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**NON-STEROIDAL ANTI-INFLAMMATORY DRUG MEDIATED
REGULATION OF COX-2 AND EP₃ RECEPTOR EXPRESSION IN
THE M-1 MURINE CORTICAL COLLECTING DUCT CELL LINE.**

Shawn Ferguson

**Thesis submitted to the Department of Cellular and Molecular Medicine in partial
fulfilment of the requirements for the degree of Master of Science**

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Abstract

The cortical collecting duct (CCD) is a major site of intrarenal prostaglandin E₂ (PGE₂) synthesis. We have studied the effect of non-steroidal anti-inflammatory drug (NSAID) mediated inhibition on the expression of the prostaglandin synthesizing enzymes, cyclooxygenase -1 and -2 (COX) in the CCD. By indirect immunofluorescence using isoform specific antibodies, we have localized COX-1 and -2 immunoreactivity to all cell types of the murine M-1 CCD cell line. By immunohistochemistry, both COX-1 and COX-2 were localized to the intercalated cells of the collecting duct on paraffin embedded mouse kidney sections. When COX enzyme activity was measured in the M-1 cells, both indomethacin (COX-1 and -2 inhibitor) and the specific COX-2 inhibitor NS-398 effectively blocked PGE₂ synthesis. These results demonstrate that COX-2 is a major contributor to the pool of PGE₂ synthesized by the CCD. By Western blot analysis, COX-2 expression was significantly up-regulated by incubation with either indomethacin or NS-398. These drugs did not affect COX-1 protein expression. Evaluation of COX-2 mRNA expression by Northern blot analysis following NS-398 treatment demonstrated that the COX-2 protein up-regulation occurred independently of any change in the level of COX-2 mRNA expression. The effects of NS-398 on COX-2 expression could not be attenuated by the presence of 16,16-dimethyl-PGE₂. The expression of the PGE₂ receptor EP₃ subtype mRNA is elevated in response to NS-398. In conclusion, our studies have for the first time localized COX-2 to the CCD. Furthermore, we provide evidence that the intercalated cells of the CCD express both COX-1 and COX-2. We demonstrate that in response to COX-2 inhibition

there is an increase in COX-2 and EP₃ receptor expression by the M-1 CCD cell line. The collecting duct represents the final segment for the regulation of salt and water reabsorption along the nephron. PGE₂ exerts predominantly diuretic and natriuretic effects upon the CCD. Our results which document the expression of COX-2 in the CCD provide a mechanism through which the newly developed class of COX-2 specific inhibitors could exert side effects with respect to the regulation of fluid and electrolyte homeostasis.

Dedication

To my parents, grandparents, and fiancée Agnes Rocznik whose support, interest and enthusiasm has motivated me to do my best.

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Declaration

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Abbreviations

7 TMS	7 Transmembrane Spanning
15R-HETE	15R - hydroxyeicosatetraenoic Acid
16,16-dm-PGE₂	16,16-dimethyl-prostaglandin E ₂
AA	Arachidonic Acid
ACE	Angiotensin Converting Enzyme
ANF	Atrial Natriuretic Factor
AT1	Angiotensin II type 1 receptor
APHS	o- (acetoxyphenyl)hept-2-ynyl sulfide
AQP	Aquaporin
ASA	Acetylsalicylic Acid
AVP	Arginine Vasopressin
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CCD	Cortical Collecting Duct
CD	Collecting Duct
CHO	Chinese Hamster Ovary Cell Line
COOH	Carboxyl
COX	Cyclooxygenase
cPLA₂	Cytosolic Phospholipase A ₂
CRE	cAMP response element
cTAL	Cortical Thick Ascending Limb
DAB	Diaminobenzidine
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECF	Extracellular Fluid
eNaC	Epithelial Sodium Channel
EP	E Prostanoid
ECF	Extracellular fluid
EGF	Epidermal Growth Factor
FBS	Fetal Bovine Serum
g	Gram
G-protein	Guanine nucleotide binding proteins
hr	Hour
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
IL-1β	Interleukin-1 β
IMCD	Inner Medullary Collecting Duct

Indo	Indomethacin
IP	I Prostanoid
kb	Kilobase
LPS	Lipopolysaccharide
mTAL	Medullary Thick Ascending Limb
µm	Micrometer
µM	Micromolar
min	Minute
NSAID	Non-Steroidal Anti-Inflammatory Drug
NF-κB	Nuclear factor - κB
OMCD	Outer Medullary Collecting Duct
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCT	Proximal Convoluted Tubule
PGHS	Prostaglandin Endoperoxide H Synthase
PG	Prostaglandin
PKC	Protein Kinase C
PKN	Protein Kinase N
PLC	Phospholipase C
PPAR	Peroxisomal Proliferator Activated Receptor
PSF	Penicillin Streptomycin Fungizone
PST	Proximal Straight Tubule
PAGE	Polyacrylamide Gel Electrophoresis
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulfate
SV40	Simian Virus 40
TAL	Thick Ascending Limb
TBS-T	Tris Buffered Saline - Tween 20
TLC	Thin Layer Chromatography
TP	T Prostanoid
TNFα	Tumour necrosis factor α
TXA₂	Thromboxane A₂
VSA	Valerylsalicylic Acid

Section 1: Introduction

Section 1.1: *Overview*

Non-steroidal anti-inflammatory drugs (NSAIDs) are used extensively for their anti-inflammatory, anti-pyretic and analgesic effects (DeWitt and Smith, 1995). This widespread NSAID use is emphasized by reports that in 1991 in the United States alone, 70 million prescriptions were filled for NSAIDs (Ray et al., 1993) and that U.S. sales of prescription NSAIDs totaled 1.9 billion dollars in 1997 (DeWitt, 1999). These figures exclude the purchase of over the counter NSAIDs which include such common drugs as Aspirin and ibuprofen, the purchase of which brings NSAID consumption up to 50 million Americans at a cost in excess of 5 billion U.S. dollars (Dubois et al., 1998). The therapeutic effects of NSAIDs stem from their ability to inhibit the activity of the prostaglandin forming enzymes termed cyclooxygenase (COX)-1 and -2, also known as prostaglandin endoperoxide H synthase (PGHS) -1 and -2 (DeWitt, 1999; Loll et al., 1995; Vane, 1971). In so much as the therapeutic effects of NSAIDs are valuable, their use is not without consequence. Common serious side effects of NSAID therapy include the development of gastric ulcers and renal toxicity (Murray and Brater, 1997). The renal effects of NSAIDs can be divided into two categories. The inhibition of the synthesis of vasodilatory prostaglandins can lead to acute ischemic renal insufficiency. The inhibition of the synthesis of natriuretic and diuretic prostaglandins can result in impaired blood pressure control and edema. Although these

problems have long been well documented, the specific mechanisms and the variable susceptibility towards these adverse effect of NSAIDs within different subgroups of the population remain largely unexplained. The kidney is a complex organ composed of many specialized regions characterized as having specific functions. The focus of my work is the kidney cortical collecting duct and the regulation of prostaglandin synthesis by NSAIDs in this specific structure of the kidney. More specifically, I have focused on understanding the consequences of specific COX-2 inhibition upon the steady state levels of the PGE₂ synthesizing enzymes COX-1 and COX-2 as well as the expression of the PGE₂ receptors.

Section 1.2: *The Arachidonic Acid Cascade.*

Synthesis of prostanoids by the COX isoforms is dependent upon availability of arachidonic acid which serves as substrate (Figure 1). In response to specific stimuli, an elevation in intracellular calcium concentration causes translocation of cytoplasmic phospholipase A₂ (cPLA₂) from the cytosol to the phospholipid membrane of the endoplasmic reticulum whereupon the arachidonic acid can be released from the SN2 position of membrane phospholipids. Additional arachidonic acid releasing enzymes include secretory PLA₂ (Murakami et al., 1999) as well as phospholipase D (Fujita et al., 1996). Upon being released, arachidonic acid can be acted upon by the COX isoforms to produce prostaglandin H₂ (PGH₂). PGH₂ is unstable and is rapidly enzymatically converted to a biologically active prostanoid such as PGE₂, PGF₂, PGD₂, PGI₂ or thromboxane A₂ in

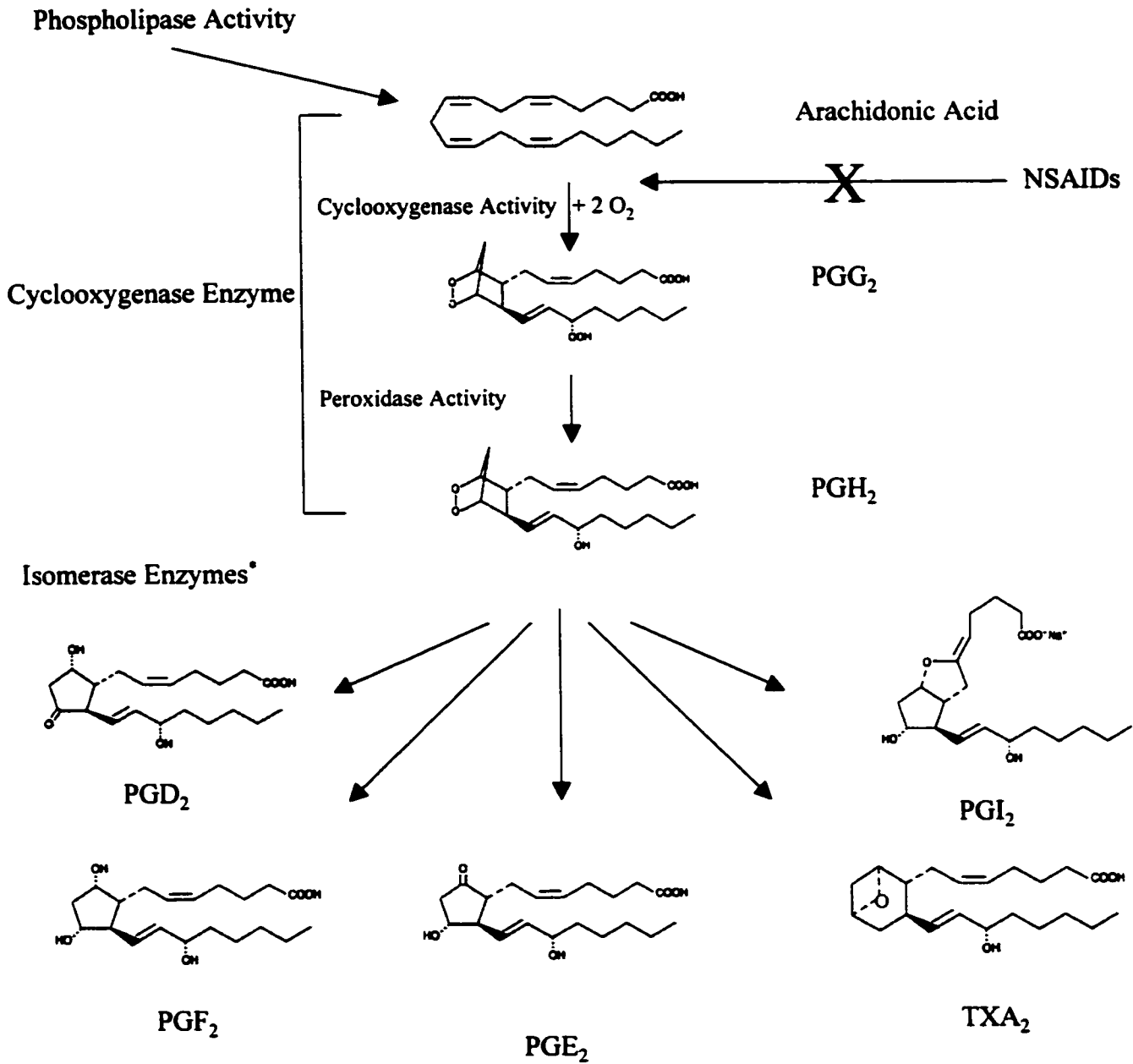
a cell specific fashion such that in a given cell type, only one of these potential products will predominate.

The synthesis of each of these prostanoids from PGH_2 is mediated by separate enzymes. The conversion of PGH_2 to PGD_2 , PGE_2 , and PGF_2 is performed by enzymes characterized as glutathione S-transferases (Watanabe et al., 1997). Meanwhile, PGI_2 and TXA_2 synthesis from PGH_2 is dependent upon two different cytochrome P450 enzymes (Shyue et al., 1997). The PGE_2 synthase activity which is expressed in the kidney is glutathione dependent and like the COX isoforms is found in the microsomal fraction (Watanabe et al., 1997).

There is evidence that the identity of the prostanoid synthesized in a particular cell type can be regulated. For example, in response to tumour necrosis factor, murine macrophages shift prostanoid production from predominantly PGD_2 to PGE_2 in a manner which is dependent upon the up-regulation of PGE_2 synthase and the down-regulation of PGD_2 synthase (Fournier et al., 1997). There may be a specific functional coupling between COX-2 and PGE_2 synthase and between COX-1 and PGD_2 synthase and TXB_2 synthase (Naraba et al., 1998). Treatment of rat peritoneal macrophages with bacterial lipopolysaccharide (LPS) led to an increase in PGE_2 production which could be blocked by NS-398, meanwhile, PGD_2 synthesis was unaltered in response to LPS and was not inhibited in the presence of NS-398 but was inhibited in the presence of aspirin (Matsumoto et al., 1997). Although these initial findings indicate that the final active prostanoid product

generated by a specific cell type is variable and regulated, more work is required in order to understand the role this regulation may play in the various pathophysiological processes in which prostanoids are implicated.

Figure 1. The arachidonic acid cascade. This simplified pathway presents the sequence of reactions occurring from the release of arachidonic acid from membrane phospholipids to the synthesis of an active prostanoid. The major prostanoid which is synthesized in a specific cell type will depend upon the identity of the predominant isomerase acting on PGH_2 .



* In any given cell type, one isomerase will predominate so as to give one major prostanoid product

The physiological effects of prostanoids are mediated by autocrine and paracrine signaling mechanisms. For this to occur, these compounds must leave the cell. Diffusion of prostanoids across membranes is slow, therefore, their escape from the cell is dependant upon the action of prostanoid transporters (Chan et al., 1998). In epithelial cell types where there is polarized release of PGE₂ (Coffey et al., 1997), this phenomenon could be explained by polarized distribution of the prostanoid transporters. Prostanoid receptors are members of the 7 trans-membrane spanning domain (7 TMS) G-protein coupled receptor family. Specific receptors exist for each of the separate prostanoid products which can be derived from PGH₂. A system of nomenclature has been developed whereby the PGE₂ receptors are referred to as E-prostanoid or simply EP receptors, the PGI₂ receptor as an IP receptor, the TXA₂ receptor as TP receptor and so on with the same pattern for the D and F prostanoids (Coleman et al., 1994).

Section 1.3: *Structure and Activity of COX-1 and COX-2.*

The two COX isoforms are derived from two different genes found on separate chromosomes (Herschman, 1996). The structures of these two genes have similar organization of introns and exons. The structure of the 11 exon 22kb COX-1 gene differs mainly from the 10 exon 8kb COX-2 gene with respect to the organization of the first exon and the size of the introns (Smith, 1992). The first 2 exons of the COX-1 gene are represented by a single exon in the COX-2 gene. As the remaining exons are similarly

arranged, the larger size of the COX-1 gene is due to longer introns. The mouse COX-1 protein consists of 576 amino acids and the COX-2 protein is composed of 568 amino acid. The COX-1 and COX-2 proteins from the same species share 60 % amino acid identity (Smith, 1992).

The structures of both COX-1 and COX-2 have been determined by x-ray crystallography (Kurumbail et al., 1996; Loll et al., 1995; Picot et al., 1994). The structures of COX-1 and COX-2 are very similar. They both function as homodimers and are composed of three domains: an EGF-like binding domain, a membrane binding domain and a heme containing catalytic domain possessing two distinct active sites. The EGF-like binding domains suggest that COX isoforms may interact with other proteins, however, a functional role for these domains remains to be determined. The membrane binding domains of the COX isoforms which consist of 4 hydrophobic α -helices which are novel in that they allow for the COX homodimer to intercalate itself within just one layer of the phospholipid membrane bilayer (Li et al., 1998). The catalytic domain is comprised of two separate active sites. The cyclooxygenase active site catalyzes the bis-oxygenation of arachidonic acid to generate prostaglandin G_2 (PGG_2). Access to the cyclooxygenase active site is via a long narrow channel which extends from the membrane binding domains to within close proximity of the heme group at the center of the COX monomer (Picot et al., 1994). The entrance to this channel is positioned within the interior of the phospholipid bilayer. Acetylation of serine 530 of COX-1 by aspirin effectively blocks entry of substrate into this channel. Acetylation of serine 516 blocks the normal COX-2 activity. In the

peroxidase active site, PGG₂ undergoes a reduction to prostaglandin H₂ (PGH₂). With respect to catalytic activity, the two COX isoforms have a K_m of 5.6 and 5.4 μM for arachidonic acid (Laneuville et al., 1994). With respect to the cyclooxygenase active site, x-ray crystallography has revealed that COX-2 possesses a slightly larger binding pocket for arachidonic acid (Kurumbail et al., 1996). This larger binding pocket has implications for the selectivity of NSAIDs for one COX isoform over the other. In fact, there are two isoleucine residues in the COX-1 which are represented by valines (Val 434 and Val523) in the COX-2 protein binding pocket (Vane et al., 1998). When the COX-1 isoleucine 523 is mutated to the smaller valine residue, this mutant COX-1 is sensitive to COX-2 specific inhibitors. Both COX isoforms possess a threonine-glutamine-leucine (TEL) sequence at their carboxyl terminal which could mediate their targeting to the endoplasmic reticulum (Smith, 1992). Retention within the lipid bilayer of the endoplasmic reticulum is dependant upon the helical membrane binding domains (Li et al., 1998). In chimeras which did not possess these domains, endoplasmic reticulum targeting occurred, but the proteins did not associate with the membranes. The COX-2 protein has an 18 amino acid sequence immediately prior to the COOH - terminal which has no known role with respect to protein function but which has proved useful for the generation of COX-2 specific antibodies (Smith, 1992). There is an N-terminal 17 amino acid cassette in COX-1 which is not found in COX-2 and which is of unknown significance (Vane et al., 1998).

Section 1.4: Regulation of COX isoform expression.

It is at the level of regulation of expression of the two COX isoforms where they differ sharply. The COX-1 enzyme is constitutively expressed in most cell types where it plays a housekeeping role. COX-2 has been termed the inducible cyclooxygenase due to its rapid up-regulation in response to a variety of stimuli including: growth factors (Coffey et al., 1997), cytokines (Martin-Sanz et al., 1998), hypoxia (Schmedtje et al., 1997), peroxisomal proliferators (Callejas et al., 1999; Meade et al., 1999), LPS (Inoue et al., 1995) and phorbol esters (Inoue et al., 1995). These variabilities between COX-1 and COX-2 regulation can be explained by the structures of their respective promoter regions. The COX-1 promoter is typical of housekeeping genes in that it lacks a TATA box (Smith and Dewitt, 1996). Elevations in COX-1 mRNA have been associated with differentiation but these changes are slow and modest compared to the rapid up-regulation of COX-2 in response to the appropriate stimuli (Smith and Dewitt, 1996). The induction of COX-2 expression by the above-mentioned factors can be explained by the structure of the COX-2 promoter region (Fletcher et al., 1992). The mouse COX-2 promoter possesses the following response element motifs upstream of the start site: SP-1, ATF/CRE, NF-IL6 (C / EBP), E - Box and a TATA box (Herschman, 1996). This promoter region confers upon COX-2 the characteristics of an immediate early gene.

Section 1.5: *Subcellular localization of COX isoforms.*

Determining the intracellular localization of the 2 COX isoforms is of interest as a means of answering questions concerning the role of the prostanoids produced by the separate isoforms. Evidence indicates that the 2 isoforms can make use of separate pools of arachidonic acid (Herschman, 1996). COX-1 and COX-2 have been described as being localized to the membranes of the endoplasmic reticulum and nuclear envelope (Morita et al., 1995). By immunofluorescence and cytochemical techniques, Morita et al found COX-1 to be distributed equally between the endoplasmic reticulum and the nuclear envelope. Meanwhile COX-2 content was found to be twice as concentrated within the nuclear envelope. A more recent study from the same group has reported that by immunoelectron microscopy and Western blotting of subcellular fractions, COX-1 and COX-2 were found to be equally represented within the luminal membrane of the endoplasmic reticulum as well as within the inner and outer membranes of the nuclear envelope (Spencer et al., 1998). Interestingly, it was observed using confocal immunofluorescence microscopy in a human colon cancer epithelial cell line that upon epidermal growth factor (EGF) treatment there was an accumulation of COX-2 immunoreactivity within the nucleus (Coffey et al., 1997). To further complicate this story, it has recently been reported that in response to high concentrations (>100 μ M) of the NSAID diclofenac, there was an induction of a COX-2-luciferase reporter chimera which localizes predominantly to the cytosol even though the chimera possesses both the COX-2 membrane binding and EGF-like domains (Simmons et

al., 1999). The N-terminal of the COX-1 and COX-2 proteins which contains the membrane binding domain, the EGF like domain and the glycosylation site are sufficient when fused to green fluorescent protein (GFP) to ensure proper localization of the COX proteins (Li et al., 1998). Although it remains unclear as to whether or not the steady state localization of COX-1 and COX-2 differ, it is becoming evident that the localization of COX-2 is dynamic and can shift in response to the appropriate stimuli. The functional significance of the different subcellular localizations of COX-2 are unknown. These observations are interesting because they add another layer of complexity to the regulation of this enzyme.

