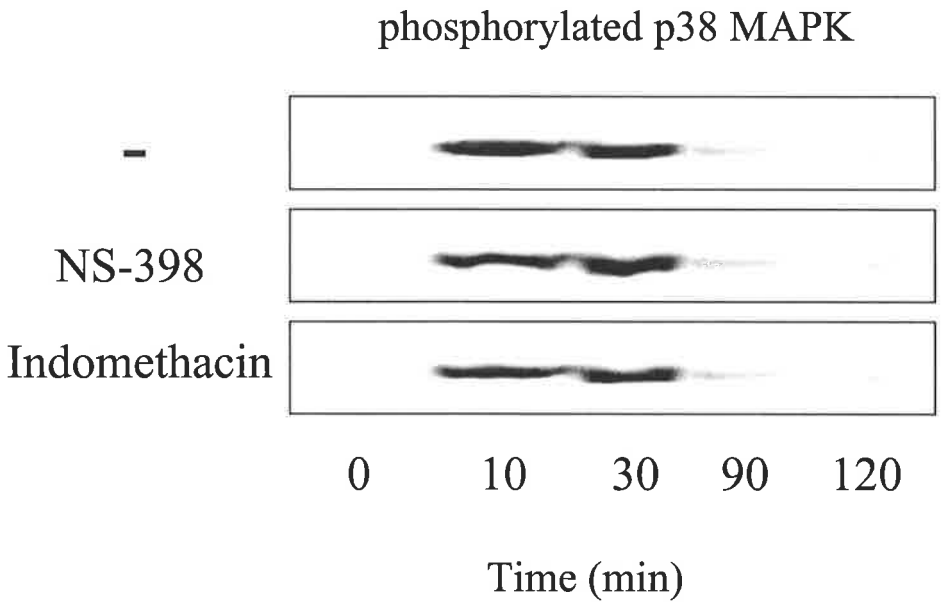


Figure 5.4
Effect of COX inhibitors on p38 MAPK phosphorylation in monocytes
Monocytes ($5x10^6$) were pre-incubated (15min, 37°C) with NS-398 (1μM) or indomethacin (10μM) prior to LPS stimulation for up to 120min. Cell pellets were processed for western blot analysis. Blot is representative of 3 separate experiments.

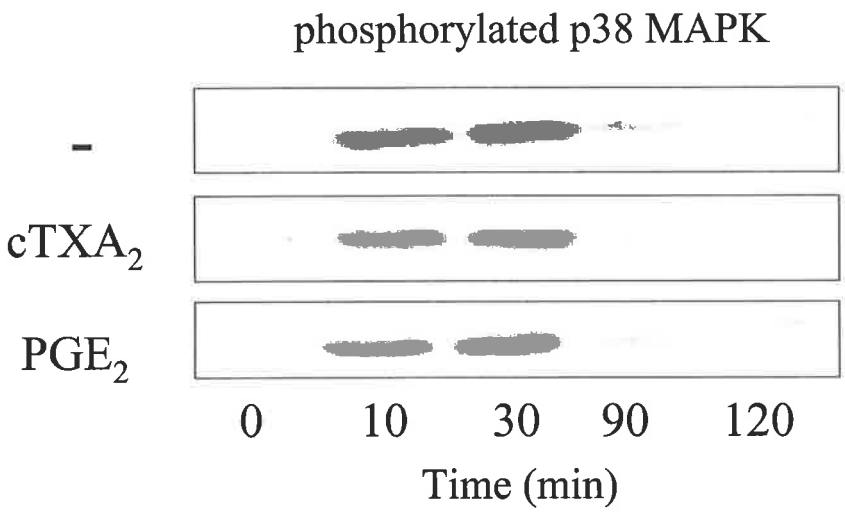


Figure 5.5
Effect of exogenous eicosanoids on p38 MAPK phosphorylation in monocytes
Monocytes ($5x10^6$) were pre-incubated (15min, 37°C) with PGE_2 (100ng/ml) or $cTXA_2$ (10μM) prior to LPS stimulation for up to 180min. Cell pellets were processed for western blot analysis. Blot is representative of 3 separate experiments.

Although altered p38 MAPK phosphorylation does not appear to be the mechanism for the inhibitory effect of PGE_2 on TNFα synthesis, the data suggest strongly that endogenous PGE_2 is a regulator of TNFα synthesis, i.e. an autocrine effect. In addition, it must be considered that endogenous TNFα can upregulate COX-2 because exogenous TNFα is used often as a stimulus for COX-2 induction in inflammatory cells. Therefore, the possibility of an autocrine loop involving induction of COX-2 by endogenous TNFα, was examined.

5.3.6 Effect of monocyte derived TNFα on COX-2 expression and activity

To determine whether endogenous TNFα in monocytes has an autocrine effect on COX-2 expression and activity, monocytes were pre-incubated with a neutralising antibody against TNFα or an isotype-matched control antibody (8μg/ml) prior to LPS stimulation. Neutralising TNFα activity markedly inhibited COX-2 induction (Fig 5.6a) and eicosanoid synthesis (Fig 5.6b).

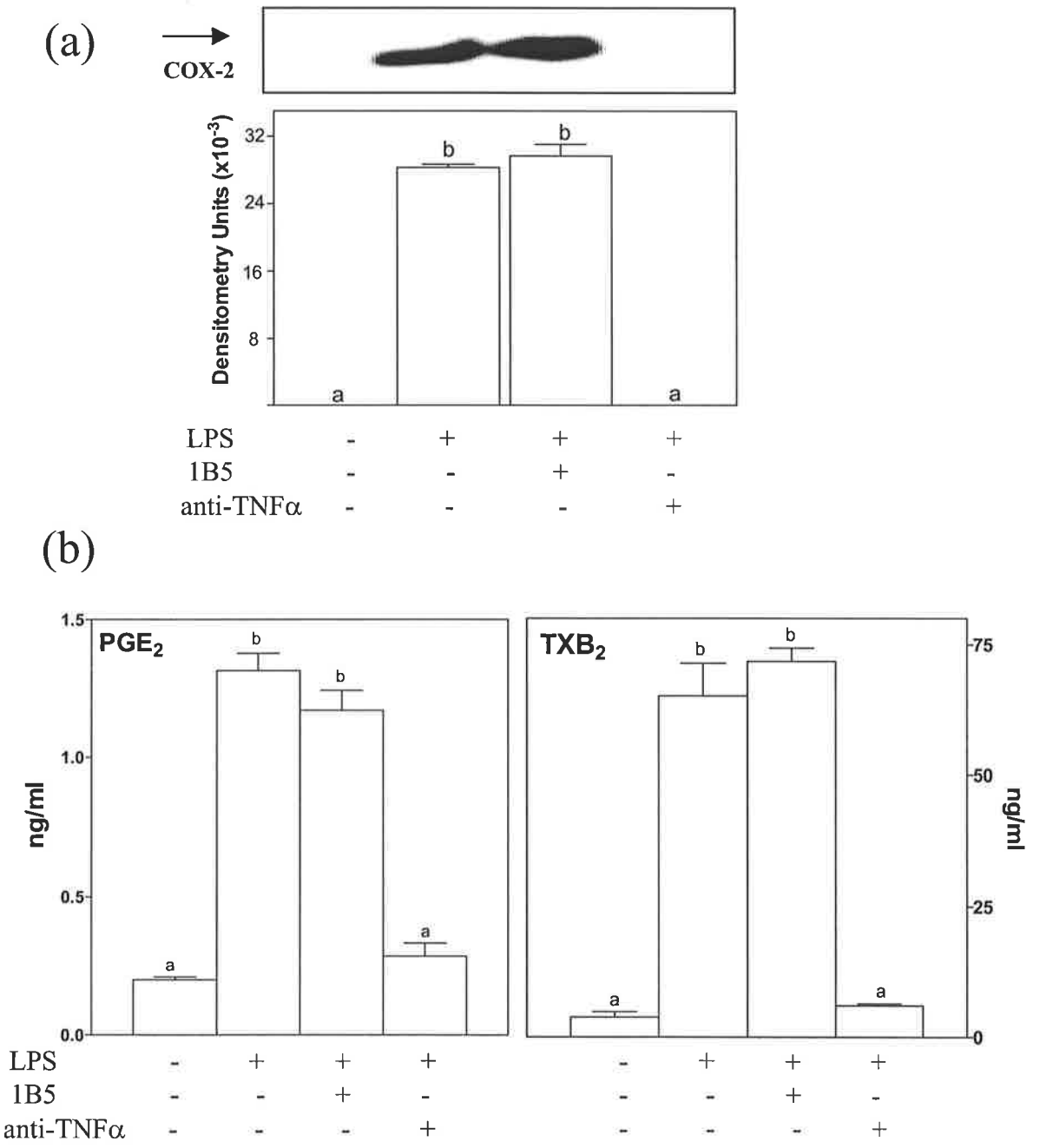


Figure 5.6

Effect of neutralising TNFα Ab on COX-2 induction and activity

Monocytes ($5x10^6$) were pre-incubated (15min, 37°C) with neutralising TNFα monoclonal antibody (anti-TNFα) or isotype matched control (1B5) (8μg/ml) prior to LPS stimulation (18h, 37°C). (a) Cell pellets were processed for western blot analysis. Blot is representative of 3 separate experiments (mean ± SEM, n=3). Bars with different letters are significantly different from each other ($p<0.05$). (b) Supernatants were collected and PGE_2 and TXB_2 were measured by RIA (mean ± SEM, n=3). Bars with different letters are significantly different from each other ($p<0.05$).

5.3 DISCUSSION

The results in this chapter demonstrate a marked increase in TNFα and IL-1β production in response to hypoxia. Previous studies have demonstrated that hypoxia can increase TNFα and IL-1β synthesis in mononuclear cells (Ghezzi et al. 1991) and human alveolar macrophages (Hempel et al. 1996) and can increase TNFα mRNA in human monocytes (Guida and Stewart 1998). The results in chapters 3 and 4 show that the increased TNFα and IL-1β release is coincident with reduced eicosanoid synthesis in hypoxic monocytes. Thus the two events may be related.

In normoxia, the increased TNFα production in the presence of NS-398 suggests that a COX-2 product may be responsible for tonic autocrine suppression of TNFα synthesis. COX-2 inhibition decreases both TXA_2 and PGE_2 synthesis. However, for the following reasons, it is the suppression of PGE_2 synthesis, which is likely to be responsible for the upregulation of TNFα synthesis. Firstly, COX-2 induction in monocytes is associated with greatly increased PGE_2 synthesis relative to that of TXA_2 (Penglis et al. 2000). Secondly, exogenous PGE_2 suppressed TNFα synthesis in a dose-dependent manner (and $cTXA_2$ did not, data not shown). Collectively, these results suggest that the hypoxia-mediated increase in TNFα synthesis may be due to the concomitant hypoxia-mediated reduction in PGE_2 synthesis. This is not the only candidate mechanism. It has also been reported that hypoxia-induced increases in TNFα synthesis in the J774.1 murine macrophage cell line, is attributable to hypoxia induced mitochondrial production of reactive oxygen species (ROS) (Chandel et al. 2000).

This thesis also examined the hypothesis that PGE_2 may inhibit TNFα synthesis by suppressing the phosphorylation of the p38 MAPK in monocytes. However, inhibition of endogenous eicosanoid synthesis or the addition of exogenous eicosanoids to monocytes did not induce any significant changes in p38 MAPK phosphorylation.

COX inhibition did not affect IL-1β synthesis in normoxia or hypoxia, indicating that, unlike TNFα, IL-1β is not regulated by eicosanoid mediated events.

While the reduction in PGE_2 synthesis observed in hypoxia was attributable to reduced p44/42 MAPK activation and subsequent AA release from membrane phospholipids, there was a concomitant increase in COX-2 expression. In normoxia, the marked suppression of LPS stimulated COX-2 expression by the addition of neutralising anti-TNFα antibody indicated that TNFα is an important autocrine or paracrine mediator of COX-2 upregulation. Therefore, the over-expression of COX-2 in hypoxia may result in part, from increased TNFα production in hypoxia. These results are at variance with a recent report demonstrating that neutralisation of TNFα does not alter COX-2 protein expression in human macrophages (Barrios-Rodiles et al. 1999).

The mechanisms for the effect of endogenous TNFα on COX-2 induction were not examined in this study. However, Chapter 3 demonstrates that early response genes may be regulated by changes in transcription rate or mRNA stability. TNFα mRNA contains multiple repeats of AU-rich motifs in the 3'-UTR of the gene that regulates stability of the message (Kruys et al. 1989; Han et al. 1990; Akashi et al. 1994). It has been reported that exogenous TNFα can increase COX-2 mRNA stability without an effect on the COX-2

transcription rate (Huang et al. 2000). However, another study showed that exogenous TNFα and IL-1β increased COX-2 transcription (Diaz et al. 1998). Thus, a coherent explanation of the mechanisms through which TNFα regulates COX-2 expression remains to be established.

Overall, the observations in stimulated monocytes suggest an autocrine feedback loop in which production of TNFα upregulates COX-2 and synthesis of PGE_2, that in turn suppresses TNFα production. It was previously demonstrated in stimulated human monocytes that whilst TXA_2 synthesis is an early COX-1 dependent response, synthesis of PGE_2 is delayed and is dependent on induction of COX-2 (Caughey et al. 1997). Thus, it is possible that COX-2 upregulation and consequent PGE_2 synthesis provides a system for a 'self-limited' monocyte response with regard to TNFα production. This proposition is supported by the increased TNFα synthesis observed with COX-2 inhibition. In hypoxia, the system is dysregulated with regard to TNFα synthesis, possibly because PGE_2 synthesis is decreased as a result of reduced AA release. A consequence of the unrestricted TNFα synthesis is sustained expression of COX-2 to levels that exceed substrate availability (Fig 5.7).

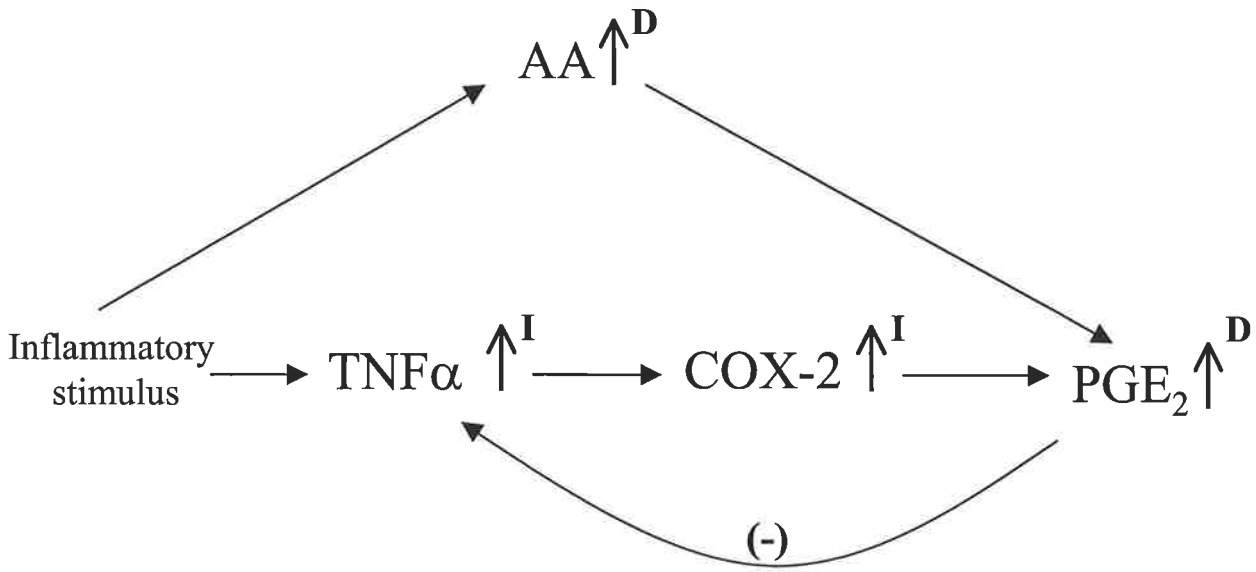


Figure 5.7
Proposed scheme for autoregulation of TNFα synthesis by a COX-2 dependent pathway
The vertical arrows indicate the changes in normoxia. The superscripts indicate how these changes are modified by hypoxia. D, decreased in hypoxia; I, increased in hypoxia.

5.5 CONCLUSION

The observed decrease in monocyte PGE_2 synthesis in hypoxia, invites consideration of implications regarding PGE_2 concentrations in inflamed lesions such as a rheumatoid joint. The rate of monocyte PGE_2 synthesis in a hypoxic rheumatoid joint can be expected to be limited by release of the COX-2 substrate, AA, which is reduced in hypoxia. However, the synovial concentration of PGE_2 derived from hypoxic monocytes would still be well above that in a healthy joint simply due to the presence of monocytes in an inflamed synovium. In addition, there is likely to be a contribution to total joint PGE_2 levels from other cell types such as synoviocytes. Thus, even in a hypoxic joint, it is probable that the PGE_2 concentration should be sufficient to contribute to swelling and pain, which can be alleviated by non-steroidal anti-inflammatory drugs (NSAIDs), including COX-2 inhibitors. Furthermore, it is possible that any increase in monocyte TNFα synthesis, which may result from reduced monocyte PGE_2 synthesis, could be an important upregulator of events that lead to joint damage.

It is clear that hypoxia is an important but often neglected determinant of inflammatory mediator production and one which can influence a broad range of events that occur in monocyte-containing lesions. An effect of hypoxia on other cells types involved in joint inflammation also warrants investigation. The following chapter characterises the effects of hypoxia on fibroblast-like synoviocytes with regard to COX-2 expression and eicosanoid production.

CHAPTER 6

THE EFFECT OF HYPOXIA ON COX-2 EXPRESSION AND ACTIVITY IN FIBROBLAST-LIKE SYNOVIOCYTES (FLS); EFFECT OF SOLUBLE MONOCYTE MEDIATORS

6.1 INTRODUCTION

Rheumatoid arthritis (RA) joint pathology is characterised by synovial hypertrophy resulting from synovial hyperplasia and leucocyte infiltration. The activities of this tissue can lead to cartilage degradation and ultimately, joint failure. Fibroblast-like synoviocytes (FLS) are believed to contribute to these events through their augmented proliferative and invasive properties following activation (Firestein 1996; Pap et al. 2000; Tolboom et al. 2002) and decreased rate of apoptosis (Aupperle et al. 1998). Their invasiveness is aided by the action of matrix metalloproteinases (MMPs), which are synthesised by many cells in the synovium, including FLS. MMPs degrade major protein components of the extracellular matrix, thereby providing space for synoviocytes to invade adjacent articular cartilage and bone (Tolboom et al. 2002).

In addition to being participants in the chronic degradative processes of rheumatoid joint disease, FLS may contribute also to the processes that underly the acute signs and symptoms of joint inflammation. Immunoreactive COX-2 has been demonstrated in synovial inflammatory cells (Lee et al. 2000) and FLS in humans with arthritis (Siegle et al. 1998) and animal models of arthritis (Kang et al. 1996). COX-2 catalyses the conversion of arachidonic acid (AA), which is cleaved from membrane phospholipids, to

form proinflammatory eicosanoids such as prostaglandin E_2 (PGE_2) and prostacyclin I_2 (PGI_2). These factors have nociceptive effects and through their vasodilatory actions, contribute to tissue oedema in RA (Robinson et al. 1975; Robinson et al. 1979; Doherty et al. 1987).

While there are many studies of eicosanoid production by FLS in vitro, they have been largely conducted in room air. However, there is considerable evidence that rheumatoid joints are chronically hypoxic (Falchuk et al. 1970; Lund-Olesen 1970; Ellis et al. 1994). I have shown that hypoxia causes upregulation of COX-2 expression in monocytes, but with a dissociation with eicosanoid production, which was decreased in hypoxia (Chapter 3-4 and (Demasi et al. 2003)). Despite extensive research on the regulation of inflammatory mediator production in synoviocytes, the effects of oxygen tensions relevant to pathology on COX-2 expression and eicosanoid synthesis in FLS, is unknown. This chapter investigates the effects of hypoxia on COX-2 expression and enzymatic activity. While a comprehensive study of MMP activity by FLS in hypoxia is beyond the scope of this study, preliminary experiments in this chapter examine MMP activity in FLS under hypoxic conditions. In addition, since monocytes and synoviocytes are present together in rheumatoid joints, the effects of soluble monocyte-derived mediators on FLS were examined under hypoxic conditions.

6.2 MATERIALS and METHODS

6.2.1 Materials

Materials were obtained from the following sources: COX-2 rabbit polyclonal Ab (Cayman Chemicals (Ann Arbor, MI)), PGE_2, rabbit anti-PGE_2, 6-Keto$PGF_{1\alpha}$, rabbit anti-6-KetoPGF_1 (Sigma Chem Co), pyrogen free Lymphoprep, (Nycomed, Oslo, Norway) protein trans-blot transfer membranes (Bio-Rad, North Ryde, AUS), RNA nylon transfer membrane (Hybond N+, Amersham Pharmacia Biotech, Pisacataway, NJ), peroxidase labelled donkey anti-rabbit Ab, peroxidase labelled goat anti-mouse Ab, [^{3}H]PGE_2, [^{3}H]6-Keto$PGF_{1\alpha}$,[^{3}H]arachidonic acid (Amersham International, Little Chalfont, ENG), Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) IL-1β (Endogen, Boston, MA) α[^{32}P]dCTP (Perkin Elmer, CT), GIGA Prime probe labelling kit, (Bresatec, Adelaide, AUS), phospho p38 polyclonal Ab, phospho p44/42 polyclonal Ab (New England Biolabs, Beverly, MA), monoclonal anti-IL-1β neutralising Ab (R&D Systems, Minneapolis, MN), 1B5 isotype control Ab (gift from GMayrhofer, Arthritis Lab, Hanson Institute, Adelaide, AUS)), MAX Efficiency ® DH5α™ competent cells, Trizol (Invitrogen, Carlsbad, CA), PCR Purification Kit, Reaction Cleanup Kit, Endofree® Plasmid Maxi Kit (Qiagen, Victoria, AUS), Jet PEI (PolyTransfection), Dual-Luciferase™ Reporter Assay kit, pGL3-Basic vector (Promega, New South Wales, AUS).

