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Characterization of the Prostacyclin/IP Receptor System in the Rat Kidney :
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**Characterization of the Prostacyclin/ IP Receptor System in
the Rat Kidney: Implications for Diabetic Nephropathy**

© Rania Nasrallah

**This thesis is submitted as a partial fulfillment of the Ph.D. program in Cellular and
Molecular Medicine.**

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DEDICATION

To my loving parents Simon and Amira Nasrallah

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I am sincerely thankful to Dr Richard L. Hébert for his supervision, guidance, and friendship, throughout the course of my graduate studies. I will always cherish the experience, knowledge and autonomy that I have acquired in his laboratory.

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ABSTRACT

This study examined the distribution of prostacyclin (PGI₂) receptors (IP) along the rat nephron, the signaling responses coupled to IP activation, and any alterations in this system in high-glucose treated (HG)-mesangial cells (MG) and streptozotocin (STZ)-diabetic rat kidneys. In situ hybridization, RT-PCR, RealTime RT-PCR, and Northern blot were used to study mRNA, and immunohistochemistry and Western blot for protein. IP mRNA was detected in glomeruli, vasculature, and cortical and medullary tubules on rat tissue sections, and in different preparations: kidney (C: cortex, OM: outer medulla, and IM: inner medulla), cultured MG (primary and transformed), proximal tubule (PT; fresh and cultured), and inner medullary collecting duct (fresh: f-IMCD and cultured: c-IMCD). IP protein was noted in PT, MG, IMCD, OM, and IM, but not cortex.

Our work suggests that IP activation increases cAMP in: primary and immortalized MG, cultured PT, f-IMCD and c-IMCD. Inhibition of vasopressin-cAMP was obtained in f-IMCD. However IP was not linked to any calcium signaling in our studies. And we did not detect any IP subtypes or spliced variants.

The role of PGI₂/IP in MG was examined following 24 hr stimulation with cicaprost (CCP). A decrease in fibronectin and p27 was observed in cell lysates, and an increase in matrix metalloproteinase-2; CCP had no effect on thymidine or leucine incorporation. In response to 24 hr and 4 day HG, CCP- and iloprost (ILP)-cAMP was attenuated in MG: immortalized and primary cultures, respectively. CCP-cAMP was also diminished by anisomycin, a MAPK activator.

PGI₂ synthesis is dependent on cyclooxygenase (COX) and PGI₂ synthase (PGIS). We show increased COX-1 and -2 in the medulla of STZ rats, as well as HG-IMCD. Only COX-2 was elevated in HG-MG. While PGE₂ synthase was augmented in the OM of STZ rats, PGIS protein was reduced in HG-MG. HG did not affect IP mRNA in vitro, but reduced IP protein. However, IP mRNA was diminished in the OM of STZ rats, and all three-kidney regions in 6-mth uni-nephrectomized/STZ rats. The attenuation of PGI₂/IP described in our work suggests that IP may serve as a target to prevent progressive injury to diabetic kidneys.

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LIST OF ABBREVIATIONS

Ang II: angiotensin II
AA: Arachidonic acid
ACE: angiotensin converting enzyme
AGE: advanced glycation end-product
AT 1: Ang II receptor type 1
ATCC: American Type Culture Collection
AVP: arginine vasopressin
cAMP: cyclic adenosine monophosphate
BM: basement membrane
BP: blood pressure
c: cultured
C: cortex
CCD: cortical collecting duct
CCP: cicaprost
CD: collecting duct
COX: cyclooxygenase
cTAL: cortical thick ascending limb of Henle
DM: diabetes mellitus
DN: diabetic nephropathy
DP: PGD₂ receptor
ECF: extracellular fluid
eNOS: endothelial nitric oxide synthase
EP: PGE₂ receptor
ER: endoplasmic reticulum
ESRD: end-stage renal disease
ET-1: endothelin-1
f: freshly isolated
FP: PGF_{2 α} receptor
GFR: glomerular filtration rate
HETEs: hydroxyeicosatetraenoic acids
HG: high glucose
IBMX: isobutyl-methyl xanthine
IC: interstitial cells
IDDM: insulin dependent diabetes mellitus
IL-1 β : interleukin-1 β
ILP: iloprost
IM: inner medulla
IMCD: inner medullary collecting duct
IP: PGI₂ receptor
ISH: in situ hybridization

Iso-PGE₂: isoprostane E₂
Iso-PGF_{2α}: isoprostane F_{2α}
KO: knockout mice
LOX: lipoxygenase
LTs: leukotrienes
MAPK: mitogen activated protein kinase
MG: mesangial cells
mIC: medullary interstitial cells
MMP: matrix metalloproteinase
mRNA: messenger RNA
mTAL: medullary thick ascending limb
NFκB: nuclear factor κB
NIDDM: non-insulin dependent diabetes mellitus
NO: nitric oxide
NSAIDs: non-steroidal anti-inflammatory drugs
OM: outer medulla
OMCD: outer medullary collecting duct
PDGF: platelet derived growth factor
PG: prostaglandin/prostanoid
PGD₂: prostaglandin D₂
PGDS: prostaglandin D₂ synthase/isomerase
PGE₂: prostaglandin E₂
PGES: prostaglandin E₂ synthase/isomerase
PGF_{1α}: PGI₂ metabolite
PGF_{2α}: prostaglandin F_{2α}
PGFS: prostaglandin F_{2α} synthase/isomerase
PGH₂: intermediate endoperoxide
PGI₂: prostacyclin/prostaglandin I₂
PGIS: prostacyclin synthase/isomerase
PGS: prostaglandin synthase/isomerase
PKA: protein kinase A
PKC: protein kinase C
PLA₂: phospholipase A₂
PMA: phorbol myristate acetate
POX: peroxidase activity of COX
PPAR: peroxisome proliferator-activated receptor
PSF: PGI₂ stimulating factor
PT: proximal tubule
RAS: renin-angiotensin system
RBF: renal blood flow
ROS: reactive oxygen species
rMG: immortalized rat mesangial cells
STZ: streptozotocin
TGFβ: transforming growth factor β

TP: TXA₂ receptor
TXA₂: thromboxane
TXAS: thromboxane synthase/isomerase
NX: uni-nephrectomy

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FORMAT

This thesis is submitted as a collection of published journal articles (Chapters 2-5). Chapter 1 contains a general introduction with a critical review of the relevant literature, while chapter 6 contains a general discussion. The references for chapters 1 and 6 are found in chapter 7.

AUTHORIZATION

Permission was obtained for the use of figures and tables from the four manuscripts included in this thesis (chapters 2-5), from the associate editors of both journals: The American Journal of Physiology (Penny Ripka) and Prostaglandins Leukotrienes and Essential Fatty Acids (Pierre Sirois). Refer to the next two pages for the permission letters.

The following four publications are credited as the source:

1. **NASRALLAH, Rania**, Zimpelmann, J, Singh, S, Hebert, R.L. Molecular and Biochemical characterization of Prostacyclin (IP) receptors in the rat kidney. Am. J. Physiol 280: F266-F277, 2001.
2. **NASRALLAH, Rania**, Anne Landry, Sonia Singh, Monika Sklepowicz, and Richard L. Hébert. Increased expression of cyclooxygenase-1 and -2 in the diabetic rat renal medulla. Am J. Physiol. 285: F1068 – F1077, 2003.
3. **NASRALLAH, Rania**, Anne Landry, James W. Scholey, and Richard L. Hébert. Characterization of the PGI₂/IP System in Cultured Rat Mesangial Cells. Prost. Leuk. Ess Fatty Acids 70 (5): 455 – 464, 2004.
4. **NASRALLAH, Rania**, and Richard L. Hebert. Reduced IP receptors in STZ-induced diabetic rat kidneys and high-glucose treated mesangial cells. Am. J. Physiol 287: F673 – F681, 2004.

CHAPTER 1: GENERAL INTRODUCTION

1.1. The kidney

Since the work of Homer Smith in the mid-30s, it is well recognized that the kidney plays a key role in the maintenance of salt and water homeostasis in the body (Berliner 1995). It also serves in the regulation of acid-base balance, the excretion of various substances, and the production and secretion of hormones. Each kidney is composed of over one million nephrons, which consist of a series of tubule segments connected to the glomerular capsule, which surrounds a bed of capillaries, altogether termed the glomerulus. The fact that the kidneys receive about 20% of the total cardiac output validates their importance in regulating the composition of body fluids as well as intracellular and extracellular environments. Several stages of branching of the renal vasculature occur from the right and left renal artery and within each lobule of the cortex, giving rise to the afferent arterioles, the blood supply entering the glomerular capillary network. Blood is then returned to peritubular capillaries exiting the glomerulus via the efferent arteriole. Projections of these capillaries into the inner regions of the kidney are termed vasa recta. Although less than 1% of the renal blood reaches these vessels, they are an essential source of oxygen and nutrients to the ascending loops of Henle and the medullary collecting duct (CD), serving an important function in concentrating and diluting the urine, and for the exchange of substances between the tubules and the cardiovascular system. (Koeppen and Stanton 1997)

The kidney is subdivided into 3 morphologically and functionally distinct regions:

the outermost cortex, the outer medulla, and the innermost region the inner medulla/papilla (reviewed in Jacobson 1981). Four major tubular segments within each of these regions: the proximal tubule (PT), loops of Henle, distal tubule, and CD, play various roles in tubular transport due to their diversity in cellular composition. The high permeability of the PT epithelial cells allows it to carry out the bulk of reabsorptive processes. On the other hand, the tight epithelium of the CD ensures a greater fine-tuning of the luminal fluid. Many hormone systems, growth factors, cytokines, and metabolic pathways influence renal function by contributing to various processes within specific nephron segments, including the renal renin-angiotensin system (RAS), arachidonic acid (AA) metabolites, interleukin-1 β (IL-1 β), and protein kinase C (PKC).

1.1.1. The glomerular mesangial cells

The glomeruli are present within the cortical region of the kidney. There are three main cell types composing the glomerular structure: mesangial (MG), epithelial (podocytes), and endothelial. All three function in unison to determine the filtration rate and composition of the filtrate. MG cells bathing in the mesangial matrix/mesangium surround the capillary loops of the glomeruli. They have several key properties that explain their numerous functions (Koeppen and Stanton 1997): they are vascular smooth muscle-like cells enabling them to contract and regulate the capillary pressure and flow, while providing structural support for the surrounding capillaries; they secrete matrix proteins (fibronectin, collagen, laminins) thereby altering the composition of the mesangium; they participate in immune responses due to their phagocytic properties; and they play an active role in the secretion of various substances including hormones:

angiotensin II (ANG II), vasopressin (AVP), PGs; growth factors: transforming growth factor β (TGF β), platelet derived growth factor (PDGF); and cytokines: IL-1 β . MG cells play a central role in the development of glomerulosclerosis in many diseases.

1.1.2. The inner medullary collecting duct (IMCD)

The CD is the ultimate regulator of filtrate composition, and hence of urine. It is anatomically divided into separate regions: cortical (CCD), outer medullary (OMCD), and inner medullary CD (IMCD). It is composed of a heterogenous population of epithelial cells, namely the principal and α -/ β -intercalated. While the principal cells are mainly responsible for the trafficking of water and electrolytes (sodium, potassium), the intercalated cells play a role in the regulation of acid-base balance by the kidney. The main function of the IMCD is to transport water via aquaporin-2 (Knepper 1996), transport sodium via apical epithelial sodium channel (Hager 2001), and urea reabsorption, as well as ammonia recycling. AVP action on the principal cells is a key regulator of CD transport processes (reviewed in Bankir 2001a). But many other hormonal systems play an active role in IMCD homeostasis including: PGs, ANG II, endothelin-1 (ET-1).

1.2. The arachidonic acid cascade

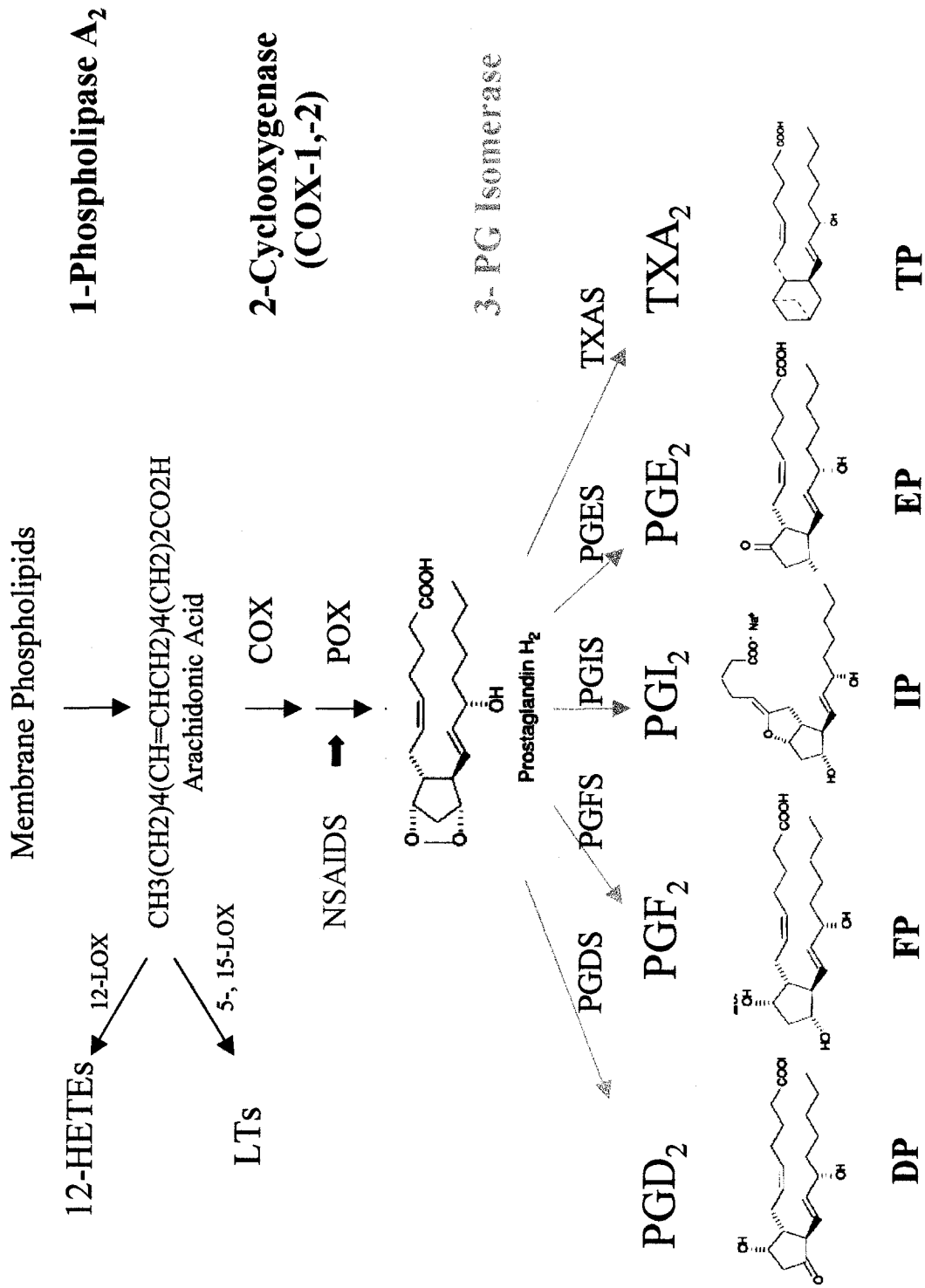
AA is released from membrane phospholipids by the action of phospholipase A₂ (PLA₂). Activation of phospholipases is highly dependent on hormonal stimulation, for example ANG II (Dulin 1998, Freeman 1998), IL-1 β (Anthonsen 2001), and PKC (Williams 1993). AA serves as a precursor for eicosanoid signaling molecules including

leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs), and PGs. The metabolic conversion of AA into these various biologically active compounds occurs via four distinct enzymatic pathways: 1) 5-lipoxygenase (LOX), 2) 12-LOX, 3) 15-LOX, and 4) cyclooxygenase (COX). The products of the COX pathways, including PGs and thromboxane (TXA₂), are collectively called prostanoids (PGs). These are 20-carbon oxygenated fatty acids. *The cyclooxygenase pathway is illustrated in Figure 1.1.*

1.2.1. The cyclooxygenase pathway: COX, isomerases, PGs

There are currently three known isoforms of COX: COX-1, -2, and the recently identified spliced variant of the COX-1 gene, COX-3 (Chandrasekharan, 2002). COX have both cyclooxygenase and peroxidase (POX) activity, that catalyze the conversion of AA into the intermediate endoperoxide PGH₂ (Smith 1996); which is then converted to 5 major biologically active PGs: PGE₂, PGF_{2α}, PGI₂, PGD₂, and TXA₂ by specific synthases/isomerases: PGES, PGIS, TXAS, PGDS, PGFS (Watanabe 1997). While COX-1 is constitutively expressed in most tissues, COX-2 is the inducible form (DeWitt 1993). Thus it is widely believed that PGs derived from COX-1 play a homeostatic role, while those from COX-2 would be harmful and thus could be therapeutically targeted in various diseases. But renal studies contradict this classification, as discussed below.

Figure 1.1. The cyclooxygenase pathway: three key steps for synthesis of prostanoids. Depicted are the three steps in the biosynthesis of prostanoids: 1) Hormonal activation of phospholipase A₂ to generate arachidonic acid from phospholipids; 2) Oxidation (POX: peroxidase activity) and reduction (COX: cyclooxygenase activity) of arachidonic acid by cyclooxygenases (COX-1 and -2) to generate an intermediate endoperoxide PGH₂; 3) conversion of PGH₂ to 5 major prostanoids (PGD₂, PGF_{2α}, PGI₂, PGE₂, TXA₂) by specific synthases/isomerases (PGDS, PGFS, PGIS, PGES, TXAS). Each prostanoid binds its own receptor to alter cell responses (DP, FP, IP, EP, and TP). Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the cyclooxygenase activity of COX. Also shown is 5-lipoxygenase (LOX), 12-LOX, and 15-LOX mediated metabolism of arachidonic acid via distinct pathways, giving rise to leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs).



COX-1 and -2 are integral membrane proteins, the translation products of both COX genes are of similar size, between 72 and 74 KDa, encoded by 2.8 kb mRNA and 4-4.5 kb transcripts, respectively. While COX-1 is mainly associated with the lumen side of the endoplasmic reticulum (ER), COX-2 is located both in the ER and the nuclear envelope. The COX-1 gene (22.5 kb) contains 11 exons and ten introns. On the other hand COX-2 contains 10 exons and 9 introns. The COX-2 gene (8 kb) is much smaller than COX-1, because the introns of COX-2 are substantially smaller, (reviewed by Smith and DeWitt 1995). Despite the fact that the catalytic activity of both isoforms is quite similar, they only share about 66% homology in their amino acid sequence (Harris 1994). Also, there is a great deal of variability in their respective promoter regions, suggesting that the regulation of COX-2 expression is much more composite. Growth factors or other agents may regulate COX-1, but it mainly serves a housekeeping function with constant levels of expression in most tissues. Several features distinguish COX-2 from COX-1: the presence of a TATA box in the 5' promoter region of COX-2 makes it readily inducible by different transcription factors (Appleby 1994); presence of a nuclear factor κ B (NF κ B) motif that mediates the stimulatory effects of IL-1 β on the COX-2 gene (Roshak 1996); down-regulation of COX-2 by TGF- β (Reddy 1994). Interestingly, microarray analysis recently revealed that COX-2 is induced by the PGI₂ analogue iloprost (ILP), providing a positive feedback mechanism to upregulate its own synthesis in human vascular smooth muscle cells (Meyer-Kirchrath 2004)

Since in certain cells such as the macula densa cells of the kidney COX-2 is constitutively expressed and not COX-1 (Harris 2000), the current understanding regarding their respective roles becomes more complex, especially with respect to the

renal system. Insights into the importance of each isoenzyme came about by the generation of isoform-specific deficient mice. While no major renal pathology was documented for the COX-1 knockout (Langenbach 1995), COX-2 lacking mice display abnormalities in renal development and severe nephropathy (Dinchuk 1995, Morham 1995): reduced number of glomeruli, immature glomeruli and tubules, tubular atrophy, and interstitial inflammation and fibrosis. Certain studies have investigated the expression profile of COX-2 throughout the kidney. Harris et al (1994) first showed constitutive COX-2 expression in the macula densa, juxtaglomerular apparatus, epithelial cells of the cortical thick ascending limb of Henle (cTAL), and medullary interstitial cells (mIC), but no other structures (glomeruli, CD, arterioles). On the other hand, a study by Ferguson et al (1999) indicates constitutive COX-2 in the mouse CD, which was previously not observed in the rat kidney (Harris 1994). Other studies also suggest that the renal localization differs somewhat between species. In a recent study of 53 normal human nephrectomy specimens, COX-2 was constitutively expressed in arterioles and glomeruli, in cTAL and macula densa, in vasa recta endothelial cells, and in CD (Adegboyega 2004). But recent work in mouse and rat kidney did not detect COX-2 in distal tubules or CD (Campean 2003).

While separate genes encode COX-1 and COX-2, COX-3 is a spliced variant of the COX-1 gene isolated from canine brain that retains intron 1. Unlike COX-1 and COX-2, this isoenzyme is highly responsive to acetaminophen (Chandrasekharan 2002), hence the enthusiasm surrounding its discovery. It was also detected in human cerebral cortex and heart, however insertion of intron 1 caused a frameshift possibly resulting in a non-functional protein in humans (Shwab 2003). Therefore the relevance of COX-3 to

human pathophysiological processes remains questionable. The main idea is that COX-3 is probably more important in the termination phase of the inflammatory response (Warner 2002), hence the weak anti-inflammatory effect of acetaminophen. If so, targeting COX-3 to enhance its function may prove to be a useful anti-inflammatory tool in various diseases. The identification of COX-3 is especially promising for PGI₂ biology, as suggested by Warner and Mitchell (Warner 2002), since acetaminophen has been shown to reduce circulating PGI₂ levels independent of actions on COX-2. Since both COX-1 and -2 are constitutively expressed in renal tissue, but also induced in pathophysiological states, it will be interesting to see whether COX-3 is present in the kidney, and if any novel renal processes may be associated with this third isoform. Furthermore, whether an antagonistic relationship exists between COX-3 and the other COX isoforms. Generation of a COX-3 deficient mouse in the future will surely shed some light on its importance to various biological processes.

The intermediate endoperoxide, PGH₂, generated by the action of COX was initially thought to be inactive. However, Soler et al. (2001) showed that untransformed PGH₂ is an important AA metabolite found in rat glomeruli and MG cell suspensions, and IL-1 β enhances its production. Not only is it quite abundant, but also accounts for the vasoconstrictor function of AA in the isolated perfused rat kidney (Quilley 1989). Quilley et al (2003) showed that this enhanced AA vasoconstrictor effect in the diabetic rat kidney is dependent on COX-2 and not COX-1. It has long been recognized that PGH₂ shares the same receptors as TXA₂ (Halushka 1989); therefore, future studies will surely examine the role of PGH₂ in other renal processes. In a recent review by Ferrario et al. (2004), the antagonistic property of losartan (an ANG II antagonist) at TXA₂/PGH₂

receptors is highlighted, bringing forth new avenues to be explored with respect to PGH_2 and TXA_2 function. The clinical relevance awaits further investigation; especially since ANG II receptor blockers like losartan are recommended as the first line of defence in patients with type II diabetes mellitus and hypertension (Rayner 2004).

1.2.1.1. Non-steroidal anti-inflammatory drugs

The significance of PGs to many biological functions are clearly demonstrated by the adverse effects, especially in the gut and kidneys (Rocha 2001, DeWitt 1999) associated with the use of non-steroidal anti-inflammatory drugs (NSAIDs). Unfavourable renal effects of NSAIDs, for example, include abnormalities in water and sodium metabolism, acute renal failure, chronic renal injury, to name a few. The prostaglandin biosynthetic pathway is also gaining its fame in the area of cancer biology and metastasis (Tsuji 1997, DuBois 2003). NSAIDs inhibit the cyclooxygenase activity of COX. But it seems the effects of certain NSAIDs are more complex, beyond the inhibition of PG synthesis. They may directly activate peroxisome-proliferator activated receptors (PPARs) and alter the expression of various genes (Guan 2002b), and the export of PGs from cells via the human multi-drug resistance protein MRP4 is also a target for NSAIDs (Reid 2003). Since chronic use of NSAIDs is linked to gastrointestinal and nephron toxicity, a great deal of research has focussed on unravelling the cell biology related to individual COX enzymes.

The advent of specific COX-2 inhibitors this past decade (Pennisi, 1998), has led to substantial progress in the area of COX/PG physiology. While there does not appear to be an advantage of these over non-selective NSAIDs in terms of nephrotoxicity and loss

of renal function (K^+ and Na^+ retention), Celecoxib and other COX-2 inhibitors are widely used nowadays (Rossat 1999). In the cardiovascular system, a homeostatic balance between PGI_2 and TXA_2 is crucial for the prevention of hypertension, stroke, atherosclerosis, myocardial infarctions, as well as other vascular diseases (deLeval 2004). Numerous studies stress the role of COX-2 in maintaining the PGI_2/TXA_2 ratio (McAdam 1999, Cheng 2002, deLeval 2004), thereby cautioning against the use of selective COX-2 inhibitors in susceptible individuals. Likewise, in the renal system more pronounced complications, especially in the elderly and in patients with pre-existing renal disease, seem to be associated with high doses of the COX-2 inhibitor Celecoxib (Whelton 1999, Giovanni 2002). In a recent study it was shown that COX-2-specific inhibitors play a role in the development of renal papillary necrosis (Akhund, 2003). Clearly the idea that COX-1 produces PGs that are important for normal function, and that COX-2 derived products play a pathological role, is not so obvious in the renal system. Each enzyme produces a separate pool of PGs, and depending on the cell type, the balance between cytoprotection and damage will determine the outcome and contribution to kidney disease.

1.2.1.2. Prostaglandin isomerases and prostaglandins

PGs are implicated in the maintenance of homeostasis and the pathogenesis of certain diseases, in the vasculature and in specific organ systems throughout the body: vasodilatory and constrictor functions, angiogenesis, platelet aggregation, pain, fever, respiratory functions, reproduction, gastric functions, sleep, immune responses, apoptosis, tumorigenesis etc. For instance, Sheng et al (2001) proposes a pro-neoplastic

role for PGE₂ in regulating the growth and motility of colorectal carcinoma cells, while PGI₂ may be anti-neoplastic for murine lung cancer (Keith 2002). The same antagonistic relationship can be seen in the cardiovascular system between PGI₂ and TXA₂, serving to regulate vascular tone and thrombosis (Cheng 2002). Furthermore, embryo implantation is highly dependent on COX-2-derived PGI₂ acting on PPAR δ (Lim 1999). The generation of each PG is dependent on the preferential coupling of a COX isoform with a specific isomerase. The significance of the coupling of COX to different PG isomerases (PGS) is just being defined. However, this seems to occur in a species and tissue-specific manner, but also depends on subcellular compartment co-localization. It is this variable coupling that determines which PG will predominate in a given cell. For example, microsomal PGES functionally couples to COX-2 in the perinuclear environment, while cytosolic PGES will couple to COX-1 (Tanioka 2000, Murakami 2000). Similarly, various studies indicate that the synthesis of PGI₂ is highly dependent on the expression of COX-2 in many instances and not COX-1: reproductive system (Helliwell 2004) and vasculature (McAdam 1999, Norata 2004). This same preferential coupling of enzymes is seen in other AA pathways, for example the synthesis of LTB₄ involves the action of both 5-LOX and LTA₄ hydrolase (Brock 2001).

Despite the significant role of PGs in many biological processes, and the extensive work in the field of COX and NSAIDs, there is a vast gap in the literature regarding the expression of specific PGS in the kidney. Recently, a comprehensive study undertaken by Vitzthum et al. (2002) examined the expression of different PGS in microdissected rat nephron segments. Their findings can be summarized as such: 1) PGDS is found in the proximal convoluted tubule, TAL, distal convoluted tubule, CCD,

and OMCD. 2) PGES is restricted to the entire CD. 3) PGIS mRNA was present in the whole kidney but not in any nephron segment examined. 4) TXAS was found only in glomeruli. This study contradicts previous reports, especially with respect to PGE₂ and PGI₂ synthesis along the nephron. For example, Yanagisawa et al (1993) showed enhanced activity of COX, 5-LOX, PGES, PGIS, and TXAS in glomeruli isolated from rats with bilateral ureteral obstruction. Also MG cells are an important source of both PGI₂ (Klein 1998) and PGE₂ (Guan 1997). Moreover, PG synthesis along the rabbit nephron has been previously described by Farman et al (1987), indicating that the IMCD is the major contributor to overall PG synthesis, with PGE₂>PGI₂>TXA₂ all along the tubule segments. Glomerular PGE₂ synthesis was also prevalent over other PGs. And finally, Hassid (1979) showed that in rat glomeruli, several PGs were produced: PGF_{2α}>PGE₂>PGF_{1α}>TXB₂>PGD₂. In a recent study by Campean et al (2003), microsomal-type PGES expression was investigated along the rat and mouse nephron showing parallel expression with COX-2 in the mTAL and macula densa and co-expression with COX-1 in distal convoluted tubule, connecting tubule, and non-intercalated CD cells.

Interestingly, a series of PG-like compounds were discovered over a decade ago by Morrow and co-workers (1990), termed isoprostanes. These AA derivatives are formed by the free radical-catalyzed peroxidation of arachidonate, independent of enzyme activity. The most important isoprostane produced in humans is 8-iso-PGF_{2α}, which is the most accurate in vivo marker of oxidative stress. It acts in part through the vascular TXA₂/PGH₂ (TP) receptor (Takahashi 1992, Janssen 2002), and through a putative 8-iso-PGF_{2α} specific receptor (Fukunaga 1997). Actually, levels of iso-PGF_{2α} are

significantly elevated in smokers (Morrow 1995). In asthmatic patients, TXA_2 is not involved, but TP receptor antagonists are therapeutically effective (Fujimura 1991); since isoprostanes can act on TP receptors, the beneficial effect is likely due to increased oxidative stress generating significant amounts of these compounds. Also, elevated levels of 8-iso-PGF_{2 α} are seen in hemodialysis patients with end-stage renal disease (ESRD), in association with cardiovascular complications due to oxidative stress (Lim 2002a). Though not many studies have examined the role of isoprostanes in the renal system, Takahashi et al. (1992) reported a vasoconstrictor effect of iso-PGF_{2 α} in rat kidney. The same effect was seen with iso-PGE₂ (Morrow 1994). The isoprostane pathway has also emerged as a new contributor to COX-independent generation of PGE₂ and PGD₂ in vivo (Gao 2003). In this recent report, the investigators suggest that “cis” isoprostanes produced by the non-enzymatic conversion of AA are readily epimerised into their respective “trans” configuration, resulting in the generation of PGE₂ and PGD₂ identical to those produced via the COX pathway. The significance of this alternate source of PG-like compounds and their relative contribution to overall cellular effects of PGs in the kidney is not yet appreciated, but surely warrants further investigation. Since a growing body of evidence supports the role of oxidative stress in a variety of diseases, this brings forth the possibility of improving therapy by combining antioxidants with NSAIDS.

1.3. Prostaglandin receptors and signaling pathways

While PGE₂ is the most prominent PG produced in the kidney, there is also significant synthesis of PGI₂ and TXA_2 (Bonvalet 1987). The highest level of PG synthesis occurs in the glomerular regions, and in the inner medulla, in particular in the

IMCD. However, species differences do exist in terms of the respective levels of each PG (Hassid 1979, Sraer 1982, Farman 1987). Although a plethora of renal functions have been proposed for the different PGs, the underlying mechanisms are poorly documented. At the glomerular level they not only maintain renal blood flow (RBF) and glomerular filtration rate (GFR), but also affect the secretion of renin. In addition, all along the tubules, they differentially regulate transport of H₂O and solute in the specific segments of the nephron (Breyer 1998, Schramek 1995, Harris 1998). In part, these diverse cellular effects of PGs are mediated by distinct G-protein coupled receptors (Toh 1995), each one having a greater affinity for a respective PG. These receptors are typical rhodopsin receptor-like structures with 7 transmembrane domains, an extracellular NH₂-terminus and an intracellular COOH-terminus (Wise 1996). They couple to different G-proteins thereby activating various intracellular signaling pathways. There are various classification schemes used to categorize the PG receptors. The general nomenclature is simple, PGE₂ receptors are termed E-type PG receptors or EP, PGI₂ receptors are IP, TXA₂ receptors are TP, and so on (Coleman 1994). However, a phylogenetic classification of the receptors arranges them into three separate clusters based on their ability to relax or constrict vessels. Therefore, cluster 1 (EP₂, EP₄, IP, DP) receptors are mainly vasodilatory receptors and stimulate adenylate cyclase, cluster 2 (TX, EP₁) receptors have constrictor effects by increasing intracellular calcium, and cluster 3 (EP₃) receptors inhibit cAMP and mainly elicit vasoconstrictor responses (Toh 1995).

1.3.1. Intrarenal localization of PG receptors and their function

By far the most extensively studied of these receptors in the kidney are the EP

receptors (reviewed in Sugimoto 2000, Breyer 2003). To date there are four cloned EP receptor subtypes in humans and rodents: EP₍₁₋₄₎. The EP₁ receptor couples to G_q-protein and activates PKC. EP₁ mRNA is most abundant in the medullary region of the kidney, and is involved in the inhibition of sodium reabsorption by PGE₂ in the entire CD, thus accounting for its natriuretic and diuretic effects. It may also be present in the glomerular region (MG cells) and vasculature (afferent arteriole), but this has not been clarified across species. The EP₂ receptor couples to G_s-protein and activates adenylate cyclase, to date it remains unclear where this receptor is expressed in renal tubules and what role it plays in tubular physiology, but it is mainly found in the renal vasculature and IC in humans (Morath 1999). The EP₃ receptor couples to G_i-protein and inhibits adenylate cyclase. By this mechanism PGE₂ inhibits AVP-dependent water transport in the CD as well as salt. This subtype is abundant in the mTAL again regulating transport of solute in this segment (Sugimoto 2000). Also, PGE₂ vasoconstricts the rat afferent arteriole via an EP₃ receptor spliced variant coupling to G_i-protein (Tang 2000). And finally, the EP₄ receptor couples to G_s-protein and stimulates adenylate cyclase. It is expressed on the apical membrane of cells in the rodent CD and stimulates sodium reabsorption by increasing cAMP levels. EP₄ receptors are also quite abundant in glomerular MG cells and podocytes (Bek 1999, Ishibashi 1999). Jensen et al (1999) showed that EP₄ receptors were also present in rat juxtaglomerular cells, consistent with the stimulatory effect of PGE₂-cAMP on renin secretion. Tang et al (2000) confirmed the expression of this subtype in the rat afferent arteriole; and PGE₂ was shown to elicit its vasodilator effect via EP₄-cAMP and not EP₂ receptors. Furthermore, recent work by Therland et al (2004) showed colocalization of EP₄ receptors with COX-2 in the human kidney vasculature.

EP₄ receptors are insensitive to the EP₂ agonist butaprost, and the EP₁₊₃ agonist sulprostone. TP receptor mRNA is mainly found in the glomerular region and arterial smooth muscle cells of the rat kidney (Studer 1994, Abe 1995). To date the localization of DP receptors in the kidney has not been determined in any species (Hirata 1994, Boie 1995), however indirect evidence does suggest their presence. For example Rao et al. (1987) infused PGD₂ into the kidney and showed hemodynamic effects (increased RBF, urine output, creatinine clearance) as well as altered tubular transport (sodium and potassium excretion), but this may be due to actions on other receptors. Though PGF_{2α} is a major urinary AA metabolite, FP receptor localization is also not as clear; it is mainly expressed in the distal convoluted tubule and CD principal cells in the mouse (Saito 2003). In addition to the four subtypes identified for the EP receptors, alternative splicing in the COOH-terminus tail of the EP₃ receptor gives rise to distinct isoforms that couple to different G-proteins in expression systems, but this is dependent on agonist concentration (Namba 1993). To date only two of these EP₃ spliced variants have been detected in the rat kidney (Takeuchi 1994), in AVP-sensitive segments of the nephron (TAL, CD): one coupling to G_i-protein and the other altering intracellular calcium.

The past decade has led to many advances in the study of these receptors, and the cellular responses linked to each one, and more and more insight is now available with respect to their involvement in certain renal pathology (Ishibashi 1999, Breyer 2003). However, it is becoming quite evident that under normal conditions, PGs mainly participate in homeostatic functions, thus serving to antagonize or enhance the cellular responses to other factors, for example ANG II (Schramek 1995, Scharschmidt 1983). Thus it is conceivable that a disturbance in PG signaling will alter this balance and

perpetuate a disease state, like diabetic nephropathy where increased ANG II levels in the early nephron lead to various complications (expansion of matrix, hypertrophy).

To gain insight into the importance of each PG receptor system, targeted mutations have been performed for each receptor subtype generating knockout mice (KO). The DP receptor KO indicates an inflammatory role in the lung, mediating allergic asthmatic reactions (Matsuoka 2000). A targeted deletion of TP receptors in the mouse resulted mainly in reduced platelet aggregation and longer bleeding time (Thomas 1998). No major renal disturbances have been reported thus far, with comparable vasoconstrictor responses (Schnermann 2000) to wild type mice. The FP-mutant mice exhibit parturition failure due to sustained progesterone levels, stressing the role of the $\text{PGF}_{2\alpha}$ /FP system in induction of labour (Sugimoto 1997). Although the kidney is a major site of FP receptor expression, there is no obvious defect in kidney function in these FP-deficient mice but further confirmation is needed. The EP_1 KO shows signs of diminished pain-sensitivity responses, but also alterations in blood pressure regulation. A disturbance in renal function has not been delineated (Stock 2001). Kennedy et al. (1999) produced an EP_2 receptor-deficient mouse that presented an important defect in the reproductive system, and linked this receptor subtype to fertility in these transgenics. Administering a PGE_2 bolus to these mice resulted in a hypertensive response compared to the hypotensive effect in wild type littermates, indicating that the vasoconstrictor action of PGE_2 mediated by EP_{1+3} is left unopposed in KO mice in the absence of the vasodilator EP_2 . Though the renal expression of EP_2 receptors remains controversial, a renal effect is suggested since the EP_2 KO develops salt sensitive hypertension (Kennedy 1999, Hébert 2003). On the other hand, Fleming et al (1998) studied urinary concentrating functions in

EP₃ KO and found no major abnormality compared to wild type littermates. The main phenotype of the EP₃-deficient mice is an impaired febrile response (Ushikubi 1998). The study of EP₄ deficiency is hindered by the fact that these mice die within 72 hrs of birth due to patent ductus arteriosus (Nguyen 1997), but studies in surviving KO mice reveal a role for this subtype in bone resorption (Miyaura 2000). Thus it seems that due to compensation by the remaining PG pathways, no major renal deficiencies were noted in any of these transgenic animals. This compensatory effect is also seen in PGS deficient mice, for instance in PGIS KOs there is increased synthesis of PGE₂ and TXA₂ counteracting the lack of PGI₂ (Yokoyama 2002), though these mice exhibit severe kidney injury possibly due to unopposed actions of these increased PGs. Also, COX-2 compensates for the lack of COX-1 in reproductive processes (Reese 1999).

1.3.2. Prostaglandins: intracrine signaling pathways

Although to date most of the effects of PGs have been dependent on autocrine and paracrine modes of signaling, recent evidence indicates that PGs can affect cellular function via intracrine effects. This is not unforeseen, since all the synthetic machinery is present in the vicinity of the nucleus. For example, certain EP receptors were located to the nuclear envelope in brain endothelial cells by Bhattacharya et al. (1998, 1999), showing that nuclear Ca²⁺ mobilization in response to PGE₂ occurs via EP₁ receptors, and regulation of endothelial nitric oxide synthase (eNOS) and other target genes by perinuclear EP₃ receptors (Gobeil, 2002). Nuclear DP receptors were also observed. It is unclear what contribution these intracrine effects of PGE₂ would have to renal homeostatic processes and to disease.

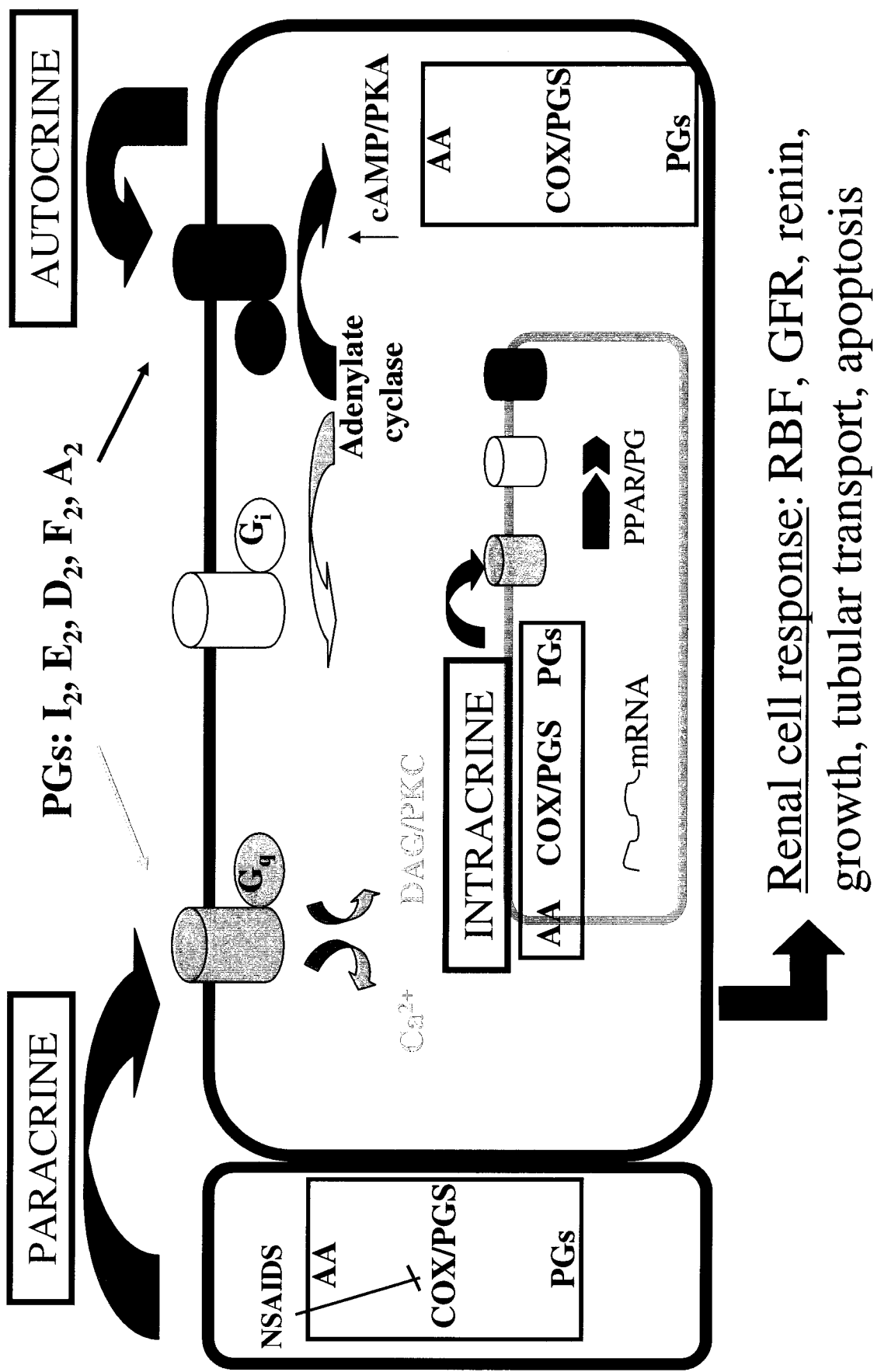
In addition, a new family of nuclear receptors has been identified for PGs, the PPAR (α , γ , δ) pathways. Until the cloning of PPARs a decade ago, it was thought that biological activities of PGI₂ were exclusively mediated by the cell surface IP receptor. Yet, current studies reveal that PGI₂ may be acting via other receptors like EP (Coleman 1994, Narumiya 1999) and PPARs or even un-identified mechanisms. For example, it was shown that PGI₂ regulates gene expression by activating the PPAR δ pathway (Hertz 1996, Guan 2002b). Although thus far PGE₂ itself has not been linked to activation of PPARs, the extent to which these pathways interact with the “classical” PG signaling mechanisms remains to be determined. For instance Hatae et al. (2001) studied the PGI₂/PPAR δ effects on cell apoptosis, demonstrating that the PGI₂/IP/cAMP pathway antagonizes the effects mediated by the PPAR δ pathway. A study by Guan et al (1997) investigated the distribution of different PPARs in the rabbit kidney, but PPAR δ was only present at low levels, with no significant expression in the cortex or medullary CD. In contrast, Yang et al (1999) found that PPAR δ mRNA was abundant in all micro-dissected nephron segments examined from Sprague-Dawley rats. Therefore, in rodent kidneys it appears to be the most abundant PPAR throughout the nephron (Guan 2002b). Yet very little is known about its role in the renal system. In a study by Hao et al (2000), it was demonstrated that COX-2-derived PGI₂ activates downstream PPAR δ to protect cultured mIC from hypertonicity-induced cell death. To date the study of PPAR δ -deficient mice is problematic due to developmental defects, thus future attempts will investigate the use of variable genetic backgrounds to generate these knockouts (Peters 2000, Lim 2002b). It will be of interest to determine what interaction exists between these “intracrine PG pathways” and the classical “cell surface” effects, and the overall contribution to renal

cell processes in health and disease. *A summary of the signaling pathways associated with prostaglandin action is illustrated in Figure 1.2.*

1.4. Prostacyclin and the IP receptor

PGI₂ was first isolated by Vane and coworkers (Bunting 1976), and has been implicated in many biological processes throughout the body. Initially, it was most recognized for its potent vasodilatory effects, and its ability to inhibit aggregation of circulating platelets. Nowadays, it is quite evident that not only does it play a key role in the vasculature, but it also contributes to the maintenance of homeostatic functions of many organ systems, and the pathogenesis of certain diseases. In the brain for instance, several studies confirm the role of PGI₂ as a cytoprotective factor, preventing neuronal damage and even death under certain circumstances (Sato 1999). It is also implicated in reproductive processes, whereby it regulates embryo implantation (Lim 1999, Reese 1999). In addition, recent advances are considering its potential uses in the treatment/prevention of strokes and other cardiovascular diseases (Cheng, 2002), and pulmonary hypertension (Wise 1996, Vachieri 2004), preventing thrombosis and the formation of vascular lesions (Wang, 1996). Finally, in the kidney it may be involved in ischemic renal disease (Yokoyama 2002) and chronic renal failure.

Figure 1.2. Diverse signaling options for prostanoids: paracrine, autocrine, and intracrine mechanisms. As illustrated prostanoids can elicit cellular responses by paracrine, autocrine, and intracrine mechanisms. PG production in a cell is dependent on COX and isomerase (PGS) colocalization: cytoplasm and/or perinuclear sub-compartments. This PG can then act on neighboring cells in a paracrine fashion by binding to cell surface G-protein coupled receptors and activate various second messenger systems: cAMP/PKA, calcium/DAG/PKC. Alternatively, binding to perinuclear PG receptors (eg. EP, DP, or PPAR) can influence nuclear responses and affect target gene transcription. Altogether, renal prostanoids regulate renal blood flow (RBF), glomerular filtration rate (GFR), renin secretion, glomerular and tubular growth, tubular transport processes, and cell fate.



The synthesis of PGI₂ along the nephron is quite significant. The highest regions are the glomeruli and the inner medulla, especially the IMCD. However the extent of contribution of each region to the overall renal PGI₂ synthesis varies among species. For example, in humans, the majority of PGI₂ is produced by the glomeruli, but in rodents the inner medulla prevails (Bonvalet 1987). The intermediate endoperoxides, produced by the action of COX, are acted upon by PGIS to produce PGI₂. Since it is a member of the cytochrome p450 family, PGIS is also known as CYP8A (Wang 1996). The PGIS cDNA were cloned from different species, and in rat it contains a 1503 bp open reading frame encoding a protein of about 500 amino acids (Tone 1997). The renal expression was lower than other tissues, but significant expression of PGIS mRNA was noted in inner medullary tubules and mIC by in situ hybridization.

Although many other studies indicate that PGI₂ is a major product of AA throughout the kidney, PGIS mRNA was not detected by RT-PCR in glomeruli or microdissected tubular segments of the rat nephron (Vitzthum 2002). The reasons for this discrepancy are not clear, but it is possible that other PGIS isoforms are present that were not detected with the primers utilized in this study. However, this is not the first report of such a nature, with species differences accounting for most of the variability in the literature. Keith et al (2002) demonstrated that disruption of PGIS in mice leads to a significant reduction in lung cancers, yet PGIS expression was insignificant in human lung tumours (Ermert 2003). In addition to PGIS, a PGI₂ releasing factor (PSF) has been identified in the glomerular vasculature (Ono 1998) regulating PGI₂ production by endothelial cells, and PSF levels in the renal vasculature are diminished in STZ-diabetic rats. Hata et al (2000) suggests that changes in PSF levels could account for biphasic

(early drop followed by later increase) alterations in retinal blood flow that are characteristic of diabetic retinopathy in STZ-rats. How PSF contributes to overall PGI₂ levels in the kidney, and PGI₂-mediated effects is not apparent at this time.

The importance of PGI₂ to renal function is clearly demonstrated by the phenotype of PGIS deficient mice (Yokoyama 2002), which resembles somewhat the renal abnormalities reported in COX-2 deficient mice (Dinchuk 1995, Morham 1995, Norwood 2000). Gross morphological assessment showed atrophy, surface irregularities and cysts in PGIS knockouts. As well, PGIS-deficiency induced renal fibrosis and vascular injury including arteriosclerosis and wall thickening in the kidney and aorta. Furthermore, fibrotic and necrotic lesions were distributed from the medulla to the cortex, along tubules and vessels, with increases in collagen IV. Renal fibrosis may have contributed to the enlargement of Bowman's space and defluxion of the renal tubular cells observed in the cortex of these mice. The fact that the renal phenotype closely resembles the one reported in the COX-2 KO further confirms the preferential association between COX-2 and PGIS/PGI₂ that is seen in the cardiovascular (Cheng 2002) and reproductive systems (Lim 1999).