Section 1.6: *COX-1 and COX-2, what are their roles in health and disease?*

Prostanoids serve as modulators of a myriad of physiological processes including but not limited to: inflammation, pain perception, fever, gastric cytoprotection, renal salt and water reabsorption, ovulation, parturition, wound healing, and platelet aggregation (Herschman, 1996). Since the discovery of COX-2 in 1991 (Kujubu et al., 1991), interest has been focused on understanding the contributions made by each COX isoform to the abovementioned processes. One hypothesis which predominated much of this research was that COX-2 produces the prostanoids associated with inflammation, pain perception and pyresis while COX-1 produces the prostanoids required for normal physiology (Seibert et al., 1999). This hypothesis flowed from the many studies which characterized COX-2 expression to be low or non-existent in unstimulated tissues but that the mRNA and protein

are rapidly up-regulated in response to cytokines (Ristimaki et al., 1994) and LPS (Inoue et al., 1995). Meanwhile, COX-1 is constitutively expressed in most mammalian tissues (DeWitt and Smith, 1995) and is well known to produce gastric cytoprotective prostanoids as well as prostanoids involved in the regulation of renal hemodynamics and transport.

The apparently different roles of the two COX isoforms led to the search for a COX-2 specific inhibitor with the aim of controlling inflammation, pain and fever while sparing the cytoprotective prostanoids produced by COX-1. However, as time has passed, the issue has been complicated by the fact that both constitutive and inducible COX-2 expression have been implicated in normal physiological processes including: renal development (Morham et al., 1995), renin secretion (Cheng et al., 1999; Harris et al., 1994), insulin secretion (Robertson, 1998), ovulation (Davis et al., 1999; Lim et al., 1997) and wound healing (Mizuno et al., 1997). COX-2 is constitutively expressed in the human thyroid epithelium (Smith et al., 1999). This study also found that in a human thyroid epithelium cell line, this constitutive expression of COX-2 was found to be interleukin - 1 α dependant. There is even a recent report wherein it is demonstrated that COX-2 can produce both inflammatory and anti-inflammatory prostaglandins at different time points in a rat model of inflammation (Gilroy et al., 1999). The selective COX-2 inhibitor celecoxib has been found to decrease the urinary excretion of prostacyclin metabolites in human volunteers (McAdam et al., 1999). This further implicates a possible role for COX-2 in normal control of platelet stickiness and raises questions about the appropriateness of combining low dose

aspirin therapy with COX-2 inhibitor therapy. This could be of consequence in elderly individuals taking low doses of aspirin to inhibit platelet aggregation and COX-2 inhibitors to alleviate the symptoms of arthritis.

Increased COX-2 expression has also been associated with several disease processes. The best characterized disease model of COX-2 overexpression is colon cancer. Compelling evidence for a role for COX in colon cancer comes from human epidemiological studies which have shown 40-50 percent reductions in the risk of developing colon cancer in regular users of NSAIDs (Dubois et al., 1998; Thun et al., 1991). Eberhart et al found that approximately 90 percent of colon primary tumours are COX-2 positive (Eberhart et al., 1994). Furthermore, a separate study demonstrated that overexpression of COX-2 in rat intestinal epithelial cells was found to confer resistance to apoptosis as well as decreased cell adhesion (Tsujii and DuBois, 1995). Additionally, COX-2 overexpression has also been associated with increased invasiveness and metastatic potential (increased metalloproteinase activity) in a human colon cancer cell line (Caco-2) (Tsujii et al., 1997). More recently, it has been demonstrated that COX-2 produces prostaglandins involved in the regulation of angiogenesis as evaluated in a colon cancer cell/endothelial cell co-culture model (Tsujii et al., 1998).

NSAID intake has also been inversely correlated with the risk of developing Alzheimer's disease and dementia in human epidemiological studies. The Rotterdam study (cross sectional design) found a relative risk of developing Alzheimer's disease to be 0.38

in a comparison of NSAID users to non-users (Andersen et al., 1995). In a longitudinal study, the relative risk for Alzheimer's disease decreased to 0.40 in people with greater than 2 years of NSAID use (Stewart et al., 1997). In addition to these epidemiological studies, Western blots on autopsy samples from the brains of Alzheimer's disease patients have detected elevated COX-1 and COX-2 expression in the temporal cortex of Alzheimer's disease sample versus healthy controls (Kitamura et al., 1999).

The chronic pain and loss of mobility associated with osteoarthritis are treated extensively with NSAIDs. In human osteoarthritic cartilage specimens there has been observed a 50 fold increase COX-2 mediated PGE₂ compared to cartilage from healthy control specimens (Amin et al., 1997).

Section 1.7: *COX-1 and COX-2 knock-out studies.*

The study of COX-1 and COX-2 knockout mice generated some interesting observations. Although it had been widely hypothesized that the gastric toxicity of NSAIDs is due to their inhibition of COX-1 in the stomach, the COX-1 knock-out mouse did not exhibit an increased susceptibility to ulcers (Langenbach et al., 1995). It was confirmed that gastric cytoprotection was not being conferred by compensatory up-regulation of COX-2. In fact, these mice were resistant to induction of ulcers by NSAIDs. These mice also exhibited an altered inflammatory response to arachidonic acid suggesting that COX-1 does play a role in inflammation. The most striking defect in the COX-1 knockout mice was that

mating between COX-1^{-/-} mice produced decreased numbers of live offspring due to a defect at the level of parturition. Litter size was unaltered, however, pup survival was decreased.

The phenotype of the COX-2 knockout mouse was surprising, these mice exhibited abnormal renal development (Morham et al., 1995). At birth, the kidneys were normal, but they did not continue to develop. This resulted in nephron hypoplasia and as a consequence a small number of functioning nephrons. These effects proved to be more severe in males and led to death between 8 and 16 weeks of age. The female COX-2^{-/-} mice were impaired with respect to ovulation, fertilization, implantation and decidualization (Lim et al., 1997). It has recently been shown that the anovulation of these mice can be corrected by the administration of PGE₂ or interleukin-1 β (IL-1 β) (Davis et al., 1999). Although no compensatory mechanism made up for the lack of COX-1 in the stomach of the COX-1^{-/-} mice (Langenbach et al., 1995), in a study of cultured fibroblasts from either COX-1 or COX-2 null mice, there was compensatory up-regulation of the remaining COX isoform (Kirtikara et al., 1998).

Section 1.8: COX inhibitors- NSAIDs .

NSAIDs only inhibit the cyclooxygenase activity and not the peroxidase activity of the COX isoforms (DeWitt, 1999). The most well known NSAID, acetylsalicylic acid (ASA, Aspirin) is atypical in that it irreversibly inhibits COX activity by acetylating a serine in the active site of the both COX isoforms (Lecomte et al., 1994; Smith and Marnett, 1991).

Other inhibitors which function by acetylating COX isoforms include valerylsalicylic acid (VSA) and o- (acetoxyphenyl)hept-2-ynyl sulfide (APHS). VSA is an irreversible COX-1 specific inhibitor (Bhattacharyya et al., 1995). Recently, a COX-2 specific irreversible inhibitor, APHS, which preferentially acetylates COX-2 has been described (Kalgutkar et al., 1998). Interestingly, when COX-2 has been acetylated, it can acquire the ability to convert arachidonic acid to 15R-hydroxyeicosatetraenoic acid (15R-HETE) (Lecomte et al., 1994) which can serve as a precursor to lipoxin synthesis (Gronert et al., 1998).

Unlike the abovementioned examples, the vast majority of NSAIDs are reversible competitive inhibitors of the COX isoforms. The drugs can further be divided into two classes. The class described as completely reversible COX inhibitors includes popular over the counter pain relievers which contain ibuprofen (Gierse et al., 1999). The equation below describes the freely reversible nature of the enzyme/inhibitor (EI) interaction:



The second group can be described as time dependant reversible COX inhibitors. This group contains the well known non-selective inhibitor indomethacin. Upon rapid formation of a freely reversible EI complex, there is a time dependant transition to a more tightly bound EI complex as described by the schematic:



This time dependant inhibition may require a structural change in the COX protein. Pre-incubation of COX-1 with indomethacin conferred resistance to trypsin mediated proteolysis (Kalgutkar et al., 1996; Kulmacz, 1989).

Since the discovery of the COX-2 isoform, there has been considerable interest in the development of a COX-2 specific inhibitor (Laneuville et al., 1994). This work has been driven by the hypothesis that while COX-2 generates prostanoids associated with inflammation, COX-1 produces the prostanoids associated with normal gastric and renal physiology (Hawkey, 1999). In December 1998, approval was given in the United States for the use of celecoxib (Celebra, Monsanto) the first COX-2 specific inhibitor approved for human use. The specificity of celecoxib for COX-2 stems from the fact that while it is a freely reversible inhibitor of COX-1, it inhibits COX-2 in a time dependant reversible manner (Gierse et al., 1999). Due to its tighter binding to COX-2, COX-2 inhibition by a time-dependant inhibitor can be achieved at lower concentrations than those required for COX-1 inhibition.

Glucocorticoids are capable of reducing inflammation in part due to their effects on COX-2 expression. As opposed to the NSAIDs which diminish inflammation through the inhibition of COX-2 activity, glucocorticoids inhibit COX-2 transcription and destabilize the COX-2 mRNA (Newton et al., 1998; Ristimaki et al., 1996). The inhibition of COX-2 expression by glucocorticoids is not universal. Glucorticoids may stimulate the production of prostaglandins associated with parturition (Economopoulos et al., 1996). The human

COX-2 promoter does not contain a glucocorticoid response element (Appleby et al., 1994). The inhibition of COX-2 expression by glucocorticoids occurs via an inhibition of nuclear factor- κ B (NF- κ B) mediated stimulation of COX-2 transcription (Inoue and Tanabe, 1998). The anti-inflammatory effects of glucocorticoids are not solely mediated by the inhibition of COX-2 expression (Minghetti et al., 1999). In this study, the inducible isoform of nitric oxide synthase was also down-regulated by glucocorticoids.

Section 1.9: *The EP receptors.*

PGE₂ exerts diverse and at times contradictory effects through a family of four pharmacologically distinct receptors which have been named EP₁, EP₂, EP₃, and EP₄ (Coleman et al., 1994). The diverse effects of the PGE₂ receptors flow from their ability to signal via different heterotrimeric guanine nucleotide binding protein (G-protein) coupled pathways (Coleman et al., 1994). Via these different receptor mediated pathways, PGE₂ can have the effect of stimulating phosphatidyl inositol turnover and/or stimulating and/or inhibiting cAMP synthesis.

Section 1.10: *EP₁ Receptor.*

In 1993, Watabe et al, reported the cloning of a mouse cDNA encoding a 405 amino acid protein which when expressed in a chinese hamster ovary cell line (CHO) exhibited the characteristics of the pharmacologically defined EP₁ receptor (Watabe et al., 1993). The

cloned human EP₁ receptor cDNA also encodes a 405 amino acid protein which has similar characteristics when expressed in COS cells (Funk et al., 1993). Signaling by this EP receptor is via G_q resulting in phosphatidylinositol hydrolysis, mobilization of intracellular Ca²⁺, and protein kinase C (PKC) activation (Funk et al., 1993). Northern blot analysis found the mRNA for this receptor to be most highly expressed in the kidney and the lung. In the kidney, the EP₁ receptor has been demonstrated to be responsible for the PGE₂ mediated inhibition of Na⁺ reabsorption in the CCD (Guan et al., 1998). Interestingly, the EP₁ gene is completely overlapped by the protein kinase N (PKN) gene (Batshake and Sundelin, 1996). This finding explains the 6 kb band which was observed above the 2.4 kb EP₁ band on the EP₁ Northern blotting results presented by Watabe et al (1993).

Section 1.11: *EP₂ Receptor.*

Prior to the cloning of the EP₂ receptor by Regan et al in 1994, reports of EP₂ receptor cloning were actually referring to what is now named the EP₄ (Regan et al., 1994). The EP₂ receptor is pharmacologically defined as being sensitive to the PGE₂ analogue butaprost. Butaprost or PGE₂ stimulation of the EP₂ receptor results in G_s mediated stimulation of adenylate cyclase activity (Katsuyama et al., 1995). Northern blotting revealed the EP₂ receptor mRNA to be most highly expressed in the uterus (Katsuyama et al., 1995). The cAMP accumulation associated with activation of this receptor helps to mediate the relaxation of smooth muscle induced by PGE₂. A recent study by Kennedy et

al revealed that EP₂ receptor knock-out mice exhibited impaired female fertility, elevated baseline systolic blood pressure and salt-sensitive hypertension (Kennedy et al., 1999). The reproductive abnormalities in the EP₂ receptor null mouse are consistent with those described for the COX-2^{-/-} mice (Morham et al., 1995).

Section 1.12: *EP₃ Receptor.*

The EP₃ receptor is responsible for the pyretic effects of PGE₂. In EP₃^{-/-} mice neither PGE₂, interleukin-1 (IL-1), or LPS could generate a febrile response (Ushikubi et al., 1998). The EP₃ receptor is frequently referred to as having a G_{i/o} mediated inhibition of adenylate cyclase activity (Sugimoto et al., 1992). However, there is evidence for other EP₃ mediated signaling pathways involving: stimulation of adenylate cyclase, phosphatidyl inositol turnover and interaction with the small GTPase Rho (Katoh et al., 1996; Namba et al., 1993). The EP₃ signaling pathway depends not only on which EP₃ isoform is expressed but also upon the cellular milieu in which it is found (Katoh et al., 1996; Namba et al., 1993). Multiple EP₃ isoforms arise from the single EP₃ receptor gene through alternative splicing. The mouse EP₃ receptor consists of at least 3 isoforms (Irie et al., 1993). The nomenclature which is used to describe these EP₃ splice variants is confusing as splicing varies from species to species. The three EP₃ splice variants which have been described in the mouse have been named EP_{3α}, EP_{3β}, and EP_{3γ}. These three EP₃ isoforms share a common 1005 bp cDNA which codes for a 335 amino acid protein which comprising the 7-TMS domains and

the ligand binding site. The cDNA differs at the 3' end where 3 distinct tails generate the characterized isoforms which are 1099, 1085, and 1096 bp for EP_{3α}, EP_{3β}, and EP_{3γ} respectively (Irie et al., 1993). The alternative -COOH termini of the resulting proteins help to regulate the G-protein coupling of these receptors. The EP_{3α} and EP_{3β} subtypes have both been characterized as coupling with G_i leading to an inhibition of adenylate cyclase activity albeit with different efficiencies. They bind ligands equally, but differ in their sensitivity to these agonists as assessed by their ability to inhibit adenylate cyclase (Sugimoto et al., 1993). The EP_{3α} receptor possesses significant agonist independent constitutive activity corresponding to approximately one half maximal activity (Hasegawa et al., 1996). The EP_{3γ} is capable of coupling with either G_i or G_s leading to inhibition or stimulation of adenylate cyclase activity respectively (Irie et al., 1993). Five rabbit EP₃ receptor splice variants have been described (Audoly et al., 1999). Alternative splicing of the rabbit EP₃ receptor yields receptors with different signaling characteristics than the mouse EP₃ splice variants. Each of the rabbit EP₃ splice variants is capable of stimulation of cAMP response element mediated gene transcription in a means which is independent of cAMP generation and is pertussis toxin insensitive. This EP₃ mediated effect on cAMP mediated gene transcription is associated with a rise in intracellular [Ca²⁺] and is partially bisindolylmaleimide sensitive, therefore, suggesting a role for PKC in this signaling pathway (Audoly et al., 1999). The human EP₃ receptor also undergoes alternative splicing to yield at least 4 isoforms with alternative cytoplasmic COOH termini (An et al., 1994). As for the

mouse and rabbit EP₃ receptor splice variants, these tails can confer the ability to interact with different G-proteins.

Section 1.13: *EP₄ Receptor.*

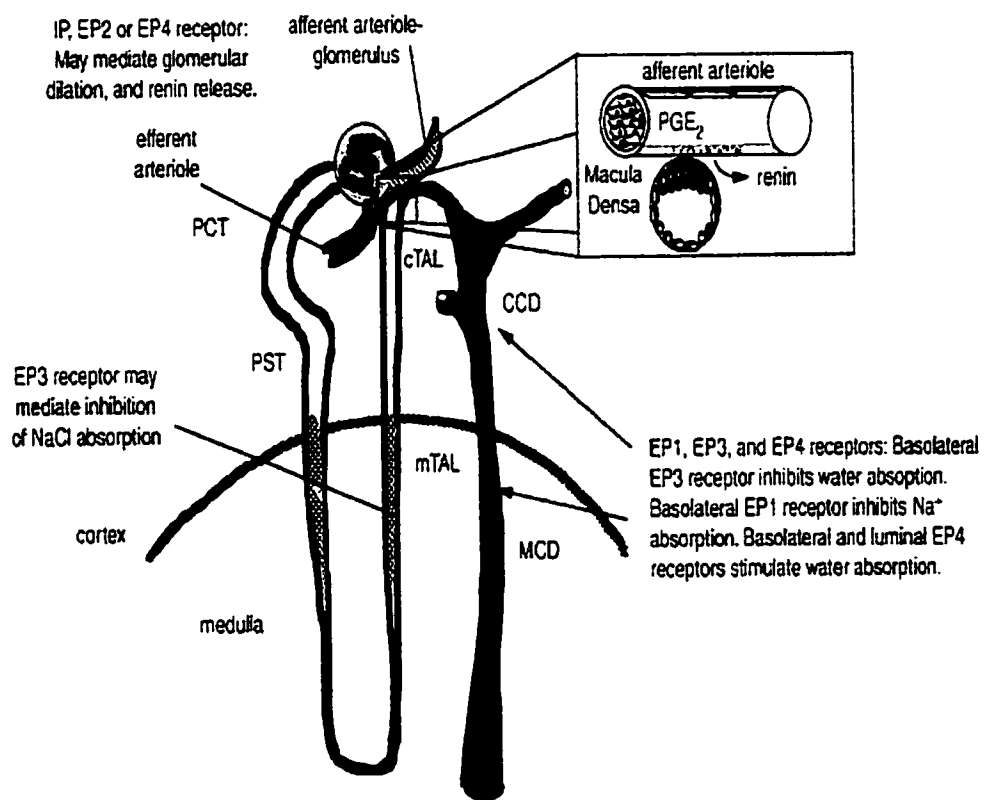
The EP₄ receptor stimulates adenylate cyclase via G_s. It is, therefore, similar to the EP₂ receptor described above which originally resulted in confusion over naming and references to these two receptors. Upon cloning, the receptor which is now defined as EP₄, was referred to as EP₂ (Honda et al., 1993). It is, however, derived from a separate gene (Arakawa et al., 1996), and unlike the EP₂ receptor, the EP₄ receptor is insensitive to the PGE₂ analogue butaprost (Breyer et al., 1996b). The major abnormality in the EP₄^{-/-} mice was that following birth, the ductus arteriosus failed to close resulting in pulmonary edema and death. Patent ductus arteriosus was not reported in either of the COX knockout models which suggests the occurrence of a compensatory mechanism. Another role for this receptor is the mediation of the effects of PGE₂ on osteoclast differentiation (Weinreb et al., 1999).

Section 1.14: *The Kidney.*

A detailed explanation of general physiology of the kidney is well presented elsewhere (Seldin and Giebisch, 1992). Briefly, we possess a pair of kidneys whose complex structure allows for the performance of a multitude of functions. Regulated water and electrolyte secretion and reabsorption by the kidneys controls extracellular fluid volume and

composition. Endocrine roles of the kidneys include vitamin D hydroxylation, renin release, and erythropoietin synthesis. Blood flow to the kidneys represents approximately 20 % of cardiac output. The kidney cortex is rich in cytochrome P450 enzymes which coupled with the tremendous blood flow to this region make it an important site for the metabolism of some drugs.

Figure 2. Nephron structure and distribution of prostaglandin receptors and their functions along the length of the nephron (Breyer et al., 1998). Proximal convoluted tubule (PCT), proximal straight tubule (PST), medullary thick ascending limb (mTAL), cortical thick ascending limb (cTAL), cortical collecting duct (CCD), and medullary collecting duct (MCD).



(Breyer et al., 1998)

The nephron is the functional unit of the kidney, approximately 1 million nephrons make up the human kidney. Each nephron is composed of distinct segments composed of specialized epithelial cells (Figure 2). In brief, within the capillary bed of the glomerulus, blood is filtered. The ultrafiltrate which is generated collects in Bowman's space and enters the proximal tubule. Approximately, 2/3 of water and solute reabsorption takes place in the proximal tubule. Water and solutes reabsorbed by the tubules are returned to the circulation via a second capillary bed: the peritubular capillaries. The proximal tubule is followed by the loop of Henle which travels deep into the medulla before returning to the cortex. The low oxygen environment of the medulla does not favor metabolic activity within the thin descending or thin ascending limbs. Rising up from the medulla, the thick ascending limb (TAL) is responsible for reabsorption of 20 % of the filtered salt and water. Within the distal tubule, a further 5 % of filtered salt and water is reabsorbed. The distal tubules of many nephrons converge upon and branch into a single collecting duct which is left to handle between 5 and 10 % of the filtered load. The collecting duct travels from the cortex into the renal papilla and can be divided into three distinct segments: the cortical collecting duct (CCD), the outer medullary collecting duct (OMCD) and the inner medullary collecting duct (IMCD). Upon leaving the IMCD, filtrate which has not been re-absorbed gathers in the renal pelvis and exits the kidney bound for the bladder via the ureter.