6.2.2 Methods

Cell culture and incubations

Fibroblast-like synoviocytes (FLS) were isolated as described previously (Neidhart et al. 2003) from synovial fluid collected from patients with inflammatory joint effusions following signed informed consent. Briefly, FLS were cultured by adherence to plastic T75 flasks, then seeded in 6-well culture dishes at $5x10^5$ cells / 2ml and allowed to reach confluency within 24h. FLS experiments were performed in RPMI 1640 containing 10% FCS and stimulated with IL-1β (2ng/ml).

Fresh human monocytes were isolated by counter current centrifugal elutriation as described in section 2.2.1. The purity of all preparations was assessed by FACS analysis and typically >85% of cells were monocytes, with the remainder lymphocytes.

For experiments in which conditioned medium (CM) from monocytes was used, CM was prepared as follows. Resting monocytes ($2x10^6$ / ml) were incubated in normoxia or hypoxia for 18h after which the CM was collected. The monocyte CM was equilibrated in normoxic or hypoxic conditions and then added to FLS ($5x10^5$ / 6 well plate) in normoxia or hypoxia for 18h.

Northern Analysis

Northern membranes were processed by hybridising membranes with a COX-2 cDNA probe overnight at 43°C on a rotator. Initial washes began at RT with low stringency wash buffer (2x SSC, 0.1% SDS) for 30min. A Geiger counter was used to determine the extent of background radioactivity on the membrane. If the membrane required further washing a higher temperature (43°C) and more stringent wash buffer (0.1x SSC, 0.1% SDS) was used. Radiation sensitive film was exposed to the membrane for 24h at –80°C.

Western Analysis

Membranes were treated with COX-2 (1:1,000), phospho p-38 MAPK (1:500, phospho p-44/42 MAPK (1:500) and β-actin (1:2,500) Abs in TBS-Tween o/n at 4°C. Following washing, the membrane was treated with anti-rabbit or anti-mouse horseradish peroxidase (HRP) Ab at 1/10,000 dilution in TBS-Tween for 45min. The chemiluminescent substrate solution was added to the membrane (5min) prior to exposure to light sensitive film for 2-5min.

Transcription Studies

A COX-2 promoter-reporter construct was designed as indicated in section 2.2.15 in the methods section. Briefly, this comprised a region –922bp upstream from the transcription start site of the COX-2 gene ligated into the pGL3-Basic vector (Promega), which contains a luciferase reporter gene downstream from the site of ligation. Transfection of the reporter construction into FLS was performed using 4μg of the pGL3-COX-2-922 construct and 4μl of Jet PEI, each suspended in 75μl of sterile NaCl solution (150mM). The Jet PEI / NaCl solution was then added to the DNA/NaCl solution and incubated at RT for 30min. The medium in the wells was then changed to fresh medium, and 150μl of the DNA/Jet PEI was added to each well. The transfection was allowed to proceed for 5h then the medium was replaced again with either hypoxic or normoxic medium and stimulated with IL-1β (2ng/nl) for 8h. Following this, the medium was removed and cell lysates were assayed for luciferase activity.

Matrix metalloproteinase (MMP) determination

Gelatin zymography was used to detect total MMP activity in FLS conditioned medium as described in methods section 2.2.12. Broad range molecular weight markers were used to identify the molecular weight of the digested band.

Statistical Analysis

Results are expressed as the mean ± S.E.M of triplicate incubations. Analysis of variance followed by the Neuman-Keuls multiple comparisons test was used to identify statistically significant changes in eicosanoid production between treatments using WINKS (Texasoft, Cedar Hill, TX).

6.3 RESULTS

6.3.1 Effect of hypoxia on COX-2 mRNA and protein in FLS

FLS ($5x10^5$/ 6 well plate) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxia for 18h. IL-1β (2ng/ml) transiently induced COX-2 mRNA and sustained an increased in COX-2 protein expression in FLS over a study period of 18h. The upregulation of COX-2 mRNA and protein was potentiated by hypoxia (Fig 6.1). FLS were not responsive to TNFα stimulation in terms of COX-2 expression (data not shown).

6.3.2 Effect of hypoxia on transcription of COX-2 in FLS

In Chapter 3-4, it is reported that hypoxia can increase transcription of COX-2 in monocytes and therefore this mode of regulation was examined in FLS.

FLS ($2x10^5$ / 12 well plate) were transfected with a -922bp segment of a COX-2 promoter/luciferase reporter construct. Hypoxia resulted in a two-fold increase in transcription rate (Fig 6.2).

In Chapter 3, it was reported that hypoxia also stabilised COX-2 mRNA in monocytes. Therefore, this was examined in FLS.

6.3.3 Effect of hypoxia on COX-2 mRNA stability in FLS

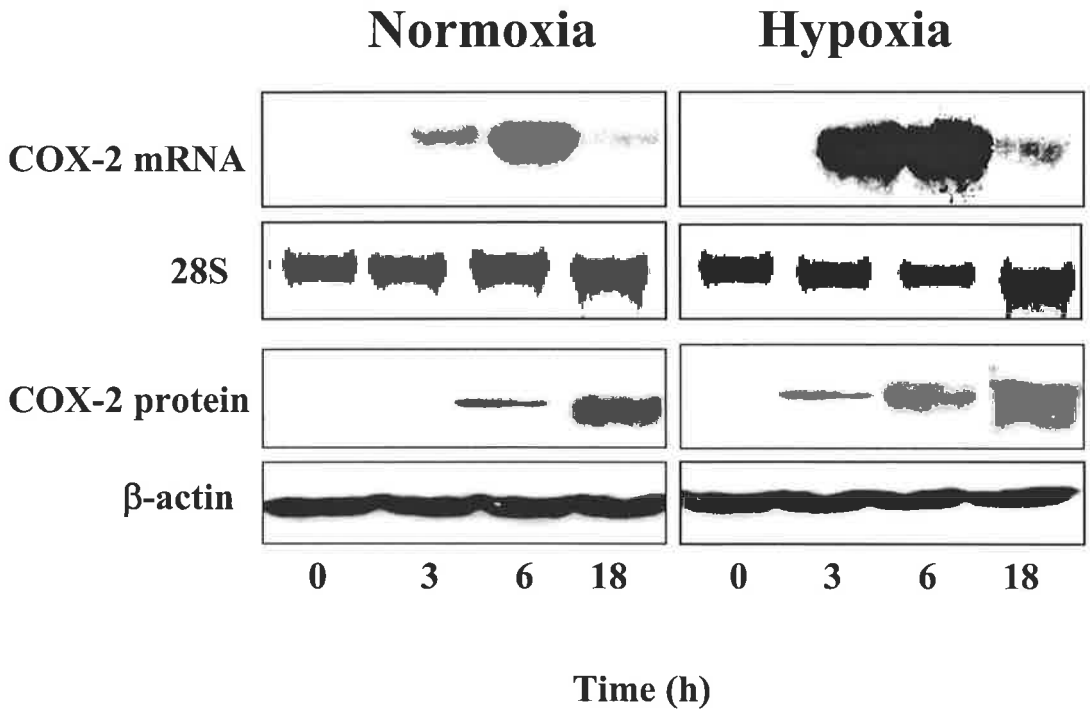


Figure 6.1
Effect of hypoxia on COX-2 mRNA and protein in FLS
FLS ($5x10^5$) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxia over 18h. Cells were processed for northern blot and western blot analysis as described in the Methods section. The 28S band and β-actin staining confirmed similar RNA and protein loading, respectively, in each lane. Results are representative of 3 separate experiments.

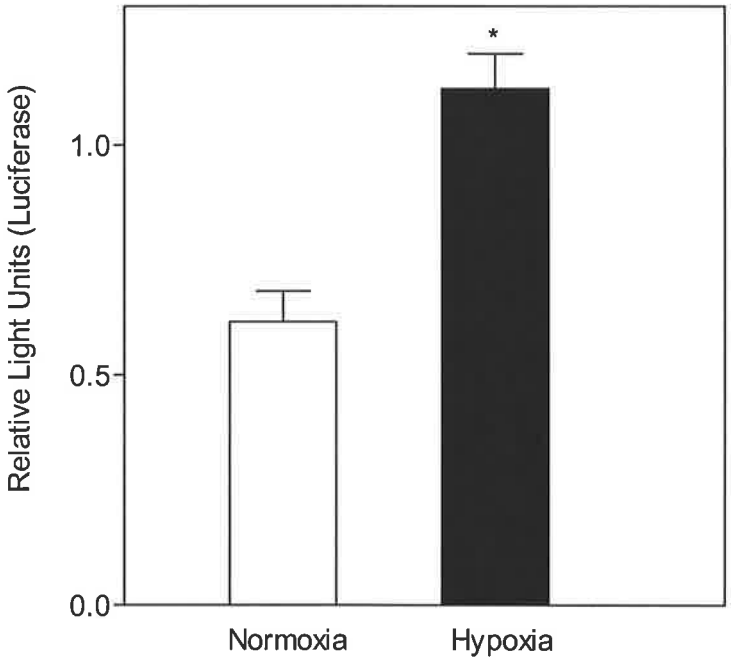


Figure 6.2

Effect of hypoxia on COX-2 transcription in FLS

FLS ($2x10^5$cells/mL) were transfected with COX2 -922 construct (4μg) using Jet PEI and incubated for 5h. The media was changed, and stimulated with IL-1β (2ng/ml) for 8h in normoxia (open bar) or hypoxia (solid bar). Cells were then lysed and assayed for luciferase activity. Results are expressed as the ratio of firefly/*Renilla* luciferase activity (mean ± SEM; n=3). *$p<0.05$, by comparison to normoxic FLS.

FLS ($5x10^5$ / 6 well plate) were stimulated with IL-1β for 3h to induce COX-2 mRNA. Actinomycin D (AD, 5μg/ml) was added to inhibit further transcription and the level of COX-2 mRNA was measured for a further 3h. Unlike monocytes, the rate of COX-2 mRNA decay was similar in normoxic and hypoxic conditions (Fig 6.3).

6.3.4 Effect of hypoxia on phosphorylation of p44/42 and p38 mitogen activated protein kinase (MAPK) in FLS

p38 MAPK activation is involved in the induction of COX-2 in monocytes (Pouliot et al. 1997) whereas activation of p44/42 MAPK is involved in the induction of COX-2 in endothelial cells (Caughey et al. 2001a). With an increase in transcription rate, it was important to determine whether an increase in the phosphorylation of these pathways contributed to the increased expression of COX-2 in hypoxia.

IL-1β stimulated phosphorylation of p44/42 and p38 MAPK (Fig 6.4). However, there was no change in the phosphorylation of either p38 or p44/42 MAPK or in the time course of decay of phosphorylated forms of these enzymes in normoxic or hypoxic conditions (Fig 6.4).

6.3.5 Effect of hypoxia on prostaglandin synthesis in FLS

To determine whether increased COX-2 expression in FLS resulted in an increase in activity, the effect of hypoxia on 6-KetoPGF$_{1\alpha}$ and PGE_2 accumulation was examined.

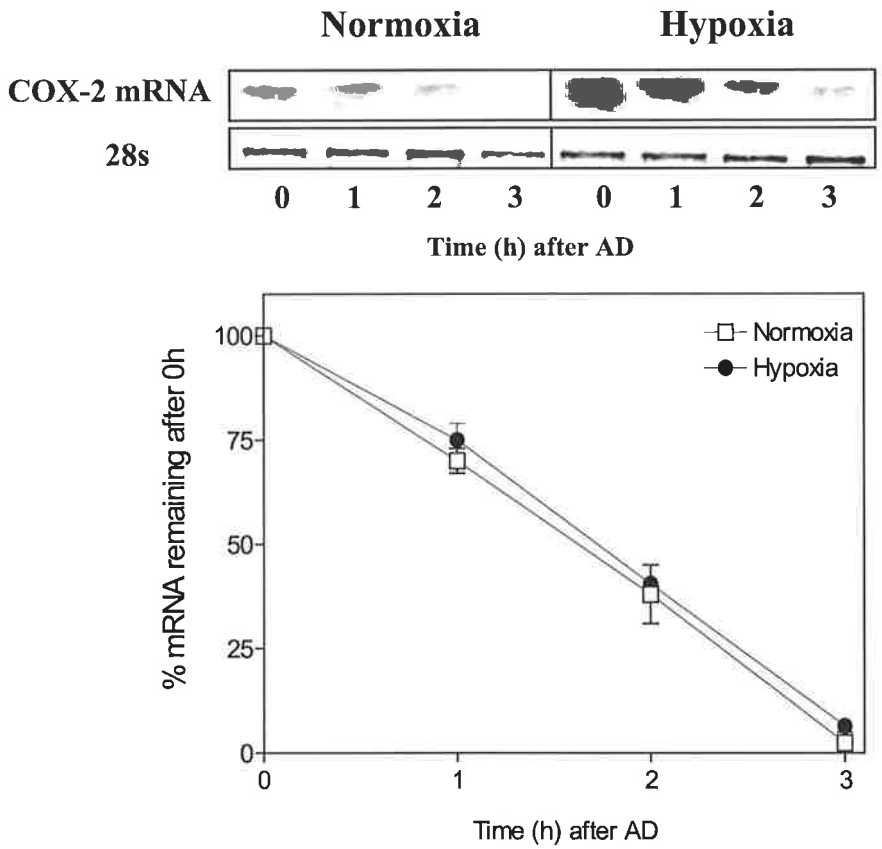


Figure 6.3

Effect of hypoxia on COX-2 mRNA stability in FLS

FLS ($5x10^5$) were stimulated with IL-1β (2ng/ml) for 3h in normoxia or hypoxia. AD (5μg/ml) was then added and the level of COX-2 mRNA was assessed for a further 3h by Northern analysis. In the graphic depiction of densitometry results, COX-2 mRNA is normalised against the 28S band and the rate of decay is shown with values at 1-3h expressed as a % of value at 0h (mean ± SEM; n=3); normoxia (□) and hypoxia (●).

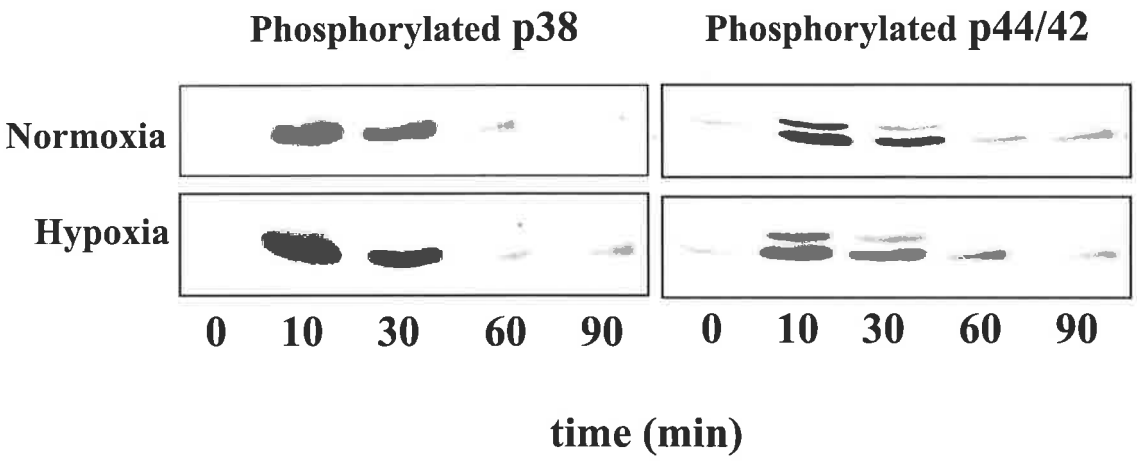


Figure 6.4
Effect of hypoxia on phosphorylated p44/42 MAPK and p38 MAPK in FLS
FLS ($5x10^5$) were stimulated with IL-1β (2ng/ml) under normoxic or hypoxic conditions for up to 90min. At each time indicated, cells were processed for western blot analysis. Results are representative of 3 experiments.

FLS were treated with IL-1β in normoxia or hypoxia over 18h and the accumulation of PGE_2 and 6-Keto$PGF_{1\alpha}$ in the supernatants was measured. There was a significant increase in the rate of PGE_2 and 6-Keto$PGF_{1\alpha}$ synthesis in hypoxia (Fig 6.5). FLS did not synthesise TXA_2 (data not shown). The increased synthesis of PGE_2 and 6-Keto$PGF_{1\alpha}$ parallelled the increased COX-2 protein expression seen in Figure 6.1.

6.3.6 Effect of hypoxia on endogenous arachidonic acid (AA) release in FLS

In monocytes, decreased arachidonic acid (AA) release in hypoxia was observed (Chapter 3-4 and (Demasi et al. 2003)). Therefore, the effects of hypoxia on AA release in FLS, were examined.

FLS were pre-incubated with [^{3}H]AA in normoxia (18h, 37°C) to incorporate labelled AA into cell membranes. The cells were then washed (3x) and incubated with IL-1β in normoxia or hypoxia for 30h. In addition, some [^{3}H]AA pre-labelled cells that had been hypoxic for 9h were returned to normally oxygenated medium for the following 21h.

In normoxia, there was a time dependent release of labelled AA from FLS when stimulated with IL-1β (Fig 6.6). Neither hypoxia nor re-oxygenation after hypoxia, resulted in any significant change in AA release from IL-1β stimulated FLS (Fig 6.6).

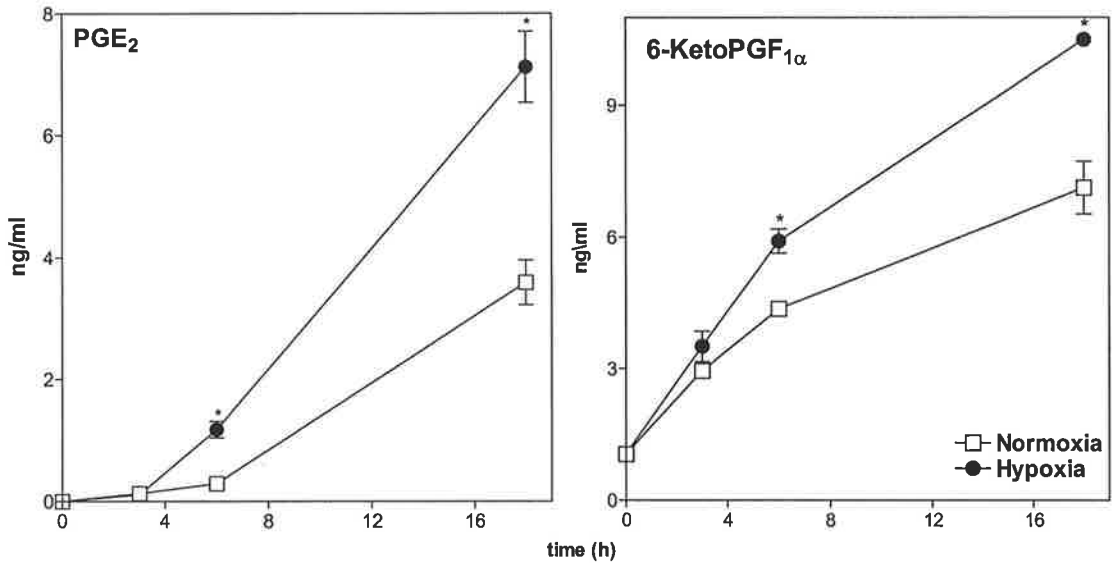


Figure 6.5

Effect of hypoxia on prostaglandin synthesis in FLS

FLS ($5x10^5$) were stimulated with IL-1β (2ng/ml) in normoxia (□) or hypoxia (●) over 18h. Supernatants were collected and assayed for PGE_2 and 6-keto $PGF_{1\alpha}$ by RIA (mean ± SEM; n=3) *$p<0.05$, by comparison to normoxic FLS.

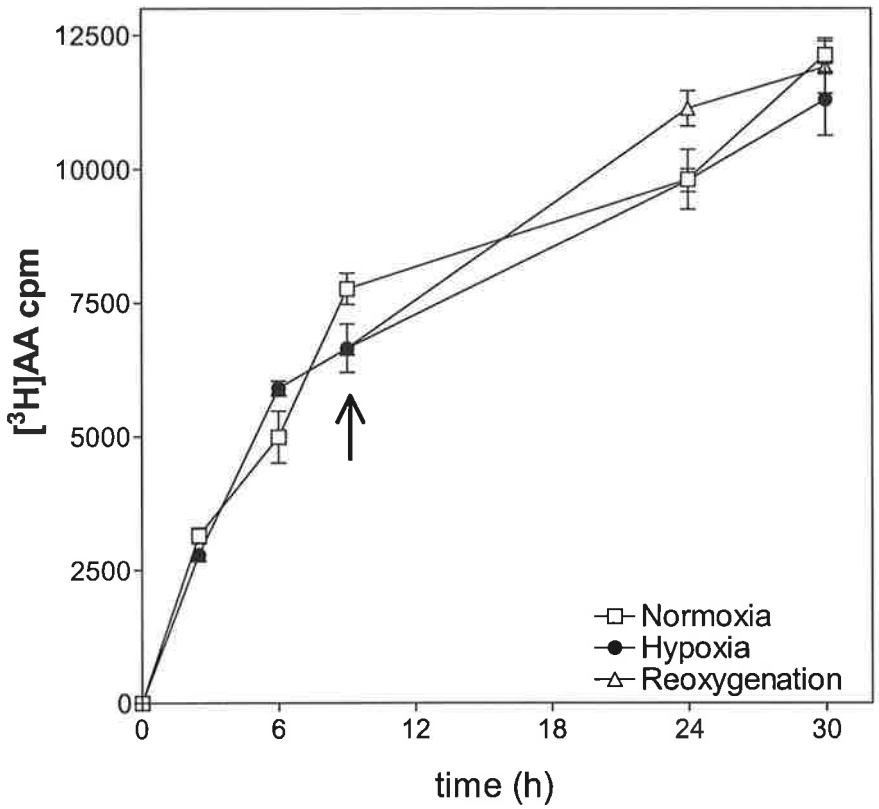


Figure 6.6

Effect of hypoxia on arachidonic acid (AA) release in FLS

FLS ($5x10^5$) were incubated with [^{3}H]AA (2μCi/ml) overnight. The following day the cells were washed 3x and stimulated with IL-1β (2ng/ml, 30h, 37°C) in normoxia (□) or hypoxia (●). In addition, cells were incubated under hypoxic conditions for 9h (↑) then reoxygenated for a further 21h incubation (Δ). Supernatants were collected and [^{3}H]AA release was determined using a scintillation counter (mean ± SEM; n=3).