PGI₂ is very labile and is rapidly metabolized into a nearly inactive product, PGF_{1α}. This rapid inactivation of PGI₂ has been a major limitation in the initial studies of PGI₂ biology, but several pharmacological analogues are now available, including cicaprost (CCP), which is a highly selective IP agonist, and iloprost (ILP), which is less selective owing to its potency for the EP receptors (Wise 1996). Several other formulations are currently in use, including beraprost sodium, UT-15, and taprostene, highly recognized for their improved stability (Clapp 2002, Olschewski 2004). For

instance, treprostinil infused intravenously has a half life of 34 min compared to the 2 - min half life of PGI₂, and is considered for the treatment of pulmonary arteriolar hypertension (Olschewski 2004). Despite their random use in various studies, new reports suggest that certain agonists can distinguish between the different IP receptors among species (Chow 2004). Thus care must be taken in comparing data between species, and the effects obtained from one agonist to another. Another shortcoming for the use of PGI₂ analogues is their ability to activate PPAR δ , with the exception of CCP (Hatae 2001). Other factors hindering certain studies, as with other PGs, are the lack of specific antagonists for the IP receptor and the unavailability of rodent antibodies that detect the receptor. Clark et al. (2004) just reported the development of a series of selective, high affinity IP receptor antagonists with analgesic properties in the rat, which will surely be useful in future studies once thoroughly characterized. In spite of these drawbacks, PGI₂ analogues have proven to be assets in the various studies investigating the mechanisms of action of PGI₂ in a plethora of biological processes, and there have been many advances linking PGI₂ to certain pathological events.

1.4.1. The IP receptor

PGI₂ elicits most of its cellular effects by binding to the cell surface IP receptor. The first IP receptor was cloned from mouse thymus in 1994 (Namba 1994) and shortly thereafter from rat (Sasaki 1994), human (Boie 1994), and other species. The cloned cDNA in rats comprises a 1248 bp open reading frame encoding a protein of 416 amino acids, molecular weight about 45 KDa (Sasaki 1994). The mRNA is a 3.7 kb transcript detected by Northern blotting. The rat and mouse IP receptors are 94% homologous in

their amino acid sequence, but IP has low homology with other prostanoid receptors. By northern blot analysis and in situ hybridization (ISH) in the mouse, IP mRNA was detected in the thymus, spleen, heart, lungs, and high levels in the vasculature and the brain (Namba 1994). While IP was not detected by northern blotting in the mouse kidney, it was localized by ISH in the vasculature and glomeruli (Oida 1995). Further studies also examined the expression of IP receptors in other species. However, within the kidney these findings remain controversial. For instance, high levels of IP mRNA are present in Tamm-Horsfall positive tubules (mTAL) of the rat outer medulla (Hébert 1998), but human studies indicate that both IP protein and mRNA are only found in non-mTAL tubules of the outer medulla (Komhoff 1998). In addition, this study localized the expression of IP receptors to specific structures within the human kidney. There was a great abundance within the vasculature throughout the kidney, mainly in the glomerular regions of the cortex, but very little in the inner medulla. While extensive work has examined the extra-renal expression of the rat IP receptor, to date very little is known about the expression of IP receptors in the rodent kidney. A study by Yamashita et al (2002) reports the expression of IP receptor protein in the rat afferent arterioles and glomeruli, but no reference is made to localization in other parts of the rat nephron.

1.4.1.1 Signaling pathways coupled to IP receptors

Like other PG receptors, IP is a G-protein coupled receptor, thereby activating intracellular signaling pathways. The main signaling linked to PGI₂ binding to IP is the stimulation of adenylate cyclase via coupling to G_s-protein, thereby increasing intracellular cAMP levels. To date there is no molecular evidence for the presence of IP

receptor subtypes or spliced variants. Nonetheless, various biochemical and functional studies in different tissues do suggest that they may exist. For example, functional studies in isolated perfused rabbit CCD showed that PGE₂ and iloprost inhibit AVP-dependent water transport by activating two different receptors (Hébert 1995), possibly through an “IP₃” subtype of the IP receptor (consistent with the EP₃ receptor). While in the rat IMCD PGI₂ stimulates cAMP but no inhibitory response was obtained (Veis 1990), the reverse occurred in the rat mTAL. Both ILP and CCP inhibited AVP-dependent cAMP stimulation, but no stimulatory response with these compounds was achieved (Hébert 1998). Also, using different PGI₂ analogues (not CCP), conflicting binding affinities were observed in the rat CNS, suggesting a different IP receptor subtype is located in specific regions of the brain such as the hippocampus (Takechi 1996). Furthermore, using two new CNS-specific IP ligands: 15R-TIC and 15-deoxy-TIC, studies indicate that an IP receptor subtype exists in the rostral region of the brain with different ligand specificity than the peripheral IP; and that signaling via this subtype does not involve cAMP or calcium (Sato 1999).

On the other hand, many studies indicate that IP receptors may be coupling to different G proteins and hence activating multiple signal transduction pathways. This is illustrated in the following studies. In cultured adipose cell lines, PGI₂ was shown to increase free calcium ion concentrations (Vassaux 1992). Furthermore, after cloning the mouse IP cDNA, it was expressed in Chinese Hamster Ovary (CHO) cells and found to increase both cAMP and inositol trisphosphate levels in these cells in response to ILP (Namba 1994). Also, Chu et al (2004) recently showed in CHO cells that IP-receptor mediated activation of extracellular signal-regulated kinases (ERK) 1 and 2 occurs via a

Gq/PKC dependent process. Similarly, in HEK-293 cells, reports of mouse and human IP receptor switching coupling between G_{i-} , G_{q-} , and G_s -protein were documented. But this differential G-protein-IP receptor coupling is dependent on protein kinase A (PKA) and protein kinase C (PKC)-mediated events (Miggin 2002).

1.4.1.2. Regulation of the IP receptor

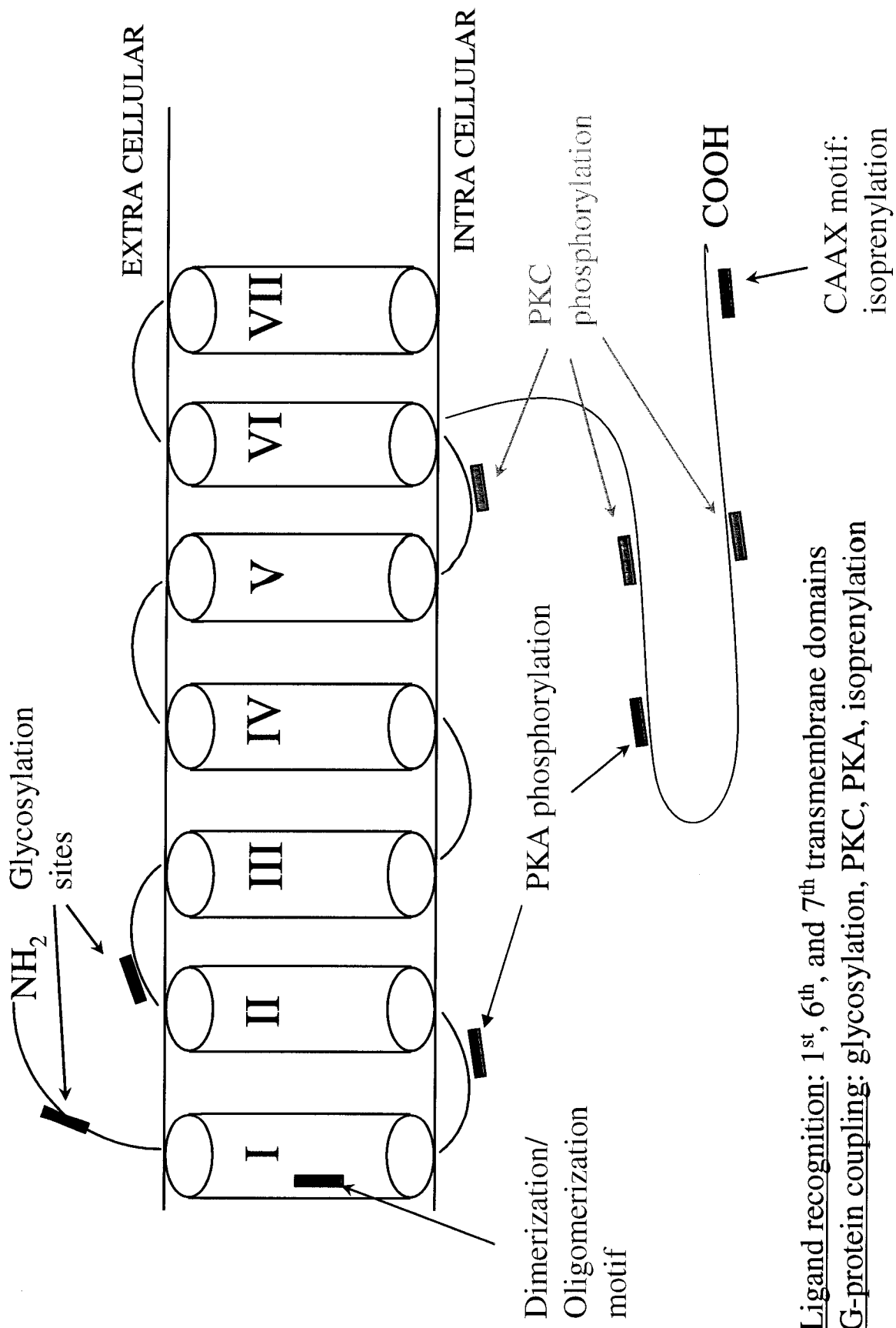
Several studies have examined the regulation of IP receptor function. Hayes et al (1999) showed that IP receptors from human and mouse contain conserved putative isoprenylation CAAX motifs in their COOH-terminus, and that isoprenylation does not influence ligand binding but is required for efficient coupling to adenylyl cyclase and phospholipase C. Another study by Smyth et al (1998) indicates that PKC-dependent phosphorylation of the human IP receptor is critical for homologous desensitization, uncoupling of receptor-G protein interactions and subsequent internalization for degradation or recycling. In contrast Sobolewski et al (2004) studied the mechanism of IP receptor desensitization in rat pulmonary artery smooth muscle cells. They showed that heterologous desensitization of CCP-cAMP (and to other $G_{\alpha s}$ -protein coupled agonists like bradykinin) occurred within 6 hrs of CCP exposure, and is mediated predominantly by a PKA-inhibitable isoform of adenylyl cyclase. Actually, the phosphorylation state of the IP receptor is an important determinant of G-protein coupling. For instance, the switching mechanism reported for mouse IP receptors suggests that IP-adenylyl cyclase activation induces PKA-dependent phosphorylation of the receptor on COOH-terminus tail serine 357 directing subsequent coupling to G_{q-} and G_{i-} proteins (Lawler 2001). In addition to phosphorylation, other post-translational modifications such as NH_2 -terminus

glycosylation are also central elements in the regulation of IP receptor function in humans (Smyth 1998), mice, and rats (Sasaki 1994). Another interesting feature of the human IP receptor is that it can form dimers/oligomers by forming disulfide bonds between four extracellular cysteine residues, in an agonist-independent fashion (Giguere 2004). A dimerization motif is located in the first transmembrane domain of the human IP receptor (Smyth 2002). The significance of dimerization to IP mediated signaling has not been clarified, but surely would account for some of the controversy with respect to PGI₂/IP effects in different cells or species differences observed. *The structure of the IP receptor and its putative sites of regulation are illustrated in Figure 1.3.*

1.4.1.3. The IP knockout

Murata et al. (1997) produced a targeted deletion in the IP cDNA to generate a recombinant mouse lacking the IP receptor. As expected, this mouse displayed certain characteristic features, including an increased susceptibility to pain, due to a recognized interaction between PGI₂ and the “serotonin” pathway in the brain. It was also more prone to thrombus formation, due to the potent anti-aggregatory action of PGI₂ on platelets, as well as enhanced inflammatory responses. However, these mice were normotensive and fertile, and displayed no obvious renal pathology. Upon further characterization, Yahata et al (1998) reported that IP KO mice exhibit salt sensitive hypertension and enhanced renin release following water deprivation, although COX-2 derived PGI₂ is recognized as a stimulator of renal renin secretion. The mechanisms underlying these observations need further investigation.

Figure 1.3. Structure of the IP receptor and its putative regulatory sites. This diagram shows the 7-membrane spanning domains of the IP receptor (I – VII), with an extracellular NH₂-terminus and intracellular COOH-terminus tail. Also depicted are the putative regulatory sites identified in IP receptors from mouse, rat and human studies. Two potential glycosylation sites are present in the NH₂-terminus and 1st extracellular loop. Potential phosphorylation sites for PKA and PKC are indicated in the 1st and 3rd intracellular loops and COOH-terminus tail. Also, IP receptors across species contain an isoprenylation motif in the COOH-terminus tail. Finally, a dimerization motif has been located to the 1st transmembrane domain of the human IP receptor. The 1st, 6th and 7th transmembrane domains confer ligand binding specificity, and G-protein/effector coupling is regulated by glycosylation, PKA and PKC phosphorylation, and isoprenylation.



The mild renal phenotype in the IP KO compared to the PGI₂-deficient mice (Yokoyama 2002) suggests that PGI₂ interactions in the kidney are a great deal more elaborate, and PGI₂ acting on IP-independent pathways like activation of PPAR δ or other signaling systems may be more important to certain aspects of renal pathology. This hypothesis requires further investigation, but surely will provide interesting insight into the interaction between these other pathways and the PGI₂/IP system in renal disease.

1.5. Diabetic nephropathy

FG Banting, CH Best, and JJR Macleod first showed in the early 1900's that insulin, a hormone that they extracted from the pancreas, was the most effective treatment for diabetes. For this, their work was awarded the Nobel Prize for Medicine in 1923. Diabetes is a general term, which encompasses two types of disease: defect in vasopressin biology (insipidus) or impaired insulin production/response (mellitus). Diabetes mellitus (DM) in turn comprises an insulin-dependent (IDDM) and non-insulin dependent (NIDDM) form of the disease, formerly referred to as juvenile- and adult-onset DM, respectively. IDDM or type I DM results from a destruction of the insulin-producing pancreatic β -cells via an autoimmune response. On the other hand, NIDDM or type II DM mainly results from insulin insensitivity and is highly linked to genetics and obesity. This latter type is the most common and accounts for over 90 % of diabetic cases (Encyclopaedia Britannica 2003).

The complications of diabetes are multi-faceted. It is linked to many other diseases: doubled chance of liver disease and liver cancer (El-Serag 2004), greater possibility of developing Alzheimer's and other forms of dementia (Arvanitakis 2004),

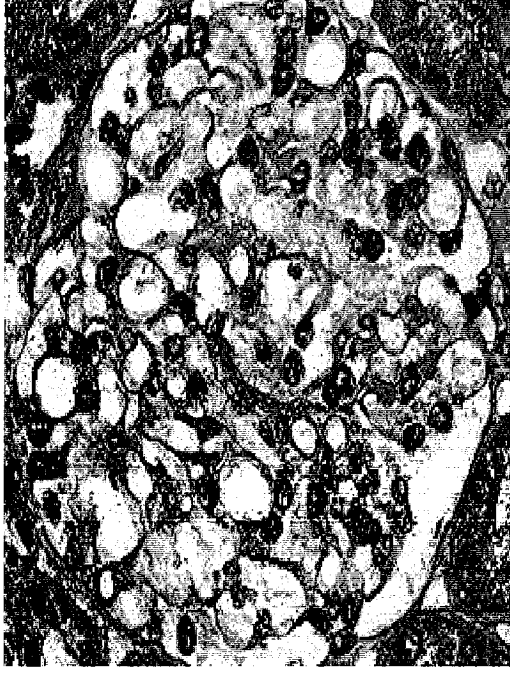
increased risk of cardiovascular disease: pulmonary hypertension, stroke/myocardial infarctions (Mazzone 2004), and is related to lower limb amputations (Duthois 2003), also leads to cataracts and blindness (Ruberte 2004). It is a major contributor to morbidity and mortality in industrialized nations.

Diabetes is also a leading cause of chronic kidney disease resulting in ESRD. Renal changes linked to diabetic nephropathy (DN) encompass glomerular, vascular, and tubulo-interstitial events. Kriz et al. (1998) provides an excellent overview of the proposed mechanisms underlying the histopathological features that link glomerular injury to tubulo-interstitial fibrosis. The renal complications include: an initial hyperfiltration, associated with a preferential vasodilatation of the afferent arteriole; also, hypertrophy of the glomerulus and tubules; expansion of the mesangial and tubular matrix (altered protein degradation and/or synthesis) leading to glomerular sclerosis and interstitial fibrosis; followed by tubular lesions and atrophy in later stages of the disease (recently reviewed by: Ziyadeh 2004). A recent review by M Nangaku (2004) emphasizes the role of tubulo-interstitial injury in progression to ESRD; a focus is made on the effects of filtered macromolecules and hypoxia as key injurious mechanisms. Also, Thomson et al. (2004) propose that the initial injury seen in the diabetic kidney is due to an “integrated system of parts rather than specific cellular mechanisms that comprise those parts”, suggesting that feedback mechanisms from the macula densa serve as a relay from the tubule to the glomerulus. *The histopathological changes in diabetic glomeruli are shown in Figure 1.4.* The clinical manifestations associated with diabetes-related chronic kidney disease include: proteinuria, metabolic acidosis, extracellular fluid (ECF) expansion (hypertension and edema), and hyperkalemia. To date the most

common management of the disease, aimed at slowing the development of diabetic complications and ESRD is decreasing the blood pressure (BP), normalizing glucose levels, diet/exercise (salt and protein restriction), ACE inhibitor/AT1 receptor antagonist (Canadian Diabetes Association 2003, American Diabetes Association 2003). But also many other targets are in trial, such as anti-TGF β antibodies (Ziyadeh 2004) and PKC β inhibitors (Koya 2000).

High glucose is the main determinant of the changes associated with the pathogenesis of DN. Its effects are mediated by several different mechanisms: polyol pathway, advanced glycation end products (AGE), stimulation of growth factors, activation of metabolic pathways, oxidative stress (generation of reactive oxygen species), and osmotic effects (Sheetz 2002). To add to this complexity, the induction of numerous downstream effectors of these pathways results in a vicious cycle of events, all uniting to perpetuate the extent of renal injury. Other factors are also important mediators of diabetic change, and are increased in response to high glucose. Activation of a renal RAS, TGF β , as well as PKC, is key to the glomerular and PT events that contribute to changes in GFR and tubular handling of solute and H₂O. This includes hypertrophic responses, accumulation of matrix proteins, fibrosis, epithelial-mesenchymal transition, etc...(recently reviewed by Liu 2004). To better appreciate the cellular interactions and contribution of these various systems, several rodent models of diabetes are in use, and have facilitated the study of the mechanisms underlying the injury to the kidney.

Figure 1.4. Histopathological features of diabetic glomeruli: key changes in mesangial cells. This picture compares A) a healthy glomerulus to B) diabetic nephropathy. As shown, there is prominent thickening of the basement membrane (BM), mesangial hypercellularity, diffuse mesangial matrix expansion, and mild nodular sclerosis. Periodic acid-Schiff stain, original magnification X400. This picture was adapted from [http:// www2.us.elsevierhealth.com/ajkd/atlas/34/5/atlas34_5.htm](http://www2.us.elsevierhealth.com/ajkd/atlas/34/5/atlas34_5.htm).



A



nodular sclerosis

mesangial
hypercellularity

B

BM thickening

mesangium
expansion

Adapted from: http://www2.us.elsevierhealth.com/ajkd/atlas/34/5/atlas34_5.htm

1.5.1. Diabetic animal models: streptozotocin-induced diabetes

In the literature, a number of models are utilized to mimic human diabetes, however the advanced renal pathological changes such as the presence of Kimmelstein-Wilson nodular lesions (Dolan 2003, Gross 2004, Sharma 2003) and renal failure do not occur in these animals. While some rodent models more closely display the progressive characteristics of the disease, these tend to require tedious genetic manipulations and maintenance, and can be quite costly. Examples of popular models include: 1) db/db mouse a model of type II DM (Sharma 2003), 2) the nonobese diabetic mouse (NOD) a hypertensive model of spontaneous type I DM (Maeda 2003), 3) spontaneously hypertensive obese rat (SHR/N-cp) which is a model of type II diabetes (Gross 2004), 4) BioBreeding spontaneously diabetic rat is a model of type I DM (Cohen 1987).

So far, the most popular method of inducing type I diabetes in rodents is the injection of streptozotocin (STZ). This compound induces a diabetic state by targeting the β -cells of the pancreas (Schlein 1974), thus inhibiting the secretion of insulin. The high recognition of this model stems from its low cost and low maintenance, even though the nephropathy per se is quite mild, with only moderate changes in GFR, mesangium expansion, and proteinuria. Even after extended periods, severe nephropathy does not occur. Gross and coworkers (2004) recently compared renal morphological changes in the STZ model of type I DM and the spontaneously hypertensive obese rat model of type II DM to their respective controls. They report only modest glomerulosclerosis and tubulointerstitial lesions in the 6-mth STZ-diabetic rats. In a study by Yong and Bleasel (1986), STZ-diabetic rats were maintained for up to 30 wks and still only sparse fibrinoid glomerular lesions were observed, though degenerative changes were found in the distal

tubule as early as 2 weeks following the onset of diabetes. In another study by Hirose et al. (1982), even after 18 mths of diabetes no advanced glomerular lesions were noted. Though the morphological aspects of the disease in STZ rats do not correspond to those seen in diabetic patients, biochemical studies indicate the presence of various pathological features such as activation of the local RAS (Burns 2000) in glomeruli and PT, increased levels of advanced glycation end products (Soulis-Liparota 1991), accumulation of reactive oxygen species (ROS) (Palm 2003), contribution of polyol pathway (Palm 2004), activation of PKC (Koya 1997), and elevated TGF- β (Young 1995, Makino 2002).

Since the renal injury induced by STZ-diabetes is quite mild, it is not uncommon for investigators to generate a more severe insult by superimposing other factors such as hypertension, obesity, nephrectomy, and ischemia. These artificial models have been shown to hasten the progression of kidney disease, resulting in more pronounced glomerulosclerosis and tubulointerstitial changes, a greater rise in GFR and more severe proteinuria. For example, Melin et al (1997) assessed the extent of kidney change in STZ-diabetic rats followed by 30 min of ischemia in the left kidney only. Compared to non-ischemic diabetic kidneys, there was a substantial loss of renal function by 8 wks, with extensive inflammation and tubulo-interstitial fibrosis, tubular atrophy and substantial loss of renal mass. The non-diabetic kidneys completely recovered from the ischemic insult, providing a useful model to study progressive kidney disease, which resembles human DN. Another recognized model is the unilateral nephrectomy-STZ rat first described by Steffes and coworkers (1978). While the extent of tubulo-interstitial nephropathy is not as severe as Melin's unilateral ischemia-STZ model, these rats do

display more pronounced glomerular disease than STZ-diabetes alone: hypertrophy, mesangium enlargement, hyperfiltration, and severe proteinuria.

1.6. Cyclooxygenases, prostaglandins, and diabetes

Various studies allude to the putative role of COX and PGs in DN. Certain investigators examined the beneficial effects of targeting specific PG pathways to alleviate the manifestations of the disease, including EP₁ receptor antagonists (Makino, 2002) and IP receptor agonists (Owada 2002, Koh 1999, Robles 1993). Also, TXA₂ synthase inhibitors have proven to be beneficial in both rodent and human models of DM (Tajiri 1994, Umeda 1995), suggesting that a change in TXA₂/PGI₂ ratios may be relevant to diabetic injury. But the underlying mechanisms of PG involvement remain uncertain. In diabetic kidneys, it has been clearly demonstrated that COX enzymes are elevated, and glomerular PG production in most species is increased, as well as in STZ-rats (Kreisberg 1983, Schambelan 1985, Komers 2001). Several pathways are stimulated by hyperglycemia (see figure A.1) that may alter the levels of AA (PLA₂ activity), COX, and PGs, including: activation of PKC (Porte 1996), p38 mitogen-activated protein kinase (Guan 1997, Cheng 1999), and IL-1 β (Guan 1998). Furthermore, inhibitors of COX-2 (NS-398) have been used to reverse some of the renal complications of STZ-diabetes, such as altered GFR, without affecting mean arterial pressure or renal plasma flow (Komers 2001). Interestingly, the COX-2 inhibitor NS-398 also prevented low-dose STZ-mediated destruction of pancreatic β -cells in mice (Tabatabaie 2000).

Since a great deal of the nephropathy in diabetes is due to alterations in the mesangial matrix, it is of great interest that PGs regulate MG cell function. For instance,

MG cell-derived PGE₂ and PGI₂ modulate the constrictor actions of hormones such as ANGII, norepinephrine, and AVP (Scharschmidt 1983). Furthermore, Ishibashi et al (1999) examined EP₁ and EP₄ levels in MG cells exposed to high glucose, and showed attenuation of PGE₂-dependent cAMP production. In addition, Mahadevan et al (1996) showed that PGE₂ contributes to mesangial hypercellularity in diabetes. Moreover, TXA₂ has been shown to increase MG cells fibronectin via PKC activation, a mechanism dependent on cGMP levels (Studer 1994).

1.6.1. The PGI₂/IP system in diabetes

There are many references to a defect in PGI₂ biology in diabetes. In a study by Funakawa et al (1983), suppression of the circulating RAS in 8 wk STZ-diabetic rats was related to PGI₂ biosynthesis. Reductions in PGI₂ synthesis are notable in the vasculature; Ono et al. (1998) describes a significant reduction in the levels of PSF in the renal vasculature of diabetic rats, consistent with reports of diminished circulating PGI₂ in diabetes (Harrison 1978, Silberbauer 1980). Also, several investigators suggest that the interaction between the PGI₂/IP system and other pathways may be relevant in DM. Yamashita et al (2002) showed that chronic beraprost sodium administration reduced the glomerular hyperfiltration in 4 wk STZ-diabetic rats by attenuating endothelial cell nitric oxide synthase (ecNOS) expression in afferent arterioles and glomeruli. Likewise, a study by Itoh et al (2001) reported a role for PGI₂ in reducing endothelin-1 (ET-1) levels in STZ-diabetic rats, and its relevance to the prevention of disease progression. Similarly, PGI₂ analogues were tested and their renal benefits compared to ACE inhibitors (Villa 1997). However, to date the role of PGI₂/IP in MG cells has not been elucidated, nor its

contribution to changes in diabetes; and the levels of IP receptors in the diabetic kidney have not been investigated.

1.7. Hypothesis, rationale, and objectives

We hypothesize that distinct IP receptors are expressed throughout the rat nephron, mediating the signaling responses to PGI₂; and a disturbance in the PGI₂/IP system contributes to mesangial cell changes characteristic of diabetic injury.

This hypothesis will be addressed by five main objectives:

- 1) While biochemical and functional studies indicate that IP receptors are present in the rat kidney, to date the renal distribution has only been performed in other species (human, mouse, rabbit). Thus the first objective of this work examines the intrarenal expression of IP mRNA: on rat kidney tissue sections by in situ hybridization; in different kidney tissue preparations by RT-PCR: cortex (C), outer medulla (OM), inner medulla (IM), IMCD (fresh vs. cultured), MG cells (primary vs. immortalized), PT (fresh vs. cultured); in different regions of the kidney: C, OM, IM and in cultured rat MG cells by Northern blotting. And to determine the distribution of IP protein by Western blotting in various tissue preparations: C, OM, IM, IMCD, MG, PT, and spleen.
- 2) Multiple signaling options have been described for PGI₂, but its actions on the IP receptor are well documented, mainly coupling to G_s-protein to increase cAMP. However, the signaling pathways coupled to this interaction in the rat kidney

require further clarification since species and cell-type specific differences have been noted: activation of G_i - and G_q -protein coupled signaling pathways. Therefore the purpose of the second objective is to determine whether alternatively spliced forms or subtypes homologous to the cloned rat IP cDNA can be identified by RT-PCR followed by TA cloning and sequencing, in different segments of the nephron: cortex, OM, IM, mTAL, IMCD, MG. And to characterize the signaling pathways linked to the PGI_2 /IP system in renal cells (IMCD, MG, PT): cAMP stimulation/inhibition, and calcium; using different PGI_2 analogues (CCP and ILP).

- 3) The glomerulus is a major site of renal PG synthesis. Though the paracrine and autocrine regulation of glomerular function by PGI_2 has been proposed, to date the role of the PGI_2 /IP system in MG cells has not been determined. Since MG cell change is involved in diabetic injury to the glomerulus, alterations in the PGI_2 /IP system may contribute to the pathogenesis. Therefore the third objective of this work is to clarify the effect of 24 hour CCP on MG cell function related to diabetes: p27 levels, matrix synthesis (fibronectin) and degradation (MMP-2); and determine whether CCP stimulation alters MG cell growth responses: thymidine incorporation (DNA synthesis) and leucine incorporation (protein synthesis).
- 4) Glucose is a main determinant of diabetic renal injury, but other glucose-dependent factors (PKC, ANGII, MAPK, and $TGF\beta$) are also involved. These compounds have been shown to alter AA release, COX expression, and PG levels.

But their effects on IP mediated signaling have not been investigated. Therefore the fourth objective of this study is to determine the effects of high glucose on the expression of PG synthesis enzymes (COX-1, COX-2, PGIS) in MG cells, as well as COX-1, COX-2, and PGES in cultured IMCD cells; and the levels of PGI₂ and PGE₂ in high-glucose treated IMCD. Furthermore, to determine if signaling via the IP receptor is altered upon exposure of MG cells to a high glucose environment, and changes in IP receptor protein and mRNA expression (primary cultures versus immortalized cells). Also, to examine the effect of high glucose on EP₁ and EP₄ receptors in cultured IMCD. And finally, to determine the effect of various glucose-dependent factors (ANG, TGFβ, PKC, MAPK) on CCP-IP-cAMP responses in immortalized rMG cells.

- 5) No major renal abnormalities have been observed in IP-receptor deficient mice suggesting that the PGI₂/IP system may be more relevant in a disease state. Numerous studies allude to a decreased bioavailability of PGI₂ in the diabetic milieu, but they fail to establish a defect in PGI₂/IP mediated effects and downstream mechanisms. Therefore the last objective is aimed at studying the expression of IP receptors in different kidney regions of STZ-diabetic rats, at different stages of diabetes (2, 4, 6, 8, 12, and 16 wks), by Northern blot analysis; and in 6-mth uni-nephrectomized STZ-diabetic rats by RealTime RT-PCR.

1.8. Significance of research

Diabetes is threatening to reach epidemic proportions, thus finding a unified approach to study its pathogenesis, and the common link between the various complications of the disease is bewildering scientists all over the world. About 30-40% of diabetic patients progress to ESRD. Current modes of therapy are ineffective in preventing this course, without much success in providing a means for a cure.

The balance between vasodilator and constrictor hormones plays an important role in determining various aspects of renal function: renal hemodynamics and tubular transport, as well as the outcome of renal disease. The antagonistic relationship between PGI₂ and vasoconstrictors such as TXA₂ is well recognized in the cardiovascular system, contributing to both cardiovascular and pulmonary disease. If such a relationship exists between PGI₂ and vasoconstrictors in the kidney, a disruption of this balance can underlie the changes seen in diabetic nephropathy. There is ample evidence suggesting that alterations in both TXA₂ and PGI₂ biology is implicated in the pathogenesis of diabetic nephropathy, however a direct outline of the role of the PGI₂/IP system in the specific renal changes has not been established, especially with respect to the mesangial cell changes that alter glomerular function. Therefore it is essential to examine the role of the PGI₂/IP receptor system in mesangial cells and determine how this system is altered in the diabetic environment. Thus a defect in the PGI₂/IP system could be rectified to oppose the damaging effects of many injurious diabetic factors. Hence the completion of this work should shed some light on the usefulness of specifically targeting IP receptors to shift an imbalance between vasodilators and constrictor hormones that influence the evolution of diabetic nephropathy and other chronic kidney diseases.

1.9. Outline of research approach

The research presented in this thesis consists of 4 published manuscripts, each dealing with characterizing the PGI₂/IP system in the normal kidney and/or the diabetic environment. The first manuscript (chapter 2) examines the distribution of the IP receptor mRNA along the rat nephron, and characterizes the signaling responses to IP receptor agonists in cultured and freshly isolated IMCD. The second manuscript (chapter 3) deals with the expression of COX-1, COX-2, and PGES in STZ-diabetes, and examines the synthesis of PGE₂ and PGI₂ in high-glucose treated IMCD, as well as changes in EP₁₊₄ receptors. The third manuscript (chapter 4) describes the expression of COX and PGIS in response to prolonged high glucose in cultured primary MG cells, and the effect of prolonged high glucose on IP receptor expression and signaling. Finally the fourth manuscript (chapter 5) outlines the role of the PGI₂/IP system in transformed MG cells, examines the effect of 24 hr glucose on IP-receptor mediated signaling and IP mRNA and protein expression, as well as the expression of IP mRNA in STZ-diabetic rats.

CHAPTER 2

Molecular and Biochemical Characterization of Prostacyclin Receptors (IP) in the Rat Kidney

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Rania Nasrallah wrote the manuscript. Rania Nasrallah performed all the experiments except the IMCD microdissections, which were performed by Joseph Zimplemann. Sonia Singh assisted with the cAMP assays, and Joseph Zimplemann assisted with the calcium measurements.

**MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF
PROSTACYCLIN RECEPTORS (IP) IN THE RAT KIDNEY**

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Running Title: Prostacyclin expression in rat IMCD

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ABSTRACT

The IP message was detected by RT-PCR in the renal cortex, outer (OM) and inner medulla (IM), and in IMCD-f (freshly isolated) and IMCD-c (cultured); also the EP_{1,3,4} receptor subtypes, but not EP₂. Digoxigenin ISH localized IP mRNA in the tubules of the OM and IM, and the vasculature. Also in the glomeruli, arteries and tubules of the cortex. IP splice variants or subtypes could not be detected by RT-PCR followed by TA cloning, though several non-functional point mutations or single base pair deletions were observed. Iloprost (ILP), cicaprost (CCP), PGE₂ and arginine-vasopressin (AVP) stimulated cAMP on both IMCD preparations. In addition, AVP-stimulated cAMP in IMCD-f was inhibited by all three prostanoids, but not in IMCD-c. Calcium experiments were performed on IMCD-c or microdissected IMCD (IMCD-m). CCP, ILP, and PGE₂ did not alter [Ca⁺⁺]_i in IMCD-c. However, on IMCD-m, both PGE₂ and ILP increased [Ca⁺⁺]_i levels equipotently and CCP had no effect. Pre-treatment with the EP₁ antagonist AH-6809 indicates that the response to ILP and PGE₂ is mediated via EP₁. These results suggest that IP receptors in the rat IMCD mediate the cAMP but not calcium signaling linked to PGI₂, to date no subtypes or splice variants were identified.

KEYWORDS: cAMP measurements, in situ hybridization, intracellular calcium, IP receptor, prostacyclin, rat inner medullary collecting duct, RT-PCR, TA cloning

INTRODUCTION

Comparable to PGE₂, prostacyclin is a major product of the arachidonic acid cascade within the kidney (3). Its production is dependent on the action of cyclooxygenases, as well as prostacyclin synthase. This enzyme converts the intermediate endoperoxides into prostacyclin in the cyclooxygenase pathway (35). Since its discovery in 1976 (32), it has been implicated in the maintenance of homeostatic functions and the pathogenesis of many diseases, and plays various roles in different organ systems. Although initial studies to investigate the role of prostacyclin were limited by the fact that it is quite labile and rapidly metabolized into 6-keto-PGF₁, many pharmacological analogues are now available to facilitate these endeavors (36). The most common of these are iloprost (ILP) and the more selective IP agonist cicaprost (CCP). Like other members of the prostanoid family, prostacyclin elicits its effects by binding to G-protein coupled cell surface receptors, IP receptors (6, 16). However, activation of other signaling pathways has been observed in many studies. For instance, in cultured adipose cell lines, prostacyclin was shown to increase free calcium ion concentrations (33). Furthermore, after cloning of the mouse IP cDNA, it was expressed in Chinese Hamster Ovary cells and found to increase both cAMP and inositol trisphosphate levels in these cells in response to ILP (20). Moreover, in the rat kidney, multiple signaling is observed in different segments of the nephron. While in the rat IMCD prostacyclin stimulated cAMP but no inhibition of AVP-dependent cAMP stimulation was obtained (34), the reverse occurred in the rat mTAL: both prostacyclin analogues, ILP and CCP, inhibited

AVP-dependent cAMP stimulation, but no stimulatory response with these compounds was achieved (11)

In 1994, the IP cDNA was cloned from mouse (20), human (2), and rat (25). IP mRNA was detected in several tissues using various molecular biology techniques. By Northern blot analysis and in situ hybridization in the mouse, the message was detected in the thymus, spleen, heart, lungs, and high levels in the vasculature and the brain (20). While IP was not detected by Northern blotting in the mouse kidney, it was localized by in situ hybridization in the vasculature and glomeruli (22). Further studies also examined the expression of IP receptors in other species (11, 16). However, within the kidney these findings are controversial. For instance, using ISH on rat tissue sections and RT-PCR, it was shown that high levels of IP mRNA are present in Tamm-Horsfall positive tubules of the outer medulla. This is a specific protein expressed exclusively in the mTAL, hence serves to identify this segment (11). In contrast to findings in the rat, human studies using both ISH and immuno-histochemistry with an antibody to detect the IP protein, showed that IP receptors, both mRNA and protein, are only found in Tamm-Horsfall negative tubules of the human outer medulla (16). In addition, this study localized the expression to specific structures within the human kidney. There was a great abundance within the vasculature throughout the kidney, mainly in the glomerular regions of the cortex, but very little in the inner medulla. While extensive work has examined the extra-renal expression of the rat prostacyclin receptor, to date very little is known about the expression of IP receptors in the rodent kidney. Moreover, to date there is no molecular evidence for the presence of IP receptor subtypes or spliced variants. Nonetheless, various biochemical and functional studies indicate that prostacyclin and PGE₂ activate

different receptors, even suggesting an "IP₁" and "IP₃" subtype (10) of the prostacyclin receptor (consistent with the EP receptors). Altogether, it remains controversial by which processes IP is evoking these various effects. Whether it is acting through different IP receptor subtypes or splice variants, coupling to different G proteins, or simply acting through EP receptors or other prostanoid receptors, remains to be determined. The main hypothesis of this work is that distinct IP receptors mediate the renal effects of prostacyclin in the rat kidney. Therefore, in order to gain further insight into the role of prostacyclin along the length of the rat nephron, this study examines the renal distribution of the prostacyclin receptor mRNA using both RT-PCR and in situ hybridization. Also, the possibility that prostacyclin receptor subtypes or splice variant can be detected in different regions of the rat kidney was explored. Moreover, the cAMP and calcium signaling in response to prostacyclin analogues in the rat IMCD was assessed.

MATERIALS AND METHODS

Tissue preparation:

A bilateral nephrectomy was performed on male Sprague-Dawley rats (175-200 g), and the kidneys were immediately placed in a beaker on ice, containing 300 mOsm Krebs-ringer buffer pH 7.4, composed of: 118 mM NaCl, 14 mM glucose, 25 mM NaHCO₃, 4.7 mM KCl, 1.8 mM MgSO₄ (7H₂O), 1.8 mM KH₂PO₄ and 2.5 mM CaCl₂. A small superficial incision was then made into each kidney and the renal capsule was removed. Under a dissecting microscope, on ice, the kidney was divided into 5 transverse slices each 5 mm thick. Using high intensity light, each region of the kidney was then separated with dissecting scissors based on colour differences between the various regions: brownish cortex, pinkish outer medulla, and whitish inner medulla. The tissue was placed into Petri dishes on ice containing Krebs buffer. Following the dissection, the Krebs solution was poured off the fragments of tissue, and discarded. The tissue was then homogenized and RNA was isolated using the Trizol method (Gibco-BRL).

IMCD isolation:

The kidneys were cut in half longitudinally, and the inner medulla/papilla (white region) was dissected out and placed in a Petri dish on ice, containing Krebs solution (as small a volume as possible). The tissue was minced using a one-sided razor blade and transferred to a 50 ml sterile polypropylene tube containing 20 ml of: 0.05% (w/v) collagenase A (Boehringer Mannheim) and 0.01% (w/v) DNase I (Boehringer Mannheim). The tissue was then digested for 1.5-2 hrs at 37°C, in bubbling 5% CO₂/air.

Following the digestion, the tubule suspension was centrifuged at 800 rpm for 2 min, washed in Krebs buffer (x3), and placed in a hypo-osmotic solution (1:4, Krebs buffer: H₂O) for 3 min, while continuously inverting the tube gently. The combination of collagenase digestion and osmotic shock destroys all the cells in the inner medulla (i.e. medullary interstitial cells, vasa recta) with the exception of the IMCD (15, 37). The suspension was then centrifuged once more as described above, and the IMCD pellet was collected. Previous immunocytochemical analysis of these cells in our laboratory, using specific antibodies for the detection of principal and α -intercalated cells, and peanut lectin agglutinin β -intercalated cells, revealed that all three collecting duct cell types were present in this preparation, with the majority (over 50%) of cells being of the principal cell type (data not shown).

IMCD culture:

After having obtained the IMCD pellet as described above, the pellet was resuspended in the desired volume of DMEM-F12 culture media (Gibco), pH 7.4, containing: 10% fetal bovine serum (Gibco-brl), 1% penicillin-streptomycin-fungizone (Gibco-brl), 5 μ g/ml insulin (Sigma), 5 μ g/ml transferrin (Sigma), 5 ng/ml selenium (Sigma), 2.5 nM Triiodothyronine/sodium salt (Sigma), and 50 nM hydrocortisone (Sigma). The rat IMCD cells were plated on Petri dishes and grown for 3 days at 37°C and 5% CO₂. Prior to the experiments, once the cells have reached 90-100% confluence, they were serum deprived overnight.

RNA isolation and RT-PCR:

Total RNA was isolated from the various tissues using the Trizol method, as described by the manufacturer (Gibco-brl); and was DNase (Boehringer Mannheim) treated to eliminate genomic DNA. 3 μ l containing 1 μ g of DNase-treated RNA was used for each sample. The RNA was reverse transcribed into cDNA using MuLV reverse transcriptase and random hexamers, provided in the Gene-AMP RNA PCR core kit (Perkin Elmer). Samples were prepared in duplicate for each reaction, the duplicates serving as controls for the reverse transcription since the reverse transcriptase was omitted in these tubes. The upstream and downstream primers used for PCR amplification of each cDNA are: IP receptor (407 bp; nucleotides 856-1263) FF-1 5'-GGCACGAGAGGATGAAGTTTACC-3' and FF-2 5'-GTCAGAGGCACAGCAGTC-AATGG-3'; EP₁ receptor (336 bp; nucleotides 865-1201) EP_{1,1} 5'-CGCAGGGT-TCACGCACACGA-3' and EP_{1,2} 5'-CACTGTGCCGGGA ACTACGC-3'; EP₂ receptor (401 bp; nucleotides 757-1158) EP_{2,1} 5'-AGGACTTCGATGGCAGAGGAGAC-3' and EP_{2,2} 5'-CAGCCCCTTACTTCTCCAATG-3'; EP₃ receptor (437 bp; nucleotides 538-975) EP_{3,1} 5'-CCGGGCACGTGGTGCTTCAT-3' and EP_{3,2} 5'-TAGCAGCAGATAAA-CCCAGG-3'; and EP₄ receptor (423 bp; nucleotides 941-1364) EP_{4,1} 5'-TTCCGCTCGT-GGTGCGAGTGTTTC-3' and EP_{4,2} 5'-GAGGTGGTGTCTGCTTGGGTCAG-3' (1). The DNA amplification was performed using a thermal cycler, the Perkin Elmer Gene-AMP PCR System 2400. Depending on the primers used, the PCR reaction consisted of the following parameters: denaturing at 94°C for 2 min; followed by 35 cycles of: denaturing at 94°C for 30 sec, annealing at 63°C for 45 sec, and extension at 72°C for 60 sec; extension at 72°C for 10 min, and finally cool-down to 4°C. The given amplification

products are then separated by gel electrophoresis, on a 1.5-2% agarose gel, for size determination with standards, and visualized under UV light using ethidium bromide.

Endonuclease restriction digest:

In order to verify the identity of the amplified fragment of the prostacyclin receptor, endonuclease digestion was performed. The rat prostacyclin receptor contains an NcoI restriction site in its COOH-terminus tail, located within the 407 bp product amplified using the IP primers mentioned above. This endonuclease generates two fragments from the IP cDNA fragment: 256 bp and 151 bp. In order to purify the amplified PCR product prior to digestion, the 407 bp band was isolated from an agarose gel using the BIO 101 GeneClean kit, which consists of separating the DNA from agarose using NaI, and then binding the DNA to a glassmilk matrix, followed by an elution with H₂O. The restriction digest was carried out by incubating the restriction enzyme with the sample at 37°C for 90 min. The digested products were then separated on a 3% agarose gel. Since the EP₃ receptor PCR product amplified does not contain a restriction site for this enzyme, it was used as a negative control for the digest.

In situ hybridization:

The kidneys were removed from male Sprague-Dawley rats and immediately frozen with CO₂ powder on dry ice, and then stored at -80°C until needed. In order to localize the expression of the prostacyclin receptor within the rat kidney, longitudinal rat kidney cryosections, 8-10 μm thick, were fixed in 4% paraformaldehyde and treated with proteinase K and 0.1% active DEPC (diethyl-pyrocabonate). A 40 bp sense and anti-

sense oligonucleotide sequence, 5'-AAGTTCTGGTTGTGTTGTCTGTGTGCCCGTT-CTGTCCATG-3', with no homology to any known rat kidney mRNA (Geneblast), was selected. This corresponds to a region following the 7th trans-membrane domain in the C-terminus of the prostacyclin receptor cDNA. Using a non-radioactive labelling method, the oligoprobe was labelled using the digoxigenin (DIG) oligonucleotide tailing kit (Boehringer Mannheim). The hybridization consisted of incubating the tissue sections with DIG labelled sense and anti-sense probes for 18 hrs at 42°C. The hybridization buffer contains: 25% formamide, 5x SSC (saline sodium citrate buffer), Tris-HCl (pH 7.5), Denhardt's solution, sodium dodecyl sulphate, and DEPC-H₂O. Following the hybridization, the sections were washed twice in 2x SSC for 10 min, and then in 0.5x SSC at 55°C for 15 min, and finally in 0.5x SSC at room temperature for 15 min. Afterwards, DNA-RNA hybrids within the kidney section were visualized using the DIG nucleic acid detection kit (Boehringer Mannheim), which consists of incubating the hybridized tissue sections with an anti-alkaline phosphatase antibody-conjugate for 2 hrs. Detection is based on an enzyme catalyzed colorimetric reaction between the anti-DIG alkaline phosphatase antibody-conjugate and the NBT/BCIP substrate for 16 hrs, which forms a bluish/brown precipitate. This signal can then be seen using light microscopy.

TA cloning and sequencing:

Total RNA from different regions of the kidney was obtained as described above. This includes the cortex, outer medulla, inner medulla, mTAL and IMCD. In order to determine whether IP receptor subtypes or spliced variants can be identified in any of these regions, fragments of the rat IP cDNA were amplified by RT-PCR, purified, then cloned and sequence analyzed. By this method, any sequence with some homology to a region of the IP cDNA, can be detected. Briefly, four sets of primers were selected spanning the entire length of the rat prostacyclin receptor cDNA. The primer map is shown in figure 1. Each of these primer sets, and combinations thereof, were used to amplify various fragments along the entire cDNA, within each region.

Following separation of the fragments by gel electrophoresis, the desired band was excised and purified from the agarose using glassmilk (as described above). Next, 5-10 ng of the DNA was incubated for 18 hrs at 14°C with T₄ DNA ligase, which ligates the DNA fragment into pCR2.1 vector using the TA cloning method (Original TA cloning kit, Invitrogen). Also, specific features of the pCR2.1 vector facilitate the insert analysis: ampicillin resistance gene for selection and maintenance in *E. coli.*, and T7 promoter and M13 Reverse priming sites for sequencing of the insert. Using a heat shock technique, TOP10F' competent cells (a strain of *E. coli.*), were transformed with plasmids containing the given insert. Next, they were placed for 1 hr at 37°C with continuous shaking at 225 rpm. Aliquots were plated on ampicillin-coated agar plates for selection of positive colonies using blue-white screening. After which the plasmid DNA was purified and sequenced using a dye-chemistry sequencing method.

Figure 2.1. Primer map for amplification of IP receptor cDNA fragments. Illustration of the various sets of primers used for amplification of different fragments spanning the entire length of the IP receptor cDNA. The IP cDNA is represented in black and a color scheme is utilized to identify individual sets of primers and their location along the cDNA. The various products obtained for each set, as well as combinations thereof, are shown.

- 1.1 (10-33)
- 1.2 (462-483)
- 2.1 (448-471)
- 2.2 (939-960)
- 3.1 (917-939)
- 3.2 (1318-1341)
- FF-1 (856-878)
- FF-2 (1241-1341)

NH₃ ————— COOH

— 1.1 — 1.2
— 2.1

— 3.1 — 2.2
— FF-1

— 3.2 — FF-2

————— 474 bp

————— 513 bp

————— 425 bp

————— 407 bp

————— 950 bp

————— 893 bp

————— 1331 bp

————— 1255 bp

————— 817 bp

————— 345 bp

————— 485 bp

cAMP radioassays:

For experiments on cultured IMCD, cells were grown to confluence in 24 well plates for 3 days, and serum starved 24 hrs. The cells were then pre-treated for 15 min in DMEM-F12 containing 0.5 mM IBMX (Sigma) and 10 μ M indomethacin (Sigma). At timed intervals, the cells were then stimulated with 1 μ M to 0.1 nM of: arginine-vasopressin (AVP), PGE₂, iloprost (ILP), and cicaprost (CCP). The samples were all prepared in duplicates. To stop the reaction, 300 μ l of 10% trichloro-acetic acid (TCA) was added to each well. Following a 30 min incubation on ice, the samples in TCA were transferred to eppendorf tubes and centrifuged for 10 min at 4000g. Next, 250 μ l of each sample was transferred to glass test tubes and four ether extractions of TCA were performed using 4 times the volume of H₂O-saturated diethyl-ether per extraction. 1 M Tris-HCl was used to bring the pH of the samples to 7-8. Using the cAMP radioassay kit (Intermedico), cAMP levels in each sample were then measured in 100 μ l of sample according to the manufacturer's instructions. For experiments on freshly isolated IMCD, the final IMCD pellet obtained was resuspended in an appropriate volume of DMEM-F12 containing IBMX and indomethacin (as above), ensuring 180 μ l per sample in addition to 2 samples for protein determinations. In the second set of experiments, in order to verify the ability of the various agonists to inhibit the AVP-dependent increase in cAMP, the cells were pre-treated for 15 min with the above-mentioned concentrations of agonists; and then treated with 0.1 μ M AVP, in the presence of the agonist, for 7 min.