Section 1.15: *COX isoforms and the kidney.*

COX activity is not uniform throughout the nephron. Radioimmunoassay experiments on microdissected nephron segments revealed prostanoid production to be highest per gram (g) of protein in the collecting duct (Bonvalet et al., 1987; Farman et al., 1987). Within the collecting duct, there was a gradient of increasing prostaglandin production going from the cortex to the inner medulla. The thick ascending limb also had high levels of prostaglandin production. In both of these regions, PGE₂ is the major prostanoid product. The individual COX isoforms have been localized in the kidney by in situ hybridization and immunohistochemistry on paraffin embedded kidney slices. COX-1 has been localized to the collecting ducts, interstitial cells, and endothelial cells (Komhoff et al., 1997). Meanwhile, COX-2 expression has been demonstrated in the macula densa, medullary interstitial cells and the IMCD (Harris et al., 1994; Yang et al., 1998). The macula densa is a specialized group of 20-30 columnar epithelial cells found on the wall of the cortical TAL where it passes in close proximity to the glomerulus and afferent and efferent arterioles. This complex is referred to as the juxtaglomerular apparatus. One key function of this structure is mediation of tubular glomerular feedback, whereby solute delivery to the macula densa results in signaling to the afferent arteriole to contract or relax. In this way, filtration is matched to reabsorption on a second to second basis in each individual nephron. Furthermore, signals from the macula densa can also mediate the release of renin by the afferent arteriole. Low salt diets enhance macula densa COX-2 expression and under these conditions, COX-2 inhibition decreases renin release (Harding

et al., 1997). This interaction with the renin-angiotensin system has consequences on long term regulation of extracellular fluid (ECF) volume. There exists a negative feedback loop whereby angiotensin II via the angiotensin type 1 receptor (AT1 receptor) down-regulates macula densa COX-2 expression, meanwhile, angiotensin converting enzyme (ACE) inhibitor therapy increases macula densa COX-2 expression and plasma renin activity (Cheng et al., 1999). The regulation of COX-2 expression in the macula densa has been well studied. Macula densa COX-2 expression is elevated in rats in response to restricted sodium intake and decreased in response to salt loading, these effects of dietary salt intake on COX-2 expression are reversed in the inner medulla (Harris et al., 1994; Yang et al., 1998).

Section 1.16: *The cortical collecting duct: structure and function.*

The collecting duct possesses a tight, polarized epithelium composed of a heterogeneous population of principal, α - and β -intercalated cells (Verlander et al., 1996). The proportion of principal cells in the CCD is about 60%, this number rises moving down the collecting to the IMCD. The reabsorption of salt and water by the CCD principal cell is tightly regulated. The α - and β -intercalated cells are responsible for proton secretion and reabsorption respectively. As mentioned above, the CCD is a mosaic of principal and intercalated cells which form a tight polarized epithelium. This polarity is essential for vectorial transport of water and solutes across CCD cells (Xu et al., 1997).

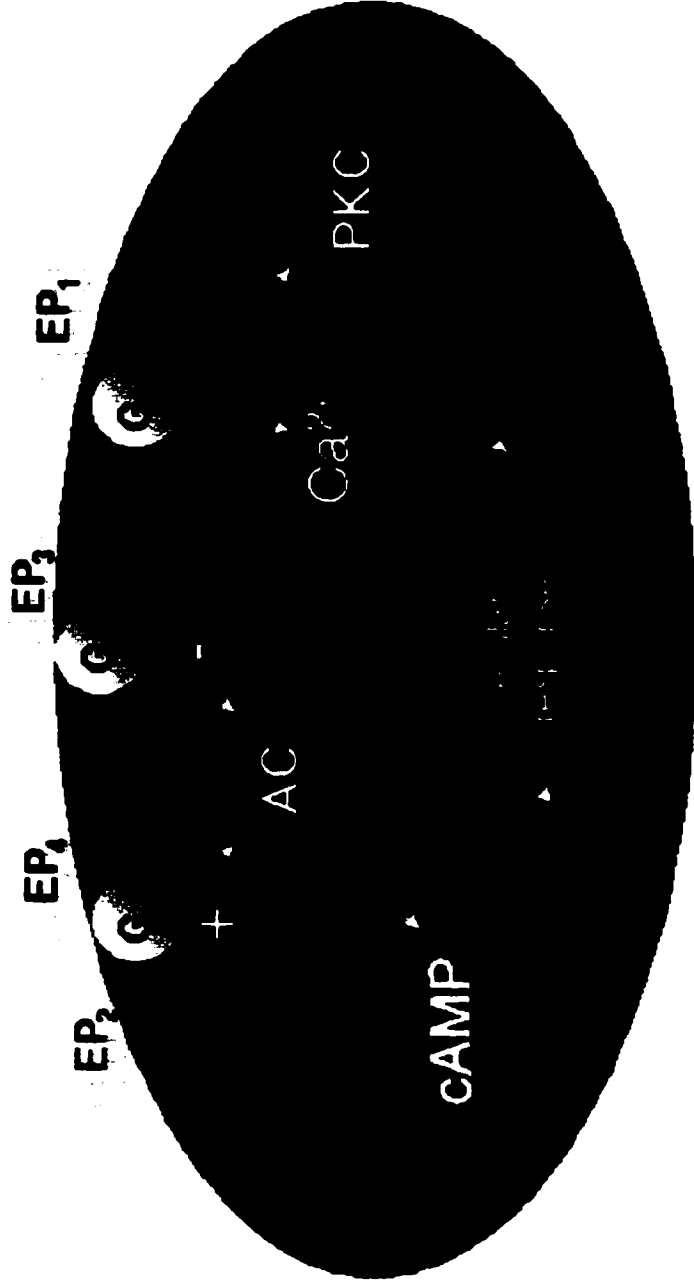
The main driving force for transport in the principal cell is the $\text{Na}^+ \text{-K}^+$ ATPase which is strictly localized to the basolateral membrane and which serves to maintain a low

intracellular $[Na^+]$. Localization of the epithelial sodium channel (eNaC) to the apical membrane of principal cells allows for reabsorption of Na^+ from the lumen. The eNaC represents a key site whereby the hormones aldosterone and AVP stimulate principal cell Na^+ reabsorption. Amiloride, a diuretic, functions by blocking the eNaC. Reabsorption of sodium via the eNaC generates a lumen-negative potential difference which facilitates the escape of potassium from the principal cell via a specific channel (Xu et al., 1997).

The water permeability of the CD apical membrane is regulated by the expression, insertion and removal of the aquaporin 2 (AQP2) water channel in the collecting duct apical membrane (Nielsen et al., 1999). Sub-apical vesicles containing AQP2 are inserted within the apical membrane in response to AVP - V_2 receptor mediated stimulation of cAMP synthesis (Valenti et al., 1998). Stimulation of the V_2 receptor also acts to promote increased AQP2 transcription (Yasui et al., 1997). AQP3 and AQP4 are constitutively expressed in the principal cell basolateral membrane allowing for vectorial flow of the water entering the cell via AQP2 (Nielsen et al., 1999).

Figure 3. PGE₂ receptor signaling pathways (Breyer et al., 1998). The figure illustrates the best characterized signaling pathways of the EP receptor subtypes. The EP₃ and EP_{2/4} receptors exert opposing effects on the activation of adenylate cyclase via G_i and G_s coupled signaling respectively. EP₁ receptor stimulation mediates an increase in phospholipase C (PLC) activity via coupling to G_q which results in an increase in intracellular [Ca²⁺] and PKC activation.

PGE₂
↓



(This figure is re-printed with permission from C.J. Kennedy)

Section 1.17: Prostanoid signaling in the CCD

The CCD expresses the EP₁, EP₃, and EP₄ receptors as determined by in situ hybridization (Breyer et al., 1996a; Sugimoto et al., 1994) and functional studies (Hébert et al., 1990; Hébert et al., 1991; Hébert et al., 1993). Each one of these receptors is found in the basolateral membrane of the CCD whereas only EP₄ is also found on the luminal membrane (Figure 3) (Sakairi et al., 1995). Functional evidence suggests that this pattern of EP receptor distribution is limited to principal cells (Figure 3) (Breyer et al., 1998). The EP₁ receptor mediates the natriuretic effects of PGE₂ on the CCD via Ca²⁺ mediated signaling (Guan et al., 1998). In the CCD, the EP₃ receptor is best characterized as having an inhibitory effect on the induction of AQP2 mediated osmotic water permeability by AVP (Fleming et al., 1998). This effect is largely due to EP₃ coupling with G_i, however, there is also a PKC mediated component associated with the inhibition of cAMP accumulation by PGE₂ in the rabbit CCD (Noland et al., 1992). PKC signaling can lower cAMP levels via type IV phosphodiesterase activation in the medullary collecting duct (Tetsuka et al., 1995). The situation of the EP₄ receptor on the luminal membrane of the CCD allows it to respond to urinary PGE₂ which is shown to be elevated in cirrhosis of the liver and congestive heart failure (Sakairi et al., 1995). Stimulation of this luminal PGE₂ receptor causes G_s mediated elevations in cAMP resulting in increased osmotic water permeability.

Section 1.18: NSAIDs and the kidney.

NSAIDs ingestion can result in multiple renal side effects including: acute renal insufficiency, impaired regulation of sodium, water and potassium homeostasis, acute interstitial nephritis and papillary necrosis (Murray and Brater, 1997). These side effects can be caused by two different mechanisms: 1) toxicity of individual drug metabolites and 2) reduced synthesis of prostaglandins. A loss of the diuretic and natriuretic tubular effects of PGE₂ upon NSAID administration could be responsible for the edema and increases in blood pressure which can accompany these drugs. In fact, there is evidence that the sodium retaining effects of NSAIDs are due to COX-2 inhibition (Catella-Lawson et al., 1999). Many human studies have documented an increase in mean arterial pressure in response to NSAID therapy (Fierro-Carrion and Ram, 1997). Although these short term effects on blood pressure are modest, NSAID intake has been determined to be an independent risk factor for the subsequent need for anti-hypertensive therapy in the elderly (Gurwitz et al., 1994). Given the role played by prostanoids in renin release, hyporeninemic hypoaldosteronemia could result as a consequence of blocking macula densa COX-2 activity (Cheng et al., 1999; Harris et al., 1998). In situations characterized by high circulating levels of angiotensin II, such as volume depletion, NSAIDs can cause acute renal failure due to a loss of the

vasodilatory prostaglandins required to overcome the vasoconstrictive effects of angiotensin II on the renal vasculature (Smith and DeWitt, 1995).

Section 1.19: *The M-1 cell line as a model for study of the CCD.*

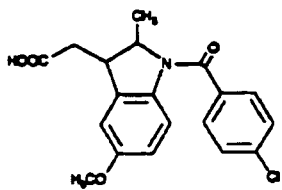
The M-1 CCD cell line is an immortal cell line derived from the kidney of an SV40 transgenic mouse. These cells have been characterized to functionally, morphologically, and immunologically reflect the native CCD (Stoos et al., 1991). These cells form a tight membrane with a high transepithelial resistance. They exhibit amiloride sensitive sodium reabsorption and potassium secretion as well as the AVP responsiveness typical of principal cells. The cultured M-1 cells also demonstrate electrogenic proton secretion which is characteristic of α -intercalated cells. Within this cell line it has been shown that both principal and α -intercalated cells are derived from β -intercalated cell precursors (Fejes-Toth and Naray-Fejes-Toth, 1992). The M-1 cell line has been studied extensively since it was initially characterized. It is responsive to both atrial natriuretic factor (ANF) and bradykinin which act together to inhibit sodium transport (Stoos et al., 1992). These cells have been extensively studied with respect to regulation of solute transport (Chalfant et al., 1996; Letz and Korbmacher, 1997; Nakhoul et al., 1998; Volk et al., 1995), hormone responsiveness (Sayegh et al., 1999), and second messenger signaling (Schnermann et al., 1996; Todd-Turla et al., 1996; Wong et al., 1995).

Section 2: Rationale

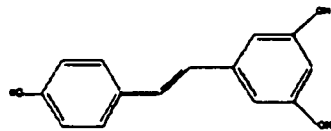
NSAIDs are valuable tools in the acute treatment of inflammation, fever, and pain. Evidence suggests that long term NSAID therapy confers protection against cardiovascular disease, colon cancer, and Alzheimer's disease (Dubois et al., 1998). Unfortunately, in susceptible individuals, these drugs can cause side effects associated with inhibition of renal prostaglandin synthesis. The tremendous beneficial effects of NSAIDs lead to their widespread use. Any side effects associated with these drugs no matter how infrequent, are of considerable interest and consequence due of their extensive use. Very little is known about the cellular adaptations which occur subsequent to NSAID use. A better understanding of these adaptations could lead to the different susceptibility to side effects from one NSAID to the next as well as between different people. Compensatory up-regulation of the remaining COX isoform has been observed in cultured cells from COX-1 and COX-2 knock-out mice (Kirtikara et al., 1998). It is unknown as to whether this compensatory up-regulation can occur in response to NSAIDs. At the level of PGE₂ receptors, NSAID treatment has been demonstrated to increase EP and FP receptor densities in synaptosome preparations from newborn pigs (Li et al., 1995). The arrival of COX-2 specific NSAIDs has been hailed with much fanfare touting their advantages over the older non-selective NSAIDs. Yet in clinical trials, the specific COX-2 inhibitors rofecoxib was found to increase the incidence of edema (DeWitt, 1999). A human study involving the

COX-2 specific inhibitor MK-966 found this drug to decrease urinary sodium excretion (Catella-Lawson et al., 1999). These findings indicate that this new class of NSAIDs is not totally benign with respect to the kidney. The CCD is a site where prostaglandins are known to regulate salt and water reabsorption and is, therefore, a putative site for the action of COX-2 specific inhibitors upon the regulation of sodium, potassium, and water homeostasis (Breyer et al., 1998). The M-1 CCD cell line represents a powerful, well characterized tool for the study of the interaction between NSAIDs and the prostaglandin synthesizing and signaling mechanisms in the CCD. We have chosen to investigate the effects on M-1 cell COX isoform content and activity of indomethacin (Smith et al., 1998), NS 398 (Futaki et al., 1993), and resveratrol (Jang et al., 1997) as representatives of non-selective, COX-2 specific and COX-1 selective inhibitors respectively (Figure 4).

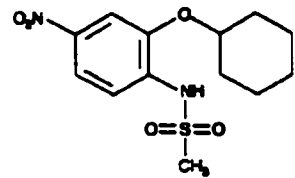
Figure 4. Structures of the NSAIDs employed in our experiments.



Indomethacin
COX-1 + COX-2 Inhibitor



Resveratrol
COX-1 Selective Inhibitor



NS-398
COX-2 Selective Inhibitor

Section 3: Hypothesis

It is hypothesized that the inhibition of prostaglandin synthesis with COX isoform specific NSAIDs will result in compensatory up-regulation of COX isoform levels and EP receptor expression in the M-1 cortical collecting duct cell line.

Section 4: Objectives and Approach

Section 4.1: Objectives

- 1) To assess via immunohistochemistry the distribution of COX-1 and COX-2 proteins in the native murine collecting duct.
- 2) To assess the levels of COX-1 (protein) and COX-2 (protein and mRNA) in the M-1 cell line before and after NSAID exposure.
- 3) To identify the prostanoids synthesized by the M-1 cells.
- 4) To characterize M-1 cell EP receptor expression before and after exposure to NSAIDs.

Section 4.2: Approach

The following strategies were implemented:

- 1) **Immunohistochemistry.**
- 2) **Immunofluorescence.**
- 3) **Thin layer chromatography.**
- 4) **Western Blotting.**
- 5) **Northern Blotting.**
- 6) **Reverse transcriptase - polymerase chain reaction (RT-PCR).**

Section 5: *Materials and Methods*

Section 5.1: *Cell Culture*

M-1 mouse cortical collecting duct cells (CRL-2038, ATCC, Rockville, MD) passages 18-30 were grown at 37 °C in 100mm dishes in a media consisting of DMEM/F12 supplemented with 5% fetal bovine serum (FBS) and 1% penicilin, streptomycin, Fungizone (PSF) antibiotic-antimycotic solution (Gibco-BRL, Burlington, ON) in a humidified atmosphere containing 5% CO₂. For the purpose of evaluating the effect of various NSAIDs on COX isoform levels, the respective drugs: indomethacin (Sigma Chemical, Mississauga, ON), NS-398 (Cayman Chemical, Ann Arbor, MI), and resveratrol (Sigma Chemical) were added to the media dissolved in dimethyl sulfoxide (DMSO). Drug concentration was in each case 10⁻⁵ M and DMSO concentration was 0.02 % (v/v).

Section 5.2: *COX-1 and COX-2 Immunofluorescence- M-1 Cells.*

M-1 cells were grown to confluence on glass coverslips, rinsed with phosphate buffered saline (PBS) and fixed for 30 min at room temperature with 1:1 (v/v) PBS: 10% phosphate buffered formalin. The fixative was washed off and the primary antibodies were applied diluted 1:50 in PBS + 10% FBS + 0.2% saponin (Sigma Chemical). The cells were incubated with the primary antibody solution at 37 °C for 1 hr. The COX-1 antibody was raised in a rabbit against a peptide corresponding to ovine COX-1 amino acids L274-A288

and was provided by Drs. Smith and DeWitt (Michigan State University, East Lansing, MI). Two COX-2 antibodies (2.5 mg of antibody per ml) raised in rabbits against different peptides from the murine COX-2 protein were purchased from Cayman Chemical (Catalog #160106 and #160116) and both were effective at specifically detecting murine COX-2 by our methodology. The coverslips were washed with PBS + 0.2% saponin prior to application of the secondary antibody. The secondary antibody, CY3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Bio/Can Scientific, Mississauga, ON) was diluted 1:75 in PBS + 10% FBS + 0.2% saponin, applied to the coverslips and incubated at 37 °C for 30 min. The coverslips were washed with PBS + 0.2% saponin before being mounted on slides with PermaFluor fade resistant mounting medium (Immunon Lipshaw, Pittsburgh, PA). In separate experiments, the saponin was not used and permeabilization of the cells was performed via a 15 min room temperature incubation in PBS + 0.1% Triton X-100 (Sigma Chemical). The specificity of the COX -2 antibodies was tested by pre-incubation with the cognate peptide (Cayman Chemical, Catalog #360106) (5:1 (w/w) peptide to antibody in PBS 18 hrs at 4° C). The COX-1 detection was controlled for by omission of the primary antibody. The results were captured under an Axiophot™ microscope (Carl Zeiss Inc., Thornwood, NY.) using Northern Eclipse 5.0 imaging software.

Section 5.3: *Immunohistochemical localization of COX-1 and COX-2 on mouse kidney slices.*

Mice (CD-1, 25-30g, 10-12 weeks) from Charles River were sacrificed and the kidneys were immediately removed. The kidney was rinsed in PBS, decapsulated, sliced transversely and placed in fixative. The fixative consisted of 2 % paraformaldehyde and 0.2 % picric acid in PBS (pH 7.4) and the fixation lasted 18 hrs at 4 °C. The kidneys were dehydrated, paraffin embedded and 4 µm microtome sections were mounted onto SuperFrost Plus slides (VWR Canlab, Mississauga, ON). The sections were re-hydrated and incubated in methanol + 0.3% H₂O₂ for 30 min at room temperature. The cells were permeabilized for 15 min in PBS + 0.3 % Triton X-100 and blocked for 30 min in PBS + 1 % skim milk powder. The COX-1 or COX-2 primary antibody was diluted in PBS + 0.1 % Triton X-100 + 1 % bovine serum albumin (BSA). For identification of the CCD via aquaporin 2 (AQP2) labeling, a specific antibody which was obtained from Dr. Mark A. Knepper (National Institutes of Health, Bethesda, MD) was diluted to 40ng/ml (Fushimi et al., 1993; Marples et al., 1995). The sections were incubated with these antibody solutions for 18 hrs at 4 °C. The secondary antibody was biotinylated- anti-rabbit IgG (Amersham Canada, Oakville, ON) which was diluted 1:100 in PBS + 1 % BSA + 0.1 % Triton X-100, this incubation lasted 30 min at 37 °C. After 10 min in 3% H₂O₂/PBS the slides were incubated with streptavidin linked horseradish peroxidase (HRP)(Amersham Canada) diluted 1:50 in PBS. Diaminobenzidine (DAB) substrate (Sigma Chemical) was used to visualize the signals. The slides were counterstained with Mayer's hematoxylin (VWR Canlab) before

dehydration and mounting with Permout (Fisher Scientific, Nepean, ON). For double labeling of COX-1/AQP2 and COX-2/AQP2, the COX isoform labeling was performed first as described above, up to and including the visualization with DAB. Following this step, HRP activity was quenched by incubating the slides in PBS + 3% H₂O₂ for 10 min at room temperature. The slides were rinsed with PBS and were incubated for 30 min with an unconjugated donkey anti-rabbit IgG Fab fragment (Jackson Immunoresearch Laboratories) to block further detection of the COX isoform primary antibody. The AQP2 antibody incubations were carried out as described above. For detection of the AQP2 localization, metal enhanced DAB (Sigma Chemical) was used. This reagent allowed for the rapid detection of AQP2 via the development of an insoluble blue precipitate which is easily discernible from the DAB (brown color) employed for the COX-1 and COX-2 localization. Following the AQP2 detection, the slides were rinsed in H₂O, dehydrated and mounted with Permout. The results were analyzed and captured using an Axiophot™ microscope (Carl Zeiss Inc., Thornwood, NY.) and Northern Eclipse 5.0 imaging software.

Section 5.4: *Enzyme Activity.*

This radio-thin layer chromatography (radio TLC) methodology which we have optimized for the measurement of COX activity in the M-1 cells has been previously described (Laneuville et al., 1995; Lecomte et al., 1994). These experiments were necessary to identify the prostanoids produced by the M-1 cells. Additionally, these experiments afforded us an opportunity to evaluate COX activity in the presence of various NSAIDs.