6.3.7 Effect of hypoxia on matrix metalloproteinase (MMP) activity in FLS

Preliminary studies to examine the effects of hypoxia on MMP activity were conducted. FLS were treated with IL-1β in normoxia or hypoxia over 18h and the accumulation of MMP activity in the supernatants was measured. A significant increase in MMP activity at 55kDa was detected in normoxia at 18h and this was greatly potentiated in hypoxia (Fig 6.7).

6.3.8 IL-1β concentration in monocyte conditioned medium (CM)

Resting monocytes ($2x10^6$ / 2.5ml) were incubated in normoxia or hypoxia for 18h. IL-1β was measured in the monocyte CM and compared with FLS CM ($5x10^5$ / 2ml) derived from identical incubation conditions.

IL-1β was detected in normoxic monocyte CM and this was significantly increased by hypoxia (Table 6.1). IL-1β was not measurable in FLS CM.

6.3.9 Effect of monocyte CM on COX-2 expression and activity in FLS in hypoxia

The monocyte CM was pre-equilibrated in normoxic or hypoxic conditions then added to FLS ($5x10^5$/ml) overnight in normoxia or hypoxia.

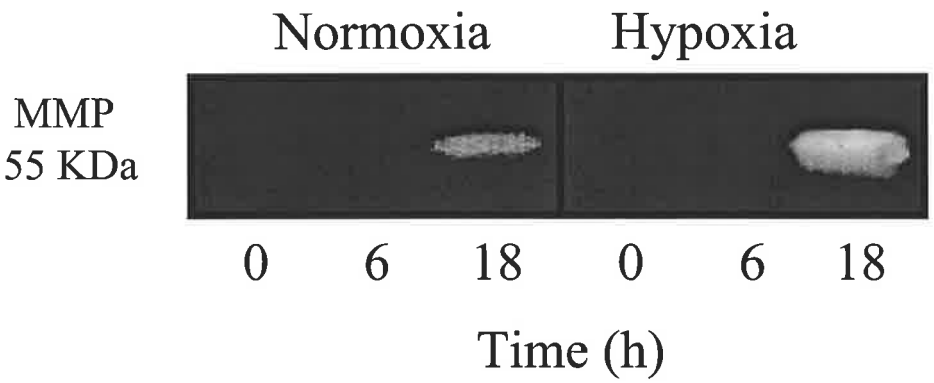


Figure 6.7
Effect of hypoxia on MMP activity in FLS
FLS ($5x10^5$) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxia over 18h. Supernatants were collected at 6 and 18h then assayed for MMP activity by gelatin zymography. Results are representative of 3 separate experiments.

Table 6.1 - IL-1β in conditioned medium (CM) from unstimulated human FLS (5 x 10^5/2ml) and monocytes (5 x 10^6/2.5ml) in normoxia or hypoxia

FLS CM		Monocyte CM	
Normoxia	Hypoxia	Normoxia	Hypoxia
(ng/ml)		(ng/ml)	
nd*	nd	3.4 ± 0.1	8.2 ± 1.6

* not detected

FLS or monocytes were incubated without deliberate stimulation under normoxic or hypoxic conditions. Supernatants were assayed for IL-1β (ng/ml) ELISA after 18h.

Monocyte CM induced COX-2 expression in normoxic FLS (Fig 6.8). This effect was augmented when the monocyte CM was derived under hypoxic conditions (Fig 6.8). In normoxic FLS, monoclonal antibody against IL-1β, inhibited the increase in FLS COX-2 expression. 1B5 isotype control monoclonal antibody had no effect (data not shown). When FLS were incubated in hypoxia, the expression of COX-2 following addition of normoxia- and hypoxia-derived monocyte CM was augmented (Fig 6.8). In hypoxic FLS, neutralising IL-1β reduced COX-2 expression induced by normoxic or hypoxic monocyte CM.

FLS synthesised PGE_2 and PGI_2 (measured as 6-ketoPGF$_{1\alpha}$). However, no TXA_2 (measured as TXB_2) was measurable. The synthesis of both PGE_2 and 6-ketoPGF$_{1\alpha}$ changed in a manner consistent with the changes in COX-2 expression (Fig 6.8).

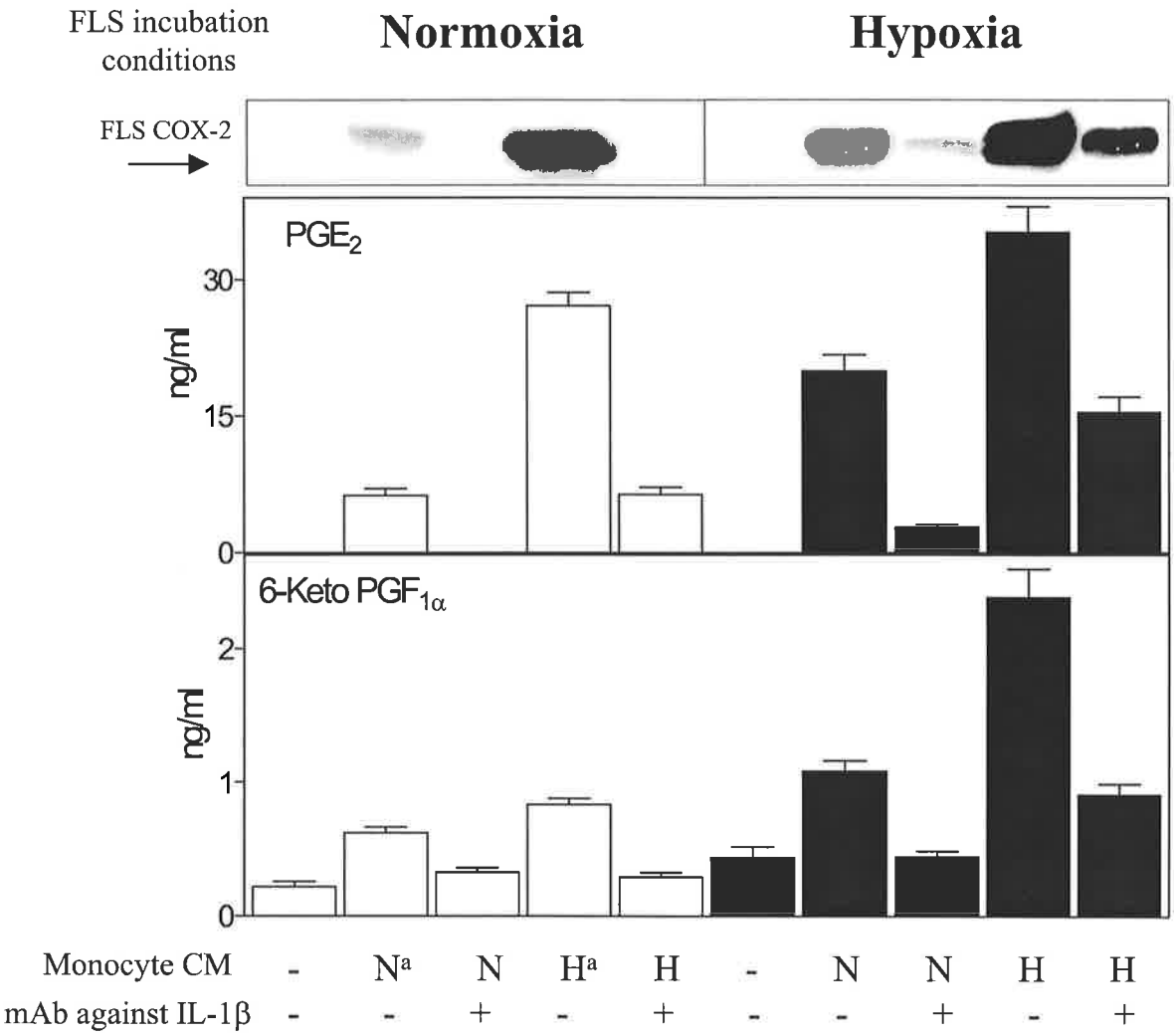


Figure 6.8

Effect of monocyte CM on COX-2 expression and activity in hypoxia in FLS

FLS ($5x10^5$) were incubated with monocyte CM in the absence or presence of neutralising IL-1β Ab (8μg/ml) or isotype control 1B5 (8μg/ml) under normoxic or hypoxic conditions. After 18h, FLS were processed for western blot analysis and PGE_2 and 6-Keto $PGF_{1\alpha}$ were measured by RIA (mean ± SEM; n=3). [a]N or H indicates the monocyte CM was derived in normoxic or hypoxic conditions, respectively.

6.4 DISCUSSION

Rheumatoid synovium has been shown to be hypoxic (Falchuk et al. 1970; Lund-Olesen 1970; Ellis et al. 1994). This can be explained by a mismatch between metabolic demand, which is increased (Dingle and Page-Thomas 1956; Naughton et al. 1993) and vascular perfusion. The latter is compromised by synovial swelling and effusion which can result in increased intra-articular pressure above capillary closure pressure (30mmHg) (Geborek et al. 1989). This tamponade effect is accentuated by certain postures and by muscle action (James et al. 1990).

To date, *in vitro* studies of inflammatory mediator production in FLS have been well characterised in normoxic conditions (20% O_2). However this does not adequately represent the conditions of oxygenation that synovial lining cells or infiltrating leucocytes encounter in an inflamed joint with an effusion (Falchuk et al. 1970; Lund-Olesen 1970; Ellis et al. 1994).

In this study, I examined the hitherto unexplored regulation of COX-2 expression and activity in FLS exposed to pathologically relevant levels of O_2 detected in inflamed joints. Hypoxia resulted in a significant upregulation of COX-2 expression attributed to an increase in transcription rate. This was demonstrated by transfecting FLS with a COX-2 promoter-luciferase reporter gene and comparing transcription rates in normoxic and hypoxic conditions. In addition, I investigated the effect of hypoxia on COX-2 mRNA stability given that the 3'-UTR of the COX-2 gene contains 22 copies of the AUUUA motif which is related to mRNA stability (Kruys et al. 1989; Akashi et al. 1994; Cok and

Morrison 2001). However, COX-2 mRNA exhibited a similar rate of decay in normoxic and hypoxic conditions. This is unlike the observation in monocytes where hypoxia markedly increased COX-2 mRNA stability (Chapter 3).

Signalling mechanisms that may potentially upregulate COX-2 expression in hypoxia could involve the p44/42 or p38 MAPK pathways. However, the results demonstrated no significant changes in the activation of either enzyme, as assessed by phosphorylation, in hypoxia compared to normoxia in FLS.

In addition to the differences between monocytes and FLS with regard to the effect of hypoxia on COX-2 mRNA stability, there were other points of difference between FLS and monocytes in their response to hypoxia. In monocytes, upregulated COX-2 expression in hypoxia was accompanied by a decrease in PGE_2 and TXA_2 production. This disparity was attributable to limited release of AA substrate, which in turn was explained by decreased phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$). By comparison, there was a hypoxia-induced increase in COX-2 expression and eicosanoid synthesis in FLS. Unlike the situation in monocytes, there was no effect of hypoxia on AA substrate release in FLS.

Numerous studies have demonstrated that co-culture incubations or soluble mediators released by other cells types may induce changes in FLS. For example, CM derived from LPS-stimulated mononuclear cells increased cellular autofluorescence and induced changes in the morphology of mitochondria in normal FLS (Pulkki et al. 1988). Co-culture of synovial fibroblasts with monocyte/macrophage cells resulted in increased IL-6 (Scott et al. 1997; Chen et al. 1998), IL-1 and TNF synthesis (Elias et al. 1988; Scott et al. 1997).

These factors increase monocyte adhesiveness to cartilage (Ishikawa et al. 1991) and promote invasiveness (Khalkhali-Ellis et al. 1997), proliferation (Wharton 1983) and release of degradative factors (Janusz and Hare 1993; Scott et al. 1997) by FLS. In addition, CM derived from mixed monocytes and lymphocyte cultures induced PGI_2 and PGE_2 synthesis in FLS derived from synovial explants of arthritic and non-arthritic donors (Hamilton et al. 1985). Despite these reports, there is a relative paucity of studies on the effect of monocyte-derived mediators on COX-2 expression and activity in FLS and there are no published studies on the effects of hypoxia on these events. This study shows that the response of FLS to IL-1β in terms of COX-2 expression, PGE_2 and PGI_2 release and MMP activity is increased under hypoxic conditions. This study reported preliminary data on total MMP activity. However, MMP activity is specifically controlled by tissue inhibitor of metalloproteinases (TIMP) (Nagase and Woessner 1999). Therefore, further studies are needed to determine to what extent hypoxia-induced increases in total MMP activity translated into increased net MMP activity. Recently, it was demonstrated that COX-2 derived PGE_2 and PGE_1 could down regulate MMP-1 (55-60kDa) via the ERK pathway in FLS (Pillinger et al. 2003). This suggests that hypoxia-induced changes in eicosanoid synthesis may regulate MMP activity in FLS.

We have also shown the COX-2, PGE_2 and PGI_2 responses of FLS to CM from normoxic and hypoxic monocytes, is largely due to IL-1β and that the response is increased when the FLS are hypoxic. The observation that hypoxic FLS are more responsive to recombinant IL-1β or monocyte derived IL-1β raises the hypothesis that surface receptors for IL-1β are upregulated in hypoxic conditions and this possibility merits investigation.

6.5 CONCLUSION

Overall, it is clear that hypoxia is an important but often neglected determinant of inflammatory mediator production and one which can potentially influence a broad range of events that occur in an inflamed synovium. These studies suggest that frequent aspiration of joints with effusions, particularly weight bearing joints, in order to alleviate intra-articular pressure and restore synovial perfusion may be a worthwhile adjunct to systemic therapy. Finally, transcription factors that control gene expression in response to hypoxia may be a potential target for therapy. For example, hypoxia inducible factor (HIF) which is a transcription factor upregulated in response to low oxygen tensions may control the expression of COX-2 in hypoxic conditions. The COX-2 promoter-luciferase reporter construct used in these studies contains a putative hypoxia response element (HRE), which is a binding site for HIF-1α and HIF-2α heterodimers with HIF-1β/ARNT. An investigation into whether COX-2 is regulated by HIF warrants investigation.

CHAPTER 7

EFFECT OF HYPOXIA ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELL COX-2 EXPRESSION AND ACTIVITY

7.1 INTRODUCTION

Endothelial cell (EC) function is central to vascular homeostasis. The ability of endothelial cells to synthesise many vasoactive, platelet active and angiogenic mediators, enables endothelial cells to play a critical role, not only in normal vascular homeostasis but in tumorigenesis, and inflammatory lesions such as a rheumatoid pannus (Folkman 1995).

Being the first cell layer in contact with blood, endothelial cells deal with a multitude of changes occurring in disease, including changes in oxygen tension which could occur with vessel spasm, shunting or thrombotic occlusion. Reduced oxygen tensions (hypoxia) have also been demonstrated in atherosclerotic plaques (Heughan et al. 1973; Bjornheden et al. 1999). These considerations provide a strong rationale for characterising the effects of hypoxia on inflammatory mediator production by endothelial cells.

Hypoxia can augment the expression of COX-2 in human monocytes (Chapter 3-4, (Demasi et al. 2003)), human synoviocytes (Chapter 6), a rabbit corneal epithelial cell line (Bonazzi et al. 2000) and human umbilical vein endothelial cells (HUVEC) (Schmedtje et

al. 1997). However, the latter study did not examine eicosanoid synthesis resulting from upregulated COX-2 expression following hypoxic exposure. This is important because early studies with endothelium or whole vessels have reported varying effects of hypoxia on prostaglandin synthesis. Increased prostaglandin synthesis has been reported in response to hypoxia in human pulmonary artery endothelial cells (Martin et al. 1992) and HUVEC (Michiels et al. 1993; Windischbauer et al. 1994). However, in some experimental models such as pulmonary arterial ECs (Farber and Young 1981), bovine aortic ECs (Patton et al. 1997) and a rabbit corneal epithelial cell line (Bonazzi et al. 2000), a decrease in prostaglandin synthesis was observed. Discrepancies in results may be due to differences in cell types or the degree of hypoxia. Also, the recent report with HUVEC did not examine alternative mechanisms for hypoxia-mediated increases in COX-2 expression, other than increased transcription.

Chapter 3-4 describe upregulated COX-2 expression in human monocytes following hypoxia (1% O_2), which was associated with both increased message stability and increased transcription (Chapter 3-4, (Demasi et al. 2003)). However, there was a paradoxical decrease in eicosanoid production, despite upregulated COX-2 induction. The dysjunction in the effect of hypoxia on COX-2 expression and eicosanoid synthesis was attributable to limited phospholipase A_2 activity and consequent limited release of arachidonic acid (AA), the substrate of COX-2 (Chapter 3-4, (Demasi et al. 2003)). Therefore, it was important to determine the effect of hypoxia on COX-2 expression and eicosanoid synthesis in HUVEC, and assess the mechanisms for COX-2 alterations in hypoxia.

Although an increase in transcription of COX-2 in hypoxic HUVEC has been reported (Schmedtje et al. 1997), other mechanisms involved in the regulation of COX-2 expression and eicosanoid synthesis in hypoxia have not been clearly elucidated.

This chapter describes the effects of hypoxia (1% O_2) on COX-2 expression in endothelial cells and the mechanisms involved in COX-2 regulation. In addition, I characterise endothelial cell eicosanoid production in hypoxia and investigate mechanisms for changes in eicosanoids.

7.2 METHODS and MATERIALS

7.2.1 Materials

Materials were obtained from the following sources: COX-2 rabbit polyclonal Ab (Cayman Chemicals), 6-KetoPGF$_{1\alpha}$ and PGE$_2$ rabbit antiserum (Sigma Chem Co), rabbit TXB$_2$ antiserum was prepared from rabbits immunised with TXA$_2$ conjugated to human thyroglobulin as used in previous studies (James and Walsh 1988), pyrogen free Lymphoprep, (Nycomed, Oslo, Norway) protein trans-blot transfer membranes (Bio-Rad, North Ryde, AUS), RNA nylon transfer membrane (Hybond N+, Amersham Pharmacia Biotech, Pisacataway, NJ), peroxidase labelled donkey anti-rabbit Ab, peroxidase labelled goat anti-mouse Ab, [^{3}H]PGE$_2$, [^{3}H]TXB$_2$ [^{3}H]6-KetoPGF$_{1\alpha}$ (Amersham International, Little Chalfont, ENG), Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) IL-1β, TNFα (Endogen, Boston, MA), α[^{32}P]-dCTP (Perkin Elmer), GIGA Prime probe labelling kit, (Bresatec, Adelaide, AUS).

7.2.2 Methods

HUVEC Incubations

HUVEC were obtained from Assoc Prof Jenny Gamble (Hanson Institute, Adelaide, Aust).

Fresh human monocytes were isolated by counter current centrifugal elutriation as described in section 2.2.1. Endothelial cell growth factor (50μg/ml) and heparin (50μg/ml) were included in the medium of each incubation, unless otherwise stated.

Northern Analysis

Northern membranes were processed by hybridising membranes with a COX-2 cDNA probe overnight at 43°C on a rotator. Initial washes began at RT with low stringency wash buffer (2x SSC, 0.1% SDS) for 30min. A Geiger counter was used to determine the extent of background radioactivity on the membrane. If the membrane required further washing a higher temperature (43°C) and more stringent wash buffer (0.1x SSC, 0.1% SDS) was used. Radiation sensitive film was exposed to the membrane for 24h at –80°C.

Western Analysis

Transfer membranes were treated with rabbit polyclonal COX-2 Ab at 1/1,000 dilution in TBS-Tween for 60min at RT or monoclonal β-actin Ab at 1:2,500 dilution in TBS-Tween o/n at 4°C. Following washing, the membrane was treated with anti-rabbit or anti-mouse horseradish peroxidase (HRP) Ab at 1/10,000 dilution in TBS-Tween for 45min. The chemiluminescent substrate solution was added to the membrane (5min) prior to light sensitive film for 2-5min.

Immunohistochemistry

Immunohistochemistry was performed on HUVEC ($2x10^4$/well) plated on a fibronectin coated 8-well chamber slide (Nalge Nunc, Naperville, IL). Following incubation with IL-1β in the presence or absence of hypoxia, cells were fixed with 96% ice cold ethanol for 30sec, before washing 2x with FACS wash. 150µl of rabbit anti-human COX-2 polyclonal Ab at 20µg/ml was added to cells (or isotype control) for 30min at 4°C. Cells were washed 2x gently with FACS wash to avoid cells detaching. 150µl of anti-rabbit-FITC was added for 40min at 4°C followed by 2x washing, the walls of the chamber were removed and a coverslip on the slide allowed the fluorescence to be detected by microscopy. Five fields/ treatment were photographed and analysed for intensity of fluorescence using V++ Precision Digital Imaging System (Auckland, NV)

Transcription studies

A COX-2 promoter-reporter construct was designed as indicated in section 2.2.15 in the methods section. Briefly, this comprised a region –531bp upstream from the transcription start site of the COX-2 gene ligated into the pGL3-Basic vector (Promega), which contains a luciferase reporter gene downstream from the site of ligation.

