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Prostaglandins and Kidney Function: A Comparison Between the Cortical and Inner Medullary Collecting Duct on Prostaglandin Production

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PROSTAGLANDINS AND KIDNEY FUNCTION: A COMPARISON BETWEEN THE CORTICAL AND INNER MEDULLARY COLLECTING DUCT ON PROSTAGLANDIN PRODUCTION

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PROSTAGLANDINS AND KIDNEY FUNCTION: A COMPARISON BETWEEN THE CORTICAL AND INNER MEDULLARY COLLECTING DUCT ON PROSTAGLANDIN PRODUCTION

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Prostaglandins are paracrine and autocrine signaling molecules that play important roles through various physiological and pathophysiological functions in the human body including the inflammatory response, control of blood pressure, and water and salt homeostasis in the kidney. Prostaglandins have been shown to have great influence on kidney function. In particular, prostaglandin E_2 influences the function of the collecting duct, primarily decreasing vasopressin-stimulated collecting duct water permeability. The aim of this study was to see how various treatments to cells, as well as different growth times, impacted prostaglandin production between two different collecting duct cell lines, one arising from the cortical collecting duct and the other arising from the inner medullary collecting duct. This was accomplished by growing and culturing the cells on 6-well plates, collecting the cell media and measuring prostaglandin accumulation via Enzyme-linked immunosorbent assay (ELISA) kits. Results showed that longer growth times decreased prostaglandin E_2 and $F_2\alpha$ accumulation in both cell lines. The effect of dDAVP, a vasopressin analog, increased prostaglandin accumulation in the inner medullary collecting duct cells, and increased $PGF_2\alpha$ while having virtually no effect on PGE_2 in the cortical collecting duct cells. The addition of a cyclooxygenase-1 and cyclooxygenase-2 inhibitor significantly decreased prostaglandin accumulation in both cell lines. Finally, changing the osmolarity of the media by adding sodium chloride (NaCl) increased prostaglandin accumulation

in both cell lines. Prostaglandin synthesis has relevant clinical implications, as non-steroidal anti-inflammatory drugs, a common over-the-counter group of pain medications, inhibit cyclooxygenase, an enzyme involved in the biosynthesis of prostaglandins.

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LIST OF ABBREVIATIONS

AVP	Arginine vasopressin
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
dDAVP	1-deamino-8-D-arginine vasopressin
EP1	Prostaglandin E_2 receptor 1
EP2	Prostaglandin E_2 receptor 2
EP3	Prostaglandin E_2 receptor 3
EP4	Prostaglandin E_2 receptor 4
FP	Prostaglandin $F_2\alpha$ receptor
IP	Prostaglandin I_2 receptor
mIMCD3	mouse Inner Medullary Collecting Duct 3 cell
mpkCCD	murine immortalized cortical collecting duct cell
PG	Prostaglandin
PGE_2	Prostaglandin E_2
$PGF_2\alpha$	Prostaglandin $F_2\alpha$
PGI_2	Prostaglandin I_2
PLA	Phospholipase A

CHAPTER 1: INTRODUCTION

Overview of prostaglandins

Biosynthesis

Prostaglandins are lipid structures derived from arachidonic acid that are formed when arachidonic acid is released from the plasma membrane by phospholipases with subsequent peroxidase activity by a class of cyclooxygenase (COX-1 and COX-2) enzymes forming prostaglandin H_2 (Ricciotti, 2011). Tissue specific synthase enzymes subsequently form the more stable and biologically active prostaglandins such as prostaglandin E_2 (Li, 2018). There are four biologically active prostaglandins generated in vivo: prostaglandins: D_2 (PGD_2), E_2 (PGE_2), I_2 (PGI_2), and $F_2\alpha$ ($PGF_2\alpha$). Thromboxane A_2 (TxA_2), a prostanoid, is also produced via a similar mechanism, as shown below in Figure 1 (Ricciotti, 2011). Upon being synthesized, prostaglandins then bind to a specific G-protein coupled receptor to exert its particular effect. As seen below in Figure 1, prostaglandins have effects on several tissues throughout the body.