Confluent dishes of M-1 cells were harvested and re-suspended in DMEM/F12 media. The NSAIDs to be assessed were added dissolved in DMSO to give a final NSAID concentration of 10^{-5} M and a DMSO concentration of 0.02 % (v/v). The following drugs were studied: indomethacin, NS-398, resveratrol and the combination of NS-398 + resveratrol. The cells were incubated with the respective drugs for 30 min at 37 °C before [$1-^{14}$ C]-arachidonic acid (AA) (specific activity 42 μ Ci/mmol) (Amersham Canada) was added to give a final concentration of 10 μ M and the cells were further incubated at 37 °C for 40 min. At the end of the incubation, the samples were centrifuged (5 min, 2000g) and the supernatant was retained for measurement of prostanoid content. Protein samples from the cellular pellet were measured by the Bradford method (Loffler and Kunze, 1989) to normalize each sample for total cellular protein. The lipid fraction was extracted from the supernatant with diethyl ether/methanol/ 0.2M citric acid solution (30:4:1 v/v/v). This prostanoid and unreacted AA containing fraction was separated on Silica Gel 60 thin layer chromatography plates (VWR Canlab) using the organic phase of an ethyl acetate/ 2,2,4-trimethyl pentane/acetic acid/H₂O (11:5:2:10 v/v/v/v) solvent system. The presence of radio-labeled products was evaluated by autoradiography using Kodak BioMax MR film. Cold PGE₂ standard (Cayman Chemical) visualized with iodine vapor was used to confirm the identity of the major prostanoid produced by the M-1 cells to be PGE₂. Our lab has demonstrated that using this solvent system PGF_{2 α} and 6-keto-PGF_{1 α} migrate more slowly and do not overlap with the signal generated by PGE₂. Scanning densitometry was performed using the Image 1.47 program

and PGE₂ signal density was normalized to total protein content as determined by the Bradford method..

Section 5.5: *Western Blotting.*

These experiments were performed to assess the effect of COX isoform inhibition on the levels of the COX isoform proteins. NSAIDs were added to the media when the cells were plated and the cells were grown to confluence (approximately 72 hrs). Dishes of confluent cells were rinsed with cold phosphate buffered saline (PBS) and harvested in PBS by scraping with a cell lifter. The harvested cells were centrifuged (2000g, 5 min), the supernatant was aspirated and the cell pellet was resuspended in 100 mM Tris (pH 7.4) containing 1 mM EDTA and 1mM EGTA. The resulting solution was sonicated for 5 sec with an Ultrasonics cell disrupter to lyse the cells. The cell lysates were spun at 10 000g for 10 min to pellet the nuclei and insoluble cytoskeleton. The supernatants were removed and assayed for protein content by the Bradford method (Bio-Rad Laboratories, Mississauga, ON). Twenty-five µg of protein from each sample was denatured in boiling Laemmli buffer (Laemmli, 1970) for 5 min and resolved by SDS-PAGE on a polyacrylamide gel consisting of a 4% stacking and a 10% resolving layer using a Mini-PROTEAN II apparatus (Bio-Rad Laboratories). Following electrophoresis the proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Canada) with the Mini-Trans blot system (BioRad). The membranes were blocked overnight at 4 °C in Tris buffered saline-0.1 % TWEEN-20 (TBS-T) supplemented with 5 % or 10 % fat free dried milk for COX-1 and COX-2

detection respectively. After rinsing away the blocking solution with TBS-T, the membranes were incubated with primary antibody diluted 1:2000 in TBS-T-2 % milk for 90 min at room temperature. The COX-1 and COX-2 antibodies are the same as those which were used for the immunofluorescence and immunohistochemistry experiments. The specificity of the COX-2 isoform specific antibodies was tested by Western blotting using 50 ng of purified COX-1 and -2 electrophoresis standards per lane (Cayman Chemical). In addition, the COX-2 antibodies were tested following pre-incubation with their respective immunizing peptides as per the immunofluorescence experiments. Following washes with TBS-T, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Promega, Madison, WI) diluted 1:2000 in TBS-T for 1 hr at room temperature. Excess secondary antibody was washed away with TBS-T. The results were visualized following developing with the Amersham enhanced chemiluminescence (ECL) reagents as per the manufacturer's directions (Amersham Canada). To ensure equal protein loading, the membranes were stripped as per the Amersham protocol, blocked for 3 hrs at room temperature in TBS-T-10% milk, rinsed with TBS-T and incubated with anti-mouse β -actin antibody (Sigma Chemical) diluted 1:5000. After rinsing the membranes, the HRP-conjugated anti-mouse IgG secondary antibody (Amersham Canada) was diluted 1:2000 and incubation took place for 30 min at room temperature. Following thorough washing, the results were visualized by the Amersham ECL protocol. Scanning densitometry was performed using the Image 1.47 program and COX signal density was normalized to β -actin density.

Section 5.6: Northern Blotting.

Total RNA was isolated from M-1 cells via the TriZol method (Gibco BRL). PolyA⁺ RNA isolated from 100 µg of total RNA with Qiagen Oligotex™ columns was loaded onto 1% denaturing agarose gels and electrophoresed. The RNA was transferred to nylon membranes (Boehringer Mannheim, Laval, QC) and fixed by baking at 80 °C under vacuum. A probe of 1.3 kb (25 ng) complementary to the portion of the mouse COX-2 cDNA between the *XmnI* restriction site at 492 nucleotides and the 3' end was labeled with ³²P - dCTP (50 µCi) by random priming with the Prime-It kit (Stratagene Cloning Systems, La Jolla, CA). The probe was obtained from a restriction enzyme digestion of a PSVL plasmid containing a full length (1.3kb) mouse COX-2 cDNA insert. This COX-2 containing vector was isolated from transformed *E. coli* (provided by Dr. Smith and Dr. DeWitt, Michigan State University) via a plasmid mini-prep. The identity of the excised fragment was confirmed by multiple restriction enzyme digestions. Following a 2 hr at 42 °C pre-hybridization, the labeled probe was added and the membrane was allowed to hybridize for 24 hrs at 42 °C. After hybridization and high stringency washing, the radioactive signal was detected by autoradiography with Kodak X-OMAT AR film. The COX-2 probe was stripped from the membrane by boiling for 3 min in diethylpyrocarbonate (DEPC) treated water. To control for the quantity of mRNA loaded per lane, α-tubulin mRNA levels were measured using a ³²P - dCTP (50 µCi) labeled probe prepared and hybridized as for the COX-2 probe. Band intensity was quantified by scanning densitometry using the Image 1.47 program.

Section 5.7: RT-PCR

Total RNA was isolated from confluent dishes of M-1 cells via the TriZol method (Gibco BRL). The EP₁, EP₂, and EP₄ primers which we employed were designed by Arakawa et al for the detection of these receptors in murine NIH 3T3 and RAW 264.7 cells (Arakawa et al., 1996). The primers which were employed in our experiments are summarized in Table 1. The EP₃ primers which we used have previously been described as being effective for the amplification of an EP₃ mRNA fragment (206 bp) from the microdissected mouse CCD (Taniguchi et al., 1994). Reverse transcription was performed with random hexamer priming. Both the RT and the PCR were carried out with the reagents and protocols provided by the Perkin Elmer RNA PCR Core Kit using a Perkin Elmer Gene Amp PCR system 2400 thermal cycler. For EP₁ and EP₂ detection, amplification of 1 µg of total RNA was reverse transcribed followed by amplification of the resulting cDNA (PCR conditions were 94 °C for 240 sec followed by 35 cycles of 95 °C for 15 sec, 63 °C for 30 sec, and 72 °C for 30 seconds followed by 240 sec at 72 °C). Total RNA isolated from mouse spleen served as a positive control for the EP₂ message. The identity of the EP₁ PCR product was confirmed by digestion with the restriction enzyme Pst I (Gibco BRL) to generate 207 bp and 129 bp bands. Bands were purified from agarose gels via QIAquick gel extraction kit (Qiagen).

Table 1: A description of the primers employed for RT-PCR experiments.

Target of Primers	Sense Primer	Anti-sense Primer	Size of Expected Fragment
EP ₁ Receptor	5' - CGCAGGGTTCACGCACACCGA - 3' (nucleotides 865-884)	5' - CACTGTGCCGGAACTACGC - 3' (nucleotides 1182-1201)	336 bp
EP ₂ Receptor	5'-AGGACTTCGATGGCAGAGGAGAC - 3' (nucleotides 757-779)	5'-CAGCCCTTACACTTCTCCAATG - 3' (nucleotides 1136-1158)	401 bp
EP ₃ Receptor	5' - TGGCCTTCCCATCACCATGATGGTCACTG - 3' (nucleotides 86-115)	5' - CCAGCGTCGCTGTGACAGGTACACCGAGG - 3' (nucleotides 264-291)	206 bp
EP ₄ Receptor	5' - TTCCGCTCGTGGTGCGAGGTTC-3' (nucleotides 941-963)	5' - GAGGTGGTGTCTGCTTGGGTCAG - 3' (nucleotides 1342-1364)	423 bp
β-Actin	5' - TCACCCACACTGTGCCCATCTAC - 3' (nucleotides 556 to 578)	5' - GAGTACTTGCGCTCAGGAGGAGC - 3' (nucleotides 1071-1093)	537 bp

We have employed a semi-quantitative RT-PCR approach to measure the relative expression of EP₃ mRNA in NS-398 (10⁻⁵M) treated versus vehicle treated control M-1 cells. NS-398 was added to confluent dishes of M-1 cells 1.5, 4.0 and 6.0 hrs prior to RNA isolation. In each case, the NS-398 was delivered dissolved in DMSO and the final DMSO concentration was 0.02 % (v/v) For each experiment a standard curve was constructed wherein we amplified 100, 200, 400, and 600 ng of the control total RNA. The signal density was measured with a Storm PhosphoImager system and log [signal density] was plotted against log [starting total RNA (ng)]. For measurement of signal density by the Storm PhosphoImager, 15 µl of the respective PCR products was mixed with Vistra Green (1:5000 final dilution) (Amersham Canada), incubated at room temperature for 15 min and electrophoresed on a 2 % agarose gel. For the simple visualization of RT-PCR results via ultra violet transillumination, the samples were electrophoresed in 2 % agarose gels containing ethidium bromide (Sigma Chemical). A straight line was obtained by linear regression and the slope and y-intercept of this line were used to interpolate the relative strength (compared to control) of the signal for the NS-398 treated samples (200ng starting RNA). The EP₃ amplification was performed under the following PCR conditions: 94 °C for 240 sec followed by 26 cycles of 95 °C for 30 sec and 72 °C for 30 seconds followed by 240 sec at 72 °C. The cycle number was determined in preliminary experiments to be within a linear range when log [signal density] was plotted against cycle number. To minimize variability between samples within a single experiment an RT and a PCR master mix was common to all samples. The EP₃ signal was normalized to the respective β-actin signal, the β-actin

primers have previously been described (Arakawa et al., 1996). β -actin semi-quantitative PCR was performed with the following amplification conditions: 94 °C for 240 sec followed by 27 cycles of 95 °C for 15 sec, 63 °C for 30 sec, and 72 °C for 30 sec followed by 240 sec at 72 °C. For comparisons between the EP₃ receptor and β -actin, the RT step was performed in a single tube. Following the RT reaction, the contents of this tube were divided equally between the EP₃ tube and the β -actin tube. This further minimizes variability between the EP₃ amplification and the β -actin amplification. In this way, variability due to pipetting is minimized and the β -actin control more accurately normalizes for variability in the quantity of starting RNA.

Section 5.8: Statistics

Data are presented as mean \pm SEM. Statistical analysis was performed using the Prism Graphpad 2.01 software and consisted of ANOVA (TLC, Western, and Northern blot results) or repeated measures ANOVA (RT-PCR results) followed by the Bonferroni post test. Differences were accepted as statistically significant at the level of $p < 0.05$ where $n \geq 3$.

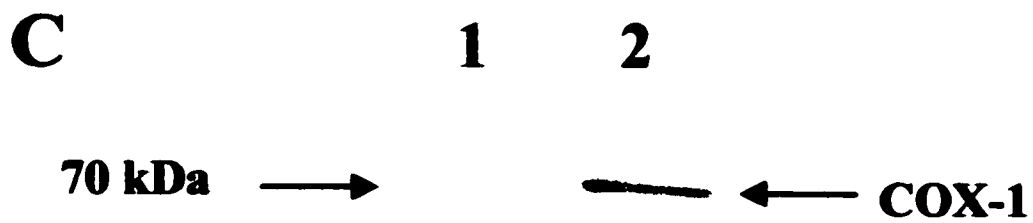
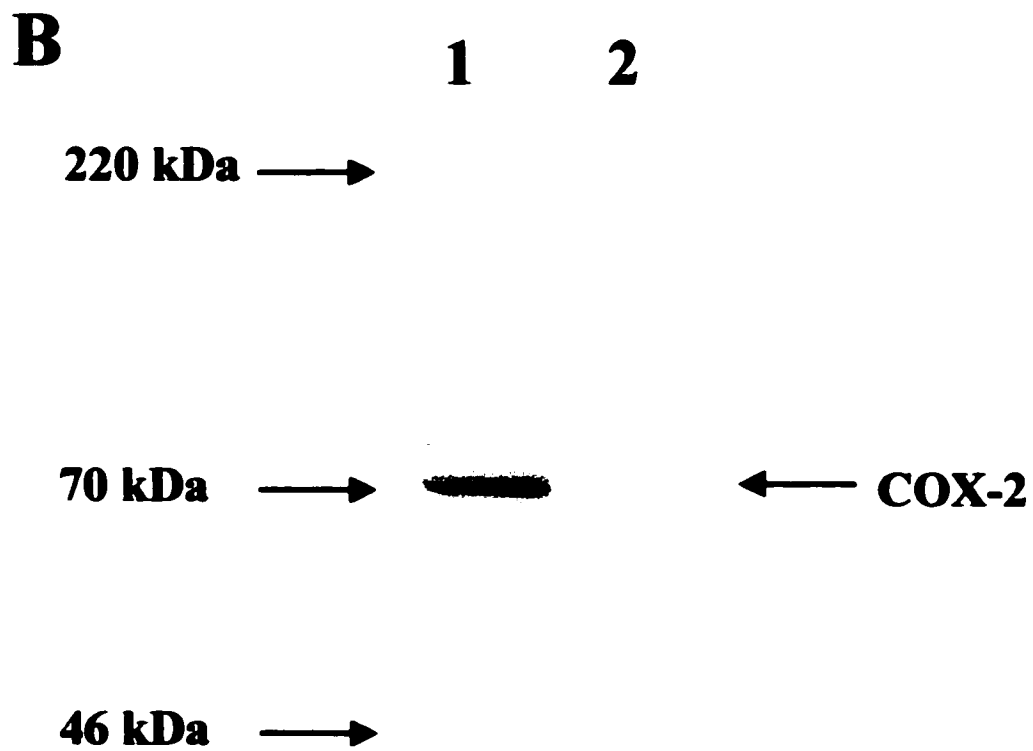
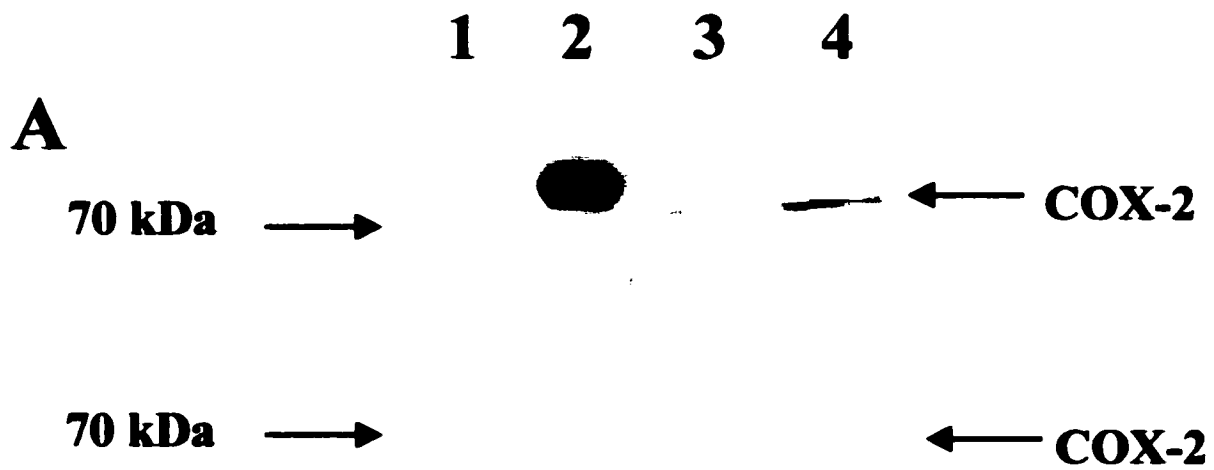
Section 6: Results

Section 6.1: *Western blotting experiments demonstrate the specificity of the COX-1 and COX-2 primary antibodies which were employed in our experiments.*

Although the two COX isoform amino acid sequences are 60 % identical (DeWitt and Smith, 1995), the divergent -COOH terminal sequence has allowed for the development of COX-2 specific antisera. In Figure 5, we demonstrate that the COX-1 and COX-2 antibodies are specific for their respective COX isoforms. In Western blotting experiments, identical results were obtained with both COX-2 antibodies obtained from Cayman Chemical (Catalog #160116 and #160116). Each of these antibodies was raised against different peptides from the murine COX-2 protein. However, for each antibody the immunizing peptide is derived from the COX-2 -COOH terminal and contains an 18 amino acid region which is absent from COX-1 (Vane et al., 1998). The immunizing peptide for one COX-2 antibody (#160116) corresponded solely to the 18 amino acid -COOH terminal sequence not found in COX-1. Therefore, it is not surprising to see in Figure 5A that this antibody detects the COX-2 standard and not the COX-1 standard. The other COX-2 (#160106) antibody which we tested was raised against a 30 amino acid peptide which encompassed this 18 amino acid cassette which is absent from COX-1. This antibody also detects COX-2 in M-1 cells

(Figure 5B). The COX-2 signal detected by both these antibodies can be abolished by pre-incubation of these antibodies with the 30 amino acid COX-2 C - terminal peptide (Figure 5, A and B). Figure 5C demonstrates that the COX-1 antibody which we have employed does not cross-react with the COX-2 standard while at the same time it does detect a band representing COX-1 in an M-1 cell lysate. Additionally, the results presented in Figures 6 and 7 reveal a different subcellular localization of the two COX isoforms which further confirms the lack of cross-reactivity of the antibodies we have selected for these experiments.

Figure 5. Western blotting experiments demonstrate the specificity of the COX-1 and COX-2 primary antibodies which were employed in our experiments. (A) This figure demonstrates the specificity of the COX-2 antibody #160116. The two pictures are of the same membrane which was first probed with the COX-2 antibody #160116 (top photo). The membrane was subsequently stripped and the detection was repeated but this time the COX-2 antibody was pre-incubated with an excess of the cognate peptide (bottom photo). Lane 1: 50 ng COX-1 standard. Lane 2: 50 ng COX-2 standard. Lane 3: 25 μ g of protein from an M-1 cell lysate, 25 μ g of M-1 cell lysate protein from NS-398 pre-treated cells. (B) Detection of COX-2 in M-1 cells with the #160106 antibody is abolished by pre-incubation of this antibody with the 30 amino acid immunizing peptide. Both lanes were loaded with 25 μ g of M-1 cell lysate prepared as described in the text. As opposed to Lane 1, prior to detection, the antibody in Lane 2 was pre-incubated with an excess (by weight) of the immunizing peptide. (C) The COX-1 antibody which was employed in our experiments does not cross-react with COX-2. Lane 1: 50 ng COX-2 standard. Lane 2: 25 μ g of protein from an M-1 cell lysate.



Section 6.2: *Indirect immunofluorescence demonstrating the pattern of COX isoform distribution in cultured M-1 cells.*

The M-1 cell line reflects the intact collecting duct in that it is a heterogenous population of principal and intercalated cells (Stoos et al., 1991). Each of these cell types has been characterized with respect to their individual roles in tubular transport. Immunofluorescence experiments with COX isoform specific antibodies were performed to investigate the distribution of the two isoforms in the M-1 cell line. Figure 6A is representative of the pattern of fluorescence associated with COX-1 immunoreactivity in a mono-layer of confluent M-1 cells. The photos in Figure 6 are representative of the results which are achieved when saponin is used to permeablize the cells. The bright perinuclear/endoplasmic reticulum pattern of fluorescence is strikingly apparent in this micrograph. Figure 6B demonstrates the lack of fluorescence when the COX-1 primary antibody is omitted. Like COX-1, COX-2 is also expressed in all cell types represented by the M-1 cell line (Figure 6C). Each cell is positive for COX-2 and the bright perinuclear ring reflects the increased content of COX-2 protein in the nuclear envelope and is consistent with previously published observations describing the intracellular location of this enzyme (Morita et al., 1995). Figure 6D establishes the specificity of the COX-2 immunofluorescence. The COX-2 signal is abolished when the primary antibody is pre-incubated with the immunizing peptide against which it was raised. The results which are presented in Figure 6 are representative of the results which are obtained when the cells are permeablized with saponin. The detergent Triton X-100 is more effective than saponin at permeablizing the nuclear envelope which is

related to the low cholesterol content of this membrane (McNulty et al., 1995; Willingham and Pastan, 1985). This yields a striking difference in the localization of COX-2 (Figure 7B). Under these conditions, COX-2 localization is predominantly nuclear. The COX-1 localization (Figure 7A) is not as affected by the change in detergents. The main difference is the loss of the perinuclear ring, however, the localization is still mainly cytoplasmic/endoplasmic reticulum associated.

Figure 6. Indirect immunofluorescence demonstrating the pattern of COX isoform distribution in cultured M-1 cells. (A) Using a COX-1 isoform specific primary antibody, a perinuclear pattern of fluorescence was observed for COX-1 which is compatible with localization to the endoplasmic reticulum and nuclear envelope. (B) Omission of the COX-1 primary antibody results in complete loss of this labeling. (C) COX-2 was found to be most densely localized within the nuclear envelope with less intense fluorescence associated with the endoplasmic reticulum. (D) Pre-incubation of the COX-2 primary antibody with the immunizing peptide abolishes all COX-2 related fluorescence. All photos in this figure, represent a magnification of 1250X.

