Cyclooxygenase activity and expression in glial cells

by

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In this study, a panel of inhibitors with a range of selectivities for the two cyclooxygenase isoforms (COX-1 and -2) was used to probe the contribution of each to the synthesis of thromboxane B$_2$ (TXB$_2$) in cultured glia after stimulation with A23187 or arachidonic acid. Mixed glial cell cultures (~95% astrocytes, 5% microglia) were prepared from the cortices of newborn rat pups and maintained in vitro for 14 days. Controversy exists as to the contribution of astrocytes and microglia to the prostanoid synthetic capacity of mixed glial cell cultures. In order to investigate this, derivative cultures were made which were enriched in either astrocytes or microglia. Both astrocytes and microglia were found to express COX protein, and TXB$_2$ production was inhibited in each by inhibitors preferential for the different COX isoforms suggesting that both COX-1 and COX-2 contributed to TXB$_2$ synthesis in both cell types. Removal of serum from the culture medium for 4 days caused a decrease in the level of basal and stimulated TXB$_2$ synthesis, with a concomitant decrease in the presence of COX protein. There was also a decrease in potency of COX-2 selective inhibitors suggesting that the removal of serum caused a down-regulation of COX-2 expression. COX protein expression and TXB$_2$ synthesis were restored to control levels 7 days after serum re-addition, but the inhibitor profile subsequently obtained showed a pattern which differed considerably from either control or serum-deprived cells. Of particular note was an increase in the potency of paracetamol after serum re-addition. The effect of nitric oxide (NO) donors on COX activity in mixed glial cells under normal culture conditions was also investigated. Although inhibition of endogenous NO production was without effect, NO donors reduced stimulus-induced TXB$_2$ release. This was found to be independent of the COX isoform present in the cells and was additive with that evoked by COX inhibitors.
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"Do not quench your inspiration and your imagination; do not become the slave of your model."

Vincent van Gogh, 1853-1890
Publications resulting from this work

Some of the results presented in this thesis have been published as abstracts and papers in the following journals:


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<td>8-bromocyclic-GMP</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CaM</td>
<td>Ca(^{2+})/calmodulin-dependent</td>
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<td>CAPE</td>
<td>caffeic acid phenethyl ester</td>
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<td>CNS</td>
<td>central nervous system</td>
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<td>COX</td>
<td>cyclooxygenase</td>
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<td>cPLA(_2)</td>
<td>cytosolic PLA(_2)</td>
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<td>CRE</td>
<td>cAMP response element</td>
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<td>1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate</td>
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<td>EBSS</td>
<td>Earle's balanced salt solution</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EET</td>
<td>epoxy-eicosatrienoic acid</td>
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<td>eNOS</td>
<td>endothelial NOS</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>Hb</td>
<td>haemoglobin</td>
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<td>HETE</td>
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<td>HIV</td>
<td>human immuno-deficiency virus</td>
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<td>HPETE</td>
<td>hydroperoxyeicosatetraenoic acid</td>
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<td>intracellular PLA(_2)</td>
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<td>LH-RH</td>
<td>leutenising hormone releasing hormone</td>
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<td>lipopolysaccharide</td>
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<td>leukotriene</td>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>MEM</td>
<td>minimum essential medium</td>
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<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<td>nNOS</td>
<td>neuronal NOS</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>NOS</td>
<td>nitric oxide synthase</td>
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<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
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<td>NSB</td>
<td>non-specific binding</td>
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<td>O$_2^-$</td>
<td>superoxide</td>
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<td>ONOO·</td>
<td>peroxynitrite</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>phosphate buffered saline</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PG</td>
<td>prostaglandin</td>
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<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<td>PLA$_2$</td>
<td>phospholipase A$_2$</td>
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<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
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<td>radioimmunoassay</td>
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<td>reverse transcriptase polymerase chain reaction</td>
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<td>SDS</td>
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<td>s-nitrosoglutathione</td>
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<td>SNP</td>
<td>sodium nitroprusside</td>
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<td>SOD</td>
<td>superoxide dismutase</td>
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<td>sPLA$_2$</td>
<td>secretory PLA$_2$</td>
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<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
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<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<td>TNFα</td>
<td>tumour necrosis factor α</td>
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<tr>
<td>TX</td>
<td>thromboxane</td>
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Chapter 1:

Introduction
Chapter 1: Introduction

Inflammation

The four classical signs of inflammation were first described by the Roman medical compiler Cornelius Celsus in AD 30. His often quoted definition “Notae vero inflammationes sunt quattuor: rubor et tumor cum calore et dolore” (the characteristics of inflammation are four: redness and swelling, with heat and pain) continues to provide an elegant and concise summary of the symptoms associated with the phenomenon.

For many centuries inflammation was thought to be a disease in its own right rather than what is now considered to be a manifestation of the tissue response to a wide variety of insults. The redness and heat are due to vasodilation resulting in increased blood flow in the vessels within the affected region, and the swelling refers to the oedema associated with exudation of plasma and migration of leukocytes from circulating blood following increased vascular permeability. The pain is caused by the release of chemical mediators which modulate nociception as well as the physical compression of nerves in the vicinity of the inflamed site.

Inflammation is the body’s response to infection or injury and is directed towards destruction of the infectious agents and repair of the damaged areas. With this in mind, although reduction of inflammation through the use of anti-inflammatory drugs is desirable for symptomatic relief, their use may diminish the effectiveness of the host’s response. In certain
situations, however, the inflammatory response can do far more harm than good, for example in the chronic inflammation associated with rheumatoid and osteo-arthritis. Inflammatory responses have also been implicated in a number of CNS pathologies in which it has been associated with the death of host cells such as in multiple sclerosis and Alzheimer’s disease. A wide range of chemical mediators regulate the inflammatory response to various degrees including histamine, kinins, cytokines and eicosanoids. In the search for effective anti-inflammatory treatments, much attention has been focussed on controlling the production of lipid mediators which are heavily implicated in the propagation and maintenance of inflammation and are synthesised rapidly within activated cells in a highly controlled way.

Lipid mediators derived from arachidonic acid

Prostaglandins (PG) and thromboxanes (TX) are pivotal regulators of the peripheral immune and inflammatory responses, mediating such effects as vasodilation and vasoconstriction, platelet aggregation, pain and pyresis. These molecules are synthesised in most cells, and in addition to their inflammatory role, they play an important part in the regulation of normal cell activities such as the control of gastric secretions, renal function, reproduction and uterine contraction. They were first discovered in the 1930s when Von Euler (reviewed by Von Euler, 1983) showed that certain constituents of semen were able to lower blood pressure in experimental animals. It was he who coined the term prostaglandin for the active
ingredient since he believed it to be produced in the prostate gland. It was another 30 years before the first prostaglandins were isolated and their structures elucidated by Bergstrom, Samuelsson and co-workers (reviewed by Vane and Botting, 1992) whose work led to the identification of the main classes of prostaglandin. In the early 1970s the isolation of the endoperoxides PGG\(_2\) and PGH\(_2\) resulted in the discovery of the thromboxanes which, along with the prostaglandins, now form the family of chemical mediators known as the prostanoids, the major constituents of which are shown in Fig 1.1.

Prostanoids, along with leukotrienes, comprise the principal metabolites of arachidonic acid (AA) and are known collectively as the eicosanoids, that is the biologically active compounds derived from carbon-20 unsaturated fatty acids. AA is found esterified to membrane phospholipids, predominantly glycerophospholipids, within cells. Other enzymatically derived products include the lipoxins and various hydroperoxy acids (HPETEs), the latter also being formed by autooxidation, as well as epoxy-eicosatrienoic acids (EETs) which are formed via cytochrome P-450.

Leukotrienes are derived from AA via the lipoxygenase enzyme which produces the 5-hydroxyeicosatetraenoic acid (5-HPETE) precursor to the leukotriene family. The biological activity of the principal leukotrienes (LTs) are as follows. LTB\(_4\) is involved in chemotaxis, adhesion of leukocytes to endothelial cells, lysosomal release, superoxide (O\(_2^-\)) generation, activation of natural killer cells and induction of the interleukin-2 receptor. LTD\(_4\) has been
Figure 1.1: Chemical structures of the major prostanoids.
implicated in bronchoconstriction, vasoconstriction and plasma exudation as has LTC\textsubscript{4}, with LTC\textsubscript{4} having additional effects on leutinising hormone release, depolarisation of Purkinje cells and opening of the atrial K\textsuperscript{+} channel (reviewed in Shimizu and Wolfe, 1990).

The prostanoids on the other hand are produced from AA via the action of the cyclooxygenase enzyme (COX). In addition to the cyclooxygenase reaction which converts AA to PGG\textsubscript{2}, this enzyme also catalyses the hydroperoxidase reaction which reduces PGG\textsubscript{2} to PGH\textsubscript{2}, thus COX is often referred to as prostaglandin H\textsubscript{2} synthase or prostaglandin-endoperoxide synthase (Fig 1.2). The fate of the PGH\textsubscript{2} depends on the relative activities of a number of enzymes which catalyse specific inter-conversions to the primary prostanoids as shown in Fig 1.1. All of the prostanoids are composed of oxygenated 20 carbon fatty acids which contain a cyclic ring, a C-13 - C-14 \textit{trans} double bond and a C-15 hydroxyl group. Prostaglandins contain a cyclopentane ring whilst thromboxanes have a cyclohexane ring. The main biological effects of some commonly occurring prostanoids are listed in Table 1.1.

The individual prostaglandins are distinguishable by the different locations of the oxygen group(s) in the cyclopentane ring which determines the letter by which they are identified. This abbreviation is followed by an index (usually a -2) which indicates the number of double bonds present in the side chains. Their effects are via both plasma membrane receptors, intracellular receptors, and by action downstream of receptor activation. Prostanoid
Figure 1.2: The mechanism of action of PGH₂ synthase (COX).
### Table 1.1: Biological effects of some commonly occurring prostanoids. (adapted from Shimizu and Wolfe, 1990)

<table>
<thead>
<tr>
<th>Prostanoid</th>
<th>Biological Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Growth inhibition, urinary excretion.</td>
</tr>
<tr>
<td>PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Growth inhibition, antiaggregation, smooth muscle contraction, hypothermia, sleep induction, narcolepsy, acetylcholine release, inhibition of prolactin release, inhibition of leutenising hormone releasing hormone (LH-RH) release, leutenising hormone release, increase of serotonin turnover.</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Smooth muscle relaxation, Na&lt;sup&gt;+&lt;/sup&gt; excretion, plasma exudation, inhibition of gastric secretion, hyperthermia, awake state, stimulation of LH-RH release, regulation of catecholamine release, inhibition of natural killer cells.</td>
</tr>
<tr>
<td>PGF&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>Bronchoconstriction, vasoconstriction, luteolysis, acetylcholine release.</td>
</tr>
<tr>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Vasodilation, antiaggregation, inhibition of gastric secretion, cytoprotection.</td>
</tr>
<tr>
<td>TXA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Bronchoconstriction, vasoconstriction, aggregation.</td>
</tr>
</tbody>
</table>

Receptors are generally G-protein coupled, or belong to the family of nuclear steroid/thyroid hormone receptors. The plasma membrane G-protein coupled prostanoid receptors are highly specific for the individual prostanoids and are expressed in a highly cell type-specific manner. They fall into 3 categories which are dependent upon the second messenger system they activate; the activation and/or inhibition of adenylate cyclase and the mobilisation of calcium. In addition to these plasma membrane receptors, 15-d-PGJ<sub>2</sub>, a metabolite of PGD<sub>2</sub>, has been shown to activate peroxisome proliferator-activated receptor-γ (PPAR-γ), a nuclear hormone receptor. This interaction is less specific than the binding to the plasma membrane receptors, but activation of PPAR-γ results in a heterodimer of the receptor with the retinoic acid receptor which can bind to specific DNA sequences activating various...
target genes (reviewed by Versteeg et al., 1999). Various observations have suggested that prostanoids also act downstream of these receptors directly as second messengers with effects on growth factor-dependent actin reorganisation, platelet-derived growth factor (PDGF)-induced rises in cAMP and cAMP-dependent kinase (PKA) activation, hepatocyte growth factor- and epidermal growth factor- induced DNA synthesis and angiotensin-mediated activation of mitogen activated protein kinase (MAPK) (reviewed by Versteeg et al., 1999).

**Cyclooxygenase**

The COX enzyme was first purified in 1976 from bovine and ovine vesicular glands (Miayamoto et al., 1976; Hemler et al., 1976) and cloned in 1988 (DeWitt and Smith, 1988). Experiments with this complementary DNA sequence revealed that rapid changes in levels of COX protein and activity were not accompanied by the expected changes in mRNA, and further studies led to the hypothesis that there must be two pools of COX; one constitutive and insensitive to glucocorticoid regulation, and the other inducible and glucocorticoid-regulable (reviewed by O'Banion, 1999). The molecular basis for this phenomenon became clear in 1991 with the cloning of a second gene whose product also had COX activity and became known as COX-2 (Kujubu et al., 1991; Xie et al., 1991; O'Banion et al., 1991).
Both enzymes have a molecular weight of between 71 and 74 kDa (variations being due to species differences and differential levels of N-glycosylation, reviewed in Otto and Smith, 1995) and similar Km and Vmax values for the metabolism of AA. They are encoded by distinct genes which map to different chromosomes, but are similarly organised at a genomic level. Both genes have been cloned from mouse, rat, human and chicken and all share the same general characteristics. The COX-1 gene spans 22-25 kb and contains 11 exons and 10 introns resulting in a 2.8 kb mRNA sequence, while the immediate early gene which codes for the inducible enzyme is shorter at 8 kb and has 10 exons and 9 introns with an mRNA product of 4.2 kb. In addition, the COX-2 gene has a long 3' untranslated region containing a large number of motifs known to confer message instability (Srivastava et al., 1994). The genes encode proteins of approximately 600 amino acids with 63% sequence homology, with the inducible isoform lacking the amino terminal hydrophobic region of COX-1, but possessing an 18 amino acid insertion near its carboxyl terminus which is not present in the constitutive isoform. These differences have allowed the development of antibodies specific for each isoform of the enzyme (Kujubu et al., 1993) and have led to suggestions about differential intracellular segregation, however both protein sequences possess a C-terminal P/STEL sequence which is responsible for anchoring the enzymes to the membrane of the endoplasmic reticulum (Song and Smith, 1996).

The X-ray crystallographic structures of both isoforms have been obtained (Picot et al., 1994; Kurumbail et al., 1996) showing them to be dimeric, with each monomer comprising
three independent folding units: an epidermal growth factor-like domain, a highly amphipathic membrane-binding motif, and a large globular haem-containing catalytic domain. The membrane binding motif is particularly novel in these enzymes since it anchors them to one leaflet of the lipid bilayer rather than employing a trans-membrane motif more typical of an integral membrane protein. The active sites for the two catalytic activities and the long hydrophobic substrate-binding channel are adjacent, with only two variations between the two isoforms: An Ile in COX-1 is substituted for Val in COX-2 at positions 434 and 523. A unique feature of this enzyme is that the hydrophobic substrate binding channel appears to be closely associated with one of the four aliphatic helices which comprise the membrane binding domain, leading to the suggestion that the substrate binding channel opening is situated deep within the lipid bilayer of the endoplasmic reticulum, perhaps facilitating the acquisition of AA directly from the membrane.

As mentioned previously the COX enzymes catalyse two separate stages in the metabolism of AA, a cyclooxygenase reaction resulting in the incorporation of molecular oxygen to form PGG\(_2\) followed by a peroxidase reaction which reduces the hydroperoxide PGG\(_2\) to the corresponding alcohol PGH\(_2\) (see Fig 1.2). The cyclooxygenase reaction begins with the removal of the (135)-hydrogen from the AA to form an arachidonyl radical. Sequential additions of oxygen at C-11 and C-15 then yield PGG\(_2\) which becomes PGH\(_2\) in the peroxidase step by the reduction of the 15-hydroperoxide group (reviewed by Smith et al., 1996). During the reduction of the hydroperoxide group of PGG\(_2\) a free radical intermediate
forms which is reduced by nicotinamide adenine dinucleotide which subsequently donates an electron to molecular oxygen to form \( O_2^- \). This can be scavenged by superoxide dismutase to form \( H_2O_2 \) which can react with further \( O_2^- \) molecules to form highly reactive hydroxyl radicals. In this way reactive oxygen species form as a byproduct of the enzymatic activity. Both isoforms of the enzyme have similar levels of catalytic activity (approximately 3500 mol of AA/min/mol of dimer) and the \( K_m \) values are about 5 \( \mu \)M for both AA and \( O_2 \) in each of the isoforms. Analysis of the kinetics of the action of the enzyme requires the accelerative actions of peroxidase intermediates in initiating the cyclooxygenase reaction to be taken into account, in addition to the self-catalysed inactivation of both the cyclooxygenase and peroxidase activities. Basically, the cyclooxygenase activity requires the presence of hydroperoxides for initiation, and the peroxidase is believed to play an important role in the provision of this, but by its very nature the peroxidase must also decompose hydroperoxides. This balance between hydroperoxide generation and removal is also believed to occur at a cellular level and may be one of the main factors in the regulation of prostanoid synthesis. Kulmacz et al. (1994) developed a model for the mechanism by which the enzyme functions, based on the idea that an enzyme-intermediate in the peroxidase cycle becomes converted to a catalytic intermediate, possibly a tyrosyl radical, which is then able to react with the AA and initiate the cyclooxygenase reaction. The elegant computer model which they proposed for the symbiotic effect of the two activities was able to account for many of the peculiarities associated with the reaction kinetics of the enzyme, in particular its inactivation by agents which reduce the available hydroperoxide, and its inhibition in the
presence of phenol by cyanide at concentrations which rule out participation of the haem group. The model allowed the group to predict a number of kinetic parameters for the enzyme which were in agreement with experimental findings.

In their 1996 review, Smith et al. suggested that the hydroperoxide requirement of the cyclooxygenase reaction was met by an alkyl peroxide or peroxynitrite which caused oxidation of the haem group at the peroxidase active site. This oxidised haem group then oxidises the neighbouring tyrosine residue (Tyr-385) located in the cyclooxygenase active site which gives the tyrosyl radical mentioned earlier responsible for the abstraction of the (13S)-hydrogen from the AA. A number of other functionally significant amino acid residues have been identified by site directed mutagenesis (reviewed in Otto and Smith, 1995). A model of the active site, first proposed by Smith and Marnett (1994), is shown in Fig 1.3. The haem group which is involved in the initial activation of the enzyme is coordinated by two histidine residues (His 388 and His 207) which are conserved in both isoforms. The cyclooxygenase active site consists of a long hydrophobic channel with its opening proximal to the membrane binding domain and the other end near the haem group. Within this channel is an arginine residue (Arg 120) which is believed to bind the carboxylate ion of the AA substrate. On the opposite side of the channel, Tyr 355 governs the stereospecificity of the enzyme towards inhibitors. The numbers quoted here are for the residues in the COX-1 isoform, but they are conserved in COX-2. The best known amino acid residue in the cyclooxygenase active site is the serine which is acetylated by the classical non-steroidal anti-
Figure 1.3. Model of the active site of ovine COX-1. Adapted from Otto and Smith (1995).
inflammatory drug (NSAID), aspirin. This is Ser 530 in COX-1 which corresponds with Ser 516 in COX-2, the different numbering being due to the absence in COX-2 of a sequence of amino acids found at the N-terminus of COX-1, which is situated such that upon acetylation the acetyl group protrudes into the active site and interferes with AA binding. The specific effects of the other NSAIDs will be discussed later.

It is believed that the 523 Ile/Val substitution mentioned previously as the primary difference between the two isoforms confers the specificity of the enzyme to the drugs selective for each isoform. (reviewed in Vane et al., 1998). The smaller size of the Val allows access to a side pocket off the main substrate channel in COX-2 which is normally blocked in COX-1 by the longer side chain of Ile. It is believed that the 434 Ile/Val substitution controls the differences in substrate specificity of the two isoforms. COX-2 will accept a wider range of substrates than COX-1, for example eicosapentaenoic acid, γ-linolenic acid, α-linolenic acid, and linoleic acid, in addition to AA and dihomo-γ-linolenate which act as substrates for both isoforms. Interestingly COX-2 which has been acetylated by aspirin will still oxidise AA, but to 15-hydroxy-tetraenoic acid (HETE).

In addition to these differences between COX-1 and COX-2 with regard to inhibitor and substrate specificity, there appears to be a difference between the two with regard to the level of hydroperoxide required for activation. COX-2 requires approximately 2 nM hydroperoxide for activation which is an order of magnitude lower than the requirement for
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COX-1 activation. As a result, in a situation where both isoforms were present, it is feasible that COX-2 could be functional whilst COX-1 remained in a latent state, and metabolism of AA would therefore be entirely via the action of COX-2 despite the presence of COX-1 (Kulmacz, 1998). Another interesting feature of the COX enzyme is that it can undergo suicide inactivation. Addition of AA to preparations of isolated COX results in a rapid increase in activity followed by a fall in activity which is not related to product inhibition. It has been demonstrated that within the catalytic lifetime of 1 mol of synthase, 1300 mol of PGG$_2$ is produced before some mechanism-based inactivation occurs (Marshall et al., 1987; Kulmacz et al., 1994).

Whilst COX is believed to be the key control step in the prostanoid synthetic pathway, phospholipase A$_2$ (PLA$_2$) has also been implicated as a regulable factor which can control the whole prostanoid synthetic cascade through limiting the release of AA from membrane phospholipids. More than 10 PLA$_2$ isoforms with distinct sequences have been identified which can be classified as either cytosolic or secretory and differ in the nature of their Ca$^{2+}$ requirement as well as their molecular weight and structure. If they are classified according to their biological properties then they can be grouped into three main categories; secretory (sPLA$_2$), cytosolic Ca$^{2+}$-dependent (cPLA$_2$) and intracellular Ca$^{2+}$-independent (iPLA$_2$). The sPLA$_2$s are found in extracellular fluids and include various snake and insect venoms as well as synovial and pancreatic fluids. They exhibit a requirement for mM Ca$^{2+}$, have relatively low molecular weights (~14kDa) and are strongly resistant to proteolysis and denaturation.
The cPLA₂ is more relevant to the control of the intracellular inflammatory response and is believed to be involved in various receptor-activated signalling cascades. It has high molecular mass (~85kDa) and can be phosphorylated by kinases of the MAPK cascade causing modulations in its activity. A Ca²⁺-lipid binding domain allows the cPLA₂ to translocate to membranes in response to increases in intracellular Ca²⁺ providing a functional activation of the enzyme in response to cellular stimulation (reviewed by Dennis, 1994). Unlike the other PLA₂s, the cPLA₂ shows a preference for phospholipids which contain AA and requires only μM Ca²⁺ for activation. The iPLA₂s share the size and intracellular localisation of the cPLA₂ but have no requirement for Ca²⁺ and no apparent specificity for phospholipids containing AA. A paradigm for the interaction of the different PLA₂s in the release of AA has been proposed wherein the cPLA₂ is activated initially through increases in Ca²⁺ or the action of phosphorylation cascades, and in cells not expressing sPLA₂s these enzymes account for the majority of AA mobilisation. In cells which are capable of expressing sPLA₂s however, the bulk of AA mobilisation appears to be mediated by sPLA₂ which once secreted associates with the outer surface of the cell, releasing AA into the extracellular matrix from which it can be obtained by surrounding cells for eicosanoid synthesis (reviewed by Balsinde et al., 1999). This hypothesis is bourne out in elegant over-expression studies which have shown that the presence of cPLA₂ activity is vital for sPLA₂ function (Murakami et al., 1998). Furthermore these studies showed that cPLA₂ was a key regulator of the ionophore-induced immediate and cytokine-induced delayed release of AA, but the enzyme only responded to the inflammatory stimuli in the
presence of serum. Interestingly it has also been shown recently that sPLA$_2$ can trigger a signalling pathway in astrocytoma cells which activates both cPLA$_2$ and the MAP kinase cascade, suggesting the potential for a mechanism of indirect self-activation by the otherwise redundant sPLA$_2$ which requires active cPLA$_2$ (Hernández et al., 1998). The iPLA$_2$ is believed not to be involved in stimulated AA release but has been implicated in phospholipid remodelling, i.e. the incorporation of AA and other fatty acids into the membrane in different regions of the cell thus maintaining the AA pools required by the other PLA$_2$s. Although this is not entirely clear, it has been shown that the AA derived from iPLA$_2$ was not metabolised to PGE$_2$ in parallel experiments to those showing the prostanoid fate of the AA products of the other PLA$_2$ enzymes (Murakami et al., 1998). The activation and up-regulation of PLA$_2$ in response to a number of inflammatory stimuli has been demonstrated (Nakazato et al., 1991; Glaser et al., 1993; Pfeilschifter et al., 1993; Tan et al., 1996; Luo et al., 1998). Interestingly the pro-inflammatory role of PLA$_2$ is not only the release of AA to be used as a COX or lipoxygenase substrate, but also the release of the 2-lysophospholipid which is the other cleavage product of the PLA$_2$ activity. 2-lysophospholipid can be used to form platelet activating factor which is another potent pro-inflammatory mediator (Snyder, 1995).

**Regulation of COX expression**

The differences in the sequences of the C-terminal amino acids of the two isoforms of COX suggest the concept of differences in segregation of the isoforms to different regions of the
cell. The idea of the two isoforms being segregated to different subcellular compartments is not unreasonable considering the differences involved in the regulation of their expression and suggestions that the two isoforms utilise different pools of AA (Reddy and Herschman, 1994). Initial studies using selective antibodies showed little difference in distribution of the two isoforms in fibroblasts, with immunoreactivity being detected in both the endoplasmic reticulum and nuclear envelope of 3T3 cells (Regier et al., 1993). Subsequent investigations using a wider variety of antibodies and cell types and a histofluorescence assay for COX activity showed that although both isoforms were detected in the endoplasmic reticulum and nuclear envelope, COX-2 protein was more concentrated in the nuclear envelope whereas COX-1 was located predominantly in the endoplasmic reticulum (Morita et al., 1995). The idea of this kind of segregation would provide an elegant model for the role of COX-2 in providing prostanoids which act locally within the nucleus using targets such as the PPAR-γ system mentioned earlier. However, this apparent segregation of isoforms was not confirmed by immunoelectron microscopy studies which showed roughly equal amounts of each isoform distributed between the inner membrane of the nuclear envelope and the outer membrane which is contiguous with the endoplasmic reticulum (Spencer et al., 1998). In addition, the high lipid-solubility of the AA makes the idea of the existence of subcellular compartments of the substrate which are accessible to discrete populations of enzymes less feasible.

It has been well established the COX-1 is expressed in a constitutive way in most mammalian
tissues, so most studies into the control of expression of COX have focussed on the inducible isoform which will be discussed shortly. However, although COX-1 mRNA and protein levels do not appear to fluctuate in response to mediators in the same way as COX-2, there must be some level of regulation of this isoform since it is found in some but not all cell types, and is expressed at varying levels in different tissues. This control of expression of COX-1 is believed to occur developmentally (reviewed by Otto and Smith, 1995) in response to cell differentiation and hormonal changes.

In contrast, COX-2 protein is not detected in most tissues under normal physiological conditions and its induction, certainly in cells of the periphery, is usually associated with an inflammatory response. High levels of COX-2 expression have been widely investigated in rat models of inflammatory arthritis, and induction of COX-2 has been detected in cartilage and synovial tissues from human patients suffering from osteo- and rheumatoid arthritis (reviewed in Dubois et al., 1998). The inducible nature of this isoform is a result of certain features of its genetic code. The length of the COX-2 gene is about 8 kb long in mouse, rat and human and the promoter region contains a TATA box and several transcription factor binding sites, which makes COX-2 a typical immediate early response gene with transcription regulation being the key mechanism for control of COX-2 protein levels. The expression of COX-2 has been shown to be rapidly induced or up-regulated in various cell types by a number of substances including phorbol ester (Kujubu et al., 1991), lipopolysaccharide (LPS), forskolin (Kujubu and Herschman, 1992), cAMP (Fiebich et al.,
1996a), tumour necrosis factor-α (Cao et al., 1998), Freund’s complete adjuvant (Hay et al., 1997), transforming growth factor-β (TGF-β), IL-1 (Maier et al., 1990), the product of the v-src oncogene (Xie and Herschman, 1995), PDGF (Lin et al., 1989) and serum (Rich et al., 1998). COX-2 induction in fibroblasts by serum and PDGF has been shown to involve activation of a cAMP response element (CRE) in the COX-2 promoter (Xie and Herschman, 1996). The promoter region for COX-2 has also been shown to include consensus sequences for NF-IL6, PEA-1, myb, GATA-1, xenobiotic-response element, nuclear factor-κB (NF-κB), PEA-3, Sp-1 and 12-O-tetradecanoyl-phorbol-13-acetate-response element (Kosaka et al., 1994). In vascular endothelial cells, LPS and phorbol ester were shown to induce COX-2 up-regulation via trans-acting factors such as C/EBPδ binding to the NF-IL6 site and the CRE, although some activity could only be explained by the action of additional cis-acting elements (Inoue et al., 1995).

Despite the presence of an array of promoter sites for the initiation of COX-2 transcription, most of the experimental evidence to date suggests that most of the established stimuli act via various cascades which ultimately converge on the activation of NF-κB. NF-κB is a member of the Rel family of transcription factors which consists of five proteins, RelA (p65), c-Rel, RelB, NF-κB1 (p50/p105) and NF-κB2 (p52/p100), all of which possess a conserved amino-terminal DNA binding and dimerisation domain called the Rel-homology domain. NF-κB exists in the cytoplasm of most cells in the form of homo- or hetero-dimers which are maintained in an inactive form by association with inhibitory proteins of the IκB family, in
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particular IκBα. These proteins exert their inhibitory effects by masking a nuclear localisation signal located within the Rel-homology domain of the NF-κB, thus preventing nuclear localisation and DNA binding. Activation of NF-κB is achieved through the phosphorylation of two serine residues on IκB which leads to its ubiquitination then degradation via the 26S proteosome, whereupon the NF-κB is translocated to the nucleus where it binds to target genes to initiate transcription (Simeonidis et al., 1999). The key step in this activation of NF-κB is the phosphorylation of IκB which has been shown to be achieved by the action of IκB kinases known as IKKs, which are found within a high molecular weight complex known as the IKK signalsome. These IKKs are believed to be activated through phosphorylation by upstream kinases, the products of a number of possible signal transduction cascades (Mercurio and Manning, 1999).