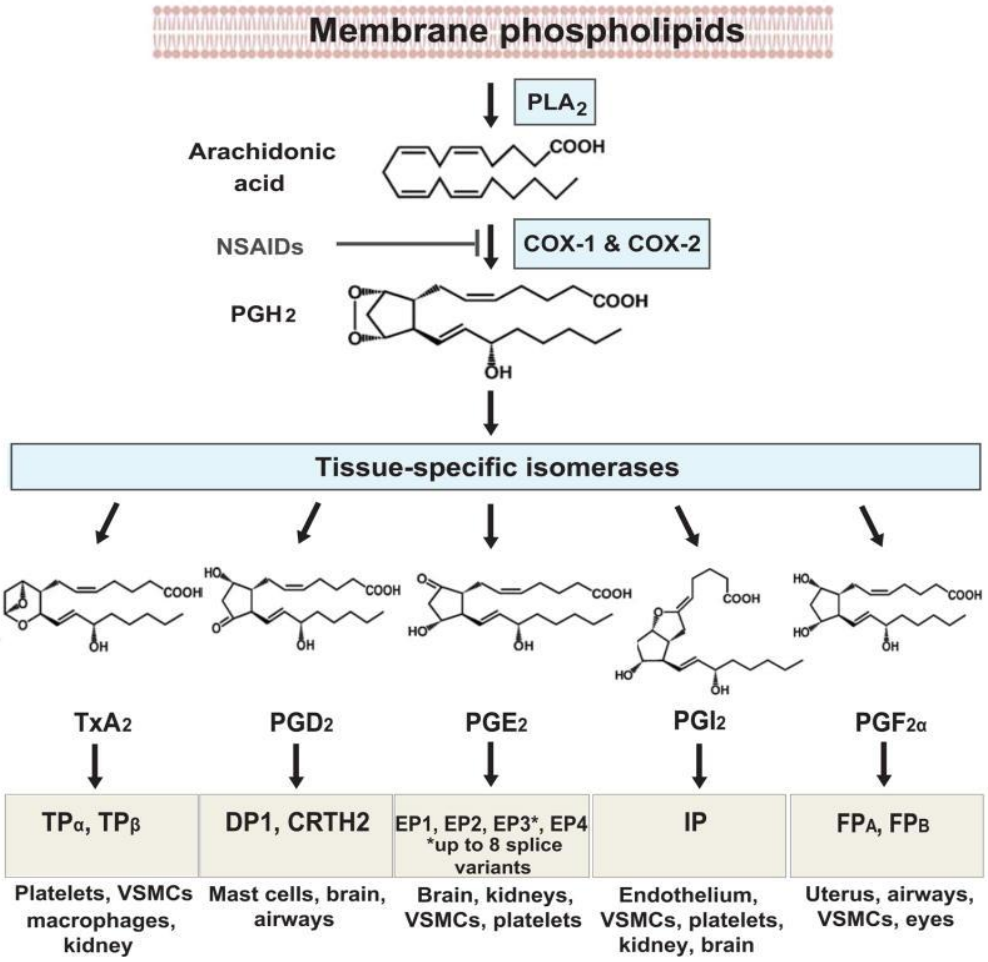

Figure 1: Biosynthetic pathway of prostanoids (Ricciotti, 2011)

The role of Phospholipase A in the biosynthesis of prostaglandins

Phospholipase A (PLA) comprises a family of enzymes which play a key role in the production of prostaglandins. One of the isoforms of PLA is cytosolic PLA (cPLA). The primary function of cPLA is the liberation of arachidonic acid from membrane phospholipids. cPLA activity depends on an increase in intracellular calcium as calcium binds to the enzyme at its C2 domain, facilitating the translocation of the enzyme to intracellular phospholipid membranes. Once bound to the phospholipid membrane, many other enzymes can help to regulate the activity of cPLA through phosphorylation (Casale, 2018).