Calcium measurements:

IMCD cells, isolated from 175-200 g Sprague-Dawley rats, were cultured on round coverslips for 3 days, and serum starved overnight. The cells were then loaded for 45 min with the calcium indicator fura-2. The dual-wavelength deltascan 1 spectrophotometry system was used to measure changes in calcium levels within the cells. The tracings were monitored by computer using the Felix software. The technique is based on a shift in the excitation/emission wavelength of fura-2 upon binding of the dye to the ionic species (calcium), therefore the calcium concentration can be determined based on changes in the ratio of the dye's fluorescence intensity at the two wavelengths: 340 and 380 nm. Different agonists were applied to the cells using a 450 mOsm solution containing 0.1% albumin (w/v) and (in mM): 190 NaCl, 25 NaHCO₃, 5 KCl, 1.2 MgCl₂, 8 glucose, 5 HEPES, 10 Urea, 1.5 CaCl₂, and 5 NH₄Cl; and cellular responses were assessed. The compounds tested were 0.1 μ M of: AVP, PGE₂, ILP, CCP, angiotensin IV, endothelin, ATP.

The second set of experiments consisted of micro-dissecting IMCD from the kidneys of 75 g male Sprague-Dawley rats. The single tubule isolated was then inserted at each end into glass pipets and was perfused lumenally with the solution mentioned above (9). Afterwards, the tubule was loaded with fura-2 for 30 min prior to stimulation with the desired agonists. The compounds of interest, namely 10 nM of PGE₂, ILP, CCP, and AVP were applied to the basolateral surface of the tubule and the cellular responses were measured.

Finally, to determine whether the calcium effect obtained with iloprost is mediated by the IP or EP₁ receptor, the tubules were pre-treated for 3 min with AH-6809,

an EP₁ receptor antagonist. Next 10 nM of PGE₂ or ILP were added to the tubule in the presence of the antagonist, and the calcium response was assessed.

Statistics:

Experiments were performed using duplicate samples and repeated 3-6 times each. The SigmaPlot software for windows version 4.01 (1986-1997) was used for data analysis. Results are expressed as mean \pm SEM.

RESULTS

Detection of IP mRNA by RT-PCR:

Since prostacyclin is a major renal product of the arachidonic acid cascade, and very little is known about its role in the kidney, this study localized the expression of prostacyclin receptors within different regions of the kidney, in order to understand the renal effects of prostacyclin. As shown in figure 2, IP mRNA is detected in all three regions of the kidney: cortex, outer medulla, and inner medulla. The expression pattern of the four different EP receptor subtypes was also examined in these tissue preparations. While EP₁, EP₃, and EP₄ mRNA was amplified from all three kidney regions, EP₂ receptor mRNA was not amplified even by altering PCR conditions: increasing cycle number, decreasing annealing temperatures, varying the amount of starting RNA. Next we isolated total mRNA from preparations of cultured IMCD, freshly isolated IMCD tubules, and mTAL. Once again, the IP receptor was detected in all three cellular preparations; and with the exception of EP₂, consistent with previous findings (5), the aforementioned EP receptors were also found as shown in figure 3.

Analysis of 407 bp PCR product:

In order to verify the identity of the IP product amplified by RT-PCR, endonuclease digest and sequencing analysis were performed. Since the 407 bp IP fragment amplified contains a restriction site for the restriction enzyme NcoI, we performed a restriction digest and found that indeed the expected digest products were obtained: 256 and 151 bp, as shown in figure 4. As expected, the absence of an NcoI

restriction site within the EP₃ product served as a negative control for the digest. Also, we used TA cloning to isolate and express the 407 bp fragment in a plasmid, which was then transfected into bacteria, and grown. Then the DNA was purified and sequenced. Indeed the product amplified corresponds to the published sequence for the rat IP receptor cDNA (data not shown).

Localization of renal IP mRNA by ISH:

In addition to the RT-PCR experiments, the expression of the IP receptor within the kidney was analysed by in situ hybridization. Using a digoxigenin-labelled oligoprobe, the IP message was detected on rat kidney tissue sections as indicated in figure 5. First, the IP mRNA is present throughout the kidney: cortex, outer medulla, and inner medulla. Although the most intense signals were found within the tubules of the outer medulla, diffuse tubular staining was also visible within the cortex, and inner medullary regions. In the cortex, glomerular staining is obvious as indicated by the arrow, with intense signals in the vasculature. These results are consistent with previous findings in various species. For instance, in the mouse, ISH revealed intense IP signals within the renal vasculature (22). Also, in the rat, high levels of IP mRNA were detected in the mTAL of the outer medulla (11). And finally, in human kidney, IP expression was found in all regions, with staining in the vasculature, glomeruli, Tamm-Horsfall-negative distal tubules, and the collecting ducts (16).

Figure 2.2. Detection, by RT-PCR, of EP and IP receptor mRNA, in different rat kidney tissue preparations. Gel electrophoresis of the amplified RT-PCR products, from DNase-treated total RNA of different rat kidney tissue preparations: A) cortex, B) outer medulla, and C) inner medulla/papilla. The sets of primers for the four EP receptor subtypes and prostacyclin receptor were used. Lane number: 1-100 bp DNA ladder, 2-IP (407 bp); 3-EP₁ (336 bp); 4-EP₂ (401 bp); 5-EP₃ (437 bp); 6-EP₄ (423 bp). n = 4 – 5.

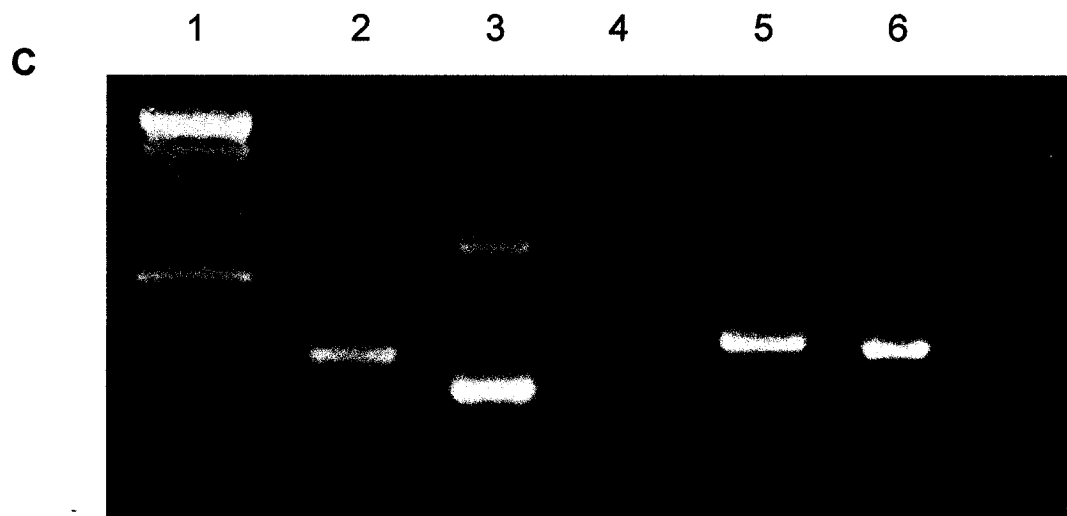
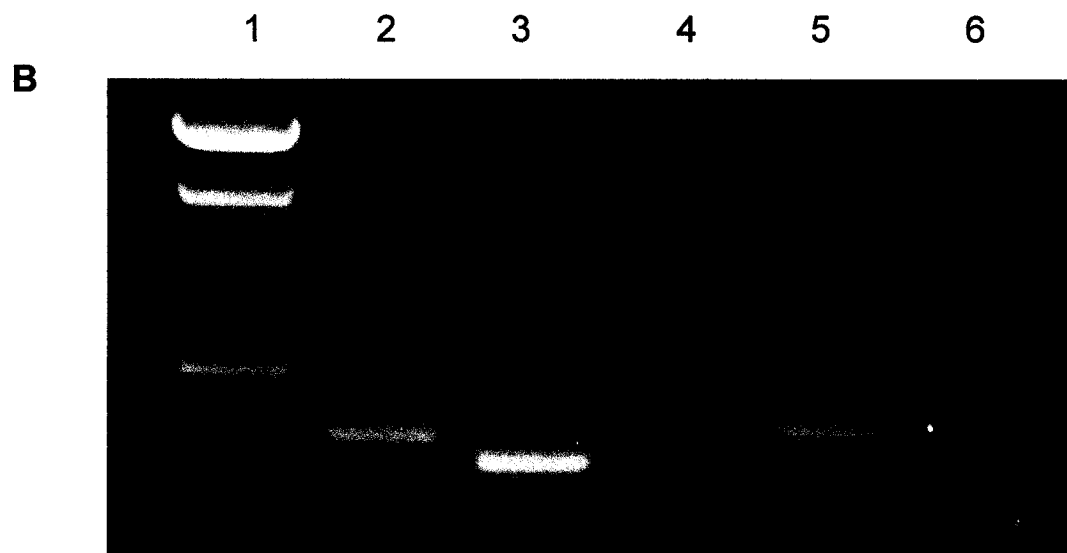
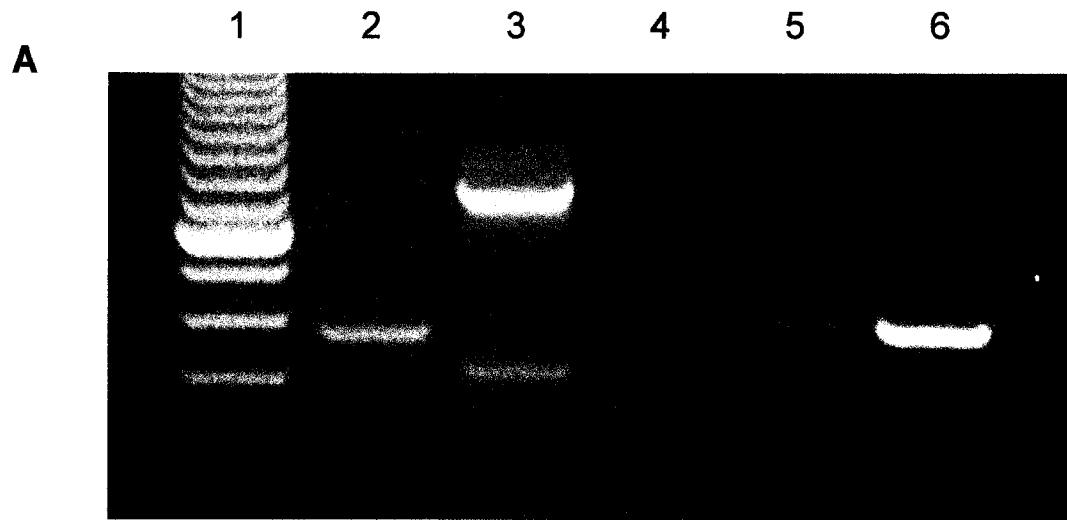


Figure 2.3. Detection of prostanoid receptor mRNA by RT-PCR in different rat kidney cell preparations. Gel electrophoresis of the amplified RT-PCR products, from DNase-treated total RNA of A) freshly isolated rat IMCD, B) cultured rat IMCD, and C) rat mTAL. Lane number: 1- 100 bp DNA ladder; 2- IP (407 bp); 3- EP₁ (336 bp); 4- EP₂ (401 bp); 5- EP₃ (437 bp); 6- EP₄ (423 bp). n = 3 – 6.

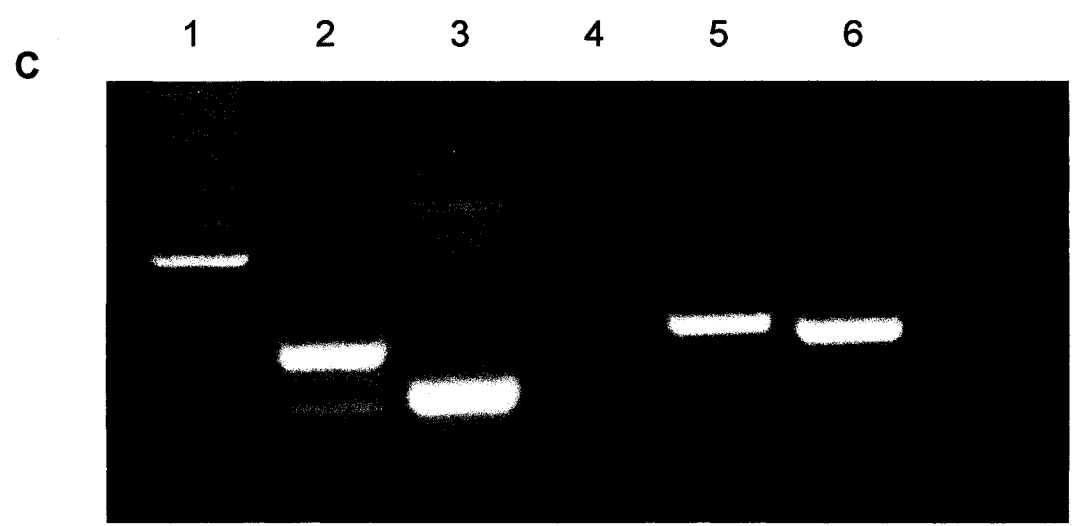
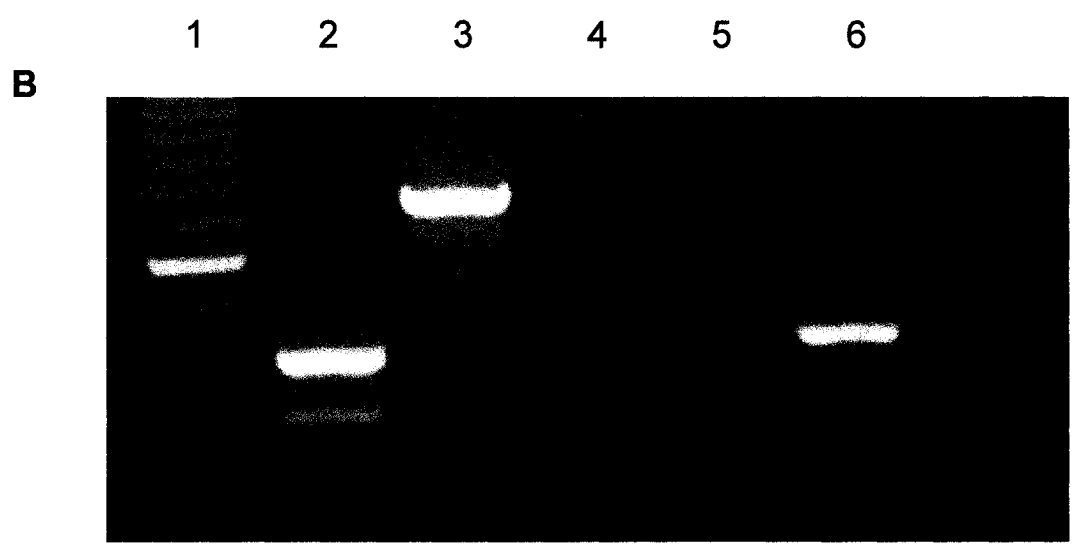
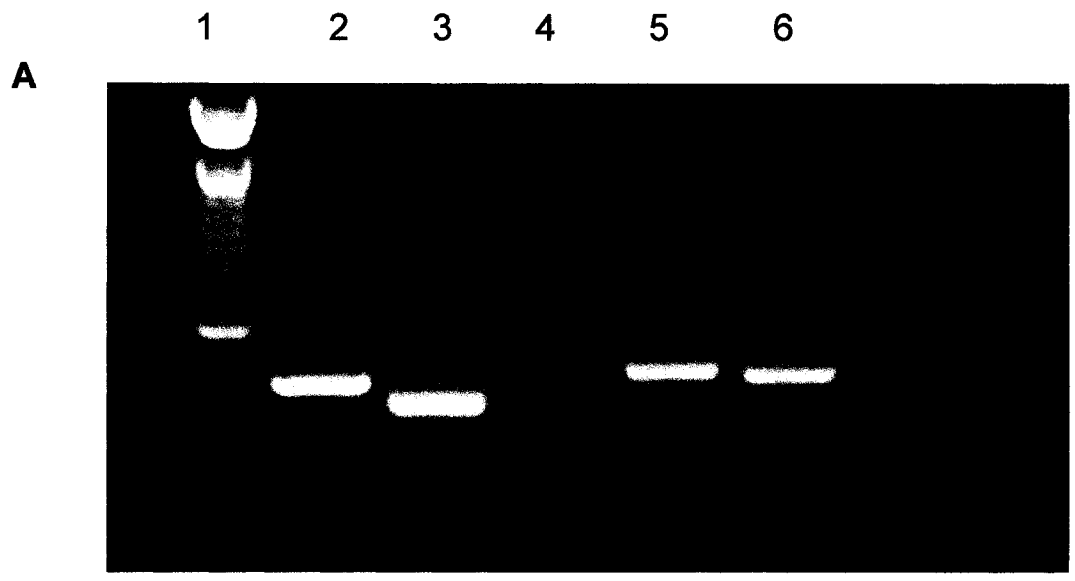


Figure 2.4. Endonuclease digestion of RT-PCR products. Gel electrophoresis of the amplified IP and EP₃ receptor products from freshly isolated rat IMCD and spleen (positive control). The PCR products were digested with the restriction enzyme NcoI, for which a restriction site is present within the amplified IP receptor product. Since the fragment from the EP₃ receptor does not contain a restriction site for this endonuclease, it serves as a negative control for the digest. The samples are shown with and without NcoI digestion. Lane number: 1-100 bp DNA ladder; 2-IP IMCD, no NcoI, 437 bp; 3-EP₃ IMCD, no NcoI, 437 bp; 4-IP spleen, no NcoI, 407 bp; 5-IP IMCD, with NcoI, 256 and 151 bp; 6-EP₃ IMCD, with NcoI, 437 bp; 7-IP spleen, with NcoI, 256 and 151 bp. n = 3.

1 2 3 4 5 6 7

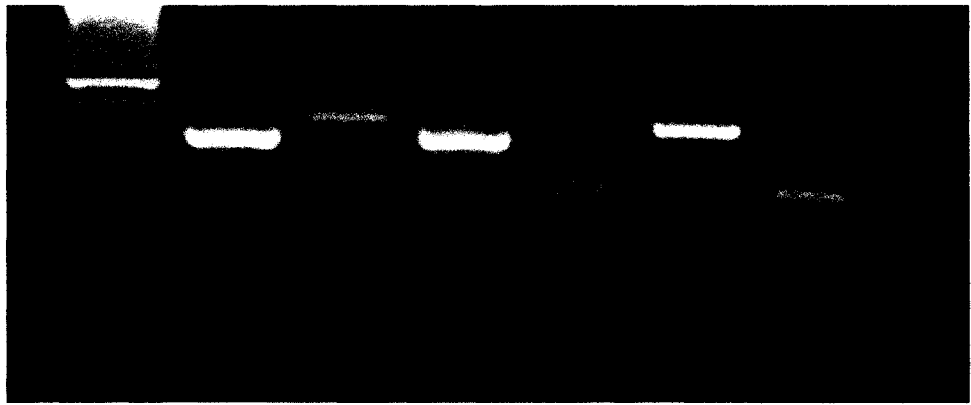
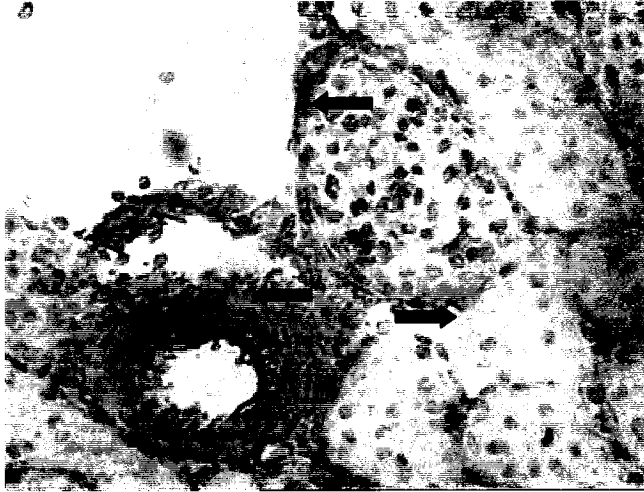
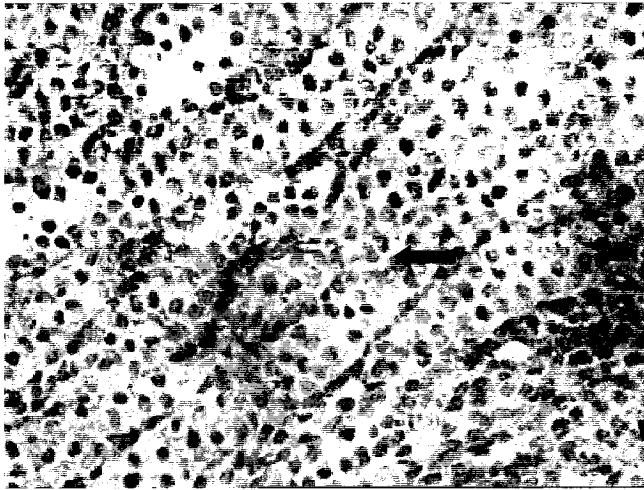


Figure 2.5. Detection of prostacyclin receptor mRNA by In Situ Hybridization. The detection of IP mRNA was done using an anti-digoxigenin alkaline phosphatase antibody conjugate that binds to digoxigenin labeled RNA-DNA hybrids, followed by an enzyme-catalyzed color reaction forming a bluish/brown precipitate. The IP message can be seen: A) in the rat cortex (glomerulus and vasculature); B) in the rat outer medullary tubules; and C) in the rat inner medulla. Images were taken at a 40 X magnification. No signal was detected in hybridizations with sense oligoprobes. n = 3.

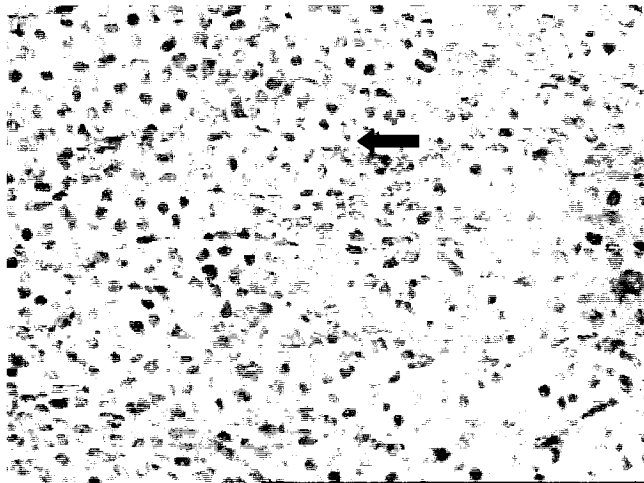
A



B



C



Detection of IP receptor subtypes and/or splice variants:

Since there is a great deal of controversy in the literature regarding the mechanisms by which prostacyclin elicits its various effects, RT-PCR and TA cloning methods were employed to determine whether IP subtypes or alternatively spliced forms of this receptor can be detected within the different regions of the kidney. By selecting four different sets of primers (figure 1) spanning the entire length of the rat prostacyclin receptor cDNA, we first amplified fragments of the cDNA using these primer sets, and then combinations thereof, to ascertain whether products similar to the published IP sequence exist. Table 1 summarizes the data obtained from the initial experiments using the four primer sets. As shown, no positive colonies (containing an insert) were obtained out of 15 selected in the cortex, therefore sequence analysis was not done for this region. In the outer medulla any product sequenced was either identical to the published sequence of the IP cDNA, or contained single mutations or deletions that do not alter the protein itself (open reading frame analysis). Two of the plasmid preparations analysed did not contain any insert; and no amplification product was obtained by PCR with the IP₂ set of primers regardless of the PCR conditions used i.e. annealing temperature, number of cycles, starting RNA concentration, and primer concentration. The same results can be seen for the inner medulla, where all the sequences were identical to the published IP cDNA. In contrast, in the mTAL using the FF-1/2 primers, a product was amplified corresponding to 300 bp in addition to the expected 407 bp. This same product was also noted in other tissue preparations, inconsistently. However, sequencing analysis revealed that it is a product with very little homology to the IP cDNA, but stronger homology at the primer sequences. Therefore we conclude that it is a result of non-

specific amplification. Likewise, in the IMCD a product of 600 bp was obtained with the IP₃. But in this case, sequencing of the product unveiled a 400 bp sequence identical to the published IP cDNA, and not a 600 bp product. In the IMCD, a product was amplified using the IP₂ primers, confirming the quality of the primers and the adequacy of the PCR conditions employed.

cAMP assays:

With the exception of previous work on cultured rat IMCD (34) very little is known about the signalling pathways linked to the IP receptor in the rat IMCD, and studies in other organ systems remain contradictory. Therefore these experiments measured the cellular levels of cAMP in response to stimulation of both freshly isolated and cultured IMCD with various prostanoids: PGE₂, ILP, and CCP; as well as AVP. The first set of experiments consisted of examining the stimulatory effect of the various agonists on the cAMP pathway. In cultured rat IMCD, as shown in figure 6, while all compounds tested stimulated cAMP levels, the greatest stimulation at all concentrations was obtained with AVP at percentages of control ranging from $27 \pm 2\%$ at 0.1 nM to $62 \pm 4\%$ at 1 μ M. The stimulation with the different prostanoids was comparable at agonist concentrations between 1 nM and 10 μ M. Despite a greater stimulation with AVP on freshly isolated IMCD, from $61.3 \pm 6\%$ to $72 \pm 7\%$, as opposed to the cultured cells, the stimulation obtained with the different prostanoids was similar to, if not less than, that of the cultured IMCD. However, once again all three prostanoids comparably stimulated cAMP production in the rat fresh IMCD: from $6.8 \pm 3\%$ to $46 \pm 2\%$ with PGE₂, from $4 \pm 2\%$ to $38 \pm 6\%$ with ILP, and from $6 \pm 4\%$ to $42 \pm 3\%$ with CCP. It is of interest to note

that a stimulatory effect was only obtained at concentrations of PGE₂ greater than 100 nM. Furthermore, decreasing the concentrations of AVP to 0.01 nM did not decrease the stimulatory response in both fresh and cultured IMCD (data not shown).

In the second set of experiments, we examined the ability of the three prostanoid analogues to inhibit the AVP-stimulated cAMP production in cultured and freshly isolated IMCD. While all the compounds tested did not inhibit the stimulatory effect of 100 μM AVP in cultured cells (data not shown), an inhibitory effect was obtained in response to all three prostanoids in freshly isolated IMCD, as shown in figure 7. Although the smallest inhibition was achieved with ILP at all agonist concentrations, there was a comparable inhibition with CCP and PGE₂ at higher concentrations, above 10 nM. But at 1 nM the inhibitory effect on AVP achieved with CCP was much greater than the two other prostanoid analogues, reaching $70 \pm 15\%$, suggesting that the CCP effect may be mediated by a distinct IP receptor subtype or splice variant. A decrease in agonist concentrations to 0.01 nM did not result in a further inhibitory response with any of the prostanoids above (data not shown).

Calcium measurements:

To further clarify the prostacyclin signalling mechanisms in the IMCD, calcium responses linked to the IP receptor were examined using analogues of prostacyclin, ILP and CCP. First, changes in intracellular calcium ($[Ca^{++}]_i$) were measured on cultured IMCD cells grown on coverslips. None of the various compounds tested: 100 nM AVP, 100 nM PGE₂, 100 nM ILP, 100 nM CCP, 1 μM endothelin, 100 μM ATP, and 10 μM Ang IV, produced a rise in $[Ca^{++}]_i$ levels (data not shown). Some of these compounds

have previously been shown to trigger a rise in calcium in other cell preparations. For instance, PGE₂ is known to increase calcium via the EP₁ receptor in rabbit cortical collecting duct (RCCD) cells (5, 10). Also, ILP has been shown to increase [Ca⁺⁺]_i levels in isolated perfused rabbit CCD (9). In addition, 100 μM ATP has previously been shown to elicit a calcium response in cultured IMCD (26), however no effect was detected in our experiments using any of these compounds.

Since a positive calcium response was not obtained on cultured IMCD cells with any of the compounds tested, calcium levels were measured on micro-dissected rat IMCD. This is represented in figure 8. The mean baseline level of calcium was 25.4 ± 18 nM. When 10 nM ILP was added to the tubule, an increase in calcium to a peak of 1.35 ± 0.4 μM was obtained within 30 - 60 sec. This was followed by a shoulder reaching a plateau at 229 ± 56 nM. This same pattern was obtained when 10 nM PGE₂ was added to the rat IMCD. The peak calcium level attained was slightly lower at 560 ± 149 nM, which rapidly decreased to a plateau at 201 ± 68 nM.

Because no calcium signal was obtained with CCP, and ILP is a less selective agonist of the IP receptor with greater affinity for the EP₁ receptor (21), it is difficult from these studies to conclude that the calcium response observed is attributable to the IP receptor. Therefore, in order to determine whether or not the effect obtained with ILP is linked to the EP₁ receptor, AH-6809, an EP₁ receptor antagonist was used to inhibit the calcium response. Once again, experiments were performed on micro-dissected rat IMCD and the calcium responses were noted in the presence of 10 μM AH-6809. Compared to a baseline value of 25 ± 18 nM, the calcium responses to both PGE₂ and ILP were completely inhibited in the presence of the EP₁ receptor antagonist: 23 ± 2 nM with 10

nM PGE₂ + AH-6809, and 52 ± 31 nM with 10 nM ILP + AH-6809. These results are demonstrated in figure 8, which is a representative tracing of the three experiments performed. In order to assess whether or not the effect of AH-6809 was reversible at the end of the experiment, 10 nM ILP was added once more and a peak calcium response of 387 ± 73 nM was obtained, followed by a plateau at 112 ± 32 nM. These results suggest that the rise in $[Ca^{++}]_i$ in response to ILP is in fact mediated by the EP₁ receptor, and not through an alternate receptor such as an IP subtype or splice variant.

Region	Primers/Predicted Size of Fragment	Size of Fragment Analyzed	Number Sequenced	Results of Sequencing		
				Published Sequence	No Result	Difference from published sequence
Cortex	FF1/2 (407 bp) IP1 (474 bp) IP2 (513 bp) IP3 (425 bp)	ND	No positive colonies (of 15 selected)	ND	ND	ND
Outer Medulla	FF1/2 IP1 IP2 IP3		7 2 0 (PCR) 4	2 2 4 1	2 (Vector)	3 (del, 2 μ , 1 μ)
Inner Medulla	FF1/2 IP1 IP2 IP3		2 1 0 (PCR) 1	1 1 1 1	1 (Vector)	
mTAL	FF1/2 IP1 IP2 IP3	407/300	1/1 5 0 (PCR) 0 (no colonies)		1 (DNA) 1 (Vector)	300 bp 4 (1 μ)
IMCD	FF1/2 IP1 IP2 IP3		2 1 1 1	2 1 1 1 (400)		1 (2 DNA sequences)

Table 2.1. TA Cloning analysis of amplified DNA fragments from different kidney tissues. Summary of results obtained by dye-chemistry sequencing. For each region of the kidney, four different primer sets were used spanning the entire length of the published cDNA, and the size of the fragment analyzed is indicated only when aberrant from the predicted size. The number of bacterial colonies examined for each region is indicated, as well as the sequencing results obtained i.e. whether or not it differs from the published IP cDNA and the difference noted. No result indicates that no insert was present in the vector, or the DNA purity was too poor for sequencing.

Figure 2.6. Measurement of cellular cAMP levels by radioimmunoassay in response to stimulation with various agonists. A) Cultured rat IMCD and B) freshly isolated rat IMCD, were stimulated with four different agonists: AVP, PGE₂, ILP, and CCP, at concentrations ranging from 10 μ M to 0.1 nM; and the percentage of cAMP stimulation relative to control was plotted. Samples are presented as mean \pm SEM, duplicates for each condition were performed. n = 3 – 5.

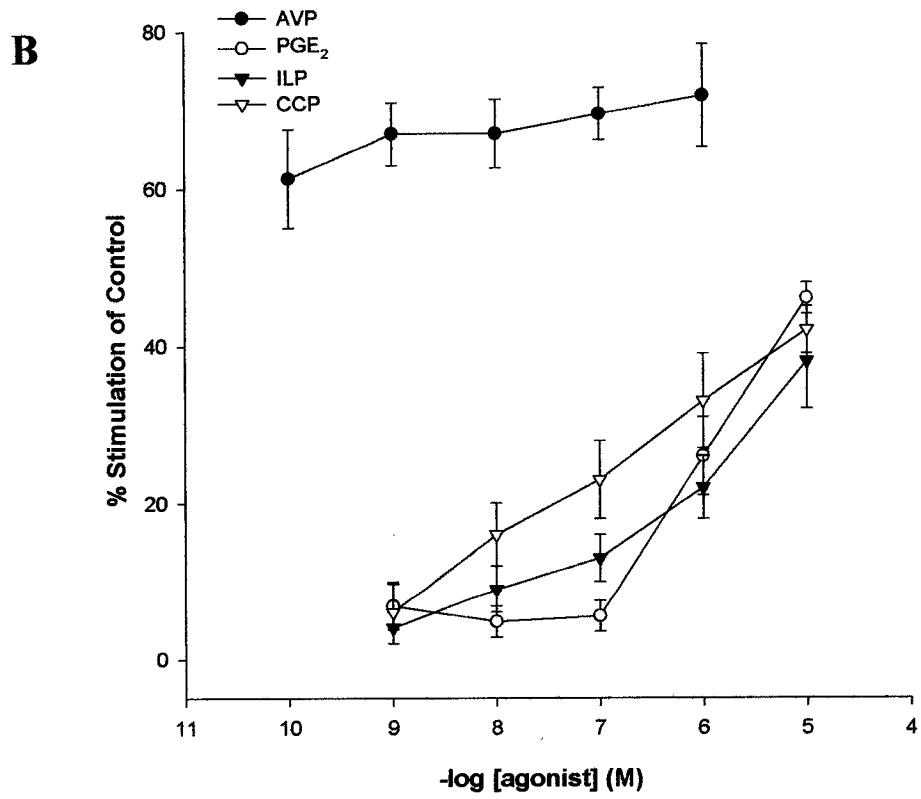
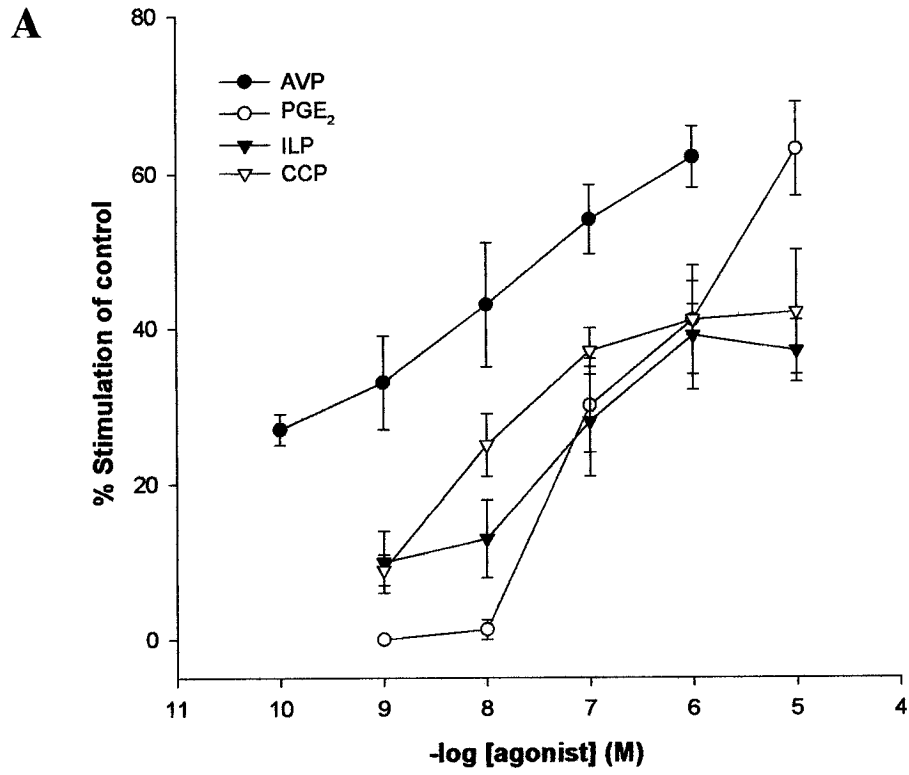


Figure 2.7. Inhibition of AVP-dependent cAMP stimulation by prostanoid analogues. Freshly isolated rat IMCD were pre-treated for 15 min with either PGE₂, ILP, or CCP at concentrations ranging from 10 μ M to 1nM, in the presence of indomethacin and IBMX. 100nM AVP was then added for 7 min, and the inhibitory effect of the prostanoids on cAMP stimulation by AVP was determined. Data is presented as mean \pm SEM of percentage inhibition of AVP. Each sample was prepared in duplicate. n = 3 - 5.

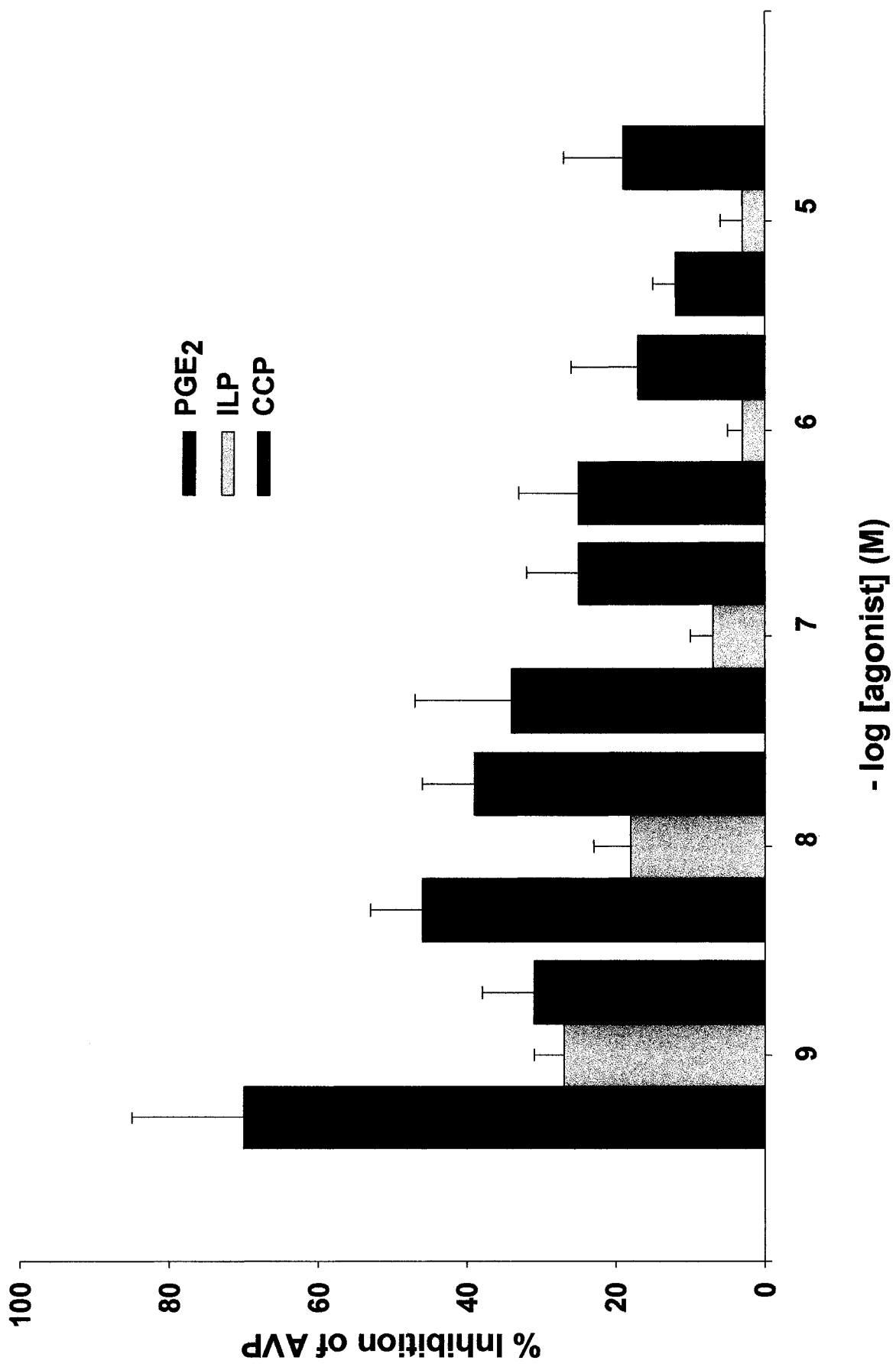
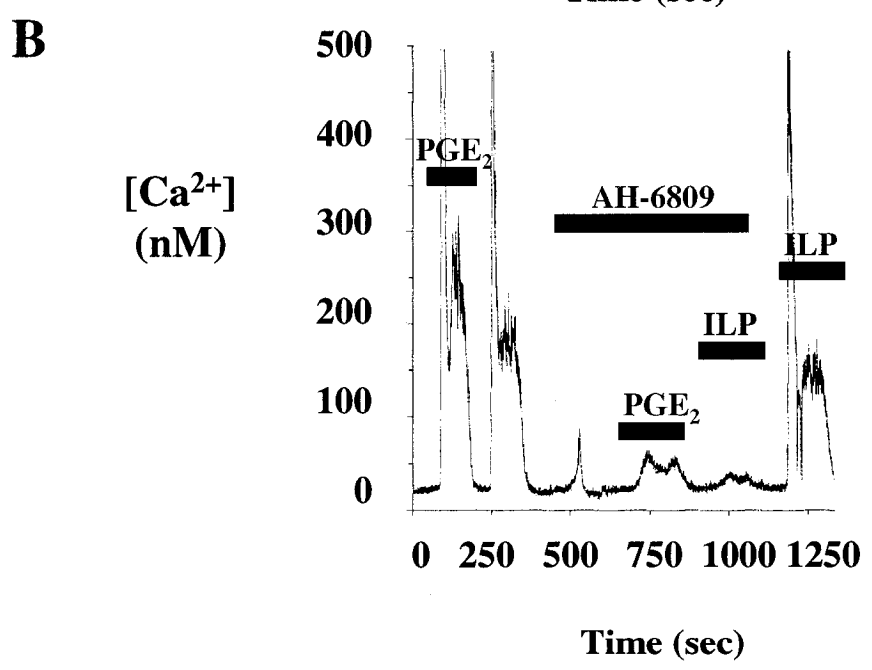
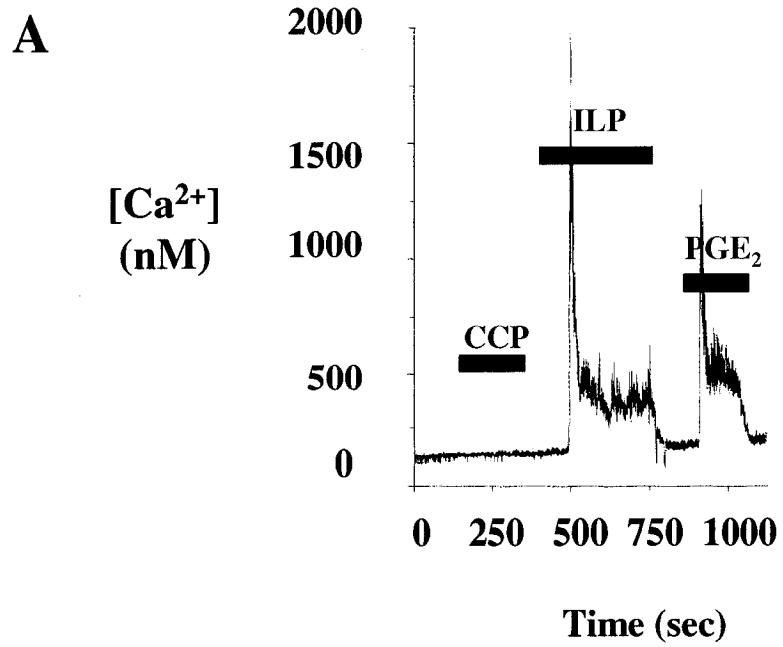


Figure 2.8. Changes in intracellular calcium levels in response to prostanoids.

Microdissected rat IMCD were loaded with fura-2 for 45 min. A) rat IMCD were exposed to 10 nM of CCP, ILP, and PGE₂. Changes in calcium concentrations are shown over time. The bar indicates the period of exposure to each prostanoid. n = 3. B) IMCD tubules were exposed to 10 nM of either PGE₂ or ILP, in the absence or presence of 10 μM AH-6809. n = 4.



DISCUSSION

We know that prostaglandins are an important group of biologically active compounds, implicated in the maintenance of homeostasis and the pathogenesis of certain diseases, both in the vasculature and in specific organ systems throughout the body. Their significance in the kidney is clearly demonstrated by the undesirable renal effects, associated with the use of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the production of prostaglandins. Over the past decade or so, the cell surface receptors that mediate the effects of prostanoids, in particular those of thromboxane (TP receptors) and PGE₂ (EP receptors) have been cloned and characterized (5, 6, 21). Within the kidney, these receptors have been localized to the glomerular region, the renal vasculature, and the distal segments of the nephron including the medullary thick ascending limb (mTAL) and the collecting duct (CD). This was accomplished using a variety of techniques: binding studies, immuno-histochemistry, in situ hybridization, RT-PCR, and Northern blot analysis. The presence of prostanoid receptors within these regions is consistent with the fact that they have been implicated in various renal functions such as: regulating glomerular filtration rate, glomerular hemodynamics, renin release, and salt and water transport in the collecting duct. However, to date, there remains some controversy in the literature regarding these findings, and also with respect to differences between species. For instance, it was shown by immuno-histochemistry that the EP₄ receptor is not present in the human collecting duct (17), but several biochemical studies indicate that this receptor subtype, via coupling to G_s, mediates the stimulatory effects of PGE₂ on cAMP production throughout the rodent collecting duct

(5, 11). Also, the EP₃ mRNA was not detected in the human IMCD (4), whereas in rodents it is expressed in this segment of the tubule (29). Also of interest, are the conflicting results found in human kidney between localization of the mRNA and protein for the EP₃ receptor in the outer medulla: mRNA was detected in the mTAL (4) but the protein was only found in Tamm-Horsfall-negative tubules (17). The same is true for the prostacyclin (IP) receptor. While it is highly expressed in the mouse (22) and human (16) kidney, very little is known regarding its distribution within the rat kidney. Previous work by Hébert et al. (11) gave the first indication of the expression of IP receptors in epithelial cells, and localized this message to the mTAL in the outer medullary region of the rat kidney. In contrast, in the human kidney (16), the outer medullary expression of the IP receptor was restricted to Tamm-Horsfall-negative tubules (non-mTAL). Therefore to pursue these findings, and enlighten the investigation of EP and IP receptor expression in the rat kidney, this study used RT-PCR to determine whether the four EP receptor subtypes and the IP receptor mRNA can be detected within the three regions of the rat kidney (cortex, outer medulla, inner medulla), and in specific tissue preparations of the distal nephron (fresh IMCD, cultured IMCD, mTAL). With the exception of the EP₂ receptor, the mRNA for the EP_{1,3,4} and IP receptors were observed in all tissue preparations examined. The expression pattern of EP receptors within the kidney is consistent with the role of PGE₂ along the nephron. For example in the rodent collecting duct, acting through the EP₁ receptor it is thought to be involved in the inhibition of sodium transport by PGE₂, through EP₃ it inhibits AVP-dependent water reabsorption, and finally through the EP₄ receptor it stimulates water reabsorption (5). The lack of EP₂ receptors within the kidney is in accordance with previous findings indicating that a

butaprost-insensitive mechanism through the EP₄ receptor, and not the EP₂ receptor, mediates the stimulatory action of PGE₂ on cAMP production in the rodent collecting duct (4, 5), also that no G_s-coupled EP₂ mRNA was detected in the human kidney by in situ hybridization (4). Since our results corroborate previous work showing a lack of EP₂ in the rat kidney, we suggest that it is via the EP₄ receptor that PGE₂ elicits its effects in the renal cortex.

In order to localize the IP receptor mRNA within the kidney, in situ hybridization was employed. Our findings demonstrate that all three regions of the kidney contain the IP message. In the cortex, the signal is observed within the glomerular region, in the vasculature, and in tubular structures. The highest levels are located within the tubules of the outer medulla, and there is diffuse staining in tubules of the inner medullary region. This is in agreement with previous in situ hybridization studies in rodents showing for instance staining of the renal vasculature and glomeruli in the mouse (22). But also confirms the finding of rat IP receptor mRNA in Tamm-Horsfall-positive tubules of the outer medulla i.e. the mTAL (11). This is consistent with the RT-PCR results of this study, localizing the IP message within the mTAL. However, further investigation into the role the prostacyclin/IP signalling mechanisms in this nephron segment is needed in order to account for the discrepancies noted between rodents and human kidneys, since in human studies the IP mRNA expression was restricted to the Tamm-Horsfall-negative tubules i.e. non-mTAL segments of the outer medullary region (16).

Altogether, these findings substantiate the implication of prostacyclin and the IP receptor in various renal functions. Being a potent vasodilator (6, 16, 20, 21), prostacyclin acting through vascular IP receptors can alter renal blood flow and

glomerular filtration rate. Also, it is known to be a greater stimulator of renin release than PGE_2 (7). Given that there is high expression of IP in the outer medullary tubules, it surely may participate in the modulation of sodium and water transport in these segments. Furthermore, it may play a role at the level of the macula densa thereby regulating renal function of these cells. In cultured rat IMCD for instance, PGI_2 did not inhibit AVP-dependent cAMP stimulation (34), but in isolated perfused rabbit cortical collecting duct, ILP did inhibit AVP-stimulated water flow (10). Other putative functions of the prostacyclin/IP system may include the regulation of gene transcription in the kidney, whether it's a direct effect, or via an indirect mechanism is still uncertain. Finally, reports have been made that prostacyclin may activate the peroxisome-proliferator-activated-receptor (PPAR) transduction pathway, thereby altering gene transcription (12).