A number of studies have shown the involvement of elements of the MAPK cascade in the up-regulation of COX-2 induced by various stimuli. The MAPKs are serine/threonine kinases which are distinguished from tyrosine kinases by their requirement for dual phosphorylation on distinct threonine and tyrosine residues. In mammalian cells five distinct MAPK cascades have been identified, which are typically organised in a three-kinase sequence consisting of a MAPK, a MAPK activator (MAP kinase kinase) and an activator of the MAPKK (MAP kinase kinase kinase) (Fig 1.4)

The up-regulation of COX-2 by bacterial LPS was shown to be regulated by mediators of
Figure 1.4: MAP kinase cascades. Three of the best characterised MAPK cascades are shown with each showing the typical three kinase cascade organisation. The MAPK 1/2 cascade is activated in response to growth factors and results in cell proliferation. The stress activated JNK and P38 MAPK cascades are two independent, parallel pathways that are activated by extracellular stimuli and cellular stresses including inflammatory cytokines, heat shock, high osmolarity, ceramides and TGF-β.
two MAPK cascades (p42/44 MAP kinase and p38 MAP kinase) in macrophages (Paul et al., 1999). These two cascades were also implicated in the induction of COX-2 by IL-1β in cardiac myocytes (LaPointe and Isenovic, 1999), and the p38 MAP kinase system was also responsible for the induction of COX-2 in myometrial smooth muscle by IL-1β (Bartlett et al., 1999). In studies using kinase-dead forms of MAPK cascade constituents, Guan at al. (1998) showed that activation of both the JNK/SAPK and the p38 MAPK signalling cascades was required for IL-1β-induced COX-2 expression in renal mesangial cells and NIH 3T3 cells. Interestingly, the role of p38 MAP kinase in the initiation of transcription has been brought into question. Ridley et al. (1998) showed that it played no part in transcription but that it was an important mediator in the induction of COX-2 expression in HeLa cells through a post-transcriptional role. An inhibitor of p38 MAPK rapidly destabilised COX-2 mRNA suggesting that p38 MAPK functions to regulate mRNA stability rather than participating in a cascade resulting in activation of the COX-2 promoter. A similar mechanism has been suggested to explain the effects of the synthetic glucocorticoid dexamethasone which causes complete abolition of induced COX-2 expression but only a minor part of this action is attributable to down-regulation of transcription. Experiments using IL-1β-induced COX-2 expression in A549 cells showed that dexamethasone acted post-transcriptionally to enhance degradation of COX-2 mRNA (Newton et al., 1998).

Despite these post-transcriptional effects of p38 MAPK, there is compelling evidence to link a number of MAPK signalling cascades directly to the promotion of COX-2 transcription.
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So far four different MAPKKKs have been identified which can activate IKK, thus resulting in an activation of NF-κB. These are MEKK1, MEKK2, MEKK3 and NIK (Zhao and Lee, 1999). Interestingly, other MAPKKKs identified as activators of the JNK or p38 pathways, such as MEKK4, ASK1 and MLK3, failed to cause NF-κB activation in this study suggesting the presence of a highly specific sequence of mediators which link the MAPK cascades to COX-2 induction via NF-κB.

It was mentioned previously that derivatives of PGD had been shown to act as peroxisome proliferators leading to the regulation of transcription at specific promoter sites. The COX-2 promoter region has been shown to contain a peroxisome proliferator response element which when activated enhances the expression of COX-2 (Meade et al., 1999). The PPAR-γ once activated forms a heterodimer with isoforms of the retinoid X receptor which can then bind to the peroxisome proliferator response element and enhance gene expression. In addition to the activation of PPAR-γ by prostanoid derivatives and NSAIDs, compounds such as fatty acids, plasticizers and anti-diabetic drugs have been shown to be peroxisome proliferators. The modulation of COX-2 expression by fatty acids, prostanoids and NSAIDs is of particular interest in investigations of the role of dietary fat in colon and breast cancer. Meade et al. (1999) demonstrated that unsaturated fatty acids which have been implicated in the progression of these cancers were able to induce COX-2 expression in mammary epithelial cells, and also showed that a number of other compounds including PGA₂, 15-d-PGJ₂, 15- and 8-HETE, fenamates, ibuprofen and NS398 were all able to increase COX-2
expression. An interesting cell-specific effect observed with the NSAID sulindac sulphide, is that it strongly enhanced COX-2 expression in mammary epithelial cells but had a greatly reduced effect in colonic epithelial cell lines. The implications for the induction of COX-2 by via PPAR-γ through such a diverse array of stimuli are widespread, not only with regard to the treatment of cancer and the development of new NSAIDs, but also with a view to understanding the complex feedback or feedforward effects of COX products and substrates on the expression of the inducible isoform.

Other signal transduction pathways have been shown to cause induction of COX-2 by activating the CRE in the promoter. In particular two pathways have been shown to converge on the CRE after activation with the product of the v-src oncogene, pp60^src, a non-receptor tyrosine kinase (Xie and Herschman, 1995). It was shown that v-src activated the Ras/MEKK1/JNKK(SEK1)/JNK(SAPK) signal transduction pathway leading to the phosphorylation of c-Jun and subsequent transcription of COX-2 from the CRE. In addition, a Ras/Raf/MEK/ERK pathway was shown to be another route by which the v-src signal caused increased c-Jun activity at the CRE, although rather than directly phosphorylating c-Jun this pathway caused increased expression of c-Jun. It is interesting to note that COX-2 expression following v-src treatment was persistent whereas most other stimulators, such as inflammatory cytokines, caused a transient increase in COX-2 expression. It has been suggested therefore that v-src activation of this latter pathway may induce AP-1 expression, causing a transcription-dependent mechanism of increased COX-2 expression which differs
from the effects of other inducers. The CRE site on the COX-2 promoter has also been implicated in the increased expression of the enzyme as a result of the action of ceramide, serum and PDGF via similar Ras mediated pathways (Xie and Herschman, 1996; Subbaramaiah et al., 1998). It has also been shown to be activated by the protein phosphatase inhibitor okadaic acid (Mahboubi et al., 1997) which inhibits PP-1 and -2A resulting in a shutdown of the MEKK1/MEK1/ERK cascade (probably as a result of increased PKA activity which inhibits the MEKK1 kinase Raf-1) and a concomitant activation of the MEKK1/JNK/SAPK/JNK cascade causing activation of both CRE and AP-1 promoter elements (Miller et al., 1998). Finally, recent work with mesangial cells has shown that 5-HT produces an increase in COX-2 expression via the p42/44-MAPK pathway, but also through a Ca²⁺/calmodulin-dependent (CaM) kinase pathway which possibly leads to the activation of the transcription factor C/EBPβ (Goppelt-Struebe et al., 1999). In these cells, abolition of basal COX-2 was detected after incubation with inhibitors of CaM kinase II, suggesting that this pathway may also be responsible for mediating the basal expression of COX-2.

Investigations of the pathways which mediate the ligand-induced expression of COX-2 have revealed an increasingly more complex network of cascades acting directly and indirectly upon different promoter regions. Studies of this kind are far from elucidating a complete and reliable model for this process, which undoubtedly differs depending on the stimulus, the cell type, and quite possibly the state of activity of the cell. While the ultimate goal of such
investigations must be to fully dissect and understand the interrelations between all of the cascades involved, in the short term much work has focussed upon identifying therapeutically beneficial substances. These could potentially inhibit the up-regulation of expression of COX-2 and therefore act to attenuate its pathogenic effects. Perhaps the best example of a substance which has been shown to suppress COX-2 induction is salicylate which has been used as an anti-inflammatory agent for many years. Both salicylate and its acetylated derivative aspirin have been shown to inhibit COX-2 transcription in peritoneal macrophages after stimulation with LPS, phorbol 12-myristate 13-acetate (PMA) and IL-1β (Xu et al., 1999) although the mechanisms for this action are not entirely clear. Salicylate has been shown to inhibit AP-1- and NF-κB-mediated gene transcription, but at doses in excess of those found to inhibit COX-2 induction (Dong et al., 1997; Kopp and Ghosh, 1994), although some inhibition of IκB kinase by aspirin and salicylate at pharmacological concentrations has been reported and could help to explain the phenomenon (Yin et al., 1998). This inhibition of COX-2 transcription by aspirin and salicylate has recently been shown to occur with the salicylate derivatives triflusal and 2-hydroxy-4-trifluoromethylbenzoic acid, but in the case of these agents the COX-2 suppression is more readily attributed to direct inhibition of the NF-κB levels (De Arriba et al., 1999). Salicylic acid has also been shown to act on MAPK cascades upstream of the IκB level. It was shown to inhibit the activation of p42/p44 MAPK by TNF, but not the EGF-induced MAPK activation, suggesting the presence of distinct MAPK pathways which are activated in response to these stimuli (Schwenger et al., 1996).
Further evidence for the activation of NF-κB via different second messenger pathways specific for different inducers of COX-2 comes from the inhibition of NF-κB by the benzophenanthridine alkaloid sanguinarine (pseudocheletherine), a known antiinflammatory agent. This potent inhibitor of NF-κB activation was shown to somehow block the phosphorylation and subsequent degradation of IkBα after stimulation with IL-1, phorbol ester, okadaic acid and TNF, but not after stimulation with hydrogen peroxide or ceramide, suggesting that these different stimuli activate distinct cascades which converge on the activation of NF-κB (Chaturvedi et al., 1997).

**COX Inhibitors**

Inhibition of COX is central to the action of the NSAIDs, a class of drugs which have become the most frequently consumed drugs in the world throughout the latter part of the 20th century. The classical member of this family of drugs is aspirin which is a derivative of salicylate, a naturally occurring substance whose medicinal properties have been exploited for many thousands of years. About 3500 years ago the Ebers papyrus mentioned the use of dried Myrtle leaves in the relief of rheumatic pains from the womb, and a thousand years later Hippocrates suggested the use of willow bark for the relief of pain in childbirth and fever. The first published clinical trial of salicylates was in 1763 in England when the Reverend Edward Stone reported to the Royal Society the success of using dried, pulverised willow bark for the treatment of fever in about 50 patients. The gradual identification and
purification of salicylic acid took place over the next 100 years followed by a search for an alternative compound which would cause less gastrointestinal irritation. This goal was finally achieved by Felix Hoffmann in 1897, working for the Bayer company in Germany, with the acetylation of salicylic acid to form aspirin. During the last century the use of aspirin became widespread and a number of "aspirin-like" drugs including paracetamol, phenylbutazone, indomethacin and naproxen were discovered, all of which in some way alleviated the swelling, redness, fever or pain of inflammation. Because of the differences between these drugs and the glucocorticoids, the name NSAID became appropriate. It wasn't until 1971 when John Vane and colleagues at the Royal College of Surgeons discovered that aspirin and similar substances inhibited COX that the widely accepted mode of action for these drugs as inhibitors of prostanoid synthesis was proposed. There are now a great many NSAIDs which have a diverse variety of structures leading to a range of selectivity for the different COX isoforms and major differences with respect to gastrointestinal, pulmonary and renal side effects.

The mechanism of action of COX inhibitors can be broadly divided into three categories;

1. Substrate analogues that cause rapid, reversible, competitive inhibition such as ibuprofen, mefenamic acid, flufenamic acid and sulindac.

2. Free radical scavengers and anti-oxidants which act rapidly as non-competitive inhibitors by blocking the peroxide tone required for COX activity. It has been proposed that paracetamol might act in this way.
3. Irreversible inactivators which modify the enzyme in a time-dependent way. An example is aspirin which acetylates the active site and indomethacin which diminishes the catalytic effect through conferring some kind of conformational change.

After the discovery of the inducible COX isoform and its association with inflammatory events it was suggested that the therapeutic efficacy of NSAIDs in terms of anti-inflammatory and analgesic effects was due to their ability to inhibit COX-2, whilst the accompanying inhibition of COX-1 which is critical for gastric cytoprotection accounted for their deleterious gastrointestinal side effects. The chemical structures of some common NSAIDs are shown in Fig 1.5. It has become common to classify these inhibitors in terms of their relative selectivity for COX-1 or COX-2 rather than by their chemical compositions. This is more useful with a view to balancing the potential therapeutic effects verses detrimental effects of the drugs and their relative positions in this series may be determined through observed effects on platelet aggregation, renal or gastrointestinal damage and antiinflammatory/analgesic properties (Table 1.2).

A number of tests have been developed in an attempt to assess the selectivity for one or the other isoform in order to investigate the therapeutic potential for novel and classical NSAIDs. These tests have tended to utilise either purified/recombinant enzymes, cultures of intact cells, or human whole blood. In this way, the NSAIDs have become classified as belonging to one of a number of functionally defined classes; COX-1 selective (aspirin,
Figure 1.5: Structures of some common NSAIDs
### Table 1.2: NSAID classification based on the selectivity of drugs for COX-1 or COX-2.

<table>
<thead>
<tr>
<th>Category</th>
<th>Inhibit platelet aggregation</th>
<th>Renal or GI side effects</th>
<th>Antiinflammatory/analgesic</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selective COX-1 inhibitors</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Low-dose aspirin</td>
</tr>
</tbody>
</table>
| Nonselective COX-1 inhibitors| +                            | +                        | +                          | Piroxicam
Indomethacin
Diclofenac
Ibuprofen                   |
| Selective COX-2 inhibitors   | ±                            | ±                        | +                          | Salicylate
Nimesulide
Meloxicam                     |
| Highly selective COX-2       | -                            | -                        | +                          | NS398
Celecoxib
MK966
DuP697                        |

Adapted from O’Banion, 1999.
sulindac), COX-1 preferential (piroxicam, indomethacin), non-selective (diclofenac, ibuprofen), COX-2 preferential (nimesulide) and COX-2 selective compounds (NS398, DuP697). Although the relative selectivities of the drugs remain reasonably consistent throughout these studies, there are discrepancies in the reported potencies of the drugs for each of the isoforms depending on the system used. Moreover, due to the rather artificial environment in which COX functions in many of these assays, their effectiveness for assessing the potential *in vivo* attributes of the drugs has been called into question. Assays using broken cells, for example, failed to account for intracellular drug distribution and cell penetration, and many systems using purified cell preparations failed to take into account plasma binding. Human blood based assays overcame these problems to a certain extent but have introduced other drawbacks such as the different incubation times which are often required for the assay of the activities of the different isoforms. This issue has been addressed recently with the development of an assay in which the NSAID activity in the plasma of animals after intravenous injection with drugs was assayed *ex vivo* using platelets and macrophages (Giuliano and Warner, 1999), revealing previously unaddressed differences in the time-course of bioavailability of some commonly used drugs.

The search for COX-2 selective inhibitors has resulted in the emergence of a family of second-generation NSAIDs almost exactly a century after the introduction of aspirin. The first to be approved was celecoxib (SC58635; Celebrex™) in December 1998, a structural derivative of one of the prototypical COX-2 selective inhibitors DuP697. Other COX-2
selective drugs on the market include Mesulid™ (nimesulide), Mobic™ (meloxicam), and Rofecoxib (MK0966; Vioxx™). So far these drugs have proved to be as efficacious as their less selective predecessors in the reduction of inflammation but have shown significantly reduced levels of gastrointestinal toxicity (Hawkey, 1999). The implications of the reduction of NSAID-associated toxicity must not be underestimated, indeed it has been suggested that the number of deaths due to NSAID-induced bleeding ulcers in the USA alone is comparable to those from AIDS and violent crime (Marnett and Kalgutkar, 1999), so any reduction in that number would be greatly desirable. In addition it is believed that a great many sufferers of chronic inflammatory-related disorders, such as arthritis, who currently eschew NSAID treatment on the grounds of the potential toxicity, would be more willing to use COX-2 selective anti-inflammatories.

Although the COX-2 selective NSAIDs seem to be effective in relieving inflammation without gastric side-effects, the paradigm in which COX-2 is responsible for inflammation and hyperalgesia whilst COX-1 provides the prostanoids for normal healthy physiology may be an oversimplification. The gastric toxicity of classical NSAIDs appears to occur in subsets of patients who for some reason are more susceptible to the damaging action of the drugs. Interestingly, animal models of chronic inflammation also appear to show this increased susceptibility, and in these cases inhibition of COX-2 caused a marked increase in the severity of the damage (reviewed by Wallace, 1999). It has been proposed, therefore, that COX-2 plays an important role in the healing of gastric ulcers, so the treatment of patients
with ulcerated or inflamed gastric mucosal linings with COX-2 inhibitors could exacerbate the condition.

In addition to the role of COX-2 in the maintenance of gastric integrity, COX-1 has been implicated as having a substantial role in the inflammatory response. Perhaps the most striking evidence comes from investigations using knockout mice which lack one or other of the isoforms. COX-2 knockout mice showed normal inflammatory responses to common tests such as oedema following treatment with phorbol ester, AA or carageenan. This oedema was readily attenuated by NSAIDs suggesting that COX-1 generated prostanoids were sufficient for the inflammatory response in these models (reviewed in O'Banion, 1999). In addition, a number of reports have shown that COX-2 selective inhibitors only show anti-inflammatory efficacy at doses high enough to inhibit COX-1 (reviewed in Wallace, 1999).

A pharmacological approach to resolving this issue demonstrated that although COX-1 was involved in models of skin inflammation (ie the Arthus reaction triggered by antibody/antigen interaction), it did not make a significant contribution to the carrageenan-induced rat footpad model of inflammation, an effect believed to be mediated predominantly by centrally produced products of COX-2 (Smith et al., 1998). Although the vast body of evidence corroborates the role of COX-2 in inflammation and COX-1 in mucosal maintenance, these observations suggest that the contribution of the two isoforms to prostanoid production under different circumstances may be far more complex than many people would like to admit.
Another area which is often overlooked with regard to the NSAIDs is their widespread nonprostaglandin-related actions. All of the NSAIDs interfere with cell membrane associated functions to various degrees, including generation of hydrogen peroxide in neutrophils which is inhibited by piroxicam but not ibuprofen, and inhibition of phosphodiesterase by indomethacin and diclofenac leading to an increase in intracellular cAMP. NSAIDs have also been implicated in the disruption of signal transduction via G-proteins, an event which might be responsible, at least in part, for the analgesia elicited by COX. The antinociceptive abilities of some NSAIDs have also been attributed to prostanoid-independent effects. There is evidence to suggest that some NSAIDs act directly on opioid receptors since both lysine acetylsalicylate and diclofenac have been reported to reduce the heroin withdrawal syndrome in humans (reviewed by Cashman, 1996). In addition, the antinociceptive effect of diclofenac has been attributed to its ability to reduce 5-HT and 5-hydroxyindoacetic acid levels in the brain stem and spinal cord (McCormack, 1994). A further mechanism has been proposed which suggests that NSAIDs can antagonise the hyperalgesia induced by activation of glutamate or substance P receptors (reviewed by Cashman, 1996).

Finally, it has also been shown that NSAIDs can influence the expression of COX-2, probably via activation of the PPAR pathway in a similar way to the PGD derivative 15-d-PGJ$_2$ (Lehmann et al., 1997; Minghetti et al., 1997) leading to the search for NSAIDs which could potentially target both the expression and activity of COX-2 simultaneously. It is clear that the effects of the NSAIDs are not limited to the inhibition of COX activity alone and
their analgesic effects in particular are probably due to a combination of other events superimposed upon the reduction in prostanoid synthesis.

**COX and its products in the CNS**

The prostanoids are a vital means of cellular communication in the CNS and have been shown to play important roles in the regulation of many centrally mediated activities. The classical role of prostanoids in the CNS is their regulation of cerebral blood flow, in particular the involvement of PGE$_2$ in the progression of vascular headaches and migraine. PGE$_2$ is a potent hyperalgesic agent potentiating pain induction by tachykinins and causing dilation of the temporal artery (reviewed by Shimizu and Wolfe, 1990; Wolfe, 1982). PGD$_2$ has also been shown to be involved in the vasodilation and headache associated with the administration of vitamin B (Morrow et al., 1989). Prostanoids have also been heavily implicated in the cerebral vasospasm which occurs after subarachnoid haemorrhage, TXA$_2$ and PGF$_{2a}$ in particular have been shown to act along with various other chemical mediators to mediate this major cause of human morbidity and mortality (Chehrazi et al., 1989; Yokota et al., 1991). Another central role ascribed to prostanoids is the maintenance of the sleep/wake cycle. PGD$_2$ and PGE$_2$ have been shown to promote sleep and wakefulness, respectively, by exercising an excitatory influence on the appropriate neurons (Koyama and Hayaishi, 1994; Sri Kantha et al., 1994; Hayaishi, 1994). These two prostaglandins are also involved in thermoregulation, with increased PGD$_2$ leading to hypothermia (Ueno et al.,
1982) and increased PGE$_2$ associated with hyperthermia. This role of PGE$_2$ is of particular importance since production of this molecule seems to be pivotal to the generation of fever, probably mediated by the activation of sympathetic outflow via the ventromedial hypothalamus, and possibly acting directly on thermosensitive neurons to stimulate thermogenesis in brown adipose tissue (reviewed by Rothwell, 1992).

Elevated central levels of prostanoids have been associated with various pathological states and they are believed to mediate a wide range of central responses to damage and disease. In ischaemic brain injury for example, the reduction in cerebral blood flow results in increased intracellular Ca$^{2+}$ levels, release of the excitatory amino acid glutamate, acidosis and production of toxic free-radicals which induce neuronal cell death by necrosis. This initial response is followed by an activation of glial cells and a chronic inflammatory response which leads to a demarkation of infarcts and removal of debris, and further neuronal death by apoptosis (Stoll et al., 1998). Prostaglandins are released in hypoxic brain regions at micromolar concentrations (Huttemeier et al., 1993) and are believed to play a predominantly cytoprotective role in the progression of ischaemia. In particular PGI$_2$ has been shown to protect cultured neurons from damage during hypoxia and reoxygenation and glutamate-induced injury (Cazevielle et al., 1993), as could most other prostanoids (Cazevielle et al., 1994). Interestingly, those prostanoids which were neuroprotective in vitro included Carba-TXA$_2$ which is generally believed to have a deleterious effect in ischaemia due to its vasoconstrictive and platelet aggregatory properties (Chen et al., 1986).
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It is also apparent that prostanoids play an important role during the induction and progression of seizures, although the exact nature of their contribution is not clear. It has been shown that reducing the level of PGE$_2$ in the brain of rats undergoing seizures evoked by tacrine and lithium chloride abolishes the expression of the seizures (Paoletti et al., 1998), and elevated PGE$_2$ levels have often been associated with various other types of seizure (Busija and Leffler, 1989; Naffah-Mazzocoratti et al., 1995). In a similar way to the antagonism between PGE$_2$ and PGD$_2$ observed for thermoregulation and sleep/wakefulness, PGD$_2$ has been implicated as having anti-convulsant effects upon seizures evoked in rats by pentylenetetrazol. However, measurement of prostanoid levels in the hippocampus during pilocarpine-induced seizures, a common model of epilepsy, showed a more complex alternating pattern of prostaglandin levels (Naffah-Mazzocoratti et al., 1995), and focal administration of PGD$_2$, PGE$_2$ and PGF$_{2\alpha}$ had no anti-convulsive effect on electrically kindled seizures (Croucher et al., 1991).

PGE$_2$ has been indicated to be of benefit in ameliorating the destructive autoimmune effects in multiple sclerosis and post-infectious encephalomyelitis. It is interesting that administration of a long-acting PGE analogue in animal models inhibited the inflammatory response, and the effect was enhanced by the co-administration of the COX inhibitor indomethacin, suggesting that the pathological effects were to some degree dependent on the synthesis of prostanoids (Reder et al., 1994). These findings were corroborated by the relief of pain in multiple sclerosis patients suffering from trigeminal neuralgia using the same
long-acting PGE analogue (Reder et al., 1995). In addition, high cerebrospinal fluid levels of 8-epi-PGF$_{2a}$ were recently correlated with the degree of disability in multiple sclerosis sufferers (Greco et al., 1999).

Another important role for CNS prostanoids seems to be in the dementia associated with the later stages of HIV infection. Dementia of this type is believed to be caused by infection and activation of macrophage or glial cells in the brain which release various pathogenic mediators causing neuronal dysfunction and death. Increased levels of PGD$_{2},$ PGE$_{2},$ PGF$_{2a},$ and TXB$_{2}$ have all been recorded in the cerebrospinal fluid of HIV positive patients with dementia and/or myelopathy (Froldi et al., 1992; Griffin et al., 1994). The role of these increased prostanoids in the progression of the dementia is not clear, since the administration of glucocorticoids, which decrease prostanoid levels, resulted in increased neuropathy with astrogliosis and neuronal apoptosis in HIV-infected mice (Limoges et al., 1997).

The intoxicating effects of alcohol on the brain have also been ascribed to a prostaglandin-mediated mechanism. In particular, levels of the PGE series were seen to increase with the absorption phase of alcohol administration, while those of the PGF series remained elevated in correlation with blood ethanol levels. It is believed that the various CNS effects associated with alcohol, in particular intoxication and hangover, are due predominantly to potentiation of prostanoid actions such as the regulation of vascular tone and water and electrolyte balance (Parantainen, 1983; George et al., 1986).
Recent work has focussed on the apparent involvement of the prostanoid synthetic cascade in the progression of Alzheimer's disease. This is because much of the neurodegeneration associated with this condition may be due to an inflammatory response which damages host cells. This condition will be discussed below with regard to the involvement of glial cells in the brain's inflammatory response. There has been some interesting speculation as to the precise involvement of prostanoids in Alzheimer's disease. Elevated levels of PGD$_2$ and TXB$_2$ have been detected in postmortem brains of Alzheimer patients (Iwamoto et al., 1989) whilst others have recorded decreased levels of prostanoids associated with the condition (Wong et al., 1992), although there is some speculation as to the effect of postmortem delay on these results (McGeer and McGeer, 1995). More recently, an increase in levels of PGE$_2$ and 6-keto-PGF$_{1\alpha}$ has been recorded in the cerebrospinal fluid of patients with probable Alzheimer's disease with no overall increase in total eicosanoid concentration (Montine et al., 1999). Moreover, activation of PGE$_2$ receptors on cultured astrocytes has been shown to stimulate overexpression of amyloid precursor protein, raising the possibility that PGE$_2$ might be involved in the formation of the amyloid plaques associated with this condition (Lee et al., 1999). This further supports the proposal by Prasad et al. (1998) that prostanoids are one of the major extracellular signals that initiate neurodegeneration through increased levels of amyloid-β and ubiquitin via elevation of intracellular cAMP, in contrast to the many examples of neuroprotective effects mediated by prostanoids discussed already. Another hypothesis is that the presence of β-amyloid plaques exacerbates the stimulation of glial cells by local foci of inflammation such as trauma, ischaemia or infections causing the
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release of inflammatory mediators, particularly PGE$_2$, which evoke a self-perpetuating inflammatory loop resulting in neurodegeneration (Landolfi et al., 1998). In either case it is likely that the progression of Alzheimer's disease is associated with changes in the levels of prostanoids in the brain.

It is clear from these examples that the prostanoids play a pivotal role in a great many CNS functions, with subtle changes in the levels of the specific mediators making significant changes in the activity of the cells in the brain. Not only are they a means of intercellular communication in the healthy brain, they also manifest themselves as integral mediators in the brain's inflammatory response to damage and disease. It is apparent that the synthesis of different prostanoids can cause a wide range of often opposing downstream effects, yet there has been little work to show how the expression or activity of the individual isomerase enzymes which convert the PGH$_2$ to the different prostanoids is regulated in cells. It has been shown that stimuli such as bacterial endotoxin or the inhibition of protein kinase C (PKC) may increase the level of PGE$_2$ synthase activity in rat Kupffer cells, whilst activation of PKC by substances such as phorbol ester suppressed PGE$_2$ synthase activity resulting in a concomitant functional increase in PGD$_2$ synthase activity (Grewe et al., 1992). These, and other, observations do not suggest any control of specific prostanoid production regulated at the level of expression of the specific isomerases. They are believed to be expressed in a cell specific manner which explains the production of different profiles of prostanoid production from various cell types, for example the differential capacity for astrocytes and
neurons to produce TXs and PGF₂₅₆ (reviewed by Murphy et al., 1988). In addition, it has been suggested that functional coupling may exist between various specific synthases and distinct pools of COX (Kudo and Murakami, 1999; Naraba et al., 1998; Brock et al., 1999). Although the capacity for control of the production of individual prostanoids may exist at this level, it is widely accepted that the predominant target for the regulation of the production of all of the prostanoids is upstream of the isomerases. Regardless of the end result, prostanoid synthesis depends on the presence of active COX and free AA substrate. Therefore the method by which the level of prostanoid release is controlled is generally accepted as being at the level of the PLA₂ and COX enzymes.

From the diverse array of functions ascribed to prostanoids in the normal and pathological maintenance of the CNS, it is clear that the control of the release of these mediators, and therefore the regulation of the expression and function of the COX enzyme, is fundamental to brain function. The CNS is one of the few tissues consistently shown to possess some basal COX-2 immunoreactivity (Yamagata et al., 1993; Breder et al., 1995; Peri et al., 1995). Most of the early work on COX-2 in the CNS focussed on the neuronal expression of the enzyme with a detailed study by Breder et al. (1995) reporting COX-2 immunoreactivity in dendrites and cell bodies of distinct populations of neurons confined predominantly to the cortex, hypothalamus, hippocampus and spinal cord. Despite being consistently expressed, COX-2 should not be considered to be constitutive since many investigations have shown the expression of COX-2 in neurons to be a dynamic process.
which varies with development and brain activity. COX-2 was confirmed to be the predominant isoform contributing to prostanoid production in the cortex and microvasculature of neonatal pigs, with a more even contribution of the two isoforms in juvenile animals (Peri et al., 1995), although COX-2 immunoreactivity was undetectable in embryonic brain (Yamagata et al., 1993). Kaufman et al. (1996) demonstrated that COX-2 immunoreactivity was localised to the dendrites of excitatory neurons, particularly the dendritic spines which are associated with synaptic signalling. This not only suggested a role for COX-2 and its prostanoid products in post-synaptic signalling, it also demonstrated the ability of COX-2 to be compartmentalised to a discrete subcellular location. Despite the many observations of COX-2 expression in normal CNS tissue, the purpose of the expression of this isoform in neurons remains a mystery. Experiments using COX-2 knockout mice have not revealed any vital role for the inducible isoform since no gross abnormalities of the brain have been detected (O'Banion, 1999). These early investigations failed to report any basal COX-2 immunoreactivity in glial cells despite many investigators claiming these cells to be the primary compartment for prostanoid production in the CNS based on their greater capacity for prostanoid synthesis when compared to neurons in culture (Bruner et al., 1993; Keller et al., 1985). More recently, the presence of COX-2 has been shown in astrocytes in normal neonatal pig brain (Degi et al., 1998) and adult rat brain (Hirst et al., 1999) suggesting that both astrocytes and neurons may contribute to the basal COX-2 expression detected in CNS tissue. The reasons for basal COX-2 expression by CNS cells are not clear and often overlooked, despite the fact that much of the reported work in the
field has focussed on the factors which modulate the levels of COX-2 in these cells both in culture and in situ.