HUVEC were transfected using Amaxa HUVEC (Vs.2) Nucleofector™ kit (Amaxa, Maryland, USA) according to the manufacturer's instructions. Briefly, cells were plated at 80% confluency and the next day the medium was changed to EGM-2 medium (Clonetics). After an overnight incubation at 37°C, the cells were trypsinised and resuspended at $1x10^6$ / 100µl HUVEC Nucleofector Solution with a total concentration of 5µg plasmid DNA with a 4:1 ratio of COX-2-531 promoter reporter-construct:control *Renilla* plasmid. This solution was then transferred to an Amaxa cuvette and subjected to electroporation. Cells

were then resuspended in an appropriate volume of EGM-2 medium and transferred to 24-well plates. Cells were allowed to recover overnight, after which the medium was changed to normoxic or hypoxic RPMI + 10% FCS in the presence of IL-1β (2ng/ml) at 37°C for 18h. Cells were then assayed using the Dual-Luciferase™ Reporter Assay kit (Promega, New South Wales, AUS).

7.3 RESULTS

7.3.1 Effect of hypoxia on HUVEC COX-2 mRNA and protein

HUVEC at ($5x10^5$/ 6 well plate) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxia. IL-1β induced COX-2 mRNA and protein in normoxic endothelial cells (Fig 7.1a). However, hypoxia induced no significant change in COX-2 expression under these conditions (Fig 7.1a). HUVEC were then stimulated with IL-1β in the presence of endothelial cell growth factor and heparin in the culture medium. Under these conditions, hypoxia augmented the effect of IL-1β on COX mRNA and protein (Fig 7.1b). This augmentation confirmed findings of Schmedtje et al (Schmedtje et al. 1997).

Immunohistochemistry of adherent HUVEC with an anti-human COX-2 PAb followed by an anti-rabbit FITC-labelled PAb confirmed the augmentation of IL-1β induced COX-2 protein in HUVEC cultured in the presence of endothelial cell growth factor and heparin (Fig 7.1c). The COX-2 protein appeared to be diffusely distributed throughout the cell cytoplasm (Fig 7.1c).

All further studies were conducted with HUVEC cultured in the presence of endothelial cell growth factor and heparin.

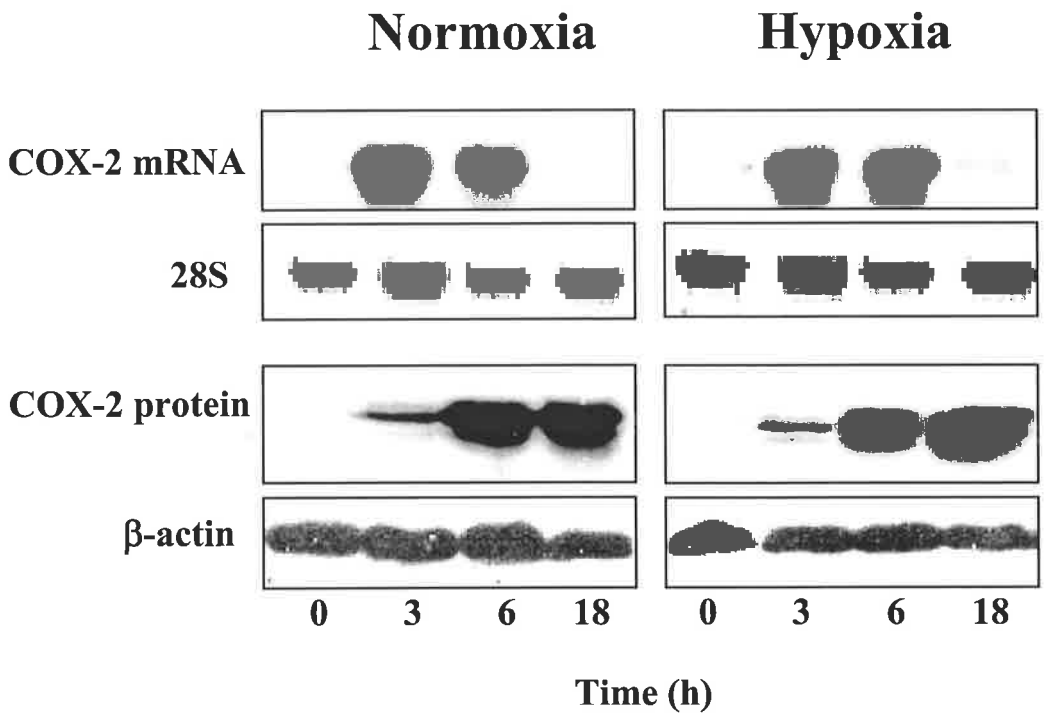


Figure 7.1a

Effect of hypoxia on COX-2 mRNA and protein in HUVEC

HUVEC ($5x10^5$) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxia over 18h. Cells were processed for northern blot and western blot analysis. The 28S band and β-actin confirmed similar RNA and protein loading, respectively, in each lane. Results are representative of 3 separate experiments.

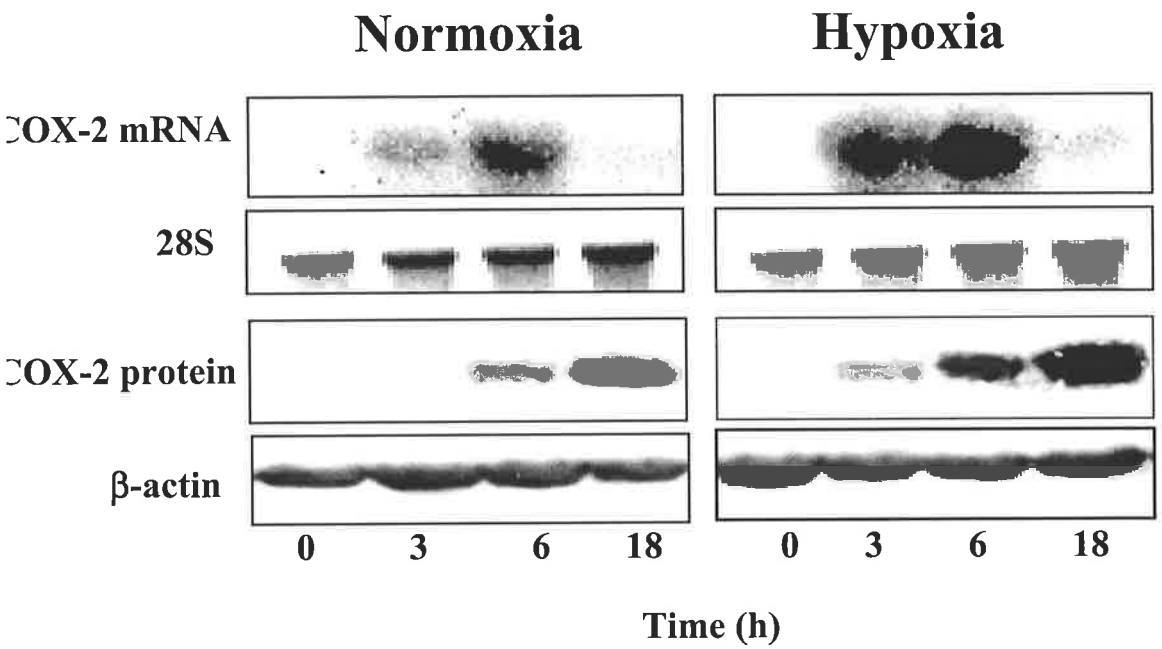


Figure 7.1b

Effect of hypoxia on COX-2 mRNA and protein in HUVEC cultured in the presence of endothelial cell growth factor and heparin

HUVEC ($5x10^5$) grown in the presence of endothelial cell growth factor (50μg/ml) and heparin (50μg/ml) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxia over 18h. Cells were processed for northern blot and western blot analysis. The 28S band and β-actin confirmed similar RNA and protein loading, respectively, in each lane. Results are representative of 3 separate experiments.

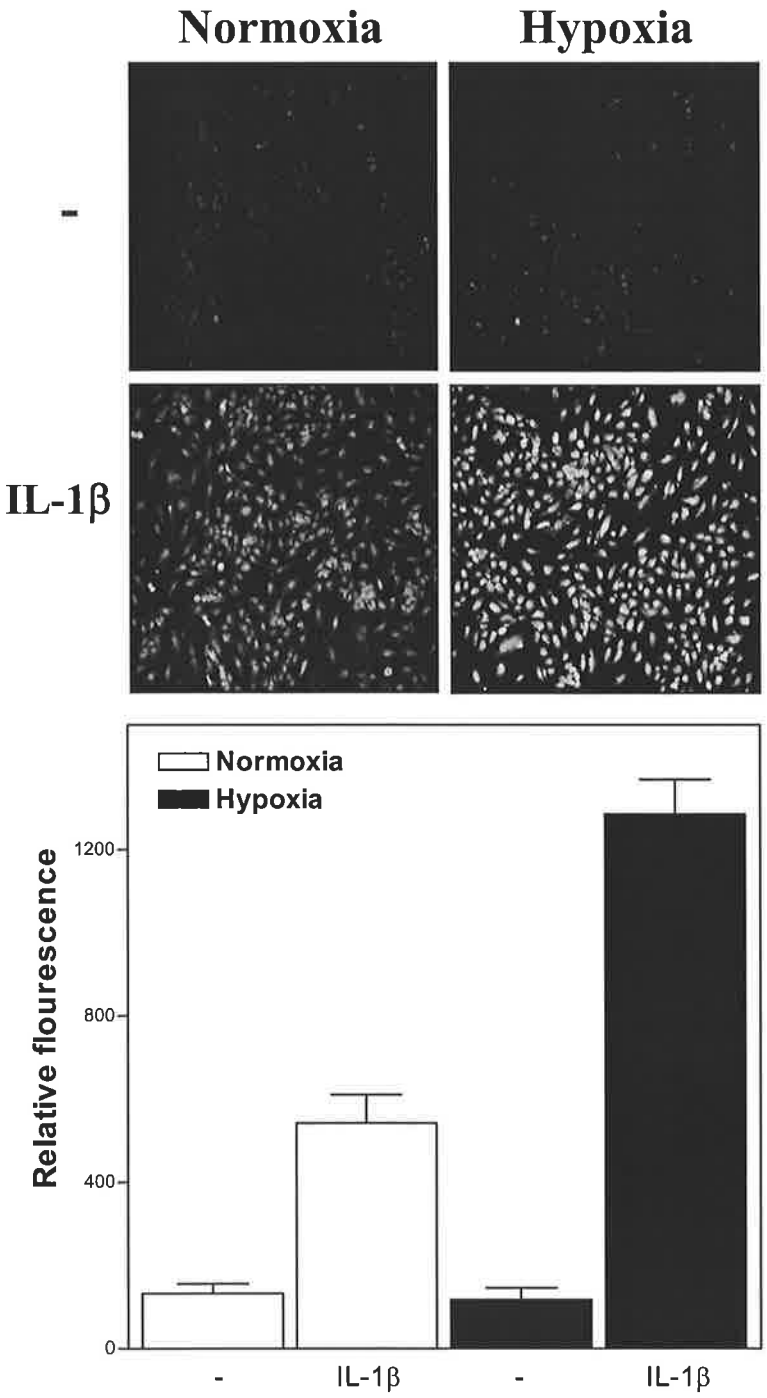


Figure 7.1c

Effect of hypoxia on COX-2 protein HUVEC by immunohistochemistry

HUVEC ($2x10^4$) in the presence of endothelial cell growth factor (50μg/ml) and heparin (50μg/ml) were seeded in a fibronectin coated 8-well chamber slide and stimulated with IL-1β (2ng/ml) in normoxia or hypoxia over 18h. Cells were treated with anti-human COX-2 PAb followed by anti-rabbit FITC labelled PAb as described in the methods. The extent of exposure of each photograph was quantified (20x magnification). The graph demonstrates the average of 5 separate slides (mean ± SEM).

7.3.2 Effect of hypoxia on transcription of COX-2 mRNA in HUVEC

HUVEC ($5x10^5$/ 6 well plate) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxia following transfection with the –531bp COX-2 promoter-luciferase reporter construct. Hypoxia resulted in a significant increase in the activity of the -531bp segment of the COX-2 promoter compared to normoxia (Fig 7.2)

7.3.3 Effect of hypoxia on COX-2 mRNA stability in HUVEC

HUVEC ($5x10^5$/ 6 well plate) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxia for 3h to allow synthesis of COX-2 mRNA. Actinomycin D (AD) was added to inhibit further transcription, then the level of COX-2 mRNA was measured by Northern analysis for a further 3h. In normoxia, there was a time dependent degradation of COX-2 mRNA levels over time 3h following AD addition (Fig 7.3). In hypoxia, the 'rate' of decay was similar to cells in normoxia (Fig 7.3).

7.3.4 Effect of hypoxia on phosphorylation of p38 mitogen activated protein kinase (MAPK) in HUVEC

The activation of the p38 MAPK pathway is essential for the induction of COX-2 in monocytes (Pouliot et al. 1997). Therefore, it was important to determine whether an increase in the phosphorylation of p38 MAP kinase contributed to the augmented expression of COX-2 in hypoxia in HUVEC.

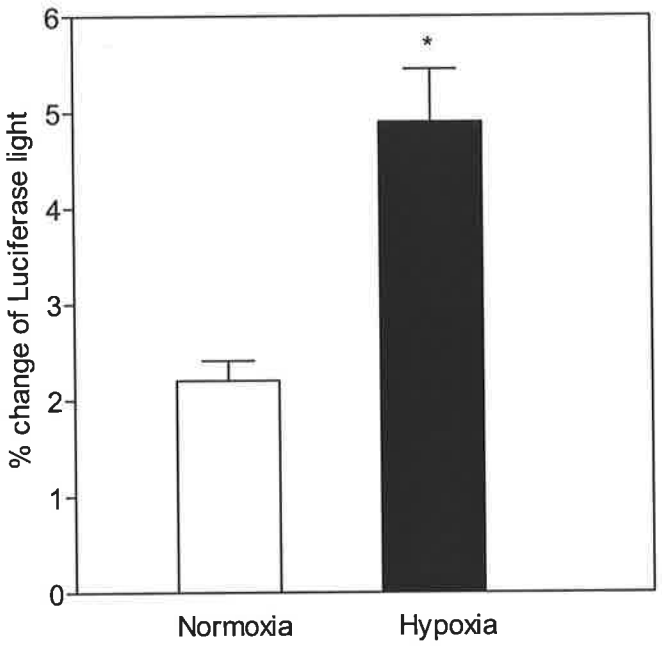


Figure 7.2

Effect of hypoxia on COX-2 transcription in HUVEC

HUVEC ($2x10^5$) were transfected with COX2 -531 construct using Amaxa HUVEC (Vs.2) Nucleofector™ kit as per manufacturers instructions. HUVEC were stimulated with IL-1β (2ng/ml) for 18h in normoxia (open bar) or hypoxia (solid bar). Results are expressed as the ratio of firefly/*Renilla* luciferase activity (mean ± SEM, n=3). *$p<0.05$, by comparison to normoxic HUVEC.

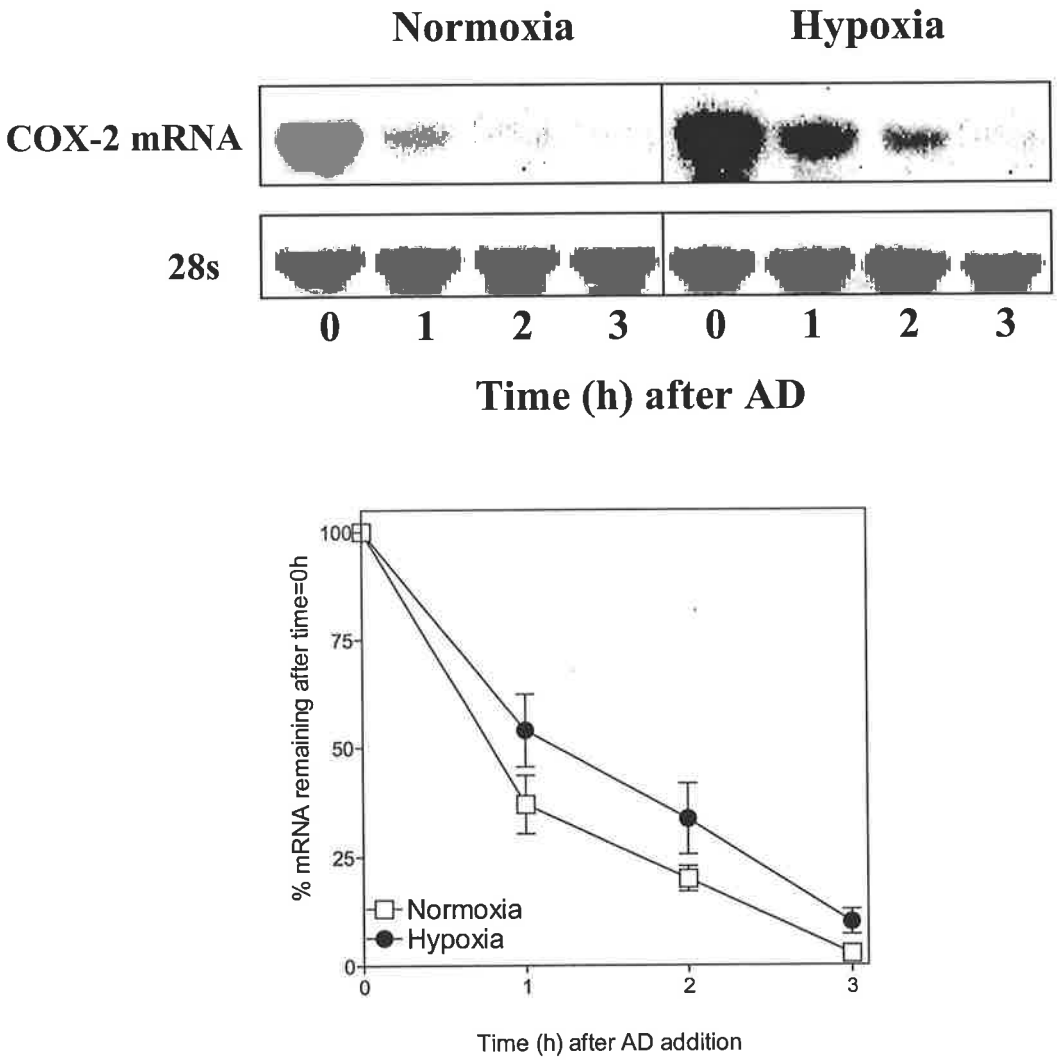


Figure 7.3

Effect of hypoxia on COX-2 mRNA stability in HUVEC

HUVEC ($5x10^5$) in the presence of endothelial cell growth factor (50μg/ml) and heparin (50μg/ml)were stimulated with IL-1β (2ng/ml) for 3h in normoxia or hypoxia. Actinomycin D (5μg/ml) was then added and the level of COX-2 mRNA was assessed for a further 3h by Northern analysis. In the graphic depiction of densitometry results, COX-2 mRNA was normalised against the 28S band and the rate of decay is shown with values at 1-3h expressed as a % of value at 0h (mean ± SEM; n=3); normoxia (□) and hypoxia (●).

HUVEC were stimulated with IL-1β for up to 60min. There was no change in the extent of phosphorylation of p38 MAPK or time course of decay in the amount of phosphorylated enzyme in hypoxia relative to normoxia (Fig 7.4).

7.3.5 Effect of hypoxia on COX-2 activity in HUVEC

To determine whether the hypoxia-induced increase in COX-2 expression led also to an increase in enzyme activity, the effect of hypoxia on synthesis of the major eicosanoids synthesised by HUVEC i.e. PGE_2, 6-Keto$PGF_{1\alpha}$ and TXB_2 was examined.

HUVEC ($5x10^5$/ 6well plate) were stimulated with IL-1β in normoxia or hypoxia over 18h. In normoxia, IL-1β stimulation was associated with accumulation of eicosanoid synthesis, which appeared to persist throughout the study period, at least for PGE_2 and 6-Keto$PGF_{1\alpha}$ (Fig 7.5). In hypoxia, there was a significant increase PGE_2 and 6-Keto-$PGF_{1\alpha}$, but not TXB_2 accumulation relative to normoxia (Fig 7.5).

After 18h, hypoxia had increased IL-1β stimulated PGE_2 and 6-Keto$PGF_{1\alpha}$ synthesis by 2 to 4-fold, that mirrored the increased expression of COX-2 protein (3-6h), described in figure 7.1. By comparison, the increased COX-2 expression was not associated with increased accumulation of TXB_2.

An increase in eicosanoid production is likely to be due to increased COX-2 protein. However, substrate availability can be limiting under hypoxia, as I have shown in monocytes (see chapter 4). Therefore, the effect of hypoxia on endogenous AA release was examined.

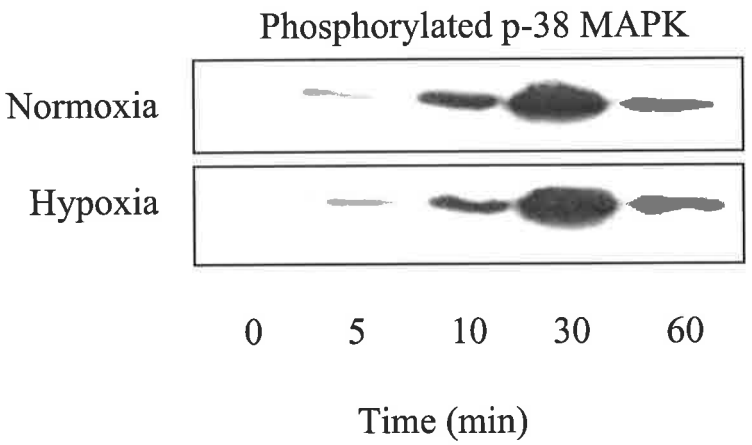


Figure 7.4
Effect of hypoxia on phosphorylation of p38 MAPK in HUVEC
HUVEC ($5x10^5$) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxia up to 60min. Protein was extracted for western analysis. Results are representative of 3 separate experiments.