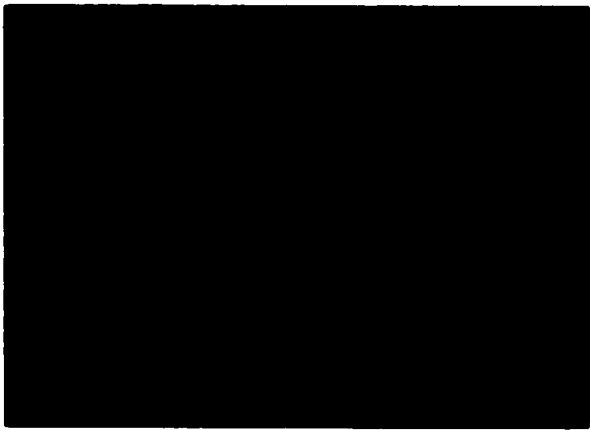
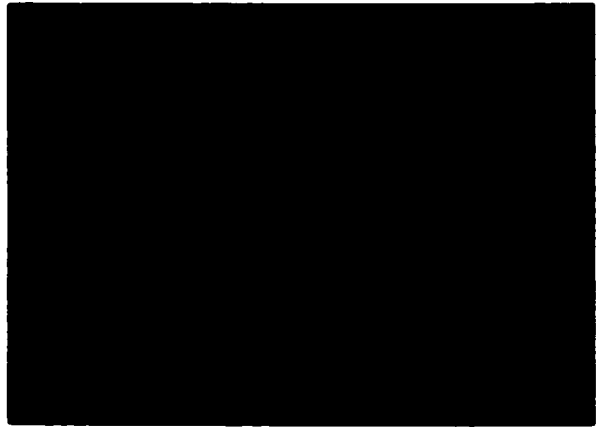
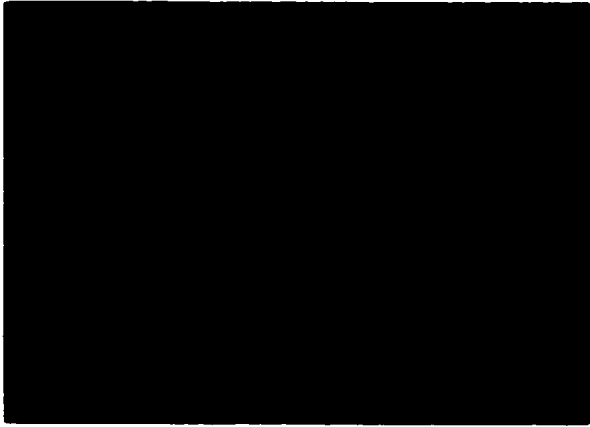
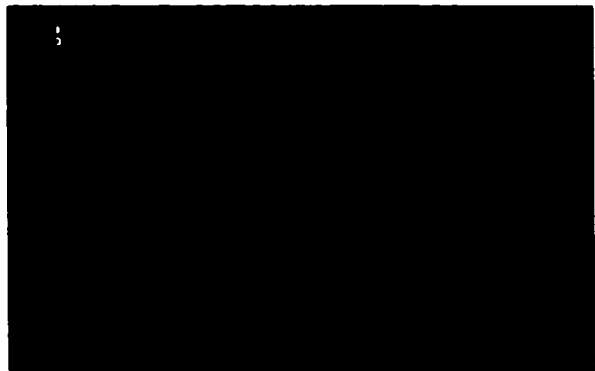
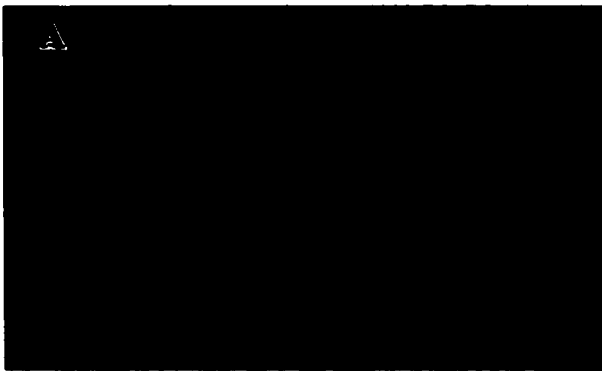


Figure 7. Permeablization of M-1 cells with Triton X-100 yields different results than saponin with respect to COX isoform localization in M-1 cells. (A) COX-1 localization is diffuse and predominantly cytosolic in Triton-X100 permeablized cells. (B) Triton X-100 permeablization of M-1 cells results in a predominantly nuclear localization of COX-2.



Section 6.3: *Immunohistochemical localization of COX-1 and COX-2 to the intercalated cells of the collecting duct.*

The distribution of COX-2 in the mouse kidney has not been characterized, therefore, in order to demonstrate that the constitutive presence of COX-2 in the M-1 cells is not an artifact of the M-1 cell line immortalization or culture conditions, COX-1, AQP2, and COX-2 immunoreactivity was localized on 3 serial 4 μm paraffin embedded transverse sections of mouse kidney (Figure 8). Figure 8A illustrates COX-1 immunoreactivity in a sub-population of cells in the cortex compatible with localization within CCDs (indicated by arrows). The aquaporin 2 (AQP2) water channel has been well characterized as the mediator of vasopressin induced principal cell apical membrane water permeability (Fushimi et al., 1993; Marples et al., 1995). We have used AQP2 as a marker of collecting ducts in order to clearly establish the identity of the COX isoform positive tubules. By labeling 3 serial sections with COX-1, AQP2, and COX-2 antibodies it was possible to confirm the localization of both COX-1 and COX-2 to the CCD. The AQP2 immunohistochemistry result displayed in Figure 8B was obtained on a serial section to that presented in Figure 8A for COX-1 (the arrows have been added to indicate AQP2 positive tubules). The glomerulus on the right hand side of the picture serves as a reference point between these 2 sections as well as the following section where COX-2 has been localized. Figure 8C clearly demonstrates that in addition to COX-1, COX-2 is also expressed in the CCD. Since only a sub-population of the CCD cells expressed the COX-1 and -2 isoforms, we sought to

determine the identity of these cells through double labeling for AQP2 with each of the COX isoforms. In Figures 8D and 8E we have localized both COX-1 and COX-2 respectively to the intercalated cells of the CCD. In each of these photos the COX isoform was detected with DAB as the chromogen (brown color, indicated by arrows) whereas the AQP2 was visualized using metal enhanced DAB as the chromogen (blue colored cells). Two important observations can be made from both 8D and 8E. Firstly, all CCD cells label for either AQP2 or the respective COX isoform. Secondly, no cells label for both AQP2 and a COX isoform. From these two observations we conclude that because there are no unlabeled CCD cells, both COX isoforms are expressed by the α - and β -intercalated cells. The lack of COX/AQP2 co-localization demonstrates the absence of expression of either COX isoform in the principal cells. As there is no AQP2 /COX isoform co-localization we can also conclude there is no significant cross-reactivity between the two detection systems which we have employed. The evidence which is provided in Figure 8 presents for the first time the finding that the intercalated cells of the CCD constitutively express both COX-1 and COX-2.

Figure 8. Immunohistochemical localization of COX-1 and COX-2 to the intercalated cells of the CCD. Pictures (A), (B), and (C) depict a series of three serial 4 μm mouse kidney sections. (A) This photo demonstrates COX-1 localization to a sub-population of CCD cells (500X). (B) The identity of these CCDs has been confirmed by detection of AQP2 in the same tubules on the next 4 μm section (500X). (C) The subsequent serial section has been labeled for COX-2, demonstrating the distribution of COX-2 in a sub-population of CCD cells (500X). (D) Double immunohistochemistry localization of COX-1 (brown, arrows) and AQP2 (blue) reveals the exclusive localization of COX-1 to intercalated cells (1250X). (E) As for COX-1, double immunohistochemical localization of COX-2 (brown, arrows) and AQP2 (blue) demonstrate that COX-2 is exclusively and consistently expressed in intercalated cells (1250X).

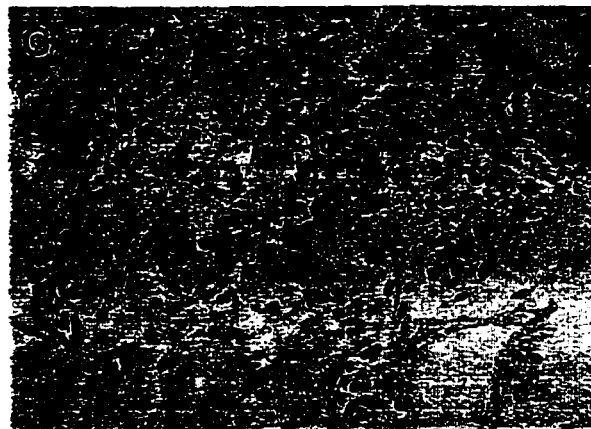
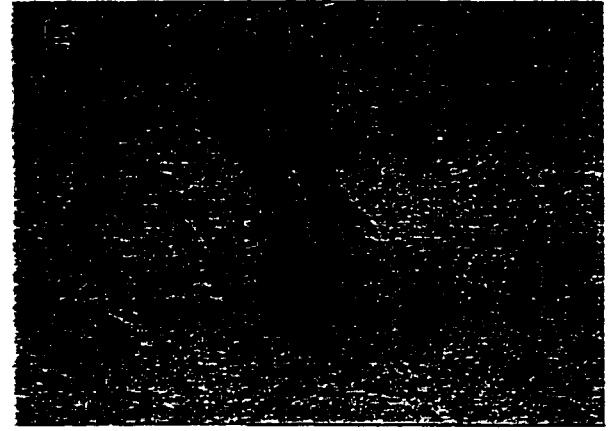
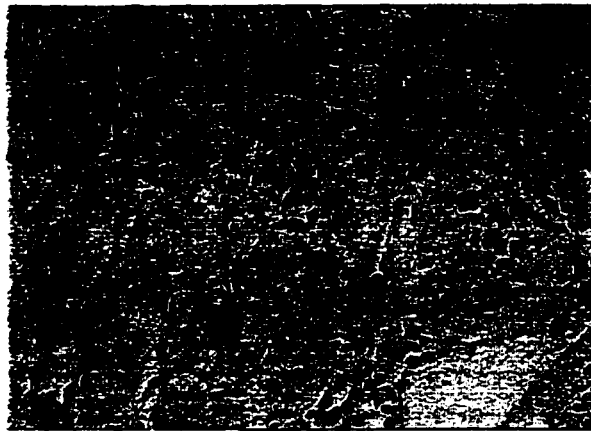
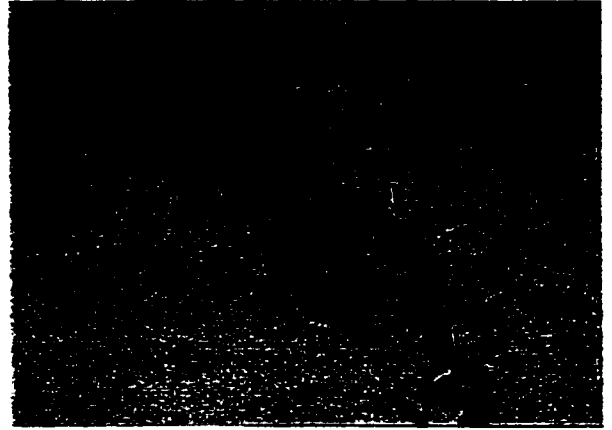
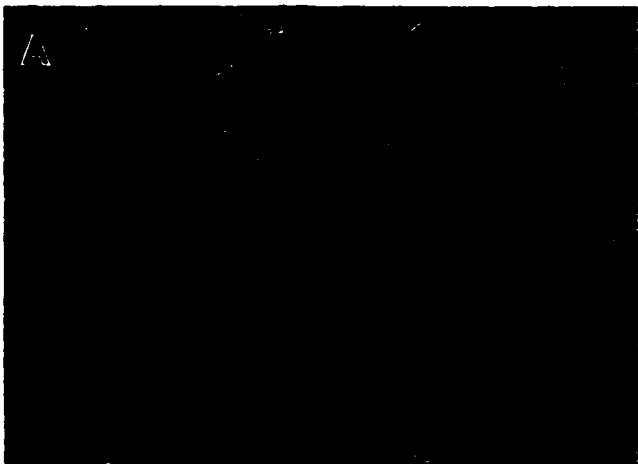


Figure 9. Immunohistochemical localization of COX-1 and COX-2 to the intercalated cells of the IMCD. Pictures **A)** and **B)** show localization of COX-1 and COX-2 respectively to the intercalated cells of the IMCD (Magnification 1250X). In both photos, the principal cells have been labeled for AQP2 (blue precipitate). Note the intense staining of the apical membrane of these cells. In part **A)**, the COX-1 distribution (brown precipitate) is limited to the intercalated cells. The same is true in part **B)** for COX-2 localization. These photos are representative of multiple observations that there are no unlabeled IMCD cells and in no cases does the COX isoform co-localize with AQP2.



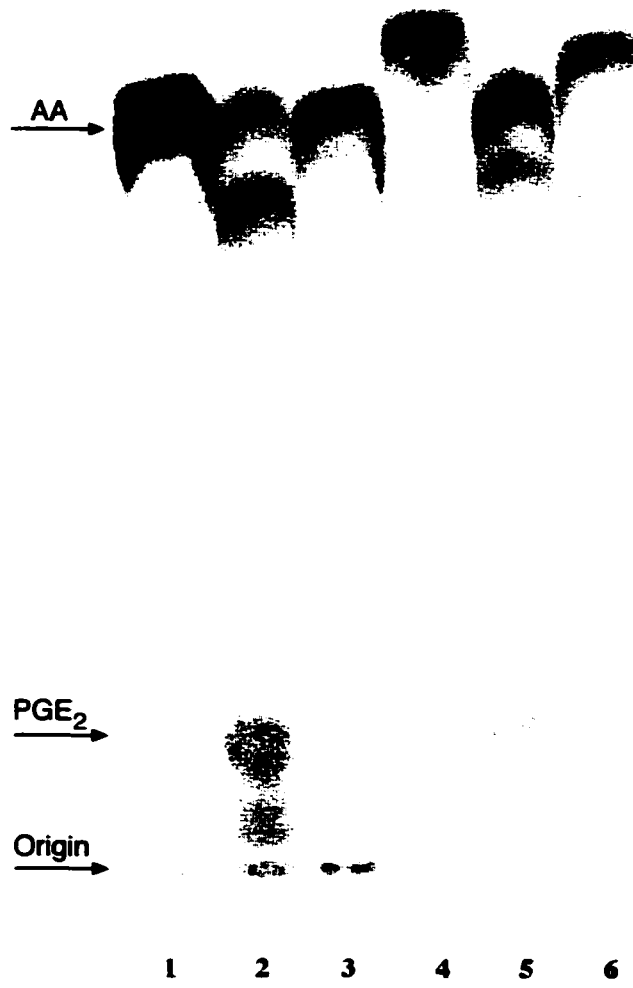
In Figure 9A, evidence is presented which localizes COX-1 to the intercalated cells of the IMCD (brown chromogen). In these cross sections of the IMCD it is possible to observe the predominantly apical localization of the AQP2 on the principal cells (blue/black chromogen). Figure 9B shows the corresponding localization of COX-2 to the IMCD intercalated cells.

Section 6.4: *Effect of NSAIDs on COX isoform activity in M-1 cells.* The immunofluorescence micrographs (Figures 6 and 7) demonstrate that both COX isoforms are expressed by M-1 cells, however, it is also relevant to determine how much each of these isoforms contributes to the total prostanoid pool produced by these cells. This was addressed by measuring the activity of each isoform as evaluated by the conversion of [1-¹⁴C]-AA to ¹⁴C-PGE₂ in the presence of the COX isoform specific inhibitors. The lipid fraction isolated from M-1 cells was separated by thin layer chromatography and the resulting signal was detected by autoradiography. The Bradford method was used to determine the protein content of each sample and the densitometric signal for each PGE₂ was normalized to the protein content for that sample. PGE₂ was the major prostanoid produced by this CCD cell line (Figure 10). Therefore, quantifying PGE₂ synthesis gives a measurement of COX enzyme activity. The localization of the PGE₂ band was performed by visualizing cold PGE₂ on lane 1 using iodine vapor. The R_f value calculated for PGE₂ was 0.14 ± 0.004. Indomethacin (10⁻⁵ M) was clearly effective at inhibiting both COX isoforms as it reduced

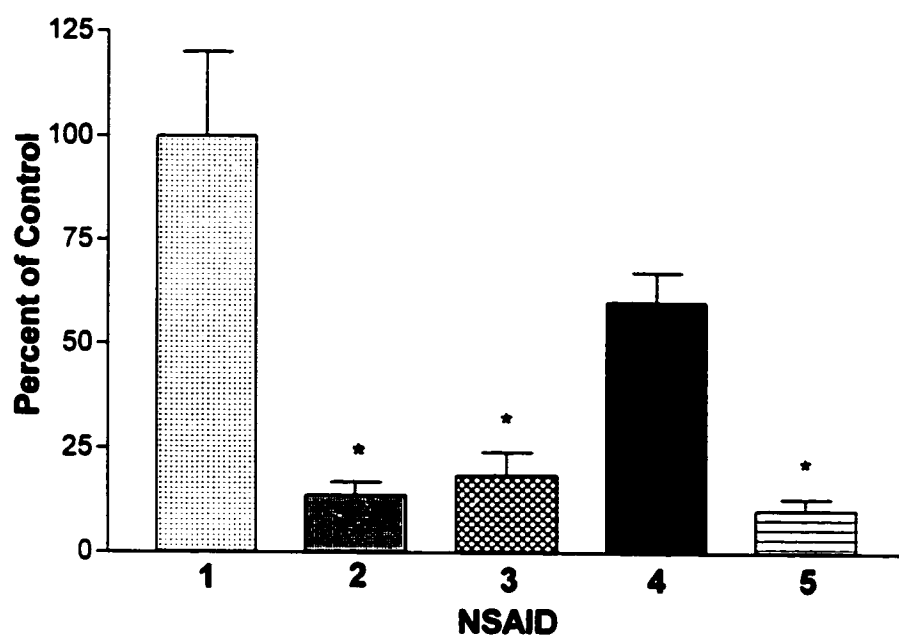
PGE₂ production to 13.7 ± 3.2 % of control ($p < 0.01$, $n = 3$). NS-398 effectively lowered PGE₂ synthesis to 18.5 ± 5.6 % of control ($p < 0.01$, $n = 3$) indicating that COX-2 is the major prostaglandin producing enzyme in these cells. Meanwhile, resveratrol treatment lowered M-1 cell COX activity to 60.0 ± 7.3 % of control ($p > 0.05$, $n = 3$). This reduction in PGE₂ synthesis reflects the inhibition of COX-1 activity by resveratrol. The combination of inhibition with both NS-398 and resveratrol yielded a reduction in COX enzyme activity to 10.3 ± 2.7 % of control ($p < 0.01$, $n = 3$). The combination of the two isoform specific inhibitors to inhibit PGE₂ production as much as does indomethacin indicates that each COX isoform is maximally inhibited by this concentration of the respective specific inhibitors.

Figure 10. Effect of NSAIDs on COX isoform activity in M-1 cells. **(A)** Representative autoradiograph of a TLC plate demonstrating radio-labeled PGE₂ production from ¹⁴C-arachidonic acid substrate following pre-incubation with respective NSAIDs. Lane 1: Cold PGE₂ standard and ¹⁴C-arachidonic acid (No M-1 cells) Lane 2: Control- No NSAID. Lane 3: Indomethacin (10⁻⁵ M). Lane 4: NS-398 (10⁻⁵ M). Lane 5: resveratrol (10⁻⁵ M). Lane 6: NS-398 (10⁻⁵ M) + resveratrol (10⁻⁵ M). **(B)** Bar graph depicting quantification of PGE₂ production by densitometry. (Bars 1-5 are representative of lanes 2-6 on the autoradiograph in part **(A)**). Results are means ± SEM (n = 3), * p < 0.01 vs control.