The first demonstration of the modulation of COX-2 levels in the brain was through experiments in which electroconvulsive seizure resulted in rapid and transient increases in neuronal COX-2. This increased COX-2 reached maximal levels between 1 and 2 h following seizure and returned to basal levels by 24 h. The effect was abolished after the administration of MK-801, an NMDA receptor antagonist, indicating the involvement of NMDA receptor activation (Yamagata et al., 1993). Increased COX-2 has also been shown to be associated with ischaemic injury in the cortex of rats after middle cerebral artery occlusion and spreading depression. Again the induction of COX-2 was blocked by the administration of MK-801 and quinacrine suggesting the involvement of NMDA receptor and PLA₂ activity (Miettinen et al., 1997). The induction of COX-2 after ischaemia has been associated with those neurons most likely to undergo cell death following ischaemic insult (Miettinen et al., 1997) and the products of increased COX-2 expression have been reported to be deleterious to the ischaemic brain (Nogawa et al., 1997) with increased PGE₂ being associated with increased infarct volume. More recently the induction of COX-2 in human brain following middle cerebral artery infarction has been shown, with COX-2 immunoreactivity being present in neutrophils, vascular cells and neurons in the area of damage (Iadecola et al., 1999). The idea that COX-2 expression might contribute to neuronal vulnerability is given further credit by its involvement in the neuronal death which
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followed prolonged kainic-acid induced seizures (Chen et al., 1995; Tocco et al., 1997), and also the observation that inhibition of the up-regulated COX-2 increased the rate of survival of hippocampal neurons after global ischaemia in rats (Nakayama et al., 1998). COX-2 expression has been induced in the brain of animals after peripheral injection of LPS, but this was localised to endothelial cells and perivascular monocytes rather than neurons or astrocytes (Quan et al., 1998; Breder and Saper, 1996). These authors postulated that elevation of prostanoid levels throughout the brain caused by the induction of COX-2 in this way may modulate specific neural pathways such as those governing the febrile response.

One significant area of study in recent years has not been the basal levels of COX-2 detected for whatever reason in the brain, or the up-regulation of COX-2 in neurons following seizures or other brain activity, but the role of COX as a mediator of the inflammatory response in those cells of the CNS which respond to physical trauma or neurodegenerative diseases, that is the astrocytes and microglia. This response, known as reactive gliosis, involves an increase in the number of glial cells accompanied by their transformation into a reactive phenotype. The role of prostanoid synthesis in astrocytes and microglia as a pivotal feature of the brain's response to damage will be discussed after a short description of the general features of glial cells.

The discovery of the brain's non-neuronal cells is usually attributed to Virchow (1846) who used the term Nervenkitt, later translated to Neuroglia, to describe "a connective substance
formed in the brain and the spinal cord in which the nervous system elements are embedded.” Glial cell involvement has subsequently been implicated in many functions of healthy and damaged brain, but research into glial cells has always lagged behind that of neurons. The glial cell family consists of astrocytes, microglia and oligodendrocytes in the CNS, as well as the Schwann cells of the peripheral nervous system. Glial cells also exist in invertebrate nervous systems and their evolutionary conservation and diversification reinforces their importance in the advanced mammalian nervous system. The roles of oligodendrocytes and Schwann cells will not be discussed here, and this discussion will focus on the astrocytes and microglia.

**Astrocytes**

Astrocytes were first identified by W.L. Andriezen in 1893 who showed the presence of fibrous glia in white matter as being distinct from protoplasmic glia in grey matter. This was later substantiated by Ramón y Cajal who is credited with first using the name “astrocyte” to describe the distinctive star-shaped morphology of the cells. The understanding of the morphological diversity of astrocytes was advanced by the discovery of glial fibrillary acidic protein (GFAP) in the early 1970s which is the basis of the gliofilaments detected initially only in fibrous astrocytes but later also shown to be present in the protoplasmic phenotype. GFAP is widely used as a marker to distinguish astrocytes from neurons and other glial cell-types, but it is important to note that many astrocytes in normal CNS tissue, especially the
grey matter, are GFAP-negative. GFAP has been shown to be up-regulated in response to CNS damage and is now widely used as a marker for "reactive" astrocytes (Eng and Lee, 1995). Because of the difficulties associated with attempting to study the biology of these cells in the functioning brain, \textit{in vitro} studies have predominated in the course of research into astrocytes. Astrocytes in culture adopt features of reactive astrocytes, in particular the expression of GFAP (discussed in Levison and Goldman, 1993). Antibodies against gangliosides have shown the existence of two classes of astrocytes \textit{in vitro}. Raff \textit{et al.} (1983) showed that prenatal optic nerve astrocyte cultures (termed type 1 astrocytes) showed no immunoreactivity with the A2B5 antibody, whilst astrocytes cultured from postnatal optic nerve (type 2 astrocytes) showed strong immunoreactivity for the antibody, as did oligodendrocytes and a common precursor cell. Although these studies suggested a distinct pattern for the development of the two distinct classes of astrocytes in the CNS, type 2 astrocytes have not been shown to be present \textit{in vivo}. The lineage of astrocytes and oligodendrocytes, both of which are formed from neuroectoderm, has been the subject of much speculation over the course of two decades, but the picture which unfolds seems to be that \textit{in vitro} oligodendrocytes and astrocytes may be derived from a bipotential O-2A progenitor cell, whilst \textit{in vivo} post-natal O-2A cells only seem to generate oligodendrocytes suggesting an earlier functional divergence of the precursors for the two glial cell types. This is just one example of the factors which must be taken into consideration when attempting to interpret findings of glial cell culture experiments in terms of the behaviour of the equivalent cells \textit{in vivo}. Not only do the cells in culture lack the normal developmental
interactions with other cell types, it is quite possible that cell lines created are entirely artificial and share nothing with their in situ counterparts save a common progenitor.

**Microglia**

The origin of the other glial cell type implicated in reactive gliosis, the microglia, has also been the subject of much debate. Microglia comprise as much as 12% of the cells of the CNS and are distributed throughout the brain in an orderly manner, yet it is still unclear whether these cells originate from the neuroectoderm as do the other brain glia, or whether they form from haemopoietic stem cells. Since the first description of them by Rio-Hortega in the 1930s their origin and ontogeny has been the subject of much debate, mainly due to the difficulties presented to investigations by the lack of truly microglial-specific antibodies and the polymorphism and antigenic plasticity displayed by the various sub-populations of these cells. Proliferation of microglia is the earliest cellular response in reactive gliosis, but it is unclear whether the increased microglial population originates from migration of the parenchymal microglia which are resident in the CNS but have a relatively low turnover rate, or whether the reactive microglia are derived from the perivascular pools of microglia which have a high turnover rate and are believed to be replenished by monocytes from the peripheral circulation (González-Scarano and Baltuch, 1999).

After brain trauma microglia which have migrated to the site of injury show an activated
phenotype in which they express increased levels of major histocompatibility complex antigens and become phagocytic, much like macrophages responding to inflammation in other tissues. They release an array of inflammatory cytokines which serve to recruit other cells to the site of damage and amplify the inflammatory response, and can also release a number of potential neurotoxins such as TNFα implicating them as being involved in neuronal cell death subsequent to the initial trauma.

It has been established that resting microglia posses a ramified phenotype, and can exist in a hyper-ramified intermediate state following the mildest injury. Most neuronal injury however results in the ramified cells becoming reactive microglia with a somewhat bushy phenotype, which, following neuronal death, can take on the appearance of phagocytic brain macrophages in order to remove the dead cells (Streit et al., 1999). It is interesting to note that this functional plasticity is also apparent on a more chronic timescale during normal ageing in the human brain. Age seems to cause microglia to become progressively more activated, with a hyper-ramified state achieved by many cells by mid-life. By late life microglia become quite hypertrophic and begin to fuse into clusters believed to eventually result in the formation of senile plaques (Streit and Sparks, 1997). Microglia appear to respond differently to different injuries in vivo, apparently facilitating both neuronal repair and degeneration. Following facial nerve axotomy, activated microglia proliferate and tightly ensheath injured motoneurons which later survive, whilst following an irreversible neuronal injury the microglia instead become macrophage-like and phagocytose the dying neurons.
Despite their obvious involvement in the brain's inflammatory response, and their similarity to macrophages of the peripheral tissues, there has been little indication of any COX-2 immunoreactivity in microglia in the brain. The only evidence to date is Elmquist et al. (1997) who showed the up-regulation of COX-2 in perivascular microglia in the rat after injection with LPS. More recently, COX-2 immunoreactivity has been detected in reactive microglia from the brains of patients suffering from chronic cerebral ischemia, in particular Binswanger's disease, a condition characterised by cerebrovascular white matter degeneration and scattered lacuna infarcts. This investigation showed that microglia were the primary glial cell type expressing COX-2 in the white matter and cerebral cortex of diseased, and to a lesser extent, control brains (Tomimoto et al., 2000). In one of the few studies which has investigated the expression of COX-1 in the human brain following focal cerebral ischaemia, COX-1 was shown to be expressed by microglia in normal and infarcted brains (Schwab et al., 2000). Microglia expressing COX-1 accumulated in and around the infarction and persisted for several months post infarction, leading these authors to infer a paracrine role for microglial COX-1 metabolites in the modification of the post-ischaemic environment.

It is believed that astrocytes become activated in response to various cytokines, growth factors, serum factors and adhesion molecules which are released from injured neurons,
reactive microglia, endothelial cells, extravasated serum and other reactive astrocytes following brain injury (Eddleston and Mucke, 1993; Ridet et al., 1997). In particular, the proinflammatory cytokines IL-1\(\beta\) and TNF\(\alpha\), which are released from reactive microglia, have been implicated in this activation. Reactive astrogliosis is believed to follow the initial microglial response and, like microglia, these cells show a spectrum of adaptive plasticity with a heterogeneity of phenotypes apparently dependent on the nature of the injury and their proximity to it. Reactive astrocytes in the immediate vicinity of a lesion lead to anisomorphic gliosis, that is the formation of a permanent glial scar which distorts the structure of the tissue in the region, whilst distal astrocytes undergo isomorphic gliosis, due presumably to less severe changes in microenvironment, without the formation of a permanent scar. In an interesting comparison with the various degrees of plasticity observed in microglia, the reactive astrocytosis which follows axotomy and results in neuronal survival is of the isomorphic type (Ridet et al., 1997).

Since an up-regulation of COX-2 is invariably detected during peripheral inflammatory responses, and brain injury produces elevation of prostanoid levels as discussed previously, it is not surprising that reactive astrocytes have been shown to express high levels of COX-2. \textit{In vivo} studies have shown that reactive astrocytes up-regulate both COX-2 and cPLA\(_2\) following kainic-acid induced brain lesions in adult rats, and this up-regulation lasts for up to 11 weeks post-trauma (Sandhya et al., 1998). The increased levels of the two enzymes suggests that it is likely that these cells are responsible for the enhanced generation of
prostanoids described earlier for such injuries, and since the activities of these enzymes also produce reactive oxygen species and free radicals, it is possible that this pathway also contributes to the neurodegeneration previously attributed to the production of free-radicals after kainate lesions (Cheng and Sun, 1994). In another study, increased PGE₂ production was attributed to the reactive astrocytes which were implicated in the vacuolation following prion protein deposition in murine scrapie (Williams et al., 1997), although no attempt was made to investigate the expression of COX isoforms in these cells. More recently, an up-regulation of COX-2 mRNA and protein and a concomitant increase in PGE₂ synthesis was demonstrated in reactive astrocytes in the cerebellum of kainate lesioned rats (Hirst et al., 1999). In human brain collected from subjects who died two or more weeks after resuscitation, the expression of COX-2 following ischaemia-reperfusion was investigated. Results showed that in the late period following global ischaemia, hypertrophic astrocytes located at the margins of necrotic areas expressed COX-2, as did macrophages, polymorphic cells and leukocytes found at the centre of the necrosis (Maslinska et al., 1999). In another study using infarcted human brain, COX-2 immunoreactivity was shown to be present throughout the brain in both neurons and glia in accord with infarct duration (Sairanen et al., 1998). This observation caused the authors to suggest that whereas the early induction of COX-2 following focal ischaemia may lead to tissue damage through the production of prostanoids and free radicals, delayed induction in remote brain areas may promote repair and reconstitution. Interestingly, in a model of traumatic brain injury caused by fluid percussion in rats, increased levels of NF-κB were localised to both microglia and astrocytes.
Chapter 1: Introduction

This increased expression of the nuclear factor implicated in the up-regulation of COX-2 persisted for up to a year post-trauma and the immunoreactive glial cells were primarily distributed to the margins of areas of persistent necrosis (Nonaka et al., 1999). This investigation provides an insight into how the expression of COX-2 might be up-regulated in the brain following trauma, particularly since in neurons NF-κB expression was not detected after 2 weeks. The NF-κB expression in microglia was maximal between 48 h and 1 week post injury, whereas in astrocytes the most robust level of immunoreactivity was after 1 week, a finding remarkably consistent with the idea that microglia are responsible for the initial response to CNS injury with astrocytes becoming involved later.

One of the most significant findings in relation to the expression of COX isoforms in glial cells in terms of clinical implications was the extensive epidemiological data which correlated the chronic use of COX inhibitors with a reduced incidence of Alzheimer’s disease (Andersen et al., 1995; Breitner et al., 1995; Rich et al., 1995; McGeer et al., 1996). Along with the characteristic β-amyloid plaques and neurofibrillary tangles associated with the disease, a local inflammatory response has been implicated in the pathogenesis of the lesions (Kalaria, 1993). Immunohistochemistry has shown that reactive astrocytes form an outer shell around Alzheimer’s disease lesions with fibrils extending into the lesion, and microglia present around the core of insoluble amyloid fibrils (McGeer and McGeer, 1995). This evidence coupled with the release of proinflammatory prostanoids from astrocytes in vitro in response to β-amyloid (Landolfi et al., 1998) has led to much interest in the field of glial
inflammatory responses.

Inferences drawn from the studies into Alzheimer’s disease show the potential effects of COX inhibitors on glial prostanoid production. In addition, much speculation has taken place with regard to the theory that the CNS may contain a distinct isoform of COX which might account for the unexplained therapeutic effects of these and other drugs (Flower and Vane, 1972; Mitchell et al., 1994). Most of the detailed investigations into COX in astrocytes and microglia to date has been carried out in vitro due to the difficulties associated with manipulating distinct classes of glial cells in situ, leading to the accumulation of a large body of data implicating an array of factors in the activation, inhibition and expression of COX isoforms in these cells. Many studies have attempted to elucidate the COX isoform responsible for the production of prostanoids in glial cells and have shown the up-regulation of COX mRNA and protein in response to a variety of stimuli. The mere presence of COX mRNA and protein however does not necessarily reflect the prostanoid synthetic characteristics of cells, and fails to account for factors which regulate the activity of the expressed enzyme. With the availability of a range of COX inhibitors with apparent selectivity for the different isoforms, it should be possible to conduct a thorough investigation of the functional properties of the COX isoforms expressed in glial cells. Furthermore, the difficulty associated with obtaining homogeneous cultures of glial cells has meant that although the role of glia as a primary source of prostanoids in the CNS has been widely documented, the relative contributions of the different glial cell types are still far from
In this study, attempts were made to:

1. Explore the expression of COX isoforms in mixed glial cultures and purified astrocyte and microglial preparations both in terms of protein expression and functional contribution to prostanoid synthesis as determined pharmacologically using a range of COX inhibitors.

2. Investigate factors which may modulate the expression and function of the COX isoforms in glial cells, particularly the presence of serum and nitric oxide (NO).
Chapter 2: Materials and Methods
Glial Cell Culture

Mixed glial cell cultures from neonatal rat cerebral cortex were prepared according to the method of Dutton et al. (1981). Following decapitation of 1 to 2 day old rat pups, the cerebral cortices were removed, thoroughly cleaned of meninges and vasculature with fine forceps, and placed in a few drops of sterile disaggregation medium of the following composition: glucose (14 mM), bovine serum albumin (BSA) (3 mg/ml) and MgSO$_4$ (1.5 mM) in Ca$^{2+}$- and Mg$^{2+}$- free Earle's Balanced Salt Solution (EBSS, Gibco). The tissue was then coarsely chopped with a sterile scalpel and transferred to a flask containing 250 μg/ml trypsin in 10ml of disaggregation medium and placed in a shaking water bath at 37°C for 15 min. Ten ml of a solution containing soybean trypsin inhibitor (192 μg/ml), DNase (6.4 μg/ml) and MgSO$_4$ (240 μM) in disaggregation medium was then added and the resulting suspension transferred to 50 ml sterile plastic tubes and centrifuged at 100x g for 5 s (Denley, BS 400) to sediment cell bodies. The supernatant was removed and the cell pellet resuspended in a few drops of a solution containing soybean trypsin inhibitor (1.2 mg/ml), DNase (40 μg/ml), and MgSO$_4$ (1.5 mM) in disaggregation medium. The tissue was then mechanically dissociated by gentle trituration through a 1.5 mm diameter stainless steel cannula attached to a 5 ml sterile syringe, and the cells allowed to settle. The supernatant was removed and transferred to a sterile 15 ml plastic tube. This step was then repeated. The final cell suspension obtained was underlaid with a 4% (w/v) BSA solution in disaggregation medium, and the intact cells were pelleted through the BSA underlay by centrifugation at
100x g for 5 min. The supernatant, which contained cell debris, was removed and the cell pellet resuspended in a small volume of growth medium of the following composition: Minimum Essential Medium with Earle’s Salts (glutamine free, Gibco) supplemented with foetal calf serum (10% v/v), L-glutamine (2 mM), D-glucose (33 mM) and antibiotic/antimycotic solution with 10 000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B /ml (1% v/v, Sigma). The cell suspension was appropriately diluted in growth medium to give a seeding density of 100,000 cells per well, then seeded onto poly-D-lysine (50 μg/ml) coated 6-well (35 mm diameter) plates (Nunc). The cells were maintained for 14 days in vitro in a humidified atmosphere containing 5% CO₂ in air at 37°C. Growth medium was changed every 7 days.

**Microglial cell culture and astrocyte subculture**

Mixed glial cell cultures were prepared in T60 flasks (Greiner) and maintained in vitro for 14 days. Microglia were detached from the astrocyte monolayer by shaking the flasks for 2 h at room temperature on a rotary shaker (Luckham R100, 70 rpm), the medium was then collected and transferred to 6-well plates. The bathing medium from 5 flasks was used to prepare four 6-well plates. These microglial cultures were maintained for 3 days in vitro before being used in experiments.

The remaining cells in the T60 flasks were washed with sterile EBSS then incubated at 37°C
with 5 ml of a solution containing D-glucose (14 mM), MgSO$_4$ (2.25 mM), ethylenediamine-tetraacetic acid (EDTA, 0.6 mM) and trypsin (0.5 mg/ml) in EBSS. After 1 h, 10 ml of growth medium was added to stop the enzyme reaction, the cells were then harvested with a sterile cell scraper and transferred to 50 ml tubes. The cell suspension was gently triturated using a pipette then centrifuged for 5 min at 100x g and the supernatant discarded. The cell pellet was resuspended in an appropriate volume of growth medium containing 5 mM leucine methyl-ester to prevent the survival of remaining microglia (Giulian and Baker, 1986) then seeded onto poly-D-lysine (50 μg/ml) coated 6-well plates as for the primary glial cell culture. These astrocyte-enriched sub-cultures were then maintained *in vitro* for 7 days before being used in experiments.

**Immunofluorescence**

Cells were prepared as before then seeded onto poly-D-lysine (50 μg/ml) coated 13 mm diameter glass coverslips in 24-well plates. After being maintained *in vitro* for the length of time prescribed for the equivalent cultures prepared for experimentation, the presence of astrocytes and microglia in each of the preparations was examined by immunofluorescent detection of GFAP a specific astrocyte marker, or uptake of labelled low density lipoprotein by microglia.
GFAP - labelling

All procedures were carried out at room temperature. Growth medium was removed and cultures were washed three times with 0.5 ml volumes of PBS (1.5 mM KH$_2$PO$_4$, 154 mM NaCl, 5.1 mM Na$_2$PO$_4$) then fixed for 10 min in 0.5 ml PBS containing paraformaldehyde (4% w/v). Cells were washed as before then permeabilised for 5 min in 0.5 ml PBS containing 0.2% Triton X-100. After washing, the cells were blocked for 10 min in 0.5 ml PBS containing 5% (v/v) swine serum, washed, then incubated for 1 h in 0.5 ml PBS containing primary antibody (rabbit anti-cow GFAP, 1:300 dilution, DAKO). Cells were then washed in PBS as before and incubated for 30 min in 0.5 ml PBS containing the secondary antibody (anti-IgG-FITC, swine anti-rabbit, 1:30 dilution, DAKO). The cells were then washed, the coverslips then mounted on glass slides in Hydromount and viewed under fluoresceine optics.

Dil-Ac-LDL labelling

Growth medium was removed and the cells washed three times with 0.5 ml volumes of MEM then incubated for 4 h at 37°C in 0.5 ml MEM containing 10 μg/ml acetylated low density lipoprotein labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-perchlorate (Dil-Ac-LDL, Molecular Probes). Cells were washed with MEM as before then fixed for 10 min in 0.5 ml PBS containing 4% (w/v) paraformaldehyde. The cells were then washed in MEM, the coverslips then mounted on glass slides in Hydromount and viewed
under rhodamine optics.

In some cases cells were double labelled, first with Dil-Ac-LDL then, after fixation, with the GFAP-label as described above. In all cases cells were visualised by fluorescence microscopy (Nikon Microphot FXA microscope, objective magnification x10) using different barrier filters to select for fluoresceine or rhodamine wavelengths. The video signal from the microscope was digitised using Flashpoint software and captured into Paint Shop Pro 5.

**Treatment of cultures with serum-free medium**

Primary glial cell cultures were prepared as described previously. After at least 14 days the growth medium was removed and replaced with Dulbecco’s Minimum Essential Medium/Nutrient mix Ham’s F12 (1:1) (without glutamine, Gibco) supplemented with D-glucose (33 mM), L-glutamine (2 mM) and antibiotic/antimycotic solution (1% v/v, Sigma). Cultures were maintained *in vitro* for a further 4 days before use. In some cases the serum-free medium was then removed and replaced with growth medium and the cells were thereby maintained in the presence of serum for up to 14 days *in vitro*.

**Measurement of stimulated thromboxane B$_2$ release**

Cultures grown on 6-well plates were washed twice in warmed buffer of the following
composition: NaCl (116 mM), NaHCO$_3$ (26 mM), NaH$_2$PO$_4$ (1 mM), MgSO$_4$ (1.5 mM), KCl (5 mM), CaCl$_2$ (1.3 mM) and D-glucose (20 mM) pregassed with 5% CO$_2$ in O$_2$ then left to equilibrate in 2 ml of this buffer at 37°C for 20 min. In those experiments where inhibitors of prostanoid production were investigated, drugs were added to the bathing medium and incubated for 10 min. Prostanoid production was then initiated by addition of various drugs to the bathing medium and the incubations continued for a further 30 min. At the end of the incubation 1ml aliquots were removed from each of the wells and stored at -20°C prior to analysis. In some cases samples of the cells were scraped up in a small volume of cold buffer and sedimented into pellets by centrifugation for 5 min at 12 000 rpm (MSE Micro Centaur). The supernatant was discarded and the pellets were stored at -20°C for analysis of proteins.

All aliquots were assayed in duplicate for TXB$_2$, the stable metabolic product of TXA$_2$ using a specific radioimmunoassay (RIA) (Jeremy et al., 1985; 1986) with a detection limit of 0.01 ng/ml. To make a standard curve, a stock solution of 1 μg/ml TXB$_2$ was appropriately diluted to 10 ng/ml with chilled RIA buffer of the following composition: Tris (6.9 g/l) and gelatin (500 μg/l), buffered to pH 7.4. The stock was further diluted in RIA buffer to obtain standard solutions of the following concentrations: 5, 2.5, 1.25, 0.625, 0.312, and 0.156 ng/ml. Routinely, 100 μl of sample or TXB$_2$ standard were added to 3.5 ml polystyrene test tubes (Greiner) and diluted with 100 μl of chilled RIA buffer. The contents of each tube were mixed and incubated for approximately 18 h at 4°C with 100 μl rabbit anti-TXB$_2$ antiserum (Sigma) and 50 μl of 0.125 μCi/ml [³²H]TXB$_2$ (215 Ci/mmol, Amersham)
(approximately 10,000 dpm per sample). A duplicate non-specific binding (NSB) sample containing 300 µl RIA buffer and 50 µl \[^{3}H\]TXB\(_{2}\) was included in the incubation, as was a duplicate total binding (B\(_{o}\)) sample containing no sample or TXB\(_{2}\) standard.

At the end of the incubation period, 500 µl of chilled dextran-coated charcoal solution (1.0 g/l dextran and 8.0 g/l activated charcoal) in RIA buffer was added to each tube, mixed and allowed to equilibrate for 10 min at 4°C. Tubes were then centrifuged at 1000x g at 4°C for 5 min (Beckman GS-6KR), the supernatant was decanted into scintillation vials, and the radioactivity determined by liquid scintillation counting. Standard curves were compiled and fitted using GraphPad Prism software and unknown concentrations of TXB\(_{2}\) determined from the following formula:

\[
\% \text{ Binding} = \frac{\text{Counts in sample (dpm)} - \text{NSB}}{B_o \text{ (dpm)}} \times 100
\]

A representative standard curve is shown in Figure 2.1.

The standard curve was essentially linear for % bound values of between 100 and 25 % corresponding to between 0 and 1.5 ng/ml of TXB\(_{2}\). Any samples with higher concentrations of TXB\(_{2}\) were diluted so that the % bound fell within the linear portion of the curve and the resulting concentration value was then corrected for this dilution.
Chapter 2: Materials and Methods

Figure 2.1: Standard curve for the determination of TXB$_2$ concentration. Various concentrations of TXB$_2$ were obtained by dilution of 1 µg/ml stock TXB$_2$ in RIA buffer. There were assayed as described and the resulting % bound values used to create a standard curve for each separate assay.

In some cases the samples were assayed for the presence of PGE$_2$ using a similar RIA with the following differences. 300 µl of rabbit anti-PGE$_2$ (Sigma) was added and the samples incubated for 30 min at 4°C prior to a 1 h incubation at 4°C in the presence of 100 µl 0.5 µCi/ml $[^3]$H]PGE$_2$ (165 Ci/mmol, Amersham) (approximately 10,000 dpm per sample). After incubation, 0.2 ml cold dextran coated charcoal suspension was added before incubation and centrifugation as before. Analysis was carried out as for the TXB$_2$ assay using a standard curve derived from PGE$_2$ standard solutions over the range 0.15 to 5 ng/ml.
Chapter 2: Materials and Methods

Sources of drugs and reagents

Drugs and reagents used in these experiments were obtained from Sigma or BDH unless otherwise stated. NS398 and nimesulide were from Cayman Chemicals, ONO-RS-082 was from Cascade Biochemicals and SNOG was from Affiniti Research Products.

Electrophoresis and Western Blotting

Cell pellets were resuspended in 100 μl of phosphate buffered saline containing NaCl (550 mM), KCl (10 mM), Na₂HPO₄ (90 mM) and KH₂PO₄ (7 mM), buffered to pH 7.5 and the total protein concentration determined using the method of Bradford (1976) which determines the binding of the azo dye Coomassie Brilliant Blue G-250 to basic and aromatic residues of proteins. Upon binding the red form of the dye (absorbance maximum 470 nm) is converted to the blue form (absorbance maximum 595 nm) so detection of the level of absorbance at 595 nm gives an indication of the amount of protein present. This may be quantified by comparing test samples to standard samples of known protein concentration using a calibration curve. Coomassie Brilliant Blue dye solution was prepared by dissolving 30 mg of Coomassie Brilliant Blue G-250 in 100 ml of absolute ethanol. 50 ml of concentrated phosphoric acid was added and the solution diluted to 1 l with water and filtered to remove any undissolved dye.
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A calibration curve was determined using known concentrations of BSA. Varying concentrations of BSA from 1 to 6 μg per 100 μl of water were added to 1 ml of Coomassie Brilliant Blue dye solution and their absorbance at 595 nm measured using a spectrophotometer (LKB Biochrom Ultrospec II) against a blank sample containing 100 μl water with 1 ml Coomassie Brilliant Blue dye solution.

An appropriate amount of the unknown protein sample was diluted to 100 μl with water and mixed with 1 ml Coomassie Brilliant Blue dye solution and the absorbance at 595 nm measured against the blank. The concentration of the protein was adjusted by dilution with water such that it fell within the limits of the calibration curve, then a value was obtained for the protein concentration in the original sample by correcting the value obtained from the calibration curve by the dilution factor. This allowed a known concentration of protein to be loaded into each well of the electrophoresis gel.

*SDS - Polyacrylamide gel electrophoresis*

Polyacrylamide gels are useful supporting media for electrophoresis since they are chemically inert and can be readily formed in a range of pore sizes by varying the concentration of acrylamide and methylene bis-acrylamide. The anionic detergent sodium dodecyl sulphate (SDS) disrupts non-covalent interactions in native proteins and renders the negative charge on the proteins insignificant since that acquired on binding is much greater. Therefore SDS-PAGE is a means to separate proteins purely on basis of size with the smaller molecules
travelling fastest through the gel.

Gel electrophoresis was carried out according to Laemmli (1970) using an SDS Tris-glycine discontinuous buffer system. A 10 % acrylamide vertical slab gel (6 cm x 8 cm) was prepared with 30 ml running gel containing acrylamide/bis-acrylamide 35.5:1 (10 ml), water (12.5 ml), N, N, N', N'-tetramethylethylenediamine (TEMED) (15 µl) and 7.5 ml buffer consisting of Tris buffered to pH 8.8 (1.5 M), EDTA (8 mM) and SDS (0.4%) and degassed under vacuum for 30 min. TEMED caused cross-linking of the acrylamide monomers in a process which was catalysed by the addition of 10% (w/v) ammonium persulphate (150 µl) just prior to casting. The gel was cast vertically between glass plates (which had been cleaned with ethanol and acetone then dried) using the BioRad casting stand to maintain the shape of the gel until it had set. The gel was poured to a height of 6 cm and a thin layer of hydrated butanol was applied to the top surface of the resolving gel at this stage to prevent meniscus formation during casting. After 45 min the hydrated butanol was washed from the surface of the polymerised resolving gel, the stacking gel was poured onto the surface and a 10 well comb placed at the top. The stacking gel (4 ml) consisted of acrylamide/bis-acrylamide 37.5:1 (0.65 ml), water (2.3 ml), TEMED (5 µl), 0.5 M Tris buffer at pH 6.8, EDTA (8 mM) and SDS (0.4% w/v) and was degassed under vacuum for 15 min before the addition of 10% (w/v) ammonium persulphate (80 µl). After 10 min the stacking gel was set and the comb could be removed, the gel was then placed in the electrophoresis assembly according to the manufacturers instructions (mini-Protean II system, BioRad). The electrophoresis reservoirs were filled with electrode buffer containing
Tris-base pH 8.8 (6 g/l), glycine (28.8 g/l), EDTA (0.675 g/l) and SDS (1 g/l).