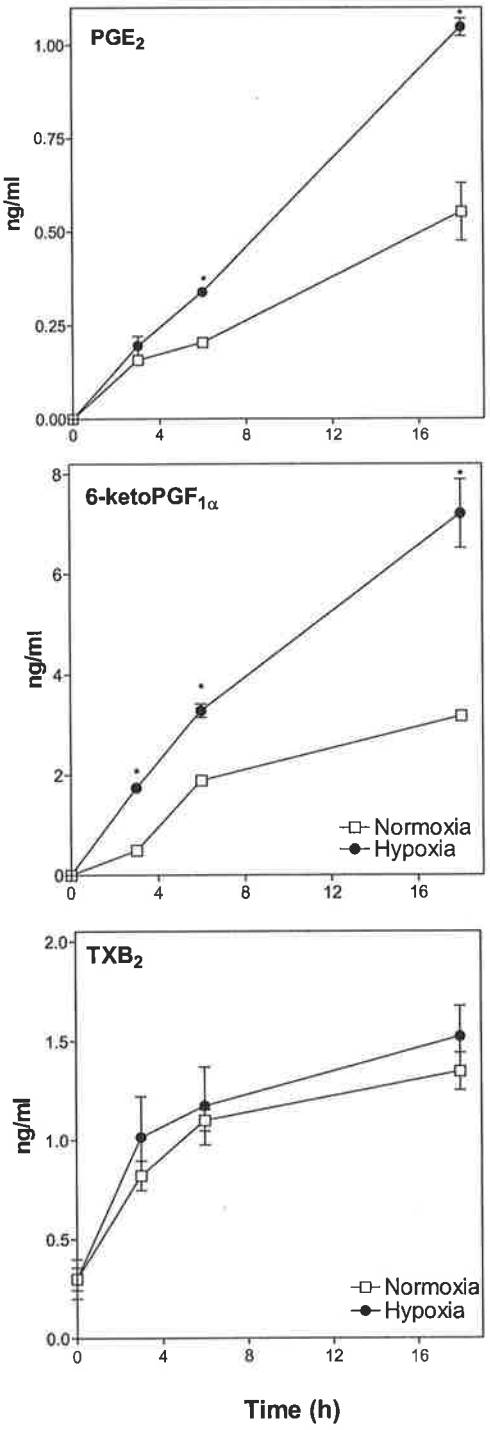


Figure 7.5

Effect of hypoxia on COX-2 activity in HUVEC

HUVEC ($5x10^5$) were stimulated with IL-1β (2ng/ml) in normoxia (□) or hypoxia (●) over 18h. Supernatants were collected and assayed for PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 by RIA (mean ± SEM; n=3). *$p<0.05$, by comparison to normoxic HUVEC.

7.3.6 Effect of hypoxia on endogenous arachidonic acid (AA) release in HUVEC

HUVEC ($5x10^5$/ 6 well plate) were incubated with [^{3}H]AA in normoxia (18h, 37°C) to incorporate labelled AA into cell membrane phospholipids. After 18h, cells were washed (3x) and incubated with IL-1β in normoxia or hypoxia for 30h. In addition, some cells were incubated in hypoxia for 9h, then returned to oxygenated conditions for a further 21h.

In normoxia, accumulation of labelled AA from IL-1β stimulated HUVEC occurred steadily throughout the incubation period (Fig 7.6). In hypoxia, accumulation of endogenous AA was greater throughout (Fig 7.6). When hypoxic cells were re-oxygenated after 9h, there was no change in the rate of AA release.

Cytosolic PLA_2 is the major PLA_2 responsible for stimulus induced release of AA from cell membranes. Therefore, the effect of hypoxia on the phosphorylation of $cPLA_2$ was examined.

7.3.7 Effect of hypoxia on cytosolic phospholipase A_2 ($cPLA_2$) phosphorylation in HUVEC

HUVEC ($5x10^5$/ 6 well plate) were stimulated with IL-1β up to 90min. In normoxia, maximal phosphorylation of $cPLA_2$ occurred at 30-60min and dephosphorylation occurred soon after (Fig 7.7). In hypoxia, the extent of phosphorylation was markedly increased and

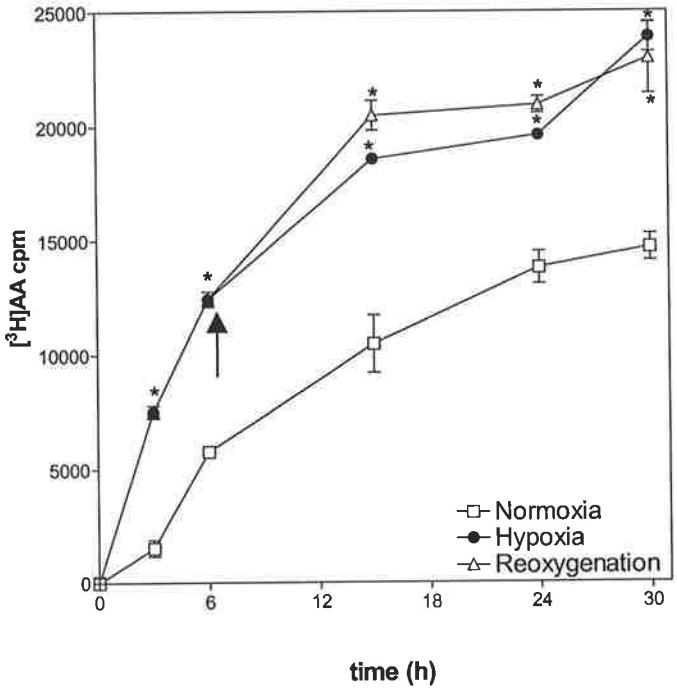


Figure 7.6

Effect of hypoxia on arachidonic acid release in HUVEC

HUVEC ($5x10^5$) were incubated with [^{3}H]AA (2μCi/ml) overnight. The following day the cells were washed 3x and stimulated with IL-1β (2ng/ml, 30h, 37°C) in normoxia (□) or hypoxia (●). In addition, some cells were hypoxic for 9h and reoxygenated (Δ) for a further 21h (↑). Supernatants were collected and [^{3}H]AA release was determined using a scintillation counter (mean ± SEM; n=3) *$p<0.05$, by comparison to normoxic HUVEC.

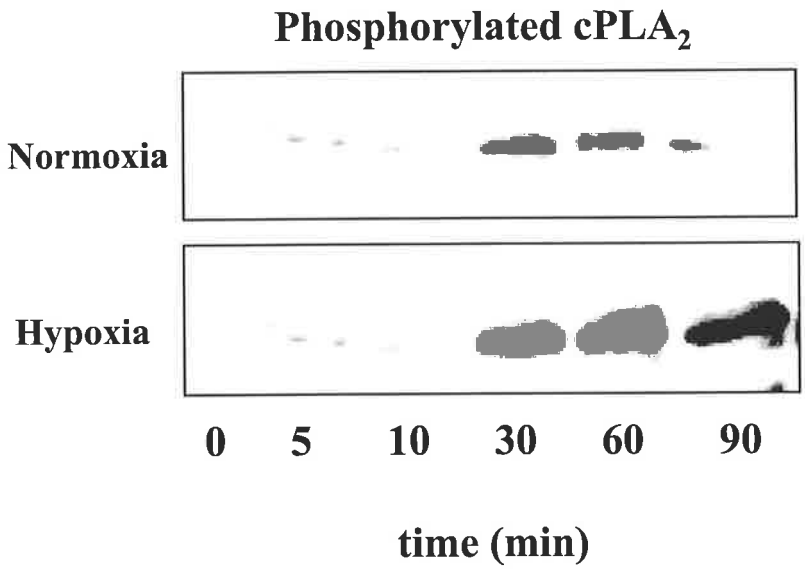


Figure 7.7
Effect of hypoxia on phosphorylated $cPLA_2$ in HUVEC
HUVEC ($5x10^5$) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxic conditions for up to 90min. At each time point, cells were processed for western blot analysis. Results are representative of 3 experiments.

prolonged (Fig 7.7). These results are consistent with an increase in AA release following IL-1β stimulation in hypoxia.

7.3.8 Effect of NS-398 on 6-KetoPGF$_{1\alpha}$ and TXB$_2$ synthesis in HUVEC

A disruption in the balance of COX-derived products, PGI_2 and TXA_2 has been associated with the development of atherosclerosis (Sinzinger et al. 1991). Whilst selective COX-2 inhibitors can relieve pain associated with inflammatory diseases, they can alter the balance of eicosanoids in inflammatory foci and in the vasculature, depending on whether the eicosanoid is COX-1 or COX-2 derived. Therefore, we investigated the effect of the selective COX-2 inhibitor, NS-398 on the ratio of 6-Keto-$PGF_{1\alpha}$ to TXB_2 in normoxia or hypoxia.

HUVEC ($5x10^5$) were pre-incubated for 15min with or without NS-398 (1μmol/L) prior to stimulation with IL-1β (2ng/ml) in normoxia or hypoxia for 18h. The results are expressed as % of eicosanoid remaining from normoxic HUVEC (at 100%) after 18h (Fig 7.8). Hypoxia augmented 6-KetoPGF$_{1\alpha}$ synthesis by ~2.5-fold, but did not alter TXB_2 synthesis (Fig 7.8). The selective COX-2 inhibitor, NS-398 resulted in complete inhibition of 6-KetoPGF$_{1\alpha}$ synthesis in both normoxia and hypoxia whereas TXB_2 was only modestly inhibited in normoxia and was not altered at all in hypoxia (Figure 7.8).

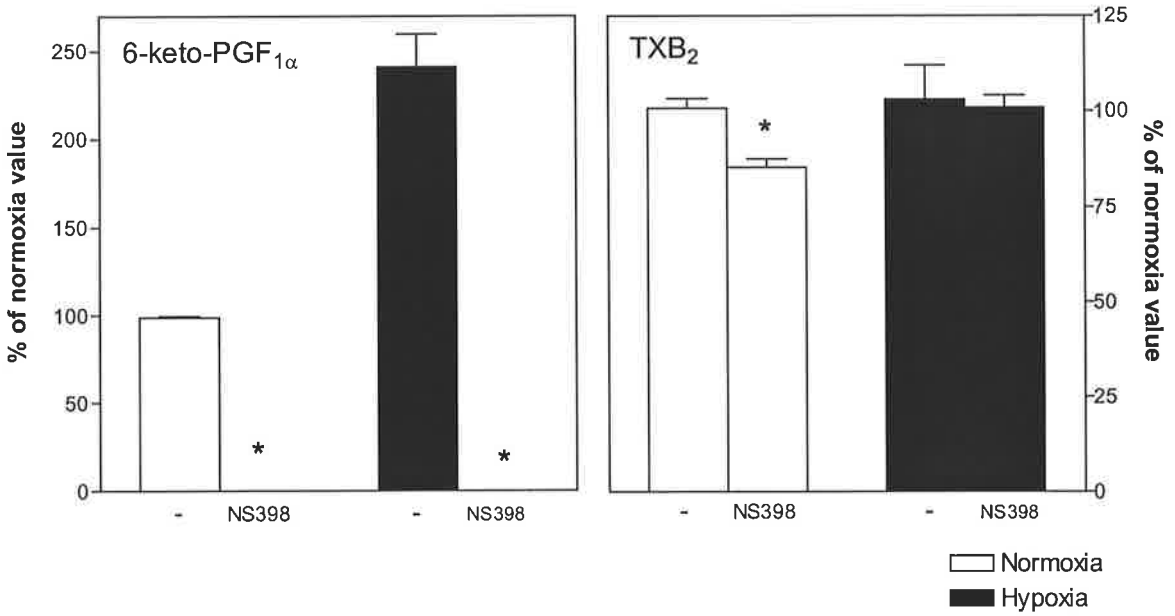


Figure 7.8

Effect of NS-398 on 6-keto-$PGF_{1\alpha}$ and TXB_2 in normoxia and hypoxia in HUVEC

HUVEC ($5x10^5$) were stimulated with IL-1β (2ng/ml) in normoxia or hypoxia in the absence or presence of NS-398 (1μmol/L). After 18h, supernatants were collected and 6-Keto$PGF_{1\alpha}$ and TXB_2 were measured by RIA (mean ± SEM; n=3). The graphs represent the % of eicosanoid remaining from normoxia. *$p<0.05$, statistically significant from cells with no NS-398

7.4 DISCUSSION

The location of endothelial cells and their ability to synthesise many vasoactive mediators, suggest that they play an important role in vascular homeostasis both in health and disease, such as atherosclerosis. The arterial wall depends on diffusion for its supply of oxygen and nutrients. However in atherosclerosis, diffusion across the thickened arterial wall becomes impaired (Morrison et al. 1972; Bjornheden and Bondjers 1987). This results in a disparity of energy metabolism that is believed to support the development of the plaque. Oxygen microelectrodes have been used in vitro and ex vivo to demonstrate decreased oxygen tensions in atherosclerotic plaques (Heughan et al. 1973; Bjornheden et al. 1999). Therefore it is important to consider oxygen tensions when conducting studies of vasoactive and platelet-active mediators.

Several studies have focused on the effects of hypoxia on COX-2 expression. In human endothelial cells, hypoxia increased COX-2 expression through increased binding of the transcription factor, NF-κB to one of two cognate binding sites in the COX-2 promoter region (Schmedtje et al. 1997). Other mechanisms of increased COX-2 expression in hypoxia were not explored. Cobalt-simulation of hypoxia resulted in the sustained upregulation of COX-2 expression, prostaglandin synthesis and VEGF expression in prostate cancer cells (Liu et al. 1999). Similarly, PPAR activation in hypoxia upregulated COX-2 expression in a rabbit corneal epithelial cell line (Bonazzi et al. 2000). This chapter has demonstrated a significant increase in COX-2 expression in HUVEC following hypoxic exposure using a –531bp region of the COX-2 promoter attached to a luciferase reporter gene, thereby showing that the mechanism for this upregulation was due in part, to

an increase in transcription rate. The implication is that the element responsible for induction of COX-2 under hypoxic conditions resides within the –531bp region of the COX-2 promoter. Possible hypoxic response elements (HRE) within this region warrant investigation.

Hypoxia did not alter COX-2 message stability or phosphorylation of p38 MAPK in HUVEC, suggesting that these two factors were not responsible for the augmented COX-2 expression observed in hypoxia.

The hypoxia-induced increase in HUVEC COX-2 expression was parallelled by a 2.5-fold increase in PGI_2 synthesis. The increased accumulation of PGI_2 (and PGE_2) in hypoxia was enabled by an increase in the release of AA substrate, attributable to increased activation of $cPLA_2$. However, there was no significant increase in TXA_2 synthesis in hypoxia. The COX product, PGH_2, is the common substrate for both TX and PGI synthase and the concentrations of PGH_2 will be increased in hypoxia by the increase in AA release and the upregulation of COX-2. The hypoxia induced augmentation of PGI_2 (and PGE_2), but not TXA_2 synthesis can be explained by the observation that the K_m for TX synthase is considerably less than PGI (and PGE) synthase (Penglis et al. 2000; Caughey et al. 2001b). Thus TX synthase is saturated at a lower concentration of PGH_2 than PGI synthase (Penglis et al. 2000). Therefore, increased PGH_2 above the concentration at which TX synthase is saturated could increase PGI_2 synthesis with little or no change in TXA_2 synthesis.

The results of this study suggest that the endothelial response to hypoxia is to increase the ratio of ambient PGI_2 relative to TXA_2 through increased endothelial COX-2 expression

and $cPLA_2$ activation. This will have the effect of promoting vascular patency and mitigating thrombotic events. Thus, selective COX-2 inhibition may have undesirable effects on this homeostatic vascular response to hypoxic stress since it will suppress the increase in PGI_2 synthesis with little or no effect on TXA_2 synthesis. This thesis is supported by studies that suggest a protective role for COX-2 in the vasculature and the myocardium in vivo. Ingestion by healthy volunteers of the selective COX-2 inhibitors, celecoxib and rofecoxib, decreased PGI_2 synthesis without inhibiting TXA_2 production by platelet COX-1 (Catella Lawson et al. 1999). In an animal model of vascular injury, ablation of prostacyclin receptors caused an exaggerated and pathological response to injury which was abrogated by antagonism of TXA_2 (Cheng et al. 2002). Also protection from oxidant damage, as well as protection by ischaemic pre-conditioning from ischemic damage, was abolished by selective COX-2 inhibitors in cardiomyocytes and in a rabbit model of myocardial ischemia (Adderley and Fitzgerald 1999; Shinmura et al. 2000; Bolli et al. 2002). In a 12 month double- blind safety study in rheumatoid arthritis, there was a 4-fold increase in myocardial infarction in the group allocated to rofecoxib, compared with the group receiving the comparator NSAID, naproxen (Bombardier et al. 2000). Furthermore, an accelerated accrual of serious thrombotic cardiovascular events since late in the study suggests a long-term effect of selective COX-2 inhibition on the vascular pathology. Also, an increase in coronary occlusive events was found in recipients of prescriptions for rofecoxib in a large cohort study (Ray et al. 2002). The present study suggests that the endothelial response to hypoxia is a protective one and is dependent on COX-2 upregulation and activity.

7.5 CONCLUSION

Overall, this study suggests that hypoxia is an important determinant of vasoactive and platelet-active mediator production. Furthermore, it is evident that selective COX-2 inhibition with NS-398 can alter the balance of eicosanoids. The clinical implications include the creation of a pro-thrombotic and pro-atherogenic environment in the vasculature.

Atherosclerotic plaques are characterised by smooth muscle cell proliferation and monocyte infiltration. These monocytes are capable of synthesising inflammatory cytokines that may influence lesion initiation, progression, or complication. The following chapter will determine whether interactions between monocytes and endothelial cells regulate changes in inflammatory genes and elucidate possible monocyte derived mediators involved in the regulation of HUVEC eicosanoid synthesis.

CHAPTER 8

ENDOTHELIAL-MONOCYTE INTERACTIONS; EFFECT OF HYPOXIA

8.1 INTRODUCTION

The interactions between monocytes and vascular wall cells have a pivotal role in atheromatous plaque formation. Therefore it is important to consider the mechanisms for leukocyte recruitment and activation in local regions of vascular inflammation.

During vascular inflammation, monocyte rolling, adherence and transmigration between endothelial cells (ECs) is mediated by a complex array of adhesion molecules (Beekhuizen et al. 1990; Takahashi et al. 1994; Takahashi et al. 1996a) and cytokines (Fan et al. 1993; Takahashi et al. 1996a). Thus, monocytes recruited in this manner interact with endothelial cells and because they are capable of synthesising soluble mediators that augment inflammation, such as prostanoids (Koll et al. 1997) and cytokines (Chuluyan et al. 1995; Takahashi et al. 1996b) and chemoattractants (Wempe et al. 1997), there is the potential for monocytes to influence endothelial functions.

It has been clearly demonstrated that in vascular diseases such as atherosclerosis, hypoxia occurs in conjunction with or as a result of the inflammatory process. Therefore, endothelial cells are exposed to soluble mediators from adjacent cells, including monocytes under hypoxic conditions. Hypoxia may affect fatty acid synthesis in aortic tissue cultures (Howard 1972; Filipovic and Rutemoller 1976) and cholesterol synthesis in monocyte-

derived macrophages (Matsumoto et al. 2000) as well as inducing increases in aortic lactate dehydrogenase synthesis (Lindy et al. 1974) and oxygen consumption (Bjornheden and Bondjers 1987). While it is not known to what extent these factors contribute to the development of atherosclerosis, the data clearly suggest that hypoxia can alter endothelial biochemistry. Despite these findings, there is no definitive evidence regarding the influence of monocyte-derived mediators on endothelial cell COX-2 expression and activity in hypoxia. Therefore, this chapter examines the effect of monocyte and HUVEC co-culture, as well as conditioned medium (CM) from each cell type, on COX-2 expression and activity in HUVEC and monocytes, respectively.

8.2 METHODS and MATERIALS

8.2.1 Materials

Materials were obtained from the following sources: COX-2 rabbit polyclonal Ab, (Cayman Chemicals, Ann Arbor, MI), VEGF rabbit polyclonal Ab (Peprotech), 6-KetoPGF$_{1\alpha}$ and PGE$_2$ rabbit (Sigma Chem), rabbit TXB$_2$ antiserum was prepared from rabbits immunised with TXA$_2$ conjugated to human thyroglobulin as used in previous studies (James and Walsh 1988), pyrogen free Lymphoprep, (Nycomed, Oslo, Norway), protein trans-blot transfer membranes (Bio-Rad, North Ryde, AUS), peroxidase labelled donkey anti-rabbit Ab, peroxidase labelled goat anti-mouse Ab, [^{3}H]PGE$_2$, [^{3}H]TXB$_2$, [^{3}H]6-KetoPGF$_{1\alpha}$ (Amersham International, Little Chalfont, ENG), Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL), IL-1β, TNFα (Endogen, Boston, MA), anti-IL-1β and anti-TNFα neutralising Abs (R&D Systems, Minneapolis, MN), tissue culture inserts (0.4μM) (Nalge Nunc International, Naperville, IL), 1B5 isotype control Ab (gift from GMayrhofer, Arthritis Lab, Hanson Institute, Adelaide, Aust)).

8.2.2 Methods

Cell culture and incubations

HUVEC were obtained from Assoc Prof Jenny Gamble (Hanson Institute, Adelaide, Aust).

Fresh human monocytes were isolated by counter current centrifugal elutriation as described in section 2.2.1. The purity of all preparations was assessed by FACS analysis and typically >85% of the cells were monocytes with the remainder lymphocytes.

Co-cultures were performed in duplicate. HUVEC ($2x10^5$/well) were cultured in 24-well plates with transwell inserts (10mm transwell cell culture inserts, 0.4μM) containing monocytes ($1.5\text{-}3x10^6$/transwell) (Fig 8.1). Also, HUVEC or monocytes were incubated alone under identical conditions. After 18h, both cell types were prepared for COX-2 analysis. Following incubation overnight at 37°C, tissue culture inserts were removed and the two cell populations were collected separately. The common supernatants were collected for measurement of eicosanoids and cytokines by RIA and ELISA, respectively.