After having localized the renal expression of the prostacyclin receptor in the rat, the next study examined whether or not IP receptor subtypes or splice variants can be detected in various regions of the kidney, since to date there is no molecular evidence for their existence. Nevertheless, many findings suggest that these forms may exist (10, 23, 24, 30). In this study, TA cloning and sequencing analysis revealed no product homologous to any fragment spanning the length of the published rat IP receptor cDNA. This finding is not surprising, considering that a phylogenetic classification scheme places the IP receptor in cluster 1 with DP, EP_2 and EP_4 receptors (31), all of which do not have any subtypes or splices; however, no alternative signaling pathways for those receptors have been suggested other than the stimulation of cAMP through coupling to G_s . In contrast, IP is known to activate various intracellular signaling messengers. Both functional and biochemical studies support this argument. In isolated perfused rabbit

CCD, ILP inhibits AVP-dependent water flow in a manner independent of the EP₃ receptor subtype, possibly acting through an "IP₃" subtype of the prostacyclin receptor (10, 11). Further-more, characterization of the prostacyclin receptor by binding analysis using various prostacyclin analogues showed conflicting binding affinities in the rat central nervous system (CNS) for each analogue, suggesting that a different IP receptor subtype is located in certain regions of the brain such as the hippocampus (30). Although these studies support the hypothesis, there remains the possibility that prostacyclin and its analogues are acting through other receptors such as the EP receptors, or simply that these effects are mediated by coupling of the existing IP receptor to different G proteins. Evidence for the latter stems from the initial work done to characterize the receptor by Namba et al., showing that expression of the cloned mouse IP receptor cDNA into Chinese Hamster Ovary cells gave rise to both an increase in cAMP and inositol trisphosphate levels upon stimulation with ILP (20). It is still possible that prostacyclin action is mediated by all three mechanisms. While the current study does not provide evidence for the presence in the rat kidney of IP receptor subtypes or alternatively spliced forms that are homologous to the published IP cDNA, it does not exclude the possibility that sequences with low homology to the cloned IP receptor do exist.

To further examine the possibility that multiple signalling pathways are associated with the binding of prostacyclin to the IP receptor in the rat kidney, cAMP assays were performed in the IMCD. While a stimulatory response to CCP, ILP, PGE₂, and AVP was obtained in both fresh and cultured IMCD, an inhibition of AVP-dependent cAMP stimulation by the three prostanoids was only observed in freshly isolated IMCD. This lack of inhibitory response in cultured IMCD cells is consistent with previous work by

Sonnenberg and Smith in RCCD cells, suggesting the possibility that the G_i -protein signalling pathway is aberrant in cultured cells (28). Whether this defect is due to a lack of receptor coupling to G_i , to an inactivation of one of the subunits, or to an absence of G_i due to a protein down-regulation in response to culture conditions, is not clear for this system. Another interesting aspect of these results is that the stimulation of cAMP production in response to PGE_2 is only seen at higher agonist concentrations. This is also consistent with previous findings showing that at 1 nM PGE_1 inhibits AVP-stimulation of cAMP, whereas it increases cAMP levels at 100 nM in rabbit CCD cells (27). The stimulatory effect obtained with the prostacyclin analogues, CCP and ILP, is consistent with the fact that the major signaling pathway linked to the IP receptor is the activation of adenylate cyclase (20), but also with previous work in cultured rat IMCD showing an increase in cAMP levels in response to PGI_2 (34). However, the demonstration in this study of an inhibitory effect of CCP and ILP on AVP-dependent stimulation of cAMP provides further evidence for the existence of IP receptor subtypes or splice variants in the rat freshly isolated IMCD. It is also of interest to note the difference in cellular response to both CCP and ILP; CCP is by far a more potent inhibitor of the AVP-dependent stimulation of cAMP, again supporting the aforementioned argument that these two compounds are activating different receptors within the same cells.

The final part of this work examined the calcium signaling pathways linked to the prostacyclin/IP system in the rat IMCD. While no $[Ca^{++}]_i$ changes were observed in cultured rat IMCD in response to many compounds: CCP, ILP, PGE_2 , AVP, endothelin, angiotensin IV, ATP; both ILP and PGE_2 increased $[Ca^{++}]_i$ in micro-dissected rat IMCD. Of interest is the fact that CCP did not increase $[Ca^{++}]_i$ in micro-dissected tubules as well

as in cultured IMCD. Very little is known about the calcium signaling mechanisms in the cultured IMCD, but previous work did show an increase in $[Ca^{++}]_i$ upon stimulation with 100 μ M ATP (26). In addition, it was shown that AVP increases calcium in renal papillary collecting tubule cells in culture (13). At this time we cannot account for this discrepancy in cultured cells to explain the lack of response in our preparation. However, several other studies support the use of the above-mentioned compounds as positive controls in our study. For instance, in isolated perfused rabbit CCD, both PGE₂ and ILP increased $[Ca^{++}]_i$ (10); whether or not this effect was mediated by the EP₁ receptor only, or by two different receptors is uncertain. Nonetheless, the highest levels of EP₁ are found in the collecting duct, and this receptor does couple to G_q to increase calcium levels (5). Also, previous work in rat terminal IMCD showed that PGE₂ caused a rapid increase in $[Ca^{++}]_i$ (18). Furthermore, endothelin is known to increase calcium via the ET_B receptor (19), which is highly expressed in the IMCD (8, 14). An increase in calcium in response to endothelin was observed in rat terminal IMCD (19). To further clarify the calcium signaling in response to prostaglandins in the rat IMCD, we used the EP₁ antagonist AH-6809 to block the increase in calcium obtained in response to PGE₂ and ILP. The calcium spike was in fact abolished in the presence of AH-6809 for both compounds, and this effect is reversible. Although it appears that ILP increases calcium in the rat IMCD via the EP₁ receptor, we cannot yet dismiss the possibility that other receptors also mediate its effect on calcium metabolism.

Therefore, in conclusion, this study localized the expression of EP receptor subtypes and the IP receptor in the rat kidney. The IP receptor is clearly expressed in the

rat IMCD and mediates the effects on cAMP signaling pathways in response to prostacyclin analogues, but not the calcium response in this segment of the nephron.

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CHAPTER 3

Increased Expression of Cyclooxygenase-1 and -2 in the Diabetic Rat Renal Medulla

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Rania Nasrallah wrote the manuscript, designed all the experiments, and assisted with the troubleshooting. Also, Rania Nasrallah performed all the experiments with the technical assistance of Anne Landry, with the exception of: Sonia Singh performed the in vivo work with the assistance of Rania Nasrallah, Anne Landry performed the enzyme immuno-assays with the assistance of Rania Nasrallah, and Monika Sklepowicz performed the EP₄ receptor Western blots.

**Increased Expression of Cyclooxygenase-1 and -2 in the Diabetic Rat
Renal Medulla**

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Running Title: Altered COX in diabetic rat IMCD.

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ABSTRACT

Alterations in renal prostaglandins may contribute to some of the renal manifestations in diabetes leading to nephropathy. Prostaglandin production is dependent upon the activity of cyclooxygenases (COX-1 AND -2) and prostaglandin synthases. Our current study has investigated levels of these enzymes in rat streptozotocin-diabetes at 2, 4, 6 and 8 weeks of diabetes. Immunohistochemical analysis revealed an increase in COX signal in the inner and outer medulla of the diabetic rats. This was confirmed by Western blotting, showing up to a 4 –fold increase in both COX isoforms at 4-6 weeks of diabetes. Also, Western blot analysis revealed a 6-fold increase in PGE₂ synthase expression in the outer medullary region of 6 weeks diabetic rats, but no difference in the inner medulla. In cultured rat IMCD, levels of COX were increased 2-3 –fold in cells exposed for 4 days to 37.5 mM glucose in comparison to control of 17.5 mM. While no change in PGE₂ synthase levels was noted, PGE₂ synthesis was increased. Furthermore, levels of EP₁ and EP₄ mRNA were increased, as well as a 2 –fold increase in EP₄ protein levels. Future studies will determine which COX isoform is contributing to the majority of PGE₂ produced in the diabetic IMCD, and the significance of these findings to disturbances in IMCD function and to the progression of diabetic nephropathy.

KEYWORDS: COX, PGE₂, STZ-diabetic rats, IMCD, EP₄ receptor

INTRODUCTION

Prostaglandins (PG) are involved in diverse renal functions regulating hemodynamics and tubular transport processes. Prostaglandin E₂ (PGE₂) is by far the most prominent prostanoid (PG) produced in the kidney (3), particularly in the glomerular regions and the inner medulla. In part, the ubiquitous involvement of PGs in renal function is dependent on distinct G protein-coupled receptors, each one having a greater affinity for a respective prostanoid. For instance, PGE₂ elicits cellular responses by binding at least four EP receptor subtypes, EP₁₋₄ (8, 31). The past decade has led to many advances in the study of these receptors, and the cellular responses linked to each one, especially in the kidney. Many investigators have localized each subtype to specific cell types all along the nephron (4, 29, 45, 46), and more recently nuclear localization of these receptors has been documented (1, 2). In addition, more and more insight is now available with respect the involvement of PGs and their receptors in certain renal pathology (12, 25, 35). However, it is becoming quite evident that under normal conditions, PGs mainly participate in homeostatic functions, therefore serving to antagonize or enhance the cellular responses to other factors, for instance angiotensin II (19, 40, 42). Thus it is conceivable that a disturbance in PG production and signalling will alter this balance and perpetuate a disease state.

The medullary region is an important contributor to the overall pool of renal prostanoids, in particular the inner medullary collecting ducts (IMCD). Renal PGE₂ production is dependent upon the activity of two enzymes: cyclooxygenases (COX -1 and -2), and PGE₂ synthase (44, 49). Although it is believed that COX-1 is a constitutively

expressed form of the enzyme and COX-2 is the inducible form (10), this has been proven untrue in the kidney. For instance studies in the M-1 mouse cortical collecting duct cell line have shown that the intercalated cells of the collecting duct constitutively express both COX isoforms, and that COX-2 contributes to the majority of the PGE₂ produced in these cells (11). Likewise, it has been noted that while COX-1 is present in collecting ducts, interstitial cells, and endothelial cells (23, 43), COX-2 is constitutively found in the macula densa cells, cortical thick ascending limb, medullary interstitial cells, and inner medullary collecting ducts (16).

Insights into the importance of each COX isoform came about by the generation of isoform-specific deficient mice. While no major renal pathology was documented for the COX-1 knockout (26), COX-2 lacking mice display abnormalities in renal development and severe nephropathy (30). It is only when other underlying conditions exist, that the significance of COX becomes recognizable. For example, after dehydration, COX-dependent prostaglandin production becomes an important survival mechanism in renal medullary interstitial cells (15). Thus, it is notable to look at both enzymes as contributing to separate pools of PGs, and depending on the cell type, the balance between cytoprotection and damage will determine the outcome and contribution to renal diseases.

Diabetic nephropathy is a leading cause of end-stage renal disease. While alterations in prostaglandin levels have been implicated in the pathogenesis of diabetic nephropathy (20, 24, 27), resulting in hemodynamic changes and structural variations (9), the contribution of individual COX isoforms needs further clarification. Thus the purpose of this work is to study the expression of COX-1 and COX-2 in the outer and inner medulla

of the kidney at 2, 4, 6, and 8 weeks in streptozotocin (STZ)-diabetic rats: by immunohistochemical analysis, Western blotting, and Northern blot analysis. Also to determine whether PGE₂ synthase levels are altered in STZ-diabetic rats. Finally, since PGE₂ is a key regulator of the natriuretic and diuretic functions of the kidney collecting ducts, the second part of this work will focus on changes in cultured rat inner medullary collecting ducts (IMCD) exposed to high glucose, studying PGE₂ levels and EP receptor expression.

MATERIALS AND METHODS

Diabetic rat model:

Tissue from different kidney regions (cortex, outer medulla, inner medulla) of adult male Sprague-Dawley rats weighing 200 - 300g was isolated under bright light using a dissecting microscope. The diabetic model utilized in the studies is the Streptozotocin (STZ)-diabetes, a widely used model resembling Type 1 diabetes mellitus. STZ is an N-nitroso derivative of D-glucosamine that is utilized to induce diabetes in a variety of experimental animals, and to determine the long-term complication of diabetes. It basically functions as a toxin that selectively destroys the insulin producing cells of the pancreas, rendering the rat diabetic within 24 hrs of injection (41). The animal care facilities at our disposal have a current protocol for inducing diabetes and maintaining the rats. Three different groups of rats were employed: 1) vehicle-treated control (administered 0.1 mol/L sodium citrate buffer, pH 4.0); 2) STZ-diabetic rats (administered 65 mg/kg STZ (Sigma, St.Louis, MO, USA), in 0.1 mol/L sodium citrate buffer, pH 4.0; hyperglycemia were maintained between 11 - 17 mmol/L with daily subcutaneous injections of 1 - 2 units of insulin); 3) STZ-insulin (same as group 2 except for maintenance of euglycemia by subcutaneous implantation of a sustained release insulin implant; Linplant, Linshin, Scarborough, ON, Canada). The day after STZ administration, a urine analysis was performed for glucose and ketones using a Keto-Diastix reagent strip (Bayer Inc., Etobicoke, ON, Canada). If the glucose level was above 111 mmol/L, the animal automatically received about 1.5 units of insulin. If however the urinary glucose was at 56 mmol/L, a blood glucose test was performed and when levels

were 27-44 mmol/L the animal was given a dose of insulin. Otherwise blood glucose was not tested daily. Animals with sustained glucosuria were assigned to STZ-Diabetic or Insulin groups. Throughout the study, 1.5-2 U of insulin was sufficient to maintain blood glucose levels between 10-17 mmol/L. Experiments were performed on rats in early stages of diabetes at 2, 4, 6 and 8 weeks following STZ injections, as well as matched controls for each stage. Our project follows the guidelines from the Canadian Council on Animal Care, and meets the ethical guidelines for our institution. The body weights of each animal were recorded daily, and kidney weights were measured once the animal was sacrificed. This data is summarized in Table 1.

Immunohistochemistry:

Kidneys were removed from control, diabetic and insulin-treated rats at 2, 4, 6, 8 weeks after STZ injection, and fixed in 4 % paraformaldehyde/0.2 % picric acid in PBS for 18 hrs at 4 °C. Paraffin embedded longitudinal sections 4 µm thick, were then permeabilized for 15 min in 0.3 % Triton X-100/PBS and incubated with COX-1 or COX-2 polyclonal antibodies (Cayman) for 18 hrs at 4 °C. Following incubation with biotinylated anti-rabbit IgG for 30 min at 37 °C, the sections were incubated with streptavidin-linked horseradish peroxidase, and diaminobenzidine substrate was used to visualize the signals. Counterstaining was performed with Mayer's hematoxylin and sections were analyzed using a Zeiss microscope.

Table 3.1. Summary of body weights and kidney weights for rats at different stages of STZ-diabetes.

W E E K S	Body Weights (g)			Kidney Weights (g)			Kidney/body weights ($\times 10^{-3}$)		
	Control	Diabetic	Insulin	Control	Diabetic	Insulin	Control	Diabetic	Insulin
2	348 \pm 7	278 \pm 1.9	320 \pm 16	1.2 \pm 0.05	1.5 \pm 0.03	1.3 \pm 0.08	3.4	5.4	4.1
4	401 \pm 15	323 \pm 16	395 \pm 16	1.3 \pm 0.06	1.8 \pm 0.04	1.2 \pm 0.04	3.2	5.6	3.0
6	468 \pm 16	367 \pm 14	411 \pm 11	1.5 \pm 0.1	2 \pm 0.1	1.5 \pm 0.1	3.2	5.4	3.6
8	474 \pm 15	366 \pm 21	451 \pm 13	1.5 \pm 0.06	2 \pm 0.1	1.5 \pm 0.05	3.2	5.5	3.4

Daily measurements of body weights were recorded for each animal in all three groups (control, diabetic, insulin) at different stages post-STZ treatment (2, 4, 6, 8 weeks). The mean for each group is presented, as well as the corresponding kidney weights. The ratio of kidney/body weight is increased in diabetics compared to controls and insulin groups throughout the course of the study.

IMCD cell culture:

The inner medullary regions from 5 rats were pooled, and minced on ice in a Petri dish. IMCD were isolated as previously described (32) by bubbling in 5 % CO₂/air at 37 °C in a solution of collagenase/DNase, followed by osmotic shock. IMCD cells were cultured in DMEM-F12 containing: 10 % (1st day) then 2 % FBS, 1 % P/S, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 2.5 nM triiodothyronine/sodium salt, and 50 nM hydrocortisone (Sigma). Cells were grown at 37 °C and 5 % CO₂ in media containing either: 1) control (17.5 mM glucose in DMEM-F12), 2) 30 or 37.5 mM glucose, 3) 30 or 37.5 mM mannitol (osmolarity control). The glucose in the media remained constantly above 35 mM until 48 hrs after initial plating where it dropped to about 25-30 mM (measured using Keto Diastix glucose indicators). Therefore the culture media was changed at two days to ensure exposure of cells to 37.5 mM glucose over the 4-day period. Following 3 days, the cells were serum starved in similar media for 24 hrs prior to experiments.

Western blotting:

Protein lysates from outer and inner medulla were obtained by homogenizing the tissue in a 25 mM Tris-HCl lysis buffer. For IMCD cell cultures, protein samples were prepared by lysing cells in 100 mM Tris-HCl (pH 7.4), containing 1 mM EDTA and 1 mM EGTA, followed by sonication for 5 sec using an Ultrasonics cell disrupter. The cell lysates were then centrifuged at 10 000 g for 10 min, and the supernatant were removed. 25 µg of each sample were resolved by SDS-PAGE on a polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking O/N in 5 % milk/TBS-T, the

membrane was incubated with either anti-COX-1 or anti-COX-2 polyclonal antibody. Following incubation with an HRP-conjugated goat anti-rabbit IgG secondary antibody, ECL was used to visualize the signals. A single band of 70 or 72 KDa was obtained for COX-1 and COX-2, respectively. The samples were then normalized with detection of β -actin, and a densitometric analysis was performed. Tables 2 and 3 summarize the data obtained at 2, 4, 6, and 8 weeks of STZ-diabetes. PGE₂ synthase levels were examined in outer and inner medullary samples at 6 weeks of diabetes, and in cultured IMCD treated with either glucose or mannitol, using an anti-PGE₂ synthase antibody (Cayman) detecting a 16 kDa product. Also in cultured IMCD, the EP₄ receptor was detected using a human polyclonal α -EP₄ IgG (Cayman), diluted 1:5000 in 10 % milk in TBS-T.

Northern blotting:

Kidneys were removed from control, diabetic, and insulin-treated rats at 6 weeks after STZ injection. Total RNA was isolated from samples of outer and inner medulla using the Trizol method, as described by the manufacturer (Gibco-brl); and was DNase-treated (Boehringer Mannheim) to eliminate genomic DNA. 10 μ g of total RNA from each sample was loaded onto a formaldehyde gel. RNA was then transferred to a nitrocellulose membrane. After baking for 2 hrs/80°C/vacuum, the membrane was incubated O/N with a [³²P]dCTP-labeled human COX-2 cDNA probe, then exposed to film for 1 week. To normalize the samples, the expression of β -actin was determined for the same membrane by re-probing with a human anti- β actin cDNA (Cayman) after stripping membranes in boiling 0.5 % SDS. IMCD cells were grown to confluence in 100 mm Petri dishes and scraped off then centrifuged at 1100 rpm. The collected pellet was

resuspended in 1 ml Trizol reagent (Gibco), and total RNA was isolated using the Trizol method, as described by the manufacturer (Gibco-brl); and was DNase-treated (Boehringer Mannheim) to eliminate genomic DNA. Densitometric analysis was used to compare the relative expression COX-2 in freshly isolated medullary tissue, and of EP₁ and EP₄ receptor mRNA in each IMCD sample using mouse EP₁ and EP₄ cDNA probes (a gift from Matthew Breyer at Vanderbilt University). Data is presented as mean (fold of control) ± SEM.

Enzyme immunoassays:

Cultured rat IMCD were exposed to either 37.5 mM glucose or mannitol, and PGE₂ levels in these samples were compared to control (17.5 mM glucose). In order to quantify the amount of each prostanoid (PGE₂, 6-keto-PGF_{1α}) being produced, the supernatant was removed from each culture dish, and competitive enzyme immunoassays (EIA, Cayman Chemical, Ann Arbor MI) were performed. Production of prostanoids in IMCD at 2 days and 4 days were analyzed by EIA following the manufacturer's instructions. Briefly, the assay is based on a competitive binding of PGE₂ or 6-keto-PGF_{1α} and their respective acetylcholinesterase conjugate (tracer) for a limited amount of monoclonal antibody. Since the tracer concentration is held constant, the amount of tracer bound to the antibody will be inversely proportional to the amount of PG in the sample. Detection is based on a colorimetric reaction using Ellman's Reagent, which contains the substrate to acetylcholinesterase. The intensity is then determined by spectrophotometry.

Statistics:

The SigmaPlot software for windows version 4.01 (1986 - 1997) was used to analyze data. Results are expressed as mean \pm SEM. A one-way ANOVA were performed to assess the statistical significance between data points, followed by the Tukey test for comparison of values.

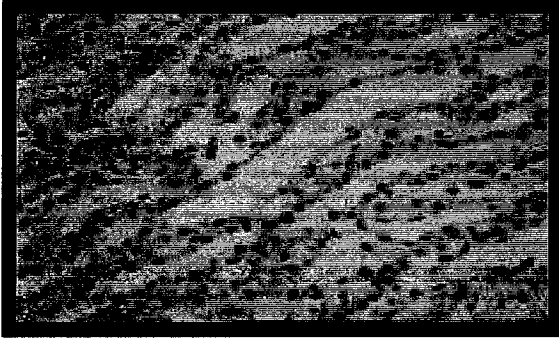
RESULTS

COX-1 and COX-2 are increased in the STZ-rat medulla:

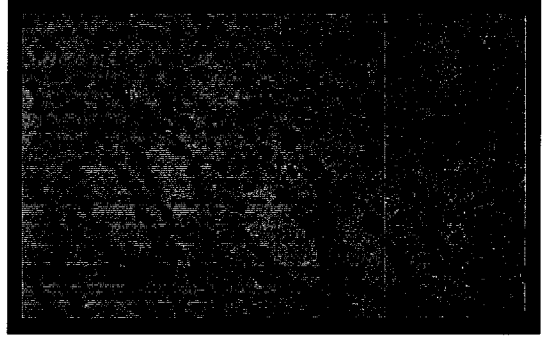
Immunohistochemical analysis of COX-1 and COX-2 revealed that both are present in tubular segments of the outer (outer medullary collecting ducts, medullary thick ascending limb) and inner (inner medullary collecting ducts) medulla in control rats. Although there were no obvious changes in distribution of COX isoforms throughout the medullary regions upon injection of STZ, there was clearly an increase in the staining intensity at 4 and 6 weeks of STZ-diabetes (figs 1 and 2). And this increased intensity is reversed in the insulin-treated group. Previous experiments in our laboratory confirmed that each antibody is specific for its respective COX isoform (11). As indicated in figure 3, Western blot analysis confirms the immunohistochemistry findings, showing a 2 - 4 fold increase in COX-1 between 2 and 6 weeks of STZ-diabetes, and about a 3 -fold increase in COX-2 at 6 weeks (fig 4). A summary of the levels of COX-1 and -2 at different stages of diabetes is shown in Tables 2 and 3. In contrast to changes in protein levels, Northern blotting indicates that COX-1 and -2 mRNA is unchanged in the medullary region of 6 week STZ rats (data not shown).

Figure 3.1. Immunohistochemical analysis of COX-1 at 4 weeks. Longitudinal sections of paraffin embedded kidneys were used to localize COX-1 in the medullary regions of the kidney of 4 weeks STZ diabetic rats compared to controls. COX-1 was detected using a human polyclonal antibody. Increased tubular staining is shown by arrow in diabetic sections. A-C) outer medulla; D-F) inner medulla; Control (A, D), Diabetic (B, E), Insulin (C, F). N = 3.

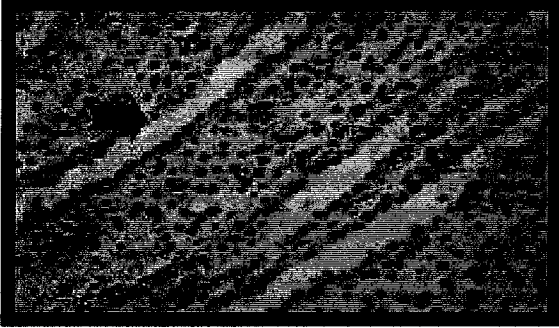
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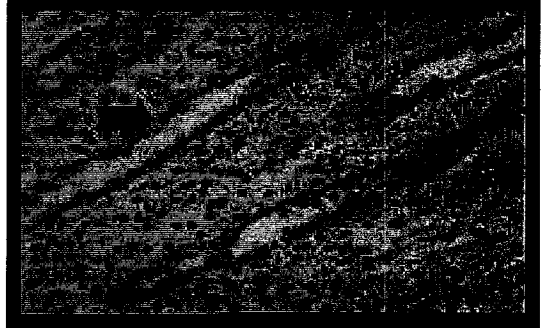
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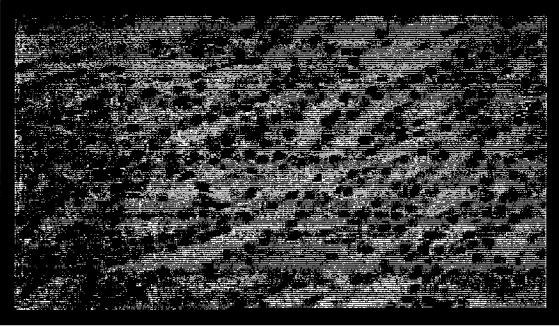
B



E



C



F

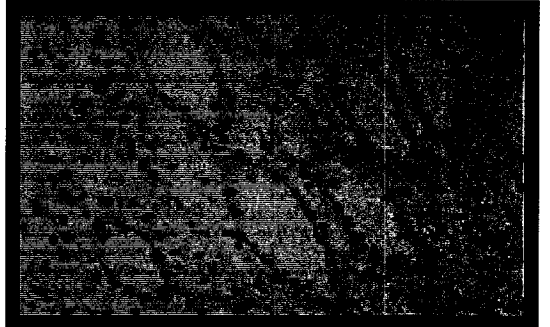


Figure 3.2. Immunohistochemical analysis of COX-2 at 6 weeks. Longitudinal sections of paraffin embedded kidneys were used to localize COX-2 in the medullary regions of the kidney of 6 weeks STZ diabetic rats compared to controls. COX-2 was detected using a human polyclonal antibody. Increased tubular staining is shown by arrow in diabetic sections. A-C) outer medulla; D-F) inner medulla; Control (A, D), Diabetic (B, E), Insulin (C, F). N = 3.

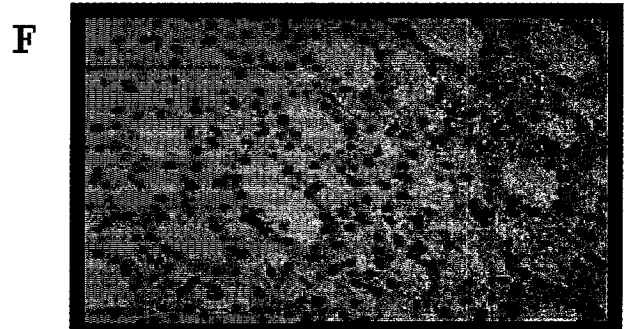
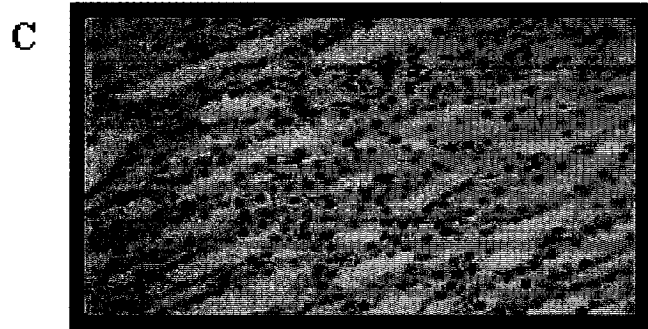
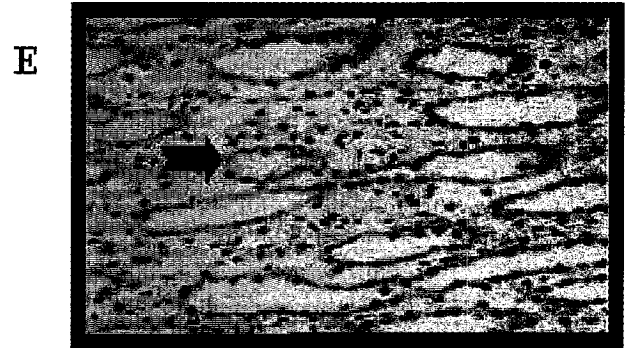
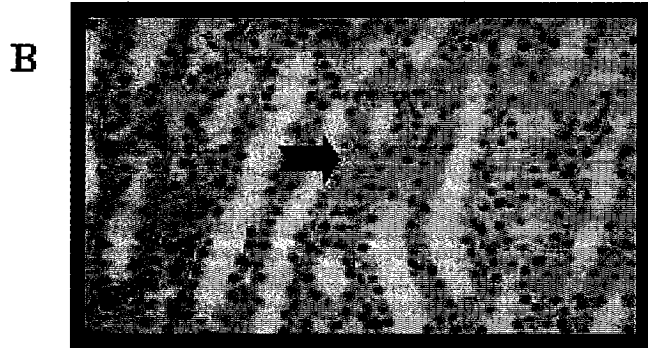
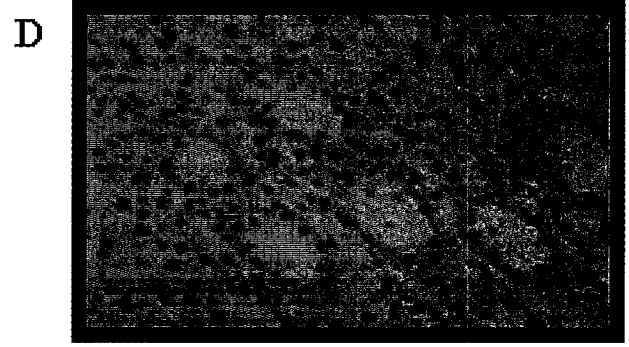
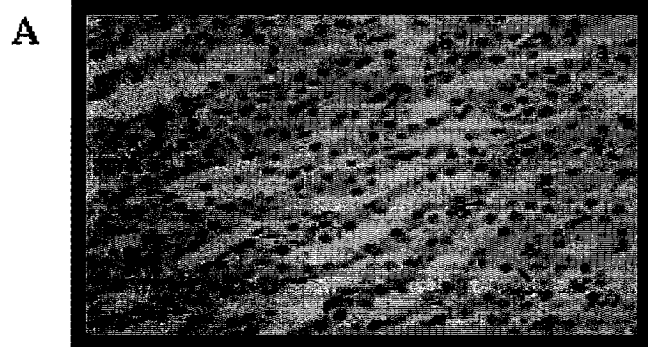


Figure 3.3. COX-1 protein is increased at 6 weeks diabetes. A) Representative autoradiograph of COX-1 (70 KDa) in outer medulla. Lane number: 1- control, 2- diabetic, 3- insulin. B) Detection of β -actin to normalize samples. C) Densitometric analysis. Results shown as fold of control. Data presented as mean \pm SEM, n=6. *p<0.05.

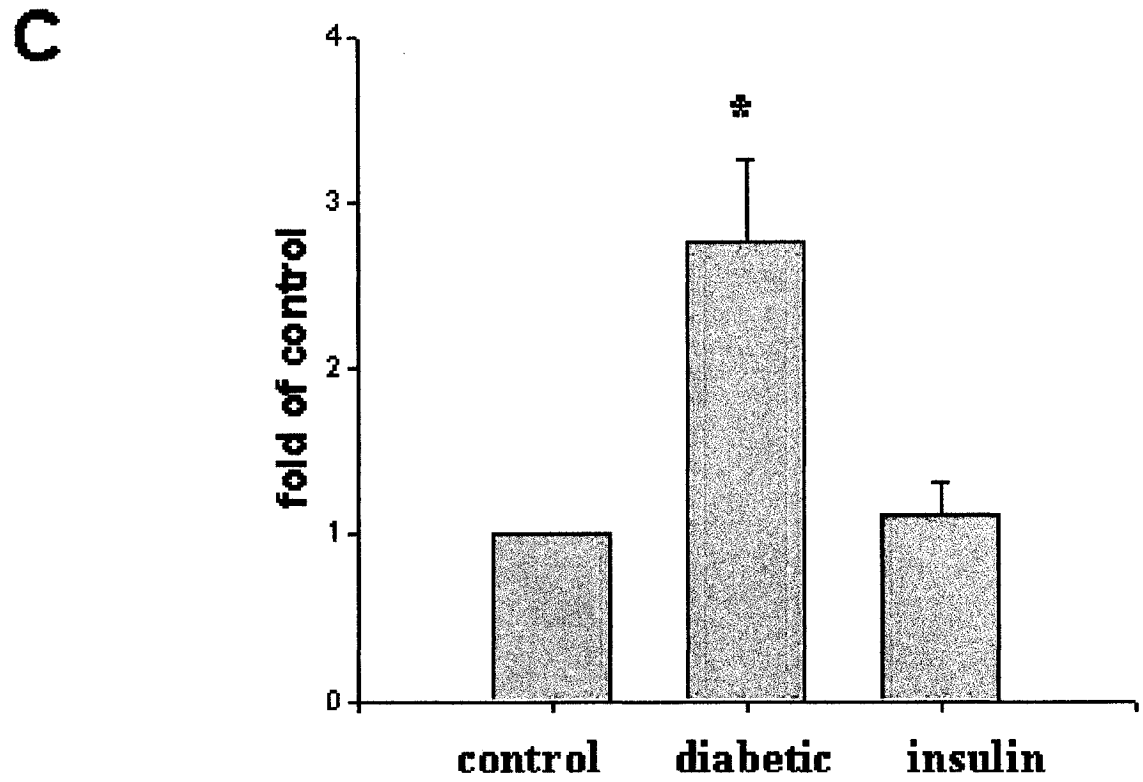
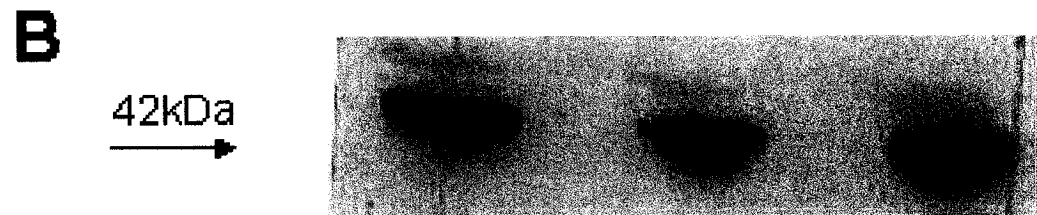


Figure 3.4. COX-2 protein is increased at 6 weeks diabetes. A) Representative autoradiograph of COX-2 (72 KDa) in outer medulla. Lane number: 1- control, 2- diabetic, 3- insulin. B) Detection of β -actin to normalize samples. C) Densitometric analysis. Results shown as fold of control. Data presented as mean \pm SEM, n=5.*p<0.05.

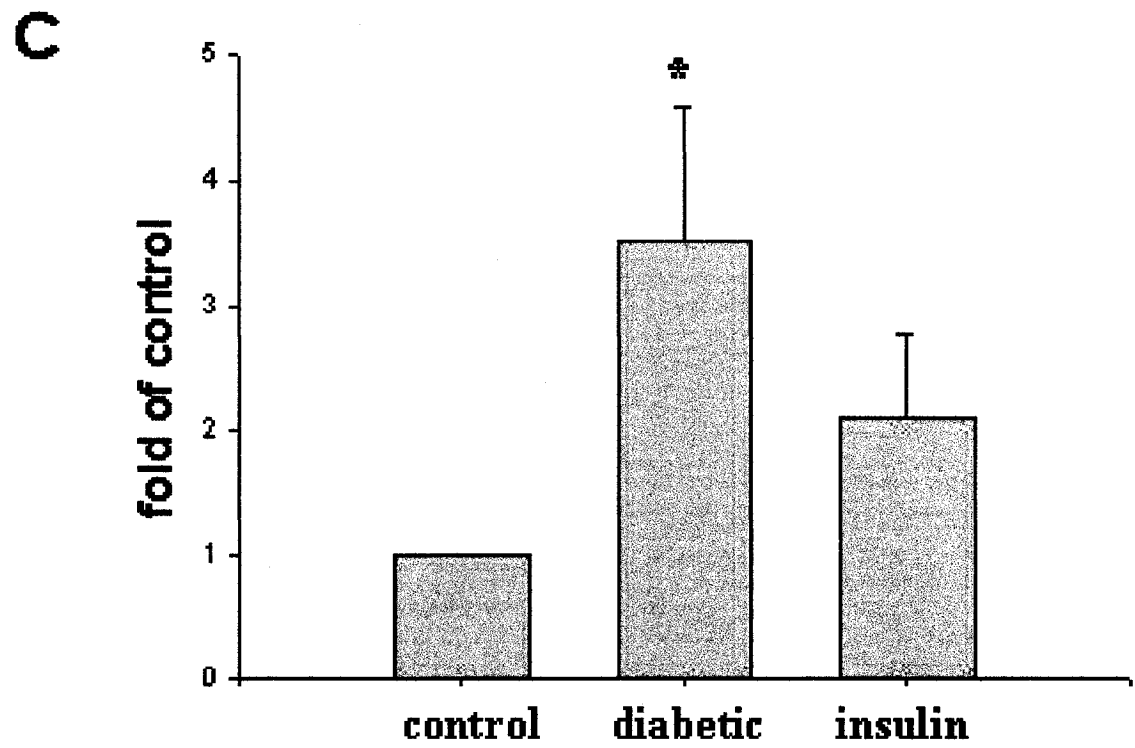
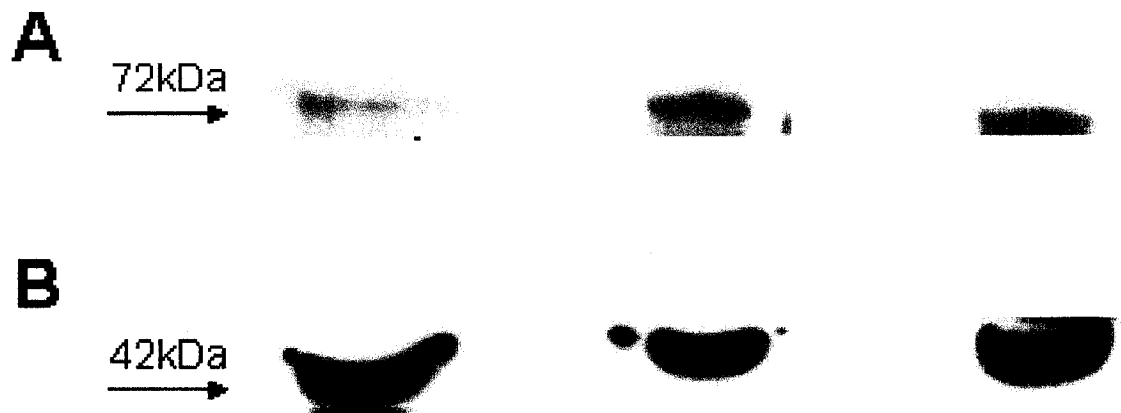


Table 3.2. Summary of COX-1 levels at different stages of STZ-diabetes.**A**

Weeks	Control	Diabetic	Insulin	N
2	1	2.31 ± 0.47*	2.29 ± 0.61	4
4	1	3.74 ± 0.41**	1.37 ± 0.32	5
6	1	3.00 ± 0.68*	2.02 ± 0.49	5
8	1	1.37 ± 0.42	0.82 ± 0.20	5

B

Weeks	Control	Diabetic	Insulin	N
2	1	1.16 ± 0.08	0.9 ± 0.33	3
4	1	4.28 ± 1.03*	3.85 ± 1.43	5
6	1	1.74 ± 0.24*	1.39 ± 0.3	6
8	1	2.32 ± 0.21**	1.18 ± 0.11	5

Protein was isolated from medullary regions of the kidney from 2, 4, 6, and 8 weeks STZ-rats: A) outer medulla, B) inner medulla. Western blot analysis was performed to quantify levels of COX-1 in each region. Results are presented as fold of control. Data shown as mean ± SEM. N = 3-6. *p<0.05 **p<0.001

Table 3.3. Summary of COX-2 levels at different stages of STZ-diabetes.

A

Weeks	Control	Diabetic	Insulin	N
2	1	1.49 ± 0.39	1.68 ± 0.91	3
4	1	1.26 ± 0.23	1.53 ± 0.42	5
6	1	3.62 ± 0.41**	0.90 ± 0.35	5
8	1	2.00 ± 0.69	0.95 ± 0.40	5

B

Weeks	Control	Diabetic	Insulin	N
2	1	1.94 ± 0.37	1.21 ± 0.42	3
4	1	2.01 ± 0.62	2.10 ± 0.59	5
6	1	2.55 ± 0.66*	2.65 ± 0.74	5
8	1	1.20 ± 0.11	0.64 ± 0.21	5

Protein was isolated from medullary regions of the kidney from 2, 4, 6, and 8 weeks STZ-rats: A) outer medulla, B) inner medulla. Western blot analysis was performed to quantify levels of COX-2 in each region. Results are presented as fold of control. Data shown as mean ± SEM. N = 3-5. *p<0.05 **p<0.001

PGE₂ synthase levels are increased in outer medulla of 6 weeks STZ rats:

A secondary enzyme in the synthesis pathway of PGE₂ is PGE₂ synthase, which converts the inactive intermediate PGH₂ produced by the action of COX on arachidonic acid. Western blotting was utilized to measure protein levels of this enzyme in diabetic rats compared to controls. A 5.9 ± 1.9 -fold increase in PGE₂ synthase protein was observed in the outer medulla of 6 weeks STZ-diabetic rats (fig 5), but no change in enzyme levels was found in the inner medulla. Consistent with this latter finding, the levels of PGE₂ synthase were unchanged (1.1 ± 0.2 -fold control; n = 4) in cultured rat IMCD exposed to 37.5 mM glucose for 4 days, compared to 17.5 mM controls.

COX-1 and COX-2 are increased in IMCD upon exposure to high glucose:

To determine whether the increase in COX observed in the medulla of 4-6 week STZ-rats also occurred in the IMCD exposed to high glucose, we cultured IMCD cells in 37.5 mM glucose for 4 days. As illustrated in figure 6, both COX-1 and COX-2 levels were augmented 2.1 ± 0.4 -fold and 3.1 ± 0.4 -fold, respectively, in high glucose-treated cells compared to controls. As noted above for 6 weeks STZ rats, no change in COX-1 and -2 mRNA was noted by Northern blotting in cultured rat IMCD exposed to high glucose (data not shown).

Figure 3.5. PGE₂ synthase is increased in outer medulla of STZ-diabetic rats. Protein lysates were obtained from medullary regions of 6 weeks rats. A) Representative autoradiograph of PGE₂ synthase (16 KDa) in outer medulla. Lane number: 1- control, 2- diabetic, 3- insulin. B) Detection of β -actin to normalize samples. C) Densitometric analysis of PGE₂ synthase in outer and inner medulla. Results shown as fold of control. Data presented as mean \pm SEM, n = 5-6. * p<0.05.

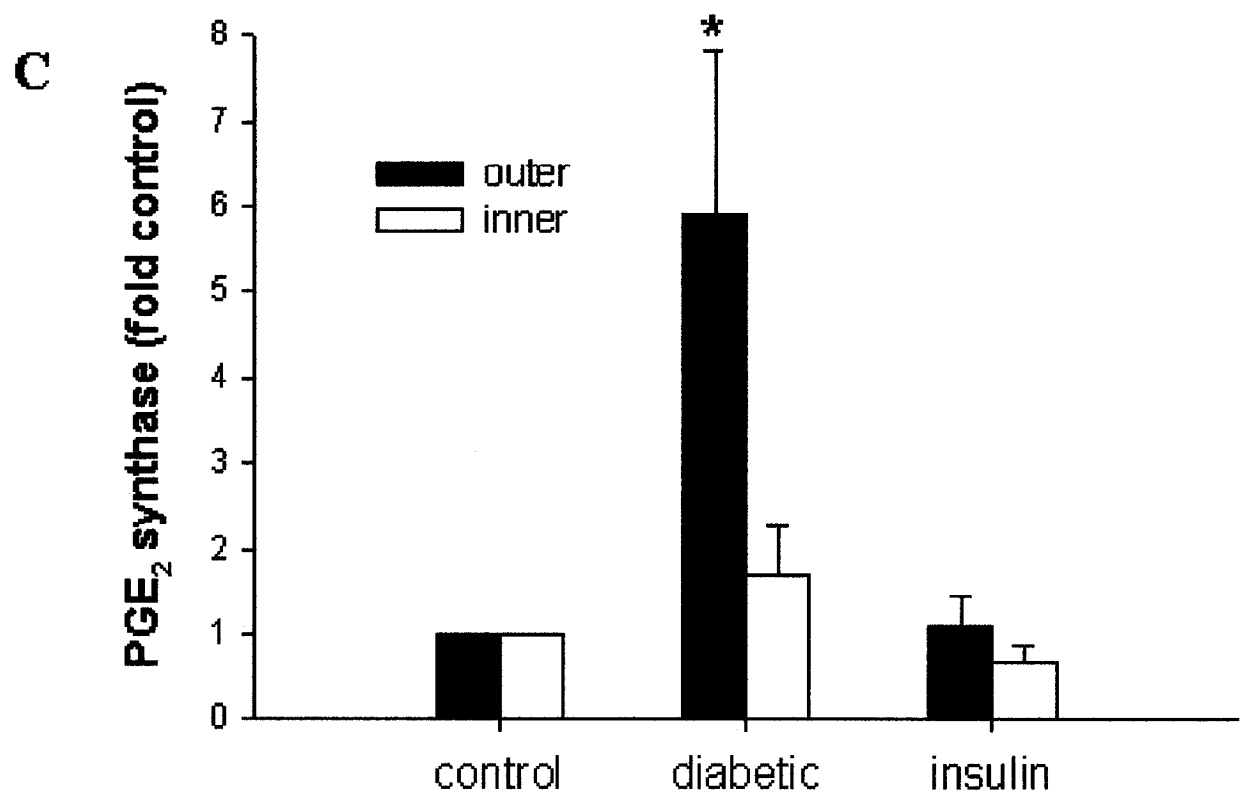
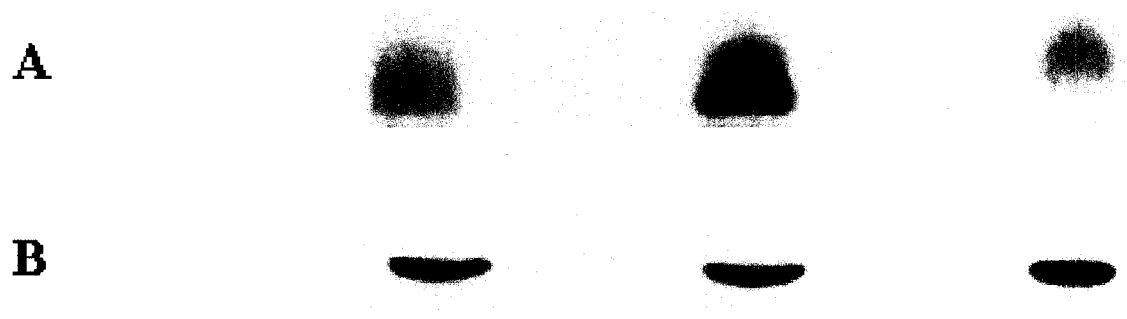
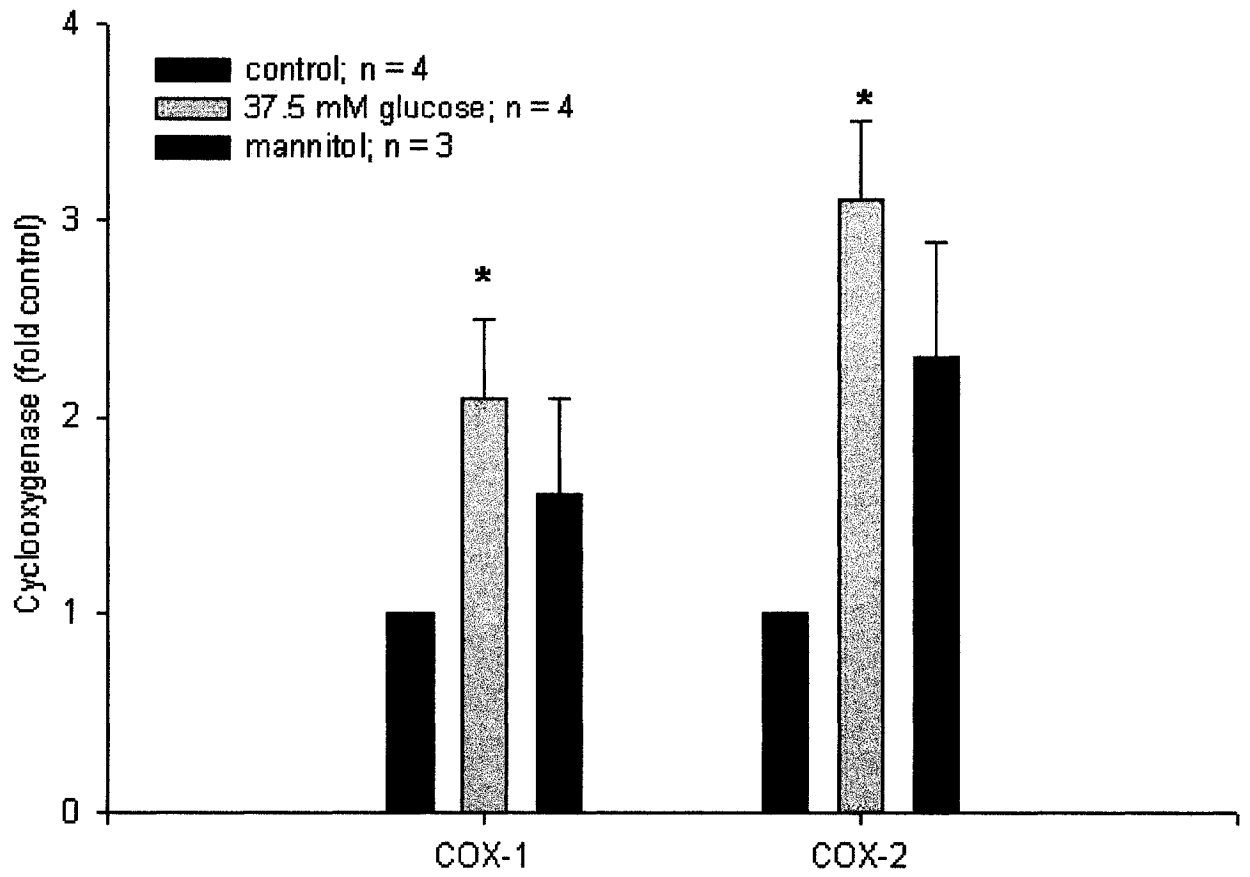


Figure 3.6. COX-1 and -2 are increased in cultured rat IMCD exposed to high glucose. Protein was isolated from IMCD exposed to either: 1- control, 2- 37.5 mM glucose, 3- 37.5 mM mannitol, for 4 days; and quantified by Western blotting. Densitometric analysis of COX, results shown as fold of control. Data presented as mean \pm SEM, n = 4. * p < 0.05.