Five μg protein samples were prepared in 1.5 ml plastic tubes and combined with water and sample buffer to give a 15 - 25 μl preparation for loading with the following final composition: NaH₂PO₄ (10 mM), glycerol (10% v/v), bromophenol blue (0.017 % w/v) and SDS (2.5 % w/v) in PBS pH 7.0. Dithiothreitol (10 % w/v) was added and the samples were vortex mixed, boiled for 5 - 10 min, then allowed to cool prior to loading into the wells. 20 μl buffer solution was loaded into any empty wells to maintain consistency of sample migration within the gel. Protein standards of known molecular weight were included in each experiment. 1 μl of biotinylated protein standard sample (Amersham) was diluted in 10 μl sample buffer with 2-mercaptoethanol (5 % v/v) then boiled and loaded in the same way as the protein samples. The protein standards used were of the following molecular weights (kDa) 97.4, 68, 46, 31, 20.1 and 14.4. In some cases commercially purified standard COX-1 and COX-2 proteins (both from ovine origin, Cayman Chemical) were included in the electrophoresis. These were initially used at a concentration of 0.5 μg per lane as suggested by the supplier, although it was subsequently found that 50 ng was sufficient to produce a detectable band, and were prepared for loading following the same procedure as the test samples. A voltage was applied across the gel (150 V) and the samples were allowed to move through the gel until the visible band of blue dye reached the bottom of the resolving gel. The resolving gel was then removed from the glass plates and immediately used for immunoblotting.
Western Blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane by Western blotting using a semi-dry transfer cell with a platinum-coated titanium anode and a stainless steel cathode (BioRad “Trans-Blot SD”). Initially both the gel and the nitrocellulose membrane (Amersham “Hybond ECL”, 6 x 8 cm sheets) were allowed to equilibrate for 15 min at room temperature in transfer buffer: Tris-base pH 8.5 (25mM), glycine (192 mM) and methanol (20% v/v) in water. Two pieces of thick filter paper (10cm x 7.5 cm x 0.3 cm) were thoroughly soaked in transfer buffer and used to sandwich the gel against the nitrocellulose membrane between the electrodes of the transfer cell as shown in Fig 2.2.

![Diagram of the semi-dry transfer cell used for Western blotting.](image)

Care was taken to ensure that there were no air bubbles trapped between any of the layers which would have resulted in inconsistencies in transfer. It was also important to avoid any contamination of the nitrocellulose membrane with exogenous proteins so handling was
minimised and gloves were worn. Protein was transferred from the gel to the nitrocellulose membrane at 10 V for 30 min.

Immunochemical detection

Immediately after transfer, the non-specific binding sites on the nitrocellulose membrane were blocked by overnight incubation at 4°C in 5% (w/v) dried non-fat milk in PBS-T which contained 0.1% polyoxyethylenesorbitan monolaurate (Tween 20) in PBS. After blocking, the nitrocellulose membrane was washed twice with PBS-T, washed for 15 min then twice for 5 min on an orbital shaker using fresh PBS-T each time. Sufficient liquid to allow complete immersion of the nitrocellulose membrane was used in all washes and incubations (minimum 10 ml). The membrane was incubated with a primary antibody specific for either COX-1 (Cayman Chemical, mouse anti-ovine monoclonal antibody, 5 μg/ml) or COX-2 (Transduction Laboratories, mouse anti-rat monoclonal antibody, 0.625 μg/ml) in PBS-T for 1 h at room temperature on an orbital shaker. After washing as before the membrane was incubated with a horseradish peroxidase (HRP) conjugated secondary antibody (Transduction Laboratories, goat anti-mouse IgG polyclonal antibody, 0.2 μg/ml), which showed cross-reactivity with both primary antibodies for 1 h at room temperature on an orbital shaker. Streptavidin-HRP conjugate (Amersham, 0.1% v/v) was included in this incubation to allow later detection of the biotinylated protein standards. After washing as before, the immobilised protein samples and standards were detected via their HRP labelling using the Amersham ECL™ system, the principles of which are outlined in Fig 2.3.
Figure 2.3: Principles of Western blot analysis using enhanced chemiluminescence.

In this system the HRP which is attached to the proteins via the antibodies acts as a catalyst for the oxidation of luminol, a process which subsequently emits small but sustained quantities of light. This light was specifically enhanced up to 1000 fold by the other constituents of the Amersham ECL reaction mixture which allowed detection by exposure of the nitrocellulose membrane to Polaroid™ film in an ECL mini camera (Amersham) for up to 15 min. After exposure nitrocellulose membranes were stored moistened with PBS-T and sealed within PVC film at 2 - 8°C for future re-probing.

Stripping and re-probing membranes

In some cases it was necessary to probe a single nitrocellulose membrane with antibodies specific for both COX-1 and COX-2. The entire immunochemical detection procedure described above was carried out using antibody specific for one isoform, then the membrane was stripped of all antibodies and probed in the same way for the other isoform. In these
cases the membrane was first incubated at 50°C for 30 min with occasional agitation in
stripping buffer containing 2-mercaptoethanol (100 mM), SDS (2% w/v) and Tris (62.5
mM) buffered to pH 6.7. The membrane was then washed for 2 x 10 min in large volumes
of PBS-T and blocked in 5% (w/v) non-fat dried milk in PBS-T for 1 h at room temperature.
Incubation with ECL detection reagents and exposure to film at this stage ensured the
removal of antibodies. The immunodetection protocol was then performed as described
previously using the other primary antibody.

*Western blotting - preliminary studies*

Initial experiments were performed in order to establish the parameters described here for
optimal detection of COX protein. Antibodies were generally used according to the
concentrations suggested by the manufacturers, although in all cases a range of alternative
titres were investigated and found to be less effective (data not shown). In order to allow
comparison between samples of cells after various treatments it was important to load an
equivalent amount of material in each lane as determined by normalising the samples
according to their total protein content. In order to establish an appropriate concentration
of total cellular protein which would provide a detectable amount of COX, various amounts
of protein from normal mixed glial cell cultures were loaded onto a single gel and detected
for using the anti-COX-2 antibody as shown in Fig 2.4. A clear band indicating the presence
of COX-2 protein migrated to a similar position as the 68 kDa molecular weight marker in
most of the samples. COX-2 immunoreactivity was detected in all of the samples containing
Figure 2.4: Western blot to establish the optimal amount of cellular protein to load for use in the detection of COX-2. Various amounts of normal mixed glial cell samples were loaded into the wells to give the following protein concentrations (in μg per well): (1) 0.2, (2) 0.39, (3) 0.78, (4) 1.56, (5) 3.13, (6) 6.25, (7) 12.5, (8) 25, (9) 50.
between 0.78 and 50 μg of protein, whilst in the samples containing less than 0.78 μg of protein the COX-2 level was below the limit of detection. The presence of a faint band of COX-2 immunoreactivity in the higher concentration samples was probably due to the migration of some COX-2 in its native dimeric conformation. From this experiment it was decided that 5 μg of total protein would give a clear indication of the presence of COX-2 in future experiments.

Attempts at stripping and re-probing membranes in order to allow the detection of COX immunoreactivity with antisera specific for COX-1 or COX-2 are shown in Fig 2.5. The protocol used, which was based on that supplied by Amersham, seemed to result in a substantial reduction in immunoreactivity upon re-probing when compared to the levels apparent in the initial detection. This effect was observed regardless of the order in which the two antisera were used and suggested that the stripping procedure, which was designed to remove antibody from the immobilised protein, resulted in modification or detachment of the protein which was bound to the membrane. In the light of these findings this process was deemed to be unsuitable as a means of comparing the levels of COX-1 and COX-2 immunoreactivity present on a single nitrocellulose membrane.
Figure 2.5: Stripping and re-probing Western blots. A and B are images of the same membrane probed first with anti-COX-2 antiserum (A) then stripped and re-probed with anti-COX-1 antiserum (B). Lanes contained 5 μg protein from (1) mixed glia, (2) subcultured astrocytes, (3) microglia, (4) COX-2 standard, (5) COX-1 standard, (6) molecular weight marker proteins. B and C are images of another membrane probed first with anti COX-1 antiserum (C) then stripped and re-probed with anti-COX-2 antiserum (D). Lanes contained protein from mixed glial cultures (1) 5 μg, (2) 15 μg, (3) 30 μg, (4) 5 μg, (5) molecular weight marker proteins, (6) 50 ng COX-1 standard, (7) 50 ng COX-2 standard.
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Introduction

The role of glia as a primary source of prostanoids in the CNS has been widely documented, but the specific contributions of the different glial cell types, and the relative contributions of the different COX isoforms therein, are not entirely clear. Investigators of glial cell types in vitro have long argued that the role of prostanoid production be attributed either to astrocytes or to microglia. Many have shown that cultured astrocytes are capable of prostanoid synthesis and release (Keller et al., 1987; Seregi et al., 1987; Murphy et al., 1988; Petroni et al., 1991), while more recently, others have suggested that it is in fact microglia that are responsible for the prostanoid forming capacity of these cultures (Matsuo et al., 1995, Giulian et al., 1996). Both Matsuo et al. (1995) and Giulian et al. (1996) argue that previous work in the field has overlooked the fact that microglia invariably contaminate (5-20 % of total cell numbers) what others have assumed to be predominantly astrocyte preparations, and they suggest that upon closer examination these microglia can be demonstrated to be the predominant source of prostanoids in the resulting cell cultures. However, the former group failed convincingly to show the production of prostanoids by microglia, basing their hypothesis instead on the apparent lack of visible microglia in astrocyte cultures which failed to produce prostanoids in response to stimuli. On the other hand, the latter group used extremely long incubation periods which failed to show clearly the production of prostanoids immediately following stimulation, thus the issue of which glial cell type is responsible for the production of prostanoids in vitro remains to be elucidated fully.
Another area which has provoked much debate has been the identification of the isoform of COX responsible for the production of prostanoids by glial cells. The CNS is known to contain the inducible isoform, COX-2, in the absence of the stimuli required for its expression in the periphery (Yamagata et al., 1993; Breder et al., 1995; Kaufmann et al., 1996), a phenomenon which was originally thought to be due to neuronal expression but has more recently been suggested to be due also to the presence of COX-2 in glia. In vivo experiments have detected the presence of COX-2 immunoreactivity in normal neonatal porcine astrocytes (Degi et al., 1998), kainate lesioned rat hippocampal astrocytes (Sandhya et al., 1998) and both normal and lesioned rat cerebellum and cerebral cortex (Hirst et al., 1999). Interestingly, the presence of COX-2 in microglia in vivo has been detected only after injection of LPS (Elmquist et al., 1997), and others have failed to detect any expression of COX-2 by microglia in normal and lesioned rat brain (Hirst et al., 1999) although some microglial COX-2 expression has recently been demonstrated in normal and diseased human brain (Tomimoto et al., 2000). Despite the expression of COX-2 under basal conditions in astrocytes in vivo, most investigations have focussed on demonstrating that this expression may be greatly up-regulated by a wide range of activators including pro-inflammatory cytokines, physical trauma and various disease states. It has also been shown that these activators confer a reactive phenotype on astrocytes and microglia which has been argued to be their status under normal culture conditions (McMillian et al., 1994; Kimelberg, 1998), so it would be expected therefore that glial cells might express elevated levels of COX-2 under normal culture conditions in the absence of any further stimulus.
COX-2 protein has been detected in both cultured astrocytes (Hirst et al., 1999; Koyama et al., 1999; O'Banion et al., 1996; Luo et al., 1998), and microglia (Fiebich et al., 1996; Minghetti et al., 1997; Bauer et al., 1997; Slepko et al., 1997) but these investigations have mainly focussed on exploring the up-regulation of COX-2 by various stimuli. All of them have detected some low level basal COX-2 expression under normal culture conditions, but interestingly only one investigation showed the presence of COX-1 (Luo et al., 1998) in astrocytes, the others presumably accepting the presence of this isoform as inevitable given its constitutive nature in other cell types. The expression of COX-1 by microglia has been implicated as being important in the response of the human CNS to focal cerebral ischaemia (Schwab et al., 2000). Microglial COX-1 expression was also mentioned by Slepko et al. (1997) who reported that whilst its expression was not altered by the effects of LPS, there was a slight increase in the level of COX-1 in those microglia which had been maintained in culture for 7 to 14 days and which appeared to resemble morphologically the activated microglia found in vivo (Slepko and Levi, 1996). This group also showed that under resting conditions, stimulation of microglial COX activity with LPS caused a greater release of TXB₂ than PGE₂. This effect was reversed in more “activated” cells with an increase in COX-2 expression leading to an enhanced production of PGE₂ whilst TXB₂ expression remained constant, leading to the hypothesis that TXB₂ synthase was unable to cope with the increased levels of PGH₂ thus providing increased substrate for PGE₂ synthase (Slepko et al., 1997).

Many of the studies which have been undertaken to explore COX-2 in glial cells have
used an approach whereby COX-2 expression is detected using techniques such as Northern blotting, RT-PCR or in situ hybridisation which measure mRNA levels, these data being corroborated by the detection of COX-2 protein using immunoblotting with COX-2 specific antibodies. To date none of these investigations have undertaken to investigate changes in functional COX activity which are the presumed outcome of increased COX-2 expression.

In this study a variety of inhibitors were selected from the ranks of the NSAIDs in order that the relative contribution of each COX isoform could be investigated fully. Aspirin was used as a classical COX-1 selective inhibitor whose mechanism of action has been well established. It inhibits COX-1 in intact cells at micromolar concentrations which accounts for its antithrombotic abilities as an inhibitor of platelet TXA₂ release, but it only inhibits COX-2 at much higher concentrations (Mitchell et al., 1994), although in certain assay systems aspirin has been shown to inhibit COX-2 with a potency similar to that established as being selective for COX-1 (Giuliano and Warner, 1999). Two non-selective COX inhibitors, piroxicam and indomethacin, which show greater selectivity for COX-1 than COX-2 were also used. Piroxicam is one of the most potent NSAIDs (Carty et al., 1980; Fröhlich, 1997) which, together with indomethacin, has been used clinically in the treatment of inflammation. Ibuprofen was chosen as another example of a non-selective inhibitor which is commonly used in the relief of pain and inflammation and has been shown to have little preference for either isoform of COX (Mitchell et al., 1994). Nimesulide and NS398 were chosen as readily available and
widely used experimental inhibitors of COX-2. Nimesulide has been shown to have between 5 and 16 fold selectivity for COX-2 in various studies (Hawkey, 1999; Tavares et al., 1995). NS398 is a highly selective COX-2 inhibitor which is commonly used as the archetypal blocker of this isoform (Koyama et al., 1999; Brambilla et al., 1999). It was one of the first COX-2 selective compounds to be identified and has been shown to be 80- to 1000- fold more selective for COX-2 than COX-1 (Gierse et al., 1995). The potencies reported for these drugs, as determined in different assay systems, tend to vary, and often they can only be considered to be selective for one or the other isoform at low concentrations. If high concentrations of any of the preferential or selective drugs are used they have the ability to inhibit either isoform indiscriminately, and in many cases clinical doses of selective inhibitors are believed to result in tissue concentrations of drugs which cannot be considered to be acting selectively (Hawkey, 1999). This has often been overlooked, particularly by researchers attempting to selectively block COX-1 or COX-2 in vitro, where a single high concentration of an inhibitor is chosen which has been shown to block selectively an isoform in one particular assay system, whilst that same concentration may be considered high enough to inhibit both isoforms in other assays (Brambilla et al., 1999; Koyama et al., 1999; Hewett, 1999; Janabi et al., 1996). In the light of these caveats, the approach used in this investigation required that a full inhibition profile be obtained for each of the drugs thus allowing a reliable comparison of their potencies.

In order to stimulate the prostanoid synthetic cascade, cells were treated with either AA
or the calcium ionophore A23187. AA is the substrate for COX so adding exogenous AA bypasses PLA₂ and results in an increase in COX activity and subsequent elevation in the level of prostanoid production. A23187 has been used widely as a stimulant of prostanoid synthesis in cultured cells (Pressman, 1976; Murphy et al., 1985; Keller et al., 1987; Petroni et al., 1991). It causes an increase in intracellular Ca²⁺ concentration which leads to the activation of PLA₂ which, in turn, mobilises AA from membrane phospholipid stores in a way not dissimilar to the physiological cascade. In order to investigate prostanoid synthesis in response to these stimuli, the level of TXB₂ released by cultured cells was measured by radioimmunoassay. TXB₂ is the stable metabolite of TXA₂ and is the prostanoid of choice since not only is it known to be one of the major products of glial prostanoid synthesis both in vivo and in vitro (reviewed by Murphy and Pearce, 1988), it is not released by meningeal cells which are a minor contaminant of primary glial cultures (Murphy et al., 1985).

In order to address the issue of how the different cell types contribute to COX activity in mixed glial cell cultures, derivative cultures were obtained in an attempt to isolate purified preparations of astrocytes and microglia. These were first characterised by immunofluorescence microscopy in order to determine their homogeneity compared with the mixed glia, then the relative levels of COX protein in each cell type were investigated by Western blotting. The panel of inhibitors described above was used to examine the functional contribution of the different COX isoforms to TXB₂ synthesis in mixed glial cell cultures, astrocytes and microglia.
Results

Detection of COX isoforms in mixed glia by Western blotting

Before attempts were made to detect the presence of COX isoforms in glial cell cultures it was important to ensure the specificity of the antibodies for COX-1 and COX-2 protein. The commercially available antibodies did not distinguish between purified COX-1 and COX-2 standards, also obtained from commercial suppliers, from ovine sources as shown in Fig 3.1 (a) and (b). In addition, both antibodies cross-reacted with COX protein in a preparation of mouse macrophages which was, according to the suppliers, supposed to contain only COX-2 (Fig 3.1; c). The difference in the position of the ovine COX-1 and COX-2 bands reflects their different molecular weights (70 kDa for COX-1, 72 kDa for COX-2). This difference in their relative positions confirmed that the observed lack of specific detection was due to the antibodies cross-reacting with both isoforms rather than contamination between the two standards. The information supplied with the COX-1 antiserum suggested that there may be some cross-reactivity with ovine COX-2 but not with murine or rat COX-2. Since this antibody also cross-reacted with the mouse macrophage sample supplied as a COX-2 positive control, it was likely that this antibody would show cross-reactivity with COX-2 from species other than sheep. These observations suggested that both the COX-1 and COX-2 antibodies used in this investigation, and used in previous work (Luo et al., 1998), could not be relied on to distinguish between the isoforms. Therefore antibodies were used in these experiments
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Fig 3.1: Western blot characterisation of COX antibody specificity. In (A) and (B) lane 1 contained molecular weight marker proteins, lanes 2 and 3 contained 5 μg of COX-1 and COX-2 standard protein respectively. COX immunoreactivity was detected with anti-COX-1 (A) or anti-COX-2 (B) antiserum. In (C) lanes 1 and 3 contained 5 μg COX-1 standard protein, lanes 2 and 4 contained 5 μg COX-2 standard protein. COX immunoreactivity was detected with anti-COX-1 (lanes 1 and 2) or anti-COX-2 antiserum. Lane 5 contained molecular weight marker proteins.
merely to detect the presence of COX protein and not to explore the relative expression of one or the other isoform. Fig 3.2 shows the presence of a clearly visible band of protein in mixed glial cell cultures with immunological reactivity detectable with the anti-COX-2 antibody. The molecular weight is equivalent to that of the ovine COX-2 standard. This would suggest the presence of COX-2 protein in the mixed glial cells but since the COX-2 antibody used was shown to cross-react with COX-1 protein, and both isoforms are known to have similar molecular weights, it is not possible to state unambiguously that glia possess one or the other, or indeed both, COX isoforms.

Stimulus-evoked release of TXB$_2$ from glial cell cultures

In order to establish appropriate parameters for later experiments, a number of preliminary experiments were undertaken. Fig 3.3 shows that A23187 was able to elicit a concentration-dependent release of TXB$_2$ from glial cell cultures within the range of concentrations used. A maximum of 1000 % stimulation over basal was achieved at 10 μM and although saturation of the response was not evident, a sub-maximal dose of 3 μM was selected as an appropriate concentration for the investigation of the time-course of release shown in Fig 3.4. TXB$_2$ accumulation increased rapidly for the first 15 min then reached a maximum by 30 min. It was this 30 min incubation which was deemed to be an appropriate length for the A23187-induced release of TXB$_2$ to be used in subsequent experiments. Fig 3.5 shows the concentration-dependent release of TXB$_2$ in response to AA. This stimulus caused a maximal stimulation of approximately 800 %.
Figure 3.2: Western blot analysis of COX protein in mixed glial cell cultures. Lanes contained (1) 5 µg protein from mixed glial cells, (2) 50 ng COX-2 standard protein, (3) molecular weight marker proteins. COX immunoreactivity was detected by incubation with anti-COX-2 antiserum. This blot is representative of results obtained in three experiments.
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Figure 3.3: Concentration-dependence of A23187-stimulated TXB$_2$ release from cultured glia. Results show the release of TXB$_2$ elicited by 30 min incubations with various concentrations of A23187 as a % over basal release. Basal release over 30 min was 0.39 ± 0.04 ng/ml. Data are means ± SEM of at least 3 determinations.
Figure 3.4: Time course of A23187-stimulated TXB$_2$ release from cultured glia. Cultures were incubated for various periods in the presence or absence of 3 μM A23187. Data are means ± SEM of at least 3 determinations.
Figure 3.5: Concentration-dependence of AA-stimulated TXB$_2$ release from cultured glia. Results show the release of TXB$_2$ elicited by 30 min incubations with various concentrations of AA as a % over basal release. Basal release over 30 min was 0.58 ± 0.02 ng/ml. Data are means ± SEM of 4 determinations.
over basal at a concentration of 30 μM. The estimated EC$_{50}$ value was 10 μM which was a suitable dose to use to elicit a sub-maximal response in subsequent experiments. This dose was used in the time-course investigation shown in Fig 3.6 which shows a linear increase in TXB$_2$ formation over the 30 min incubation period.

The mode of action of A23187 was explored further by investigating the dependence of stimulated release on the presence of extracellular Ca$^{2+}$ and the action of PLA$_2$. Fig 3.7 shows that upon removal of Ca$^{2+}$ from the bathing medium, both basal and A23187-stimulated production of TXB$_2$ were reduced by 51 % and 87 %, respectively. Fig 3.8 demonstrates the concentration-dependent reduction of the A23187-stimulated response in the presence of the PLA$_2$ inhibitors quinacrine and ONO-RS-082. Both inhibitors reduced the response but interestingly ONO-RS-082 showed a much steeper inhibition profile with an apparent IC$_{50}$ of 25 μM, whilst quinacrine (estimated IC$_{50}$ = 1.4 μM) had a more shallow inhibition profile which appeared to level off at approximately 70 % inhibition. Neither of these inhibitors had a significant effect on the response due to stimulation with AA as shown in Fig 3.9, although incubation with 30 μM ONO-RS-082 alone for 40 min caused a slight (12 %) stimulation, whilst 30 μM quinacrine caused a 20 % reduction in basal TXB$_2$ release.
Figure 3.6: Time course of AA-stimulated TXB₂ release from cultured glia. Cultures were incubated for various periods in the presence or absence of 10 μM AA. Data are means ± SEM of at least 3 determinations.
Figure 3.7: Ca\(^{2+}\)-dependent nature of A23187-stimulated TXB\(_2\) release from cultured glia. Cultures were incubated for 30 min in the presence or absence of 3 µM A23187 in Ca\(^{2+}\)-containing medium or Ca\(^{2+}\)-free medium supplemented with 0.2 mM EGTA. Data are means ± SEM of three determinations.
Figure 3.8: Inhibition of A23187-stimulated TXB\(_2\) release from cultured glia by PLA\(_2\) inhibitors. Cultures were incubated in the presence of various concentrations of quinacrine or ONO-RS-082 for 10 min before the addition of 3 \(\mu\)M A23187 for 30 min in the continued presence of inhibitor. Data are means ± SEM of four determinations and are expressed as a % of the response elicited by A23187 in the absence of drug. Basal and A23187-stimulated TXB\(_2\) levels were 0.29 ± 0.05 ng/ml and 2.70 ± 0.07 ng/ml, respectively (n = 6).
Figure 3.9: Effect of PLA₂ inhibitors on AA-stimulated TXB₂ release from cultured glia. Where appropriate, cultures were incubated with 30 μM quinacrine or ONO-RS-082 for 10 min prior to incubation with 10 μM AA or vehicle for 30 min in the continued presence of inhibitor. Data are means ± SEM of 3 determinations.
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Immunofluorescence

The mixed glial cultures were examined by immunofluorescence microscopy for the presence of astrocytes and microglia. Astrocytes were detected by immunofluorescent labelling of GFAP which was detectable under fluoresceine optics. Microglia were labelled with an internalised low-density lipoprotein (Dil-Ac-LDL) which was detectable with rhodamine optics. The mixed glial cell cultures contained a confluent layer of astrocytes with an approximate cell density of 1000 cells/mm$^2$, on the surface of which there were 60-120 microglia/mm$^2$. These values are very approximate since there was difficulty distinguishing individual astrocytes due to their highly confluent flattened phenotype, and there was a good deal of variation in the numbers of microglia between the various fields of view. Representative examples of mixed glial cell cultures are shown in Fig 3.10 and the microglia typically comprised between 5 and 15 % of total cells in any given area. Fig 3.11 shows double-labelled images of two fields viewed under either fluoresceine or rhodamine optics. The background light detected under rhodamine optics in this figure is due to the leeching through of fluorescence signal from the underlying cells labelled for GFAP and does not represent uptake of Dil-Ac-LDL by astrocytes since it is not apparent in single-labelled samples (Fig 3.10; B, D and F). It is notable that the GFAP-positive astrocytes in Fig 3.11 appear to have taken on a more stellate morphology than their single-labelled counterparts. This is likely to be a result of the 4 h incubation in serum free medium which was necessary to allow accumulation of Dil-Ac-LDL in the microglia. Serum deprivation (Chapter 4) was found to cause a similar
Figure 3.10: Fluorescence photomicrographs of mixed glial cultures. Panels A, C and E show representative fields of cells labelled with an antibody against GFAP and viewed under fluoresceine optics. Panels B, D and F are representative fields of the same cultures incubated with Dil-Ac-LDL then viewed under rhodamine optics. Images were taken at x 10 magnification. The scale bar = 100 μm.
Figure 3.11: Double label fluorescence photomicrographs of mixed glial cultures. Panels A and B show a field of cells viewed under fluoresceine (GFAP) and rhodamine (Dil-Ac-LDL) optics, respectively. Another field of cells viewed in the same manner is shown in panels C and D. Images were taken at x 10 magnification. The scale bar = 100 μm.
change in morphology which was apparent during routine inspection of cultures using phase contrast microscopy, but after approximately 24 h these cells returned to their previous protoplasmic morphology.

In subcultured astrocytes which were grown in the presence of leucine methyl-ester, the monolayer of GFAP-positive astrocytes persisted whilst no microglial labelling was apparent after incubation with Dil-Ac-LDL (Fig 3.12). Interestingly a small number of cells in the subcultured astrocyte preparation (as low as 4 - 5 cells per 25 mm\(^2\)) were observed to have taken up the microglial marker, but these cells were much larger than the microglia normally found to be present in glial cell cultures and had a number of short processes (images not shown). Conversely, the microglial cell cultures contained approximately 150 - 200 cells/mm\(^2\) which were positive for the microglial marker and were devoid of any GFAP-positive cells (Fig 3.13). The cultures used in the immunofluorescence investigations were prepared in an identical manner to those used in the Western blotting and pharmacological procedures and were observed to be of an equivalent composition when viewed under phase contrast optics, with no distinguishable cells present other than those which displayed labelling with one or the other marker.
Figure 3.12: Fluorescence photomicrographs of astrocyte-enriched cultures. Panels A and B show representative fields of cells labelled with an antibody against GFAP and viewed under fluoresceine optics. Panels C and D show a field of cells double labelled with an antibody against GFAP and Dil-Ac-LDL viewed under fluoresceine (GFAP) and rhodamine (Dil-Ac-LDL) optics, respectively. Another field of cells viewed in the same manner is shown in panels E and F. Images were taken at x 10 magnification. The scale bar = 100 μm.
Figure 3.13: Fluorescence photomicrographs of microglia-enriched cultures. Panels A-D show representative fields of cells labelled with Dil-Ac-LDL and viewed under rhodamine optics. Panel E shows a representative field of the same culture labelled with an antibody against GFAP and viewed under fluoresceine optics. Images were taken at x 10 magnification. The scale bar = 100 μm.
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Detection of COX in astrocytes and microglia

Fig 3.14 shows the presence of protein which was immunoreactive with COX-2 antibody in each of the enriched preparations alongside that detected in mixed glia. Although blots such as these cannot be considered to be quantitative, it is clear that the band visible in the microglial lane is fainter than that in the astrocyte lane, which in turn is fainter than the band seen in the mixed glial lane, despite equivalent amounts of total protein being loaded in each case. This would suggest the presence of more COX-2 immunoreactive protein in the mixed glia than in either of the enriched cultures, and also more COX-2 immunoreactivity in astrocytes than in microglia. Since the COX-2 antibody was shown to cross-react with COX-1 standard protein (Fig 3.1) these data could be taken to indicate that different levels of COX per total cell protein were present in each of the different preparations.

Effect of COX inhibitors on stimulus-evoked TXB₂ release

The functional contribution of the different COX isoforms to prostanoid synthesis in mixed glia and astrocyte or microglia enriched preparations was investigated using the panel of inhibitors described in the introduction to this Chapter. Concentration-response relationships for the inhibitors in mixed glia are shown in Fig 3.15. All of the inhibitors were able to reduce stimulated TXB₂ production in a concentration dependent manner with the following rank order of potency (estimated IC₅₀ values in μM are shown in
Figure 3.14: Western blot detection of COX immunoreactivity in different glial cell preparations. Lanes contained protein from (1) mixed glial cell cultures, (2) astrocyte-enriched cultures, (3) microglial cultures, (4) 50 ng COX-2 standard, (5) molecular weight markers. In each of the lanes containing glial cell samples, 5 μg protein was loaded. COX immunoreactivity was detected by incubation with anti-COX-2 antiserum. This blot is representative of results obtained in three experiments.
Figure 3.15: Concentration-response relationships for the effects of various COX inhibitors on A23187-stimulated TXB$_2$ production in mixed glial cell cultures. Cultures were pre-incubated with one of the inhibitors for 10 min prior to a further 30 min incubation in the presence of 3 μM A23187. Results are expressed as a % of the stimulation over basal levels evoked by A23187 and are means ± SEM from at least 4 determinations. Basal and A23187-stimulated levels of TXB$_2$ were 0.53 ± 0.05 ng/ml and 2.26 ± 0.12 ng/ml, respectively (n = 12).
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parentheses): indomethacin (0.06) ≥ piroxicam (0.10) > NS398 (0.18) > ibuprofen (0.25) >> nimesulide (2.00) >> aspirin (130).