A

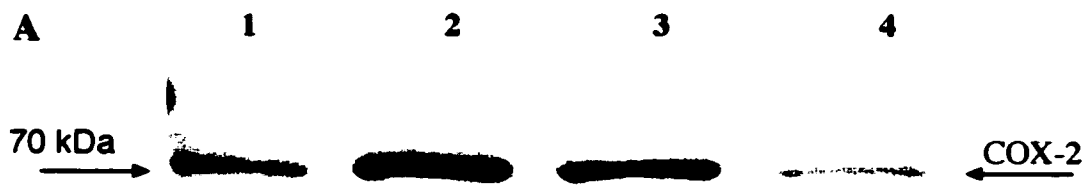


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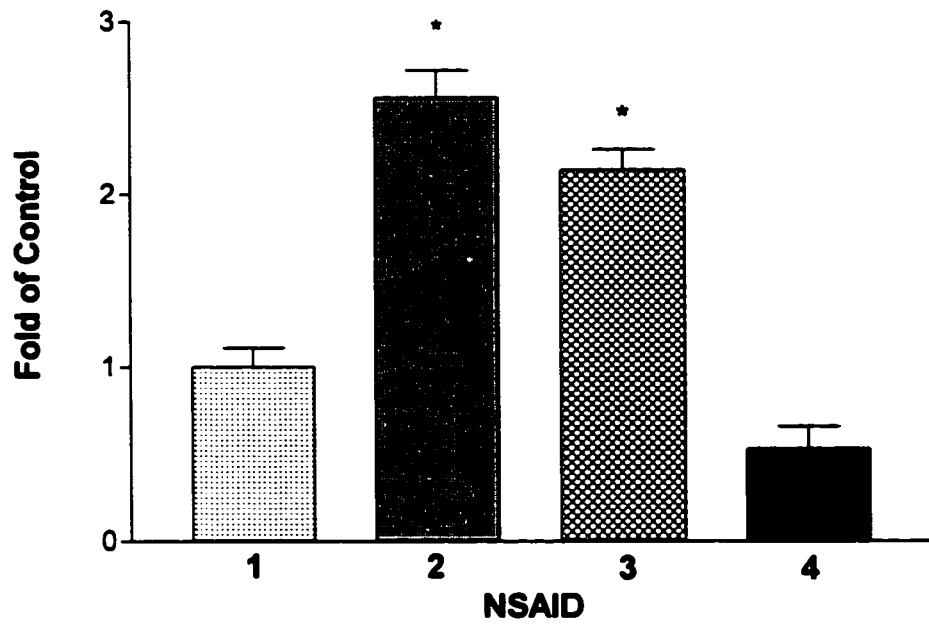


Section 6.5: *Inhibition of COX-2 activity leads to an increase in COX-2 enzyme levels.* M-1 cells constitutively express COX-1 and COX-2 protein (Figures 6 and 7). To assess the ability of these cells to compensate for reductions in prostaglandin synthesis, the M-1 cells were grown in the presence of non-specific (indomethacin 10^{-5} M) and COX isoform specific inhibitors (NS-398 10^{-5} M and resveratrol 10^{-5} M). Confluent cells were harvested and steady state levels of COX isoforms were evaluated by Western blotting using COX isoform specific antibodies as described in the Materials and Methods section. Following culture in the presence of COX-2 inhibition there was an up-regulation in the levels of COX-2 protein (Figure 11). The extent of this COX-2 up-regulation was 2.6 ± 0.2 and 2.1 ± 0.1 fold for indomethacin and NS-398 respectively ($p < 0.001$ versus control, $n = 4$). While there was a trend for a greater effect in the presence of indomethacin (inhibits both isoforms), the difference between the effect of indomethacin and NS-398 (specific COX-2 inhibitor) was not statistically significant. The COX-1 specific inhibitor resveratrol did not have a statistically significant effect on COX-2 content (0.5 ± 0.1 fold of control). The increase in COX-2 protein content associated with COX-2 inhibition was measured and found not to be significantly different at time points from 4 to 72 hrs of NSAID exposure. The quantification of COX-2 protein content as presented in Figure 11C was performed by scanning densitometry. In each lane, the COX-2 level was normalized to the β -actin signal (COX-2 signal / β -actin signal). COX-2 up-regulation was then expressed as a fold of the control values.

Figure 11. Inhibition of COX-2 activity leads to an increase in COX-2 enzyme steady state levels. (A) Representative autoradiograph of a Western blot detecting COX-2. **(B)** The corresponding bands for β -actin protein content were used to normalize for variations in loading the gels. Lane 1: Control- No NSAID. Lane 2: indomethacin (10^{-5} M). Lane 3: NS-398 (10^{-5} M). Lane 4: resveratrol (10^{-5} M). **(C)** Bar graph depicting quantification of COX-2 Western blot results by densitometry. Results are means \pm SEM (n = 4), *p < 0.001 vs control.



C

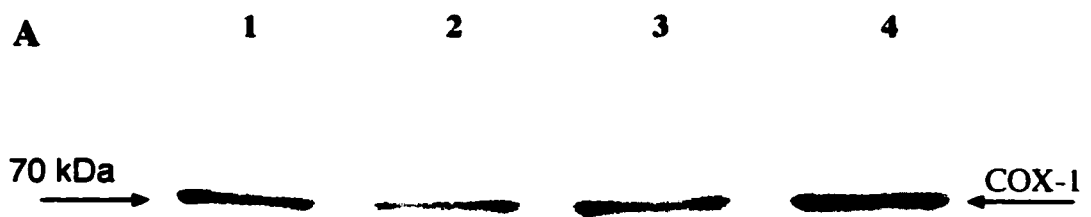


Section 6.6: *M-1 CCD cell COX-1 levels are not affected by COX isoform inhibition.*

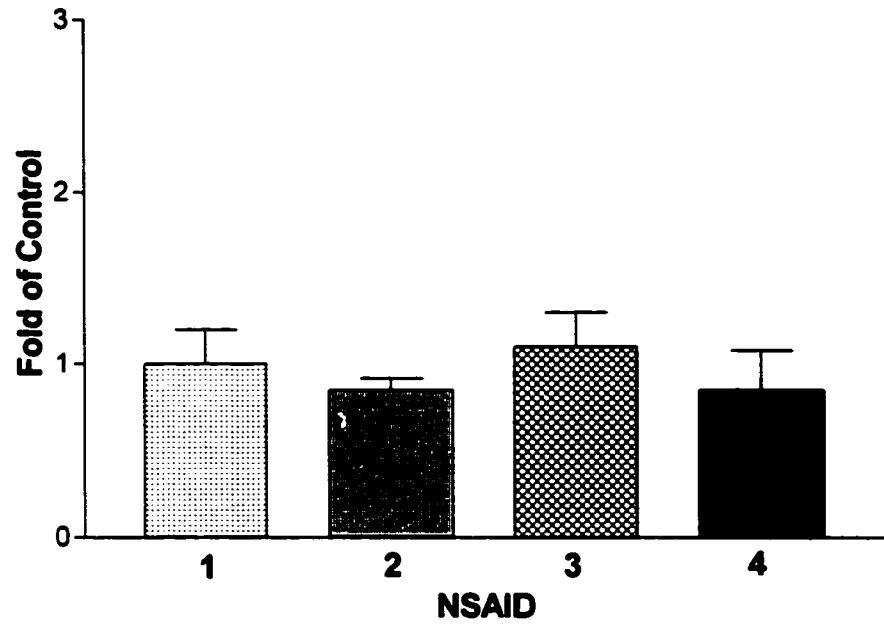
Western blotting was performed to determine if COX-1 is up-regulated in response to NSAID mediated inhibition of M-1 cell prostanoid synthesis. Following the same 72 hr NSAID treatment described for COX-2 Western blot analysis, it was found that COX-1 isoform levels are not affected by the presence of the NSAIDs studied. Figure 12 demonstrates that while M-1 cells do express COX-1 protein constitutively, the levels of this COX isoform are not regulated by culture in the presence of the NSAIDs tested. The values for COX-1 expressed as a fold of the control which were obtained for M-1 culture in the presence of indomethacin, NS-398 and resveratrol were 0.9 ± 0.1 , 1.1 ± 0.2 , and 0.9 ± 0.2 respectively ($p > 0.05$ versus control). This COX-1 antibody which was raised against a peptide corresponding to mouse COX-1 amino acids L274-A288 does not cross react with the COX-2 (Figure 5). This conclusion can be drawn from the lack of any effect of indomethacin or NS-398 on the band intensity for COX-1 as compared to the up-regulation of COX-2 by these same treatments (Figure 11). The lack of cross-reactivity of this antibody for COX-2 is further confirmed by the intracellular pattern of localization for COX-1 (Figures 6 and 7) which is clearly different from that obtained for the COX-2 protein.

Figure 12. M-1 CCD cell COX-1 levels are not affected by COX isoform inhibition.

(A) Representative autoradiograph of a Western blot detecting COX-1. Lane 1: Control- No NSAID. Lane 2: indomethacin (10^{-5} M). Lane 3: NS-398 (10^{-5} M). Lane 4: resveratrol (10^{-5} M). **(B)** The corresponding bands for β -actin protein content were used to control for variations in loading the gels. **(C)** Bar graph depicting quantification of COX-1 Western blot results by densitometry. Results are means \pm SEM (n = 4).



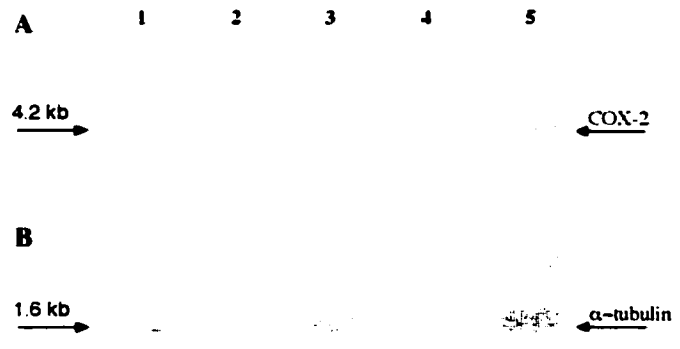
C



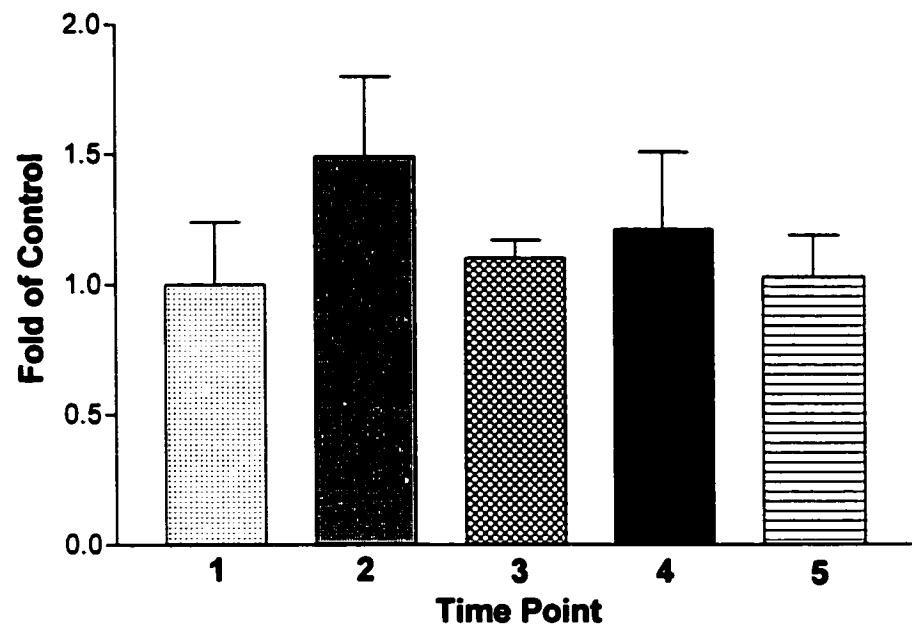
Section 6.7: *The increase in COX-2 protein content is not correlated with an increase in COX-2 mRNA levels.*

Given the preceding observations regarding the up-regulation of COX-2 protein by the inhibition of COX-2 activity, it was of interest to study the effect of NS-398 treatment on levels of COX-2 mRNA expression. To assess whether the increase in COX-2 protein is dependant on an increase in COX-2 mRNA levels, Northern blot analysis was performed. Using a ³²P-labeled 1.3 kb fragment of the murine COX-2 cDNA as a probe, it was possible to detect a 4.2 kb signal matching the known size of the COX-2 mRNA (Figure 13A). In addition to the time points depicted in Figure 13, COX-2 mRNA levels were measured 2 hrs after addition of NS-398 to the culture media. At no time point was there a significant increase in COX-2 mRNA expression compared to the control (n = 3, p > 0.05 versus control). In the histogram (Figure 13C), COX-2 mRNA levels have been normalized to the corresponding α -tubulin band (Figure 13B). α -Tubulin levels were measured in order to control for variability in the efficiency of isolating poly-A⁺ RNA from one sample to the next as well as to control for variability in sample loading. Although the differences in α -tubulin levels are considerable, these variations were random from one experiment to the next. This observation indicates that the variability is derived from the isolation/loading of the samples and not from an effect of NS-398 on the levels of α -tubulin mRNA expression.

Figure 13. The increase in COX-2 protein content is not correlated with an increase in COX-2 mRNA expression. This figure presents a time course for the regulation of M-1 cell COX-2 mRNA expression by the COX-2 specific inhibitor NS-398 (10^{-5} M). (A) Lane 1: Control - No NS-398 Lane 2: 8 hr. Lane 3: 18 hr. Lane 4: 30 hr. Lane 5: 54 hr. (B) Corresponding signals for α -tubulin mRNA detection. (C) Histogram depicting quantification of COX-2 mRNA signal by densitometry followed by normalizing with the α -tubulin signal. Results are means \pm SEM (n = 3).



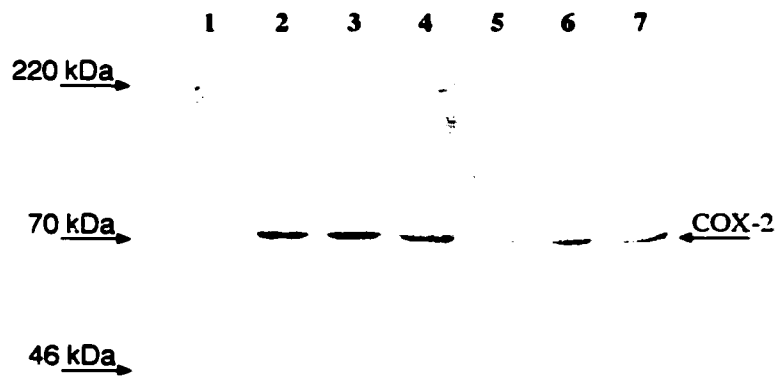
C



Section 6.8: *Co-incubation with the stable PGE₂ analogue, 16,16 - dimethyl -PGE₂, does not prevent the COX-2 up-regulating effect of NS 398.*

Having observed that NS-398 treatment of M-1 cells resulted in an increase in COX-2 protein levels in the absence of an increase in COX-2 mRNA we wished to further our understanding of the mechanism responsible for this phenomenon. In order to determine whether the increase in COX-2 protein is due to the loss of a PGE₂ response, M-1 cells were co-incubated with NS-398 (10⁻⁵ M) + 16,16-dimethyl-PGE₂ (16,16-dmPGE₂) (10⁻⁵ M). This incubation lasted 4 hrs. This was the minimum incubation time required in order to see the effects of NS-398 alone on COX-2 protein content. The 16,16-dmPGE₂ concentration of 10⁻⁵ M was chosen based on a published report wherein this concentration was used to overcome the effects of inhibition of PGE₂ synthesis by NS-398 in a porcine brain microvessel cell culture model (Li et al., 1997). In Figure 14, a Western blot of representative of the results of three separate experiments is presented. When the effect of NS-398 (lane 2) on COX-2 content is compared with the effect of NS-398 + 16,16-dmPGE₂ (lane 3), it is apparent that the intensity of both COX-2 bands is similar and is elevated compared to the vehicle treated control (lane 1). Not only does 16,16-dmPGE₂ not reverse the effect of NS-398, but on its own 16,16-dmPGE₂ causes an increase in COX-2 content (lane 4). Furthermore, sulprostone, an EP_{1,3} selective agonist (Dumont et al., 1998) is also able to up-regulate COX-2 protein content (lanes 5-7) suggesting that the effects of 16,16-dmPGE₂ on COX-2 content are mediated by one of these two EP receptors.

Figure 14. Co-incubation with the stable PGE₂ analogue, 16,16 - dimethyl - PGE₂, does not prevent the COX-2 up-regulating effect of NS 398. Lane 1: Cell lysate from untreated M-1 cells. Lane 2: Cell lysate from NS 398 (10⁻⁵ M) treated cells. Lane 3: Lysate from NS 398 (10⁻⁵ M) + 16,16 dmPGE₂ (10⁻⁵ M). Lane 4: Lysate from 16,16 dmPGE₂ (10⁻⁵ M) treated cells. Lanes 5-7: These cells received a 4 hr pre-treatment with sulprostone (10 nM, 100 nM and 10μM respectively). Each lane in this figure was loaded with 25 μg of protein from the respective cell lysates. The data in this figure is representative of three separate experiments.



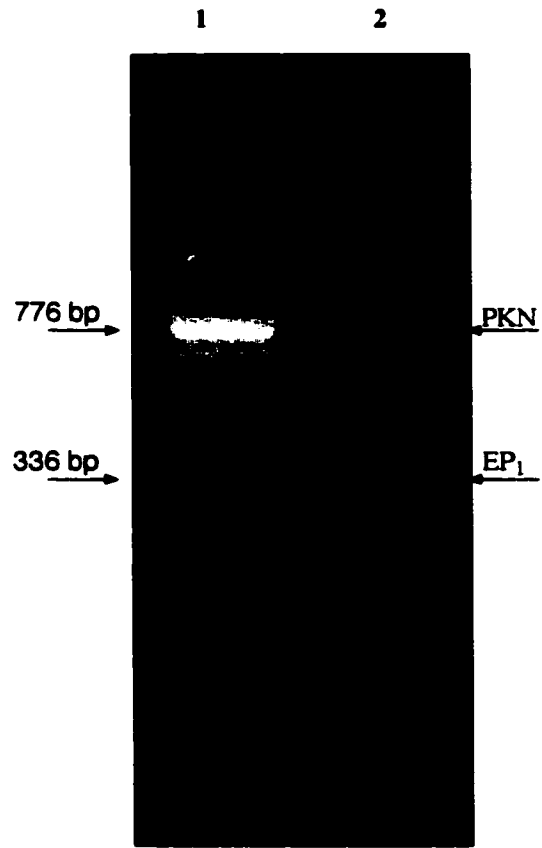
Section 6.9: *The M-1 cell line expresses both the EP₁ receptor mRNA as well as the protein kinase N (PKN) long transcript.*

In our hypothesis, we proposed that compensation for the effects of NSAIDs could occur at the level of the COX enzymes or via alterations in the levels of prostaglandin receptors. Having addressed the issue of the COX enzymes, our attention now shifts toward the PGE₂ receptors (EP receptors). In vivo, the EP₁ receptor is highly expressed in the collecting duct (Breyer et al., 1996a). We must confirm that under the culture conditions which we have chosen that the EP₁ is still expressed. When RT-PCR was performed with the EP₁ primers described in Table 1, the results consistently yielded a 776 bp band in addition to the expected EP₁ 336 bp band (Figure 15). The EP₁ receptor primers which we used spanned two exons of the EP₁ gene. The 776 bp band corresponded to the predicted product from the amplification of the EP₁ genomic DNA. However, when the reverse transcriptase was omitted, this band was absent. A literature search revealed that there exists a long transcript of PKN which possesses an exon which completely overlaps the whole EP₁ gene in a "tail to tail" manner (Batshake and Sundelin, 1996). The identity of each of these two bands (Figure 15A) was confirmed by purifying each band from the gel and subjecting them to restriction enzyme digestion with Pst I (Figure 15B). These digestions yielded a 207 bp band which was visible for EP₁ as well as 3 bands for PKN at 388, 202 and 186 bp. These bands all matched the predicted sizes for the Pst I digestion of the respective EP₁ and PKN PCR products. The 336 bp EP₁ fragment was predicted to give a 129 bp and a 207 bp band when digested with Pst I. The 129 bp band is not visible in Figure 15 most likely

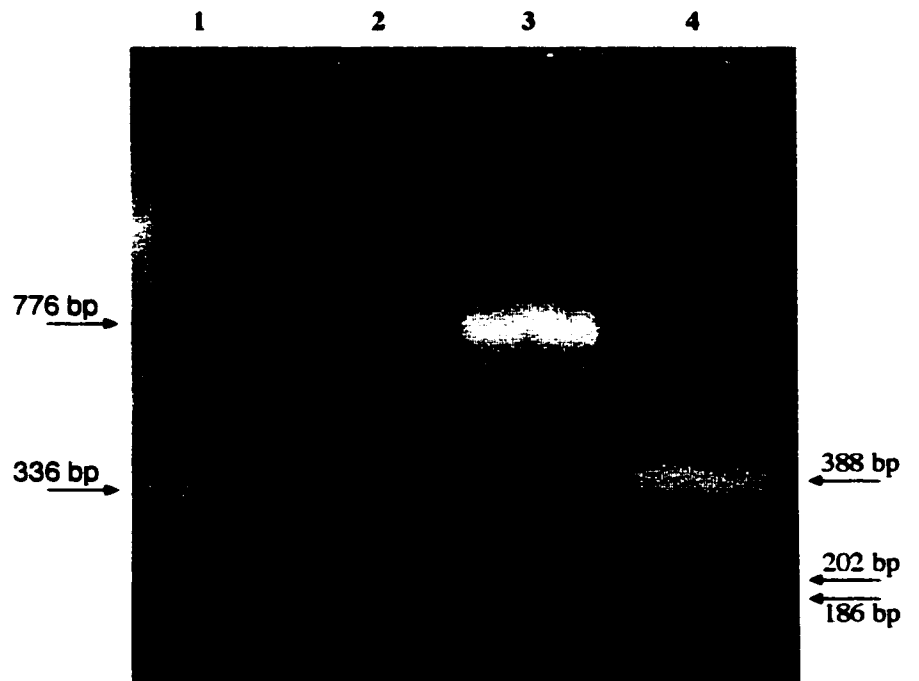
because it is a weak band and is obscured by the bromophenol blue from the loading buffer which runs at this size.

Figure 15. The M-1 cell line expresses both the EP₁ receptor and protein kinase N as assessed by RT-PCR. (A) Lane 1: RT-PCR with EP₁ specific primers amplifies major bands at 776 bp and 336 bp (35 cycles of PCR). Lane 2: These bands are absent when the reverse transcriptase enzyme is omitted. (B) Lane 1: The 336 bp band (EP₁) presented in (A) was excised, gel purified and re-electrophoresed. Lane 2: Pst I digestion of the 336 bp EP₁ band to yield a 207 bp fragment. Lane 3: The 776 bp band (PKN) presented in (A) was excised, gel purified and re-electrophoresed. Lane 4: Pst I digestion of the 776 bp PKN band yield fragments of 388, 202, and 186 bp. The samples presented in this figure were run on 2 % agarose gels containing ethidium bromide.