PGE₂ synthesis is increased in IMCD upon exposure to high glucose:

In spite of the lack of change in PGE₂ synthase levels in the inner medulla and IMCD (as noted above), a significant increase in COX was observed. Since COX is the rate-limiting enzyme in the PGE₂ synthesis pathway, we measured PGE₂ levels by enzyme immunoassay. As shown in figure 7, PGE₂ levels are elevated 2.05 ± 0.46 –fold in IMCD exposed to high glucose for 4 days, but no change in 6-keto-PGF_{1 α} levels was observed. In comparison, the levels of both prostanoids were elevated to a similar extent after 2 days of high glucose exposure: 1.7 ± 0.3 –fold control for PGE₂, and 1.6 ± 0.17 –fold control for 6-keto-PGF_{1 α} . It is noteworthy that TXB₂ levels were also measured, but they did not differ from control (data not shown).

EP₁ and 4 receptors are altered in IMCD upon exposure to high glucose:

As illustrated in figure 8, both EP₁ and EP₄ receptor mRNA is detected in rat IMCD. This medullary localization of EP receptor subtypes is in agreement with our previous work demonstrating the expression of EP_{1, 3, 4} receptors mRNA in medullary segments of the rat nephron, namely EP_{1,3} in medullary thick ascending limb (18, 33) and EP_{1,3,4} in IMCD (32). As shown in this paper, upon exposure of cells to high glucose, there is a significant increase in EP₄ receptors, of 1.8-fold. Interestingly, when mannitol was added instead of glucose there is a slight reduction in EP₄ mRNA. While a tendency for EP₁ mRNA to increase is noted, the relevance remains inconclusive due to an increase in response to mannitol as well. Whether the change in mRNA is due to an osmotic effect is noteworthy, and ongoing work in our laboratory will further examine this different effect on both receptors. Furthermore, Western blot analysis indicates that EP₄ receptors

are elevated in IMCD cultured in 37.5 mM glucose, approximately 2 -fold compared to control cells grown in 17.5 mM glucose (fig 9). This is consistent with previous reports from our laboratory indicating that a change in cortical collecting duct COX levels resulted in compensatory changes in PGE₂ receptor levels at the cell surface (33).

Figure 3.7. Prostanoid levels are increased in cultured rat IMCD exposed to high glucose. IMCD were exposed to either: 1- control, 2- 37.5 mM glucose, 3- 37.5 mM mannitol, for A) 2 days, and B) 4 days. PGE₂, and 6-keto-PGF_{1α}, production was assayed by competitive enzyme immunoassay, showing results as fold of control. Data presented as mean ± SEM. N = 6. * p < 0.05, ** p < 0.001.

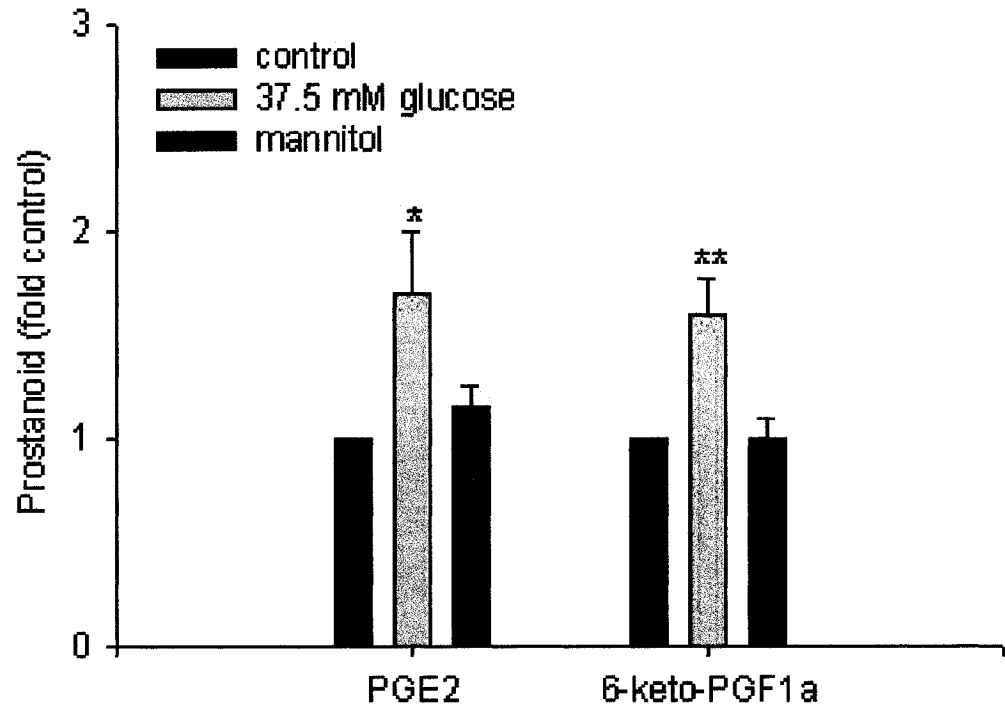
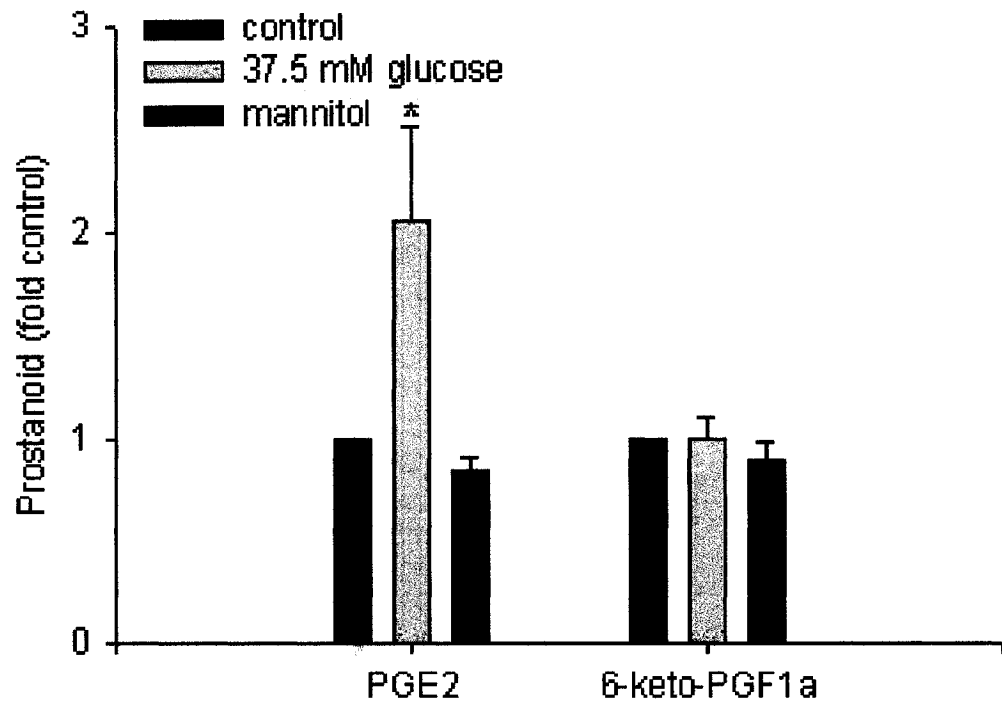
A**B**

Figure 3.8. EP receptor mRNA is increased in IMCD exposed to high glucose. Northern blot detection of EP receptor mRNA in cultured IMCD exposed to: 1) control, 2) 37.5 mM glucose, and 3) 37.5 mM mannitol, for 4 days. Densitometric analysis of EP₁ and EP₄ receptor mRNA levels, shown as fold of control. Results expressed as mean \pm SEM (n=3), p < 0.05.

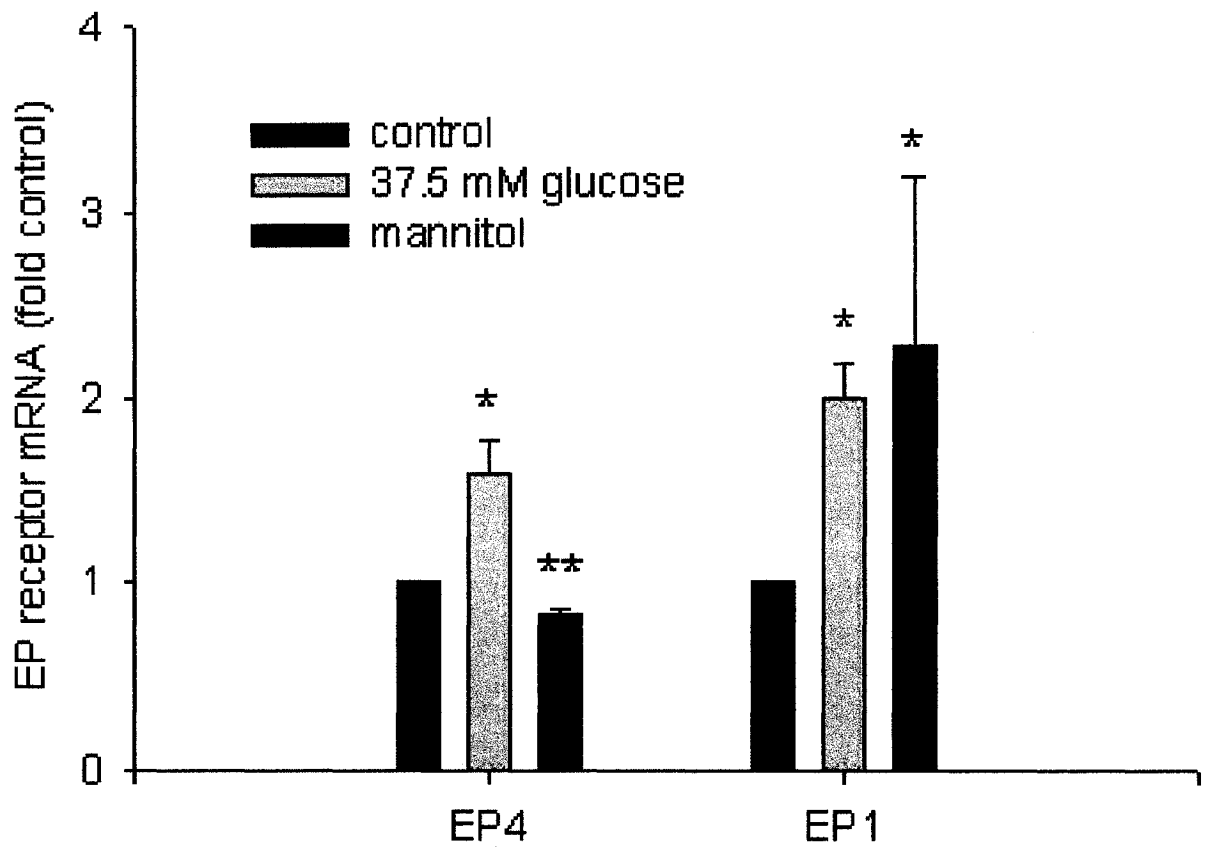
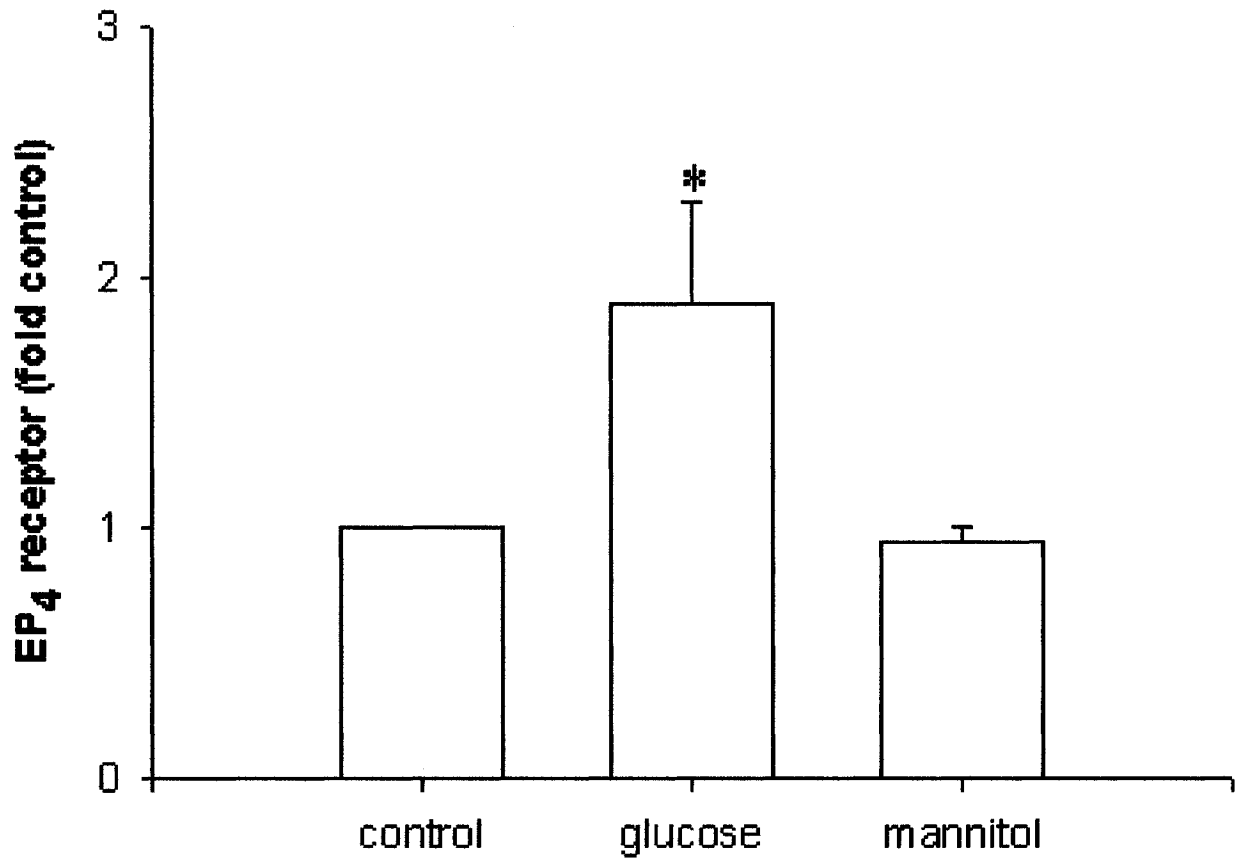


Figure 3.9. EP₄ receptors are increased in cultured rat IMCD exposed to high glucose. IMCD were exposed to either: 1- control, 2- 37.5 mM glucose, and 3- 37.5 mM mannitol. EP₄ receptors are presented as fold of control. Data presented as mean \pm SEM, n = 6. * p < 0.05.



DISCUSSION

Numerous studies elude to the putative role of prostanoids (PGs) in diabetic nephropathy (9), but a major controversy exists as to the nature of their involvement: whether they propagate the complications or serve to antagonize the deleterious effects of other agents. Moreover current work has examined the beneficial effects of specifically targeting certain prostanoid pathways to alleviate the manifestations of the disease, including EP₁ receptor antagonists (28) and IP receptor agonists (22, 48). But the underlying mechanisms of PG involvement remain uncertain. In diabetic kidneys, it has been clearly demonstrated that COX enzymes are elevated, and glomerular PG production in most species is increased (13, 20, 47), as well as in STZ-rats (25, 39). Also, several pathways are stimulated by hyperglycemia and are putatively responsible for altering the levels of COX, including: activation of protein kinase C (36), p38 mitogen-activated protein kinase (7, 14), and countless others (21, 34). Actually, selective inhibitors of COX-2 (NS-398) have been used to reverse some of the renal complications of STZ-diabetes, such as altered glomerular filtration rate, without affecting mean arterial pressure or renal plasma flow (24). Also, in the remnant kidney model, COX-2 inhibition slowed the development of proteinuria and attenuated renal structural damage in animals treated with these drugs (38). The exact mechanisms by which COX-derived prostanoids participate in the pathogenesis of the nephropathy, and the extent of contribution of individual COX isoforms surely requires further elucidation. However, it is clearly no longer valid to label COX-1 products as beneficial for normal function and COX-2 derived prostanoids as playing a pathological role.

In this study we characterized the medullary expression of COX-1 and COX-2 in diabetic rats. Tubular levels (IMCD, OMCD, mTAL) of both COX isoforms are elevated between 4 – 6 weeks after STZ injection, as well as in cultured IMCD exposed to high glucose for 4 days. This is in agreement with a previous study by Komers et al (24), showing increased cortical expression of COX-2 at 4 weeks of diabetes. However, our study clarifies the time course for each COX isoform, between 2 and 8 weeks of diabetes. Since we did not find a change in COX mRNA in either the diabetic rats or in IMCD exposed to high glucose, it is possible that there is increased protein stabilization; but whether there is also enhanced enzyme activity is uncertain. The renal manifestations, or cellular events, coinciding with this increase, and the role in the pathogenesis of the nephropathy at these stages of diabetes remains unclear at this time.

Another important enzyme in PGE₂ synthesis is PGE₂ synthase. While COX is increased in both the inner and outer medulla of the diabetic kidney, changes in PGE₂ synthase were only observed in the outer medulla. Consistent with this finding, we show no change in this enzyme in cultured IMCD exposed to high glucose. Thus the contribution of PGE₂ synthase to diabetic alterations in PG levels in the inner medulla seems insignificant. To verify whether the increase in COX in IMCD exposed to high glucose resulted in changes in prostanoid levels, despite the lack of change in PGE₂ synthase, we measured the production of PGE₂ and prostacyclin in the media; these are the two major prostanoids produced in the rat inner medulla (3). Our work indicates an increase in both PGE₂ and prostacyclin (6-keto-PGF_{1α}) in IMCD exposed to high glucose for 2 days, and an increase in only PGE₂ at 4 days. However at this time it is not clear which COX isoform contributes to the majority of PG production in the rat IMCD. In a

previous study by our group (11), it was demonstrated that in a mouse cortical collecting duct cell line, the majority of PGE₂ produced was dependent on COX-2 rather than COX-1. Future studies using NS-398 (a selective COX-2 inhibitor) will determine whether this holds true in the rat IMCD as well.

To further characterize changes in the IMCD exposed to high glucose, we examined the expression of PGE₂ receptors in these cells. We show by Northern blot analysis that both EP₁ and EP₄ receptor mRNA is present in cultured rat IMCD, as previously observed by RT-PCR in our laboratory (32). While exposure to high glucose increased the expression of EP₁, the response was similar upon exposure to mannitol, indicating a possible osmotic effect. Ongoing studies in our laboratory will address this issue. On the other hand, EP₄ mRNA was significantly increased, as well as EP₄ protein levels in the IMCD exposed to high glucose. It is noteworthy that work looking at the effects of high glucose on the PGE₂ response in primary cultures of MG cells showed an inhibition of PGE₂ –stimulated cAMP, with no change in EP₄ receptor mRNA (20). The reason for the discrepancy in regulation of EP₄ mRNA by glucose in IMCD versus MG cells is unclear at this time. Future studies in our laboratory will examine the cellular signaling in response to PGE₂ in order to clarify the effects of glucose in the IMCD. For instance, increases in cAMP are known to inhibit the proliferative state of cells (6, 20), determine cell fate and apoptotic responses (17), and alter gene transcription via cAMP-responsive elements in a large number of target genes (34).

As observed in this study, rather than making up for increased PGE₂ production by down-regulating EP₄ receptors, IMCD cells exposed to high glucose have an enhanced cellular response, by increasing both COX (PGE₂ production) and cell surface binding

sites for PGE₂. Whether this would result in a change of IMCD function that could perpetuate tubular disease in diabetics requires further investigation. The IMCD is an important regulator of sodium, H₂O, and potassium homeostasis, three of many disturbances seen in diabetes. Since PGE₂ is a key mediator of this IMCD function, acting through at least three subtypes of the EP receptors: EP₁, EP₃, EP₄ (5, 46), defective PGE₂/EP receptor signaling pathways could interfere with the fine-tuning of salt and water transport, and these abnormalities could contribute to edema, hypertension, vascular changes, associated with diabetic nephropathy. However, we report in this study that tubular PGE₂ levels are increased in our high glucose “diabetic” model. Since PGE₂ plays an important role in regulating ion concentrations in the urine by inhibiting NaCl transport in the collecting duct favouring salt elimination in the urine together with water (5), the resultant natriuresis and diuresis may be beneficial in diabetes as a compensatory response. To further support this idea, the significance of PGE₂ to the maintenance of salt and water homeostasis is clearly demonstrated by the undesirable renal effects such as sodium and potassium retention (37), associated with the use of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the production of prostaglandins. It would be very interesting if fluctuations in PGE₂/EP signalling could be linked in time to the state of the rat in vivo, showing that at various stages of the disease levels are increased or decreased according to factors such as: GFR, BP etc...Therefore we report here that the PGE₂ system in the IMCD is playing a protective role, compensating for systemic disturbances that are associated with the disease. Adding to the complexity, PGE₂ can also play a role within the IMCD itself, independent of H₂O and electrolyte transport. It could potentially alter the expression of numerous genes via direct nuclear signalling

pathways (1, 2), and thus contribute to the nephropathy by crosstalking with nitric oxide or angiotensin II for instance. It could also alter fibrogenesis or apoptotic events that are associated with tubular atrophy and cell loss in later stages of diabetes. The possibilities are endless, and needless to say more work is required to shed some light on the mechanisms involved.

In summary, immunohistochemical analysis reveals more intense COX-1 signals in tubule segments of outer and inner medulla at about 4 weeks of diabetes; COX-2 staining is stronger at 6 weeks. COX-1 protein levels are also increased at 2 to 6 weeks of diabetes, but COX-2 is only increased at 6 weeks. No change in COX mRNA is detectable. A 6 - fold increase in PGE₂ synthase is observed in the outer medulla at 6 weeks of diabetes, but no difference is seen in the inner medulla or IMCD. Upon exposure to high glucose, PGE₂ and prostacyclin synthesis is increased in the IMCD. Likewise, EP₄ receptor mRNA and protein is increased in IMCD exposed to high glucose. Further studies will clarify which isoform of COX is contributing to increased PGE₂ in the diabetic IMCD, and the significance of these findings to disturbances in IMCD function and progression of diabetic nephropathy. Once clarified, this could lead to the advent of better combination therapy to prevent the progression of the disease.

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CHAPTER 4

Characterization of the PGI₂/IP System in Cultured Rat Mesangial Cells

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Characterization of the PGI₂/IP System in Cultured Rat Mesangial Cells.

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ABSTRACT

Mesangial cells play an important role in glomerular function. They are an important source of cyclooxygenase (COX)-derived arachidonic acid metabolites, including prostaglandin E₂ and prostacyclin. Prostacyclin receptor (IP) mRNA was amplified from cultured mesangial cell total RNA by RT-PCR. While the prostaglandin E₂ receptor subtype EP₂ was not detected, EP_{1, 3, 4} mRNA was amplified. Also, IP protein was noted in mesangial cells, proximal tubules, inner medullary collecting ducts, and the inner and outer medulla. But no protein was detected in whole cortex preparations. Prostacyclin analogues: cicaprost and iloprost, increased cAMP levels in mesangial cells. On the other hand, arginine-vasopressin and angiotensin II increased intracellular calcium in mesangial cells, but cicaprost, iloprost and prostaglandin E₂ had no effect. Moreover, a 50 % inhibition of cicaprost- and iloprost-cAMP stimulation was observed upon mesangial cell exposure to 25 mM and 35 mM glucose for 5 days. But no change in IP mRNA was observed at any glucose concentration or time exposure. Although 25 mM glucose had no effect on COX-1 protein levels, COX-2 was increased up to 50 %. In contrast, PGIS levels were reduced by 50 %. Thus, we conclude that the prostacyclin/IP system is present in cultured rat mesangial cells, coupling to a cAMP stimulatory pathway. High glucose altered both enzymes in the PGI₂ synthesis pathway, increasing COX-2 but reducing PGIS. In addition, glucose diminished the cAMP response to prostacyclin analogues. Therefore, glucose attenuates the PGI₂/IP system in cultured rat mesangial cells.

KEYWORDS: cAMP, mesangial cells, glucose, prostacyclin, IP receptor.

INTRODUCTION

Prostacyclin (PGI₂) is a major product of the arachidonic acid cascade, most renowned for its potent vasodilatory and anti-thrombotic effects in the vasculature. It is now recognized that PGI₂ can alter both intracellular and nuclear signaling by activating two distinct pathways (1). The most characterized pathway for PGI₂ action is the PGI₂/IP system, whereby PGI₂ binds to its cell surface receptor (IP) coupling to G-proteins guanine regulatory stimulating protein (mainly G_s), and activates adenylate cyclase to increase intracellular cAMP. The IP cDNA was cloned from mouse thymus in 1994 (2), rat (3), and human (4). In the rat, the 1360 bp transcript encodes a protein of 416 a.a. The second pathway for PGI₂ signaling has recently emerged, whereby PGI₂ acts as an endogenous ligand for PPAR δ (5), a ubiquitously expressed member of the peroxisome proliferator-activated receptors. PPAR δ appears to mediate the effects of PGI₂ on blastocyst implantation (6) and intestinal tumorigenesis (7), and can regulate cell fate by promoting apoptosis (8). The target genes for this pathway have yet to be identified. Interestingly, this is not the first indication that prostanoids can alter the expression of target genes by direct effects on the nucleus. A few reports have indicated nuclear signaling linked to PGE₂ pathways, which is dependent on the presence of nuclear prostaglandin receptors, EP_{1,3,4} (9, 10). Whether or not such nuclear IP receptors exist is uncertain at this time. While the contribution of the PGI₂/PPAR pathway to renal physiological and pathological processes remains uncertain, our group and others have recently characterized the renal PGI₂/IP pathway in human (11), rabbit (12), rat (13, 14), and mouse (15).

Renal PGI₂ is mainly synthesized in the glomerulus (mesangial cells) and the collecting ducts (16). Its synthesis is dependent on two enzymes, cyclooxygenases (COX) and prostacyclin synthase. Over the past decade or so, there seems to be a greater appreciation for the contribution of PGI₂ to renal biology. However, there remain numerous gaps in the current knowledge regarding the mechanisms underlying PGI₂ actions. To further clarify the role of the IP receptor, Murata et al. produced an IP knockout mouse (17). Not only were these mice normotensive, but also displayed no obvious renal pathology. This same observation was made in PGE₂ receptor subtypes (EP₁₋₄) knockout mice (18, 19, 20). Since there is quite a great deal of overlap in function among prostanoids and other hormonal systems in the kidney, it is becoming quite evident that under normal physiological states, prostaglandins play a secondary role, acting mainly as homeostatic factors. However, their contribution to disease processes may be of greater relevance. In fact, many studies suggest that prostaglandins may play a role either in the initiation or the progression of diabetic nephropathy, a leading cause of end-stage renal disease. Whether they are behaving as cytoprotectors or contributing to the renal damage is uncertain. While the disease itself is very complex, new insights into the development of the nephropathy are emerging.

Diabetic nephropathy is characterized by an initial hyperfiltration, associated with a preferential vasodilatation of the afferent arteriole (21). A prominent feature clearly involved in the progression of the nephropathy is mesangial hypercellularity and expansion of the mesangial matrix (altered protein degradation or synthesis) leading to glomerular sclerosis, and hypertrophy of the proximal tubule (22, 23). While theories are up-and-coming implicating the hyperglycemia in these events, a great body of evidence

does also implicate a multitude of other factors that mediate the indirect effects of high glucose (24). Unravelling the disturbance in the balance of these factors is likely the key to understanding the pathogenesis of this disease. The role of prostacyclin in diabetes is becoming more and more recognized: cytoprotection, contributing to hyperfiltration, to matrix protein deposition, to hypertrophy (mitotic agent), and apoptotic events (25, 8, 26, 27).

Clearly mesangial cells play a central role in glomerular function and the progression of glomerular disease. Not only is this subset of glomerular cells a large contributor to the overall production of COX derivatives, they are also susceptible to both autocrine and paracrine control by prostaglandins. Since PGI₂ is highly synthesized in the glomerular region and has been implicated in the maintenance of glomerular hemodynamics and renin secretion (28), the focus of this work is to examine the expression of IP receptors in cultured rat mesangial cells, clarify the signaling pathways linked to this receptor, and determine whether high glucose alters this signaling.

MATERIALS AND METHODS

MG cell culture:

Mesangial cells between passages 7-9 were a generous gift from Dr James W. Scholey (University of Toronto, ON, Canada). These cells were obtained from rat glomeruli isolated by standard sieving protocols (29), and stored in liquid nitrogen. The cells were rapidly thawed out by agitation in a 37°C H₂O bath, washed in DMEM media (Sigma), and grown to confluence in a 100 mm Petri dish at 37°C and 5% CO₂. The culture media consisted of DMEM media (Sigma), pH 7.4, supplemented with: 20% fetal bovine serum (FBS), HEPES, Sodium pyruvate (Sigma), and 1% penicillin-streptomycin (Gibco-brl). The cells were utilized between passages 11-15 for all the experiments described below. For high glucose experiments, the media was changed the day following plating to media containing either high (15, 25, 35 mM) or control (5.6 mM) glucose, and maintained in culture for 5 days. Mannitol was added instead of glucose to control for osmotic effects.

RNA isolation and RT-PCR:

MG cells were grown to confluence in 100 mm Petri dishes and the cells were scraped off and centrifuged at 1100 rpm. The collected pellet was resuspended in 1 ml Trizol reagent (Gibco), and total RNA was isolated using the Trizol method, as described by the manufacturer (Gibco-brl); and was DNase-treated (Boehringer Mannheim) to eliminate genomic DNA. The RNA was reverse transcribed into cDNA using MuLV reverse transcriptase and random hexamers, provided in the Gene-AMP RNA PCR core

kit (Perkin Elmer). Samples were prepared in duplicate for each reaction, the duplicates serving as controls for the reverse transcription since the reverse transcriptase was omitted in these tubes. The upstream and downstream primers used for PCR amplification of each cDNA are: IP receptor (407 bp; nucleotides 856-1263) FF-1 5'-GGCACGAGAGGATGAAGTTTACC-3' and FF-2 5'-GTCAGAGGCACAGCAGTCAATGG-3'; EP₁ receptor (336 bp; nucleotides 865 - 1201) EP_{1,1} 5'-CGCAGGGTTCACGCACACGA-3' and EP_{1,2} 5'-CACTGTGCCGGAACTACGC-3'; EP₂ receptor (401 bp; nucleotides 757 - 1158) EP_{2,1} 5'-AGGACTTCGATGGCAGA-GGAGAC-3' and EP_{2,2} 5'-CAGCCCCTTACACTTCTCCAATG-3'; EP₃ receptor (437 bp; nucleotides 538-975) EP_{3,1} 5'-CCGGGCACGTGGTGCTTCAT-3' and EP_{3,2} 5'-TAGCAGCAGATAAA-CCCAGG-3'; and EP₄ receptor (423 bp; nucleotides 941 - 1364) EP_{4,1} 5'-TTCCGCTCGTGGTGCGAGTTTC-3' and EP_{4,2} 5'-GAGGTGGTGTCTGCTTGGGT-CAG-3'. The DNA amplification was performed using a thermal cycler, the Perkin Elmer Gene-AMP PCR System 2400. Depending on the primers used, the PCR reaction consisted of the following parameters: denaturing at 94°C for 240 sec; followed by 35 cycles of: denaturing at 94°C for 15 sec, annealing at 63°C for 30 sec, and extension at 72°C for 30 sec; then 72°C for 10 min, and finally cool-down to 4°C. The given amplification products are then separated by gel electrophoresis, on a 1.5 % agarose gel, for size determination with standards, and visualized under UV light using ethidium bromide.

cAMP radioassays:

Cells were grown to confluence in 24 well plates for 3 days, and serum starved 24 hrs. The cells were then pre-treated for 15 min in DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 10 μ M indomethacin (Sigma). At timed intervals, the MG cells were then stimulated for 10 min with 1 nM to 1 μ M of agonist: AVP, CCP, ILP, PGE₂. The samples were all prepared in duplicates. To stop the reaction, 300 μ l of 10% trichloro-acetic acid (TCA) was added to each well. Following 30 min incubation on ice, the samples in TCA were transferred to eppendorf tubes and centrifuged for 10 min at 4000g. Next, 250 μ l of each sample was transferred to glass test tubes and four ether extractions of TCA were performed using 4 times the volume of H₂O-saturated diethyl-ether per extraction. 1 M Tris-HCl was used to bring the pH of the samples to 7-8. Using the cAMP radioassay kit (Intermedico), cAMP levels in each sample were then measured in 100 μ l of sample according to the manufacturer's instructions.

In the second set of experiments, in order to verify the effect of high glucose on the cAMP response to CCP and ILP in MG cells, cells were exposed to low (15 mM), moderate (25 mM), or high (35 mM) glucose; for varying periods: 15, 30, 60 min, 12, 24 hrs and 5 days. Then IBMX and indomethacin were added for 15 min prior to stimulation with increasing concentrations of different agonists for 10 min: PGE₂, AVP, CCP, ILP from 10⁻¹⁰ M to 10⁻⁶ M. Samples were performed in duplicates and cAMP content was assayed using the Intermedico radioassay kit. Mannitol was used instead of glucose as an osmotic control.

Protein isolation and Western Blotting:

Protein samples were prepared by lysing cells (cultured proximal tubule: PT, cultured mesangial cells: MG, cultured inner medullary collecting duct: IMCD) in 100 mM Tris-HCl (pH 7.4), containing 1 mM EDTA and 1 mM EGTA, followed by sonication for 5 sec using an Ultrasonics cell disrupter. The cell lysates were then centrifuged at 10 000 g for 10 min, and the supernatants were removed. For tissue protein isolations, fragments of tissue (outer medulla, inner medulla, cortex) were homogenized in a hypotonic Tris-HCl solution (25 mM), containing 100 mM phenylmethyl-sulfonyl fluoride. The BioRad reagent was added to analyze the protein content using the Bradford method (Bio-Rad Laboratories, Mississauga, ON). After determining the protein concentrations, 50 μ g of protein were combined with Laemmli buffer and denatured by boiling for 3 min followed by 2 min incubation on ice. The samples were then loaded onto a polyacrylamide gel: 4 % stacking and 10 % resolving layers, and resolved by SDS-PAGE using a Mini-PROTEAN II apparatus (Bio-Rad laboratories). The protein was then transferred to a Hybond ECL nitrocellulose membrane (Amersham) using the Mini-Trans Blot system (Bio-Rad). After 4 - 5 hrs of blocking in 5 % milk/TBS-T, the membrane was incubated O/N at 4 °C with a human polyclonal anti-IP antibody (Obtained from Rolf M. Nusing, Germany). Following a series of washes in TBS-T for 1 hr, an HRP conjugated goat anti-rabbit IgG antibody (Promega, Madison, WI) diluted at 1:2000 was added for 90 min, the membrane was again washed for 2 hrs in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Enhanced chemiluminescence (ECL) was used to visualize the results as directed by the manufacturer (Amersham). To control for

the specificity of the primary antibody, IP protein was detected in lysates isolated from the rabbit spleen.

For analysis of COX-1, COX-2, and prostacyclin synthase (PGIS) levels, cells were cultured in control, 25 mM glucose, and mannitol, for 4 days. Lysates were prepared by sonication in RIPA buffer consisting of: 1%NP-40, 1% sodium deoxycholate, 0.1% (w/v) SDS, 0.15 M NaCl, 50 mM Tris pH 7.4, 2 mM EDTA, 0.01 M sodium phosphate pH 7.2) containing 0.5 mM PMSF, protease inhibitor cocktail (Sigma), 100 μ M orthovanadate, 1 mM sodium pyrophosphate, and 10 mM sodium fluoride. Human polyclonal antibodies for COX-1 and COX-2 were used (Cayman Chemicals), as well as a rat monoclonal anti-PGIS antibody (Calbiochem). Detection of β -actin was performed to normalize for loading.

Ca⁺⁺ measurements:

MG cells were cultured on round coverslips for 3 days, and serum starved overnight. The cells were then loaded for 45 min with the calcium indicator fura-2. The dual-wavelength deltascan 1 spectrophotometry system was used to measure changes in calcium levels within the cells. The calcium tracings were obtained using the Felix software. The technique is based on a shift in the excitation/emission wavelength of fura-2 upon binding of the dye to the ionic species (calcium), therefore the calcium concentration can be determined based on changes in the ratio of the dye's fluorescence intensity at the two wavelengths: 340 and 380 nm. Different agonists were applied to the cells using a 300 mOsm solution containing 0.1% albumin (w/v) and (in mM): 190 NaCl, 25 NaHCO₃, 5 KCl, 1.2 MgCl₂, 8 glucose, 5 Hepes, 10 Urea, 1.5 CaCl₂, and 5 NH₄Cl;

and cellular responses were assessed. The compounds tested were 1 μ M of: PGE₂, ILP, and CCP; and 0.1 μ M of: AVP and ANG II. Experiments were performed varying the order of stimulation with each compound, to ensure that one compound does not hinder the binding of the next to its receptor.

Northern blotting:

Cells were treated with glucose and mannitol as described for cAMP experiments above, and the effects on IP mRNA levels in each preparation was examined. Total RNA was isolated (as described above) from various samples, and 10 μ g was loaded onto a formaldehyde gel. Different concentrations of rat cortex RNA (1 - 10 μ g) were examined to determine the optimal detection limit for gel loading and hybridization conditions. An untreated control was performed for each time point: 1 hr, 24 hrs, and 5 days. After running the gel for 3 hrs, the RNA was transferred onto a nitrocellulose membrane and baked for 2 hrs at 80 °C under vacuum. After a 3 hrs prehybridization at 60 °C in a formamide hybridization buffer (5x SSC, 0.1 % SDS, salmon sperm DNA, 5x Denhardt's, 50 % formamide), 25 ng of a P³²-dCTP labelled (Stratagene Prime-It II) human IP probe (920 bp; Cayman) was added to the solution and hybridization was carried out O/N. After a series of moderate stringency washes in 2x SSC/0.1 % SDS at room T°C and 42 °C, autoradiography was performed. In order to normalize the amount of RNA loaded between samples, the membranes were stripped in boiling 0.5 % SDS for 15 min at room T°C, and re-probed with a human anti- β actin cDNA (Cayman). Band intensities are compared by densitometric analysis for quantification and expressed as fold of control.

Statistics:

For cAMP, experiments were performed using duplicate samples and repeated 3 - 6 times each. For Northern blots, experiments were repeated 5 times each. The SigmaPlot software for windows version 4.01 (1986 - 1997) was used to analyze data. Results are expressed as mean \pm SEM. A Student's T test and a one-way ANOVA were performed to assess the statistical significance between data points, followed by the Tukey test for comparison of values, $P < 0.05$.

RESULTS

IP receptor mRNA and protein is expressed in cultured mesangial cells:

IP mRNA was detected in MG cells (fig.1), as well as EP₁, 3, 4. No EP₂ was detectable. This is consistent with the results obtained by in situ hybridization (14) showing the IP message in a central subset of glomerular cells, most likely MG cells. Furthermore, no IP has been detected in mice podocytes (30). On the other hand, both cell types express IP in human glomeruli (11). The reason for this discrepancy is not yet clear. Previous reports have also demonstrated the presence of EP₁ and EP₄ receptors in cultured rat mesangial cells by Northern blotting, without detection of EP₂ (31), while others only detect the EP₂ subtype by RT-PCR (32). Although we detect the EP₃ subtype in these cells, we cannot distinguish which spliced variants of the receptor it represents. As previously demonstrated in our laboratory, the bright band at 730 bp amplified with the EP₁ receptor primer pair represents a fragment of the protein kinase N (PKN) long transcript, since there is exact overlap between the C-terminus tail of EP₁ and the N-terminus of PKN (33).

A signal for IP protein is observed in all the tissue preparations examined: PT, MG, IMCD, inner medulla, and outer medulla, with the exception of the cortex. As shown in the representative autoradiograph (fig.2), a single band is seen at around 52 kDa, consistent with the predicted size for the protein (3). IP protein was also observed in rabbit spleen (serving as a positive control for the antibody). This localization is consistent with previously documented signaling responses linked to the PGI₂/IP system in the rat kidney. The lack of signal in the whole cortex is of interest, considering the

presence of IP protein in the mesangial cells and proximal tubules. The reason for this discrepancy is not yet clear. However studies are ongoing in our laboratory to clarify this issue, looking at quantifying the receptor levels in different cell preparations (CCD, cTAL, glomeruli, PT). Perhaps the level of expression in the PT and MG cells is too low to be detectable in the whole cortex preparation.

High glucose increases COX-2 but decreases PGIS protein levels:

To determine whether endogenous prostacyclin production is altered by high glucose in cultured rat MG cells, both enzymes (COX and PGIS) in the prostacyclin synthesis pathway were examined. PGIS protein was detected in MG cells, a band of about 55 KDa was obtained (fig.7). PGIS levels were decreased by 49 % in response to 25 mM glucose. In addition, both COX-1 and COX-2 were also noted in MG cells. As shown in fig.8, a single band was obtained for each enzyme, 70 and 72 KDa respectively. As illustrated, only COX-2 levels were augmented 50 % in high glucose-treated cells compared to controls, with no change in COX-1 levels.

Figure 4.1. Expression of IP and EP receptors in cultured rat mesangial cells. Gel electrophoresis of the amplified RT-PCR products, from DNase-treated total RNA of cultured MG cells. Lane number: 1- 100 bp DNA ladder, 2- IP (407 bp), 3- EP₁ (336 bp), 4- EP₂ (401 bp), 5- EP₃ (437 bp), 6- EP₄ (423 bp).

1 2 3 4 5 6

407 bp



Figure 4.2. IP receptor protein is ubiquitously expressed in the rat kidney.

Representative autoradiograph showing IP receptor protein (52 kDa) in lysates from different tissue preparations. Lane number: 1 - rabbit spleen (control), 2 - proximal tubules, 3 - mesangial cells, 4 - inner medullary collecting duct, 5- inner medulla, 6- outer medulla, 7- cortex. n = 2 – 5.

1 2 3 4 5 6 7

52 KDa



Figure 4.7. Prostacyclin synthase protein is decreased by high glucose. A) Representative autoradiograph showing PGIS protein (55 kDa) in MG cell preparations from control (C), glucose (G), and mannitol (M)-treated cells. B) Detection of β -actin. C) Densitometric analysis of PGIS levels presented as fold of control. Results expressed as mean \pm SEM (n = 3), * p < 0.05.

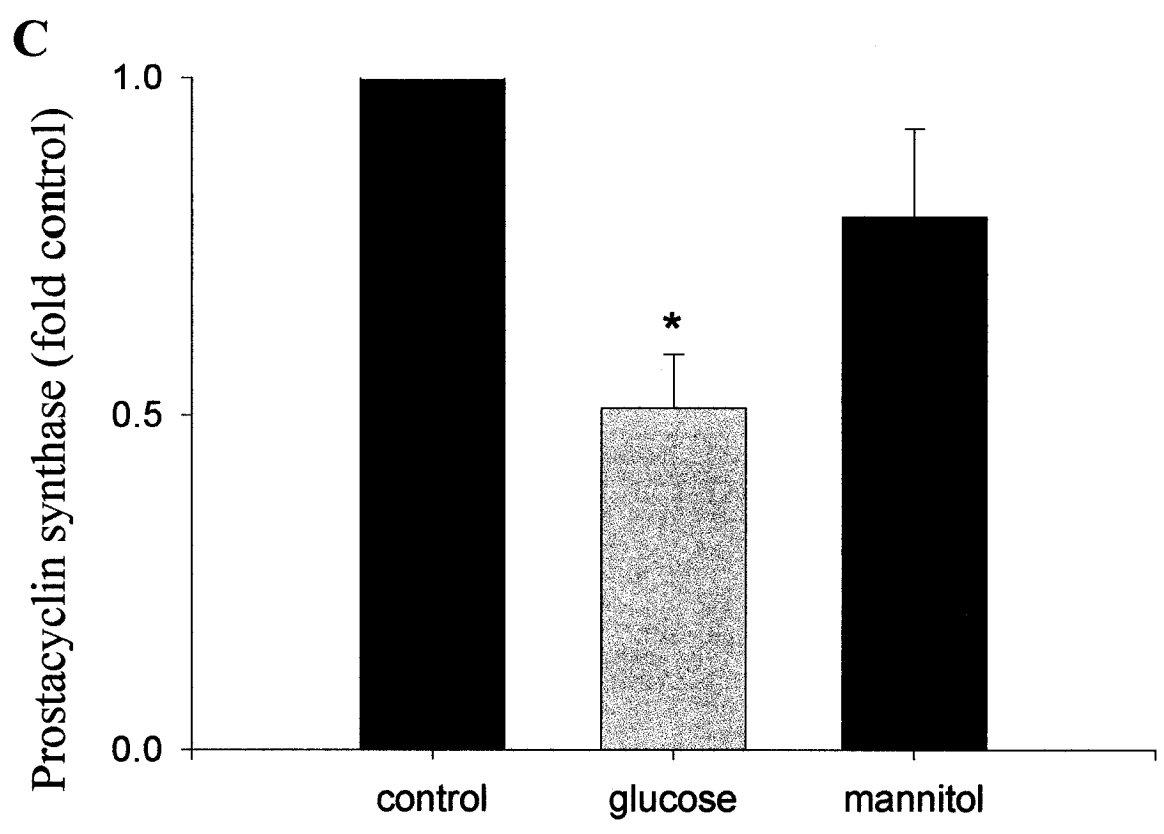
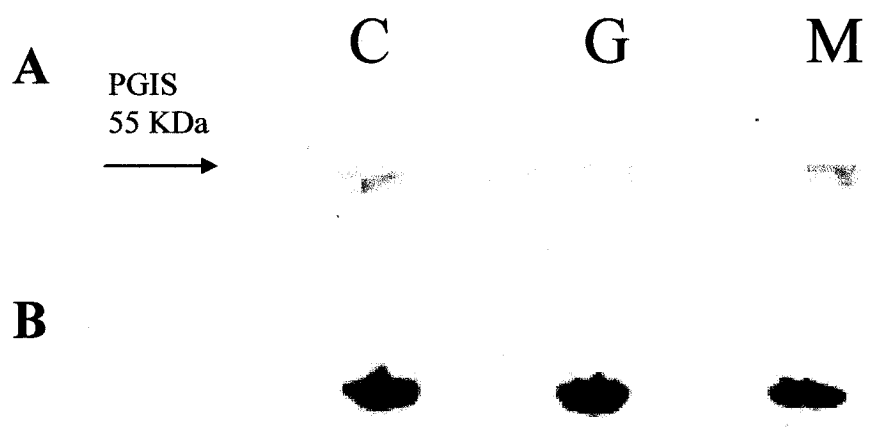
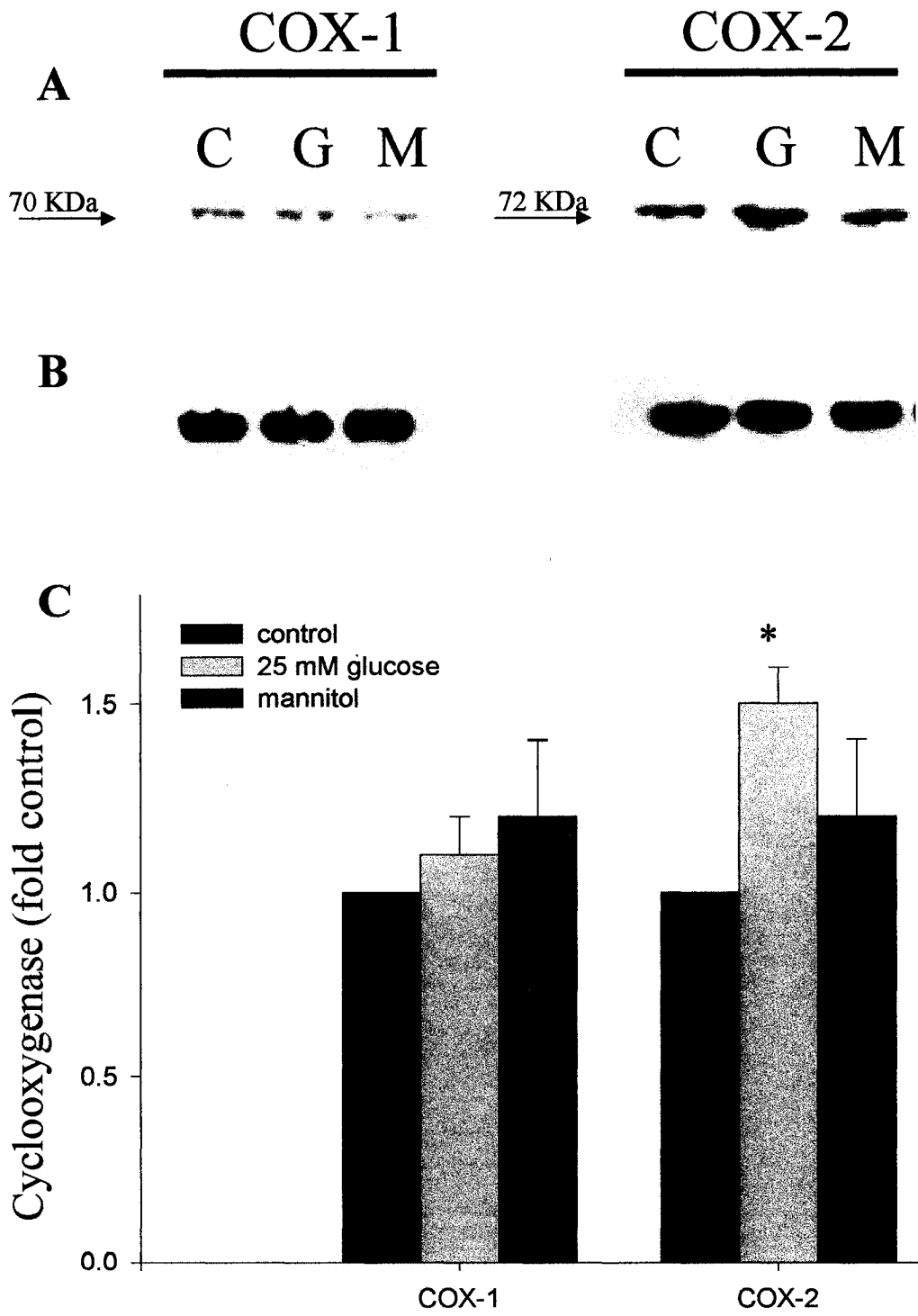


Figure 4.8. COX-2, but not COX-1, levels are augmented in response to high glucose. A) Representative autoradiograph showing COX-1 and COX-2, 70 and 72 KDa respectively in MG cell preparations from control (C), glucose (G), and mannitol (M)-treated cells. B) Detection of β -actin. C) Densitometric analysis of COX levels presented as fold of control. Results expressed as mean \pm SEM (n = 3), * p < 0.05.