The effects of the inhibitors on the release of TXB$_2$ stimulated by A23187 in subcultured astrocytes are shown in Fig 3.16. Again all of the inhibitors were able to reduce the stimulated response in a concentration-dependent manner. Interestingly the stimulation produced a 3-fold smaller response, in terms of ng TXB$_2$ per ml, in subcultured astrocytes when compared to mixed glia, but there was also a 2-fold lower basal release in these cultures. Expressing stimulated response as a percentage over basal release showed that the subcultured astrocytes had a level of stimulation of 232 %, lower than that obtained in the mixed cultures of 340 %. The relative potencies of the inhibitors were as follows for the inhibition of A23187 stimulated TXB$_2$ production in subcultured astrocytes (estimated IC$_{50}$ values in μM are shown in parentheses); indomethacin (0.03) > piroxicam (0.17) >> NS398 (2.00) > nimesulide (3.40) > ibuprofen (6.30) >> aspirin (80).

The effect of the inhibitors on the release of TXB$_2$ by microglia stimulated with A23187 is shown in Fig 3.17. As in the subcultures, A23187 elicited a lesser response in microglia, in terms of ng of TXB$_2$ detected per ml, than in the mixed glia, which is perhaps not surprising when their lower cell density is considered. Expressing the results in terms of % stimulation over basal showed that these cells had a level of stimulated release of 513 % which was considerably higher than either of the astrocyte-containing
Figure 3.16: Concentration-response relationships for the effects of various COX inhibitors on A23187-stimulated TXB₂ production in sub cultured astrocytes. Cultures were pre-incubated with inhibitor for 10 min before stimulation with 3 μM A23187 for 30 min in the continued presence of inhibitor. Results are expressed as a % of the response elicited by A23187 in the absence of drug and are means ± SEM of at least 4 determinations. Basal and A23187-stimulated TXB₂ release were 0.22 ± 0.04 ng/ml and 0.73 ± 0.07 ng/ml, respectively (n = 6).
Figure 3.17: Concentration response relationships for the effects of various COX inhibitors on A23187-stimulated TXB$_2$ production in microglia. Cultures were pre-incubated with inhibitor for 10 min before stimulation with 3 µM A23187 for 30 min in the continued presence of inhibitor. Results are expressed as a % of the response elicited by A23187 in the absence of drug and are means ± SEM of at least 4 determinations. Basal and A23187-stimulated TXB$_2$ release were 0.15 ± 0.05 ng/ml and 0.92 ± 0.13 ng/ml, respectively. (n = 7).
preparations. The relative potencies of the inhibitors were as follows for the inhibition of A23187-stimulated TXB₂ production in microglia (estimated IC₅₀ values in μM are shown in parentheses); indomethacin (0.05) > piroxicam (0.16) > NS398 (0.46) > nimesulide (0.90) > ibuprofen (1.10) >> aspirin (220).

The rank order of potency for the inhibitors was the same in both of the purified preparations but differed slightly in the mixed glia where ibuprofen was more potent than nimesulide.

The estimated IC₅₀ values for the inhibitors in the three preparations are shown in Table 3.1. It can be observed that the COX-1 selective inhibitors remained potent in both the purified cultures although aspirin seemed to be a little more potent in astrocytes than in the other preparations. The COX-2 selective inhibitors appeared to be considerably more potent in microglial cultures compared to astrocytes, but interestingly NS398 was more potent in the mixed cultures than in either of the purified preparations. The non-selective ibuprofen was also more potent in the primary mixed glia compared to the derivative cultures showing a 4-fold lower potency in microglia and a 25-fold lower potency in astrocytes.
Table 3.1: A comparison of the IC\textsubscript{50} values estimated for a panel of COX inhibitors in 3 different cultured glial cell populations. All IC\textsubscript{50} values were estimated from curves plotted using the data shown in Figs 3.15, 3.16 and 3.17 and represent the concentration of drug which caused 50% reduction in the release of TXB\textsubscript{2} after stimulation with 3 μM A23187.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC\textsubscript{50} (μM)</th>
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<tr>
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<td>2.00</td>
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Discussion

Characterisation of prostanoid production in cultured glia

The initial experiments showed that the ionophore A23187 (3 μM) and the COX substrate AA (10 μM) were able to elicit a 440% and 400% increase, respectively, in the level of TXB$_2$ released from glial cell cultures. The stimulation observed here with A23187 and AA was similar to that reported previously by others in primary astrocyte cultures (Murphy et al., 1985), where 10 μM A23187 for 60 min caused a 9-fold increase in TXB$_2$ release and 10 μM AA for 60 min caused a 7-fold increase in TXB$_2$ release. It was subsequently shown that stimulation of astrocytes with A23187 (0.01 - 10 μM, estimated IC$_{50}$ = 0.25 μM) enhanced the production of all prostanoids by 500-700% (Keller et al., 1987), although this group was unable to stimulate the production of prostanoids from primary astrocyte cultures with 3 μM AA.

The stimulatory effect of A23187 was abolished by the withdrawal of Ca$^{2+}$ from the bathing medium reflecting its dependence, as an ionophore, on the facilitation of Ca$^{2+}$ influx. The Ca$^{2+}$-dependent nature of the stimulation of prostanoid synthesis by A23187 is presumed to be due to the requirement of Ca$^{2+}$ for activation of cytosolic PLA$_2$ (Dennis, 1994) which has been shown to be present in cultured glia (Stephenson et al., 1994). Interestingly, the withdrawal of Ca$^{2+}$ also caused a decrease in the basal level of prostanoid synthesis, demonstrating that these cells show some Ca$^{2+}$-dependent
prostanoid synthesis in the absence of exogenous stimuli. The role of PLA₂ in the response to A23187 was confirmed using the PLA₂ inhibitors quinacrine and ONO-RS-082. Quinacrine is a widely used inhibitor of PLA₂ (Farooqui et al., 1999) and reduced the effect of A23187 in a concentration dependent manner. ONO-RS-082 is an inhibitor of cytosolic PLA₂ (Banga et al., 1986) and abolished the effect of A23187 over a narrow concentration range. It is interesting to note that although 3 μM A23187 was sufficient to cause a significant stimulation of prostanoid synthesis over the timescale of the incubations, others have often used much longer incubation periods, often stretching over a 24 h period (Giulian et al., 1996). Large increases in intracellular Ca²⁺ for prolonged periods have been shown to be toxic to cells through cytoskeletal disruption, protease activation, and activation of endonucleases resulting in DNA fragmentation and apoptosis (Orrenius et al., 1989), so the suitability of A23187 as an activator of PLA₂ over a sustained period is questionable.

As would be expected, AA elicited an increase in TXB₂ production, presumably through the direct supply of substrate to COX within the cells, thus effectively acting downstream of PLA₂ in the prostanoid synthetic cascade. Both stimuli caused maximal stimulation with incubation periods of 30 min, but while the time course of AA was linear, that of A23187 showed a rapid increase during the first 15 min before reaching a plateau during longer incubations. The linearity of the AA time course suggests that an increased level of COX activity resulted in a steady increase in TXB₂ accumulation in the bathing medium following diffusion of substrate into the cells. The plateau reached with A23187
treatment, on the other hand, suggests that there may be a limit to the amount of AA which can be liberated through the action of PLA₂ in response to this stimulus.

**Characterisation of cell populations**

The results from the immunofluorescence investigation into the nature of the mixed glial cultures showed that these consisted predominantly of GFAP-positive astrocytes (85 - 95 %) the remainder being phagocytic microglia. This is similar to the relative abundance of the two cell types reported in the glial cultures used by many other investigators (Petroni *et al.*, 1991; Moore *et al.*, 1991; Amruthesh *et al.*, 1993; Luo *et al.*, 1998; Pistritto *et al.*, 1998; Civenni *et al.*, 1999). Some groups using similar preparations have attributed prostanoid release to the astrocytes which are the major compartment in terms of cell numbers (Murphy *et al.*, 1985; Petroni *et al.*, 1991; Seregi *et al.*, 1987), but others have argued that microglia are responsible for the bulk of prostanoid production in these preparations (Matsuo *et al.*, 1995; Giulian *et al.*, 1996). In order to investigate this matter further, preparations enriched in astrocytes or microglia were prepared. The subcultured astrocyte cultures appeared to be entirely devoid of any microglia able to take up the fluorescent probe, presumably as a result of the combined physical removal and selective poisoning of these cells. Interestingly, Giulian *et al.* (1996) found that treatment with leucine methyl-ester only caused a small reduction in the number of Dil-ac-LDL-positive cells and further treatment using the magnetic removal of the remaining microglia after treatment with carbonyl iron was necessary in order to eliminate them.
Since the effectiveness of the Dil-Ac-LDL marker was dependent on microglial phagocytosis, as was the toxic effect of the leucine methyl-ester, the presence of non-phagocytic microglia cannot be ruled out. Their contribution to the prostanoid synthetic capacity of the cultures is unlikely, however, since it was shown (Giulian et al., 1996) that removal of phagocytic microglia was sufficient to completely abrogate TXB$_2$ synthesis. There was no evidence for the presence of cells in any of the cultures when viewed under phase contrast optics other than those subsequently shown to be positive for either the astrocyte or the microglial markers. In addition, the microglial-enriched cultures prepared from the material shaken from the astrocyte monolayer in the primary cultures comprised exclusively cells which were Dil-Ac-LDL-positive as they were completely devoid of any GFAP-positive astrocytes or other cell types visible under phase contrast. Thus it has been shown that, within the confines of the selective markers used, the astrocyte- and microglial-enriched cultures were homogenous populations of one or the other cell type providing a robust system with which to further investigate the prostanoid synthetic capacities of glial cells.

Comparison of prostanoid synthesis in different glial cell preparations

The three culture preparations showed marked differences in their level of A23187-stimulated TXB$_2$ release in terms of stimulation over basal; The mixed glia showed a 340% stimulation, of a similar order to that reported by others (Murphy et al., 1985). The astrocyte-enriched subcultures showed a slightly lower level of stimulation of 232% over
basal whilst the microglia had a higher level of stimulated release than either of the astrocyte-containing preparations of 513 %. Despite the considerably lower cell density of the microglia compared to the subcultured astrocytes, an estimation of the absolute ng/ml TXB$_2$ detected in the bathing medium of each was within a 10-fold range (approximately 1.5 ng/100,000 cells for astrocytes, 9.5 ng/100,000 cells for microglia). Cell numbers were determined by counting the number of cells in at least 10 different fields of view after immunofluorescence labelling. The average number of cells allowed an estimate to be made for the number of cells in a typical culture dish. These observations suggest that these experiments go some way towards corroborating the hypothesis posed by Giulian et al. (1996) who claimed that microglia are the principal glial source of TXB$_2$ in culture. However, expressing stimulation as the level of enhancement over basal TXB$_2$ release reveals that the differences in the prostanoid synthetic capacity of the different cell types in this investigation is considerably less than those suggested by this group. They claimed that microglia yielded >480-fold more TXB$_2$ per cell than the equivalent number of astrocytes, and that the removal of microglia from astrocyte cultures left the TXB$_2$-synthesising capacity of the remaining astrocyte-enriched preparations insensitive to stimulation with A23187. The data presented here do not support this but indicate that both cell types are capable of synthesising TXB$_2$.

Since all of the COX inhibitors were able to cause a concentration dependent decrease in stimulated TXB$_2$ release whether they were selective for one or the other COX isoform, it would be reasonable to assume that both isoforms participate to some degree
Chapter 3: Cyclooxygenase isoform expression in cultured glia

in the stimulated production of \( \text{TXB}_2 \) in all three cell preparations. Thus it would seem that COX-2 contributes to \( \text{TXB}_2 \) synthesis in both astrocytes and microglia rather than being a feature of just one of the cell types found in the mixed glial cells. The presence of COX-2 immunoreactivity in all three cell preparations as shown by the Western blot data is in agreement with this suggestion, although unfortunately the COX-2 antiserum used was unable to distinguish between the different COX isoforms. If, as suspected, the COX-2 antibody was detecting both COX isoforms it would appear that there were different levels of COX protein present in the three preparations relative to the total protein, decreasing in the order; mixed glia > astrocytes > microglia. This observation must be treated with caution however, since not only could there be some preference of the antiserum for one or the other isoform in these cells despite the cross reactivity shown with the purified standards (which could mean that the observed changes are the result of differential expression of one isoform rather than total COX protein), the three samples were standardised in terms of total protein so theoretically any major changes in the expression levels of other cellular proteins could influence the amount of material loaded and thus the apparent level of COX detected.

Comparison of the potencies of the various drugs in astrocytes and microglia reveals an interesting pattern of COX inhibition which suggests differences in the contribution of COX-1 and COX-2 to \( \text{TXB}_2 \) synthesis in each cell type. The COX-1 selective inhibitors were potent in both astrocytes and microglia suggesting that this isoform contributes significantly to the synthesis of \( \text{TXB}_2 \) in both cell types. Interestingly, the COX-2
selective drugs showed a marked increase in potency in microglia when compared to astrocytes which suggests that maybe the inducible isoform contributes to a greater extent to the TXB$_2$ synthesis in microglia than it does in astrocytes. This would support the suggestions put forward by Matsuo et al. (1995) and Giulian et al. (1996) that the microglia in cultured glial cell populations are responsible for the COX-2 activity detected therein.

One factor which must be addressed when using a panel of COX-1 and COX-2 selective inhibitors in a study such as this, is the time-dependence of the binding of inhibitors to COX. In particular, COX-2 selective inhibitors can inhibit COX-2 by a slowly reversible mechanism involving the formation of tightly binding complexes, but inhibition of COX-1 occurs via a freely reversible competitive mechanism. Thus COX-2 selective inhibitors at low concentrations are able to form COX-2-inhibitor complexes causing inactivation of this isoform, whilst minimal inhibition of COX-1 occurs (reviewed by DeWitt, 1999). One implication of this phenomenon is that if saturating concentrations of substrate are used in studies attempting to characterise the action of selective COX inhibitors then the competitive inhibition of COX-1 would not be detected. The use of submaximal levels of stimulation and a 10 min pre-incubation to allow equilibration of slow-acting COX inhibitors in this study should have minimised the time-dependent effects of the inhibitors.

If the IC$_{50}$ values of the drugs in the mixed glial cultures are compared to those for the
two more homogenous populations the trend seems to be that these primary cell cultures show a pattern of inhibition which most resembles the results seen in the microglia, with all of the drugs except nimesulide and ibuprofen showing fairly similar levels of inhibition. However, the estimated potencies of some of the drugs in mixed glia, in particular piroxicam, ibuprofen and NS398, do not fall within the range of potencies obtained for the purified cells. This suggests that the mixed glial cultures do not reflect a simple combination of microglia and astrocytes in terms of COX activity. It seems therefore that the presence of astrocytes and microglia together might result in a situation where the individual cell types no longer behave as they did in more homogenous populations with regard to the contribution of the COX isoforms to TXB$_2$ synthesis. The primary cultures were predominantly astrocytes, but their inhibitor profile was quite different to that for the astrocyte subcultures, particularly with regard to higher potencies of the COX-2 selective inhibitors and ibuprofen. In addition, the basal and A23187-stimulated levels of TXB$_2$ production were considerably higher in mixed glia than in either of the purified preparations which may not be entirely attributable to variations in cell number. Perhaps the presence of microglia in the mixed glial cultures confers a change in the expression or activation of the COX isoforms in the underlying astrocytes which can be observed as a change in inhibitor profile. Since COX-2 may be readily up-regulated in response to a variety of conditions, is reasonable to suggest that the COX-2 level in one glial cell type could be up-regulated by a signal derived from the presence of the other. COX-2 has been shown to be up-regulated in astrocytes by a number of mediators, in particular TGF-β1 (Luo et al., 1998), IL-1β (O'Banion et al.,
1996), TNF-\( \alpha \) (Mollace et al., 1998) and endothelin (Koyama et al., 1999), and in other cells COX-2 up-regulation had been demonstrated with PDGF (Lin et al., 1989) and EGF (Rich et al., 1998). Interleukin-6 is also considered to be a pro-inflammatory cytokine but does not cause elevation of COX-2 expression in hepatocytes or brain (Martin-Sanz et al., 1998; Lacroix and Rivest, 1998), although recently it has been shown to be the factor in serum from patients with preeclampsia which causes elevation of COX-2 in endothelial cells (Akarasereenont et al., 1999).

Microglia have been shown to secrete IL-1 after stimulation with zymosan (Giulian et al., 1994), LPS (Yao et al., 1992) and \( \beta \)-amyloid (Meda et al., 1999). In addition they can release TGF-\( \beta \)1 after ischemia (Lehrmann et al., 1998) and TNF-\( \alpha \) and IL-6 after trimethyltin-induced neurodegeneration (Bruccoleri et al., 1998). Since these cytokines can potentially modulate the levels of COX-2 in astrocytes, it is possible that an interaction between the cell types could be mediated by a combination of these cytokines (microglial release of cytokines is reviewed by Moore and Thanos, 1996).

There does not appear to be any evidence that COX-1 expression may be modulated by cytokines, but an interesting investigation by Pistritto et al. (1998) suggested that COX-1 was involved in the immediate production of PGE\(_2\) after stimulation of astrocytes with LPS. This suggestion stemmed from the observation that IL-1 was only able to elicit an increase in prostanoid production after 8 h, during which time it was assumed that COX-2 protein would have been synthesised. However, this group did not show that this
chronic increase in PGE$_2$ production was accompanied by an increase in COX-2 protein. Furthermore, it was assumed that since the cells had been treated with aspirin for 1 h prior to LPS addition, any effect of COX-1 would be completely blocked. This is unlikely since new molecules of this constitutively expressed isoform would undoubtedly be synthesised to some extent during 8 h of cell stimulation. In addition, it has been shown that aspirin inhibits the activity of IkB kinase-$\beta$, resulting in blockade of the up-regulation of COX-2 by the NF-kB pathway (Yin et al., 1998), so it is just as likely that the aspirin dose used had an inhibitory effect on the up-regulation of COX-2 in addition to the direct inhibition of COX-1.

Giulian et al. (1994) showed that stimulation of microglia resulted in the production of IL-1 which increased the proliferation of astrocytes in culture whilst some other soluble factor was also released which simultaneously decreased neuronal survival. Since IL-1 has been shown to cause an up-regulation in the expression of COX-2 in astrocytes (O'Banion et al., 1996), it is possible that upon stimulation microglia release this cytokine which causes astrocytes to proliferate and induce COX-2 expression. Since reactive microglia appear at nearly every type of brain insult, it is possible that this mechanism might represent one of the ways in which astrocytes and microglia interact in response to inflammatory insults in the CNS.

In summary, the data presented here has shown the presence of both astrocytes and microglia in mixed glial cell cultures, and characterised an effective way of producing
homogenous populations of each. Western blotting revealed the presence of different levels of COX immunoreactivity in each of the culture preparations which may be due to different levels of expression of one of the COX isoforms. Both isoforms of COX appear to contribute to prostanoid synthesis in astrocytes and microglia as demonstrated by the use of inhibitors preferential for each isoform, but the use of a panel of inhibitors revealed differences in the relative contribution of the isoforms in each cell type. In general, prostanoid synthesis in microglia appeared to be more sensitive to inhibition of COX-2, whilst COX-1 inhibitors were more effective in astrocytes. Upon comparison, the mixed glial cultures did not closely resemble either of the purified preparations or a simple mixture of the two suggesting that interactions between the different cell types in mixed glial cell cultures might influence COX expression or activity.
Chapter 4: Serum regulates the expression of cyclooxygenase isoforms in cultured glia
**Introduction**

Since the discovery that COX-2 can be rapidly up-regulated in many cell types in response to a range of pro-inflammatory mediators, much work has sought to determine the precise nature of this induction process. As discussed in the Introduction to this thesis, the promoter region for the COX-2 gene possesses a variety of recognition sites allowing COX-2 induction via a number of signalling cascades. Given the extensive and ever expanding range of substances which have been shown to up-regulate COX-2, the existence of a complex array of mechanisms by which the expression of COX-2 may be regulated is likely. The currently accepted model of COX expression is based on the existence of two isoforms with clearly defined roles; COX-1 is constitutively expressed and plays a role in the normal functioning of the cell, COX-2 is up-regulated in response to stimuli and is responsible for the generation of the prostanoids used in the inflammatory response. It is becoming clear that this view is an oversimplification with COX-1 having the potential to play a vital role in the inflammatory response (Langenbach et al., 1995) and COX-2 being constitutively expressed in a number of tissues in the absence of pro-inflammatory stimuli (reviewed by Wallace, 1999). Also, COX-1 expression has been shown to change in response to some factors, such as phorbol ester, in cells of a human monocytic leukaemia cell line (Smith et al., 1993). Considering the current availability of a range of COX inhibitors with selectivity for one or the other COX isoform, it is surprising that more detailed functional investigations of COX-2 up-regulation have not been performed in cells derived from the CNS.
Many of the studies which have been undertaken to explore COX-2 induction have used an approach whereby cells in vitro or in vivo receive a particular stimulus, for example LPS, growth factors or cytokines either directly, or via the stimulation of their release from other cells, thus mimicking to some extent the inflammatory response. Levels of COX-2 expression are then detected using techniques such as Northern blotting, RT-PCR or in situ hybridisation which measure mRNA levels, these data being corroborated by the detection of COX-2 protein through immunoblotting with COX-2 specific antibodies. Such studies have shown the rapid elevation of COX-2 protein and mRNA in response to phorbol ester, LPS, forskolin, cAMP, TNF-α, Freund’s complete adjuvant, TGF-β, IL-1, PDGF and serum (see Chapter 1 for references), but to date none have undertaken to investigate changes in functional COX activity which are the presumed outcome of increased COX-2 expression.

The effect of serum on COX expression

One factor which has been shown to cause the up-regulation of COX-2 expression in a number of cultured cell preparations is the presence of serum in the growth medium (Fletcher et al., 1992; O’Banion et al., 1992; Mitchell et al., 1995; Rich et al., 1996; Rich et al., 1998), making this a useful tool with which to manipulate the expression of COX isoforms. Serum contains a variety of substances which might be implicated in the induction of COX-2. These include growth factors, hormones and LPS (Gebicke-Haerter et al., 1989; Rich et al., 1998), and it has been proposed that a combination of these
constituents evoke COX-2 up-regulation rather than any particular single substance. It is apparent that there is a tissue-specific element to the modulation of COX expression by serum. One study showed that while increased serum levels caused an increase in PGE$_2$ production corresponding to an elevation of COX-2 and cPLA$_2$ protein in cultured astrocytes, little change in PGE$_2$ production or expression of either enzyme was detected in neurons (Luo et al., 1998). Furthermore, serum caused no change in COX-1 expression in this investigation although it has been shown to produce a slight up-regulation of COX-1 in cerebromicrovascular smooth muscle cells (Rich et al., 1998). In an earlier investigation, this group showed that removal of serum from cerebrovascular smooth muscle cells for 48 h caused a down-regulation of COX-2 mRNA and protein together with a reduction in PGE$_2$ production, which was restored to pre-deprived levels upon serum re-addition. COX-1 mRNA was also decreased by 40% after serum withdrawal, but although a massive increase in COX-2 message was detected after serum re-addition, COX-1 mRNA was induced by only 4-fold.

Many studies have utilised the modulation of serum levels as a means to investigate the expression of COX-2 compared with COX-1, but the expression of mRNA and protein is not sufficient to elucidate the functional activity of COX isoforms in intact cells. Observations have been made regarding changes in prostanoid synthesis after modulation with serum, in particular by Rich et al. (1996), who showed that serum deprivation in cerebrovascular smooth muscle cells caused a dramatic drop in PGE$_2$ synthesis, and Luo et al. (1998), who showed that levels of PGE$_2$ released by astrocytes were lowered in
cells grown in reduced serum conditions. However, these investigations failed to demonstrate convincingly whether the changes in prostanoid production were attributable to one or the other COX isoform, preferring instead to show complete abrogation of prostanoid production using a single high concentration of a selective COX inhibitor.

The purpose of this study was to use a panel of COX inhibitors to determine the contribution of COX isoforms to prostanoid production in glial cultures grown in serum-deprived conditions.

Results

Effects of serum removal

The effect of serum removal on the production of TXB₂ from mixed glial cell cultures was examined. Fig 4.1 shows that both basal and A23187-induced TXB₂ release were markedly reduced after 4 days of serum deprivation. Since serum withdrawal has been shown to down-regulate the expression of PLA₂ in glial cells (Luo et al., 1998), the experiments were repeated using AA as a stimulus thus bypassing the PLA₂ activity required for A23187 action. Serum withdrawal also caused a marked reduction in AA-induced TXB₂ production, suggesting that PLA₂ down-regulation was not the principal cause of the reduction in prostanoid synthesis. Another useful and widely used tool in the investigation of COX-2 expression is the anti-inflammatory glucocorticoid
Chapter 4: Serum regulates the expression of cyclooxygenase isoforms in cultured glia

Additions:
- none
- A23187
- AA

Figure 4.1: Effect of serum deprivation and dexamethasone treatment on basal and stimulus-induced TXB\textsubscript{2} production in cultured glia. Control cultures or those which had been maintained in serum free medium for 4 days or serum containing medium for 2 days in the presence of dexamethasone (10 μM) were incubated for 30 min in drug-free buffer or in buffer containing either A23187 (3 μM) or AA (10 μM). Results are means ± SEM from 4 determinations.
dexamethasone. The initial investigations which led to the discovery of COX-2 showed the presence of a pool of LPS-inducible COX which was susceptible to inhibition by dexamethasone (Fu et al., 1990). The action of dexamethasone was subsequently shown to be via inhibition of the expression of TIS10, the gene coding for COX-2 (Kujubu and Herschman, 1992). Fig 4.1 also shows that basal and stimulated TXB$_2$ production were also reduced after a 2 day incubation with dexamethasone (10 μM), suggesting that much of the prostanoid synthetic capacity of the cells was due to the presence of the inducible COX-2 isoform. A recent study which undertook to investigate whether there were differences in the profile of prostanoids resulting from the action of COX-1 compared to COX-2 suggested that COX-2 activity resulted in the production of PGI$_2$ and PGE$_2$ whilst COX-1 activity tended to result in the synthesis of TXB$_2$, PGD$_2$ and PGI$_2$, but very little PGE$_2$ (Brock et al., 1999). If AA was metabolised preferentially by the different isoforms to produce distinct prostanoid products then this could have an impact on any conclusions made here based on changes in the synthesis of TXB$_2$. In order to address this issue, the basal and stimulated levels of PGE$_2$ production were investigated in control cells and those which had undergone a 2 day treatment with dexamethasone (10 μM) (Fig 4.2). It is clear from these data that the down-regulation of COX-2 by dexamethasone caused a reduction in basal PGE$_2$ production but had no effect on A23187-induced PGE$_2$ production and only a small (~23 %) effect on that elicited by AA.

The reduction in COX-2 expression by serum withdrawal was confirmed by Western
Figure 4.2: Effect of dexamethasone treatment on basal and stimulus-induced PGE$_2$ production in mixed glia. Control cultures or those which had been maintained in serum containing medium for 2 days in the presence of dexamethasone (10 μM) were incubated for 30 min in drug-free buffer or in buffer containing either A23187 (3 μM) or AA (10 μM). Results are means ± SEM from 4 determinations.
blotting using an antibody to detect the presence of COX-2 in control and serum-deprived cultures (Fig 4.3). Although such blots cannot be considered to be quantitative, it is clear that despite equivalent amounts of cellular protein being loaded into each lane, considerably less 70 kDa protein with COX-2 immunoreactivity was apparent in the serum deprived condition when compared to control.

The apparent change in COX-2 expression and reduction in TXB$_2$ synthesis observed with serum withdrawal was further investigated by detecting changes in the contribution of the different COX isoforms to TXB$_2$ production using the selection of COX inhibitors described in the previous chapter. Figs 4.4 and 4.5 show the inhibition profiles of these inhibitors in control and serum-deprived cultures, respectively. The result obtained for control cultures in the previous chapter is shown again here for the sake of clarity. All of the inhibitors were able to reduce stimulated TXB$_2$ production in a concentration dependent manner with the following rank order of potency (estimated IC$_{50}$ values in µM are shown in parentheses): indomethacin (0.06) > piroxicam (0.10) > NS398 (0.18) > ibuprofen (0.25) >> nimesulide (2.00) >> aspirin (130). After 4 days of serum deprivation the rank order was similar to control, but a comparison of the apparent IC$_{50}$ values revealed changes in potency of some drugs. The preferential COX-1 inhibitors indomethacin, piroxicam and aspirin became more potent inhibitors of COX activity in serum-deprived cultures compared to normal cultures with estimated IC$_{50}$ values of 0.04 µM, 0.04 µM and 44 µM respectively. In contrast, the non-selective ibuprofen (0.63 µM), and the COX-2 selective NS398 (0.32 µM) and nimesulide (4.00 µM) became two-
Figure 4.3: Western blot detection of COX immunoreactivity in serum-deprived mixed glial cell cultures. Lanes contained samples from (1) 5 μg protein from control cells grown in serum-containing medium, (2) 5 μg protein from cells after 4 days in serum-free medium, (3) molecular weight marker proteins, (4) 50 ng COX-1 standard protein, (5) 50 ng COX-2 standard protein. COX immunoreactivity was detected by incubation with anti-COX-2 antiserum. This blot is representative of results obtained in three experiments.
Figure 4.4: Concentration-response relationships for the effects of various COX inhibitors on A23187-stimulated TXB₂ production in glial cell cultures. Cultures were pre-incubated with one of the inhibitors for 10 min prior to a further 30 min incubation in the presence of 3 μM A23187. Results are expressed as a % of the stimulation over basal levels evoked by A23187 and are means ± SEM from at least 4 determinations. Basal and A23187-stimulated levels of TXB₂ were 0.53 ± 0.05 ng/ml and 2.26 ± 0.12 ng/ml, respectively (n = 12).
Figure 4.5: Concentration-response relationships for the effects of various COX inhibitors on A23187-stimulated TXB$_2$ production in cultures maintained in serum-free medium for 4 days. Cultures were pre-incubated with one of the inhibitors for 10 min prior to a further 30 min incubation in the presence of 3 μM A23187. Results are expressed as a % of the stimulation over basal levels evoked by A23187 and are means ± SEM from at least 4 determinations. Basal and A23187-stimulated levels of TXB$_2$ were 0.23 ± 0.03 and 0.62 ± 0.05, respectively (n = 8).
to three-fold less potent after serum deprivation.