Enzymatic Regulation

The biological effects of prostaglandins can be regulated at the enzymatic reaction of cPLA. One isoform, $cPLA_2$, is regulated by intracellular calcium levels. Calcium binding to the C2 domain activates the enzyme, creating a conformational shift in the C2 domain, which is critical for proper orientation to bind to the phospholipid membrane. Once localized to the membrane, a catalytic domain catalyzes the hydrolysis, freeing arachidonic acid. The enzyme can be further activated by lipid second messengers such as phosphatidylinositol 4,5-bisphosphate (PIP2) or by enzymes such as mitogen-activated protein kinase (MAPK) through phosphorylation (Dennis, 2011).

The role of cyclooxygenase in the biosynthesis of prostaglandins

Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to prostaglandin H_2, a precursor to the biologically active prostaglandins. This is the rate-limiting step in the formation of prostaglandins. The conversion from arachidonic acid to PGH_2 occurs in two steps; the cyclooxygenase reaction forms the unstable PGG_2; PGG_2 is immediately converted into PGH_2 in a peroxidase reaction (Zarghi, 2011).

There are two main isoforms of COX: COX-1 and COX-2. COX-1 is expressed constitutively in many tissues (Zarghi, 2011). In the kidney, it is expressed in the afferent and efferent arterioles, medullary interstitial cells, and the cortical and medullary collecting ducts (Komhoff, 1997). Prostaglandins produced by COX-1 mediate more regulatory functions, such as regulation of renal blood flow (Zarghi, 2011). In contrast, COX-2 is not detected in most tissues, but has been detected predominantly in medullary interstitial cells and cells associated with the macula densa, with less distribution in the collecting duct (Hao, 2008). Its expression is rapidly induced by various stimuli such as proinflammatory cytokines and disorders of water-electrolyte homeostasis (Zarghi, 2011).

Various studies have been conducted to elucidate similarities and differences in function between the two isoforms. Contributing to these differences is that COX-1 and COX-2 have been demonstrated to utilize arachidonic acid generated by different phospholipases (Reddy, 1997; Langenbach, 1999). In addition, studies have indicated that the two isoforms may have different cellular localizations (Morita, 1995). COX-1 is commonly referred to as the "housekeeping" isoform, where COX-2 is commonly referred to as the "inducible" isoform, however COX-1 has been shown to be inducible under certain conditions, and COX-2 is constitutively expressed in certain tissues within the brain and kidney (Langenbach, 1999). Harris, et al. (1994) utilized various techniques to determine that COX-2 expression was localized to the macula densa, and that the level of COX-2 expression increased threefold when the rats were chronically sodium restricted. In addition, intrarenal COX-2 was shown to be upregulated during chronic angiotensin II infusion in cultured inner medullary collecting duct cells (Gonzalez, 2014). The authors also observed that COX-2 inhibition additionally decreased glomerular filtration and renal blood flow with angiotensin II infusion in anesthetized rats (Gonzalez, 2014). Within the renal medulla, COX-2 expression is regulated by dietary salt intake, and inhibition of COX-2 within the medulla has been shown to lead to sodium-sensitive hypertension in rats (Zewde, 2004). These findings note its importance in the regulation of blood volume and pressure.

Langenbach et al. (1999) found COX-1 deficient mice to be surprisingly healthy, with no gastric or kidney pathologies, however these mice did show impairment in platelet aggregation. COX-2 deficient mice had several noted pathologies; with regard to the kidney, COX-2 null mice had smaller kidneys, reduced glomeruli which were also poorly developed, and atrophied renal tubules.