High glucose attenuates CCP and ILP-cAMP stimulation:

1 μ M of cicaprost (fig.3) and iloprost (fig.4) stimulated cAMP in MG cells, from a basal level of 0.17 ± 0.02 to 4.1 ± 0.1 and 3.4 ± 0.3 (pmol/ μ g), respectively. No significant increase in cAMP was obtained in response to PGE₂ or AVP, regardless of the stimulation time or the concentration used. Also, two different PGE₂ analogues were assessed, Dinoprostone (PGE₂, Cayman) and 16,16- dimethyl PGE₂. The lack of response to AVP is in agreement with previous work that indicates the presence of V₁ and not V₂ receptors in MG cells (34). Conversely, it is surprising that a response to PGE₂ was not obtained, since we did detect the presence of the EP₄ receptor mRNA, which is known to couple to G_s-protein and increase cAMP. Furthermore, cAMP stimulation by PGE₂ has previously been demonstrated in cultured MG cells (31). However, their MG preparation was obtained from Wistar Kyoto rats instead of Sprague-Dawley rats.

To test the effect of glucose on the cAMP response to CCP and ILP, we first performed a time course for glucose exposure: 15 min to 5 days. Following 5 days exposure of MG cells to 25 and 35 mM glucose, the stimulatory effect of both CCP and ILP on cAMP production was diminished by up to 50 % (figs.3 and 4). With 25 mM of glucose, cAMP levels were diminished from 4.1 ± 0.1 to 2 ± 0.3 (pmol/ μ g) and from 3.4 ± 0.3 to 1.7 ± 0.2 (pmol/ μ g), for 1 μ M CCP and ILP respectively. Likewise with 35 mM glucose, cAMP levels were diminished to 2.1 ± 0.4 and 1.9 ± 0.2 (pmol/ μ g), for 1 μ M CCP and ILP respectively. No inhibition was observed in the presence of 15 mM glucose. No change was observed in cAMP levels using mannitol instead of glucose (data not shown). Also, mannitol had no effect on basal cAMP levels. There was no obvious difference between moderate (25 mM) and high (35 mM) glucose levels. Our data is in

agreement with previous work examining the effect of 25 mM glucose on PGE₂-cAMP in MG cells (31), also showing a diminished response.

Exposure to high glucose does not alter IP mRNA levels in cultured MG cells:

As illustrated in figure 5A, a single band at around 2.5 kbp is obtained, representing the rat IP mRNA. Furthermore, exposure of MG cells to different glucose concentrations for 5 days does not change the expression of IP mRNA (fig.5C). A summary of the results obtained for different time points is presented in Table 1. At 1hr, 24 hrs, and 5 days exposure to different glucose concentrations, the mRNA level remained comparable to control levels (5.6 mM glucose) in untreated cells.

Prostanoids do not alter Ca⁺⁺ signaling in cultured MG cells:

As illustrated in figure 6, both 0.1 μM AVP and Ang II increased [Ca⁺⁺]_i levels in cultured MG cells. The magnitude of the peak depends on the order in which the compound was added, since when Ang II was added prior to AVP the peak was higher for Ang II than AVP, and vice versa. Regardless of the order in which PGE₂, ILP, CCP were added, no effect on [Ca⁺⁺]_i was observed. Interestingly, PGE₂ has been shown to increase Ca⁺⁺ levels in cultured MG cells from the Wistar Kyoto rats (31), at this time we cannot explain the lack of effect in our preparation. Since we confirmed that the G_q pathway is intact in these cells using both AVP and Ang II, there is either a lack of EP₁ receptors coupling to G_q-protein in our preparation, or a defective coupling between this subtype and G_q-protein.

Figure 4.3. Measurement of cellular cAMP levels by radioimmunoassay in response to stimulation of cultured MG cells with CCP. CCP stimulates cAMP production in cultured rat MG cells, and this is attenuated by high glucose, and duplicates for each were performed. * $P < 0.05$, $n = 3 - 4$. The concentration of cAMP was plotted against the concentrations of CCP ranging from 10 μM to 1 nM. The cAMP response was also assessed in response to exposure to high glucose (15 mM, 25 mM, and 35 mM) for 5 days. Data is presented as mean \pm SEM.

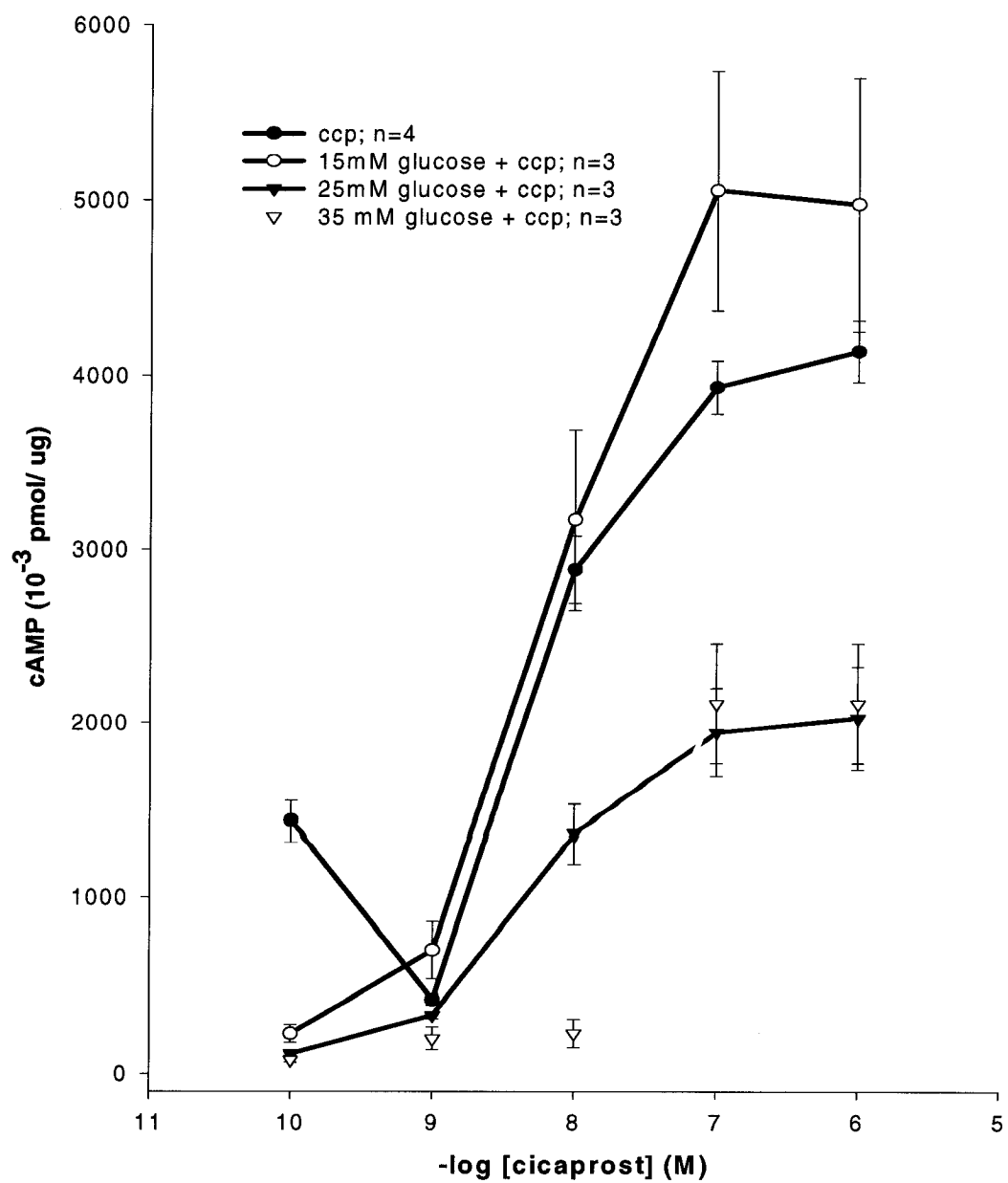


Figure 4.4. ILP stimulates cAMP production in cultured rat MG cells, and this is attenuated by high glucose. Measurement of cellular cAMP levels by radioimmunoassay in response to stimulation of cultured MG cells with ILP. The concentration of cAMP was plotted against the concentrations of ILP ranging from 10 μ M to 1 nM. The cAMP response was also assessed in response to exposure to high glucose (15 mM, 25 mM, and 35 mM) for 5 days. Data is presented as mean \pm SEM, and duplicates for each were performed. * $P < 0.05$, $n = 3-4$.

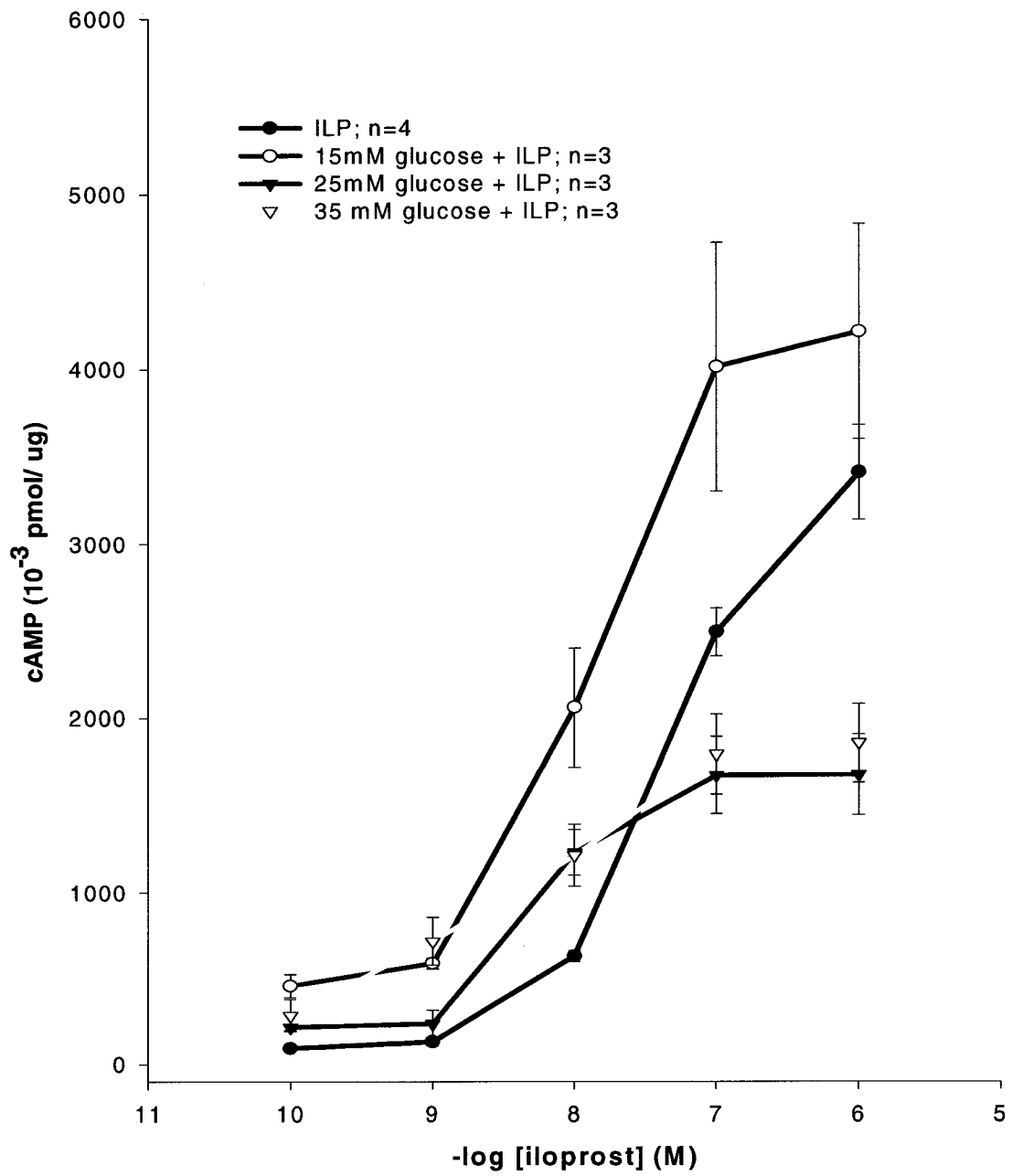


Figure 4.5. IP receptor mRNA is expressed in mesangial cells, but not altered by high glucose. Northern blot detection of IP mRNA (2.5 kbp) in cultured MG cells exposed to high glucose for 5 days. A) Representative autoradiograph of IP mRNA in different samples. Lane number: 1- control (5.6 mM glucose), 2- 15 mM glucose, 3- 25 mM glucose, 4- 35 mM glucose, 5- 15 mM Mannitol, 6- 25 mM Mannitol, 7- 35 mM Mannitol; B) Corresponding autoradiograph detecting β -actin mRNA to normalize loading; C) Densitometric analysis of IP mRNA levels for 6 different experiments. Results expressed as mean \pm SEM (n = 6), p<0.995.

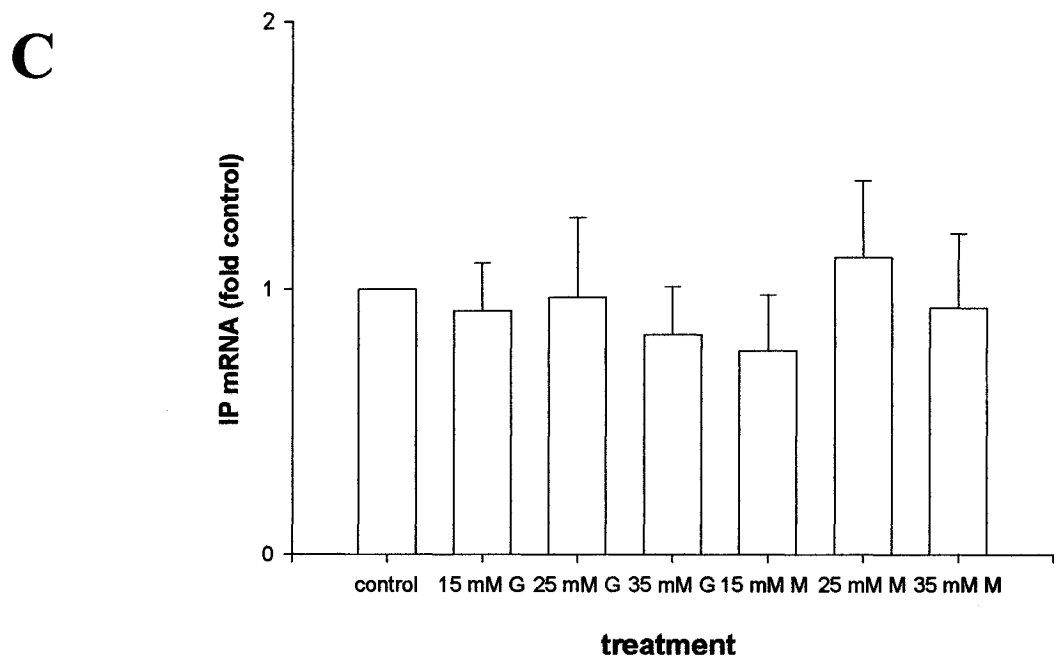
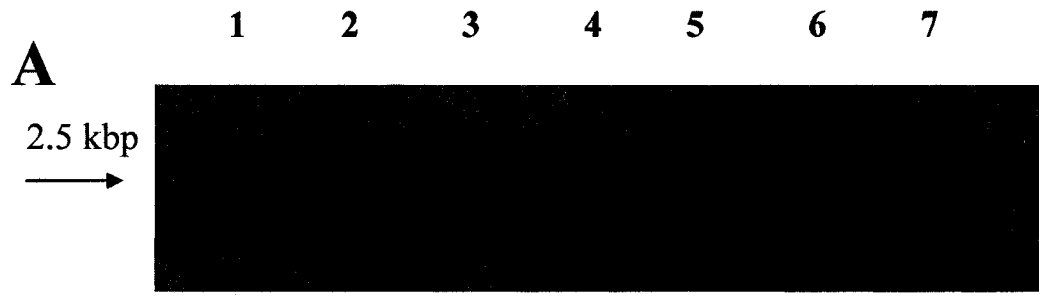
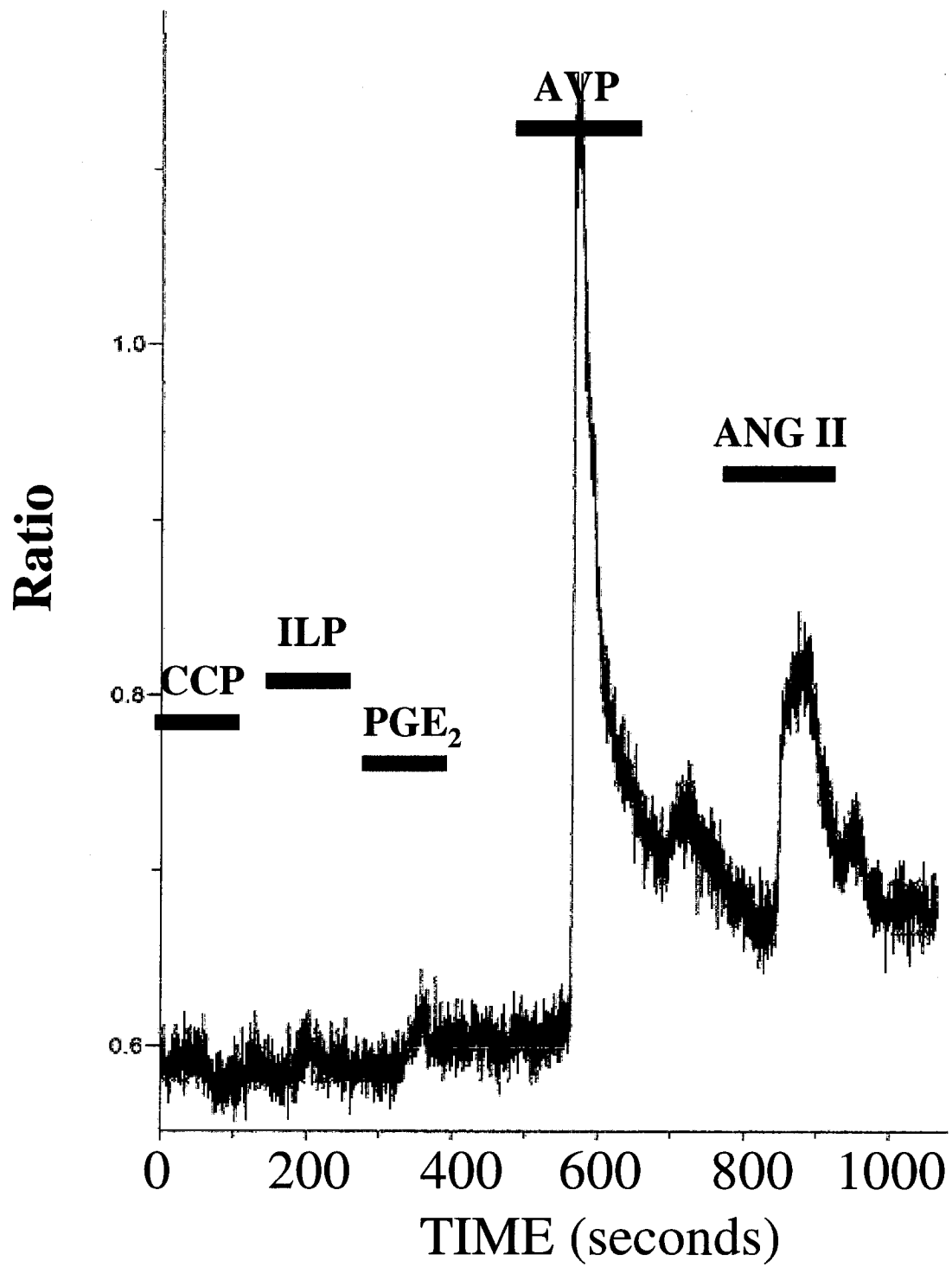


Table 4.1. Summary of the effects of high glucose on IP mRNA expression in cultured rat MG cells.

Exposure Time	IP mRNA (fold control)						
	Control	15 mM Glucose	25 mM Glucose	35 mM Glucose	15 mM Mannitol	25 mM Mannitol	35 mM Mannitol
1 hour (n = 3)	1±0	1.2±0.14	1.3±0.04	1±0.11	1.03±0.08	0.77±0.14	0.68±0.04
24 hours (n = 4)	1±0	1.4±0.16	1.15±0.16	1.25±0.13	1.15±0.11	1.08±0.09	0.9±0.09
5 days (n = 6)	1±0	0.92±0.18	0.97±0.3	0.83±0.18	0.77±0.21	1.12±0.29	0.93±0.28

MG cells were exposed to different concentrations of glucose or mannitol for 1 hr, 24 hrs, or 5 days and IP mRNA was quantified by densitometric analysis. Results are presented as fold of control, mean ± SEM for each group. No significant difference from control was obtained.

Figure 4.6. Intracellular calcium levels are not altered by prostanoids in cultured mesangial cells. Representative tracing of intracellular calcium changes in response to different agonists. MG cells stimulated with 1 μ M PGE₂, CCP, and ILP; and 0.1 μ M AVP and ANG II. Calcium levels are presented as the ratio of fluorescence at 340/380 nm. n = 3.



DISCUSSION

The contribution of mesangial cells (MG) to glomerular function as a whole is dependent on the intricate interactions between this subset of cells and the two other types of cells that comprise the glomerular region, namely the podocytes and endothelial cells. MG cells are involved in various aspects of glomerular physiology and pathology, with contractile properties to modulate adjacent capillaries, providing structural support for the capillary loops, and as hormonal mediators of glomerular hemodynamics and filtration. MG cells are also actively involved in the turnover of matrix proteins, maintaining a balance of protein synthesis and degradation (involving matrix metalloproteinases and their inhibitors). Actually, a disturbance in MG function has been implicated in many progressive renal diseases characterized by proteinuria, such as diabetic nephropathy, as evidenced by an initial mesangial hypercellularity and expansion of the mesangial matrix (35).

Not only is this subset of glomerular cells a large contributor to the overall production of cyclooxygenase derivatives, they are also susceptible to both autocrine and paracrine control by prostaglandins. For instance, MG-derived PGE₂ and I₂ modulate the constrictor actions of hormones such as Ang II, norepinephrine, and AVP (36) by increasing cAMP. Also, prostaglandins can alter MG function by inhibiting their proliferation in response to mitogenic stimuli (37). Recently, a study showed the expression of EP₁ and EP₄ receptor subtypes in cultured rat MG cells by Northern blot analysis. Also, PGE₂-dependent cAMP production is attenuated in these same cells (31). Moreover, TXA₂ has been shown to increase MG cells fibronectin via PKC activation, a mechanism dependent on cGMP levels (38).

In the present study, we characterized the expression of IP receptors in cultured rat MG cells. We show by RT-PCR and Northern blotting that IP receptor mRNA is present in MG cells. This finding confirms our previous in situ hybridization study in rat kidney, indicating that a subset of central glomerular cells contain IP receptors, while the peripheral cells do not (14). Likewise, in mouse kidney it was shown that while glomeruli do express IP receptors, these are not detectable in podocytes (15). A single product was obtained by Northern blotting, arguing against the presence of IP receptor spliced variants or subtypes. IP protein was noted in MG cells consistent with the expression of IP mRNA. Having confirmed the expression of both cyclooxygenase isoforms and prostacyclin synthase in MG cells, this study further supports the possibility of autocrine regulation of MG function by the PGI₂/IP system.

Also, we show IP protein in preparations of cultured PT cells, IMCD, and in the medullary regions of the kidney (outer and inner); however, no signal was observed in lysates from the whole cortex. The medullary localization of IP protein is in agreement with our previous work demonstrating the expression of IP mRNA in medullary segments of the nephron, namely mTAL (13) and IMCD (14). In terms of intra-renal expression of the IP receptor in other species, certain discrepancies have been noted (15, 13, 11) with respect to specific nephron segments and cell types expressing the receptor. The functional reason for this difference remains uncertain. For example, unlike rodent studies, Komhoff et al. (11) showed glomerular IP in both podocytes and MG cells of human glomeruli. A better characterization of the role of the PGI₂/IP system in various aspects of renal function across species will help enlighten this ambiguity.

Although various aspects of the role of prostaglandins in the glomerular region has been examined by numerous investigators, their involvement in proximal tubule physiology remains poorly documented possibly because this nephron segment does not contribute to their production in the kidney. Nonetheless, the PT plays a substantial role in the metabolism of prostaglandins, and due to its proximity to the glomerulus, is subject to paracrine interactions by glomerular prostanoids. Both PGE₂ and PGI₂ had a protective role on primary cultures of PT epithelial cells that were subjected to hypoxia and reoxygenation, although the mechanism of cytoprotection remains unclear (39). To the best of our knowledge, this is the first study confirming the presence of IP receptors in PT of any species. Having detected IP protein in PT cells, it will be interesting to clarify the significance of the PGI₂/IP system in PT physiology and disease, including the progression of diabetic nephropathy.

In order to clarify the signaling pathways linked to the PGI₂/IP system in MG cells, we examined the effect of the PGI₂ analogues CCP and ILP on cAMP and calcium responses. Both compounds increased intracellular cAMP levels, but did not alter intracellular calcium. Since CCP is highly selective for the IP receptor (40), it is most likely that the cAMP signaling is mediated by the IP receptor via coupling to G_s-protein and activation of adenylate cyclase. Since this is a highly recognized pathway for PGI₂/IP signaling (41, 40), we did not attempt to confirm this finding with inhibitors of adenylate cyclase. As for ILP, although it is a less selective IP agonist owing to its potency for the EP receptors, we believe the effects demonstrated in this study are linked to the IP receptor since a PGE₂ response was not obtained in our cells. The lack of PGE₂ effect was somewhat surprising since it had previously been shown that PGE₂ increases cAMP

in cultured MG via the EP₄ receptor, and increases calcium via EP₁ coupling to G_q-protein. This discrepancy may be due to species differences, since Ishibashi's group (31) used Wistar Kyoto rats instead of Sprague-Dawley rats, or to isolation procedures and culturing conditions. Of interest, in a study by Nusing et al (32), no PGE₂ receptor subtypes other than EP₂ were amplified from cultured rat MG cells by RT-PCR, and indicated EP₂-mediated increases in cAMP. Although our work confirms the expression of EP₁ and EP₄ receptor subtypes mRNA in MG cells, the PGE₂/EP₁/calcium and PGE₂/EP₄/cAMP pathways are not active. Thus the PGI₂/IP system is linked to cAMP stimulation but not calcium signaling in cultured MG cells isolated from Sprague-Dawley rats.

The next part of this work examined the effects of high glucose on the PGI₂/IP system in MG cells. Although COX-2 levels were increased in response to high glucose, we propose that prostacyclin production is diminished in these cells due to the substantial decrease in PGIS levels. This decrease provides further support for the argument that a defect in the PGI₂/IP system may account for some of the later changes in diabetic glomeruli and tubules, as proposed by Villa and co-workers (27). In fact, it is becoming quite evident that individual prostaglandin levels fluctuate throughout the course of the disease, implicating them in the progressive changes leading to sclerosis and tubular atrophy. Although no change in IP receptor mRNA was noted, there may be a decrease in IP protein levels since we observe a diminished cAMP response to both CCP and ILP in response to high glucose. However we cannot rule out the possibility that the attenuation in cAMP is due to a lack of receptor coupling to G_s-protein, or to an inactivation of one of the G-protein subunits in response to high glucose. Further studies are needed to

clarify this issue. Moreover, work looking at the effects of high glucose on the PGE₂ response in MG cells showed a similar inhibition of cAMP, with no change in EP₄ receptor mRNA (31). Perhaps glucose is directly altering cAMP levels, acting at the level of adenylate cyclase or phosphodiesterases. Studies are ongoing in our laboratory to clarify this issue and examine the significance of this diminished cAMP response linked to the PGI₂/IP system. For instance, increases in cAMP are known to inhibit the proliferative state of cells (42, 31), reduce mesangial contractility by antagonizing responses to intracellular calcium (43), determine cell fate and apoptotic responses (8), alter gene transcription via cAMP-responsive elements in a large number of target genes (32). Since the inhibition by glucose is only evident at longer exposure times, another possibility is that an intermediate factor is mediating these effects. Several compounds are potential candidates in mesangial cells: advanced glycation end products (AGEs), transforming growth factor (TGF β), PKC, to name a few. Further investigation is needed to examine these mechanisms.

In order to clarify the role of the PGI₂/IP system in the glomerular region, this study focused on examining the expression of IP receptors in cultured rat MG cells. From this work, we conclude that both IP mRNA and protein are detectable, and that this system is linked to a stimulatory cAMP-signaling pathway. Furthermore, exposure of MG cells to high glucose increased COX-2 but decreased PGIS enzymes, diminishes the cAMP response to PGI₂ analogues, but does not alter IP mRNA. This could have important implications for glomerular changes in diabetic nephropathy at later stages of the disease, especially if the PGI₂/IP system plays a protective role in antagonizing the damaging effects of other factors by increasing cAMP.

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CHAPTER 5

Reduced IP Receptors in STZ-induced Diabetic Rat Kidneys and High-Glucose Treated Mesangial Cells

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Reduced IP receptors in STZ-induced diabetic rat kidneys and high-glucose treated mesangial cells.

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ABSTRACT

Mesangial cells (MG) are an important source of renal PGE₂ and PGI₂. The purpose of this study was to examine the effects of cicaprost (CCP: PGI₂ analogue) on MG function, and the expression of IP receptors in streptozotocin (STZ)-diabetic rats and glucose-treated MG cells. CCP increased cellular cAMP in immortalized rat MG cells. Both glucose and anisomycin attenuated CCP-cAMP, but not phorbol-myristate-acetate, angiotensin II, or transforming growth factor- β . Also, IP receptor protein was reduced in response to glucose. While CCP decreased the levels of the cell cycle inhibitor p27, it did not alter thymidine or leucine incorporation. However, CCP reduced fibronectin levels by 40 %, and increased matrix metalloproteinase-2 levels by 3- fold, a key enzyme in matrix degradation. Finally, IP receptors were significantly reduced in outer medulla of 4 and 12 wks STZ-diabetic rats; and in the cortex, outer, and inner medullary regions in 6-mth uni-nephrectomized STZ-diabetic rats. The changes in the CCP/IP system observed in this study suggest that IP may serve as an alternate therapeutic target in diabetes.

KEYWORDS: cAMP, CCP/IP, glucose, immortalized mesangial cells, STZ-diabetic rats

INTRODUCTION

Prostacyclin alters cell function by two distinct mechanisms (13, 24, 41): increasing intracellular cAMP via cell surface IP receptors, and binding peroxisome-proliferator activated receptors (PPAR β/δ). While the significance of the PGI₂/PPAR pathway to renal physiological or pathological processes is still indefinable, the PGI₂/IP/cAMP system is certainly an important regulator of renal hemodynamics and tubular transport properties. Our group and others (20, 27, 28) have localized IP receptors to various cells in the kidney (endothelial, epithelial, mesangial, interstitial), but species differences have been noted in terms of expression patterns. However, using the selective IP agonist CCP, we have clearly demonstrated that distinct IP receptors mediate the signalling responses to PGI₂ in the rat and rabbit collecting ducts (28, 29), a major area of prostanoid synthesis in the kidney. While the glomerular mesangial cells are also an important source of renal prostaglandins, the effects of the PGI₂/IP/cAMP system on mesangial cell function is poorly defined. Furthermore, most studies thus far have utilized non-selective PGI₂ analogues (iloprost, carbaprostacyclin, beraprost sodium) therefore fail to distinguish between IP receptor and PPAR involvement.

Changes in mesangial cells are recognized as a major hallmark of diabetic nephropathy, proliferation, hypertrophy and mesangium expansion occur in the early phases on the path to glomerulosclerosis (2, 43). The disturbances are multifaceted, but the underlying link between different factors has yet to be defined. Glucose is surely a key determinant of the mesangial alterations, but crosstalk does occur between numerous pathways during the course of the disease. Important systems include: the polyol pathway,

the renin-angiotensin system, PKC, MAPK, TGF- β , to name a few (4, 17, 42). While the role of prostaglandins in diabetes is well recognized, their individual contribution to specific renal changes has not been thoroughly addressed. Levels of prostaglandins will fluctuate throughout the course of the disease (8, 9, 10), but how that affects the overall cell response in specific nephron segments is unclear. Surely a better understanding of how the individual prostanoid pathways, receptor expression as well as prostaglandin synthesis, are changing throughout different stages of diabetes is needed to clarify their involvement and provide more specific targets for therapeutic intervention.

Renal PGI₂ production is dependent upon the activity of two enzymes: cyclooxygenases (COX -1 and -2), and PGI₂ synthase (24, 35). We have recently shown that primary cultures of rat mesangial cells have diminished levels of PGI₂ synthase in response to prolonged high glucose, but increased levels of COX-2 (31). Similar findings were reported in human aortic endothelial cells exposed to high glucose, with increased COX-2 and thromboxane A₂ synthesis, but reduced PGI₂ release (7). We also showed that prostacyclin analogues (CCP, ILP) increase cAMP in mesangial cells without altering intracellular calcium levels, and that prolonged exposure to glucose reduced CCP-cAMP without altering IP mRNA levels (31). The effect of CCP on mesangial cell function was not investigated.

Countless studies have implicated alterations in prostacyclin levels in the pathogenesis of diabetic nephropathy. Villa et al (37) administered CCP to 8 mth uni-nephrectomized STZ-rats and showed comparable benefits (normalized GFR, reduced proteinuria) to the ACE inhibitor fosinopril. Also, long-term administration of beraprost sodium (prostacyclin analogue) to diabetic patients has proven to be beneficial in the

prevention of diabetic complications by decreasing albuminuria (32, 39). In another study, beraprost sodium attenuated glomerular hyperfiltration and macrophage infiltration in STZ-diabetic rats by modulating nitric oxide synthase expression (44). While these studies suggest a defect in prostacyclin signalling, to date there is no evidence of changes in IP receptor levels in diabetic kidneys.

Therefore the purpose of this study was to examine the effects of CCP in immortalized rat mesangial cells, looking at the expression of IP receptor mRNA and protein, growth responses (thymidine, leucine incorporation), the expression of p27, matrix proteins (fibronectin), and matrix metalloproteinase (MMP-2) levels. And the last part of this work examines the changes in IP mRNA levels in the three regions of the kidney at different stages of STZ-diabetes in rats (2-16 wks) and in advanced disease in uninephrectomized 6-mth STZ-diabetic rat kidneys. Deciphering the regional differences in receptor expression will facilitate the investigation of the contribution of this system to the nephropathy and the link to specific alterations throughout the nephron. A better understanding of these changes could lead to better avenues for the treatment or prevention of individual complications as they arise.

MATERIALS AND METHODS

Diabetic rat model:

Tissue from three kidney regions (cortex, outer medulla, inner medulla) was isolated from 200 g Sprague-Dawley rats under bright light using a dissecting microscope. The diabetic model utilized in the studies is the Streptozotocin (STZ)-diabetes, a widely used model resembling Type 1 diabetes mellitus. STZ is an N-nitroso derivative of D-glucosamine that is utilized to induce diabetes in a variety of experimental animals, and to determine the long-term complication of diabetes. It basically functions as a toxin that selectively destroys the insulin producing cells of the pancreas, rendering the rat diabetic within 24 hrs of injection (34). Although the STZ model is well recognized in the scientific community, a major drawback is that the renal phenotype does not correlate with the human disease. Proteinuria, glomerulosclerosis, and renal dysfunction are not very prominent. In order to hasten the renal manifestations of the disease, uni-nephrectomy is performed following STZ administration increasing the stress to the remaining kidney. The animal care facilities at our disposal have a current protocol for unilateral nephrectomies, inducing diabetes by administering STZ, and maintaining the rats.

Six different groups of rats were employed:

1. Vehicle-treated control (administered 0.1 mol/L sodium citrate buffer, pH 4.0).
2. STZ-diabetic rats (administered 65 mg/kg STZ (Sigma, St.Louis, MO, USA), in 0.1mol/L sodium citrate buffer, pH 4.0; hyperglycemia will be maintained between 17-25 mmol/L with daily subcutaneous injections of 1-2 units of insulin).

3. STZ-insulin rats (same as group 2 except for maintenance of euglycemia by subcutaneous implantation of a sustained release insulin implant; Linplant, Linshin, Scarborough, ON, Canada).
4. Right unilateral nephrectomy (NX)
5. NX/STZ: STZ administration (as for group 2) + unilateral nephrectomy
6. NX/STZ-insulin: same as group 3 + unilateral nephrectomy.

The day after STZ administration, a urine analysis is performed for glucose and ketones using a Keto-Diastix reagent strip (Bayer Inc., Etobicoke, ON, Canada), and animals with sustained glucosuria are assigned to STZ-Diabetic, NX/STZ or Insulin groups. Experiments were performed on STZ-rats in early stages of diabetes (2, 4, 8, 12 and 16 wks) and in later stages of disease (24 weeks) in uni-nephrectomized STZ-rats. Table 3 compares the kidney weights and body weights of the 6-mth uni-nephrectomized rat groups.

Mesangial cell culture:

Rat mesangial cells (rMG) immortalized with pSV3-Neo at passage 8 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). These cells are positive for desmin and vimentin and negative for cytokeratin 8. Work in our laboratory further confirmed their origin by immunocytochemical analysis of α -smooth muscle actin. For all experiments rMG cells were cultured on Petri dishes in Dulbecco's modified Eagle's medium (Sigma) containing 15% fetal bovine serum and 0.4 mg/ml geneticin (G-418; Gibco), and maintained at 37 °C and 10 % CO₂. The media was replaced every two days to maintain a constant growth environment, and for cAMP and

thymidine/leucine incorporation experiments the media was replaced by DMEM without serum 24 hrs and 3 days prior to stimulation of cells, respectively.

cAMP measurements:

Confluent rMG cells were pre-treated for 15 min with 10 μ M indomethacin and 0.5 mM 3-isobutyl-1-methylxanthine, then stimulated for 10 min at timed intervals with 0.1 μ M CCP or PGE₂. cAMP levels were measured using a radioassay kit. For experiments with high glucose, the cells were cultured in the presence of 5.6 mM glucose (control) or 25 mM glucose for 24 hrs prior to stimulation with prostanoids. Mannitol was used as an osmotic control. Data are presented as mean (fold of control) \pm SEM. To examine the effect of different factors on the CCP-IP-cAMP system, we measured the cAMP response to CCP in cells treated with 1 μ M angiotensin II, 2 ng/ μ l TGF- β , 100 nM anisomycin (MAPK activator, Sigma), and 100 nM PMA (PKC activator, Sigma) for various times.

RT-PCR:

Total RNA was isolated from cultured rMG cells using the TriZol Method (Gibco); and DNase treated to prevent amplification of genomic DNA. RNA was then reverse transcribed into cDNA and amplified by PCR using specific primers (listed in Table 1) for the IP receptor (407 bp), and each of the prostaglandin E₂ receptor subtypes: EP₁ (336 bp), EP₂ (401 bp), EP₃ (437 bp), EP₄ (423 bp). Samples were loaded onto a 1.5 % agarose gel containing ethidium bromide for visualization of the amplified products.

To confirm the product identity each fragment was cloned and sequenced. The negative controls consisted of omitting the reverse transcriptase for each primer pair.

Western blotting:

Cell lysates were obtained by sonicating cells in RIPA buffer containing various phosphatase and protease inhibitors. Twenty-five micrograms of each sample were resolved by SDS-PAGE on a polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking O/N in 5 % milk/TBS-T, the membrane was incubated for 3 hrs with a human anti-IP polyclonal antibody (gift from Dr RM Nusing, Germany) at a dilution of 1:4000. Following incubation with an HRP-conjugated goat anti-rabbit IgG secondary antibody, enhanced chemiluminescence was performed for signal detection. The IP level was compared in cells exposed to either: control (5.6 mM glucose), 25 mM glucose, or mannitol. A single band of 52 KDa was obtained. For detection of fibronectin, p27, and MMP-2, cells were treated for 24 hrs with 1 μ M CCP. Specific antibodies for each protein were obtained from Calbiochem.

Northern blotting:

Total RNA was isolated from rMG cells, using the Trizol method; and DNase treated. Cultured rMG cells were exposed to control, 25 mM glucose, or mannitol, and 10 μ g of total RNA from each sample was loaded onto a formaldehyde gel. RNA was then transferred to a nitrocellulose membrane. After baking for 2 hrs/80°C/vacuum, the membrane was incubated O/N with a P³²-dCTP labeled human IP cDNA (Cayman chemicals) or mouse EP₄ cDNA probe ((a gift from M.D. Breyer at Vanderbilt

University), then exposed to film for 1 week. To normalize the samples, the expression of β -actin was determined for the same membrane. Densitometric analysis was used to compare the relative expression of IP and EP₄ mRNA in each sample. Data is presented as mean (arbitrary units or fold of control) \pm SEM. n = 3. For quantification of IP mRNA in STZ-diabetic rats, kidneys were removed from control, diabetic, and insulin-treated rats at 2-16 weeks after STZ injection. Total RNA was isolated from samples of cortex, outer, and inner medulla using the Trizol method, as described by the manufacturer (Gibco-brl); and was DNase-treated (Boehringer Mannheim) to reduce genomic DNA. Ten micrograms of total RNA from each sample used and analysis of IP mRNA was performed as described above, and data is summarized in Table 2.

H³-thymidine and -leucine incorporation:

rMG cells were cultured in serum-containing growth media on 24-well plates to 50% confluence and serum-starved for 3 days, after which 1 μ M CCP or 15 % FBS were added to the DMEM for an additional 4 or 24 hrs. H³-thymidine (2 μ l/ml) was added to each well during the last 4 or 24 hrs at 37 °C, or H³-leucine for 24 hrs. Then the cells were washed 4X in ice-cold PBS and solubilized in 1N NaOH for 15 min at 37 °C. The samples were then transferred to scintillation fluid and counted. To determine whether CCP altered the effect of FBS on DNA or protein synthesis, 10 nM to 1 μ M CCP was added to the samples in the presence of FBS.

RealTime RT-PCR:

Total RNA was isolated using Trizol (Gibco) from different preparations of cortex, outer, and inner medulla from four groups of rats (ctrl, NX-STZ, NX-STZ-insulin, NX) at 24 weeks of diabetes. The relative quantity of each target nucleic acid in different samples was determined by analyzing the cycle-to-cycle change in fluorescence signal as a result of amplification during a PCR. To quantify the amount of RNA in each sample, a relative standard curve was prepared by diluting a stock of control RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is detected as an internal control to standardize the amount of sample RNA added to a reaction. The availability of distinguishable reporter dyes makes it possible to amplify and detect the target message and GAPDH in the same tube. The RT-PCR was performed using the TaqMan PCR Core Reagents Kit, and GAPDH Control Reagents Kit providing VIC-labeled GAPDH probe. The following parameters were employed: 48 °C for 30 min then 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 sec and 60 °C for 1 min. Primers and probes are selected and obtained from the Custom Oligonucleotide Synthesis Service of Applied Bio-systems. The upstream and downstream primers as well as fluorescent probes for IP and GAPDH are listed in Table 1. The data was analyzed by computer, using the ABI prism 7000 sequence detection system, and is expressed as the ratio of IP mRNA/GAPDH.

Statistics:

The SigmaPlot software for windows version 4.01 (1986 - 1997) was used to analyze data. Results are expressed as the mean \pm SEM, $p < 0.05$. Statistical significance

was determined using the unpaired t-test or one-way ANOVA followed by the Tukey test.

RESULTS

EP₄ receptor, but not IP receptor, mRNA is increased in glucose-treated rMG:

To determine whether IP receptors are expressed in immortalized rMG cells, we first examined the expression of IP and EP receptor subtypes by RT-PCR. Table 1 lists the primer pairs used for amplification of each receptor fragment. As shown in fig 1, IP as well as EP₁ and EP₄ receptor fragments were amplified and their identity was confirmed by cloning and sequence analysis. Although a band for EP₂ mRNA was detectable, the sequence did not match that published for the cloned cDNA. The EP₃ receptor was not detectable in this cell line. However, in previous work we had detected this receptor subtype in primary cultures of mesangial cells (31). To examine the effect of glucose on the expression of IP and EP₄ mRNA, Northern blotting was used for quantification and comparisons with controls. As shown in figure 2A, a single band was obtained for the IP receptor mRNA around 2 Kb. However no change in IP mRNA was noted upon culture of rMG in high glucose for 24 hrs (fig 2C), which is consistent with our work in primary cultures of mesangial cells showing no change in IP mRNA after 5 days culture in high glucose (31). On the other hand, a 2.2-fold increase in EP₄ mRNA was obtained (figure 2C), though a similar increase is seen with mannitol. Whether this change in mRNA is due to an osmotic effect is noteworthy, and ongoing work in our laboratory will further examine this issue. Interestingly, in primary cultures of mesangial cells Ishibashi et al. observed no change in EP₄ mRNA in response to high glucose exposure (15). In contrast, we have previously reported about a 2-fold increase in both EP₁ and EP₄ in cultured rat IMCD exposed to high glucose for 4 days (30).

Table 5.1. Primer pairs and probes used for RT-PCR and RealTime RT-PCR.

Target	sense primer	anti-sense primer	Probe
IP: FF1+ FF2	ggcacgagaggatgaagttacc	gtcagaggcacagcagtcaatgg	NA
EP ₁ receptor	cgcagggttcacgcacacga	cactgtgccgggaactacgc	NA
EP ₂ receptor	aggacttcgatggcagaggag	cagcccctacactctccaat	NA
EP ₃ receptor	ccgggcacgtggtgcttcat	tagcagcagataaaccagg	NA
EP ₄ receptor	ttccgctcgtggtgcgagtgtt	gaggtggtgtctgcttgggtca	NA
IP receptor	gccctcatgacaggcatca	gatggcctgcgtgaatcct	Ccgtatgctccctgcctctcacgat
GAPDH	gaaggtgaagtcggagtc	gaagatggtgatgggatttc	Caagcttcccgttctcagcc

For each IP and EP receptor subtype the sequence for the upstream and downstream primers used for amplification by RT-PCR are provided, also the primers and probes used to amplify the IP receptor and GAPDH mRNA by RealTime RT-PCR are shown. NA: not applicable.

Figure 5.1. IP receptor mRNA, as well as EP₁ and EP₄, are detected in immortalized rMG cells. RT-PCR was performed using specific primers to amplify fragments from each of the IP and EP receptors, the amplified products were confirmed by cloning and sequencing. The bands shown represent the following: lane 1- IP (407 bp); 2- EP₁ (336 bp); 3- EP₂ (401 bp); 4- EP₃ (437 bp); 5- EP₄ (423 bp); 6- omission of reverse transcriptase for IP receptor primers; 7- omission of reverse transcriptase for EP₁ receptor primers.

1 2 3 4 5 6 7

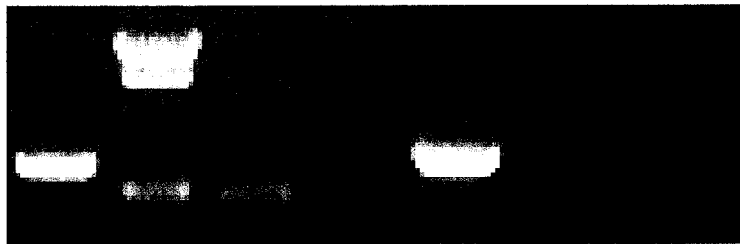


Figure 5.2. EP₄ receptor mRNA, but not IP, is increased in rMG cells exposed to high glucose. Northern blot detection of IP and EP₄ receptor mRNA in cultured rMG exposed to: 1) control, 2) glucose (25 mM), and 3) mannitol (25 mM), for 24 hrs. Representative autoradiograph showing: A) IP mRNA, and B) detection of β -actin mRNA. C) Densitometric analysis of IP and EP₄ receptor mRNA levels. Results expressed as mean (fold control) \pm SEM (n = 3), * p<0.05.

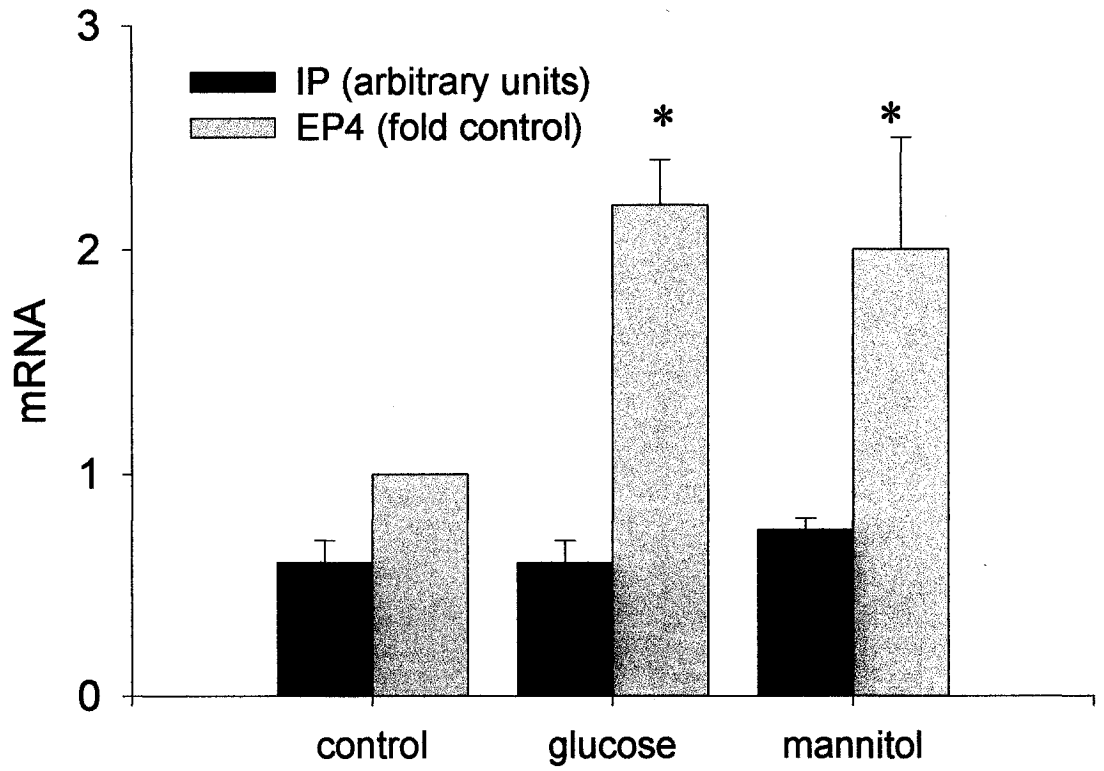
A



B



C



IP receptor protein levels are decreased in rMG exposed to high glucose:

Since no change in IP mRNA in response to high glucose was obtained, we examined whether glucose altered IP protein levels by Western blotting. As shown in figure 3, a 50 % reduction in IP protein was noted in response to 24 hr glucose treatment. This decrease was not obtained when glucose was substituted by mannitol, eliminating a possible osmotic effect on IP protein levels. Whether this decrease is due to enhanced degradation or reduced synthesis is not clear at this time.