**Effects of serum re-addition**

Having observed these changes in COX sensitivity to inhibition after serum withdrawal, the effect of replacing the serum after 4 days of deprivation was investigated. Serum re-addition restored basal and stimulated TXB$_2$ production to control levels over the time course shown in Fig 4.6. Following the first two days of serum re-addition, basal and stimulated TXB$_2$ production remained at the serum deprived level of approximately 10-fold less than control, but after 7 days both basal and stimulated TXB$_2$ production returned to control levels. Western blotting revealed that after 7 days in the renewed presence of serum, these cells showed a band of COX-2 immunoreactive protein with an increased intensity compared to that seen in the serum-deprived cells, but not as intense as in the original controls (Fig 4.7). The functional capacity of this COX re-expression was investigated using the various COX inhibitors as before, yielding the concentration-response relationships shown in Fig 4.8. Comparison of the estimated IC$_{50}$ values with those obtained for the previous conditions indicated that the preferential COX-1 inhibitors indomethacin (0.06 μM), piroxicam (0.08 μM) and aspirin (130 μM) had potencies similar to the control condition, while nimesulide (4.50 μM) displayed a potency similar to that found in serum-deprived cultures. The other COX-2 selective inhibitor NS398 (5.40 μM) together with ibuprofen (1.70 μM) were, however, considerably less potent in this condition when compared to both normal and serum-
Figure 4.6: Time course of the recovery of basal and A23187-stimulated TXB$_2$ production following serum deprivation. Cultures were deprived of serum for 4 days then challenged immediately (0 days) or following various periods of serum re-addition to the growth medium with either no additions or 3 µM A23187 for 30 min. Results are means ± SEM from at least 3 determinations. Basal TXB$_2$ production on days 1 and 2 of serum re-addition were below or on the limit of levels of detection.
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Figure 4.7: Western blot detection of COX immunoreactivity in mixed glial cell cultures after serum removal and re-addition. Lanes contained samples from (1) 5 µg protein from control cells, (2) 5 µg protein from serum-deprived cells, (3) 5 µg protein from cells deprived of serum then re-exposed to serum for 7 days, (4) COX-2 standard protein, (5) molecular weight markers. COX immunoreactivity was detected by incubation with anti-COX-2 antiserum. This blot is representative of results obtained in three experiments.
Figure 4.8: Concentration-response relationships for the effects of various COX inhibitors on A23187-stimulated TXB$_2$ production in glial cells which had been maintained in serum-free medium for 4 days prior to the re-addition of serum for 7 days. Cultures were pre-incubated with one of the inhibitors for 10 min prior to a further 30 min incubation in the presence of 3 μM A23187. Results are expressed as a % of the stimulation over basal levels evoked by A23187 and are means ± SEM from at least 4 determinations. Basal and A23187-stimulated levels of TXB$_2$ were 0.71 ± 0.09 ng/ml and 2.17 ± 0.20 ng/ml respectively (n = 6).
deprived cultures. The estimated IC$_{50}$ values are shown on Table 4.1. It is apparent from these data that despite COX re-expression coinciding with restoration of prostanoid synthetic capacity, there are distinct changes in the potencies of some inhibitors in cultures which had undergone serum deprivation then re-addition when compared to controls. In order to investigate this newly acquired variation in COX activity further, and to rule out the possibility that the effect was due to some change involving PLA$_2$, the experiment was repeated using AA to induce prostanoid synthesis in normal cultures and those which had undergone serum deprivation then re-addition (Fig 4.9 and 4.10). As was the case with A23187-induced prostanoid synthesis, all of the inhibitors were able to reduce AA-stimulated TXB$_2$ production in a concentration-dependent manner. In control glial cultures treated with AA the rank order of potency was as follows (estimated IC$_{50}$ values in µM are shown in parentheses): indomethacin (0.02) > piroxicam (0.07) > nimesulide (0.22) > NS398 (0.28) > ibuprofen (1.6) > aspirin (80). After serum deprivation and re-addition, a change in the pattern of inhibitor potency was observed which was similar to that seen when using A23187 as the stimulus. The COX-1 preferential inhibitors indomethacin (0.04 µM) and piroxicam (0.11 µM) showed only a slight change in potency although the potency of aspirin (200 µM) was more substantially reduced. As with A23187-stimulation after serum deprivation and re-addition, ibuprofen (4.4 µM) and the COX-2 selective inhibitors NS398 (7.8 µM) and nimesulide (9.7 µM) showed a marked reduction in potency. Most strikingly, the COX-2 inhibitor potencies were reduced by 30-45-fold and nimesulide failed to inhibit TXB$_2$ accumulation by more than approximately 50%.
Table 4.1: Potencies of COX inhibitors in glial cell cultures in the presence or absence of serum or in the renewed presence of serum after previous serum removal. The apparent IC₅₀ values for the various COX inhibitors used were determined from the concentration-response relationships shown in Figs 4.4, 4.5 and 4.8.
Figure 4.9: Concentration-response relationships for the effects of various COX inhibitors on AA-stimulated TXB$_2$ production in normal gial cell cultures. Cultures were pre-incubated with one of the inhibitors for 10 min prior to a further 30 min incubation in the presence of 10 μM AA. Results are expressed as a % of the stimulation over basal levels evoked by AA and are means ± SEM from at least 4 determinations. Basal and AA-stimulated levels of TXB$_2$ were 0.51 ± 0.04 ng/ml and 2.03 ± 0.33 ng/ml, respectively (n = 6).
Figure 4.10: Concentration-response relationships for the effects of various COX inhibitors on AA-stimulated TXB$_2$ production in cultures maintained in serum-free medium for 4 days prior to the re-addition of serum for 7 days. Cultures were pre-incubated with one of the inhibitors for 10 min prior to a further 30 min incubation in the presence of 10 μM AA. Results are expressed as a % of the stimulation over basal levels evoked by AA and are means ± SEM from at least 4 determinations. Basal and AA-stimulated levels of TXB$_2$ were 0.62 ± 0.03 ng/ml and 2.74 ± 0.68 ng/ml, respectively (n = 6).
Chapter 4: Serum regulates the expression of cyclooxygenase isoforms in cultured glia

Up-regulation of COX-2 expression could, as discussed in Chapter 1, be a result of activation of the PPAR or the NF-κB pathways. Since ibuprofen and other NSAIDs have been shown to cause activation of PPARγ (Lehmann et al., 1997), the effect of a 48 h incubation with 1 mM ibuprofen was investigated in mixed glial cells under basal and serum-deprived conditions (Fig 4.11). Ibuprofen treatment reduced basal and stimulated TXB$_2$ production to levels similar to those observed after serum deprivation. In cells which had undergone serum deprivation, ibuprofen treatment further reduced the basal and stimulated production of TXB$_2$. An attempt was also made to investigate the involvement of the NF-κB pathway on COX expression in these cells. Serum deprived cultures were re-expressed to serum for 7 days in the presence of 300 μM caffeic acid phenethyl ester (CAPE), a specific inhibitor of NF-κB (Natarajan et al., 1996). Unfortunately, the cells were unable to survive this treatment, complete cell death being evident within 3 days of the drug treatment.
Chapter 4: Serum regulates the expression of cyclooxygenase isoforms in cultured glia

Figure 4.11: Effect of chronic ibuprofen treatment on TXB₂ production in mixed glial cell cultures. (A) Normal mixed glial cell cultures or (B) mixed glial cell cultures which had been serum-deprived for 4 days were incubated with ibuprofen (1 mM) for 48 h before the addition of A23187 (3 μM) or AA (10 μM) for 30 min. Results are expressed as means ± SEM, n = 3/4.
Discussion

Serum removal

Removal of serum from the growth medium of cultured glia caused a change in both COX protein expression, as detected by Western blotting, and functional COX activity as assessed by stimulus-induced TXB$_2$ accumulation. This was similar to previous studies of COX in cultured astrocytes which showed that PGE$_2$ production was reduced, along with COX-2 immunoreactive protein, when the cells were grown in 1% foetal calf serum rather than the normal 10% (Luo et al., 1998). Also, studies using cultured cerebrovascular smooth muscle cells showed a similar reduction in prostanoid synthesis after 4 days of serum deprivation, corresponding to a reduction in COX-2 mRNA to below detectable levels together with a 40% reduction in COX-1 mRNA (Rich et al., 1996). Unfortunately, the antibodies used in the investigation presented here showed cross-reactivity with both purified COX-1 and COX-2, making it impossible to detect changes in the expression of the individual isoforms. Even with this lack of specificity for COX-2, it was reasonable to use the COX-2 antiserum as a means to detect changes in the general level of COX expression. The effectiveness of Western blot data for quantitative determination of protein expression is often poorly regarded, but it is clear from the results presented here that after serum deprivation there was a reduction in COX protein abundance in relation to the total cellular protein content.
Serum removal caused a reduction in TXB$_2$ production to a slightly greater extent than dexamethasone, an observation which could be considered unusual if both treatments acted solely to down-regulate COX-2 expression. This is reminiscent of the observations made by Rich et al. (1998) who showed that both dexamethasone and serum removal caused a down-regulation of COX-2 expression, but while serum removal also reduced the expression of COX-1, dexamethasone had no effect on this isoform. The greater attenuation of TXB$_2$ synthesis with serum deprivation described here could perhaps therefore be explained as a reduction in the contribution of COX-1 to prostanoid synthesis. It is important to note that dexamethasone treatment had little effect on the release of PGE$_2$ which is in contrast to the proposal by Brock et al. (1999) that PGE$_2$ is preferentially produced by COX-2. Since their hypothesis was based on observations from experiments using peritoneal macrophages, it is likely that this effect was cell type specific and perhaps reflects differences in the specific prostanoid synthase content of these cells compared to glia.

The purpose of this study was to explore changes in COX expression in terms of the effect on the relative contribution of each COX isoform to TXB$_2$ synthesis in cultured glial cells. Since all of the inhibitors were able to cause a concentration-dependent reduction of TXB$_2$ production in normal cultured glia regardless of their specificity for one or the other isoform, it would be reasonable to suggest that both isoforms contributed to prostanoid synthesis under normal (serum containing) culture conditions. The reduction in prostanoid synthesis and COX protein expression after serum removal
shown here is consistent with the hypothesis that removal of serum causes down-regulation of COX-2 expression (Rich et al., 1996; Luo et al., 1998), and that COX-2 is involved in prostanoid production in glial cells under normal culture conditions (Fiebich et al., 1996a; O'Banion et al., 1996; Minghetti et al., 1997; Bauer et al., 1997; Luo et al., 1998; Koyama et al., 1999; Hirst et al., 1999). Stimulus-induced prostanoid production which remains after serum-deprivation in glial cells has been attributed, therefore, to the activity of COX-1 (Luo et al., 1998), although previously there has been no detailed investigation of this. The results presented here provide evidence in favour of this theory since the COX-2 selective inhibitors nimesulide and NS398 became approximately 2-fold less potent after serum deprivation compared to controls, while those inhibitors which have been shown to be more selective against COX-1 than COX-2 (indomethacin, piroxicam and aspirin) became 2- to 3-fold more potent under these conditions.

Re-addition of serum

Studies using cultured cerebrovascular cells showed that following serum deprivation, re-addition of serum resulted in an initial increase in AA- and A23187-induced PGE$_2$ synthesis which peaked after 6 h at levels in excess of those achieved in control cells then decreased to control levels after 2-3 days (Rich et al., 1996). In contrast, there was an initial decrease in basal and stimulated TXB$_2$ production after serum re-addition to cultured glia, and it took at least 7 days before basal and A23187-stimulated TXB$_2$
production were restored to control levels. The increase in PGE$_2$ synthesis observed by Rich et al. (1996) after 6 h was accompanied by an increase in COX-2 mRNA and protein, but a decrease in COX-1 protein despite a slight increase in COX-1 message. The initial decrease in TXB$_2$ production reported here may therefore be a manifestation of this decrease in COX-1 protein and the longer recovery period may reflect cell-specific differences in the re-expression of COX-2 between glial cells and cells of the vasculature.

Attempts were made to investigate the nature of the transcriptional regulation of COX-2 in these cells using substances which interfere with the PPAR and NF-κB pathways. A number of NSAIDs including indomethacin, ibuprofen, fenoprofen and flufenamic acid have been shown to stimulate the PPAR pathway through binding and activation of PPAR$\gamma$ causing differentiation of fibroblasts to adipocytes (Lehmann et al., 1997). Ibuprofen at 100 μM was shown to cause a 21-fold activation of PPAR$\gamma$ to a degree comparable to that obtained with the PPAR$\gamma$ ligands BRL49653 and 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ as determined using a reporter-gene assay. In an attempt to investigate the involvement of the PPAR pathway on the re-expression of COX after serum re-addition in glial cells, ibuprofen was used to stimulate PPAR$\gamma$ in control and serum deprived cells. This treatment caused a reduction in basal and stimulated COX activity in untreated cells, and failed to increase TXB$_2$ synthesis in serum-deprived cultures. This suggests that the re-expression of COX after serum re-addition could not be mimicked by PPAR activation so is unlikely to be via this pathway. In the light of these data it was desirable to investigate any involvement of the NF-κB signalling cascade in the serum-induced
changes in COX expression. Caffeic acid phenethyl ester (CAPE), an active component of propolis from honeybee hives with antimitogenic, anticarcinogenic, antiinflammatory, and immunomodulatory properties, was shown to exert its therapeutic effects through inhibition of NF-κB activation, specifically by preventing the translocation of the p65 subunit of NF-κB to the nucleus in a human histiocytic cell line (Natarajan et al., 1996). Unfortunately, cultured glial cells failed to survive attempts to investigate involvement of the NF-κB cascade in the restoration of COX activity after serum re-addition.

Comparison of the pattern of inhibitor potencies obtained in control cells stimulated with either AA or A23187 showed that, in general, the relative potencies were of the same order regardless of the stimulus. The differences observed, such as the switching of relative positions of nimesulide and ibuprofen, may have been due to an isoform-specific effect reported recently regarding differential use of exogenously applied AA by COX-1 and COX-2 in transfected 293 cells (Murakami et al., 1999). This group suggested that low concentrations of exogenous AA (less than 10 μM) could be utilised by COX-2 whilst concentrations in excess of 10 μM were necessary for COX-1 activity. The idea that the different COX isoforms require certain levels of exogenous AA was also commented on by Reddy and Herschman (1994) who suggested that in cells expressing both COX isoforms, exogenous AA was preferentially used by COX-1. This suggestion was made after experiments in which 10 μM exogenous AA caused the synthesis of PGE\textsubscript{2} in Swiss 3T3 cells in an apparently COX-1 dependent manner, although AA-stimulated PGE\textsubscript{2} production via COX-2 was observed after inhibition of COX-1 with
aspirin. However, since the inhibitors used here displayed a similar relative order of potencies against the two stimuli it is unlikely that such a phenomenon contributed to the stimulated TXB₂ production measured in cultured glia over the incubation periods used. A more likely reason for the slight differences in the pattern of inhibitor potency between A23187- and AA-stimulated TXB₂ production could be that A23187 liberated a different amount of AA than the 10 μM which was supplied exogenously thus providing a different substrate concentration with which the inhibitors must compete.

Although the re-addition of serum for 7 days restored TXB₂ production and COX-2 immunoreactive protein expression (albeit at a slightly lower abundance than control), the profile of inhibition of COX activity after serum re-addition differed markedly from controls. Whilst the potencies of the COX-1 preferential inhibitors indomethacin, piroxicam and aspirin returned to values similar to controls, the COX-2 selective inhibitor nimesulide displayed a potency similar to that found under serum-deprived conditions. In addition, NS398 and ibuprofen were found to be considerably less potent after serum re-addition compared to either control or serum-deprived cultures. These changes in inhibitor potency were generally consistent regardless of whether A23187 or AA was the stimulus, suggesting changes in COX activity after serum deprivation and re-addition not apparent in previous studies which failed to examine the functional properties of the re-expressed COX. The reasons for these changes are not clear but suggest that the COX expressed after serum deprivation and re-addition is different in some way from that present in control cultures. This proposal is not without precedent
in the light of some recent studies. A novel transcript of COX-1 mRNA was identified in an immortalised human megakaryoblastic cell line after treatment with phorbol ester. This novel variant was subsequently shown to be expressed in a tissue-specific manner, being particularly abundant in the bladder and appendix (Plant and Laneuville, 1999). Although there was no indication that this novel transcript resulted in formation of a distinct COX isoform, another recent study has shown the induction of a COX with reduced sensitivity to certain NSAIDs. This was observed in transformed monocyte/macrophage cells treated with diclofenac for 48 h, a treatment which resulted in the expression of COX-2 activity with properties distinct from those of the established two isoforms (Simmons et al., 1999). Interestingly this proposed new COX activity was sensitive to inhibition with paracetamol, and the effect of this drug in glial cell cultures will be addressed in Chapter 5 of this thesis. These authors concluded that these treatments caused the expression of a new COX isoform or variant. The existence of a third COX isoform (COX-3) has also been proposed by Willoughby et al. (2000) to explain the generation of anti-inflammatory prostanoids during the later stages of the inflammatory response.

Thus, evidence is accumulating which suggests that various treatments of cells can result in the expression of additional COX variants or isoforms. Whilst the results presented here neither refute nor confirm these suggestions, it is clear that in cultured glial cells the removal and re-addition of serum causes the expression of a COX with reduced sensitivity to certain COX-2 selective and non-selective inhibitors whilst remaining
sensitive to the action of inhibitors preferential for COX-1.
Chapter 5: Paracetamol as an inhibitor of glial prostanoid production

Paracetamol as an inhibitor of glial prostanoid production
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Introduction

Paracetamol (4-acetaminophen) has been used in clinical medicine since 1893, yet it is an anomaly in the field of NSAID pharmacology because its exact mechanism of action remains unknown. Although it mimics classical NSAIDs in terms of its analgesic and antipyretic qualities, various studies have shown it to have little or no effect on COX activity at therapeutic doses (reviewed by Clissold, 1986; Mitchell et al., 1994). Nonetheless, paracetamol has been found to decrease the elevated levels of PGE$_2$ in the CNS following the application of a noxious stimulus (Malmberg and Yaksh, 1994; Muth-Selbach et al., 1999) which lends weight to the proposal, first put forward in the early 1970s (Flower and Vane, 1972), that paracetamol acts on an isoform of COX found only in the CNS. This is, however, likely to be too simplistic an explanation, particularly when one considers that paracetamol has effects on prostanoid levels in a variety of peripheral tissues as well (Green et al., 1989; Berg et al., 1990; Doherty et al., 1990; O'Brien et al., 1993; Pendergraft et al., 1994; Kehoe et al., 1996). In the remainder of this section I shall outline what is known about paracetamol's mechanism of action, focussing primarily on the CNS.

Perhaps the best characterised action of paracetamol on the CNS is its ability to act as an antipyretic where it is believed to reduce fever by reducing levels of PGE$_2$. Extensive studies throughout the 1970s provided compelling evidence for a model of fever involving the activation of PGE receptors in the preoptic area and anterior hypothalamus
which causes coordinated changes in heat gain and heat loss mechanisms leading to hyperthermia (reviewed by Milton, 1982). The production of PGE$_2$ necessary for the activation of these receptors was shown to be increased in response to circulating pyrogen and reduced by the administration of paracetamol (Feldberg and Gupta, 1972; 1973).

In the early work comparing the effect of paracetamol with that of aspirin and indomethacin on COX isolated from rabbit and dog brain or spleen, the lack of anti-inflammatory action observed with paracetamol was presumed to be linked to the lack of inhibition of COX from the spleen, whilst the therapeutic effects coincided with inhibition of COX isolated from brain tissue (Flower and Vane, 1972). It was this observation which first led to suggestions that different isoforms of COX might exist in discrete tissues with varying sensitivities to inhibitors, long before the identification of COX-2. The discovery of COX-2 failed to explain the paracetamol paradox since the inducible isoform was found to be present in both the CNS and peripheral sites of inflammation, findings which led to tentative suggestions that the actions of this drug may reflect the presence of a yet unknown "COX-3" present in the CNS (Mitchell et al., 1994).

The ability of paracetamol to inhibit the synthesis of PGE$_2$ at therapeutic concentrations was first shown using brain homogenates stimulated with sodium arachidonate, in the presence of glutathione and hydroquinone, wherein the reported $ID_{50}$ value was 14
μgL⁻¹ (~ 92 μM) which was of a similar order to that obtained with aspirin under the same conditions (Flower and Vane, 1972). Others subsequently showed that paracetamol was also able to inhibit the peripheral synthesis of prostanoids in bull seminal vesicle microsomes with an IC₅₀ of 1500 μM in the presence of glutathione and hydroquinone (Robak et al., 1978), although no inhibition was observed in microsomes prepared from ram seminal vesicles. Potency values for the inhibition of prostanoid synthesis in dog spleen microsomes (IC₅₀ = 662 μM) (Flower et al., 1972) and rat skin homogenates (IC₂₀ = 500 μM) (Greaves and McDonald-Gibson, 1972) were similarly higher than the plasma levels of paracetamol following ingestion of a therapeutic dose (66 - 330 μM) (Prescott et al., 1968; Glynn and Bastain, 1973). Despite this evidence, the hypothesis that the therapeutic effect of paracetamol was due to inhibition of prostanoid production in the CNS was challenged by reports that there was little difference in effect of the drug between mouse neuronal cultures, rat glial cell lines and rat kidney preparations (von Bruchlausen and Baumann, 1982), or between mouse astrocytes and peritoneal macrophages (Lanz et al., 1986).

After the identification of COX-2, paracetamol was shown to be an inhibitor of both COX-1 and COX-2 in intact cell assays (using endotoxin-activated J774.2 macrophages and bovine aortic endothelial cells) although complete inhibition of prostanoid synthesis was not obtained. In fact, results were expressed in terms of IC₃₀ values since 50% inhibition was not achieved with concentrations up to 1 mg/ml. Interestingly, these studies showed paracetamol to be a more potent inhibitor of COX-1 (IC₃₀ = 2.7 μM)
than COX-2 ($IC_{30} = 20\ \mu\text{M}$) (Mitchell et al., 1994), although a recent study using a human whole blood assay approach showed paracetamol to be more potent against COX-2 ($IC_{50} = 49$ or $64\ \mu\text{M}$) than COX-1 ($IC_{50} = >100\ \mu\text{M}$) (Warner et al., 1999).

Despite the evidence for the action of paracetamol being due to modulation of CNS prostanoid levels, little is known about the effects of the drug on glial cells, which is surprising considering the contribution these cells are thought to make to CNS prostanoid production. In one study paracetamol was shown to inhibit endotoxin-induced PGE$_2$ synthesis in microglial cells and, in another, inclusion of paracetamol in a chronic treatment with β-amyloid reduced PGE$_2$ production in a glioma cell line (Fiebich et al., 1996b; Landolfi et al., 1998). An earlier study reported inhibition of PGD$_2$ release in astrocytes after a long stimulation with A23187 (Lanz et al., 1986), but claimed that the required concentration of the drug was greater than therapeutic levels despite it being within the limits reported by Prescott et al. (1968) and Glynn and Bastain (1973).

To date no convincing evidence has been presented which completely explains the mode of action of paracetamol. It clearly has some effect on prostanoid synthesis and interacts with the COX enzyme under certain conditions, although whether the decrease in PGE$_2$ which accompanies the amelioration of fever after paracetamol administration is via a direct inhibition of prostanoid synthesis has yet to be shown. Throughout the last 30 years the many unexplained effects of this drug on prostanoid levels have alluded to the existence of further isoforms or variants of COX enzymes, possibly expressed in specific
tissues in response to stimuli, which could be inhibited by therapeutic concentrations of paracetamol. The results in the previous chapter show how the pharmacological profile of COX inhibition can be changed through modulation of the extracellular environment. Recently published work from the group of Vane has provided the most compelling evidence so far that the established model of two COX isoforms displaying rigid pharmacology is likely to be an oversimplification. This group found that treating J774.2 macrophages in culture with the NSAID diclofenac caused the induction of a COX enzyme which showed decreased sensitivity to inhibition with a number of NSAIDs, no inhibition with aspirin, and an increased sensitivity to inhibition with paracetamol (Simmons et al., 1999).

The aim of this investigation was to explore the effect of paracetamol on prostanoid synthesis in glial cells. The effect of removal and readdition of serum on the potency of the drug was explored in a similar way to the changes in pharmacology of the NSAIDs discussed in the previous chapter. In addition the effect of paracetamol on the synthesis of prostanoids in enriched preaparations of astrocytes and microglia was investigated.
Chapter 5: Paracetamol as an inhibitor of glial prostanoid production

Results

The inhibition by paracetamol of TXB$_2$ synthesis in response to A23187 and AA is shown in Fig 5.1. Paracetamol caused a concentration-dependent inhibition of TXB$_2$ production with both stimuli with estimated IC$_{50}$ values of 330 µM with A23187 and 930 µM with AA. The mode of action of paracetamol is unclear, and it could theoretically act at a number of different stages in the prostanoid synthetic cascade, perhaps at the level of PLA$_2$ or the specific isomerases in addition to COX. Furthermore, some studies have shown paracetamol to have opposing effects on the synthesis of different prostanoids, particularly TXB$_2$ and PGE$_2$ (Green et al., 1989; O'Brien et al., 1993; Berg et al., 1990; Kehoe et al., 1996; Hutchins et al., 1999). Therefore it was appropriate to investigate the effect of paracetamol on the production of PGE$_2$ in addition to TXB$_2$, as shown in Fig 5.2. Although the basal and stimulated levels of PGE$_2$ were 3-6 fold lower than those observed for TXB$_2$, paracetamol had an inhibitory effect on stimulated production of PGE$_2$ with estimated IC$_{50}$ values of 630 µM for A23187 and 500 µM for AA. Complete inhibition was not achieved against AA stimulation even at concentrations of 3 mM, the maximal inhibition being approximately 52%. The ability to inhibit both TXB$_2$ and PGE$_2$ synthesis suggested that the inhibitory action of paracetamol occurred at a site upstream of the specific isomerases and caused a general reduction in prostanoid synthesis. The upstream enzymes which are sites for the regulation of prostanoid synthesis are PLA$_2$ and COX. It was unlikely that the effect of paracetamol was due entirely to the inhibition of PLA$_2$ since inhibition occurred using exogenous AA as a
Figure 5.1: Inhibition by paracetamol of stimulated TXB$_2$ release in mixed glia. Cells were incubated for 10 min with various concentrations of paracetamol before the addition of AA (10 μM) or A23187 (3 μM) for a further 30 min in the continued presence of paracetamol. Results are expressed as a % of the response elicited by A23187 or AA in the absence of drug and are means ± SEM of at least 4 determinations. Basal = 0.53 ± 0.05 ng/ml, A23187 = 2.26 ± 0.12 ng/ml, AA = 2.30 ± 0.26 ng/ml, n = 8-14.
Figure 5.2: Inhibition by paracetamol of stimulated PGE$_2$ release in mixed glia. Cells were incubated for 10 min with various concentrations of paracetamol before the addition of AA (10 μM) or A23187 (3 μM) for a further 30 min in the continued presence of paracetamol. Results are expressed as a % of the response elicited by A23187 or AA in the absence of drug and are means ± SEM of at least 4 determinations. Basal = 146 ± 3 pg/ml, A23187 = 375 ± 10 pg/ml, AA = 757 ± 21 pg/ml, n = 8.
stimulus which bypassed the need for PLA$_2$-mediated mobilisation of endogenous AA. However, it was possible that paracetamol had an effect on both PLA$_2$ and COX so in order to further elucidate the site of inhibition by paracetamol, the effect on AA-stimulated production was examined in the presence of the PLA$_2$ inhibitor quinacrine (Fig 5.3). Once again paracetamol caused dose-dependent inhibition of stimulated TXB$_2$ release but with an estimated IC$_{50}$ value of 590 µM, showing a higher potency than in the absence of quinacrine (930 µM).

Since removal and re-addition of serum caused changes in the potency of particular NSAIDs in these cells (Chapter 4), the effect of serum removal and re-addition on the action of paracetamol was investigated and the results of these experiments are shown in Figs 5.4 and 5.5. After cells had been grown in serum-free medium for 4 days, paracetamol showed an increased potency against AA stimulation (estimated IC$_{50}$ = 340 µM compared to 930 µM in normal cultures), but remained similar (estimated IC$_{50}$ = 380 µM) to that in the control cells (estimated IC$_{50}$ = 330 µM) in response to A23187 stimulation. After re-addition of serum to the growth medium for 7 days, the potency of paracetamol against AA remained identical (estimated IC$_{50}$ = 330 µM) to that found in serum-deprived cultures, whilst there was an increase in potency with stimulation by A23187 (estimated IC$_{50}$ = 180 µM). These results are summarised in Table 5.1.

In order to examine this further, it was necessary to explore the effect of paracetamol in different glial cell types by using cultures enriched with either astrocytes or microglia. Fig
Figure 5.3: Inhibition by paracetamol of AA-stimulated TXB₂ production in the presence of quinacrine. Cells were incubated for 10 min with quinacrine (100 μM) and various concentrations of paracetamol, then AA (10 μM) was added for a further 30 min in the continued presence of the drugs. Results are expressed as a % of the response elicited by AA in the presence of quinacrine without paracetamol and are means ± SEM of at least 3 determinations. Basal (+quinacrine) = 0.20 ± 0.03 ng/ml, AA (+quinacrine) = 0.98 ± 0.21 ng/ml.
Figure 5.4: Inhibition by paracetamol of stimulated TXB$_2$ release in mixed glia deprived of serum for 4 days. Cells were incubated for 10 min with various concentrations of paracetamol before the addition of AA (10 μM) or A23187 (3 μM) for a further 30 min in the continued presence of paracetamol. Results are expressed as a % of the response elicited by A23187 or AA in the absence of drug and are means ± SEM of at least 4 determinations. Basal = 0.20 ± 0.02 ng/ml, A23187 = 0.53 ± 0.04 ng/ml, AA = 1.68 ± 0.29 ng/ml, n = 8-14.
Figure 5.5: Inhibition by paracetamol of stimulated TXB₂ release in mixed glia which had been maintained in serum-free medium for 4 days prior to the re-addition of serum for 7 days. Cultures were incubated for 10 min with various concentrations of paracetamol before the addition of AA (10 μM) or A23187 (3 μM) for a further 30 min in the continued presence of paracetamol. Results are expressed as a % of the response elicited by A23187 or AA in the absence of drug and are means ± SEM of at least 4 determinations. Basal = 0.55 ± 0.12 ng/ml, A23187 = 1.78 ± 0.23 AA = 2.68 ± 0.35, n = 8-22.
Table 5.1: Estimated IC\textsubscript{50} values for the effect of paracetamol in mixed glia. IC\textsubscript{50} values were estimated as the concentration of paracetamol (\mu M) which caused a 50% reduction in the A23187- or AA- induced release of TXB\textsubscript{2}.  