A



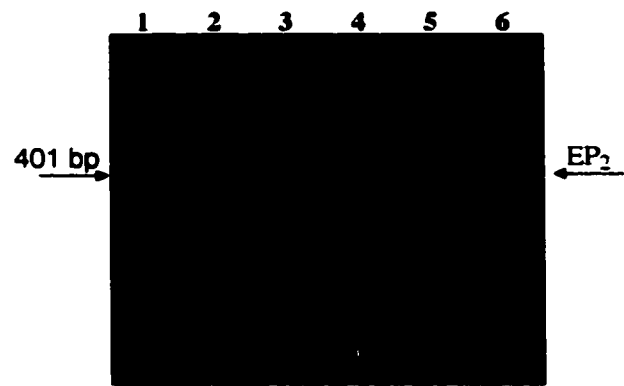
B



Section 6.10: *The M-1 CCD cell line does not constitutively express the EP₂ receptor mRNA nor does it become induced upon treatment with NS-398.*

There have not been any reports of EP₂ receptor expression in the collecting duct (Breyer et al., 1996a). However, it was necessary to ensure that the M-1 cell immortalization and/or cell culture conditions did not induce the expression of this receptor. In Figure 16, we were able to detect the 401 bp EP₂ receptor PCR product in the spleen positive control sample (Lane 1) but not in any of the samples from M-1 cells (Lanes 3 - 6). Treatment of the M-1 cells with NS-398 did not result in an induction of the EP₂ receptor mRNA.

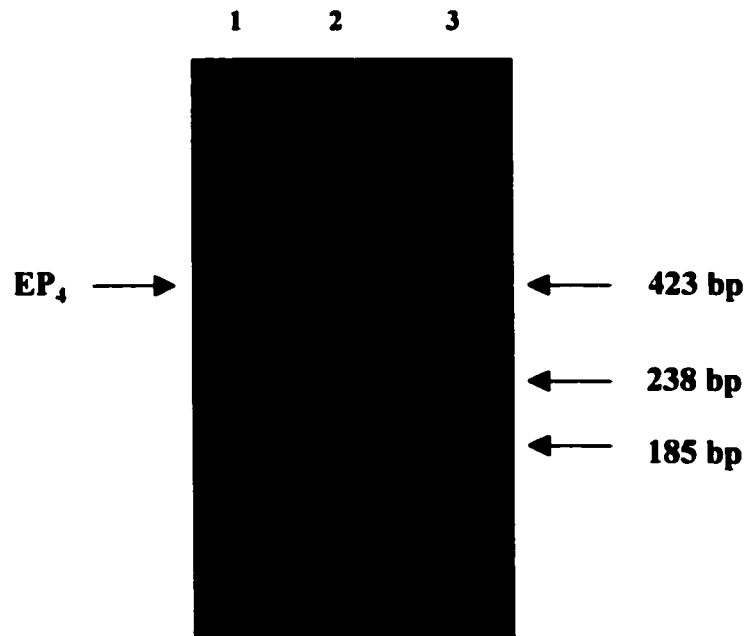
Figure 16. The M-1 CCD cell line does not constitutively express the EP₂ receptor mRNA nor does it become induced upon treatment with NS-398. Lane 1: A 401 bp product corresponding to the EP₂ receptor mRNA can be amplified from mouse spleen RNA (35 cycles of PCR). Lane 2: The EP₂ receptor signal from the spleen is not detectable when the reverse transcriptase enzyme is omitted. Lane 3: Vehicle treated M-1 cells. Lane 4: 1.5 hrs NS-398 (10⁻⁵ M). Lane 5: 6.0 hrs NS-398 (10⁻⁵ M). Lane 6: 21 hrs NS-398 (10⁻⁵ M). These samples were separated on a 2 % agarose gel containing ethidium bromide.



Section 6.11: *The M-1 cell line expresses the EP₄ receptor mRNA as determined by RT-PCR.*

The cortical collecting duct expresses the EP₄ receptor in both the apical and basolateral membranes (Sakairi et al., 1995). No antibodies are commercially available to confirm this localization in the M-1 cell line, therefore, RT-PCR was chosen as a strategy to confirm that the EP₄ mRNA is expressed by the M-1 cells in culture. In Figure 17, we demonstrate that we amplify a 423 bp PCR product corresponding to the predicted size for the EP₄ receptor our primers were designed to amplify (Lane 2). The lack of any signal when the reverse transcriptase enzyme is omitted ensures that the EP₄ receptor signal is not due to amplification of genomic DNA (Figure 17, Lane 1). Furthermore, a restriction enzyme digest (Figure 17, Lane 3) of the 423 bp EP₄ product with Hinf I yields the predicted bands of 238 and 185 bp.

Figure 17. The M-1 cell line expresses the EP₄ receptor mRNA as determined by RT-PCR. Lane 1: No EP₄ product is observed when the reverse transcriptase enzyme is omitted. Lane 2: This 423 bp product is the expected size for the EP₄ fragment we have chosen to amplify. Lane 3: Hinf I digestion of the EP₄ PCR product yields the expected size fragments of 238 bp and 185 bp. The samples presented in this figure were run on a 2 % agarose gel containing ethidium bromide



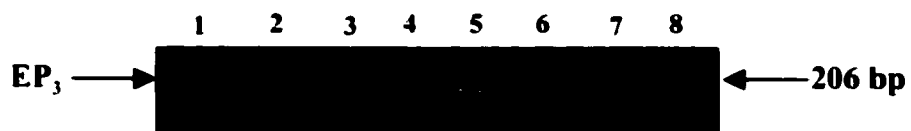
Section 6.12: Inhibition of COX-2 activity with NS-398 results in an up-regulation of the EP₃ receptor mRNA.

Up-regulation of PGE₂ receptor expression represents a putative mechanism for compensation for the inhibition of prostaglandin synthesis. The EP₃ receptor plays an important role in the collecting duct serving to attenuate the anti-diuretic effects of AVP (Hébert et al., 1993). To evaluate the effect of COX-2 inhibition on the expression of the EP₃ receptor, M-1 cells were incubated in the presence of NS-398 prior to harvesting and isolation of total RNA. In order to yield meaningful results about the relative abundance of the EP₃ receptor mRNA, it was absolutely necessary that PCR conditions were optimized to ensure that alterations in the quantity of starting EP₃ receptor mRNA would be reflected in the amount of product generated by the PCR amplification. Figure 18A lanes 1 to 4 demonstrate that under the conditions we have employed, there is a linear increase in signal intensity which is correlated with increasing the quantity of starting RNA from 100 ng to 200 ng to 400 ng to 600 ng. Lanes 5 to 7 are representative of results of 5 separate experiments in that they show an increase in EP₃ receptor mRNA compared to vehicle treated control in cells treated with 10⁻⁵M NS-398 for 1.5 hrs, 4.0 hrs and 6.0 hrs respectively. As 200 ng of starting RNA was used in lanes 5 to 7 it is appropriate to

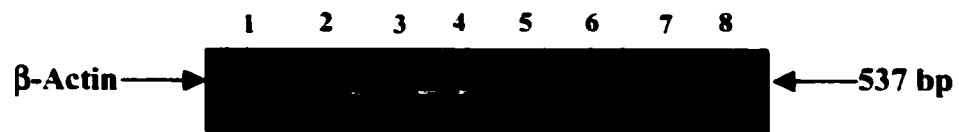
compare their signal intensity to lane 2 where 200 ng of vehicle treated control RNA was amplified. Figure 18B lanes 5 to 7 demonstrate that the treatment with the M-1 cells with NS-398 did not induce an increase in the level of β -actin mRNA. Figure 18C presents a time course for the up-regulation of EP₃ expression. The effect plateaus at 3 fold greater than the vehicle treated control after 4 hrs of incubation in the presence of NS-398. The EP₃ sequence which we have amplified does not span any introns. Therefore, it is important to note that in Figure 18A lane 8, there is no EP₃ band at 206 bp when the reverse transcriptase enzyme is omitted.

Figure 18: Inhibition of COX-2 activity with NS-398 results in an up-regulation of the EP₃ receptor mRNA. (A) Amplification of EP₃ mRNA. Lane 1: 100 ng starting RNA from vehicle treated M-1 cells. Lane 2: 200 ng starting RNA. Lane 3: 400 ng starting RNA. Lane 4: 600 ng starting RNA. Lane 5: 200 ng of starting RNA from M-1 cells treated with NS-398 (10⁻⁵ M) for 1.5 hrs. Lane 6: 4.0 hrs NS-398, 200 ng starting RNA. Lane 7: 6.0 hrs NS-398, 200 ng starting RNA. Lane 8: No EP₃ signal is visible when the reverse transcriptase enzyme is omitted. (B) Amplification of β-Actin mRNA from the same samples amplified for the assessment of EP₃ expression. Lane 1: 100 ng starting RNA from vehicle treated M-1 cells. Lane 2: 200 ng starting RNA. Lane 3: 400 ng starting RNA. Lane 4: 600 ng starting RNA. Lane 5: 200 ng of starting RNA from M-1 cells treated with NS-398 (10⁻⁵ M) for 1.5 hrs. Lane 6: 4.0 hrs NS-398, 200 ng starting RNA. Lane 7: 6.0 hrs NS-398, 200 ng starting RNA. Lane 8: No β-actin signal is visible when the reverse transcriptase enzyme is omitted. (C) Line graph depicting Storm PhosphoImager quantification of the time course of the effect of NS-398 on EP₃ receptor mRNA expression. Results are means ± SEM (n = 5), *p < 0.05 vs control. The samples presented in this figure were preincubated with Vistra Green and electrophoresed on 2 % agarose gels.

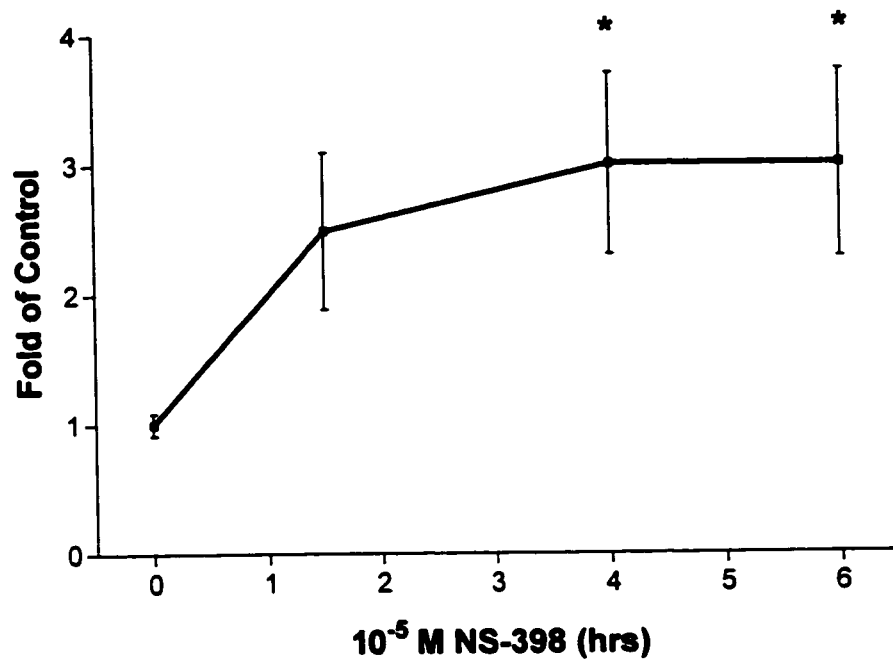
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B



C



Section 7: Discussion

Section 7.1: *Renal Localization of the COX isoforms.*

In 1978, Smith and Bell localized COX immunoreactivity to the CCD in the rabbit, cow, guinea pig, rat, and sheep (Smith and Bell, 1978). This experiment was performed before the discovery of COX-2 in 1991 (Kujubu et al., 1991) and the anti-COX antibody was raised against the whole COX protein which was isolated from sheep seminal vesicles (Smith and Bell, 1978). Given the shared 60% amino acid identity (Smith, 1992) between the two COX isoforms it is uncertain as to the selectivity of this antibody for the two COX isoforms. However, COX-2 is known to be constitutively expressed in the macula densa (Harris et al., 1998) and this antibody employed by Smith and Bell did not detect COX in the macula densa which implies that it was more specific for COX-1 than COX-2. Further studies which measured PGE₂ synthesis by the micro-dissected CCD demonstrated the robust prostaglandin synthesizing ability of the CCD (Bonvalet et al., 1987). The presence of the COX-1 isoform in the rat and human CCD has been confirmed with isoform specific antibodies (Harris et al., 1994; Komhoff et al., 1997). However, these two studies diverge with respect to the renal localization of COX-2, as COX-2 was detected in the rat macula densa but not in the human macula densa. The association between NSAID use and

hyporeninemic hypoaldosteronism is evidence that prostaglandins can play a role in renin release in humans (Harris et al., 1998; Murray and Brater, 1993). Therefore, it is quite possible that the lack of macula densa COX-2 localization to the human macula densa reflects a limitation in the methodology employed by Komhoff et al (1997). The lethal post-natal development anomalies in the COX-2 knockout mice has raised interest in the role played by COX-2 in normal renal development (Morham et al., 1995; Zhang et al., 1997). Furthermore, due to the fact that the kidneys did not develop properly, the COX-2 knockout study did not yield any information concerning the role of COX-2 in the adult kidney.

Our present study has demonstrated for the first time that in addition to COX-1, COX-2 is also expressed in the CCD. The demonstration of constitutively expressed COX-1 and COX-2 immunoreactivity in only a portion of the cells of the native CCD is not unlike the situation in the macula densa where only a small sub-population of the cells are COX-2 positive (Harris et al., 1994). However, the macula densa is composed of a single cell type.

Meanwhile, since the CCD is composed of principal, α -, and β -intercalated cell types we sought to determine whether or not the sub-population of COX isoform positive cells corresponded to a specific cell type. The AQP2/COX isoform double labeling evidence which we have presented reveals that in the native CCD under normal conditions COX-1 and COX-2 are only expressed in the intercalated cells. Full comprehension of the role of COX-1 and COX-2 in the CCD will require an understanding of the localization and regulation of the PGE₂ receptors in this segment of the nephron.

Section 7.2: *Localization of the COX isoforms within the M-1 CCD cell line.*

The M-1 cell line has been well characterized by Stoos et al (Stoos et al., 1991). These cells exhibit an amiloride sensitive Na⁺ transport and K⁺ secretion characteristic of principal cells as well as electrogenic H⁺ secretion characteristic of α -intercalated cells. The COX isoform signal in the M-1 CCD cell line (Figure 6) matches the bright nuclear envelope/endoplasmic reticulum pattern of fluorescence that is characteristic of the intracellular localization of this enzyme as reported by Morita et al (Morita et al., 1995). Each of the COX isoforms is expressed in all CCD cell types as represented by the M-1 CCD cell line (Figure 6 and 7). However, the intracellular COX-2 localization differs between Figure 6 and Figure 7. The experimental conditions under which these results were obtained differed with respect to the detergent which was used. The results in Figure 6 were obtained when the cells were permeabilized with 0.2 % saponin as per the methodology of Morita et al (Morita et al., 1995). In Figure 7, the cells have been permeabilized with 0.1 % Triton X-100. The lack of a strong intranuclear COX-2 signal under these conditions could be due to poor permeabilization of the nuclear membrane by saponin. The permeabilization of a membrane by saponin is dependent on the cholesterol content of the membrane (Willingham and Pastan, 1985). The intranuclear COX-2 signal in Figure 7 can be abolished by pre-incubation of the COX-2 antibody with the immunizing peptide. This ensures that the COX-2 signal is due to a specific antibody-antigen interaction and is not an artifact of the experimental conditions. Certainly, the COX-1 immunolocalization does not show a nuclear

signal in Figure 7. It is interesting that although both COX isoforms exhibit perinuclear localization, the precise pattern of fluorescence is not identical. In the saponin permeabilized cells (Figure 6), while the COX-2 signal demonstrates a crisp perinuclear ring, the COX-1 signal consists of punctate areas of bright signal interspersed with areas of weaker signal. Confocal microscopy might offer improved resolution for the quantification of the different intracellular localizations of the COX enzymes. A confocal image in the x-z plane demonstrating the co-localization of COX-2 with a nuclear antigen would offer confirmation of the intranuclear COX-2 localization. This type of methodology was employed to demonstrate a shift of COX-2 localization towards the nucleus in response to EGF stimulation in human colon cancer cells (Coffey et al., 1997). A mechanism or functional significance for this translocation has never been described. There is a report wherein via immunoelectron microscopy both COX-1 and COX-2 were found at the same levels in the inner membrane of the endoplasmic reticulum as well as the inner and outer membranes of the nuclear envelope (Spencer et al., 1998). This localization of the COX isoforms was performed in human umbilical vein endothelial cells, NIH 3T3 murine fibroblasts, and human monocytes but not in epithelial cells (Spencer et al., 1998). There is no known interaction between either COX isoform and another protein which could explain the variations in intracellular COX isoform localization from one cell type to the next. One published report is available wherein a yeast two hybrid assay with COX isoforms as bait,

observed protein-protein interaction between COX-1 and COX-2 with nucleobindin (Ballif et al., 1996). Given that nucleobindin binds both COX isoforms it is not a candidate for the mediation of their differential intracellular localization. Additionally, nucleobindin is predominantly secreted if not retained by binding to COX. When COS-1 cells were transfected with nucleobindin and either COX isoform, the amount of nucleobindin secretion was inversely proportional to the level of COX isoform expression. Nucleobindin has recently been renamed CALNUC due to its characterization as being the major calcium binding protein in the Golgi apparatus (Lin et al., 1999). It is unknown as to whether this nucleobindin retention by interaction with COX is functionally relevant in diseases such as systemic lupus erythematosus where serum nucleobindin levels are elevated (Kanai et al., 1993).

Section 7.3: *Limitations of COX activity and NSAID inhibition assays.*

We have measured the ability of intact M-1 cells to synthesize prostanoids from exogenous AA and have found COX-2 to be the major contributor towards M-1 cell PGE₂ synthesis. The comparison of studies of COX activity and inhibition in different cell types is complicated by several factors (Chulada and Langenbach, 1997). These factors include: the use of exogenous versus endogenous AA as substrate, purified enzyme versus broken cells versus intact cells as a source of COX enzymes, instantaneous versus time-dependant inhibition, and the study of endogenously expressed COX versus vector driven expression systems. The purified COX enzymes have near identical K_m values for arachidonic acid

(Laneuville et al., 1994). However, in cell culture assays, COX-1 shows a preferential ability to use exogenously supplied arachidonic acid (Murakami et al., 1999). Meanwhile, COX-2 is more effective than COX-1 at using endogenous arachidonic acid (Murakami et al., 1999). Moreover, COX-2 expression but not COX-1 expression is associated with increased cPLA₂ activity (Murakami et al., 1999). The results of this study suggest that not only does COX-2 depend upon cPLA₂ for arachidonic acid, but COX-2 somehow facilitates the delivery of this substrate. Our use of exogenous arachidonic acid circumvents cellular mechanisms controlling arachidonic acid release. Regardless of this limitation, the observations we make concerning COX isoform activity within the system we have employed are significant. The novel finding in our results is that both constitutively expressed COX isoforms in the CCD are functional and make contributions to PGE₂ synthesis by this nephron segment. While the Radio-TLC technique is excellent for the identification of the prostanoids produced by the M-1 cells, radioimmunoassay to detect PGE₂ produced from endogenous arachidonic acid would be more sensitive for the assessment of the contributions made by each COX isoform.

One problem with studies measuring the potency of various NSAIDs in vitro is that the conditions which are used for these assays are not representative of all the variable conditions found inside the average cell (Kulmacz, 1998; Swinney et al., 1997). Low concentrations of arachidonic acid (< 0.5 μM) as well as low concentrations of competitive inhibitors show positive allosteric regulation of COX-1 (Swinney et al., 1997). Thus arachidonic acid and NSAID binding to the COX-1 homodimer is facilitated by previous

binding. As arachidonic acid concentration decreases so will the affinity of competitive inhibitors for COX-1. COX-2 is not subject to this allosteric control and, therefore, the binding affinity of COX-2 inhibitors will not be affected by varying arachidonic acid concentrations. However, the inhibitory effects of NSAIDs on COX-2 are diminished as the concentration of arachidonic acid increases (Hamilton et al., 1999). This implies the concentration of a drug required to inhibit COX-2 at a site of inflammation will be higher than elsewhere where there is less free arachidonic acid available to act as a competitor. Therefore, there may be a preference for the inhibition of constitutively expressed COX-2 over inflammatory COX-2 at sub-maximal concentrations of COX-2 inhibitors. Another difference between COX-1 and COX-2 which may not be detected under standard in vitro assay conditions is that COX-2 activity requires approximately 10 fold lower intracellular hydroperoxide concentrations than does COX-1 (Lu et al., 1999).

Section 7.4: *Why do all cells of the M-1 cell line constitutively express COX-2?*

The lack of localization of COX-2 to the principal cells of the native CCD is at odds with our observation that COX-2 is expressed in all of the cell types represented by the M-1 cell line. While the native collecting duct only expresses COX isoforms in the intercalated cells, all M-1 cells express both of these isoforms. There are several possible explanations for this observation. It is possible that within the intact collecting duct that the principal cell do express COX-2 albeit at a level which is below the threshold of detection afforded by our immunohistochemical protocol. It is also possible that the constitutive COX-2 expression

by all of the M-1 cells is due either to the culture conditions or due to the immortalization of the M-1 cells. The M-1 cells were grown in the presence of 5% FBS which could stimulate COX-2 expression (DeWitt and Meade, 1993). However, when we starved the M-1 cells of serum for 24 hrs, Western blotting did not reveal a decrease in the content of M-1 COX-2 levels. COX-2 expression has been found to be inversely related to levels of expression of the tumor suppressor p53 (Subbaramaiah et al., 1999). p53 inhibits COX-2 transcription by binding to the TATA box of the COX-2 promoter. The M-1 cell line is derived from the collecting duct of an SV40 large T antigen transgenic mouse (Stoos et al., 1991). The T antigen interacts with and inactivates p53 (Ludlow, 1993). Therefore, COX-2 expression may be enhanced in the M-1 cell line due to a decrease in p53 mediated COX-2 suppression. Resveratrol is capable of arresting cells at the S/G2 transition (Hsieh et al., 1999; Ragione et al., 1998) and to inhibit COX-2 transcription (Subbaramaiah et al., 1998). Furthermore, the inhibition of COX-2 expression by aspirin and salicylate may be cell cycle dependant (Xu et al., 1999). Overexpression of COX-2 confers resistance to apoptosis (Tsuji and DuBois, 1995). It is uncertain as to how important the COX-2 expression by the M-1 cells is to their immortality and their ability to proliferate.