For experiments in which conditioned medium (CM) from each of the cell types were used, CM was prepared as follows. Resting monocytes ($2x10^6$ / ml) or HUVEC ($5x10^5$ / 6 well plate) were incubated in normoxia or hypoxia for 18h after which the CM was collected. The monocyte or HUVEC CM was equilibrated in normoxic or hypoxic conditions and then added to HUVEC ($5x10^5$ / 6 well plate) or monocytes ($2x10^6$ / ml), respectively, in normoxia or hypoxia for 18h.

Western Analysis

Transfer membranes were stained with rabbit polyclonal COX-2 Ab at 1/1,000 dilution or rabbit polyclonal VEGF Ab at 1/200 dilution in TBS-Tween for 60min at RT. Following washing, the membrane was treated with donkey anti-rabbit horseradish peroxidase (HRP)

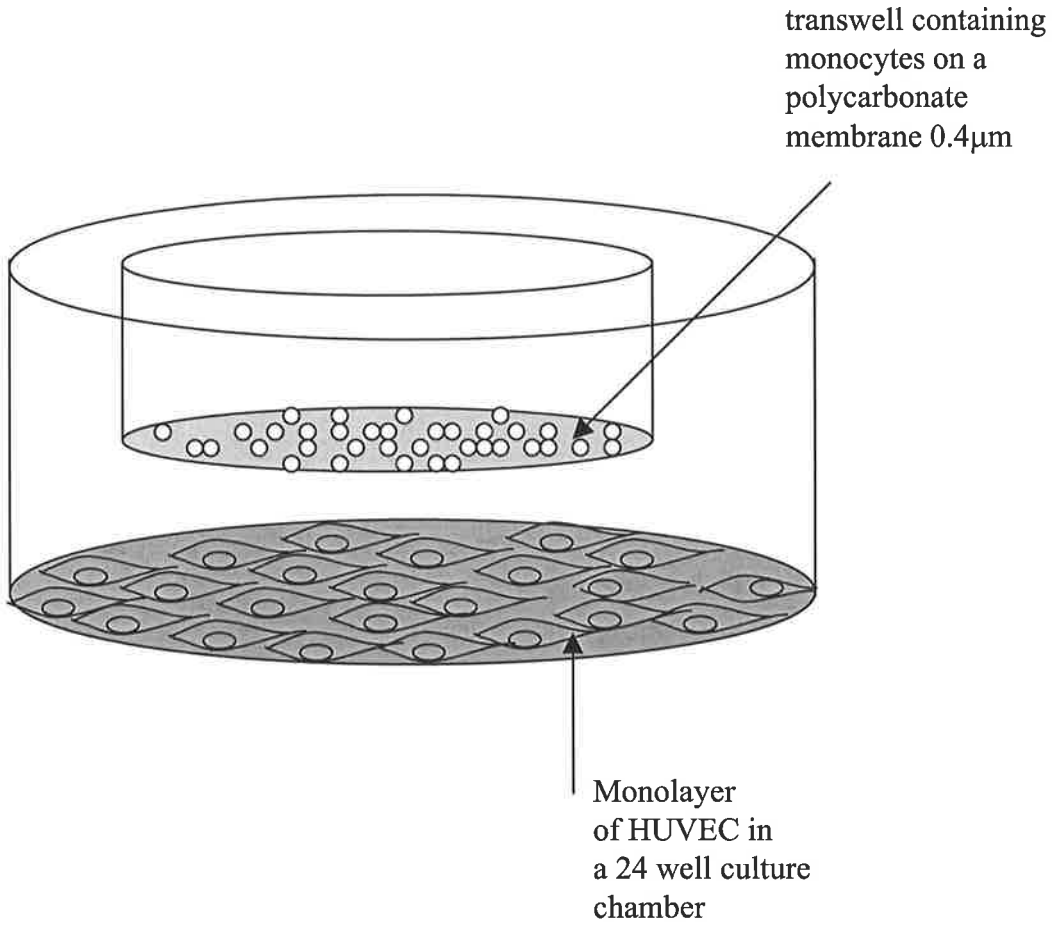


Figure 8.1

Co-culture of HUVEC and monocytes

A monolayer of HUVEC (2 x 10^5 cells) was incubated with a transwell insert containing monocytes (1.5-3x10^6/ml) in common culture medium. The porous membrane of insert (0.4μm) allowed the diffusion of soluble mediators but inhibited the passage of whole cells.

Ab at 1/10,000 dilution in TBS-Tween for 45min. The chemiluminescent substrate solution was added to the membrane (5min) prior to x-ray exposure for 2-5min.

8.3 RESULTS

8.3.1 Effect of COX-2 expression in HUVEC following co-culture in transwells with monocytes in hypoxia

HUVEC ($2x10^5$ / 24 well plate) were incubated in the absence or presence of monocytes (monocytes in transwells) in normoxia or hypoxia for 18h. In normoxia, HUVEC COX-2 protein was induced as the number of monocytes increased and this was augmented by hypoxia (Fig 8.2).

To determine whether soluble mediators derived from monocytes were capable of inducing changes in COX-2 expression and activity in HUVEC, monocyte conditioned medium (CM) was collected for experimental addition to HUVEC.

8.3.2 IL-1β and TNFα concentration in HUVEC and monocyte CM

Resting monocytes ($2x10^6$ / ml) or HUVEC ($5x10^5$ / 6 well plate) were incubated in normoxia or hypoxia for 18h after which the CM was collected and the concentration of IL-1β and TNFα in the monocyte and HUVEC CM were measured.

IL-1β and TNFα were detected in normoxic monocyte CM but not normoxic HUVEC CM (Table 8.1). Hypoxia increased the concentration of IL-1β and TNFα by approximately 2-

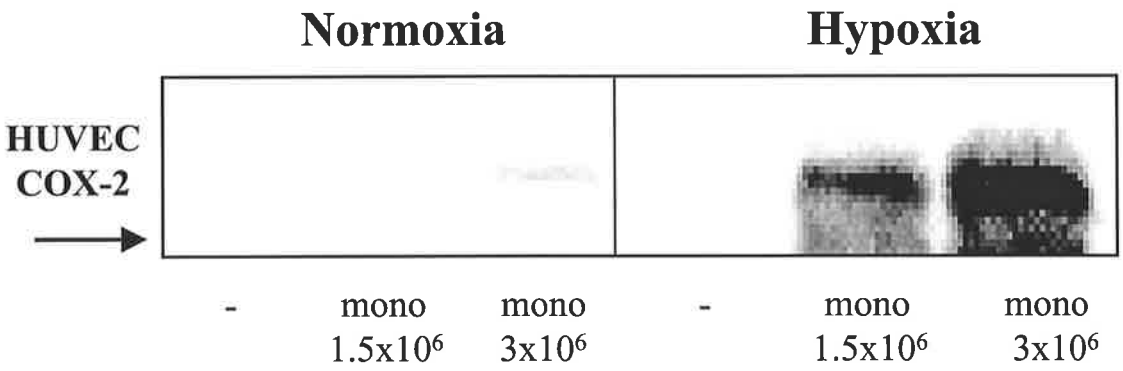


Figure 8.2
Effect on HUVEC COX-2 expression of co-culture with monocytes in normoxia and hypoxia
HUVEC ($5x10^5$) were co-incubated with or without monocytes (1.5 or 3 $x10^6$/transwell) in normoxia or hypoxia. After 18h HUVEC were processed for western blot analysis. Results are representative of 2 experiments, each of which was undertaken in duplicate.

fold and 3-fold, respectively in monocyte CM, but concentrations remained below the level of detection in hypoxic HUVEC CM (Table 8.1).

Therefore, it is possible that IL-1β and/or TNFα produced by monocytes were responsible for upregulation of HUVEC COX-2 expression in the co-cultures. In the previous chapter, COX-2 expression in HUVEC was characterised following stimulation with recombinant IL-1β only. Therefore, to assess whether TNFα was a monocyte-derived mediator involved in regulating HUVEC COX-2 expression, it was important to ensure that recombinant TNFα, at a concentration similar to those found in monocyte CM (~2ng/ml), was capable of inducing COX-2 expression in HUVEC in normoxia and hypoxia.

8.3.3 Effect of recombinant TNFα on COX-2 expression and activity in HUVEC in hypoxia

HUVEC ($2x10^5$ / 6 well plate) were incubated with recombinant TNFα (2ng/ml) in normoxia or hypoxia for 18h. TNFα induced COX-2 expression in normoxic conditions (Fig 8.3). Hypoxia greatly potentiated this response (Fig 8.3). PGE_2 and 6-KetoPGF $_{1\alpha}$ were increased but TXB_2 remained unaffected.

Having verified that IL-1β and TNFα were present in monocyte CM and that HUVEC COX-2 could be induced by either cytokine, the effects of monocyte CM on HUVEC COX-2 expression and activity were examined.

Table 8.1 - IL-1β and TNFα in conditioned medium (CM) from unstimulated human HUVEC and monocytes in normoxia or hypoxia

	HUVEC CM		Monocyte CM	
	Normoxia	Hypoxia	Normoxia	Hypoxia
	ng/ml		ng/ml	
IL-1β	nd*	nd	4.9 ± 0.1	†10.4 ± 1.6
TNFα	nd	nd	1.2 ± 0.09	†3.7 ± 0.7

* not detected

HUVEC or monocytes were incubated without deliberate stimulation under normoxic or hypoxic conditions. After 18h, CMs were assayed by ELISA for IL-1β and TNFα (ng/ml, mean ± SEM; n=3).

†$p<0.05$, significantly different from normoxic incubations

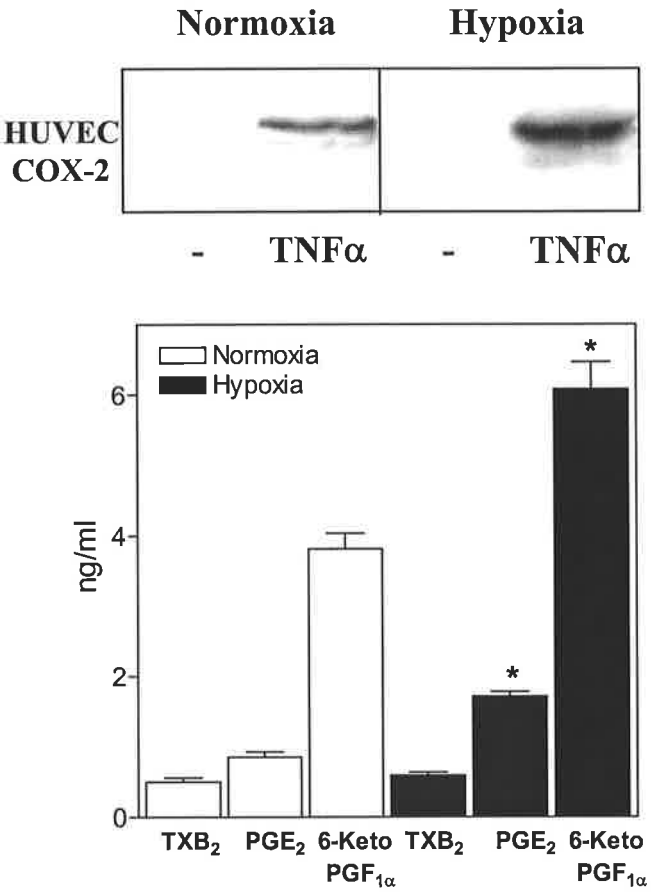


Figure 8.3

Effect of recombinant TNFα on COX-2 protein and activity by HUVEC in hypoxia

HUVEC ($5x10^5$) were stimulated with TNFα (2ng/ml) in normoxia or hypoxia over 18h. Cells were processed for western blot analysis. Supernatants were collected and TXB_2, PGE_2 and 6-Keto$PGF_{1\alpha}$ were measured by RIA (mean ± SEM; n=3). *$p<0.05$, by comparison to normoxic HUVEC.

8.3.4 Effect of monocyte conditioned medium on COX-2 expression and activity in HUVEC in hypoxia.

Monocyte CM was equilibrated in normoxic or hypoxic conditions, then added to HUVEC ($5x10^5$ / 6 well plate) in normoxia or hypoxia for 18h.

Normoxia- and hypoxia-derived monocyte CM induced COX-2 expression in HUVEC, with the hypoxia-derived CM being more potent (Figure 8.4). This pattern of interaction was similar when the HUVEC were hypoxic, although the HUVEC COX-2 response was greatly increased. Pre-incubation of monocyte CM with a cocktail of neutralising monoclonal antibodies against IL-1β and TNFα decreased the CM stimulation of HUVEC COX-2 expression in normoxia and hypoxia (Figure 8.4). 1B5 isotype control monoclonal antibody had no effect (data not shown).

The effect of monocyte CM on HUVEC 6-keto-$PGF_{1\alpha}$ and PGE_2 synthesis displayed a similar pattern to that of HUVEC COX-2 expression, including the effects of pre-incubation of monocyte CM with neutralising antibodies against IL-1β and TNFα. HUVEC TXB_2 synthesis was stimulated by monocyte CM but, in contrast to 6-Keto$PGF_{1\alpha}$ and PGE_2 synthesis, there was no additional effect of hypoxia and no inhibition by blockade of IL-1β and TNFα (Figure 8.4).

In chapter 7, it is documented that the selective COX-2 inhibitor, NS-398 inhibited 6-keto-$PGF_{1\alpha}$ synthesis, but not TXB_2 synthesis in IL-1β stimulated HUVEC. Therefore, I

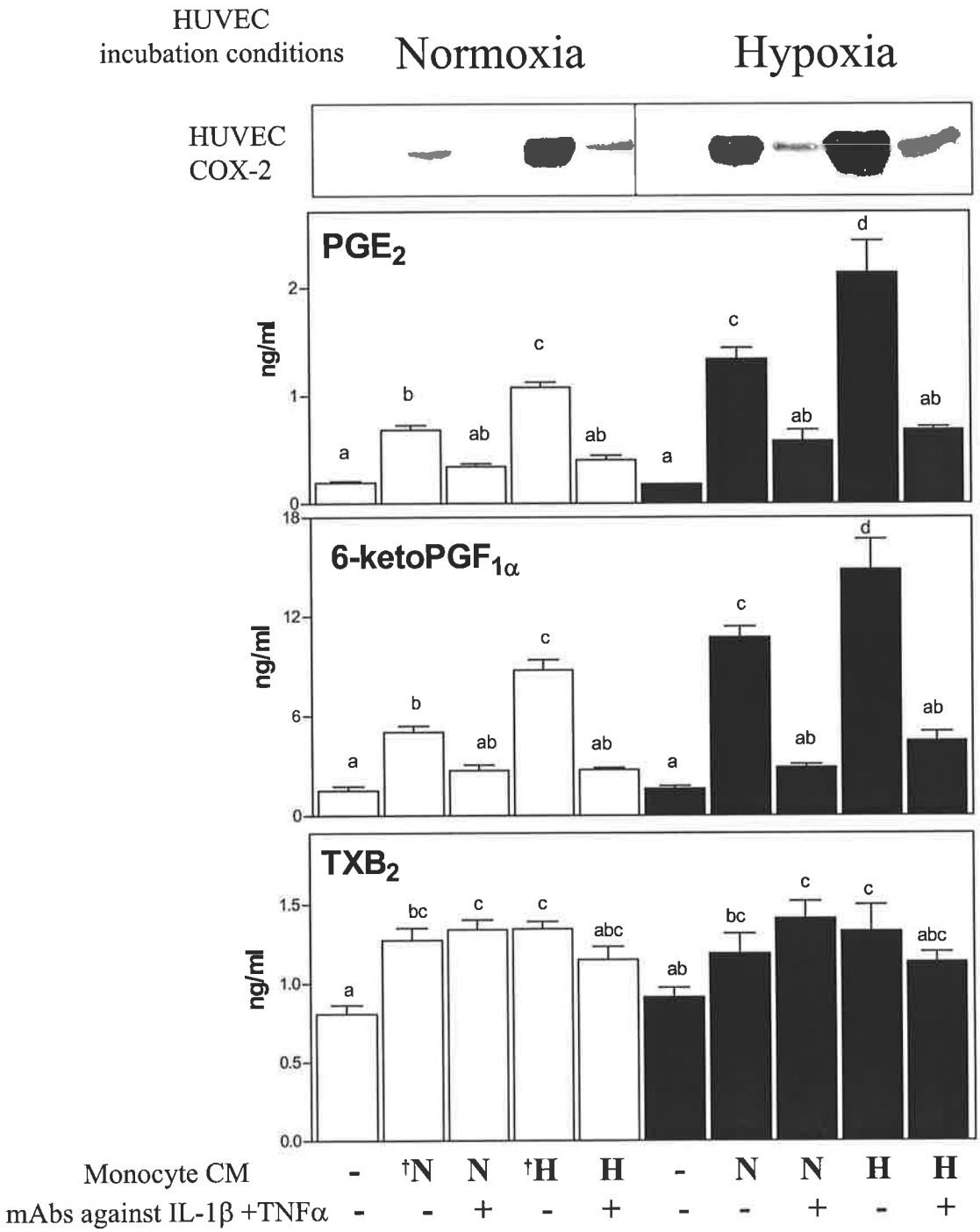


Figure 8.4

Effect of monocyte CM on HUVEC COX-2 expression and activity in hypoxia

HUVEC ($5x10^5$) were incubated with monocyte CM under normoxic or hypoxic conditions in the absence or presence of neutralising IL-1β (8μg/ml) + TNFα (8μg/ml) Ab. After 18h HUVEC were processed for western blot analysis and PGE_2, 6-KetoPGF$_{1\alpha}$ and TXB_2 were measured by RIA (mean ± SEM;n=3). Bars with different letters are significantly different from each other ($p<0.05$). †N denotes monocyte CM derived under normoxic conditions, H denotes monocyte CM derived under hypoxic conditions.

examined whether NS-398 had a similar effect of altering the synthesis of 6-KetoPGF$_{1\alpha}$ and TXB_2 following incubation with monocyte CM in normoxia and hypoxia.

8.3.5 Effect of COX-2 inhibition on HUVEC prostacyclin and thromboxane synthesis in response to monocyte CM.

HUVEC ($5x10^5$) were incubated with normoxic or hypoxic monocyte CM in the absence or presence of the selective COX-2 inhibitor, NS-398, (1μM) in normoxia or hypoxia for 18h.

The control conditions were considered to be normoxic monocyte CM added to normoxic HUVEC after 18h. The results are expressed as % of eicosanoid remaining from this control (Fig 8.5). PGI_2 synthesis was completely abrogated by NS-398 in normoxic and hypoxic conditions (Fig 8.5). Conversely, TXB_2 synthesis did not change significantly following treatment with NS-398 (Fig 8.5). These results with monocyte CM are similar to those with recombinant IL-1β stimulated HUVEC observed in the previous chapter, Figure 7.8.

8.3.6 Effect of hypoxia on COX-2 expression in monocytes following co-culture with HUVEC

Having observed that monocyte co-culture and CM could alter HUVEC COX-2, the reverse investigations into the effect of HUVEC co-culture and CM on monocyte COX-2 were performed.

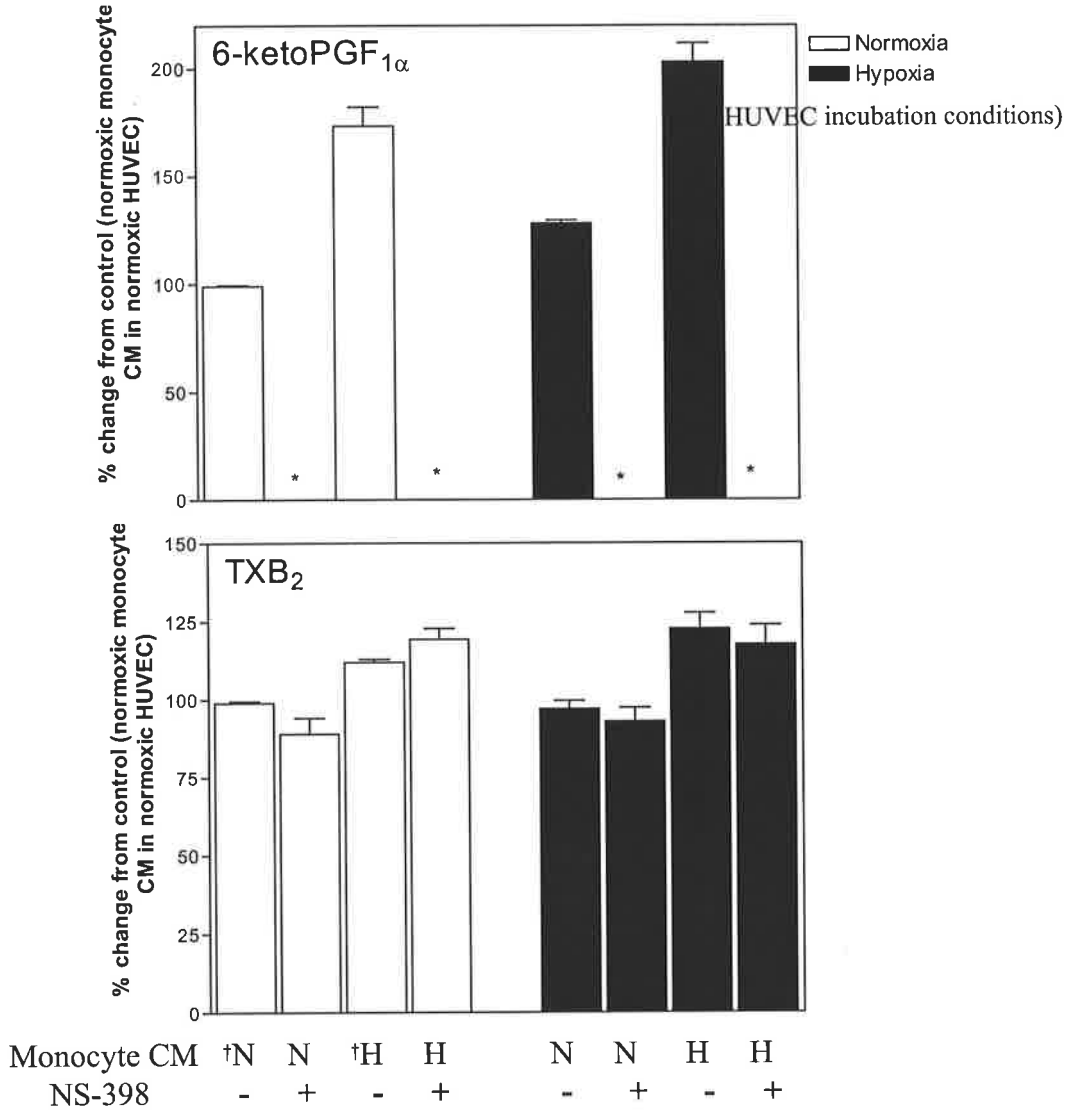


Figure 8.5

Effect of COX-2 inhibition on HUVEC prostacyclin and thromboxane synthesis in response to monocyte CM

HUVEC ($5x10^5$) were incubated with monocyte supernatants in the absence or presence of the selective COX-2 inhibitor, NS-398 (1μM) in normoxic or hypoxic conditions. After 18h, supernatants were collected and analysed for 6-ketoPGF$_{1\alpha}$ and TXB_2 by RIA (mean ± SEM;n=3). †N denotes HUVEC CM derived under normoxic conditions, H denotes monocyte CM derived under hypoxic conditions. *$p<0.05$, by comparison to equivalent incubation condition with no NS-398.