CCP-stimulated cAMP is attenuated by 24 hr glucose and anisomycin:

As can be seen in figure 4, both CCP and PGE₂ stimulated cAMP to a similar extent in cultured rMG cells, up to $56 \pm 4 \%$ and $52 \pm 5 \%$, respectively. When cells were cultured in the presence of 25 mM glucose or mannitol for 24 hrs, the stimulatory response to CCP was reduced by over 50 %, and to PGE₂ by about 40 %. Whether or not this effect of glucose is entirely osmotic requires further investigation, but given that glucose and not mannitol altered IP protein levels (see above) argues for possible glucose effects independent of osmotic changes. While the effect on both receptors was similar in response to glucose, we noted differences in the response to other glucose-dependent factors. While CCP-cAMP stimulation was greatly attenuated by 24 hr anisomycin treatment (figure 5a) to $13 \pm 1 \%$ from $56 \pm 4 \%$, PMA treatment only slightly reduced the cAMP response to CCP to $37.5 \pm 8 \%$ (figure 5b). On the other hand, a significant inhibition of PGE₂-stimulated cAMP to $14 \pm 1.5 \%$ from $52 \pm 5 \%$ was obtained in response to 15 min PMA pre-treatment. Of interest, both angiotensin II and TGF- β had no effect on the cell response to prostanoids (data not shown).

Figure 5.3. IP receptor protein levels are reduced in cultured rMG exposed to high glucose. Protein was isolated from rMG exposed to either: 1- control, 2- 25 mM glucose, and 3- 25 mM mannitol for 24 hrs, and quantified by Western blotting. Representative autoradiograph of IP protein is shown as well as detection of β -actin to normalize samples. Densitometric analysis showing IP receptor protein levels presented as arbitrary units of mean \pm SEM, n=3. * p < 0.05.

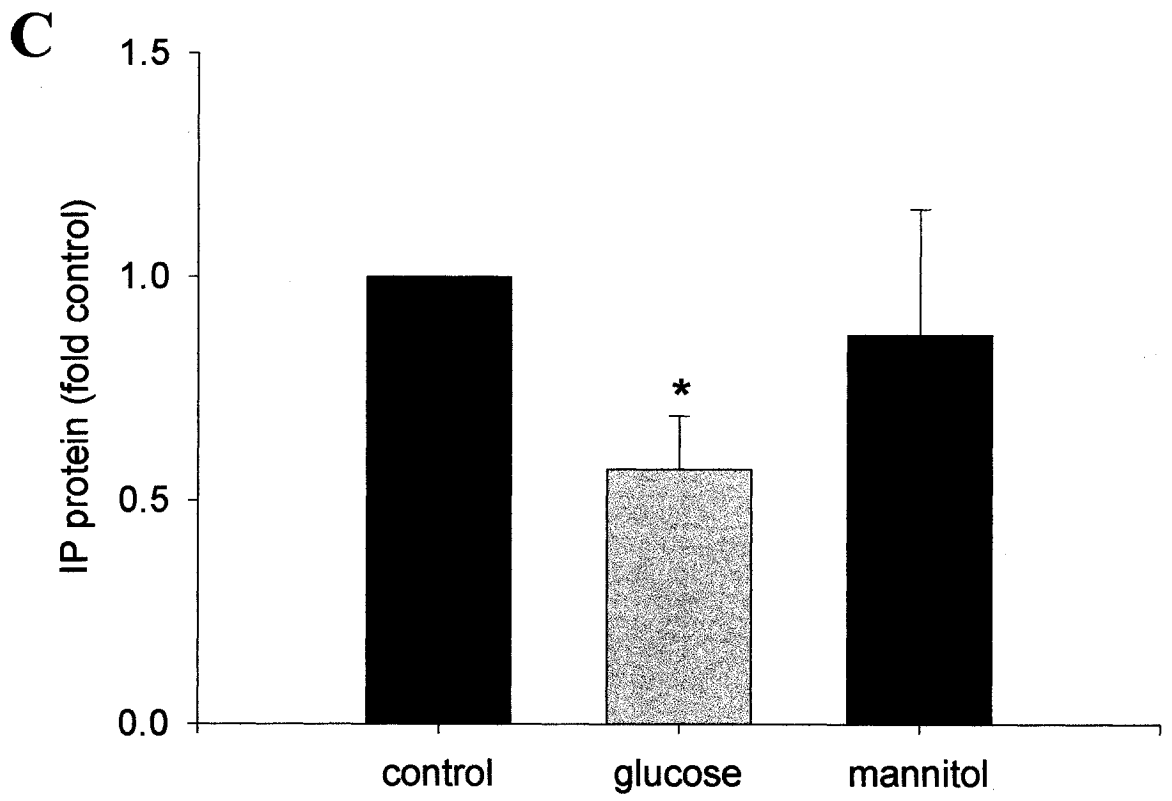
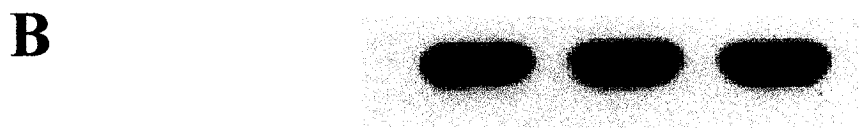
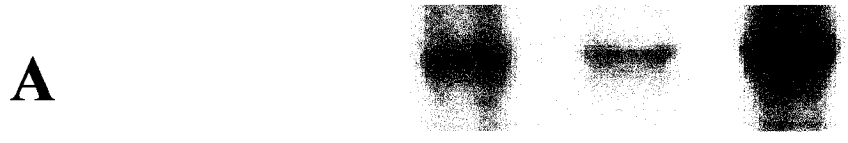


Figure 5.4. CCP-stimulated cAMP is attenuated by 24 hr glucose and mannitol. cAMP assays were performed on cells stimulated for 10 min with either 0.1 μ M CCP or PGE₂. Comparisons were made between cells cultured for 24 hrs in the presence of control (5.6 mM glucose), glucose (25 mM), or mannitol (25 mM). Data is presented as cAMP (% stimulation) \pm SEM, n = 3-6, * p < 0.05.

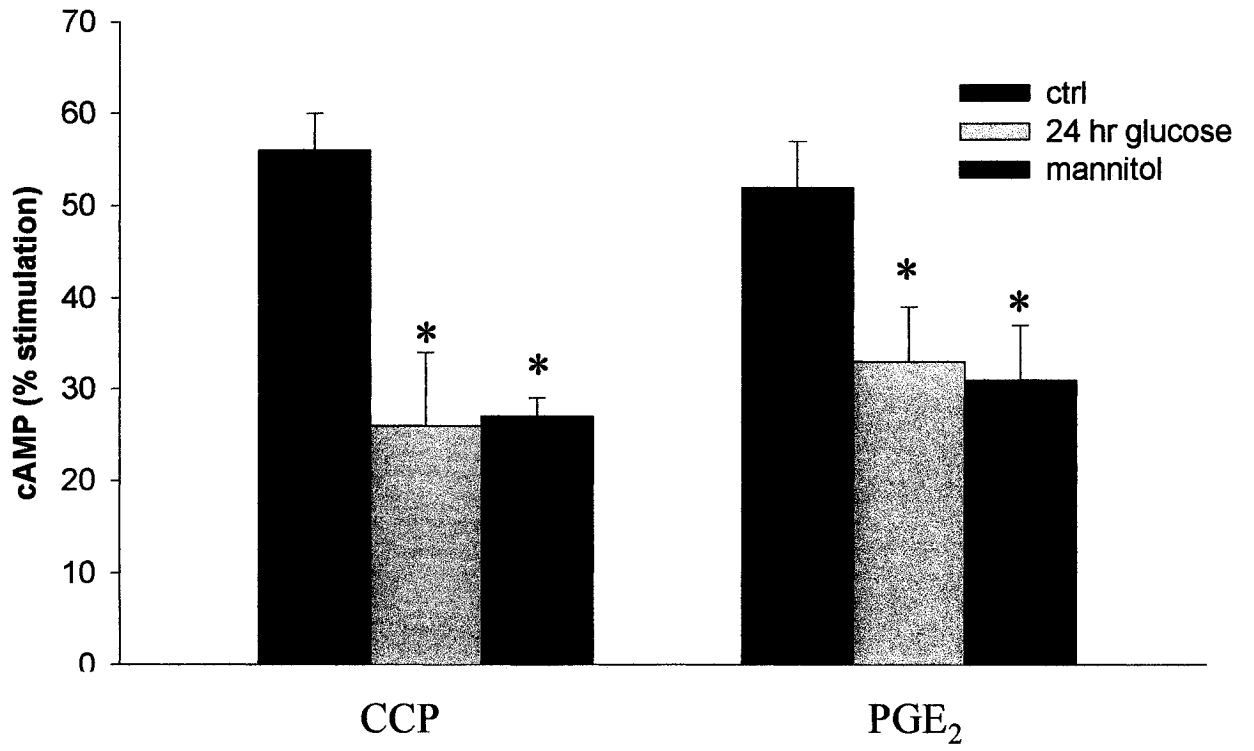
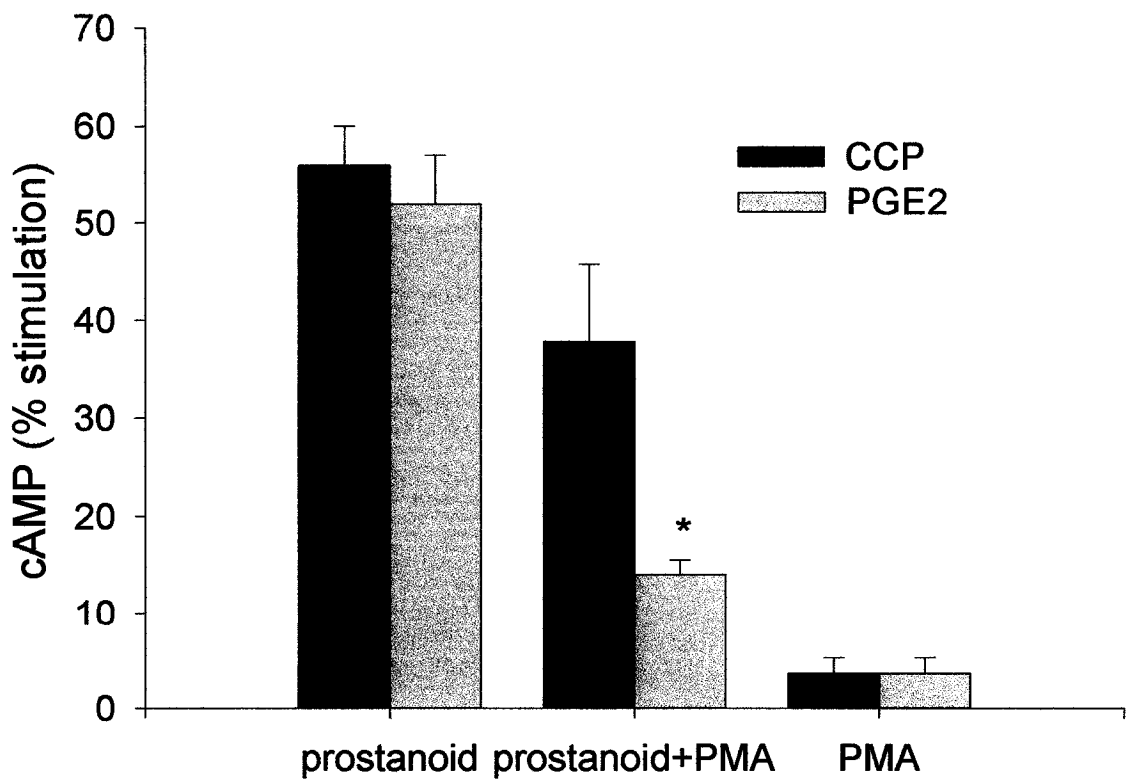
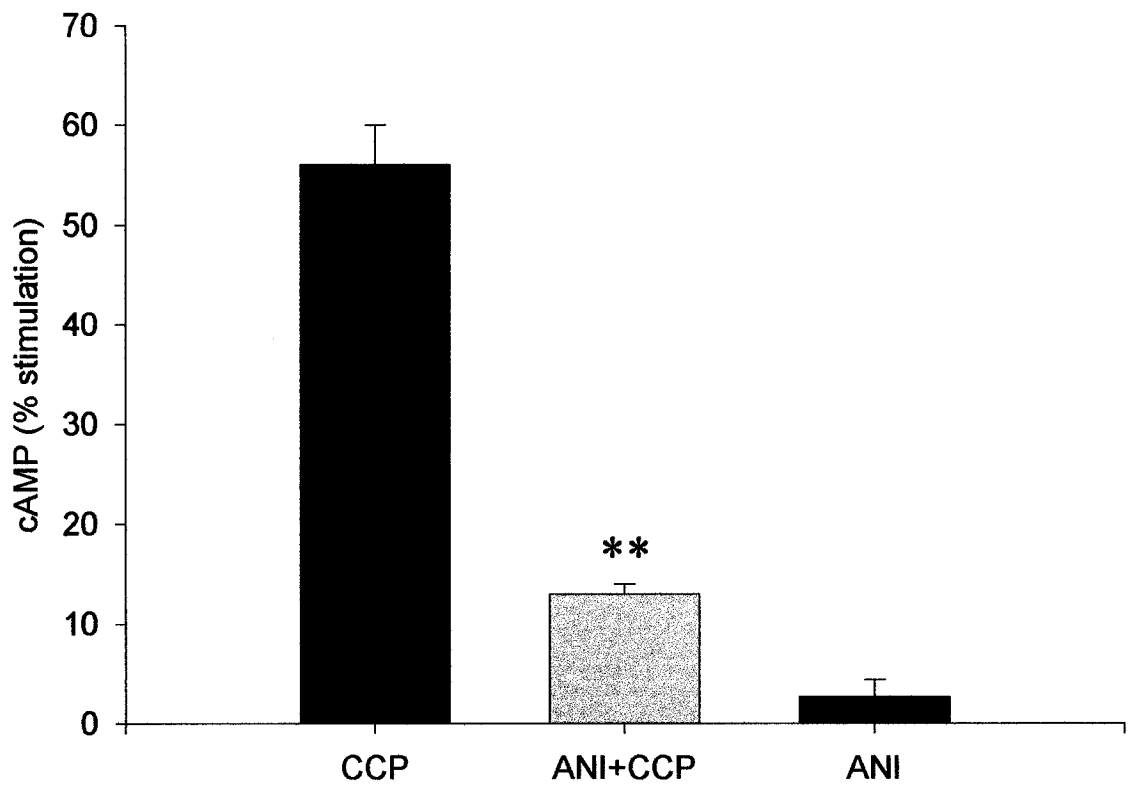


Figure 5.5. Anisomycin but not PMA altered CCP-stimulated cAMP in rMG.

Confluent rMG cells were serum starved and stimulated for 10 min with 0.1 μ M CCP, and intracellular cAMP levels were measured. The effect of 100 nM anisomycin for 24 hrs on the cAMP response to CCP is shown in A. Anisomycin alone does not alter cAMP levels. In B, the effect of 15 min PMA (100 nM) pre-treatment on the response to 100 nM CCP and PGE₂ is shown. Data presented as cAMP (% stimulation) \pm SEM, n = 3. * p < 0.05 or ** p < 0.001.



CCP decreases the levels of p27 and fibronectin, and increases MMP-2 in rMG cells:

To determine whether CCP could regulate the growth of rMG cells or matrix protein production by these cells, we measured the levels of p27 and fibronectin in cells exposed to CCP for 24 hrs. As indicated in figure 6, both p27 and fibronectin levels were significantly reduced by CCP treatment by 45 % and 55 %, respectively. Consistent with the decrease in fibronectin levels, a 3-fold increase in MMP-2 levels was obtained. This matrix metalloproteinase is a gelatinase important in the degradation of matrix proteins such as fibronectin and collagen IV (26, 38), which are major components of the mesangial matrix.

CCP does not alter growth responses in rMG cells:

Inhibitors of cyclin-dependent kinases such as p21 and p27 are important regulators of growth responses in many cell types, including mesangial cells. Both these factors are important in the progression of diabetic nephropathy (2, 23) and are thought to play a role in the mesangial cell changes (proliferation, hypertrophy) that are seen in diabetes. Despite a reduction in the levels of p27 in response to CCP, we did not obtain any changes in both thymidine or leucine incorporation by rMG cells. As shown in figure 7, FBS increased the level of thymidine incorporation 1.5-fold after 24 hrs. Not only was there no effect of CCP alone on these cells, CCP did not alter the response to FBS either. In other cell models, an inhibition of FBS-stimulated proliferation has previously been reported in response to prostanoids, including PGE₂ (14). Furthermore, CCP did not affect protein synthesis in rMG cells, but FBS treatment increased leucine incorporation by about 2.5- fold.

Figure 5.6. 24 hr CCP decreases fibronectin and p27 levels, but increases MMP-2 protein in rMG cells. Western blot analysis of p27, fibronectin, and MMP-2 was performed in rMG cells treated with 1 μ M CCP for 24 hrs. Densitometric analysis of protein levels is shown. Data presented as fold of control \pm SEM, n = 3-5, * p < 0.05.

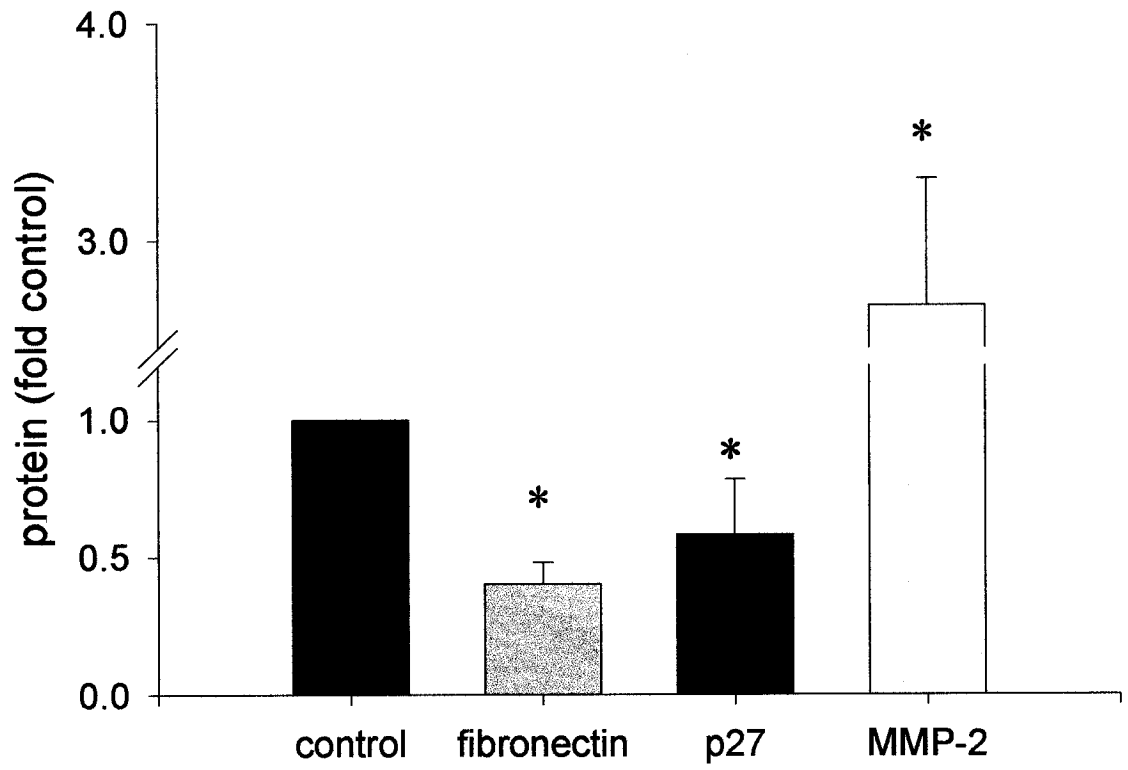
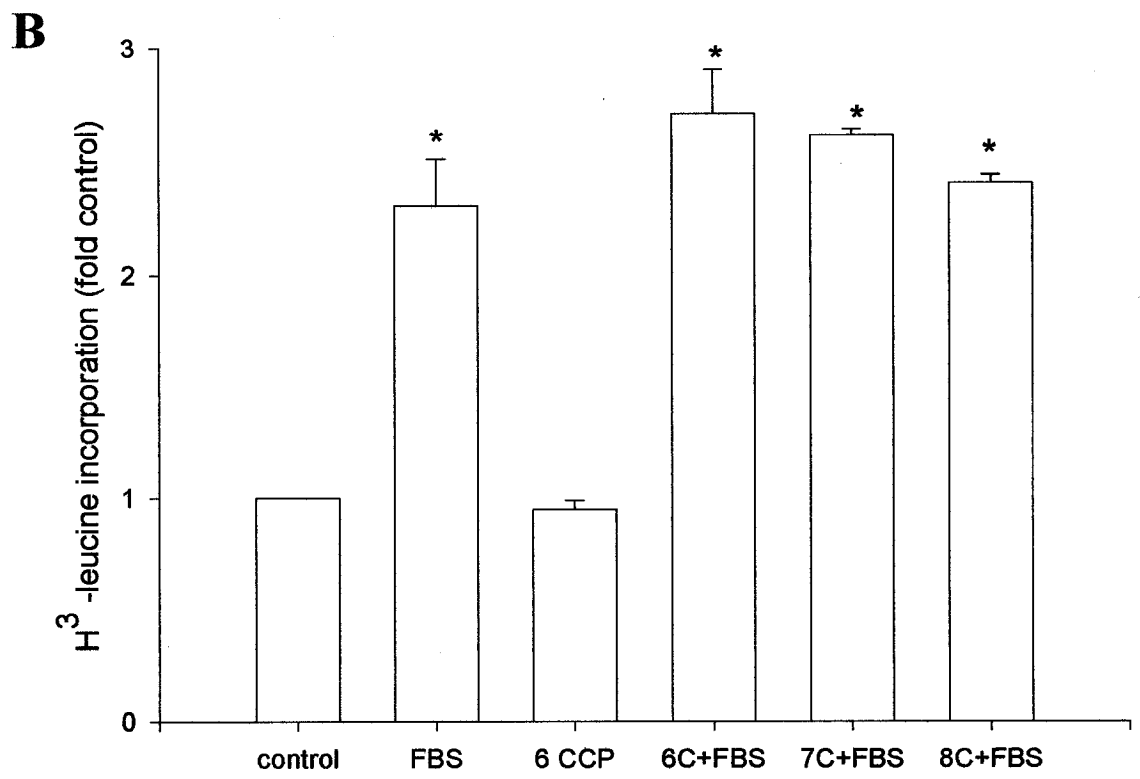
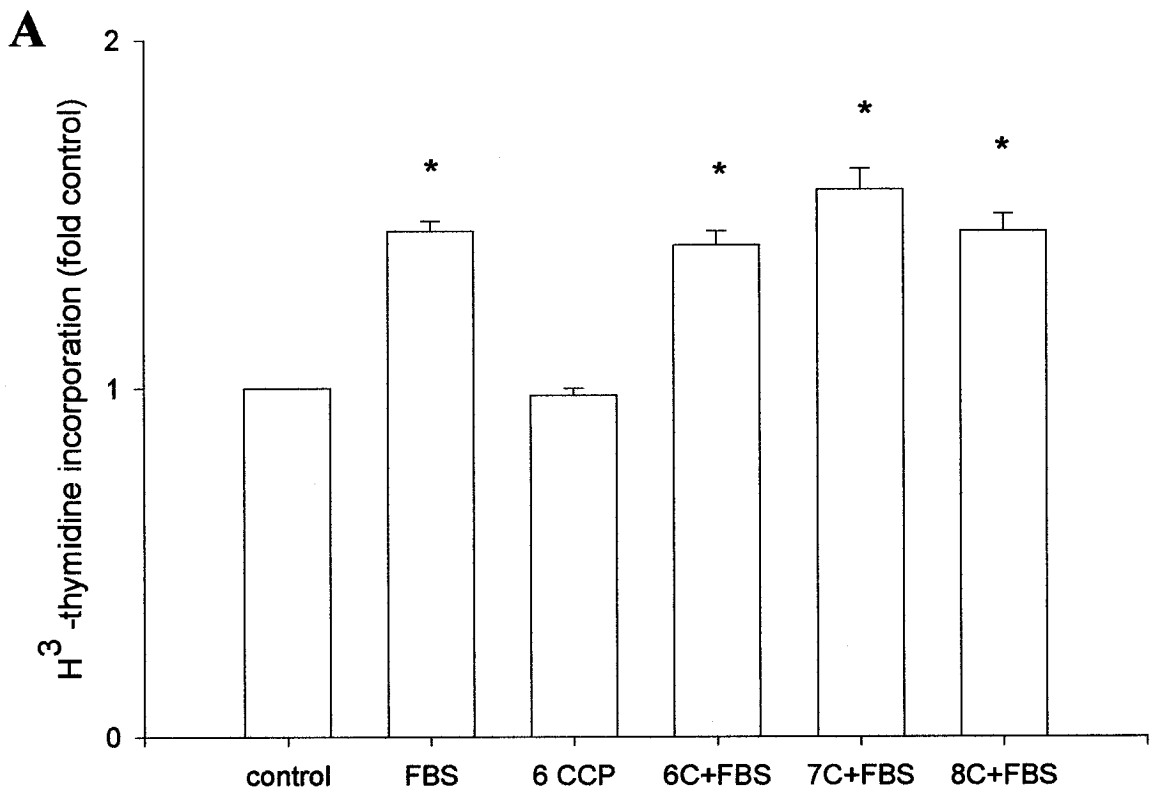


Figure 5.7. CCP does not alter thymidine or leucine incorporation by rMG cells.

rMG cells were serum starved for 3 days then cultured in the presence of DMEM containing either 15 % FBS or 1 μ M CCP (6 CCP) for 24 hrs. To determine whether CCP altered the response to FBS, cells were treated with 10 nM (8C), 100nM (7C), and 1 μ M (6C) CCP in the presence of 15 % FBS. A) H^3 -thymidine or B) H^3 -leucine incorporation by cells is presented as fold of control, n = 4, * p < 0.001.



IP receptor mRNA is decreased in diabetic rats:

The role of prostaglandins in diabetes is surely multi-faceted. However, the nature of their involvement in diabetic nephropathy remains evasive to this day. Certainly they contribute to the pathology, but they may also serve to antagonize other harmful agents. Although numerous studies have suggested a change in the levels of prostanoids or their metabolites in diabetes, to date there is very little information regarding the expression of prostanoid receptors in diabetics. Northern blotting and RealTime RT-PCR analysis was utilized to measure the relative expression of IP mRNA in different regions of the kidney of STZ-treated rats at different stages of diabetes as well as in 6 mth uni-nephrectomized STZ-diabetic rats. As shown in figure 8, a 30 % decrease in IP mRNA was only seen in the outer medulla at 4 wks and 12 wks of STZ-diabetes. A summary of other stages of diabetes is provided in table 2. As can be noted, there is no significant change in IP expression at any other stage, and none in the cortex or inner medulla. A major drawback with the STZ model is the lack of resemblance to type I diabetes in terms of renal pathology. Also, the progression of the disease is variable between animals. To hasten the development of diabetic complications the STZ-diabetic rats were subjected to additional stress by undergoing a unilateral nephrectomy following confirmation of their diabetic state, and IP mRNA levels were determined after a 6-mth period. To assess the impact of this procedure on the kidney we compared the average kidney sizes to the body weights of the different rat groups. As shown in table 3, the ratio of kidney to body weight was about 3.5 fold higher in the diabetic group compared to controls. By RealTime RT-PCR we observed comparable basal levels of IP mRNA in the cortex and inner medulla (fig 9), but about one third less in the outer medullary region of the kidney. However, a reduction

greater than 50 % of IP expression in uni-nephrectomized STZ-diabetic rats was consistently seen in all kidney regions. Maintaining euglycemia in insulin groups reversed the decrease in IP mRNA to levels comparable to controls. Only in the cortex was there an equivalent reduction of IP mRNA in uni-nephrectomized controls, with tendencies towards an increase in mRNA levels in the medullary regions for this group. However, since only two of the four animals initially assigned to this group survived to 6 months, a statistical analysis could not be performed for this group.

Figure 5.8. IP mRNA is decreased in the outer medullary region of STZ-diabetic rats. Northern blot analysis of IP mRNA expression was performed on tissue from the three regions of the kidney at different stages of STZ-diabetes. Densitometric analysis comparing IP mRNA levels in control, diabetic, and insulin rats from: A) 4 and B) 12 weeks of diabetes. Data shown as fold of control \pm SEM. * $p < 0.05$, $n = 5$.

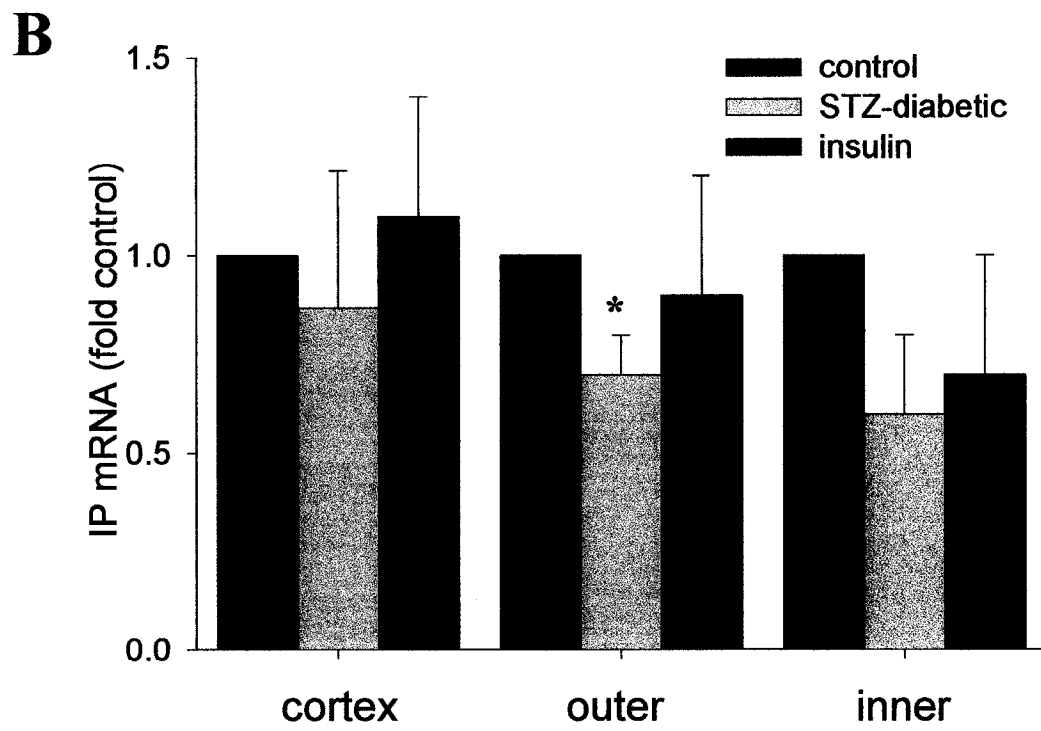
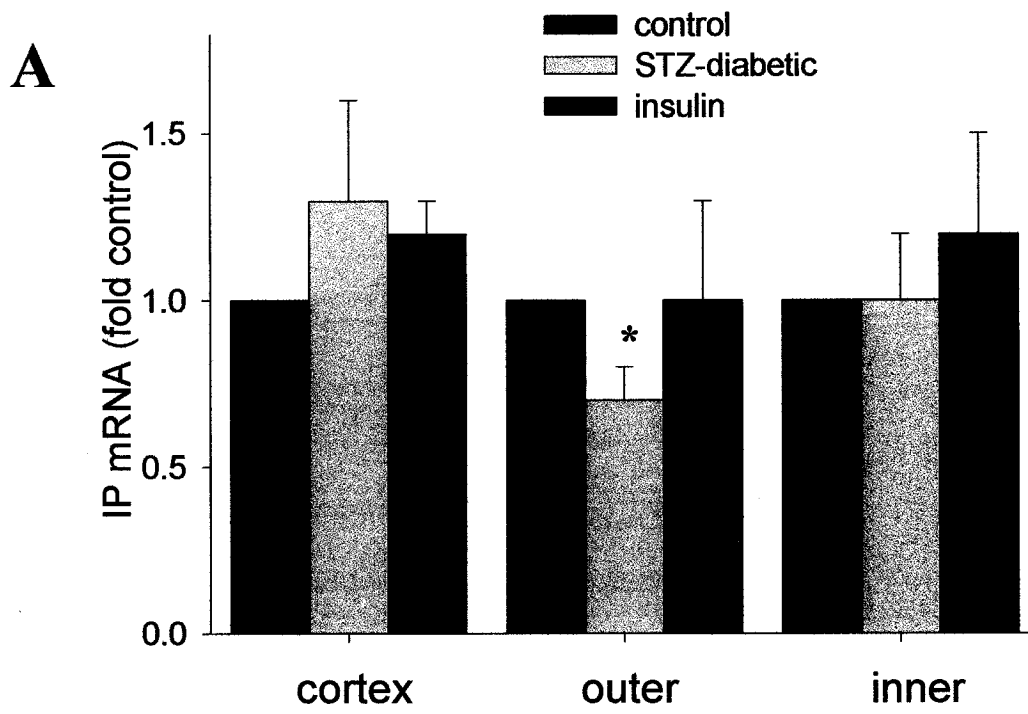


Table 5.2. Summary of IP mRNA levels at different stages of STZ-diabetes.

Cortex (Weeks)	Control	Diabetic	Insulin	N
2	1	2±0.6	0.8±0.2	5
4	1	1.3±0.3	1.2±0.1	4
6	1	2.3±0.8	1.9±0.8	5
8	1	2.6±0.8	2.4±0.6	5
12	1	0.87±0.34	1.1±0.3	5
16	1	2±0.6	0.8±0.2	5
Outer (Weeks)	Control	Diabetic	Insulin	N
2	1	1±0.3	1.4±0.2	5
4	1	0.7±0.1 *	1±0.3	5
6	1	1±0.3	1±0.2	5
8	1	0.98±0.24	0.99±0.35	5
12	1	0.7±0.08 *	0.9±0.3	5
16	1	1±0.3	1.4±0.2	5
Inner (Weeks)	Control	Diabetic	Insulin	N
2	1	0.85±0.09	0.9±0.2	5
4	1	1±0.2	1.2±0.3	5
6	1	0.7±0.2	1.1±0.8	5
8	1	1.9±0.9	1.2±0.4	5
12	1	0.6±0.2	0.7±0.3	5
16	1	0.85±0.09	0.9±0.2	5

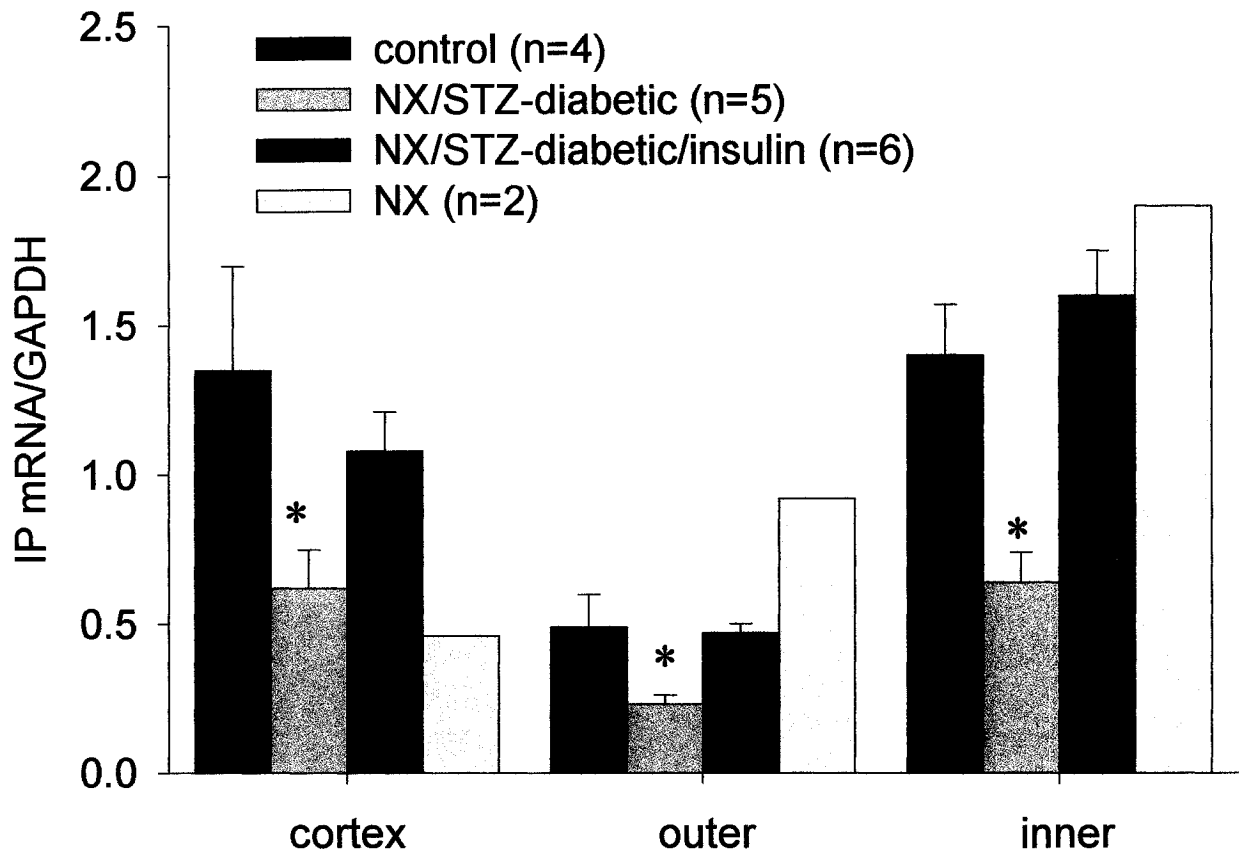
Total RNA was isolated from cortex, outer, and inner medullary regions of the kidney from 2, 4, 6, 8, 12, and 16 weeks STZ-rats. Northern blot analysis was performed to quantify levels of IP mRNA in each region using a human IP cDNA probe. Results are presented as fold of control. Data shown as mean ± SEM. N = 4-5. * p<0.05.

Table 5.3. Comparison of body weights and kidney weights for 6 mth rats.

Group (size)	Body Weights (g)	Kidney Weights (g)	Kidney/body weights ($\times 10^{-3}$)
Control (n=4)	732 \pm 49	1.8 \pm 0.1	2.5
NX/STZ-Diabetic (n=6)	433 \pm 21 **	3.8 \pm 0.2 *	8.8
Insulin (n=6)	619 \pm 23 * [†]	2.7 \pm 0.1 *	4.4
NX (n=2)	649	2.4	3.7

Daily measurements of body weights were recorded for each animal in all four groups (control, NX/STZ-diabetic, insulin, NX). The mean for each group is presented, as well as the corresponding kidney weights. The ratio of kidney/body weight is increased in diabetics compared to controls and insulin groups throughout the course of the study. * $p < 0.05$, ** $p < 0.001$, [†] $p < 0.05$ (diabetic vs insulin).

Figure 5.9. IP mRNA is decreased in the kidneys of 6 mth uni-nephrectomized STZ rats. RealTime RT-PCR detection of IP receptor mRNA in the: cortex, outer, and inner medullary regions of the kidney. Tissue was taken from 6 mth control, NX/STZ, NX/STZ-insulin, and NX rats. Results expressed as mean \pm SEM (n = 2-6), * p<0.05.



DISCUSSION

This study examined the role of the CCP/IP/cAMP system in immortalized rat mesangial cells. We show changes in rMG function in response to CCP, including decreased p27 and fibronectin levels as well as elevated MMP-2. However, CCP did not alter growth responses in rMG cells. Examining the literature available on the CCP/IP system systemically and in the kidney can provide insight into the possible significance of these findings. To date, most reports on extra-renal PGI₂ focus on its importance in balancing the constrictor effects of TXA₂. Actually, the cardiovascular complications associated with the use of selective COX-2 inhibitors may be due to a decrease in PGI₂ synthesis leaving TXA₂ un-opposed (5). Similar to this role, the IP system in glomeruli may serve a protective function in diabetes as a homeostatic modulator of constrictors such as TXA₂ and angiotensin II. Several studies indicate that both these factors are elevated in mesangial cells in response to high glucose and in diabetic glomeruli, contributing to hyperfiltration (1, 4, 8), hypertrophic events and accumulation of matrix proteins (9, 16). Therefore targeting IP may slow the progression of these complications, since both fibronectin and p27 are known to be elevated in diabetes and are important players in the development of glomerulosclerosis. Interestingly, Awazu (2) reported a defect in fibronectin translation in p27-null mice. Whether or not the decrease in fibronectin levels that we observed in rMG cells in response to CCP is partly due to its effects on p27 requires further investigation. In this regard, it is interesting that new avenues of p27 research are emerging, diversifying its role within the cell beyond the

regulation of cell cycle progression (6), and the mechanisms linking the CCP/IP/cAMP system to these events may be elucidated in the future.

An investigation of the progression of diabetic complications in STZ-diabetic mice that are deficient in p27 (2) indicates that it is key in several aspects of the nephropathy. Despite the importance of p27 in regulating proliferation and hypertrophy of cells, other inhibitors of cyclin kinases such as p21 are elevated in diabetic glomeruli and are also key to the development of diabetic hypertrophy (23). Perhaps in the rMG cells used for this study there is compensation by these other regulators of cell cycle progression and this would explain the lack of effect of CCP on thymidine or leucine incorporation by these cells. More work is needed to verify this possibility. Although the expansion of the mesangium in diabetes is mainly due to enhanced matrix protein synthesis and accumulation, there is also a decrease in protein degradation. Various matrix metalloproteinases are responsible for the cleavage of collagens, fibronectin, laminins, and other components of the matrix. Since CCP in this study increased the levels of MMP-2, it could prove to be useful in reducing the accumulation of matrix proteins and slowing the development of glomerulosclerosis. This possibility is further supported by the reduction in fibronectin observed in response to CCP in rMG cells. But it is not yet clear whether the decrease in fibronectin levels is due to defects in synthesis (dependent on p27 as mentioned above) or to enhanced degradation, or both.

Numerous studies elude to the putative role of prostanoids (PGs) in diabetic nephropathy (10), but a major controversy exists as to the nature of their involvement: whether they propagate the complications or serve to antagonize the deleterious effects of other agents. Moreover current work has examined the beneficial effects of specifically

targeting certain prostanoid pathways to alleviate the manifestations of the disease, including EP₁ receptor antagonists (25) and IP receptor agonists (18, 37, 44). But the underlying mechanisms of PG involvement remain uncertain. In diabetic kidneys, it has been clearly demonstrated that COX enzymes are elevated, and glomerular PG production in most species is increased (11, 15, 36), as well as in STZ-rats (22, 33). We recently reported elevated PGE₂ and PGI₂ synthesis in glucose-treated rat IMCD, as well as elevated COX-1 and -2 in medullary regions of 4-6 wks STZ-diabetic rat kidneys (30). Furthermore, selective inhibitors of COX-2 (NS-398) have been used to reverse some of the renal complications of STZ-diabetes, such as altered glomerular filtration rate, without affecting mean arterial pressure or renal plasma flow (19). Altogether, the work thus far tends to indicate that the alterations in prostaglandins are mainly at the level of synthesis, without any detailed studies of actual cell responses and changes in expression of different prostaglandin receptors. In the current study we characterized the expression of IP receptors in diabetic kidneys at different stages of the disease. We show that in the STZ-diabetic model, a reduced expression of IP is seen after 4 and 12 wks of diabetes, but no changes in expression were detected at other time points. Surprisingly, significant changes were only detected in the outer medullary regions. The renal manifestations or cellular events coinciding with this decrease in IP mRNA, and the significance to the nephropathy at these stages of diabetes, remains unclear at this time. On the other hand, when the diabetic kidney was subjected to additional stress to hasten the development of renal pathology, a reduction in IP expression was noted throughout the kidney. Future studies will be aimed at unravelling the significance of the decrease in IP to nephron

dysfunction in diabetes, especially changes in the distal nephron: altered electrolyte and water transport, acid-base regulation, interstitial fibrosis, and tubular atrophy.

Consistent with the decrease in IP expression *in vivo*, we report a reduced level of IP in cultured rMG cells exposed to high glucose. Accordingly, the cAMP response to CCP was attenuated in high glucose-treated cells. While no change in IP mRNA levels was noted *in vitro*, an increase in EP₄ mRNA levels was detected in response to glucose. The mechanisms underlying the inconsistent response to glucose in terms of increasing mRNA levels vs. decreased PGE₂-cAMP responses require further investigation. Especially since Ishibashi (15) reported no change in EP₄ mRNA in primary MG cell cultures, but showed the same attenuation of PGE₂-cAMP. An extensive study of how individual EP receptors are altered throughout the course of diabetes *in vivo* is lacking, and may clarify the discrepancies obtained in the two *in vitro* models: primary vs. immortalized MG cells. This highlights the importance of carefully extrapolating information from one specific model and applying it to clinical practice.

In addition to glucose, many other glucose-dependent diabetic factors have been shown to modulate cyclooxygenases and increase prostaglandin synthesis, including the glucose-induced activation of PKC as well as stimulation of MAPK pathways. An increase of both arachidonic acid release and eicosanoid production has been reported in mesangial cells in response to glucose-induced PKC activation (3, 21, 40). Similarly, interleukin-1 β (IL-1 β) is an important cytokine increased in mesangial cells in response to high glucose. Both JNK/SAPK and p38 MAPK have been implicated in COX-2 and PGE₂ increases in response to IL-1 β in rat mesangial cells (12). However, as shown in the present study, although both PKC and MAPK are involved in altered prostanoid

synthesis, they tend to attenuate the cAMP responses to both PGE₂ and CCP, respectively. The mechanisms involved need further clarification. Though there is no perfect animal model mimicking human diabetic nephropathy, and isolating mesangial cells from their glomerular setting and matrix environment surely alters their response, there is clearly sufficient evidence that the PGI₂/IP/cAMP system is attenuated in vitro. Future work examining the expression of IP protein in diabetic kidneys will confirm whether this defect also reflects the in vivo diabetic environment, thus providing a target for therapeutic intervention.

In summary, the present study demonstrates that IP receptor expression is diminished in diabetic kidneys. Though no change in IP mRNA is detectable in vitro, a significant decrease in IP protein was seen in response to high glucose. Also, upon exposure to high glucose, CCP and PGE₂ signaling is attenuated in the rMG cells. We show a decrease in p27 and fibronectin in response to CCP, as well as an increase in MMP-2 levels. Further studies will clarify the mechanisms involved in these responses and the significance of the attenuation of this pathway to the glomerular and tubular pathology seen in diabetes.

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CHAPTER 6: GENERAL DISCUSSION

The purpose of our research was to characterize the expression of the PGI₂ receptor (IP) in the rat nephron, the signaling pathways coupled to this receptor, as well as its putative role in diabetes. The most important contributions of this research are: 1) IP receptors are expressed throughout the rat nephron, including MG cells and the IMCD; 2) CCP, a selective IP agonist, stimulates cAMP without altering intracellular calcium in cultured IMCD and both primary and immortalized MG cells, as well as PT cells, and inhibits AVP-stimulated cAMP in freshly isolated IMCD; 3) CCP decreases p27 and fibronectin and increases MMP-2 in MG cells, without growth responses; 4) Both COX-1 and COX-2 are increased in STZ-diabetic rat renal medulla, as well as high-glucose treated IMCD. Only COX-2 is increased in high-glucose treated MG cells, but PGIS is decreased; 5) EP₁₊₄ receptors are increased in high glucose-treated rat IMCD and EP₄ is increased in MG cells, but IP receptors are decreased in high glucose-treated MG cells; 6) High glucose treatment attenuated the cAMP response to PGE₂ and CCP in both primary and immortalized MG cells; 7) IP receptors are decreased in the outer medulla of STZ-diabetic rats, and in 6-mth uni-nephrectomized STZ-diabetic kidneys.

6.1 Localization of IP along the rat nephron

PGs bind specific cell surface receptors that couple to different intracellular signaling pathways to exert important physiological and pathophysiological functions in the kidney. Although there is ample biochemical support implicating PGI₂ in the

regulation of renal function, to date there is little molecular evidence for distinct IP receptors along the rodent nephron. We showed the presence of IP mRNA on rat tissue sections in a subset of cortical tubules, glomeruli (mainly clusters of cells located centrally, most likely MG cells), interstitial cells and in the vasculature; in the tubular epithelial cells of the outer and inner medulla; also in various tissue and cell preparations. However, we could not identify any IP receptor subtypes or spliced variants that are homologous to the published IP cDNA in any of the kidney regions or tissue preparations examined. Yet our work does not exclude the possibility that sequences with low homology to the cloned IP receptor do exist.

Though we did provide some data that IP receptor protein is ubiquitously expressed in various segments/cells of the rat nephron: PT, MG cells, IMCD, inner medulla, outer medulla, we could not localize the IP protein on kidney sections using the human polyclonal antibody (Komhoff 1998); and to our knowledge, to date there are no rodent IP antibodies commercially available. However, Yamashita et al (2002) generated a polyclonal antibody against rat IP receptors, and confirmed the localization to afferent arterioles and glomeruli. However they did not describe the tubular expression of IP receptor protein. Although previous work in other species (Komhoff 1998, Hébert 1995, Hébert 1998, Oida 1995) did report the presence of IP in the kidney, the exact localization along the rat nephron had been lacking. Also, there seem to be several inconsistencies in the expression pattern between human and rodent studies. For instance our laboratory had reported the expression of IP receptors in the Tamm-Horsfall positive segments of the rat outer medulla (Hébert 1998), which identifies the mTAL, but in humans this segment did not express any IP receptors (Komhoff 1998). Also, in mice,

renal IP receptors were most abundant in the vasculature, with faint expression in the IMCD (Oida 1995). Moreover, IP is present in human MG cells and podocytes (Komhoff 1998) but only in rodent MG cells and not podocytes (Oida 1995, Bek 1999, Nasrallah 2001b). The reasons for these discrepancies are not yet clear, however several possibilities do arise: 1) technical differences depending on the probes used for detection; 2) actual physiological differences in function or role of IP receptors in specific tubule segments among species; 3) existence of IP subtypes in human or mice kidneys that are not detectable with probes against the “classical” IP; or 4) redundancies between receptor-pathways such that in human or mice mTAL for example, IP receptor function is taken over by another receptor like EP₄.