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Control</th>
<th>Serum deprived</th>
<th>Serum re-addition</th>
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<tbody>
<tr>
<td>A23187</td>
<td>330</td>
<td>380</td>
<td>180</td>
</tr>
<tr>
<td>AA</td>
<td>930</td>
<td>340</td>
<td>330</td>
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</table>
5.6 shows the inhibition of A23187- and AA- stimulated release of TXB₂ in astrocyte-enriched cultures. As observed in the mixed glial cultures, the potency of paracetamol was greater with A23187 stimulation than with AA, however the estimated IC₅₀ values with both stimuli were considerably increased (A23187 = 800 μM, AA = 1600 μM) compared to the mixed glial cells. In microglia (Fig 5.7), the potency of paracetamol with A23187-stimulation (estimated IC₅₀ = 390 μM) was similar to that observed in the mixed cultures (330 μM), but paracetamol failed to elicit any inhibitory effect on AA-stimulated TXB₂ release, in fact it caused a concentration-dependent increase in TXB₂ production. These estimates of the potency of paracetamol in subcultured astrocytes and microglia are summarised in Table 5.2.
Figure 5.6: Inhibition by paracetamol of stimulated TXB$_2$ release in subcultured astrocytes. Astrocyte-enriched cell cultures were incubated for 10 min with various concentrations of paracetamol before the addition of AA (10 µM) or A23187 (3 µM) for a further 30 min in the continued presence of paracetamol. Results are expressed as a % of the response elicited by A23187 or AA in the absence of drug and are means ± SEM of at least 4 determinations. Basal = 0.37 ± 0.08 ng/ml, A23187 = 0.73 ± 0.07 ng/ml, AA = 3.69 ± 1.1 ng/ml, n = 8
Figure 5.7: Inhibition by paracetamol of stimulated TXB$_2$ release in microglia. Microglial-enriched cell cultures were incubated for 10 min with various concentrations of paracetamol before the addition of AA (10 μM) or A23187 (3 μM) for a further 30 min in the continued presence of paracetamol. Results are expressed as a % of the response elicited by A23187 or AA in the absence of drug and are means ± SEM of at least 4 determinations. Basal = 0.25 ± 0.06 ng/ml, A23187 = 1.11 ± 0.07 ng/ml, AA = 1.74 ± 0.07 ng/ml, n = 6-10.
Table 5.2: Estimated IC$_{50}$ values for the effect of paracetamol in astrocytes and microglia. IC$_{50}$ values were estimated as the concentration of paracetamol (μM) which caused a 50% reduction in the A23187- or AA-induced release of TXB$_2$ from astrocyte or microglial enriched cultures.
Discussion

Effect of paracetamol on TXB$_2$ and PGE$_2$ production

Paracetamol was able to inhibit the production of both TXB$_2$ and PGE$_2$ in mixed glial cultures upon stimulation with either A23187 or AA in a concentration dependent manner over the range 0.1 - 1 mM. The inhibition of A23187-stimulated TXB$_2$ production occurred with an approximate IC$_{50}$ value of 330 µM, which coincides with the upper limit of the range of plasma concentrations of the drug following a therapeutic dose (66 - 330 µM) (Prescott et al., 1968; Glynn and Bastain, 1973). Although the estimated IC$_{50}$ values obtained for paracetamol acting on AA-stimulated release and release of PGE$_2$ lie outside this range, some inhibitory effect was observed at therapeutic concentrations in all experiments, suggesting the possibility that inhibition of prostanoid synthesis in glia may contribute to the therapeutic effects of the drug in the CNS. The estimated IC$_{50}$ values reported here are of a similar order to those reported for the inhibition of A23187-stimulated PGD$_2$ and PGE$_2$ release (134 µM and 178 µM, respectively) by paracetamol in cultured astrocytes (Lanz et al., 1986), the higher potency presumably being due to their use of lower A23187 concentrations (1 µM) and longer incubation periods (2 h). A more potent inhibition by paracetamol on PGE$_2$ release was reported in T98G glioma cells stimulated with IL-1β (between 10 and 100 µM), but 48 h incubations were used allowing the potential for changes in the expression of proteins involved in the synthetic cascade (Landolfi et al., 1998).
Chapter 5: Paracetamol as an inhibitor of glial prostanoid production

The greater potency of paracetamol as an inhibitor of A23187- rather than AA-induced TXB$_2$ production is reminiscent of the effect of ibuprofen in Chapter 3 which showed a 6-fold greater potency with A23187 than AA whilst most of the other NSAIDs were more potent with AA or approximately equipotent. The reasons for this are unclear but might reflect differences in the ability of these drugs to compete with AA from the different sources provided by the two stimuli.

Paracetamol had an inhibitory effect on both TXB$_2$ and PGE$_2$ release, with the estimated IC$_{50}$ values for PGE$_2$ after stimulation with AA and A23187 being closer to each other than those for TXB$_2$. Paracetamol failed to cause more than 52 % inhibition of AA-stimulated PGE$_2$ release, suggesting the existence of a source of AA-induced PGE$_2$ synthesis which is insensitive to paracetamol. This effect was not apparent when A23187 was the stimulus. Generally, the inhibition of both PGE$_2$ and TXB$_2$ production by paracetamol suggests that its inhibitory action is on the general synthesis of prostanoids rather than on the synthesis of the particular prostanoids measured. This view is further supported by the equivalent levels of inhibition of PGD$_2$ and PGE$_2$ release reported in astrocytes in a similar investigation (Lanz et al., 1986). This would suggest that paracetamol acts at some site upstream of the specific isomerases in the prostanoid synthetic cascade. Paracetamol could act at either the level of COX or PLA$_2$. Since PLA$_2$ is not involved in the prostanoid production stimulated by AA, it would be reasonable to reject an effect of paracetamol on PLA$_2$ since the drug appeared to be effective against either stimulus, and was in fact more potent in the presence of the PLA$_2$ inhibitor
quinacrine. The most likely explanation therefore for the inhibition of prostanoid synthesis by paracetamol in these cells is that it acts to inhibit COX in some way.

**Effect of serum removal and re-addition on inhibition of TXB$_2$ release by paracetamol**

In the previous chapter it was shown that removal of serum from the bathing medium of cells for 4 days followed by its replacement for a further 7 days caused changes both in the level of COX-protein expression and the relative contribution of each isoform to prostanoid synthesis, as determined from changes in the profile of NSAID potencies. After serum removal, the general trend was towards a system in which COX-1 selective inhibitors were more potent and those more selective for COX-2 showed decreased potency. After the re-addition of serum for 7 days the profile of the COX inhibitors differed from both the control and serum-deprived situations with a further reduction in potency of the COX-2 selective inhibitors. Paracetamol also showed marked changes in its inhibitory potency in each of these conditions. By comparing the estimated IC$_{50}$ values obtained, it is apparent that after serum removal paracetamol became almost 3-fold more potent with AA-induced release than in controls, reaching a value similar to that observed with A23187 stimulation. This increased potency was maintained after re-addition of serum whereupon an increase in the potency with A23187 occurred. It would appear from these results that the difference in the potency of paracetamol with each stimulus was removed after serum deprivation, then restored after serum re-addition but with a 2-3 fold increase in potency compared to the original situation. This is similar to
the effect reported by Simmons et al. (1999) who showed that following a 48 h treatment with the NSAID diclofenac, J774.2 cells expressed a COX activity which was sensitive to inhibition by paracetamol (estimated IC$_{50}$ 170 μM) upon stimulation with 30 μM AA. This induced COX was proposed to be a variant of COX-2 since its expression could be enhanced with LPS treatment and paralleled an increase in COX-2 protein. The induction of this potential COX-2 variant required nonphysiological concentrations (500 μM) of diclofenac which also induced apoptosis. The COX induction was suggested to be via the PPAR pathway of transcriptional activation rather than via NF-κB due to the ability of NSAIDs at similar concentrations to bind and activate members of the PPAR family of receptors (Lehmann et al., 1997). If, as these studies suggest, a catalytic variant of COX exists at which paracetamol can act as a potent inhibitor, it is reasonable to propose that, given the structural similarity of paracetamol to many of the NSAIDs (in particular aspirin), paracetamol might directly block the binding of AA to the active site of the COX variant in a similar way to that shown for other NSAIDs (Loll et al., 1995; Kurumbail et al., 1996).

Using a COX-2-luciferase reporter construct the paracetamol-sensitive COX showed a change in cellular localisation when compared to controls in that it was located in the cytosol rather than nuclear and microsomal membranes (Simmons et al., 1999). The suggestion that the change in sensitivity to paracetamol is accompanied by a change in the microenvironment of COX gives rise to another possible explanation for the results obtained here. Previous studies using purified COX have shown that the inhibitory
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potency of paracetamol is dependent on the level of peroxide present in the assay (Hanel and Lands, 1982), with the drug showing an increased potency when the peroxide level was lowered using glutathione peroxidase. Different levels of peroxide prevail within isolated subcellular compartments, so it is possible that a change in subcellular localisation of COX after serum withdrawal and re-addition would expose the enzyme to an environment of increased or reduced peroxide concentration. A microenvironment with less peroxide could cause the increase in potency of paracetamol observed here.

Glutathione peroxidase is located in the cytosol and does not react with micellar or membrane-bound peroxides (Marshall et al., 1987 and references therein), which suggests that the cytosolic hydroperoxide concentration is suppressed, adding weight to the suggestion that a translocation of COX to the cytosol could result in increased inhibitory potency of paracetamol. When comparing the effects of serum deprivation and re-addition with the diclofenac treatment reported to increase the potency of paracetamol by Simmons et al. (1999), it is important to note that the 48 h treatment of J774.2 cells with diclofenac was sufficient to induce apoptosis in those cells. Although in the present study there was no direct measurement of apoptosis, removal and subsequent re-addition of serum however did not appear to induce any cell death as determined through routine visual inspection of treated and untreated glial cell cultures using phase contrast microscopy. The morphology of the cells changed slightly immediately after serum removal, becoming more stellate in appearance, but they returned to their confluent protoplasmic state within 24 h and remained so for many weeks after treatment.
Effect of paracetamol on prostanoid synthesis in astrocytes and microglia

Previous studies have investigated the effect of paracetamol on glial prostanoid production using primary astrocyte cultures (Lanz et al., 1986), but these preparations were likely to contain a mixture of astrocytes and microglia. Since paracetamol was shown to inhibit endotoxin-induced PGE$_2$ production in microglia (Fiebich et al., 1996b), and results presented here and by others have shown differences in the ability of NSAIDs to inhibit prostanoid production in one or the other cell type, it was important to investigate the effect of paracetamol in astroglial and microglial enriched cultures.

Comparison of the estimated IC$_{50}$ values showed a much reduced potency of paracetamol in astrocytes compared to the mixed cultures. In microglia, however, the potency of paracetamol against A23187 was similar to that in mixed glia, but with AA-induced release, paracetamol appeared to have a stimulatory effect. Stimulation of prostanoid synthesis by paracetamol was also observed by Robak et al. (1978), who showed that the inhibitory effect of paracetamol was dependent on the presence of glutathione in seminal vesicle microsomes, its omission from the incubation mixture resulting in paracetamol becoming a stimulator of PGE$_2$ synthesis. Perhaps one explanation for the observed stimulation of AA-induced TXB$_2$ production in microglia could be a manifestation of this effect. A model for the action of paracetamol, as first suggested by Hanel and Lands (1982), was based on the requirement of hydroperoxide intermediates for the activation of COX, with paracetamol acting as a cosubstrate to increase the peroxidase activity of
The inhibitory effect of paracetamol occurred only when the hydroperoxide level fell below a critical value to the point where it became the limiting factor for the rate of COX activity. It is then that the radical-trapping capability of paracetamol results in its inhibition of COX-activity (van de Straat et al., 1988). Considering the opposing actions of paracetamol with respect to the two stimuli in microglia and the substantially different potencies in astrocytes, it is not unreasonable to suggest that in addition to providing a substrate for COX, exogenous AA might also raise hydroperoxide levels in the microenvironment of COX. This could then reduce the potency of paracetamol compared to that obtained with A23187 explaining the results obtained with astrocytes. In microglia the effect is such that hydroperoxide reaches a level at which paracetamol can act as a cosubstrate to enhance the peroxidase stage of the enzyme activity. This is not an unreasonable suggestion since COX itself is capable of producing hydroperoxide intermediates through the metabolism of AA, so perhaps in these cells the stimulation of COX activity with AA is such that the hydroperoxides produced are available for activation of COX, thus facilitating the stimulatory effect of paracetamol.

The hydroperoxide dependence of COX also provides a potential explanation for the difference in the effect of paracetamol on AA-induced TXB$_2$ production in astrocytes and microglia. Glutathione in its reduced form reacts with hydrogen peroxide and organic hydroperoxides and has the effect of reducing cellular levels of potentially harmful hydroperoxides in a reaction catalysed by glutathione peroxidase. Glutathione peroxidase therefore regulates the levels of hydroperoxide within cells and has been shown to
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It has been shown that microglia isolated from primary glial cultures have a higher glutathione content than astrocytes (Chatterjee et al., 1999). The high levels of glutathione found in microglia would presumably result in a reduced hydroperoxide tone compared to astrocytes and an associated increase in the inhibitory potency of paracetamol rather than the stimulation observed. In rat CNS, glutathione peroxidase levels are also higher in microglia than in astrocytes, with an elevation of immunoreactivity for the enzyme observed in excitotoxin-induced neurodegeneration (Lindenau et al., 1998). This suggests that these cells react to increased levels of reactive oxygen species through increased glutathione peroxidase expression. In this way, cellular glutathione levels could be considered indicative of the levels of hydroperoxide within the cell, suggesting that levels of hydroperoxide in microglia may be higher than in astrocytes giving rise to the observed differences in the inhibitory effect of paracetamol.

An alternative suggestion to explain the differences in potency of paracetamol in the different glial culture preparations is that there may be differences in the COX isoforms present in each cell type, as suggested in Chapter 3. It was clear from the NSAID experiments that the contribution of the different COX isoforms to TXB$_2$ synthesis varied between mixed glia, astrocytes and microglia, with the possibility that in the mixed glial preparations the presence of different cell types influences the expression of COX isoforms in some way. If, as suggested in the earlier chapter, microglia use a more COX-
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2 dependent prostanoid synthetic pathway than astrocytes, then paracetamol could be considered to be a more potent inhibitor of this combination of isoforms.

It is clear from the results presented here that the manipulation of glial cell cultures by removal and addition of serum, as well as the isolation of microglia and astrocytes, has a profound effect on the ability of paracetamol to act as an inhibitor of prostanoid synthesis. Two hypotheses have been proposed for the action of the drug, either as a direct inhibitor of COX, competing with AA for the active site in the same way as classical NSAIDs, or as an indirect inhibitor involved in the hydroperoxide-dependent activation of the enzyme. The different effects observed after different serum treatments and purification of cells could reflect changes in the hydroperoxide microenvironment of the COX isoforms involved in prostanoid synthesis, perhaps through a change in intracellular localisation or maybe through modulation of some other factor concerned with regulation of intracellular hydroperoxide tone. Alternatively, the observed differences in the effect of the drug may support the hypothesis that glial cells are able to express a third isoform or variant of COX with increased sensitivity to paracetamol.
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Chapter 6: Regulation of cyclooxygenase activity by nitric oxide

Introduction

NO, like the prostanoids, is an important mediator in many physiological and pathological processes, with particular involvement in the regulation of vascular tone and central and peripheral nervous systems. It can also cause inhibition of key enzymes involved in respiration and DNA synthesis in target cells and produce toxic substances after interaction with oxygen-derived radicals, thus participating in immunological host defence. This cytotoxic property leads to the association of NO with the pathophysiology of certain clinical conditions such as hypertension and chronic inflammation (Moncada and Higgs, 1995). In the CNS, NO has been linked to the action of excitatory amino acids. In particular, production of NO is triggered by the action of L-glutamate on neuronal NMDA receptors and is responsible for the subsequent increase in intracellular cGMP (Garthwaite et al., 1988). Interestingly this increase in cGMP occurs predominantly in glial cells, suggesting that NO may be an important mediator in the communication between neurons and glia. NO has also been associated with a variety of pathological conditions in the CNS including multiple sclerosis, ischaemia, HIV and Parkinson’s disease although there is some controversy as to whether the increased NO production associated with CNS pathophysiology is detrimental or beneficial (reviewed by Murphy, 2000).

NO is a relatively stable gaseous free radical and its highly diffusible nature provides the potential for it to act on tissues far from its site of synthesis. NO is formed by the
oxidation of the terminal guanidino nitrogen of L-arginine via the action of nitric oxide synthase (NOS) with a stoichiometric production of L-citrulline as a by-product. Three distinct NOS isozymes have been identified which are the products of separate genes. Two are constitutive and are referred to as neuronal (nNOS) and endothelial (eNOS) due to early association of the isozymes with these regions, although subsequent work has shown the presence of eNOS in both neurons and platelets and the association of nNOS with skeletal muscle, kidney, uterus, pancreas and the epithelium of bronchi. (Nakane et al., 1993; Schmidt et al., 1992; Dinerman et al., 1994). The third isoform is inducible (iNOS) and, like COX-2, is prevalent in macrophages and has been shown to be induced by a variety of stimuli, in particular inflammatory mediators, and is believed to be responsible for the generation of large amounts of pro-inflammatory and cytotoxic NO (Vane et al., 1994). Although iNOS is considered to be inducible, it appears to be expressed constitutively in the kidney and some foetal tissues, whilst the constitutive eNOS has been shown to be induced in pregnancy and during chronic exercise (Weiner et al., 1994).

Whether NO production results in physiology or pathophysiology is believed to be determined by the magnitude, duration and location of its synthesis. For example, the action of the two constitutive NOS isoforms tends to produce relatively low levels of NO for short periods of time, mediating blood flow and inhibiting platelet aggregation and leukocyte adhesion (Moncada and Higgs, 1995). The result of the induction of iNOS on the other hand is prolonged synthesis of higher levels of NO. This was originally
demonstrated in macrophages where the production of NO by iNOS contributed to the cytotoxic actions of these cells on invading microorganisms, and has subsequently been shown to occur in many other cells, particularly of the immune system, in response to activation by LPS or cytokines (reviewed by Moncada and Higgs, 1995). The cytotoxic and pathological role of NO is usually associated with its ability as a free radical to react chemically with other molecules, particularly other free radicals such as reactive oxygen species. The precise mechanisms of these cytotoxic effects, which are thought to cause tissue damage and vascular leakage in diseases such as septicaemia, rheumatoid arthritis, acute respiratory distress syndrome and inflammatory bowel disease, are unknown, but elevated levels of stable products of reactions involving NO (for example nitrotyrosine) have been detected in tissues associated with these conditions (reviewed in Darley-Usmar et al., 1995). NO elicits many of its biological effects through activation of the soluble form of guanylate cyclase resulting in increased production of the signal transduction molecule cGMP (Ignarro et al., 1987). This activation occurs through a conformational change produced upon the binding of NO to a haem group within the enzyme (Zhao et al., 1998).

Despite NO and prostanoids being well established mediators of inflammation with many similar effects, there has been much debate as to the influence each might have on the synthesis of the other. The first experimental observation of such an interaction led to the proposal that a direct activation of COX by NO was possible, since inhibition of NO release, either through the use of NOS inhibitors or the depletion of L-arginine,
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attenuated the release of PGE$_2$ from a mouse macrophage cell line after stimulation with LPS (Salvemini et al., 1993). Subsequent experiments showed that NO gas, or the NO donors sodium nitroprusside (SNP) and glycercyl trinitrate, increased the activity of COX-1 and COX-2 in various cellular and purified recombinant enzyme systems independently of soluble guanylate cyclase (reviewed by Salvemini, 1997). Moreover, after stimulation of macrophages with LPS to induce both COX-2 and iNOS expression, it was found that inhibition of iNOS activity caused a concomitant decrease in prostaglandin synthesis without affecting the level of COX-2 expression. There are, however, some inconsistencies with regard to the effect of NO on COX expression. It was recently shown that the NO donor SNP caused a concentration-dependent increase in COX-2 expression in osteoblasts, and that the induction of COX-2 expression by certain cytokines, specifically IFN$\gamma$ but not IL-$\beta$, in these cells could be blocked by the NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) (Hughes et al., 1999). This is in contrast to the reduction in COX-2 expression mediated by NO in LPS-treated J774.2 macrophages (Swierkosz et al., 1995), LPS-activated rat peritoneal macrophages (Habib et al., 1997) and LPS-treated microglia (Minghetti et al., 1996), suggesting a complex effect of NO on COX-2 expression which is specific both to the cell type and the means of induction chosen.

These initial findings were corroborated in a number of subsequent studies, for example NO donors were shown to activate both the cyclooxygenase and peroxidase activities of purified preparations of COX via an interaction with the active site haem which increased
the apparent $V_{\text{max}}$ without any effects on the $K_m$ (Maccarrone et al., 1997), and various other reports showed a similar stimulatory effect (Corbett et al., 1993; Salvemini et al., 1997). Interestingly, Curtis et al. (1996), showed that despite an increase in the peroxidase activity of COX in macrophages in response to NO, there was no corresponding increase in prostanoid production. Despite the accumulated evidence for NO-mediated activation of COX, a number of groups found that in some cases there was in fact an inhibitory effect of NO on the activity of COX (Kanner et al., 1992; Swierkosz et al., 1995; Minghetti et al., 1996; Habib et al., 1997).

One further complication in the study of the interactions between the NO and prostanoid synthetic pathways is the effects that prostanoids might have on the synthesis of NO. Some studies have shown that high concentrations of prostanoids inhibit iNOS induction without affecting enzyme activity, whilst others have shown a reduction in NOS activity after inhibition of prostanoid synthesis (reviewed in Salvemini, 1997). In most instances these actions have been attributed to activation of adenylate cyclase and the subsequent interaction of cAMP with the NOS pathway. In general, little data has been reported to suggest that inhibition of COX influences NOS function, but such an action could potentially contribute to the effects of COX inhibitors used clinically or experimentally.

As a result of numerous studies, various, and sometimes conflicting suggestions as to how NO modulates COX function have emerged. As mentioned previously, NO often achieves its effects through binding to the haem group of guanylate cyclase to induce an
increase in cGMP production. Since the inhibition of guanylate cyclase with methylene blue was shown to have no effect on the action of NO on COX (Salvemini et al., 1993), a reasonable hypothesis was that NO might exert its effects through a direct interaction with the haem group of COX. Spectral analysis revealed that NO had low affinity for the haem group of COX in its native ferric form, although NO did cause substantial conformational changes to COX in vitro which was in a reduced state and contained a ferrous haem (Tsai et al., 1994). However, the existence of COX in a ferrous state has not been shown in vivo, and if it were to exist in this form it would react readily with O₂ and CO as well as NO, in fact the conformational changes conferred through NO binding would be likely to render it inactive (reviewed by Goodwin et al., 1999). This could explain the inhibitory effect of NO on COX activity reported by some groups, but not the many examples of NO stimulation of COX. A number of suggestions have been made as to how this might occur and are summarised here.

One suggestion is that NO might nitrosylate thiol groups on the cysteine residues of COX, causing an increase in COX activity. Two cysteine residues are conserved between COX-1 and COX-2 which are potential sites for nitrosylation, and treatment of COX with NO was shown to cause an increase in activity parallel to increased thiol nitrosylation (Hajjar et al., 1995). In addition, in experiments using purified enzyme, NO caused little change in the absorption spectrum for the COX haem group, but substantial changes in the far-UV spectrum were detected indicating a change in β-sheet structure (Hajjar et al., 1995). Modification of the two conserved cysteine residues, either through
chemical means or site-directed mutagenesis, has been found to cause changes in COX activity, but the result of these approaches has been inactivation of the enzyme, making it unlikely that conformational changes cased by the nitrosylation of COX thiols could result in increased COX activity (Goodwin et al., 1999).

Apart from the interaction of NO with haem groups and thiols, NO can react with tyrosyl radicals in a reversible way, and it is by this means that it blocks the action of ribonucleotide reductase which is the enzyme which catalyses the synthesis of deoxyribonucleoside diphosphates from their ribonucleoside forerunners. Although in its resting state COX does not contain a tyrosyl radical, formation of a tyrosyl radical plays an important role in the activation process of the enzyme as discussed in Chapter 1. Evidence suggests that the COX tyrosyl radical reacts rapidly with NO, but, rather than being a reversible interaction as in ribonucleotide reductase, further oxidation occurs resulting in the formation of a radical intermediate then stable nitrotyrosine (Gunther et al., 1997). Thus it would seem that once again the action of NO would be more likely to cause an inactivation of COX than an activation if it were to act via this mechanism. The ability of NO to act as a potent reducing substrate in the COX peroxidase reaction could potentially cause an increase in activity, so perhaps a balance is achieved between these two effects giving rise to either an activation or an inhibition of COX depending on factors such as the concentration of available AA or the redox state of its micro-environment.
One final explanation for the stimulatory action of NO on COX arises from the suggestion that maybe NO is the precursor to some other molecule which, in turn, elicits the modulatory effects. Since no clear direct interaction of NO with COX has been demonstrated which would cause increased COX activity, this would seem to be a reasonable theory. One such molecule is peroxynitrite (ONOO'), a product of NO interacting with superoxide (O_2^-), which has been shown to interact with COX. ONOO' is a substrate for COX peroxidase activity and could therefore provide a means of activation due to the peroxide requirement of COX as suggested by Landino et al. (1996). This group showed that ONOO' was capable of activating COX in the presence of high concentrations of glutathione and glutathione peroxidase, a combination which normally removes any hydroperoxides and renders COX inactive. This suggests that ONOO' is a more effective activator of COX than many other hydroperoxides such as H_2O_2, PGG_2, and HPETEs. Under inflammatory conditions, cells such as neutrophils and macrophages produce both NO from iNOS and O_2^- from NADPH oxidase, so it is feasible that these could combine to form ONOO' which provides a means of activating COX.

*The effect of NO on prostanoid production in glial cells*

Cultured glia express constitutive and inducible NOS activity which can be stimulated by LPS and cytokines (Galea et al., 1992; Mollace et al., 1993; Colasanti et al., 1995). However, interactions between the NO and prostanoid synthetic pathways in these cells
remain far from clear with many conflicting reports. For example, NO was shown to activate COX in mouse astrocyte cultures after 18 h of stimulation with LPS. NOS inhibitors reduced the production of PGE$_2$ and the NO donor SNP enhanced PGE$_2$ accumulation, both in a dose dependent manner (Molina-Holgado et al., 1995). Similar activation of prostanoid synthesis mediated by NO was shown in a human astrocytoma cell line after stimulation with cytokines. The IL-1β and TNF-α stimulated release of PGE$_2$ was blocked by the presence of the NOS inhibitor L-NAME and again SNP was able to enhance PGE$_2$ production in these cells (Mollace et al., 1998). In addition, it was shown that the inhibition of COX activity in cultures of mouse astrocytes following treatment with interferon-γ (previously shown to up-regulate NO release from astrocytes) was not achieved through an inhibition of COX by NO (Hewett, 1999). This stimulatory effect of NO on COX in astrocytes is not, however, consistent as Janabi et al. (1996) showed that the inhibition of NOS in human embryonic astrocytes had little effect on prostanoid production, although it did cause a reduction of prostanoid synthesis in microglial cultures. This study also showed that whereas astrocytes in response to cytokine induction secreted NO but not O$_2^-$, microglia under similar conditions released O$_2^-$ without NO despite the expression of iNOS. It is important to note that in studies using astrocyte cell cultures, a small population of contaminating microglia are invariably present, which may or may not contribute significantly to their prostanoid synthetic capacity (discussed in Chapter 3), so these preparations could be considered to be mixed glial cell cultures. In a number of separate studies focussing on cultured microglia, Minghetti and co-workers have shown a down-regulation of LPS-induced COX
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expression and reduced prostanoid synthesis in response to endogenous NO and NO donors (Minghetti et al., 1996), a down-regulation of iNOS by the administration of exogenous PGE\textsubscript{2} (although paradoxically the inhibition of endogenous prostanoid production via COX inhibitors also caused an inhibition of iNOS expression) (Minghetti et al., 1997), and finally an increase in LPS-induced prostanoid production by inhibition of NOS together with an inhibition of prostanoid synthesis after addition of NO donors (Guastadisegni et al., 1997). Interestingly, this latter study directly compared the effects of reactive nitrogen intermediates in microglia and RAW 264.7 cells, the macrophage cell line previously used for many of the initial investigations into the effects of NO on COX. The contrary findings of this study highlight the opposing effects of similar treatments between the two cell types indicating that results obtained with RAW 264.7 cells cannot be extrapolated to microglia.

The conflicting results obtained with glial cells in culture focusses attention on the lack of understanding of the mechanisms of interaction between NO and COX. It is not even clear whether it is NO itself which interacts with COX or whether there is a more indirect process. One of the factors which appears to add to the confusion is that glial cell studies to date invariably involve pretreatment of the cells with a stimulus which induces the expression of COX-2 and iNOS, even though cultured preparations of these cells express both inducible enzymes (Murphy et al., 1993; Fiebich et al., 1996; O'Banion et al., 1996; Minghetti et al., 1997; Bauer et al., 1997; Luo et al., 1998; Koyama et al., 1999; Hirst et al., 1999). In this study the effects of NO on COX activity were investigated in mixed
Results

The effect of endogenous NO production on COX activity was investigated by inhibition of NOS with L-NAME. Table 6.1 shows that 10 min pre-treatments with L-NAME at various concentrations had little effect on either basal or A23187-stimulated TXB$_2$ production. Longer incubations (48 h) with a higher concentration (300 μM) of L-NAME also failed to elicit any substantial change in basal or A23187-stimulated TXB$_2$ release (Fig 6.1), suggesting that under these conditions the synthesis of NO by NOS did not influence COX activity in these cells.

In order to investigate the potential for the modulation of COX activity by NO, the effect of the NO donor SNP was investigated. Fig 6.2 shows that SNP elicited a concentration-dependent decrease in A23187-induced TXB$_2$ production with an estimated IC$_{50}$ value of approximately 1 mM. This inhibition was mimicked by another NO donor, S-nitrosoglutathione (SNOG), as shown in Fig 6.3. SNP at 1 mM failed to modulate basal TXB$_2$ production, whilst SNP and SNOG at 1 mM reduced A23187-induced TXB$_2$ production by 32% and 48% respectively. In order to investigate whether the inhibition elicited by SNP was due to the action of NO itself or ONOO$^-$, the effect of SNP on A23187-stimulated TXB$_2$ production was examined in the presence of either the NO scavenger haemoglobin (Hb), or superoxide dismutase (SOD) which acts to remove the
### Table 6.1: Effect of the NOS inhibitor L-NAME on basal and A23187-stimulated TXB₂ production in mixed glial cell cultures.

Cultures were incubated for 10 min with various concentrations of L-NAME before the addition of A23187 (3 μM) for a further 30 min where appropriate. Results are means ± SEM from 3 experiments.