Fresh human monocytes were incubated with HUVEC ($2x10^5$ / 24 well plate) in normoxia or hypoxia for 18h. In normoxia, co-culture with HUVEC failed to stimulate COX-2 expression in monocytes (Fig 8.6). However, in hypoxia, monocyte COX-2 was minimally upregulated and significantly augmented by co-culture with HUVEC (Fig 8.6).

To determine whether soluble mediators derived from HUVEC were capable of inducing changes in COX-2 expression and activity in monocytes, HUVEC CM was collected for addition to monocytes.

8.3.7 Effect of HUVEC CM on COX-2 expression in monocytes

HUVEC CM was equilibrated in normoxic or hypoxic conditions, then added to monocytes ($5x10^6$) in normoxia or hypoxia for 18h. In normoxia, normoxic HUVEC CM failed to induce detectable COX-2 expression in monocytes. By comparison, hypoxic HUVEC CM resulted in a significant induction of COX-2 expression and activity in normoxic monocytes (Fig 8.7). When monocytes were hypoxic, COX-2 expression with hypoxic HUVEC CM was greatly increased, although PGE_2 and TXB_2 synthesis were significantly decreased (Fig 8.7).

TNFα and IL-1β were not measurable in normoxic or hypoxic HUVEC CM (Table 8.1). Given that hypoxia is reported to upregulate VEGF synthesis by HUVEC (Namiki et al. 1995), and monocytes possess the VEGF-1 receptor, flt-1 (Barleon et al. 1996; Clauss et al. 1996), I examined whether VEGF was detectable in HUVEC CM.

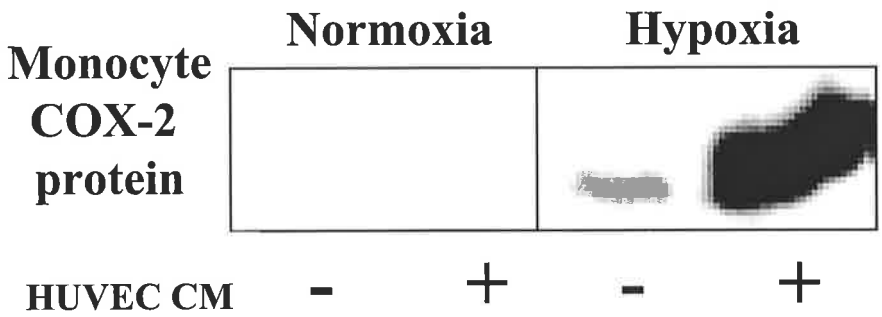


Figure 8.6
Effect of hypoxia on co-incubation of HUVEC and monocytes
HUVEC ($5x10^5$) were co-incubated with monocytes ($3 x10^6$/transwell) in normoxia or hypoxia. After 18h monocytes were processed for western blot analysis as described in the Methods section. Results are representative of 2 experiments in duplicate.

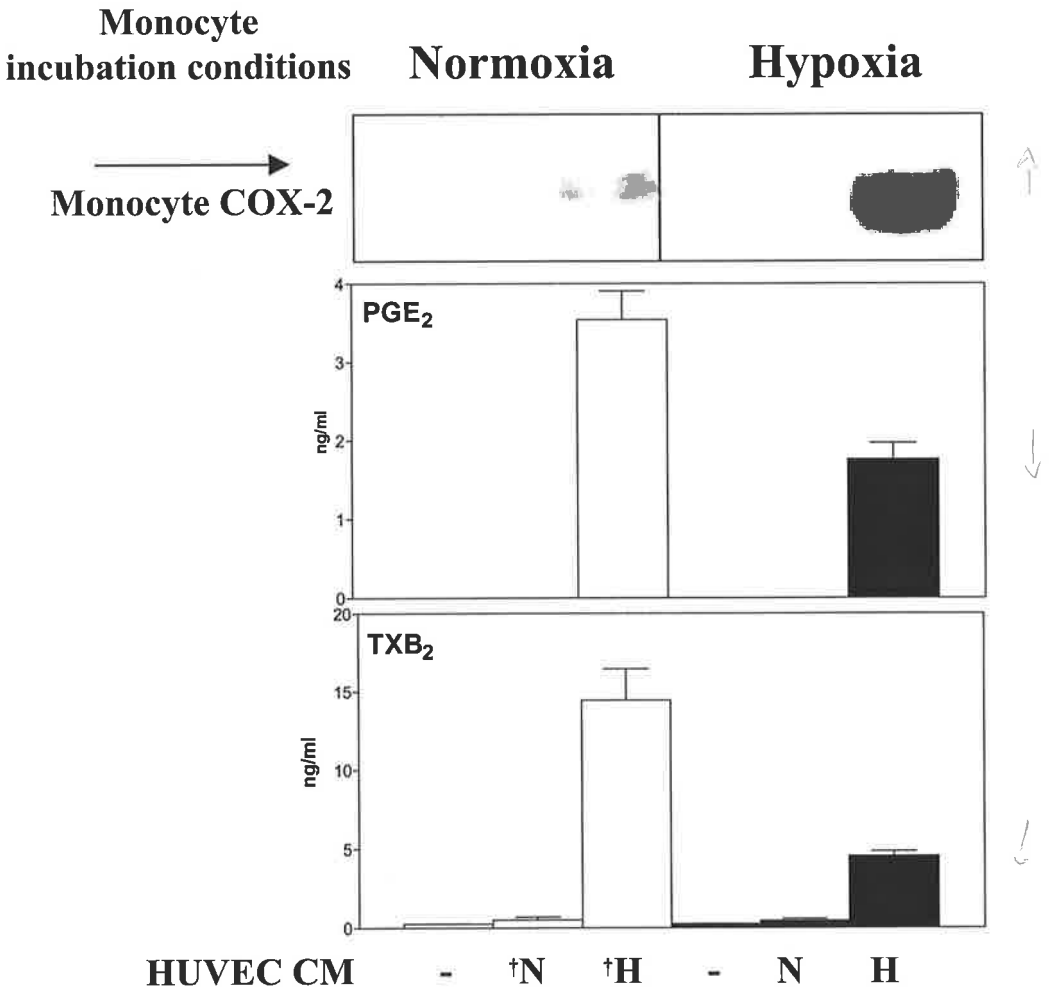


Figure 8.7
Effect of HUVEC CM on monocyte COX-2 expression and activity in hypoxia
Monocytes were incubated with normoxic or hypoxic HUVEC CM in normoxia or hypoxia for 18h. Monocytes were processed for western blot analysis and PGE_2 and TXB_2 measured by RIA (mean ± SEM; n=3). †N denotes HUVEC CM derived under normoxic conditions, H denotes monocyte CM derived under hypoxic conditions.

8.3.8 The synthesis of vascular endothelial cell growth factor (VEGF) in HUVEC and monocyte CM in hypoxia

HUVEC ($5x10^5$ / 6 well plate) or fresh human monocytes ($2x10^6$/ml) were incubated in normoxia or hypoxia for 18h without deliberate stimulation. CMs were collected and processed for western blot to determine the presence of VEGF.

In normoxia, VEGF was not detectable in any of the CMs (Fig 8.8). However following hypoxic exposure, there was a significant increase in VEGF protein in HUVEC CM (Fig 8.8).

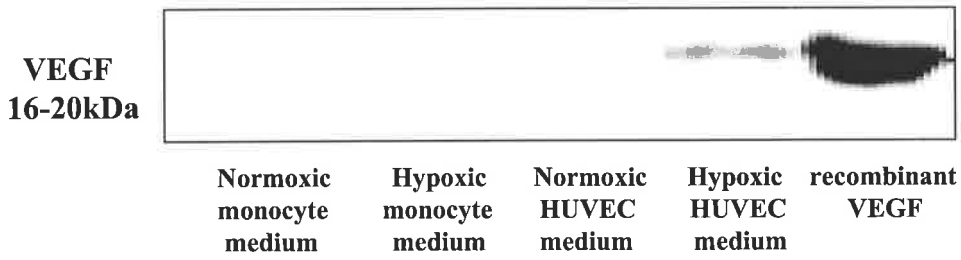


Figure 8.8

Effect of hypoxia on VEGF synthesis

HUVEC ($5x10^5$) or monocytes ($5x10^6$) were incubated in the absence of a stimulus in normoxic or hypoxic conditions. VEGF in the supernatants were detected by Western immunoblot. Results are representative of 2 experiments in duplicate.

8.4 DISCUSSION

Atherosclerotic lesions are characterised by the accumulation of monocyte derived foam cells, vascular endothelial cells and smooth muscle cells (Ross 1993). The presence of macrophages in atherosclerotic lesions is believed to originate from circulating monocytes (Ross 1993). Chemoattractants may regulate the margination and extravasation of monocytes. For example, the chemokine, monocyte chemoattractant protein-1 (MCP-1) can act as a regulator of monocyte trafficking and recruitment (Chuluyan et al. 1995; Wempe et al. 1997). In addition, the soluble mediator VEGF, produced by ECs, has potential to attract peripheral blood monocytes which have been shown to express the mRNA for VEGF receptor, flt-1 (Barleon et al. 1996; Clauss et al. 1996; Sawano et al. 2001). Other soluble mediators in the vasculature that are induced by interactions with monocytes and endothelium include platelet derived growth factor (PDGF) which is a major mitogen and chemoattractant for VSMCs (Funayama et al. 1998), MMP-1 (Hojo et al. 2000), MMP-9 (Amorino and Hoover 1998), GM-CSF and tissue factor (TF) (Wharram et al. 1991; Collins et al. 1995; Lewis et al. 1995; Lo et al. 1995; Herbert et al. 1996; Napoleone et al. 1997). Hypoxia also potentiates TF production in monocyte-endothelial cell interactions (Herbert et al. 1996).

Given that monocytes from circulating blood are recruited to lesions where the oxygen tension is low, the effect of hypoxia on macrophage-endothelial cell interactions is important. However, despite much research involving endothelial:monocyte interactions including some on the effects of hypoxia, there is a paucity of information on the effect of co-culturing monocytes and endothelial cells on COX-2 expression.

In this study, I have demonstrated that separated co-cultures of freshly isolated monocytes and HUVEC (separated using an inner well system), resulted in the induction of COX-2 expression in HUVEC in normoxia. Hypoxia augmented the effects of co-culture on COX-2 expression in both cell types. Furthermore, soluble mediators derived from monocyte CM upregulated HUVEC COX-2 expression and activity and this was potentiated by hypoxia. By using monoclonal antibodies to neutralise their activity, TNFα and/or IL-1β were identified as mediators of this induction.

HUVEC were more responsive to recombinant IL-1β or TNFα or monocyte CM under hypoxic conditions, representative of the oxygen tensions described in atherosclerotic plaques. The mechanism (s) for this are unknown and an investigation to determine whether cytokine receptors on endothelial cell membranes are upregulated in hypoxia, is warranted.

Furthermore, the addition of the selective COX-2 inhibitor, NS-398, to HUVEC following incubation with IL-1β or monocyte CM suppressed PGI_2 production and had little effect on TXA_2 production. This can be explained by the previous observations that TX synthase has a much lower K_m than PGI (or PGE) synthase (Penglis et al. 2000; Caughey et al. 2001b). Thus, when total COX activity produces high concentrations of PGH_2 (the common substrate for TX and PGI synthases), COX inhibitors can result in inhibition of PGI_2 synthesis with little affect of TXA_2 synthesis, when TX synthase remains saturated with PGH_2. Thus, PGI_2 (and PGE_2) appeared to be COX-2 products, because the selective inhibition of COX-2 left sufficient PGH_2 to saturate TX synthase. Presumably, the residual PGH_2 is COX-1 derived.

Co-incubation of HL-60 monocytic cells and iliacal endothelial cells (EC) led to increased COX-2 expression and PGI_2 synthesis in ECs but not the monocytic cells under normal oxygen tensions (Koll et al. 1997). The effects of hypoxia and selective COX-2 inhibitors were not explored. We have shown that unidentified soluble mediator(s) derived from hypoxic HUVEC CM can induce monocyte COX-2 expression. This response was potentiated when monocytes were under hypoxic conditions. Interestingly, monocytes displayed a decreased synthesis of PGE_2 and TXA_2 synthesis in hypoxia, thereby corroborating findings documented in Chapter 4 (and (Demasi et al. 2003)). In this earlier chapter, a disparity in COX-2 expression and activity in hypoxic monocytes is described and attributed to enhanced dephosphorylation of $cPLA_2$ and consequent reduced cleavage of AA from monocyte membrane phospholipids.

IL-1β and TNFα levels were not detected in normoxic or hypoxic HUVEC CM. Thus, HUVEC soluble mediator (s) that upregulated monocyte COX-2, remain unidentified. VEGF was detected in hypoxic HUVEC CM (Fig 8.8) and could be involved in the upregulation of monocyte COX-2 expression. Demonstrations of the flt-1 receptor on monocytes (Barleon et al. 1996; Clauss et al. 1996), supports the hypothesis that monocytes are responsive to VEGF stimulation. An attempt was made to examine the effect of recombinant VEGF on monocyte COX-2 expression. However, control incubations with polymyxin B, which complexes bacterial LPS, indicated that the recombinant VEGF was contaminated with LPS. Interestingly, monocytes were more responsive to hypoxic HUVEC CM in hypoxia by comparison to normoxia. Again, whether the flt-1 receptor on monocytes is upregulated in hypoxia warrants investigation.

8.5 CONCLUSION

Interactions between endothelial cells and monocytes occur in vivo normally and within pathological situations. Hypoxia can result from vascular luminal narrowing or occlusion and appears likely to aggravate inflammatory events within vessel walls or in extravascular tissues. Under hypoxic conditions, TNFα and IL-1β synthesis is induced in monocytes and these cytokines in turn stimulate COX-2 expression and eicosanoid synthesis in HUVEC. As observed in this chapter with monocyte CM and in the previous chapter with IL-1β treatment, the eicosanoid response of HUVEC to hypoxia, is to produce a vasodilatory, anti-thrombotic and perhaps anti-atherogenic mix of eicosanoids. This defensive homeostatic response is dependent on COX-2 induction and activity. Thus, the results confirm observations in Chapter 7 that selective COX-2 inhibitors have the potential to alter the balance of eicosanoids synthesised by endothelial cells unfavourably. Furthermore, soluble mediators from endothelial cells (possibly VEGF) are able to elicit changes in COX-2 expression and activity in monocytes. However, their identity remains to be defined. Although the present in vitro observations were performed on HUVEC, and not arterial endothelial cells, the results suggest an important role for monocyte-endothelial interactions in the pathogenesis of human atherosclerosis.

CHAPTER 9

CONCLUSIONS AND FUTURE DIRECTIONS

9.1 INTRODUCTION

There is evidence that hypoxia exists in a variety of inflamed lesions including rheumatoid joints (Falchuk et al. 1970; Lund-Olesen 1970; Treuhaft and McCarty 1971) and atherosclerotic plaques (Heughan et al. 1973; Bjornheden et al. 1999). To date, in vitro studies of inflammatory mediator production in cells have been well characterised in normoxic conditions (20% O_2). However this is unlikely to reflect conditions of oxygenation that prevail in inflamed lesions. This provides a strong rationale for determining the effects of reduced oxygen tensions on inflammatory mediator production. More specifically, the aim of this thesis was to examine the effect of hypoxia on the regulation of COX-2 expression and activity in monocytes/macrophages, endothelial cells and synoviocytes (prototypic mesothelial cells), which are cell types relevant to inflammatory joint disease and to other hypoxic inflammatory lesions.

This chapter presents an overview of the findings in this thesis and discusses the possibility of future studies to expand on these novel findings.

9.2 SALIENT FINDINGS & FUTURE DIRECTIONS

Monocytes, FLS and HUVEC appear to have similarities and differences in their responses to hypoxia. A summary of the salient findings of cellular responses to hypoxia is displayed in Table 9.1.

9.2.1 COX-2 expression in hypoxia

One of the consistent findings for all cell types examined is that hypoxia significantly augmented COX-2 mRNA levels and protein expression. The results indicated that hypoxia induced alterations in COX-2 gene expression, which occurred at both transcriptional and translational levels.

9.2.1.1 Transcriptional control

The rate of COX-2 transcription was examined as one possible mechanism for the upregulation of COX-2 expression in hypoxia. Transfecting U937 monocytic cells, HUVEC and FLS with a COX-2 promoter-luciferase reporter construct showed that hypoxia increased COX-2 transcription in all three cell types when compared to normoxia. The observation that hypoxia augmented COX-2 induction in HUVEC corroborated an earlier report (Schmedtje et al. 1997). This earlier report stated that the upregulation of COX-2 expression in hypoxia was due to NF-κB and did not involve HIF binding. In fact, it was stated that the COX-2 promoter region did not have an hypoxic response element (HRE), the binding site for the HIF heterodimer. This assertion was recently repeated by Liu et al, (Liu et al. 2002). However, a putative HRE site with a sequence of 5'-

Table 9.1 – Summary of similarities and differences between cell types in their responses to hypoxia.

	Monocytes	Synoviocytes	HUVEC
COX-2 mRNA	↑	↑	↑
COX-2 protein	↑	↑	↑
Transcription rate	↑	↑	↑
mRNA stability	↑	no change	no change
Eicosanoid synthesis	PGE_2 ↓ TXA_2 ↓	PGI_2 ↑ PGE_2 ↑	PGI_2 ↑ PGE_2 ↑ TXA_2 no change
Arachidonic acid release and $cPLA_2$ activation	↓ phospho-$cPLA_2$ ↓	no change	↑ phospho-$cPLA_2$ ↑
p38 MAPK phosphorylation	no change	no change	no change
Other findings	TNFα ↑	MMP-1 ↑	Eicosanoids favour vascular patency, suppressed by COX-2 inhibition

ACGTGC-3' at the position –555 to –551 bp on the COX-2 gene (Accession # L15326) has recently been identified in our laboratory. Future studies to determine whether the putative HRE site in the promoter of the COX-2 gene is functionally responsive to hypoxia, are warranted. The COX-2 promoter/luciferase reporter constructs used in this thesis could be mutated in the putative HRE sites to determine if there is loss of responsiveness to hypoxia. Verification of the presence of HIF proteins upregulated in response to hypoxia will also be important. In addition, the introduction of stable HIF expression vectors could be introduced into cells to examine further, the role of HIF on the inducibility of native COX-2. Studies on HIF induction of COX-2 may be especially relevant because experiments using HIF-1α knockout mice have shown that HIF-1α is necessary for inflammatory responses (Cramer et al. 2003a; Cramer et al. 2003b).

The COX-2 reporter-constructs used in this thesis were –531bp or –922 bp in size which were the constructs that gave the most definitive results. Comprehensive studies of other construct sizes (constructed by Rebecca Cook-Johnson in our laboratory) in various cell types were performed by Rebecca, but were not a part of these studies.

9.2.1.2 Translational control

I also found that COX-2 expression in hypoxia could be regulated by stability of the messenger RNA, although this occurred only in monocytes. Many RNAs coding for immediate-early response genes such as COX-2, are unstable and have short half-lives (Kruys et al. 1989; Cok and Morrison 2001). This is related to the presence of repeated AUUUA motifs (or AU-Rich Elements; AREs) in the 3'-untranslated region (3'-UTR) of the gene (Akashi et al. 1994). The 3'-UTR of the COX-2 gene contains 22 ARE sites.

HuR, is a nucleo-cytoplasmic shuttling protein that regulates mRNA stability by binding to AREs and increasing the half-life of the mRNA (Ma et al. 1996; Fan and Steitz 1998a; Fan and Steitz 1998b; Nabors et al. 2001). In renal mesangial cells, COX-2 mRNA stability has been shown to be regulated by HuR in response to growth factors in normoxia (Cok et al. 2003).

Hypoxia stabilised COX-2 mRNA in fresh human monocytes, but did not alter mRNA stability in HUVEC and FLS. This could be due to differences in the expression of HuR if HuR is involved in the hypoxic regulation of COX-2 mRNA. VEGF is an example of a protein regulated in this manner. Hypoxia not only upregulates VEGF gene transcription, but also increases the half-life of VEGF mRNA through the high affinity binding of HuR to AREs in the VEGF 3'-UTR (Shima et al. 1995; Stein et al. 1995; Levy et al. 1996; Claffey et al. 1998; Dibbens et al. 1999). The involvement of HuR in the 3'-UTR of COX-2 merits investigation as a possible mediator of the hypoxia-induced stabilisation of COX-2 mRNA in monocytes. Since this did not occur in synoviocytes or HUVEC, a comparison between cell types of the effects of hypoxia on a luciferase reporter / COX-2 3'-UTR construct of the type by Cok et al (Cok et al. 2003) would be of interest.