Similarly, contradicting reports have been made for EP receptor subtypes in the kidney. For example though biochemical evidence in rodents suggests that a butaprost-insensitive pathway via EP₄ receptors mediates the cAMP response to PGE₂, this subtype is not present in the human CD (Morath 1999). Likewise, human (Breyer 1996, Morath 1999) and rabbit (Guan 2002a) studies report the presence of EP₂ receptors in glomeruli and/or vasculature, but our work and others (Jensen 2001) have not been able to detect them in rodents. We show a uniform expression of three EP subtypes, EP_{1,3,4}, in all three regions of the kidney, but could not detect EP₂ in any preparations tested. This subtype is most likely only present in the renal vasculature and interstitial cells (Morath 1999, Breyer 2003), but perhaps it may be induced in tubular or glomerular cells in a pathological state. The significance of its expression and interaction with other renal PG signaling pathways is not clear at this time. Overall, the discrepancies reported in IP and EP receptor expression are not easily reconciled and further investigations are warranted

to firmly establish their role in regulating specific aspects of glomerular and tubular function.

Our work confirms the presence of IP receptor mRNA and protein in two models of cultured rat MG cells. Also, since PT make up a great percentage of the cortical tubules, it is interesting that we detected IP receptors in this part of the nephron in addition to more distal segments (mTAL and IMCD). We showed that IP protein was expressed in cultured PT cell preparations, and in unpublished studies, IP and EP₁₊₃ mRNA were detected, but not EP₂₊₄ (figure B.1.). Renin mRNA was amplified as a control for the identity of the cells, since renin is highly expressed in PT cells (Zimpelmann 2000.). Furthermore, since there is no biochemical evidence for the presence of EP₄ receptors in PT cells (this subtype is richly expressed in the distal tubules), distal tubule contamination was controlled for by the detection of EP₄, which was no longer amplified from cultured PT (figure B.1.b.), whereas it was present in freshly isolated PT preparations (figure B.1.a). This suggests that our culturing conditions indeed favor PT cell growth while restricting the survival of distal cells. The role of PGs in PT physiology and pathophysiology requires further investigation.

6.2 Signaling pathways coupled to IP along the rat nephron

Consistent with reports in the literature, our work indicates that the main signaling pathway linked to IP receptors in the kidney is the stimulation of cAMP. This was noted in MG cells, PT, as well as IMCD cells in response to both ILP and CCP. However, contrary to previous reports in isolated perfused rabbit CCD (Hébert 1995, Nasrallah

2002), or IP receptor transfected cells (Namba 1994), we did not observe any calcium signaling responses linked to IP receptors in either rat MG cells or IMCD. Although a phylogenetic classification scheme places the IP receptor in cluster 1 with DP, EP₂ and EP₄ receptors (Toh, 1995), for which splices or subtypes have not been reported to date (Coleman 1994; Narumiya 1999), no alternative signaling pathways for those receptors have been suggested other than the stimulation of cAMP through coupling to G_s-protein. However, PGI₂ analogues do activate different intracellular signaling pathways. Three possibilities have been proposed for alternative signaling responses documented for PGI₂ and its analogues: 1) the existence of IP receptor subtypes and/or spliced variants; 2) activation of IP-independent signaling pathways such as EP receptors or PPARs; 3) coupling of the existing IP receptor to different G proteins (Oka M 1993, Hébert 1995, Takechi 1996, Hébert 1998, Miggin 2002).

Only in freshly isolated IMCD did we observe an attenuation of AVP-stimulated cAMP in response to CCP, similar to previous work in rat mTAL in our laboratory (Hébert 1998) and in isolated perfused rabbit CCD where ILP inhibited AVP-dependent water flow in a manner independent of the EP₃ receptor subtype (Hébert, 1995). Though we have not yet confirmed IP coupling to G_i-protein, our TA cloning work does eliminate the existence of IP receptor spliced variants or subtypes in these two segments of the rat nephron that would account for this inhibitory effect. However, the possibility of IP receptors with low homology to the cloned IP receptor cannot be excluded. To date the reports of IP switching its coupling between G_s-, G_q-, and G_i-proteins are mainly studies where the mouse or human IP cDNA were over-expressed in various cell lines (Namba 1994, Katsuyama 1994). For example, initial work characterizing the receptor by Namba

and coworkers (1994), indicates that expression of the cloned mouse IP receptor cDNA into Chinese Hamster Ovary cells gave rise to both an increase in cAMP and inositol triphosphate levels upon stimulation with ILP. Moreover, this variable signaling seems to be species (Miggin 2002) and cell-type specific (Chow 2003). Lefkowitz et al. (2002) showed activation of phospholipase C in response to PGI₂ analogues in certain clones of HEK-293 cells but not in others. Furthermore, expression of the human IP cDNA into these same cells did not provide any Gi-protein coupled responses (Miggin 2002). Therefore, important differences are proposed between mouse and human IP in the mechanisms regulating the coupling to Gi- and Gq-protein, and on their dependence for PKA and PKC. While much work is needed to characterize these mechanisms with respect to the rat IP receptor, the inhibitory cAMP response to PGI₂ analogues reported in our laboratory (Hébert 1995, Hébert 1998, Nasrallah 2001b) is the first indication that this switching of IP receptor coupling may also occur in non-transfected rabbit and rat renal epithelial cells.

Though the role of PGs in various aspects of glomerular function has been investigated, PG involvement in PT physiology remains poorly documented. One possible explanation is the fact that this nephron segment does not contribute to their production in the kidney. Nonetheless, the PT plays a substantial role in the metabolism of PGs (Baum 2003), and due to its proximity to the glomerulus, is subject to paracrine interactions by glomerular PGs. For example, PGE₂ was shown to selectively inhibit phosphate transport in the PT (Dominguez 1984). And in another study exogenous PGE₂ attenuated the parathyroid hormone (PTH)-dependent cAMP response in PT cells (Dominguez 1988.). Whether this effect is mediated by the EP₃ receptor remains to be

determined. Paller et al (1992) showed that both PGE₂ and PGI₂ had a protective role on primary cultures of PT epithelial cells that were subjected to hypoxia and reoxygenation; the mechanism of cytoprotection remains unclear. In unpublished studies, we showed that both CCP and ILP increased cAMP levels in cultured PT cells (figure B.2.). Since V₂ receptors are abundant in the CD (Ishikawa 1988), the negative cAMP response to AVP further substantiates the purity of the preparation, excluding distal tubule contamination. Also a stimulatory response was obtained with PTH and isoproterenol. These two compounds are known to increase intracellular cAMP in this segment of the nephron (Belachgar 1995, Le Goas 1991). In spite of this, due to difficulties in culturing PT cells on coverslips, calcium measurements could not be completed. Therefore, it remains unclear whether the PGI₂/IP system in PT cells is linked to calcium signaling or other cellular responses. Further work is needed to clarify the role of this system in PT cells, and determine whether signaling linked to the PGI₂/IP system is altered in pathological states, to contribute to the disturbances in PT sodium handling or growth responses seen in diabetes for instance. Interestingly, our findings indicate that there are no EP receptors coupling to G_s in PT cells, which is in agreement with the lack of EP₄ mRNA in cultured PT cells (fig B.1.b), and confirms previous work showing only an inhibitory cAMP response to PGE₂ in rabbit PT (Dominguez 1988.).

6.3 Prostaglandin synthesis in diabetic kidneys

It is quite clear that there are notable changes in the synthesis of PGs in the diabetic kidney, as evidenced by measurements of urinary PG output (Gambardella 1988,

DeRubertis 1993, Viberti 1989). Marked changes in glomerular PG production have also been described, including that of PGE₂, PGI₂, PGD₂, and TXA₂ (Schambelan 1985, Craven 1987, Wilkes 1992). However, the synthesis of each PG in individual tubule segments has not been reported in diabetes. Furthermore, there are discrepancies with respect to the relative levels of each PG, possibly due to fluctuations occurring throughout the course of the disease. A clear temporal staging of these levels will be essential in order to better attribute their individual roles to various aspects of the nephropathy.

The synthesis of each PG is dependent on the co-expression of either COX isoform with a specific PG isomerase. In humans for instance, COX-2 preferentially couples to PGIS (McAdam 1999, Brock 1999), as well as in co-expression systems (Ueno 2001). Moreover, COX-2 inhibitors completely blocked PGI₂ synthesis in endotoxin-exposed bovine smooth muscle cells, while only partial inhibition of PGE₂ was obtained (Schildknecht 2004). Previous work by Komers et al. (2001) examined the synthesis of COX enzymes in the cortex of STZ-diabetic rat kidneys, showing an increase in only COX-2. However, our work indicates that COX -1 and -2 are both elevated in medullary regions of the nephron in STZ-rats. Also, we describe specific increases of both isoenzymes in high-glucose treated IMCD, but only of COX-2 in MG cells exposed to high glucose. Since COX is the rate-limiting enzyme in the synthesis pathway (Smith 1996), the increase in COX -1 and -2 described in our studies confirms a rise in renal PG synthesis, but the individual PG levels will also depend on the expression of isomerases.

Inflammatory processes play an important role in the pathogenesis of diabetic nephropathy, from glomerular to tubulo-interstitial changes. NSAIDS in general are good

anti-inflammatory agents, but their use is contraindicated in individuals with underlying complications such as cardiovascular and renal disease. Individually targeting each COX, especially the newly identified COX-3, may prove to be useful, if it turns out to have an antagonistic relationship with COX-1 and -2, as is proposed for the cardiovascular or central nervous system (Chandrasekharan 2002, Warner 2002, Botting 2003). However linking COX-3 and its products to the termination of an inflammatory response will need to be done first, and perhaps pharmacologically enhancing its function would be of relevance in the prevention of diabetic kidney disease. To date the presence of COX-3 in the rodent kidney and its putative role in inflammatory kidney diseases is entirely speculative, but surely worth investigating.

Very little work has examined how PG isomerases are altered in the diabetic kidney. A study by Ono et al. (1998) describes a significant reduction in the levels of a prostacyclin-stimulating factor in the renal vasculature of diabetic rats, consistent with reports of diminished circulating PGI₂ in diabetes (Harrison 1978, Silberbauer 1980). Interestingly, Saroyan and coworkers (1984) propose that any changes in PGI₂ production in diabetic human venous tissue reside higher in the AA cascade since they did not observe any alterations in PGIS activity. On the other hand, in a study by Tajiri et al (1994), TXAS activity was increased in diabetes, as evidenced by elevated urinary TXB₂ and improved renal function in response to the TXAS inhibitor (OKY-046) in 24 wk STZ rats. A similar effect was noted using a TXAS inhibitor in NIDDM patients (Umeda, 1995). Correspondingly, a rise in TXA₂/PGI₂ ratio may account for the cardiovascular complications associated with diabetes (Cheng 2002), thus a disruption in this antagonistic relationship may also contribute to diabetic kidney disease. Our work

indicates a significant augmentation of PGE₂ isomerase (PGES) in outer medullary tubules of STZ-diabetic rats, but no change in high-glucose treated IMCD. Nevertheless, PGE₂ levels were increased by high glucose in the IMCD, as well as PGI₂. Confirming the dependence of PG synthesis on COX expression in this segment of the tubule, independent of isomerase levels. On the other hand, a significant reduction in PGIS was obtained in MG cells, similar to the decrease reported in the vasculature (Harrison 1978, Silberbauer 1980). This reduction is in accordance with a decrease in the bioavailability of PGI₂ previously reported in diabetes by numerous investigators (Villa 1997, Robles 1993), which could contribute to the pathogenesis by leaving other factors such as TXA₂ and ANG II unopposed as proposed by Owada et al (2002). Moreover, the increases in vasodilatory PGs in the IMCD could have substantial relevance since both PGE₂ and PGI₂ are known to antagonize AVP action in the CD (Nasrallah 2001b, Hébert 1995). Thus a compensatory response in IMCD cells would increase PGE₂ and PGI₂ as a mechanism opposing the sustained levels of AVP for example, which are characteristic of the diabetic nephron. This is of importance since some of the clinical consequences of high AVP levels in chronic kidney disease are the changes in ECF volume (enhanced CD water permeability) and urea handling which contribute to hyperfiltration and proteinuria (Bankir 2001b).

6.4 Altered prostaglandin pathways in the diabetic IMCD

The IMCD is the ultimate regulator of urine concentration, an important transporter of H₂O and urea, as well as Na⁺. PGs play an active role in directly regulating

IMCD transport. But also indirectly by antagonizing the effects of AVP, a key hormone regulating IMCD H₂O and urea movement (Bankir 2001a), which is clearly implicated in the diabetic complications associated with chronic kidney disease. The main PG pathways present in the IMCD are the Gs-protein coupled EP₄ and IP receptors, as well as Gi-coupled EP₃ and Gq-protein coupled EP₁. Our work is the first documenting changes in PG receptor pathways in the diabetic CD, showing an increase in both EP₁ and EP₄ mRNA in the IMCD, and EP₄ protein (Nasrallah 2003). But we have not determined whether EP₁ receptor protein is also increased. Furthermore, the IMCD is composed of a heterogeneous population of cells: principal, α - and β -intercalated. Although principal cells express the EP receptors (Breyer 1998), it is not yet certain whether the intercalated cells express both these subtypes as well. And we have not yet determined whether this increase is occurring only in principal cells.

However, both EP₁₊₄ receptors do couple to different pathways (cAMP, Ca²⁺) to alter sodium transport in the CD (Breyer 2003). Whether other transport processes or gene transcription is also affected via these receptors is not clear. But surely if these antagonistic systems are both increased in diabetes, they are each serving distinct functions. A clearer picture can be painted once it has been determined which disease processes are linked to specific EP receptors, at what stage of the disease this is occurring, and which cell types they involve. To add to this complexity, these receptor subtypes are not only antagonizing each other, but are certainly cross-talking with a plethora of other pathways (ANG II, AVP, ET-1, nitric oxide...) all of which are present in the IMCD and are surely contributing to the progression of diabetic complications. For example, in STZ diabetic rats, Itoh et al (2001) reported a role for PGI₂ in reducing ET-1

levels, and its relevance to the prevention of disease progression. The level of redundancy in these systems is the main factor hindering the study of diabetic injury, due to compensatory responses by the cell.

Furthermore, we have not yet examined the levels of IP receptors in response to high glucose. Since this receptor system also increases cAMP in the IMCD, it is possible that IP will increase like EP₄. But this hypothesis has yet to be confirmed, especially since we have observed a general reduction in IP receptor expression in the diabetic kidney (Nasrallah 2004b), as well as an attenuation of the PGI₂/IP system in high glucose treated MG cells (Nasrallah 2004a,b). On the other hand, if a reduction in IP receptors is also seen in high glucose treated IMCD, then the significance of this finding to diabetic injury will be worth exploring, especially the individual contribution of both IP and EP₄-mediated pathways. It is perhaps interesting that the EP₄ increase reported could be a compensatory response to diminished IP receptors, but this hypothesis requires further investigation as well. On this note, it is entirely possible that “cross-talk” between IP and EP₄ receptors in diabetes serves completely different functions. For instance, Jones et al. (2001) showed different relaxation effects of PGI₂ analogues (like CCP) on pig mesenteric artery and rabbit aorta, in the presence or absence of EP₄ receptors. Another example of receptor “cross-talk” is that activation of DP receptors in the vasculature is linked to enhanced coupling of IP receptors to G_{as}-protein, thus potentiating IP-mediated signaling (Wise 2002). Also, antagonistic “cross-talk” between activation of TP and IP receptors is well recognized (Ullrich 2001)

The regulation of the IP and EP receptor expression in the CD is also elusive. In previous studies we proposed that a negative feedback mechanism exists in an

immortalized mouse CCD cell line (M1 cells) responsible for a rise in EP₄ expression in response to diminished levels of PGE₂ (Nasrallah 2001a), however in high-glucose treated IMCD cells we obtain both an increase in PGE₂ levels as well as increased EP₄. The significance of this discrepancy to diabetic injury requires further exploration, however it is possible that high glucose exposure or the diabetic state altered the feedback responses in these cells. It is also possible that the mechanisms of receptor regulation differ between cortical and medullary segments, or that the feedback response observed in M1 cells is cell-type specific. In support of this argument, it was shown that in inflamed human intestinal mucosa, both PGE₂ and the EP₄ receptor are significantly augmented (Cosme, 2000). Also, in our rMG cell experiments (Nasrallah 2004b), EP₄ mRNA was increased. And numerous studies indicate that PGE₂ levels were elevated in MG cells exposed to high glucose (Ishibashi 1999, Williams 1993, Kreisberg 1983), though we did not attempt to confirm these findings in our studies. Thus the negative feedback response reported in our laboratory in immortalized M-1 cells is surely not a universal mechanism regulating the expression of PGE₂ receptors in all cells or all species. In a recent report by Meyer-Kirchrath et al (2004) a positive feedback relationship was described between ILP and COX-2, showing induction of COX-2 mRNA by microarray analysis in human vascular smooth muscle cells. Perhaps a positive feedback mechanism is regulating EP₄ receptor expression in high glucose-treated rat IMCD serving to magnify the effect of PGE₂.

6.5 PGI₂/IP system alters mesangial cell function

6.5.1 Characterization of mesangial cells

MG cells regulate various aspects of glomerular function, but this is dependent on an intricate interaction with other glomerular cells, as well as the mesangium. The responses of these cells removed from the glomerular environment will likely not reflect the in vivo milieu, and care must be taken in drawing conclusions from such studies if these signals are lacking. Still, the use of isolated MG cells is highly recognized, and has been extensively characterized. In our studies two MG cell models were utilized, primary cultures (MG) up to passage 15 (gift from Dr James W Scholey) and immortalized (rMG) mesangial cells (ATCC). Upon initial characterization, we noted certain imperfections in each model that indisputably affect the interpretation of our results and the validity of these findings to actual in vivo events. First, we were not able to stain the MG cells with α -smooth muscle actin, a marker of proliferating/activated MG cells (Young 1995, Janssen 2003), but immunostaining for this protein was positive in rMG cells (figure C.1). Secondly, ANG II is known to stimulate intracellular calcium in MG cells acting on AT1 receptors (Burns 2000), and is a key regulator of glomerular and MG cell function. While our studies support this effect in MG cells, the rMG cells did not respond to ANG II. Finally, though an EP₄-mediated cAMP response to PGE₂ has been previously reported, only the rMG cells responded to PGE₂ in our studies. Whether or not the MG cells were initially responsive to PGE₂ is not known, but it is possible that increasing passage number affected this signaling pathway leaving the IP system intact. But a significant stimulation of cAMP was obtained in response to two IP agonists, and a good

calcium response is obtained in response to both AVP and ANG II. The reasons for these discrepancies are not clear at this time, but surely reflect differences in the in vitro settings and environment of the cells.

6.5.2 Role of PGI₂/IP in mesangial cells

While previous studies indicate that PGI₂ does increase cAMP in MG cells (Togawa 1997, Kitahara 2001), they fail to confirm that this response is mediated by IP. We show that MG and rMG cells both express IP receptors, which would indicate autocrine regulation of MG function by the PGI₂/IP system. Our work also demonstrates that cAMP and not calcium is altered by IP agonists like CCP, which is a highly specific/selective IP agonist (Narumiya 1999, Clapp 2002). This issue is important since it is becoming quite evident that endogenous PGI₂ can act through an intracrine fashion and activate intranuclear signaling by binding PPAR δ (Reginato 1998, Hatae 2001, Lim 2002b). The downstream targets of this interaction have yet to be determined. However most commonly used PGI₂ analogs (ILP, carba-PGI₂) do activate this alternate pathway (Hatae 2001, Lim 2002, Wise 2003) as well as EP₁ and EP₃ receptors (Narumiya 1999), and the reports in the literature on MG cells are limited to these two analogues as well as beraprost sodium (Togawa 1997). Thus the purpose of our study was to study the IP system, and exogenous CCP is the most effective method to separate IP effects vs. EP receptor or PPAR δ -mediated effects in MG cells. Moreover the existence of other as of yet unidentified signaling pathways for PGI₂ further obscures the matter.

Although other studies have proposed a role for PGI₂ and other PGs in cellular growth responses (Homma 1988, Bruggeman 1993, Koh 1999, Clapp 2002), our work

does not indicate any effect of the CCP/IP system on either protein or DNA synthesis in rMG cells. A study by Togawa et al (1997) did show an inhibitory effect of beraprost sodium on rat MG cell proliferation that is mediated by induction of mitogen-activated protein kinase phosphatase (MKP-1), which inhibits the MAPK pathway. However in a murine carcinoma cell line using another PGI₂ analogue (carba-PGI₂) no effect on cellular proliferation or thymidine incorporation was noted (Bruggeman 1993). Moreover, Clapp et al. (2002) argue that CCP is a weaker anti-proliferative agent than ILP or UT-15 for human pulmonary artery smooth muscle cells. Therefore, growth responses to PGI₂ analogues will vary in a cell-type and analogue-dependent manner. On the other hand, we did obtain a significant reduction in fibronectin levels as well as an increase in MMP-2, which is an important regulator of matrix turnover in MG cells (Dolan 2003). While a reduction in fibronectin levels was noted in response to carba-PGI₂ in a murine teratocarcinoma cell line, the opposite effect was obtained by TXA₂ stimulation (Bruggeman 1993). Furthermore, Nishio et al. (2002) demonstrated that the reduction in fibronectin synthesis by MG cells is dependent on cAMP/PKA activation, which is stimulated in response to MG cell injury from glomerular hypertension for example.

In spite of the lack of growth effects in response to CCP, we did observe a reduction in p27 opposite that previously reported in other cells, where CCP prevented p27 degradation by altering different factors such as the F-box protein, Skp2 (Stewart 2004). It is likely that differences in experimental conditions are contributing to these apparent inconsistencies with respect to p27 levels, or perhaps this is another cell-type specific effect of CCP. An additional possibility is that other inhibitors of cyclin-

dependent kinases, such as p21, are more important in regulating rMG cell growth responses. But further studies are needed to explore these alternatives.

It is of interest that recent reports suggest a more complicated role for p27 in the cell depending on compartmental localization, serving to regulate apoptotic events, cell transformation, and as a transcriptional cofactor (Coqueret 2003). Furthermore, a link has been made between p27 levels and the expression of fibronectin. Awazu et al. (2003) showed that impaired fibronectin translation occurs in the absence of p27, possibly due to alterations in the translational inhibitor 4E-BP1. Therefore, perhaps the effects of PGI₂ on matrix turnover in rMG cells are more global, with multiple pathways converging to reduce protein accumulation. Thus we propose that CCP acting on p27 indirectly lowers fibronectin expression in rMG, in parallel to increasing MMP-2, which would enhance fibronectin turnover. Although our work does not support a direct effect of CCP/cAMP/PKA on the fibronectin gene, and whether fibronectin is a target gene in the PGI₂/PPAR_δ cascade, these are other options worth exploring. Altogether, PGI₂ and its analogues seem to serve a general function to prevent matrix accumulation regardless of the cell type, thereby protecting against the development of glomerulosclerosis.

6.6 The IP system is attenuated in diabetic kidneys

6.6.1 Effect of high glucose treatment in mesangial cells

Certain studies allude to an increase in urinary PGI₂ in early diabetes (Gambardella 1988, Perico 1992), but later there seems to be a decreased bioavailability of this prostanoid (Owada 2002, Villa 1997, Robles 1993, Harrison 1978/1980). There

have also been reports of diminished levels of a prostacyclin-stimulating factor (PSF) in the glomerular vasculature of diabetic rats (Ono 1998), consistent with a general decrease in glomerular PGI₂ levels in the diabetic environment. Our studies indirectly confirm these findings in the MG cells, showing reduced PGIS expression, but increased COX-2 in response to high glucose treatment. Previous work by Cosentino et al (2003) showed similar effects on COX-2 mRNA and protein in response to high glucose in human aortic endothelial cells, with an increase in TXA₂ and a reduction in PGI₂ release.

We also show in both MG and rMG cells exposed to high glucose that not only is PGI₂ synthesis likely diminished, but also that the PGI₂/IP system is attenuated in response to glucose. Our group and others (Ishibashi 1999) consistently show a diminished response to PG-mediated cAMP. While the exact mechanism for attenuation of the PGI₂/IP system in our cells has not been elucidated, we show that the inhibition of IP-mediated signalling is partly due to changes at the receptor protein level without any effect of glucose on IP mRNA. As for the EP₄ receptor, we show attenuated cAMP responses to PGE₂ but increased mRNA levels. Whether EP₄ protein is differentially regulated is unclear at this time, and since mannitol also inhibited PGE₂-cAMP then a possible osmotic effect should be examined. However, the mechanism for glucose vs. mannitol effects is not clear, each acting via extracellular or intracellular means, similar pathways or independently. We believe that since IP receptor protein was diminished by glucose and not mannitol, this further supports the complicated nature of this regulation, and allows us to confidently suggest that glucose does affect the IP pathway at least in part by osmolality-independent mechanisms. The exact process by which mannitol is reducing CCP-cAMP responses is not apparent. It may not be entirely due to an osmotic

effect, but partially to glucose-independent effects.

A great deal of the changes in diabetes is elicited by glucose-dependent factors, such as ANG II, TGF β , PKC, MAPK, all of which are increased in the diabetic environment. Numerous studies support a role for these pathways in the regulation of prostaglandin synthesis: to alter COX (Guan 1997, Guan 1998), arachidonic acid release (Williams 1993, Huang 1999), and increase prostaglandin levels (Williams 1993, Koya 1997). However, no work has examined the effect of activation of these pathways on actual cell responses to PGs. The use of PMA and anisomycin as activators of PKC and MAPK pathways, respectively, was chosen due to the wide use of these compounds in the literature (Tsiani 2002). It is noteworthy that we also examined the effects of ANG II and TGF- β on cAMP, but both these compounds did not affect the CCP/IP/cAMP response. But as mentioned above, these immortalized cells are not entirely loyal to the "true" mesangial cell phenotype, and a defect in cell response rather than a lack of effect on the IP receptor system complicates the interpretation of these results.

However, there appears to be a general defect in the PGI₂/IP/cAMP system in diabetic mesangial cells, targeted by different diabetic factors. We report an attenuation of CCP-cAMP responses by anisomycin. Whether or not this effect results from direct receptor regulation was not confirmed. The possibility does exist that a protein intermediate is involved in the attenuation, since in addition to MAPK activation anisomycin serves as a general protein synthesis inhibitor (Kandasamy 1982). A MAPK antagonist would clarify this issue. Although PKC did not have a great effect on CCP-cAMP, it diminished the cAMP response to PGE₂. Ishibashi et al (1999) observed the

same effect of PMA on PGE₂-cAMP in MG cells without any change in EP₄ expression; and propose that activation of PKC reduces adenylate cyclase activity or modifies EP₄ receptors via post-transcriptional effects. Other studies have reported that PKC activation may have a general effect to reduce cell surface receptors, as shown for ET-1 receptors (Cozza 1990). But data also supports receptor-independent effects on hormone action, for example ANG II-induced activation of phosphodiesterases (Pfeilschifter 1987). Clearly the IP and EP₄ pathways stimulating cAMP are differentially regulated in the diabetic environment, and the significance of this to MG cell function is surely worth investigating. Furthermore, it will be necessary to confirm activation of pathways opposing the PGI₂/IP system such as TXA₂/TP/calcium and PGE₂/EP₁/calcium by diabetic factors to further validate our results, since a general stimulation in prostaglandin synthesis has been reported.

6.6.2 Expression of IP receptors in STZ-diabetic kidneys

The role of prostaglandins in diabetes is surely multi-faceted. However, the nature of their involvement in diabetic injury remains evasive to this day. Certainly they contribute to the pathology, but they may also serve to antagonize other harmful agents. Although numerous studies have suggested a change in the levels of prostanoids or their metabolites in diabetes, to date there is very little information regarding the expression of prostanoid receptors in diabetics. Wilkes et al. (1992) reported a reduction in glomerular TP receptors in 7-10 day STZ-diabetic rats, similar to the diminished expression of ANG II AT1 receptors (Burns 2000) that may account for the defect in vasoconstrictor responses resulting in early diabetic hyperfiltration. In contrast, we show reduced

expression of IP receptors in later stages of diabetes. Having said this, IP protein levels were only decreased in vitro with no change in mRNA hinting upon different regulation by glucose, but IP mRNA expression was attenuated in vivo. It will be necessary to examine the expression of IP protein in the diabetic rats to confirm an in vivo defect in PGI₂/IP/cAMP similar to that observed in vitro. Examining IP protein in vivo by either immunoblotting and/or immunohistochemistry is crucial to linking the in vitro and in vivo environments, especially since differences are seen in terms of regulation of IP mRNA vs. protein both in response to high glucose in vitro, but also since no change in mRNA was found in vitro but a decrease was observed in vivo. If the same attenuation of the PGI₂/IP/cAMP system is seen in diabetes, targeting the IP receptors could prove to be a useful tool in slowing the progression to ESRD. This is especially true if for instance a link is shown between the attenuation of this system and enhanced function of ANG II, AVP, or TXA₂ for example, left unopposed to carry out changes in glomeruli and tubule segments that hasten the progression of the disease. A study to this effect by Itoh et al (2001) proposes a role for PGI₂ in reducing the ET-1 levels that may account for development and progression of diabetic microangiopathy in STZ-rats.

While the exact mechanisms involved in IP receptor reduction in diabetes remain undetermined, the fact that the NX/STZ/insulin treatment reversed the reduction in IP mRNA further supports the “diabetic” effect on the receptor, rather than a reduction of IP mRNA as a result of direct STZ toxicity (Bennett 1981). It will be important to determine how IP and other prostanoid receptors are changing in diabetes, and how their expression varies throughout the different regions of the kidney over the course of the disease. Then,

attribute these changes in prostaglandin receptor expression to specific disturbances in cellular responses and to specific cell populations within the kidney. The attenuation of the IP receptor pathway may have important implications for the glomerular and tubular changes that contribute to later stages of kidney disease. This information could prove to be useful for the development of alternative therapeutic strategies.

6.7 Summary

The objective of our studies was to study the PGI₂/IP system in the rat kidney, and examine its expression in diabetes. Our results may be summarized as follows:

- 1) IP receptors are expressed throughout the rat nephron, and CCP alters cAMP signalling in both primary suspensions and cultured IMCD. ILP alters intracellular calcium in micro-dissected IMCD via the EP₁ receptor subtype.
- 2) Both COX-1 and -2 are constitutively expressed in the renal medulla, and are increased in diabetes. This is associated with an increase in PGE₂ and PGI₂ levels in high-glucose treated IMCD. Although EP₄ receptors are increased upon stimulation of IMCD with high glucose, EP₁ receptors are also increased by high-mannitol treatment.
- 3) IP receptors are expressed in primary MG cell cultures. CCP stimulates cAMP in these cells. High glucose attenuates the cAMP response, increases COX-2 levels, and decreases PGIS levels.
- 4) The PGI₂/IP system alters protein expression in immortalized mesangial cells. CCP decreases fibronectin and p27, and increases MMP-2. But CCP has no effect

on DNA or protein synthesis. The IP system is attenuated by high glucose treatment; anisomycin reduces CCP-cAMP, but not PMA, TGF- β , or ANG II. The PGI₂/IP system is possibly attenuated in diabetic kidneys as well, since IP mRNA expression was decreased in STZ-diabetic rat kidneys. This suggests that IP receptors may serve as a useful target in diabetes to slow the progression to ESRD.

CHAPTER 7: GENERAL REFERENCES

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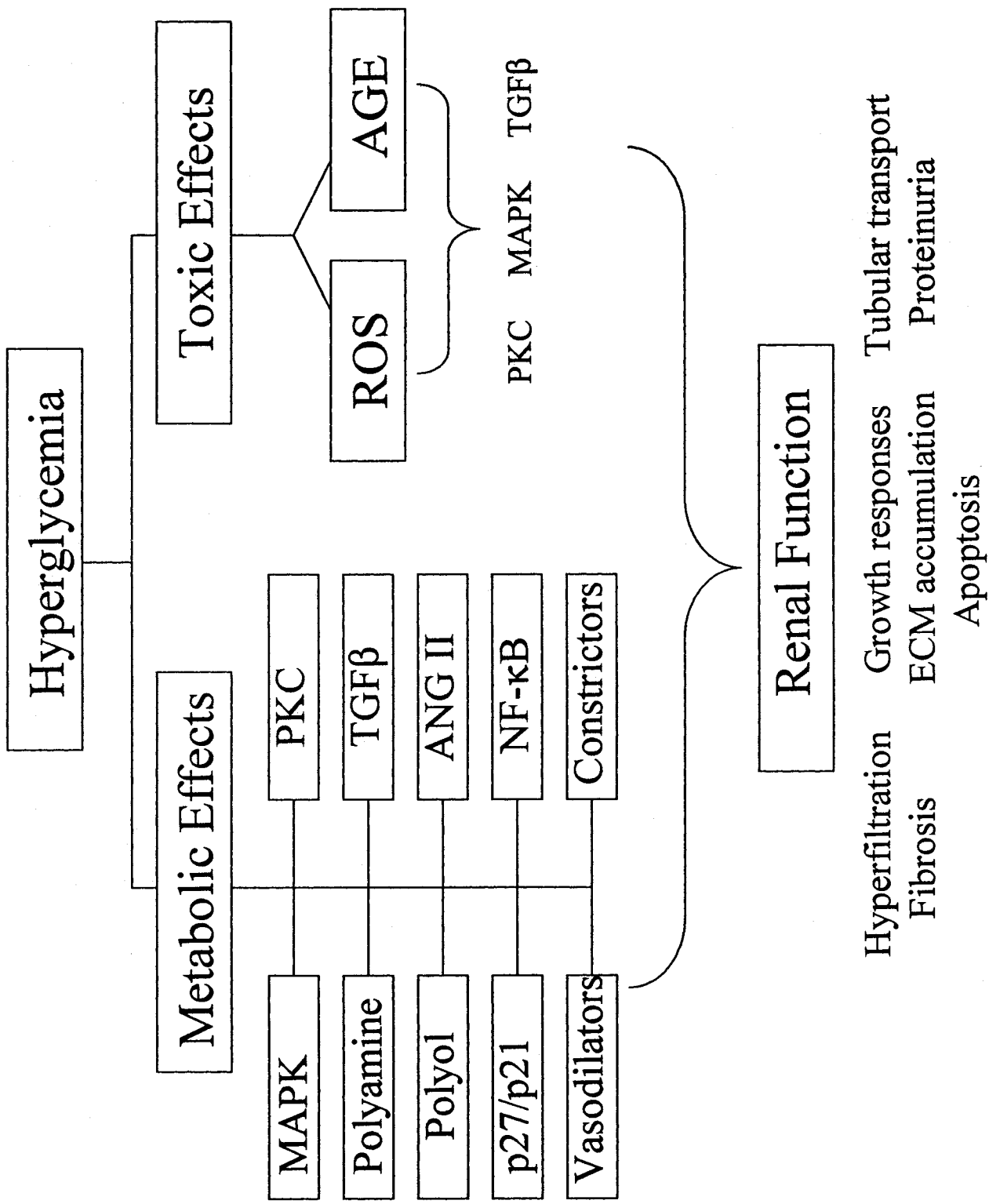
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APPENDIX A: HYPERGLYCEMIA AND RENAL **FUNCTION**

This section contains one figure illustrating the various pathways activated by hyperglycemia, all converging to alter renal function.

Figure A.1. Effects of hyperglycemia on renal function.

Figure A.1. Effects of hyperglycemia on renal function. Flowchart illustrating the various pathways activated by hyperglycemia, converging to alter different aspects of renal function. The effects of high glucose can be separated into two groups. The first are irreversible toxic effects mediated by generation of glycation end products (AGE) or reactive oxygen species (ROS). The second are reversible metabolic effects due to activation of various glucose-dependent factors.



Hyperglycemia

Metabolic Effects

Toxic Effects

MAPK

PKC

Polyamine

TGFβ

Polyol

ANG II

p27/p21

NF-κB

Vasodilators

Constrictors

Renal Function

- Hyperfiltration
- Fibrosis
- Growth responses
- ECM accumulation
- Tubular transport
- Proteinuria
- Apoptosis

APPENDIX B: PGI₂/IP IN PROXIMAL TUBULES

This section contains two figures of data from work that is not part of the manuscripts presented in this thesis, a continuation of the characterization of the PGI₂/IP system in proximal tubules.

Figure B.1. IP and EP receptor mRNA is expressed in both A) freshly isolated and B) cultured rat proximal tubule cells.

Figure B.2. PGI₂ analogues (CCP and ILP) stimulate cAMP in cultured rat proximal tubule cells. A) 15 min stimulation, B) 30 min stimulation.

Figure B.1. IP and EP receptor mRNA is expressed in both freshly isolated and cultured rat proximal tubule cells. Gel electrophoresis of the amplified RT-PCR products from DNase-treated total RNA of PT cells: A) freshly isolated and B) cultured. Renin serves as positive control for PCR reaction. IP and EP₁₋₄ receptor subtypes were amplified using specific upstream and downstream primers. Also shown is a 100 bp DNA ladder, and the arrow shows the 600 bp marker.

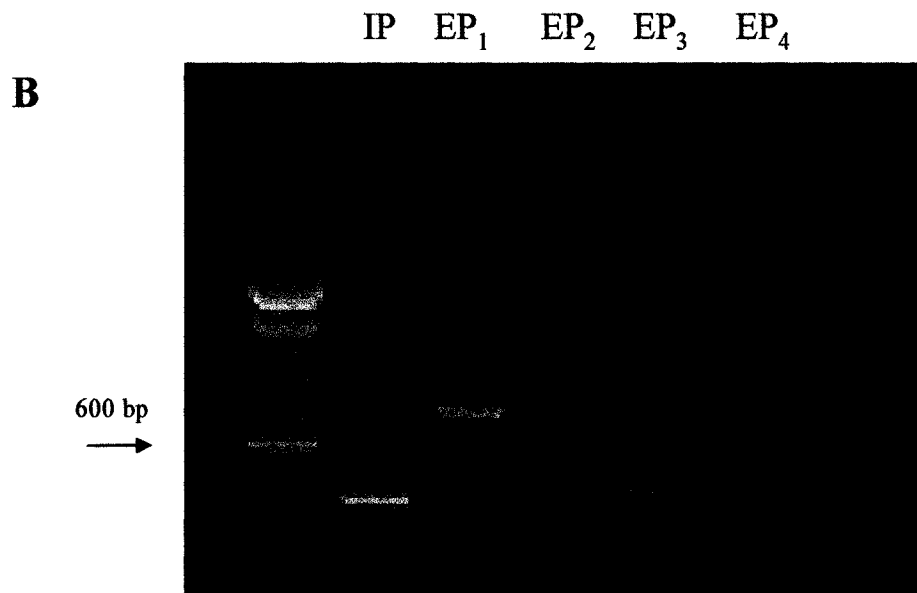
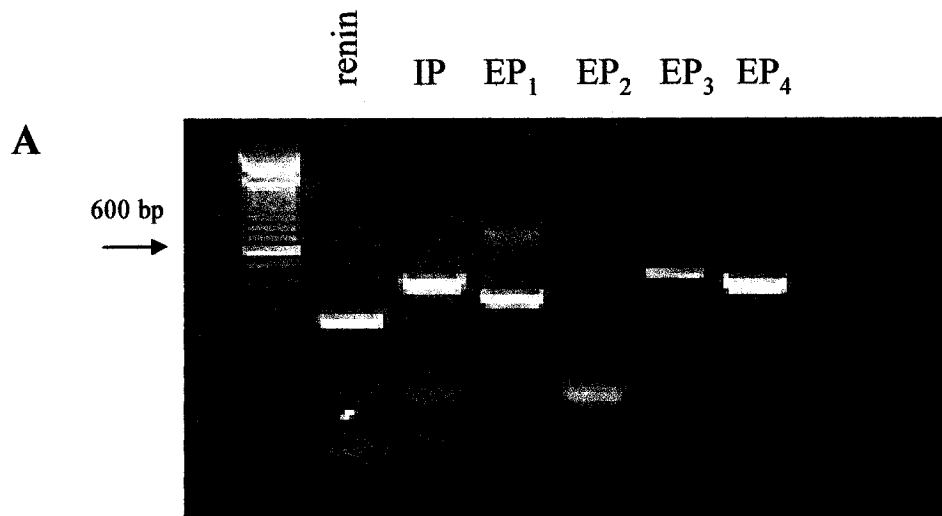
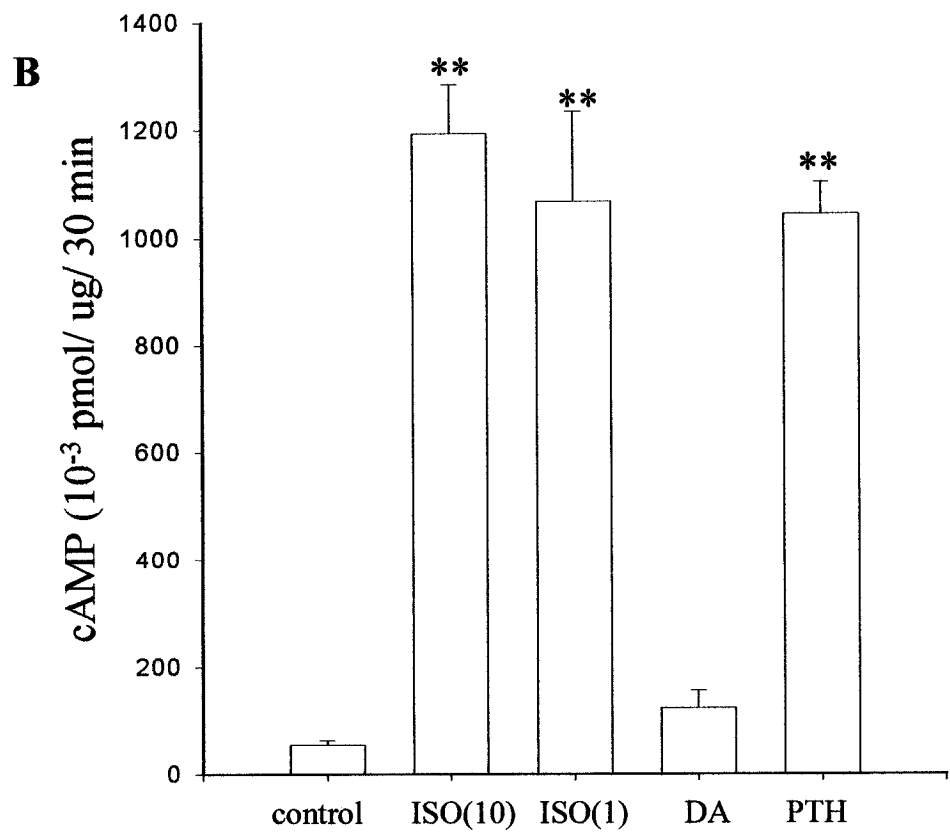
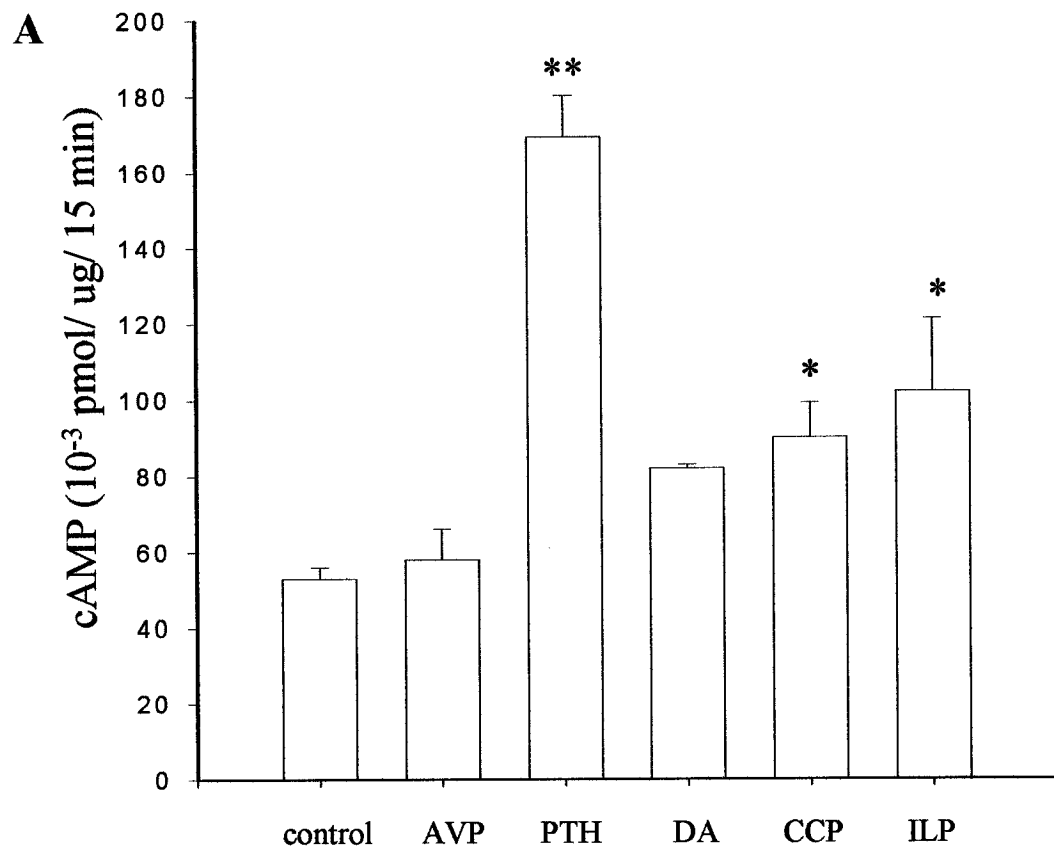


Figure B.2. PGI₂ analogues stimulate cAMP in cultured rat proximal tubule cells.

Measurement of cellular cAMP levels by radioimmunoassay. PT cells were cultured for 5 days and serum starved O/N. Cells were stimulated with different agonists for A) 15 min or B) 30 min. Results are presented as mean \pm SEM. **P<0.001, *P<0.05; n = 3. In A) AVP: arginine vasopressin (1 μ M), PTH: parathyroid hormone (1 μ M), DA: dopamine (10 μ M), CCP: cicaprost (1 μ M), ILP: iloprost (1 μ M). In B) ISO: isoproterenol (10 μ M and 1 μ M, respectively), DA: dopamine (10 μ M), PTH: parathyroid hormone (100 nM).



APPENDIX C: α -SMOOTH MUSCLE ACTIN IN rMG

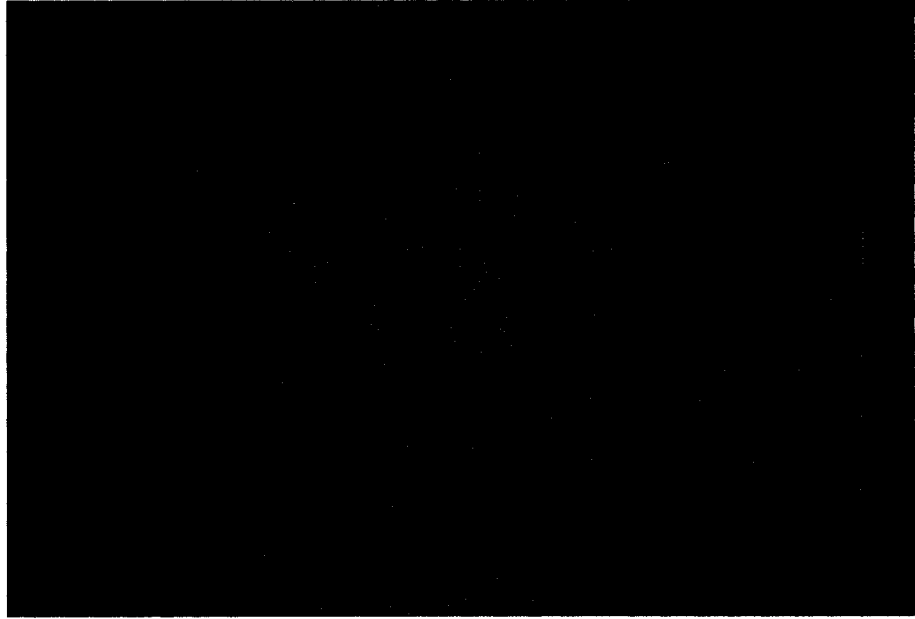
CELLS

This section contains a figure from work that is not part of the manuscripts, a characterization of the mesangial cells obtained from ATCC. α -smooth muscle actin is a marker of activated/proliferating mesangial cells.

Figure C.1. α -smooth muscle actin is detectable in cultured rMG cells. A) control and B) α -smooth muscle actin.

Figure C.1. α -smooth muscle actin is detectable in rMG cells. Immunohistochemical analysis of α -smooth muscle in immortalized rMG cells. A) control (no primary antibody) and B) α -smooth muscle actin. Cells were grown on coverslips and fixed prior to confluence with 10 % buffered formalin. A mouse monoclonal anti- α -smooth muscle actin antibody was used for detection with CY3-labelled secondary antibody. Primary antibody from Sigma (A2547) was diluted 1: 100. Magnification 40X.

A



B



APPENDIX: BIBLIOGRAPHY

Awards and Scholarships

External Awards:

<i>01/2004-08/2004</i>	OGSST scholarship
<i>01/2003-12/2003</i>	OGSST scholarship
<i>01/2002-12/2002</i>	OGS scholarship
<i>01/2001-12/2001</i>	OGS scholarship
<i>05/1998-04/2000</i>	NSERC PGS-A scholarship
<i>1998</i>	OGS scholarship (declined)
<i>1996-1997</i>	Dewaan Foundation scholarship for academic excellence
<i>1991-1992</i>	Dewaan Foundation scholarship for academic excellence

University Awards:

<i>01/2002-12/2002</i>	University of Ottawa Excellence scholarship
<i>01/2001-12/2001</i>	University of Ottawa Excellence scholarship
<i>09/2000-12/2000</i>	University of Ottawa Entrance scholarship
<i>05/1998-04/2000</i>	University of Ottawa Excellence scholarship
<i>1998</i>	Award for outstanding student seminar (Physiology program)
<i>1998</i>	University of Ottawa Excellence scholarship
<i>1997-1998</i>	University of Ottawa admission scholarship
<i>1991-1992</i>	University of Ottawa scholarship "Bourses pour études en français"
<i>1991-1992</i>	University of Ottawa Excellence scholarship

Publications

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2. **NASRALLAH, Rania**, and Richard L. Hebert. Functional role of the PGI₂/IP system in rabbit cortical collecting duct. *Recent Res. Devel. Physiol.* 2: 35 – 50, 2004.
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3. **NASRALLAH, RANIA**, Anne Landry, Monika Sklepowicz, and Richard L. Hébert. Glucose-mediated increase in cyclooxygenase-1 and -2, and EP4 receptors in rat inner medullary collecting ducts. Proceedings of the Canadian Society of Nephrology meeting, p.38A, 2003.
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