Section 7.5: *What is the mechanism by which NS-398 and indomethacin up-regulate COX-2 protein content?*

Our finding that COX-2 inhibition results in an up-regulation in COX-2 content is the opposite of what was observed with respect to compensatory up-regulation of the remaining

COX isoform in cultured fibroblast cells from COX null mice (Kirtikara et al., 1998). In addition to the compensatory COX isoform up-regulation, there is also cPLA₂ protein overexpression in these cells. We did not investigate whether or not COX inhibition affects cPLA₂ expression and/or activity in the M-1 cell line. As opposed to the compensatory up-regulation of the remaining isoform in COX null cells, our study found that COX-2 inhibition was necessary for stimulation of COX-2 up-regulation. Although not statistically significant, the trend is for M-1 cell COX-2 content to decrease in response to resveratrol treatment (COX-1 inhibition). The comparison between these two studies suggests that interactions between COX isoform expression and prostaglandin production is cell type specific. This idea is supported by the observation in COX-1^{-/-} mice that there is no compensatory COX-2 up-regulation in the stomach (Langenbach et al., 1995). In retrospect, resveratrol was not the ideal COX-1 inhibitor to use in our studies. We chose this inhibitor based on its preferential selectivity for inhibiting COX-1 activity (Jang et al., 1997). However, it has since been reported that resveratrol can inhibit COX-2 transcription in epithelial cells (Subbaramaiah et al., 1998). There is a trend towards decreasing COX-2 content in the presence of resveratrol in our experiments on the M-1 cells. Resveratrol lowers the rate of COX-2 transcription via the inhibition of phorbol ester dependant AP-1 response element activation of the COX-2 promoter CRE (cAMP response element) (Subbaramaiah et al., 1998). Therefore, the resveratrol mediated decrease in constitutive COX-2 content in the M-1 cells implies that this basal COX-2 expression is in part PKC dependant. The ideal COX-1 inhibitor for our study would be a time dependant competitive

inhibitor just like indomethacin and NS-398. However, such an inhibitor is not commercially available.

The NSAID induced up-regulation of COX-2 protein was not accompanied by any effect on COX-2 mRNA expression. This is in sharp contrast to the effect of inflammatory agents such as cytokines and phorbol esters which stimulate increased COX-2 protein and mRNA expression (Inoue et al., 1995). For example, interleukin-1 was shown to both stimulate COX-2 mRNA transcription and prolong the half-life of this message in human umbilical vein endothelial cells (HUVEC) and the human ECV324 cell line (Ristimaki et al., 1994). The mechanism responsible for the COX-2 regulation which we have documented remains unknown but is most likely post-transcriptional. Evidence is available which allows for speculation on this subject. With the murine MC3T3-E1 osteoblast cell line, NS-398 was able to block cytokine induced up-regulation of COX-2 protein (Murakami et al., 1997). This blockade was reversed by the addition of exogenous PGE₂. The PGE₂ mediated upregulation of COX-2 in the MC3t3-E1 osteoblast cell line is mediated via the EP₁ receptor (Suda et al., 1998). The M-1 cell line expresses COX-2 constitutively, thus it is possible to study COX-2 regulation without any confounding effects induced by cytokine stimulation. Unlike the previously mentioned study where NS-398 blocked cytokine stimulated COX-2 expression, in our study the NS-398 stimulates COX-2 up-regulation and is associated with an inhibition of PGE₂ synthesis. It remains unknown as to whether or not the NS-398 mediated COX-2 up-regulation is due to a decrease in PGE₂ synthesis or due to stabilization of the COX-2 protein by NS-398. We present evidence that the stable PGE₂

analog: 16,16-dmPGE₂ (10⁻⁵ M) cannot reverse the effect of NS-398 on COX-2 protein levels. This suggests that the effect of NS-398 may be through the stabilization of the COX-2 protein. However, indomethacin binds to and inhibits both COX isoforms (Laneuville et al., 1994) and yet it only induces the up-regulation of the COX-2 protein. In so much as indomethacin (10 μM) failed to up-regulate the COX-1 protein in our experiments, its ability to protect this protein from protease digestion has been documented (Kulmacz, 1989).

In order to separate the issues of NSAID mediated COX enzyme stabilization from inhibition of prostaglandin synthesis, different NSAIDs could be used. For example, VSA and APHS inhibit COX-1 and COX-2 respectively by acetylating a serine in the active site of the enzyme (Bhattacharyya et al., 1995; Kalgutkar et al., 1998). Aspirin which acetylates both COX isoforms (Bhattacharyya et al., 1995) does not confer resistance to the proteolysis of COX-1 (Kulmacz, 1989). Therefore, if the effect of NS-398 is due to stabilization of the COX-2 protein, then APHS should not have an effect on COX-2 protein levels. Meanwhile, if the effect of NS-398 is mediated by the inhibition of prostaglandin synthesis, then APHS should be equally effective at mediating this effect.

One could theorize that the inability 16,16-dmPGE₂ to reverse the effects of NS-398 is due to the fact that although PGE₂ is the major prostanoid produced by the M-1 cells there could be another prostanoid which is responsible for the effects of NS-398 on COX-2 protein levels. The radio-TLC results do reveal a faint band below that of PGE₂ which is could represent prostacyclin or 6-keto PGF_{1α} which is a metabolite of prostacyclin. However, unpublished work from our lab has failed to detect the IP receptor in the M-1 cells

by RT-PCR. Therefore, it is unlikely we are mistaken in trying to reverse the effects of NS-398 with 16,16-dmPGE₂. To be more confident in this conclusion, it would be necessary to test as to whether a stable prostacyclin analog such as iloprost could block the effect of NS-398 on COX-2 content. However, prostacyclin analogs have been shown to act as PPAR α agonists at nanomolar concentrations, therefore, incubating cells with prostacyclin analogs could have confounding effects via PPAR α activation (Hertz et al., 1996). Although 16,16-dmPGE₂ did not reverse the effect of NS-398 on COX-2 content, on it's own, 16,16-dmPGE₂ actually increased M-1 COX-2 protein content after a 4 hr incubation. This observation is compatible with the observations of Suda et al (1998) which found that EP₁ stimulation can result in increases in COX-2 expression in the MC3T3-E1 osteoblast cell line (Suda et al., 1998). We demonstrate that the M-1 cells do express the EP₁ receptor as assessed by RT-PCR. Sulprostone which is predominantly an EP₃ specific analog (Hébert et al., 1993) but which also has some EP₁ specificity (at higher concentrations) is also able to stimulate an increase in COX-2 content. The minor effect of sulprostone at 10 nM compared to it's effects at 100 nM and 1 μ M suggest that the effects of sulprostone are more likely to be mediated by the EP₁ receptor. To confirm this, sulprostone + an EP₁ receptor antagonist such AH6809 could be tested. 17-phenyl- trinor PGE₂ is a specific agonist for the EP₁ receptor (Dumont et al., 1998) which could also test the role of the EP₁ receptor in regulating M-1 cell COX-2 content. There are also reports of cAMP dependant up-regulation of COX-2 expression (Minghetti et al., 1997). Both the cAMP dependant and

calcium dependant pathways could be involved in prostaglandin mediated stimulation of COX-2 levels in the M-1 cell line.

Section 7.6: *The effects of NSAIDs may not be limited to the inhibition of COX isoform activities.*

Although we have pursued our study treating NSAIDs solely as COX inhibitors and prostaglandins as ligands for the well characterized G-protein coupled receptors, it is important to be aware that other signaling pathways exist for these molecules. It has become apparent that in addition to the effects of prostanoids which are mediated via 7-TMS G-protein coupled receptors, some prostanoids can also exert effects via peroxisome proliferator-activated receptors (PPARs). These are nuclear receptors which dimerize with retinoic acid RXR receptors (Kliewer et al., 1999). Upon ligand binding there is translocation of the receptor to the nucleus whereupon it's effects are mediated by binding to specific promoter elements. The first prostanoid ligand to be described for a PPAR receptor was 15-deoxy- Δ -^{12,14}-PGJ₂ which specifically activates PPAR γ (Kliewer et al., 1995). More recently, stable prostacyclin analogs have been shown to activate the PPAR α at nanomolar concentrations (Hertz et al., 1996). In the kidney, both PPAR α and PPAR β expression has been characterized (Guan et al., 1997). There are two pathways through which NSAIDs could affect kidney physiology via PPARs. Through inhibition of COX activity, PPAR ligand availability will decrease. However, many NSAIDs including indomethacin can also act as PPAR agonists themselves (Lehmann et al., 1997). In many

instances, the levels of NSAIDs required for PPAR activation are far above pharmacologically relevant concentrations. Therefore, effects of NSAIDs on PPAR signaling are most likely to be mediated by the inhibition of COX activity. There are some instances where NSAIDs may be relevant PPAR ligands. For example, while oral ingestion of sulindac can lead to plasma concentrations of sulindac sulfide of 10 - 15 μM , the same dose can lead to 20 fold higher concentrations in the lumen of the colon (Shiff et al., 1995).

It has long been a mystery as to how aspirin can act as an anti-inflammatory agent given that it is not a very potent COX-2 inhibitor at therapeutic concentrations (Xu et al., 1999). Moreover, salicylic acid has well characterized anti-inflammatory characteristics even though it does not inhibit COX activity (Laneuville et al., 1994). It is now known that aspirin and salicylic acid can inhibit LPS and phorbol ester mediated stimulation of COX-2 transcription starting at a therapeutically relevant concentration of 100 nM (Xu et al., 1999). The mechanism through which this occurs remains unknown. Neither aspirin nor salicylic acid act as agonists at any of the known PPARs (Xu et al., 1999). It is interesting that in 1999, people are still adding to the three decade old Nobel prize winning work of Sir John Vane on a drug which is more than a century old. Given the evidence which is available concerning NSAIDs and PPARs as well as aspirin, salicylic acid, and inflammation it is possible that some of the differences in the effects of other NSAIDs can be explained by their interactions with enzymes other than the cyclooxygenase isoforms. For example, enantiomers of ibuprofen and ketorolac can inhibit the hydrolysis of anandamide (arachidonylethanolamine) (Fowler et al., 1999).

Section 7.7: Putative compensatory mechanisms for the cellular response to inhibition of COX activity.

Compensation for NSAID mediated reductions in PGE₂ synthesis could occur either via increases in COX enzyme levels or via an increase in the expression of EP receptor subtypes. In order for prostaglandins to exert their effects on CCD salt and water transport, they must be excreted from the intercalated cell in order to act upon receptors on the principal cell (Breyer et al., 1998). The effects of PGE₂ are mediated by a distinct class of receptors referred to as E-prostanoid (EP) receptors (Coleman et al., 1994). The EP₁, EP₃ and EP₄ receptor subtypes have been localized in the CCD (Breyer et al., 1996a). Functional studies examining the effects of PGE₂ on salt and water transport have suggested that these receptors are present on the principal cells of the collecting duct (Breyer et al., 1998). Our results which localize both COX isoforms to the intercalated cells suggest that the effects exerted by PGE₂ on principal cell salt and water reabsorption represent a paracrine interaction between intercalated and principal cells. The interactions between the EP receptors, the regulation of COX isoform levels, and PGE₂ synthesis in this segment of the nephron represent an interesting field of study. Although, abundant evidence has been presented detailing the hormonal regulation of arachidonic acid release in the CCD (Lal et al., 1997), no mechanisms regulating COX isoform or EP receptor expression in the collecting duct have previously been characterized. An improved understanding of the

mechanisms which regulate prostaglandin synthesis and signaling in the CCD will help to clarify the pathways through NSAIDs can impair CCD function.

At the time which these experiments were performed, there were no commercially available antibodies against any of the EP receptors. Due to the co-expression of both the EP₁ receptor and PKN it is not possible to optimize conditions for the quantification of EP₁ receptor by RT-PCR. The PKN message is highly expressed and competes with the EP₁ message for primers and the Taq DNA polymerase. By products of the rapid PKN amplification such as pyrophosphate could also interfere with the slower amplification of the EP₁ message (Morrison and Gannon, 1994; Vander Horn et al., 1997). In order to obtain quantitative results by PCR, the reaction must be stopped while it is still proceeding linearly. However, in experiments attempting to establish these conditions for the EP₁ mRNA, the PKN signal would always plateau before the EP₁ was visible. In order to amplify the EP₁ message without the PKN message, an EP₁ specific primer could be used for the reverse transcriptase step instead of random hexamers. Alternatively, EP₁ mRNA expression could be assessed by Northern blotting using the 336 bp EP₁ PCR product as a probe. This would be expected to yield both a 2.1 kb EP₁ band which would be easily distinguishable from the 6.8 kb PKN long transcript. Given that the EP₁ receptor is responsible for the natriuretic effects of PGE₂ and that NSAIDs can cause sodium retention in some individuals but not others, a better understanding of the effect of NSADs upon CCD EP₁ receptor expression would be valuable. The EP₃ and EP₄ receptors have opposing effects on water transport which are due to their opposing effects on adenylate cyclase activity (Sakairi et al., 1995).

The extent to which NSAIDs cause water retention could be related to their effects on either one of these receptors. We present evidence that the EP₃ receptor mRNA is up-regulated three fold in response to COX-2 inhibition by NS-398 in M-1 cells. In order to measure the significance of this increase in EP₃ mRNA with respect to cAMP generation and CCD water permeability, it would be necessary to measure the inhibitory effect of PGE₂ on AVP or forskolin mediated cAMP synthesis in NS-398 treated and untreated M-1 cells. The development of EP₃ reporter constructs would allow for the investigation of the mechanism through which COX inhibition regulates EP₃ receptor mRNA expression. Co-incubation of the M-1 cells with NS-398 and EP receptor specific PGE₂ analogs would yield information concerning the particular signaling mechanism through which the EP₃ receptor is regulated by PGE₂. Previous studies have found that COX inhibition by ibuprofen is associated with an increase in PGE₂ binding in the brain of newborn pigs (Li et al., 1995). In this study by Li et al, the increased PGE₂ binding is associated with an increase in cAMP production upon administration of butaprost (EP₂ agonist) and a decrease in cAMP production in response to the EP₃ agonist M&B 28,767. In response to PGE₂, the stimulatory effect on cAMP wins out and cAMP production is elevated in the ibuprofen treated samples. In the M-1 cell line we have determined that the EP₂ receptor is not present, therefore, the inhibitory effects of the EP₃ receptor may predominate after NSAID administration. Our study compliments that of Li et al (1995) as our results suggest that the increase in PGE₂ binding and signaling that they observe in response to COX inhibition is at least in part due to increased expression of the EP₃ receptor mRNA.

Two recent papers have presented evidence that EP₁ (Bhattacharya et al., 1998), EP₃, and EP₄ (Bhattacharya et al., 1999) are present and functional within the nuclear envelope. These findings represent the first confirmation of the existence of functional G-protein coupled receptors in the nuclear envelope. These papers demonstrate that PGE₂ binding to the EP₁ and EP₃ nuclear envelope receptors can increase intranuclear Ca⁺⁺ concentrations. Furthermore, these changes in intranuclear Ca⁺⁺ concentrations caused an increase in the transcription of genes such as c-fos and inducible nitric oxide synthase. These results help to explain a role for the nuclear localization of the COX-2 isoform shown by us and others. Now that it is known that EP receptors can be found on the plasma membrane or at the nuclear envelope, it would be interesting to understand how the distribution of the EP receptors between these two locations can be regulated. With respect to the effects of NS-398 on EP₃ receptor mRNA expression, it is possible that this drug or other drugs could also affect the targeting of EP₃ receptors to the nuclear versus the plasma membrane. In the case of the EP₃ receptor, the distribution of the splice variants between the plasma membrane and the nuclear envelope needs to be characterized. This could help to explain the role of these EP₃ splice variants which do not provide any signaling pathway for PGE₂ that is not already available via the other EP receptor subtypes. The new findings concerning the perinuclear localization of EP receptors will require the re-evaluation of the effects of PGE₂ in many tissues.

The EP₃ receptor splice variants have not been quantified along the collecting duct. The primers which we used amplified a region common to all of the splice variants.

Specific primers have been designed for each of the three mouse EP₃ receptor splice variants (Irie et al., 1993). It is possible that the three fold increase in EP₃ receptor mRNA expression represents a particular subtype of this receptor. The nuclear membrane EP₃ receptor which has been described signals via a pertussis toxin sensitive increase in intranuclear calcium levels (Bhattacharya et al., 1999). Meanwhile the EP₃ receptor mediated diuretic effects of PGE₂ in the CCD are also pertussis toxin sensitive but involve the inhibition of adenylate cyclase (Bonilla-Felix and Jiang, 1996). A prostaglandin transporter has been described which can import PGE₂ into cells (Chan et al., 1998). This transporter could provide a means of controlling the proportions of plasma membrane versus nuclear membrane PGE₂ signaling.

Section 7.8: *The impact of NSAIDs on renal physiology.*

NSAIDs are a powerful tool in controlling inflammation, however, their clinical usefulness is limited by their negative side effects: primarily the induction of gastric ulcers and secondarily kidney impairment (Murray and Brater, 1997). The kidney impairment which ensues NSAID therapy can be divided into several categories. The hemodynamic effects of prostaglandins have been well studied (Zambraski, 1995). The vasodilatory effects PGE₂ and PGI₂ are important in counteracting the effects of vasoconstricting peptides such as angiotensin II and endothelin I and renal sympathetic nerve activity (Murray and Brater, 1993; Schneider and Stahl, 1998; Schramek et al., 1995). This is especially important in volume depleted states where the vasodilatory prostaglandins are critical for the

maintenance of renal blood flow (RBF) and glomerular filtration rate (GFR). Electrolyte imbalances can occur subsequent to vasoconstrictor mediated reductions in glomerular filtration rate when NSAIDs inhibit PGE₂ and PGI₂ production in mesangial cells. COX-2 has also been implicated in the regulation of renal renin levels (Harding et al., 1997). Both low salt diet (Harris et al., 1998) and renovascular hypertension studies (Hartner et al., 1998) have shown a link between macula densa COX-2 levels and renin content. In addition to the hemodynamic and endocrine effects of renal prostaglandins, they also inhibit sodium and water reabsorption in the collecting duct (Breyer et al., 1998). Our study provides evidence that the effects of PGE₂ on transport in the collecting duct represent a paracrine interaction between principal and intercalated cells. We also demonstrate that in addition to inhibiting COX-2 activity, COX-2 specific NSAIDs also rapidly stimulate an increase in COX-2 protein levels. This finding suggests that following the metabolism and clearance of a dose of an NSAID, there could be a period in which COX-2 activity rises above baseline while the increased COX-2 protein content persists in the absence of inhibitor (Callejas et al., 1999). Even during therapeutic NSAID therapy, renal prostaglandin synthesis is never 100 % inhibited (Zambraski, 1995). Measurements of urinary prostaglandin concentrations reveal that NSAIDs inhibit renal prostaglandin production by 60 - 80 % (Zambraski and Dunn, 1979). Indomethacin has been shown to have natriuretic effects in some studies and sodium retaining effects in others (Stahl et al., 1979). Based on the results of our study, I propose that these differences could be due to the degree to which indomethacin inhibits

renal prostaglandin synthesis compared to the degree to which indomethacin affects COX-2 and EP receptor expression.

Section 8: *Summary*

In summary, in the cortex of intact kidney sections, COX-1 and COX-2 are localized to the intercalated cells of the CCD. We have found that although M-1 CCD cells express both COX isoforms, COX-2 is the major COX isoform contributing to murine CCD prostaglandin production. Both the COX-1 and COX-2 isoforms were found in all cultured M-1 cell types. This is unlike the what we observed on the intact kidney slice where only the intercalated cells were COX-2 positive. When COX activity was measured in the presence of commercially available isoform specific inhibitors, it was observed that COX-2 inhibition significantly decreased PGE₂ synthesis compared to control. Meanwhile, COX-1 inhibition resulted in a smaller decrease in PGE₂ production. These results demonstrate the predominance of COX-2 activity in the production of prostaglandins by the M-1 CCD cell line. Furthermore, inhibition of the cyclooxygenase activity of COX-2 with NS-398 or indomethacin resulted in increased COX-2 protein content. This increase in COX-2 protein content occurred independently of any change in the level of COX-2 mRNA expression. COX-1 protein content was not affected by NSAID administration. 16,16-dmPGE₂ in the presence of NS-398 was unable to reverse the effects of NS-398 on COX-2 content. Moreover, 16,16-dmPGE₂ and sulprostone were both able to stimulate increased COX-2 content on their own. At the receptor level, we have shown that M-1 cell COX-2 inhibition

is associated with increased EP₃ receptor mRNA expression. Much of the previous research has focused on the role of COX-2 within the juxtaglomerular apparatus, however, within the kidney cortex, the CCD synthesizes the greatest quantity of PGE₂ per nanogram of dissected nephron segment protein (Bonvalet et al., 1987). This PGE₂ is an important mediator controlling salt and water reabsorption by the CCD (Hébert et al., 1993). Our new observations in conjunction with previously published work concerning the localization and regulation of COX-2 in the rat kidney demonstrate the importance of constitutively expressed, non-inflammatory COX-2 in the kidney. When one takes into account the role that COX-2 plays in producing prostanoids required for normal: renal development, hemodynamics, renin secretion, and now possibly collecting duct function, questions arise as to how benign the new class of COX-2 specific inhibitors will be towards the kidney. The collecting duct has been proposed to be an ideal model for the study of the regulation of prostaglandin synthesis due to the lack of any other arachidonic acid metabolizing pathways in these cells (Smith, 1992). The constitutive expression of both COX isoforms by the M-1 cell line which we have described reveals this cell line to be a useful model for the study of the cyclooxygenase pathway.

Section 9: References

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