<table>
<thead>
<tr>
<th>Additions</th>
<th>TXB₂ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.73 ± 0.11</td>
</tr>
<tr>
<td>A23187</td>
<td>4.74 ± 0.13</td>
</tr>
<tr>
<td>A23187 + L-NAME (1 μM)</td>
<td>4.42 ± 0.36</td>
</tr>
<tr>
<td>A23187 + L-NAME (10 μM)</td>
<td>4.89 ± 0.10</td>
</tr>
<tr>
<td>A23187 + L-NAME (100 μM)</td>
<td>4.83 ± 0.16</td>
</tr>
<tr>
<td>L-NAME (100 μM)</td>
<td>0.65 ± 0.07</td>
</tr>
</tbody>
</table>
Figure 6.1: Effect of long-term L-NAME pretreatment on A23187-stimulated TXB$_2$ accumulation. Cultures were maintained for 48 h in 3 ml of growth medium to which either L-NAME (300 μM) or vehicle (water) had been added. Cultures were washed in buffer then incubated for 30 min in buffer with no additions or A23187 (3 μM). Results are means ± SEM from three experiments.
Figure 6.2: Concentration dependence of SNP-mediated inhibition of A23187-induced TXB$_2$ release. Cultures were washed then incubated with various concentrations of SNP for 10 min prior to the addition of A23187 (3 µM) and further incubation for 30 min. Results are expressed as % of the response elicited by A23187 in the absence of SNP. Data are means ± SEM from three determinations. Basal and A23187-stimulated levels of TXB$_2$ release were 0.36 ± 0.09 ng/ml and 2.48 ± 0.31 ng/ml respectively (n = 4).
Figure 6.3: Effect of NO donors on A23187-stimulated TXB$_2$ accumulation. Cultures were washed in buffer then incubated with either SNP or SNOG at 1mM for 10 min. Cultures were then incubated for a further 30 min in the presence or absence of A23187 (3 μM). Results are means ± SEM from three experiments.
O$_2^-$ required for ONOO$^-$ formation. Fig 6.4 shows that the effect of SNP on A23187-stimulated TXB$_2$ production was completely abrogated in the presence of Hb, but was unaffected by the presence of SOD. In addition, 8-bromocyclic-GMP (8-BrcGMP), a membrane-permeant analogue of cGMP, failed to modulate the TXB$_2$ synthesis stimulated by A23187 (Table 6.2).

To determine whether the NO donor-mediated inhibition of stimulated TXB$_2$ production was a function of the stimulus used, the effects of SNP and SNOG on prostanoid production induced by AA were also examined. Fig 6.5 shows that SNP and SNOG reduced AA-induced TXB$_2$ production by 61 % and 78 % respectively. As before, Hb was able to prevent the inhibition of AA-induced TXB$_2$ release by SNP, with SOD causing no change in the effect of SNP (Fig 6.6). Experiments using inhibitors selective for the different COX isoforms have suggested that prostanoid production in cultured glia involves both COX-1 and COX-2 (Chapter 3). In order to investigate the effect of NO on each of these isoforms, the SNP-mediated reduction in A23187-induced TXB$_2$ synthesis was investigated in the presence of COX inhibitors. Piroxicam and nimesulide were chosen as examples of inhibitors with selectivity towards COX-1 and COX-2, respectively, together with ibuprofen which inhibits both isoforms with equal efficacy. Fig 6.7 shows that SNP, piroxicam, nimesulide and ibuprofen alone reduced A23187-stimulated TXB$_2$ production by approximately 70 %, 51 %, 63 % and 50 %, respectively, whereas the combined addition of SNP with any one of the COX inhibitors, regardless of its selectivity for either COX isoform, reduced the stimulated TXB$_2$ production by
Figure 6.4: Effect of Hb and SOD on the inhibition of A23187-stimulated TXB₂ production by SNP. Cultures were washed with buffer then incubated in 2 ml buffer containing SNP (1 mM) for 10 min in the presence or absence of either Hb (3 μM) or SOD (30 U/ml). Cultures were then incubated for a further 30 min in the presence or absence of A23187 (3 μM). Results are expressed as a % of the response elicited by A23187 in the absence of other additions and are means ± SEM from at least 3 experiments.
Table 6.2: Effect of 8-BrcGMP on A23187-stimulated TXB$_2$ accumulation. Cultures were washed in buffer then incubated for 10 min in 2 ml buffer containing various concentrations of 8-BrcGMP. Cultures were then incubated for a further 30 min in the presence or absence of A23187 (3 μM). Results are means ± SEM from at least three experiments.
Figure 6.5: Effect of NO donors on AA-stimulated TXB₂ accumulation. Cultures were washed in buffer then incubated with either SNP or SNOG at 1 mM for 10 min. Cultures were then incubated for a further 30 min in the presence or absence of AA (30 μM). Results are means ± SEM from at least 3 experiments.
Figure 6.6: Effect of Hb and SOD on the inhibition of AA-stimulated TXB₂ production by SNP. Cultures were washed with buffer then incubated in 2 ml buffer containing SNP (1 mM) for 10 min in the presence or absence of either Hb (3 µM) or SOD (30 U/ml). Cultures were then incubated for a further 30 min in the presence or absence of AA (30 µM). Results are expressed as a % of the response elicited by AA in the absence of other additions and are means ± SEM from at least three experiments.
Figure 6.7: Effect of COX inhibitors on the inhibition of A23187-stimulated TXB₂ accumulation by SNP. Cultures were washed in buffer then incubated for 10 min in 2 ml of buffer containing either SNP (1 mM), piroxicam (0.1 μM), nimesulide (2 μM) or ibuprofen (0.3 μM) alone or in combination. Cultures were then incubated for a further 30 min in the presence or absence of A23187 (3 μM). Results are expressed as a % of basal TXB₂ accumulation (0.29 ± 0.01 ng/ml) and are means ± SEM from at least three experiments.
more than 90%. Finally, in an attempt to further investigate the isoform-dependence of the effect of NO on COX, serum deprived cultures were treated with SNP before stimulation with A23187 and AA. Serum deprivation was shown to reduce expression of COX, with a reduction in the potency of COX-2 preferential inhibitors implying that this was due to reduced expression of COX-2 (Chapter 4). Fig 6.8 shows that serum deprivation resulted in a marked reduction in basal and stimulus-induced TXB$_2$ production, however SNP was found to inhibit AA- and A23187- stimulated TXB$_2$ production under these conditions to a similar extent to that found in normal cultures.
Figure 6.8: Effect of serum deprivation on the inhibition of A23187- and AA-stimulated TXB₂ accumulation by SNP. Cultures were maintained in serum-free culture medium for four days then washed and incubated in 2 ml buffer containing SNP (1 mM) for 10 min. Cultures were then incubated for a further 30 min in the presence or absence of either A23187 (3 μM) or AA (30 μM). Results are means ± SEM from three experiments.
Discussion

Effect of L-NAME

The inhibition of NOS in glial cells had no effect on basal or A23187-induced TXB$_2$ production following either short (10 min) or long (48 h) pre-incubation with various concentrations of L-NAME. This is in contrast to the work of other groups who showed that inhibition of NOS by L-NAME at similar concentrations inhibited the production of the NO product nitrite and reduced the synthesis of prostanoids in astrocytes (Molina-Holgado et al., 1995; Mollace et al., 1998) and inhibition with another NOS inhibitor, N$^\text{G}$-monomethyl-l-arginine (NMMA), inhibited prostanoid release from microglia (Minghetti et al., 1996). One reason for this difference may be that in all of these cases prostanoid production was first up-regulated by incubation with LPS or cytokines before the inhibition of NOS. Interestingly, in the study by Minghetti et al. (1996) the authors comment on the fact that the NOS inhibitor had no effect on PGE$_2$ production in microglia which had not been treated with LPS. Together with the results presented here, this would suggest that under normal culture conditions (i.e. in the absence of LPS- or cytokine-induced prostanoid production) the endogenous production of NO by NOS has little effect on COX. One other report did, however, show that inhibition of NOS in astrocytes had little effect on cytokine-induced prostanoid production, but in microglia there was a significant inhibition of PGF$_{2\alpha}$ synthesis (Janabi et al., 1996).
Despite the lack of an effect of inhibition of endogenous NO production on TXB$_2$ formation, it is apparent that the NO donors SNP and SNOG were able to inhibit the stimulated synthesis of TXB$_2$ in mixed glial cell cultures. This effect was independent of the stimulus used implying that NO acts at a later stage than that of AA liberation by PLA$_2$. In addition, the inhibition of TXB$_2$ production was not mediated by an analogue of cGMP, the second messenger whose synthesis is stimulated by NO. Together these findings suggest that the NO donors exert their effect directly on one of the elements of the prostanoid synthetic cascade downstream of PLA$_2$.

The inhibitory effect of the NO donors was abolished in the presence of the NO scavenger Hb. This suggests that it was likely to be the ability of these compounds to release NO which mediated the inhibition rather than other biologically active products which result from the metabolism of NO donors such as sodium ferrocyanide (Manzoni et al., 1992). Others have suggested that both the activation and inhibition of COX by NO could be attributed to an indirect mechanism in which NO reacts with O$_2^-$ to form ONOO$^-$ (Goodwin et al., 1999). In these cells however, the scavenging of O$_2^-$ by the addition of SOD had no effect on the inhibitory action of the NO donor, suggesting that ONOO$^-$ formation was not required for the NO-induced inhibition of COX activity and bolstering the argument in favour of a direct interaction between NO and COX. Coincubation of NSAIDs at their approximate IC$_{50}$ concentrations and SNP produced an
abolition of COX activity which was essentially additive. This effect indicates that the inhibition elicited by SNP was via a mechanism different to that elicited by the NSAIDs, all of which are believed to compete with AA at the COX active site (Mitchell et al., 1994, Kurumbail et al., 1996), thus ruling out the possibility that NO acts at the COX active site as a competitive inhibitor. Since this additive effect was evident with all three of the NSAIDs chosen, regardless of whether they had previously been characterised as being preferential inhibitors of COX-1 or COX-2 or non-selective, it seems unlikely that the effect of NO was confined to any single isoform. This was further shown by the observation that removal of serum from the growth medium, a procedure which has previously been shown to down-regulate the expression of the inducible isoform (Fletcher et al., 1992; O'Banion et al., 1992; Mitchell et al., 1995; Rich et al., 1996; Rich et al., 1998) (Chapter 4 of this thesis), did not affect the ability of SNP to inhibit TXB$_2$ synthesis.

*Comparison with other studies*

The inhibition of COX activity shown in this study is contrary to the general trend reported previously for astrocyte cultures where NO was shown to enhance prostanoid synthesis (Molina-Holgado et al., 1995; Mollace et al., 1998), however, a direct comparison with this work is difficult. Although similar mixed glial cultures were used (referred to by others as astrocyte cultures due to these being the predominant glial cell type), other investigators invariably treated cells with pro-inflammatory stimuli such as
LPS and cytokines. Whilst this treatment could be considered to recreate certain inflammatory situations, it has been shown here that glial cells release prostanoids under normal culture conditions in sufficient quantities to allow the effect of NO on COX to be studied without further activation of the cells. Another important difference between the present study and previous work with glial cells is the duration of NO treatments. Experiments performed by Mollace et al. (1998), utilised much longer incubation periods (24 h) with SNP in an attempt to investigate the effects of NO on COX. Although up-regulation of iNOS would be expected to cause prolonged synthesis of NO, the present study was concerned with the direct effects of NO on COX activity in unstimulated glial cells. Since elevations in NO have been shown to induce COX-2 expression (reviewed by Goodwin et al., 1999), long incubations with NO donors might elicit changes in COX expression which would increase the overall level of prostanoid synthesis and complicate observations of direct modulation of enzyme activity. Although Molina-Holgado et al. (1995) used very similar incubation times (30 min) and concentrations of SNP (100-200 μM) and AA (30 μM) to those described here, they reported an increase in COX activity due to NO in astrocyte cultures. The major difference between their work and the present study was 18 h of stimulation with LPS prior to treatment, which reinforces the earlier suggestion that the effect of NO on COX activity might be dependent on the state of activation of the cells. As discussed in the introduction to this chapter, the emerging trend from studies on glial cell cultures is that NO tends to increase COX activity in astrocytes and decrease it in microglia. However, the decrease in COX activity reported for microglia was observed only in LPS treated
cells after 24 h incubations with NO donors (Minghetti et al., 1996; Guastadisegni et al., 1997), so a direct comparison between their results and those presented here cannot be made.

NO has been shown to inhibit COX activity in other cell types including endothelial cells and macrophages (Doni et al., 1988; Keen et al., 1990; Swierkosz et al., 1995). A 30 min incubation with SNP caused a decrease in the AA-stimulated production of 6-keto-PGF$_{1\alpha}$ in J774.2 macrophages after LPS treatment over a similar concentration range to that reported here (Swierkosz et al., 1995). Interestingly, lower concentrations of SNP (0.001 - 1000 μM) increased COX activity in these cells. This led to the suggestion by these authors that NO might have a dual effect on the activity of COX-2 with low concentrations, such as those created by the inhibition of NOS, causing an increase in COX activity and higher concentrations causing inhibition. However, the levels of SNP which this group found to cause activation of COX in J774.2 macrophages caused an inhibition of TXB$_2$ synthesis in glial cells.

Mechanism of action of inhibition of COX by NO

Although the experiments presented here were not concerned primarily with the mechanism of the interaction between NO and COX, some insight into this area can be inferred. As discussed in the introduction to this section, various modes of interaction have been proposed including nitrosylation of thiol groups, inactivation of tyrosyl
radicals, haem iron coordination and formation of ONOO'. Since the proposed interactions which rely on a direct interaction between NO and COX are likely to be inhibitory, the action of a NO derivative such as ONOO' could explain the apparent increase in COX activity mediated by NO in some experiments. Since the inclusion of SOD did not attenuate the effects of the NO donors in these experiments, it would seem that the formation of ONOO' did not influence the inhibition of prostanoid synthesis in cultured glia. It is likely that the inhibition of TXB$_2$ production observed in these cells was due to a direct effect of NO on COX, probably via one of the three suggested mechanisms mentioned above which could lead to a conformational change and subsequent loss of COX activity. Although one cannot discount the possibility that NO might act at the level of TXB$_2$ synthase, the findings that NO has been shown to elicit changes in COX activity detected by changes in the synthesis of various other prostanoids in a wide range of cell culture and purified enzyme models (reviewed in Salvemini et al., 1997; Goodwin et al., 1999) make it likely that the main effect of NO is upon COX.

In summary it would seem from these results that, in the absence of activation of cultures with LPS or cytokines, endogenous NO synthesis had little effect on stimulus-evoked TXB$_2$ production, whereas addition of NO from donor molecules inhibited prostanoid production using a mechanism independent of O$_2$· and cGMP. The effect was additive to that caused by inhibitors of both isoforms of COX, and was not abolished by the down-regulation of COX-2, making any isoform-specific effect unlikely. This ability of NO to
inhibit the action of COX in glial cells could be a vital clue in the attempt to understand the control of glial prostanoid production, however it would be inappropriate to attach too much physiological relevance to these findings. This is because the inhibition observed with SNP occurred over the concentration range 100 - 3000 μM which, although SNP does not release a stoichiometrical amount of NO, would create NO concentrations in excess of physiological levels. Astrocytes in culture have been shown to release nanomolar concentrations of NO under basal conditions, rising to approximately 1 μM after activation with LPS as detected directly by the use of an NO-sensitive electrode (Brown et al., 1995). Studies which have measured the release of the NO metabolite nitrite as an indicator of NO production in astrocyte cultures have shown 1-2 μM nitrite under basal conditions, increasing to approximately 20 μM after stimulation with IL-1β or TNFα (Mollace et al., 1998). In the latter study, SNP at 120 μM was found to cause an increase in nitrite release of 25 μM. Since the inhibition observed here with SNP occurred at concentrations in excess of this, the levels of NO involved in the inhibition are likely to be far in excess of those created under normal culture conditions or even after stimulation with LPS or cytokines. Even if the inhibition of COX activity by SNP in these cells is a pharmacological phenomenon and may not, therefore, reflect a physiological situation, why these treatments might cause inhibition whilst being shown to cause increased COX activity in other studies still poses an interesting problem. One explanation stems from the observation that those astrocytes in which NO increased COX activity were undergoing induction of COX-2 and iNOS in response to LPS or cytokine treatment. The inducible isoforms of both enzymes can be
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up-regulated simultaneously by many common stimuli, so perhaps some mechanism exists whereby under these conditions COX exists in a state which is susceptible to activation by NO in contrast to its state under normal culture conditions. Alternatively, since NO has been shown to cause an inhibition of prostanoid synthesis in microglia, and these cells are known to be present in the mixed glial cultures used for this study, perhaps the observed effect is in fact a microglial phenomenon. This is unlikely however since microglia typically comprised less than 5% of the mixed glial cell cultures.
Chapter 7: Concluding remarks and future directions
Concluding remarks

The study described here used a panel of COX inhibitors as a means to investigate the activity of different COX isoforms in cultured glial cells. Previous reports had shown that the expression of COX isoforms in these cells was subject to modulation by a variety of factors. Most studies of this nature however first treated the cells with pro-inflammatory stimuli such as LPS or cytokines, sometimes in order to recreate certain inflammatory situations, whereas this study focussed on investigating glial cells under normal culture conditions. One of the factors implicated in causing the expression of COX-2 in cultured glia is the presence of serum in the growth medium (Slepko et al., 1997; Rich et al., 1998; Luo et al., 1998). The study described in this thesis explored the nature of this serum-dependence and showed that after serum withdrawal the level of expressed COX protein was reduced with an accompanying reduction in potency of preferential COX-2 inhibitors. This was in agreement with the generally accepted theory of serum withdrawal causing COX-2 down-regulation. However, whilst the protein expression and prostanoid synthetic capacity of COX were subsequently restored after serum re-addition, the use of selective inhibitors permitted a detailed investigation into the nature of the re-established COX activity and showed that there was a significant reduction in the potency of the preferential COX-2 inhibitors under these conditions.

Having established a means of exploring COX activity in cultured glia through stimulation with A23187 and AA, the effects of NO on COX activity were investigated.
Conflicting reports had previously shown it to be either an activator or an inhibitor of COX, and therefore to be an important mediator of prostanoid synthesis in many cells. It was shown here that the inhibition of endogenous NO had little effect on prostanoid production in cultured glial cells, although supplying exogenous NO via NO donors caused an inhibition of COX activity which appeared to be additive to the effects of NSAIDs and was not specific to either COX-1 or COX-2. The finding that NO had the potential to inhibit prostanoid production in glial cells whilst having been shown to be an activator of COX in other cells after pro-inflammatory stimulation may highlight a distinction between the COX expression in glial cells under normal culture conditions and that expressed elsewhere.

Since the approach of using a panel of selective and non-selective inhibitors provided an effective means of investigating COX activity, this strategy was used to address the controversy over which glial cell type may be responsible for prostanoid production in vitro. It would appear from these studies that astrocytes and microglia in culture were capable of prostanoid synthesis, which was reduced in both cell types by COX-1 and COX-2 preferential inhibitors. Microglia appeared to be more sensitive to inhibition by COX-2 inhibitors than astrocytes, but perhaps more importantly it was clear that the primary mixed glial cell preparations showed a pattern of COX activity distinct from either of the purified preparations. Moreover, the mixed glia did not show the characteristics expected of a mixture of microglia and astrocytes, suggesting that the expression of COX in one cell type may be modulated by a signal derived from the
An additional investigation was carried out into the effects of paracetamol on COX activity in glial cells. Despite this drug being not normally regarded as a COX inhibitor other investigators showed that certain treatments resulted in the expression of paracetamol-sensitive COX in a macrophage cell line. It was shown here that paracetamol inhibited prostanoid synthesis in mixed glial cells under normal culture conditions, and serum removal then re-addition caused an increase in its potency. Furthermore, when this drug was used in the astrocyte and microglia enriched preparations, it was found to inhibit prostanoid synthesis in both cell types except where microglia were stimulated with AA, in which case enhanced prostanoid production was detected.

Considered collectively, the results presented here give an insight into the complex nature of prostanoid synthesis in cultured glial cells, in particular the relative functional contributions of the different COX isoforms to this process. The changes demonstrated in the expression of COX after serum removal and re-addition are of particular significance. It is apparent that in response to such a treatment these cells express a COX complement with reduced sensitivity to certain inhibitors, and increased sensitivity to others, including the poorly understood paracetamol. While these changes in cultured cells do not necessarily reflect an in vivo situation, the potential for glial cells to modulate the nature of their COX activity in response to serum may be indicative of a response to
contact with certain chemical mediators endogenous to the CNS which are also contained within serum. Commercially available serum is known to contain a wide range of growth factors, cytokines, and sometimes LPS, any of which might provoke the response. In addition these changes in COX expression could reflect the means by which glial cells respond to coming into contact with blood plasma released into the CNS after damage to the vasculature.

One important consideration which appears to have been overlooked in much of the current literature is the apparent expression of COX-2 by glial cells under normal physiological conditions. While it is reasonable that many investigations should focus on the elevated levels of COX-2 associated with inflammatory conditions, the presence of COX-2 in astrocytes and microglia has been shown under resting conditions \textit{in vivo} (Hirst \textit{et al.}, 1999; Tomimoto \textit{et al.}, 2000) and \textit{in vitro} (Koyama \textit{et al.}, 1999; O'Banion \textit{et al.}, 1996; Luo \textit{et al.}, 1998; Fiebich \textit{et al.}, 1996; Minghetti \textit{et al.}, 1997; Bauer \textit{et al.}, 1997; Slepko \textit{et al.}, 1997) and is therefore not necessarily a consequence of an inflammatory response. Moreover, the inflammatory situation created by the incubation of glial cells with LPS does not necessarily re-create a CNS pathological state. Bacterial LPS rarely passes through the blood brain barrier and peripheral injection of LPS has been shown to cause COX-2 up-regulation in perivascular monocytes and endothelial cells rather than in glia or neurons (Quan \textit{et al.}, 1998; Breder and Saper, 1996). It is highly likely, therefore, that the COX-2 expressed in healthy brain is responsible for the generation of prostanoids which play a role in normal physiology, maybe in the control
of cerebral blood flow, the sleep/wake cycle, or thermoregulation. A role for COX-2 in normal CNS function may be of particular significance following the recent clinical availability of highly selective COX-2 inhibitors designed to reduce inflammation without the side-effects associated with inhibition of COX-1 involved in normal physiology. While these drugs provide the potential to reduce gastrointestinal damage wreaked by the chronic use of less selective inhibitors, the long-term inhibition of COX-2 in the brain may have unexpected consequences.

Although this work did not necessarily corroborate the existence of the hypothesised "COX-3" (Mitchell et al., 1994; Willoughby et al., 2000), it did show that under certain circumstances inhibitors can have quite unexpected effects on COX which are difficult to explain in terms of the established two isoform model. Perhaps in the near future the COX-2 variant created in macrophages (Simmons et al., 1998), or the translated protein coded for by the novel COX-1 transcript (Plant and Laneuville, 1999), or perhaps some entirely new COX entity, will be shown to exist in vivo. The existence of an alternative prostanoid synthase system in the CNS was suggested from the very outset of research into COX (Flower and Vane, 1972). Given the importance of glial cells as a major source of prostanoids in the brain, perhaps they do express such an isoform. In fact, the presence of alternative isoforms could explain many of the unusual features associated with COX in glia which have created much confusion in the field. For example, monoclonal antibodies which have been used routinely to study the expression of COX isoforms in the CNS tend to be raised against specific regions of the enzyme as purified from
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peripherally-derived cells. Perhaps under certain circumstances glial cells express COX isoforms which differ from the peripheral protein in terms of these specific regions. Slight changes in shape or amino acid sequence, or even post-translational modification might render the molecule undetectable by highly specific monoclonal antibodies. If this were the case, then some glial COX expression might show reduced immunoreactivity with certain COX antibodies, thus explaining phenomena such as the lack of detection of COX-2 in microglia in vivo until 1997 (Elmquist et al., 1997), and the early reports that COX-2 was expressed exclusively by neurons (Breder et al., 1995). On the other hand, the apparent expression of COX activity with a novel inhibitor profile shown here in vitro may be a feature of cultured glia. Since cultured astrocytes are believed to resemble the reactive phenotype of their in vivo counterparts, it could be that this finding might have little to do with the basal COX expressed in resting brain but could be created during reactive gliosis given the appropriate stimuli. The occurrence of a paracetamol-sensitive COX isoform in reactive glia and not peripheral cells could explain the antipyretic and analgesic actions of this drug as inhibitors of CNS prostanoid production with no effect on the peripheral COX-2 during an inflammatory response. Willoughby et al. (2000) proposed that a COX-3 could exist which was responsible for the synthesis of anti-inflammatory prostanoids following a period of inflammation. In comparison with this, prostanoids in the CNS have been associated with cytoprotection after injury or disease as well as being implicated in the propagation of some of the damaging effects. For example, glial cell activation during hypoxia is accompanied by the release of a number of prostanoids which have been shown to have a local neuroprotective effect (Huttemeier
et al., 1993; Cazevielle et al., 1993), whilst inhibition of prostanoid synthesis diminishes the elevated prostanoid levels associated with HIV-induced dementia but leads to an exacerbation of the damage. After cerebral ischaemia in humans, COX-2 protein is up-regulated globally in glial cells and neurons following initial acute focal expression, and it had been suggested that the elevations in prostanoids produced as a result may promote reconstruction and remodelling of the surviving neural networks (Sairanen et al., 1998). Maybe in the light of this accumulating evidence for the glial production of neuroprotective and anti-inflammatory prostanoids following CNS damage, and the speculation that such effects may be mediated in other cells by the action of a novel COX (Willoughby et al., 2000), the expression of such a COX in glial cells in response to certain circumstances could become a viable hypothesis.

This study has provided new information about the expression and activity of COX isoforms in glial cells through the use of a panel of COX inhibitors as a pharmacological probe. However, in the context of CNS prostanoid synthesis as a whole it would be inappropriate to suggest that these findings provided a significant insight into the involvement of glial cells in vivo. Cell culture studies can only ever seek to provide information regarding the potential of cells to respond in certain ways to different circumstances, and as a result many cellular responses which occur in vitro are not observed in vivo where cells interact with a diverse array of other cells and experience a variable chemical environment. In the same way, the action of COX or, indeed, the entire prostanoid synthetic pathway, can be explored in isolation but inevitably functions
as one part of a multi-faceted and highly regulated inflammatory response. The various enzymes such as COX, PLA$_2$, and the specific prostanoid synthases have all been investigated in isolation but undoubtedly act as individual nodes in a complex network of other enzymes including for instance NOS, lipoxygenase and cytochrome P450. The expression, activity, and in some cases location of these enzymes may be modulated by a phenomenal array of mediators, many of which have yet to be discovered. The only sensible approach to an investigation into such an area is to examine a manageable portion of one of these cascades and then to explore the influences which appropriate mediators can exert, but this approach can often lead to over-interpretation of the observations when attempting to put them into the context of the greater picture. Studies into the inflammatory response in general, and COX in particular have often fallen into this trap. Many of the features ascribed to the enzyme in isolated preparations differ to those in cultured cells, and undoubtedly differ still more under $\textit{in vivo}$ conditions. The diversity between the potencies of COX inhibitors in different assay systems is one typical example of this. In his commentary, Cirino (1998) warns that researchers in this field sometimes look like the blind men in the fable "the blind men and the elephant" where three men are touching an elephant but one thinks he is touching a snake, one thinks it is a bell rope, and the third believes he is touching a palm tree. It is with this in mind that one must refrain from making claims about the significance of certain aspects of COX expression and activation in cultured glia in terms of inflammatory responses in the CNS in general.
Future directions

The work presented here, and recent work by others, has provided an insight into complications in the field of inflammation which look set to change the established idea that two isoforms of COX can exist, albeit to different degrees, in the cells of the body. Perhaps the most important of the findings here is that a COX with novel pharmacology is expressed in glial cells in response to serum removal and re-addition. This is of particular importance in the light of recent work by others which has shown the existence of distinct COX isoforms or variants with altered sensitivity to inhibitors in a macrophage cell line (Simmons et al., 1999), and the discovery of alternative COX transcripts in various peripheral organs (Plant and Laneuville, 1999).

Two questions arise which could form distinct routes for further investigation. The first is to determine the identity of the COX which is produced by serum removal and re-addition. Is it a distinct COX isoform, a variant of an existing isoform, or are the changes in inhibitor profile due to the influence of some other factor such as the redox microenvironment, hydroperoxide tone, or subcellular localisation of the enzyme? Since both COX-1 and COX-2 have been cloned it would be appropriate to synthesise an array of primers which could detect the presence of mRNA coding for COX isoforms. Subsequent PCR and sequence analysis would reveal whether there were any inconsistencies in the mRNA transcripts detected which, if different from controls, could be indicative of the existence of a distinct protein product. In addition, the COX
expressed by glial cells after serum removal and re-addition could be isolated chromatographically and analysed both in terms of kinetics and inhibitor profile and compared to COX obtained from other cells. Isolation and purification of this COX would also allow analysis of the amino acid sequence and detection of any post-translational modification which might result in a distinct protein coded for by a standard COX mRNA sequence.

The second matter for further investigation could be the determination of exactly how the expression of COX is modulated in such a way as to produce the observed changes in pharmacology. Manipulation of serum levels may cause changes in the levels of an array of growth factors and cytokines and it would be useful to identify which factor in serum is responsible for the changes in COX activity. Experiments could initially take the form of depriving cells of serum for four days then adding back various different combinations of serum factors in an attempt to recreate the inhibitor profile recorded after serum re-addition. This could also be used as a means with which to explore the transcriptional regulation of COX expression through stimulation of the various promotor sites via their signalling cascades. For example, pharmacological intervention in the MAPK and NF-κB pathways after serum re-addition, or addition of activators of these and other pathways, could provide information about how this change in COX activity might be achieved at a subcellular level.

These studies have revealed further important differences between astrocytes and
microglia in culture, in particular the observation that the normal mixed glial cell cultures which are widely used as an astrocyte model do not resemble astrocyte-enriched preparations or simple mixtures of astrocytes and microglia in terms of COX inhibitor profile. It would be interesting to explore the nature of the signalling mechanisms between astrocytes and microglia which lead to the COX expression in mixed glial cell cultures. Preliminary experiments (not included here) have shown that the addition of microglial conditioned medium to subcultured astrocytes caused changes in the potency of some inhibitors with IC\textsubscript{50} concentrations of aspirin, paracetamol and ibuprofen having a greater effect after addition of microglial medium and NS398 showing little change. Further work could attempt to fully characterise the influence of microglia on astrocyte COX activity, perhaps investigating which signalling molecules released by microglia mediate this effect in an attempt to provide an insight into the interactions between these cell types in response to CNS damage.

Another area of future work would be to investigate the nature of COX expression and activity in astrocytes and microglia in vivo. As discussed previously, it is difficult to study these cells in their native environment, but molecular biological techniques, in particular the growing success of studies using knock-out mice, now provides a means with which to explore the role of COX in these cells. If further COX isoforms or variants are revealed and cloned then animals could theoretically be bred which do not express this isoform or the means to create a variant, whilst still expressing normal COX-1 and COX-2. Not only would these studies show whether any novel COX expression was an artefact
only found in culture, but the reactions of glial cells in such animals to physiological and pathological situations could reveal some functions for COX in glial cells \textit{in vivo} and perhaps provide novel therapeutic targets for the control or treatment of CNS disorders.
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