9.2.2 COX-2 activity in hypoxia

In HUVEC and FLS under hypoxic conditions, augmented COX-2 expression was associated with an increase in the eicosanoid products PGE_2 and PGI_2. Hypoxic HUVEC showed increased phosphorylation (and activation) of $cPLA_2$ and consequent increased cleavage from membrane phospholipids of AA. This increased AA release provided ample substrate for increased eicosanoid synthesis by induced COX-2 enzyme in hypoxic

HUVEC. However, increased eicosanoid synthesis occurred in hypoxic FLS in which COX-2 was induced without increased AA release from membrane phospholipids as seen in hypoxic HUVEC.

In HUVEC exposed to hypoxia, the level of TXA_2 synthesis remained unaltered despite increased PGI_2 synthesis. TXA_2 is an agonist for platelet aggregation and vasoconstriction. PGI_2 is vasodilatory and disaggregates platelets. Thus, the response of HUVEC to hypoxia is one that should favour vascular patency and suppress thrombotic events. Also, a disruption in the balance of COX-derived products, PGI_2 and TXA_2, has been associated with the development of atherosclerosis (Sinzinger et al. 1991; Cheng et al. 2002). In HUVEC, it was observed that the selective COX-2 inhibition resulted in complete abrogation of synthesis of PGI_2, whereas TXA_2, was only modestly inhibited. Thus, selective COX-2 inhibition may have undesirable effects on the homeostatic vascular response to hypoxic stress.

In monocytes, despite increased COX-2 expression in hypoxia, there was a significant decrease in the synthesis of PGE_2 and TXA_2. The dissociation between COX-2 expression and activity was corroborated by experiments with U937 monocytic cells and GM-CSF differentiated and M-CSF differentiated monocyte-derived macrophages. I demonstrated that the disparity in COX-2 expression and activity in hypoxic monocytes was due to dephosphorylation of $cPLA_2$ and consequent reduced cleavage of AA from monocyte membrane phospholipids. The extracellular signal-regulated kinases (ERKs) are reported to regulate the phosphorylation and activation of $cPLA_2$ in macrophages (Hiller and Sundler 1999; Gijon et al. 2000), neutrophils (Fouda et al. 1995; Hazan et al. 1997; Syrbu et al. 1999) and basophils (Miura et al. 1999). Therefore, the involvement of the p44/42

MAPK on AA release was examined. Inhibition of p44/42 MAPK activation by the MEK-1 inhibitor, PD 98059, inhibited the restoration of AA release following re-oxygenation, indicating that p44/42 MAPK is involved in oxygen-induced restoration of $cPLA_2$ activity. Scope for further studies involve examining the mechanisms for a hypoxia-mediated dephosphorylation of $cPLA_2$ and activation of p44/42 MAPK. Ca^{2+} influx into a cell is one of the earliest events in initiating a cascade of cellular responses. Thus, one mechanism that may control reduced $cPLA_2$ activation is reduced Ca^{2+} availability in monocytes. Hypoxia activates ATP sensitive K+ channels to hyperpolarise cell membranes, which results in extrusion of Ca^{2+} from cells (Erdemli et al. 1998; Brayden 2002). The p44/42 MAPK depends of Ca^{2+} for phosphorylation and activation. Therefore, if Ca^{2+} is reduced in hypoxia due to effects of ATP/K^+ channel hyperpolarisation, the activation of p44/42 may be reduced and hence, the activation of $cPLA_2$ would be reduced (Fig 9.1). The action of phosphatases in determining the phosphorylated (activated) state of $cPLA_2$ also warrant consideration.

9.2.3 Cytokine synthesis in hypoxia

TNFα synthesis was not detected in resting or IL-1β stimulated FLS and HUVEC. However, the synthesis of TNFα and IL-1β was detected in LPS stimulated human monocytes and was significantly potentiated by hypoxia. This occurred despite reduced PGE_2 and TXA_2 synthesis in hypoxic monocytes. TNFα expression was increased in normoxia following incubation with the selective COX-2 inhibitor, NS-398, suggesting that reduced PGE_2 (which is predominantly COX-2 derived) could be responsible in part, for the increase in TNFα synthesis seen in hypoxia relative to normoxia. In support of this consideration, exogenous PGE_2 suppressed TNFα synthesis in a dose-dependent

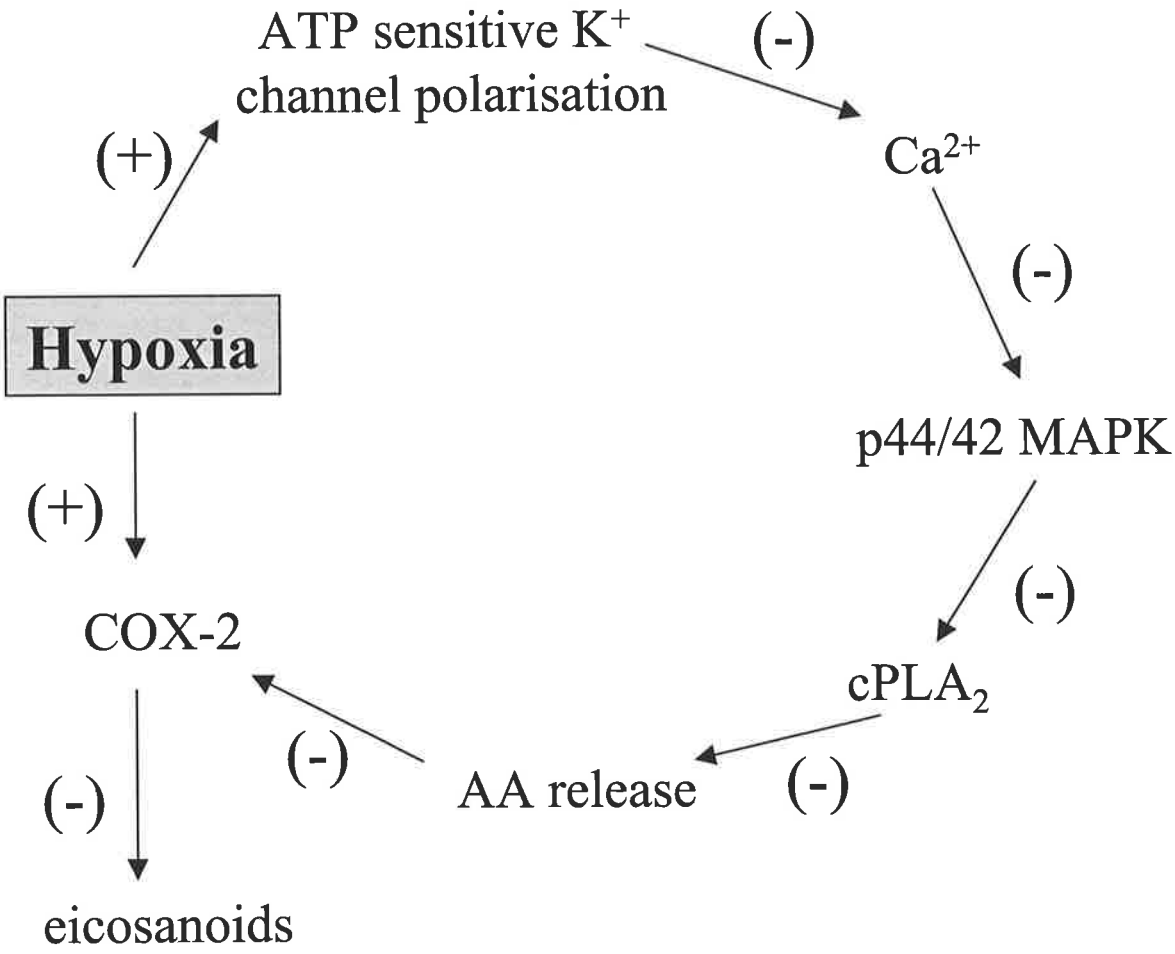


Figure 9.1
Hypothesis for the effect of hypoxia on eicosanoid synthesis in monocytes

manner. Although p38 MAPK has been identified in signalling pathways that induce expression of TNFα, PGE_2 suppression of TNFα synthesis was not attributable to an effect on the activation on p38 MAPK in monocytes, since neither inhibition of PGE_2 synthesis through COX inhibition nor the addition of exogenous PGE_2 to monocytes, resulted in a significant change in p38 MAPK phosphorylation.

The regulation of TNFα may also be under the influence of HIF activity in hypoxia. As with the COX-2 gene, our laboratory has recently identified a putative HRE binding site on the promoter of the TNFα gene. However, the function of this HRE in hypoxia remains to be determined.

On the other hand, TNFα may be involved in the regulation of COX-2. For example, LPS-stimulated COX-2 expression was suppressed by the addition of neutralising antibodies against TNFα. Therefore, the over-expression of COX-2 in hypoxia may result in part, from increased TNFα production in hypoxia. Whereas under some circumstances TNFα and COX-2 may participate in an autoregulatory feed back loop (TNFα induces COX-2 whose product PGE_2 down regulates TNFα). This loop may be partly compromised in hypoxia by reduced availability of COX substrate.

9.2.4 MMP activity in hypoxia

Preliminary studies showed that hypoxia also augmented IL-1β-induced total MMP activity in FLS. While many types of MMPs have been identified, all types play a role in tissue remodelling and angiogenesis in diseases such as RA (Visse and Nagase 2003). It is acknowledged that the net MMP activity is specifically controlled in vivo by TIMPs

(Nagase and Woessner 1999) and there would need to be further assessment to determine whether net MMP activity was also increased. However, Cha et al, reported recently that hypoxia increased MMP-1 and –3 and decreased TIMP-1 expression in human rheumatoid synovial fibroblasts, as measured by both protein and mRNA levels (Cha et al. 2003). Taken together with my findings, these results suggest that synovial hypoxia may contribute to joint damage in RA by increasing the ratio of MMP-1 and -3 to TIMP-1 production in synovial fibroblasts.

Mechanisms for the regulation of MMP activity in hypoxia have not been elucidated. In light of the fact that hypoxia induces PGE_2 and PGI_2 synthesis in HUVEC and FLS, the proposition that MMP activity is regulated by prostaglandin synthesis in hypoxia, warrants investigation. A recent report demonstrated that selective COX-2 inhibition by the compound SB203580, inhibited ERK activation and stimulated MMP-1 but not MMP-13 release (Pillinger et al. 2003). This effect was reversed by addition of exogenous PGE_1 and PGE_2 via ERK activation suggesting that COX-2 derived E prostaglandins tonically inhibit MMP-1 production (Pillinger et al. 2003).

Furthermore, it is appropriate to examine the effect of HIFs on MMP activity. Recently, our laboratory has identified two putative HRE sites on the promoter of the MMP-1 gene. An examination of HIF involvement in the MMP response to hypoxia, as suggested above with TNFα and COX-2 genes, is warranted.

9.2.5 Cell:cell interactions in hypoxia

Monocytes from circulating blood are recruited to atherosclerotic plaques and rheumatoid joints and are capable of synthesising soluble mediators that elicit changes in COX-2 expression in adjacent cell types, such as synoviocytes or endothelial cells.

In normoxia, co-culture of HUVEC and monocytes induced COX-2 protein in HUVEC but not in monocytes. Hypoxia greatly increased the effect of monocyte co-culture on HUVEC COX-2 expression. In addition, hypoxia upregulated monocyte COX-2 expression and this was greatest in the presence of HUVEC. Monocyte conditioned medium (CM) was shown to upregulate HUVEC COX-2 expression and activity and this effect was potentiated by hypoxia. The mediators in monocyte CM were identified as TNFα and IL-1β. Also, the selective COX-2 inhibitor, NS-398, completely suppressed PGI_2 synthesis induced by monocyte CM, but had no significant effect on TXB_2 synthesis in normoxic and hypoxic HUVEC. Thus, as with hypoxia alone, the HUVEC response to monocytes is one that favours vascular patency and suppression of thrombotic events.

In FLS, normoxic- and hypoxic-derived monocyte CM upregulated COX-2 expression and activity. This was largely due to IL-1β in monocyte CM and this response was augmented when FLS were hypoxic. Despite the presence of TNFα in monocyte CM, FLS did not respond to TNFα stimulation (recombinant TNFα or monocyte-derived TNFα) (data not shown). Overall, the results suggest that monocytes, once present in the joint, could exacerbate the signs and symptoms of inflammation by increasing synovial PGE_2 and PGI_2 synthesis.

9.3 CLINICAL IMPLICATIONS OF HYPOXIA

The clinical implications of hypoxia in inflamed lesions were not investigated in this thesis. However, it is possible to speculate on some possibilities with regard to disease management that takes account of tissue hypoxia at sites of inflammation.

9.3.1 Rheumatoid joints

With regard to inflamed joints, the results from this thesis suggest that hypoxia may exacerbate the signs and symptoms of inflammation by increasing prostaglandin synthesis and may potentiate the destructive processes of joint disease by increasing TNFα, IL-1β and MMP production. Frequent aspiration of joints with effusions, particularly weight bearing joints, in order to alleviate intra-articular pressure and restore synovial perfusion may be a worthwhile adjunct to systemic therapy.

In addition, further research on the mechanisms for hypoxia-mediated upregulation of COX-2, cytokines and MMPs may reveal new molecular targets for therapy. For example, if HIFs are shown to be critical mediators of the proinflammatory response to joint hypoxia, then exploration of the therapeutic potential of low molecular weight inhibitors of HIF action may be productive.

9.3.2 Atherosclerosis

Arterial wall hypoxia has been associated with many pathological processes involving the artery wall, including atherosclerosis (Hueper 1944; Crawford and Kramsch 1988; Crawford and Blankenhorn 1991; Santilli et al. 1992; Santilli et al. 1995; Bjornheden et al. 1999). It has been hypothesised that hyperbaric oxygen therapy (HBOT) may be used in the management of ischemic heart disease (Simanonok 1996). Although human data are lacking, there is supportive evidence from animal models.

Kjeldsen et al reported that arteriosclerosis was accelerated in the aortic artery of cholesterol fed rabbits following inhalation of low O_2 (hypoxia) (Kjeldsen et al. 1968). This group then demonstrated that exposing rabbits to HBOT resulted in the reversal of atheroma formation (Kjeldsen et al. 1969). It was also reported that HBOT combined with a low fat diet significantly caused atheroma regression (Vesselinovitch et al. 1974). In WHHL rabbits, hyperoxia (40% O_2) reduced atherosclerosis where as hypoxia (5-10% O_2) aggravated atherosclerotic lesions by a direct action on the vessel wall, independent of blood lipid levels (Okamoto et al. 1983).

9.4 CONCLUSIONS

This thesis highlights the differences in the response of various cell types to hypoxic conditions and explores the potential mechanisms for the regulation of inflammatory genes. The results of this thesis indicate that hypoxia is an important, but neglected determinant of inflammation and vascular homeostasis and suggest that cells can play 'inflammatory' or 'protective' roles in response to hypoxia, depending on their lineage. Lastly, this thesis provides a strong rationale for targeting factors (such as HIF) which upregulate hypoxia-responsive genes, as a potential therapeutic tool in the treatment of inflammatory disease.

APPENDIX A

Recipes for buffers and solutions not described in Chapter 2

A.1 Buffers for isolation of monocytes and preparation of STZ

Hanks Balanced Salt Solution (HBSS)

8.0g NaCl

0.4g KCl

0.35g $NaHCO_3$

0.06g KH_2PO_4

0.05g Na_2HPO_4

Dissolve all compounds in 1L Milli Q H_2O, pH 7.4

Running Buffer

1L HBSS

2.1g Tri- Sodium Citrate, pH 7.4

A.2 Buffers for Western Analysis

Resolving Gel

8.8ml Milli Q H_20

5ml 1.5M Tris Base, pH 8.8

6ml 30% Acrylamide

200µl 10% SDS (sodium dodecyl sulphate)

75µl 10% Ammonium persulphate

25µl TEMED (Bio-Rad Laboratories, Hercules, CA)

Allow 1h to set, then add stacking gel.

Stacking Gel

Insert 15-well comb

5.7ml Milli Q H_2O

2.3ml 0.5M Tris base, pH 6.8

1.7ml 30% Acrylamide

100µl 10% SDS

30µl 10% Ammonium persulphate

20µl TEMED

Allow 30min to set before loading samples.

5x sample buffer (laemilli)

100ml 3.125M Tris HCl

50ml glycerol

10g SDS

25ml β-Mercaptoethanol (Sigma Chem Co)

15ml Milli Q H_2O

Make under fume hood and store at RT

1.5M Tris Base pH 8.8

90.75g Tris Base (Sigma Chem Co)

dissolve in 400ml Milli Q H_2O, pH 8.8 with 10M HCl

complete to 500ml with Milli Q H_2O, filter 0.22μM

TrisBase 0.5M pH6.8

6.07g Tris Base

dissolve in 70ml Milli Q H_2O, pH 6.8 with 10M HCl

complete to 100ml with Milli Q H_2O

Transfer Buffer (5L)

15g Tris Base

72g Glycine

1L Methanol

Make up to 5L with RO H_2O, pH 8.3

Ponceau Red

2.5g Ponceau S

5ml Acetic Acid

Make up to 500ml Milli Q H_2O, mix well.

Lysis Buffer

10ml 10X HBSS

1ml 1M HEPES, pH 7.4

2.5ml Triton (20%)

make up to 100ml with MilliQ

A.3 Buffers for Northern Analysis

DEPC-H_2O

Eliminates RNases from solutions

500µl DEPC(diethyl pyrocarbonate)

500ml Milli Q H_2O

mix well, stand overnight at 37°C

autoclave 2x to degrade poisonous and corrosive DEPC

1% Agarose Gel

0.5g DNA grade agarose (BioRad Laboratories)

36ml DEPC-H_2O

10ml 10X MOPS

dissolve in microwave, then cool to touch

9ml 40% formaldehyde

cast gel with comb, run samples in 1x MOPS

2x loading buffer

5ml formamide

1ml 10X MOPS

1.67ml formaldehyde

1.33ml DEPC-H_2O

1ml glycerol

30µl Ethidium bromide (10mg/ml)

few granules of bromophenol blue

20x SSC

175.3g NaCl

88.2g sodium citrate

800ml DEPC-H_2O, pH 7.0 with NaOH

10X MOPS (1L)

41.86g MOPS

4.1g sodium acetate

3.72g EDTA

800ml DEPC-H_2O, pH 7.0 with NaOH

A.4 Buffers for Gelatin Zymography

Resolving gel (10%)

Heat 20mg of gelatin in 3.79ml Milli Q H_2O, then add

2.25ml Acrylamide solution, 40% (BioRad Laboratories)

1.25ml Bis-acrylamide solution, 2% (BioRad Laboratories)

2.5ml 1.5M Tris, pH 8.8

100µl 10% SDS

50µl 10% ammonium persulphate

5µl TEMED

Allow to 1h to set, then add stacking gel

Stacking Gel

Insert 10 well comb

0.59ml Milli Q H_2O

0.96ml Acrylamide solution, 40%

0.52ml Bis-acrylamide solution, 2%

2.25ml 0.5M Tris, pH 6.8

100µl 10% SDS

50µl 10% ammonium persulphate

5µl TEMED

Allow 1h to set before loading samples

2.5% Triton-X100

25ml Triton-X100

make up to 1L in Milli Q H_2O

Coomassie Blue Stain

50% (v/v) methanol

0.05% (v/v) Coomassie brilliant blue R250 – dissolve in methanol first

10% (v/v) Acetic acid

40% Milli Q H_2O

filter through Whatmans filter paper

Destain Solution (2L)

200ml Methanol

100ml Acetic Acid

1.7L Milli Q H_2O

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Erratum

Chapter 2

1. pp 63: "gentamycin" should read "gentamicin"
2. pp 65: "10mls" should be "10ml"
3. Oxygen concentrations used in the study at 1% were equivalent to ~33mmHg. This was the range of oxygen was relevant to those levels detected in hypoxic inflamed synovial lesions and atherosclerotic plaques.
4. The lowest limit of detection for the RIA was 10pg/100µl and the highest detection limit was 10ng/100µl. Cross reactivities in the TXB_2 RIA were 0.06% for PGE_2, 0.05% for 6-Keto$PGF_{1\alpha}$ <0.05%for $PGF_{2\alpha}$ and CV was 7.8 %, n=9. Cross reactivities in the PGE_2 RIA were <0.001% for TXB_2, 4.6% for 6-Keto$PGF_{1\alpha}$, 3.8% for $PGF_{2\alpha}$ and CV was 10.1 %.

Chapter 3

5. Fresh human monocytes are difficult to transfect. This is a feature of most primary cell cultures which are unlike transformed cell lines in this respect.

Chapter 4

6. pp 107: "was examined" should be deleted.
7. pp 105: A possible explanation for Chida , Matascak and Nogawa reporting differences in eicosanoid production may be due to the differences in cell types used, differences in hypoxic exposure (hypoxia vs ischaemia vs hypoxia/reoxygenation) and whether exposure was acute or chronic hypoxia.

Chapter 5

8. Frequent aspirations were suggested as a practical approach to relieving pain due to hypoxia in the joint. It is likely that patients will agree to aspiration of the joint because it relieves pain on weight bearing joints and its is usually performed when the patient receives an intra-articular steroid injection. Therefore, there is no unnecessary entry into the knee joint that may lead to increased risk of infection.

Chapter 7

4. The concentration of heparin was chosen from a previous concentration used in Schmedtje et al, (1997) *J Biol Chem.*

Chapter 8

5. Fig 8.4: The results with combined neutralising antibodies were no different from those when antibodies were used individually. Therefore, only the results of combination antibodies were shown.