Prostaglandin production and receptor distribution

As previously mentioned, prostaglandins primarily work in an autocrine or paracrine manner. In vivo, prostaglandins are rapidly degraded, limiting their effect to the immediate

vicinity of their site of synthesis (Hao, 2008). Various kidney cells produce prostaglandins: tubular epithelial cells, renal medullary interstitial cells, and cells of the glomeruli (Dunn, 1977). The main sites of prostaglandin synthesis occurs in the medullary tissue of the kidney, established by medullary slices or isolated cell preps (Bonvalet, 1987). In order for prostaglandins to exert local effects, they must bind to their receptor locally. Prostaglandin receptors are expressed throughout various parts of the kidney. PGE_2 and $PGF_2\alpha$ have long been recognized as major renal prostaglandins (Dunn, 1977). More so recently, the emergence of the importance of prostacyclin, PGI_2 has been noted (Kim, 2008). All renal cell types can synthesize PGE_2, but the highest production is seen in the glomeruli and collecting ducts (Nasrallah, 2014); it is also the major prostaglandin synthesized in the renal medulla (Kim, 2008). PGI_2 is predominantly synthesized in the endothelial and epithelial cells of the glomerulus. Although $PGF_2\alpha$ can be detected in the kidney, the enzymes catalyzing the biosynthesis of $PGF_2\alpha$ remain poorly defined (Hao, 2008).

The most abundant prostaglandin receptors in the kidney are those for PGE_2 (Kim, 2008). Four prostaglandin E (EP) receptor subtypes have been identified, and are highly expressed throughout the kidney (Breyer, 1996). Collecting ducts express all EP receptors, however, EP2 is only expressed in the cortical segment of the collecting duct (Nasrallah, 2014; Katsuyama, 1995). The medullary thick ascending limb (mTAL) expresses high levels of EP3 receptor mRNA. In addition, the glomeruli express the EP2 receptor and high levels of EP4 receptor mRNA (Kim, 2008; Nasrallah, 2014). Other expression sites of the EP receptors as well as COX-1 and COX-2 are shown in Figure 2 below.

Nephron Segment/Region	Prostaglandin E_2 receptor or COX localization
Afferent Arteriole	EP2/EP4 – vasodilators EP1/EP3 - vasoconstrictors

Glomerulus	COX-1, COX-2, EP1, EP3, EP4
Proximal Tubule	COX-1, COX-2, EP1, EP4 - transport
Thick Ascending Limb	COX-2, EP3, EP4 - transport
Macula Densa	COX-2, EP3, EP4 – renin, transport
Distal Tubule	EP3, EP4 - transport
Collecting Duct	COX-1, COX-2, EP1, EP3, EP4 - transport
Medullary Interstitial Cells	COX-1, COX-2
Vasa Recta	EP2, EP4 - vasodilators

Figure 2: Localization and physiological function of EP receptors and cyclooxygenases (COX-1/COX-2) throughout the nephron (Modified from Nasrallah, 2014 as source material)

The receptors are cell surface G-protein coupled receptors, thereby primarily influencing the action of adenylate cyclase which subsequently affects intracellular cyclic adenosine 3'5'-monophosphate (cAMP) levels. Each EP receptor not only selectively binds PGE_2, but also preferentially couples to different signal transduction pathways as shown in Figure 3 below (Modified from Li, 2018).

EP2 and EP4 activate G_s, which stimulates intracellular cAMP levels (Nasrallah, 2014). EP1 is coupled to G_q, which mobilizes intracellular calcium via phospholipase C and activates Protein Kinase C. EP3 activates G_i, which inhibits cAMP formation but increases intracellular calcium levels via phospholipase C (Breyer, 1996; Nasrallah, 2014).

As evident, PGE_2 exerts its effects through various signaling pathways, and the effect of PGE_2 can have opposite effects, depending on which receptor it binds to. For example, PGE_2 is a vasodilator in some vascular beds, however, it can also result in vasoconstriction (Breyer, 1996). This is also evident as seen from Figure 2 above: PGE_2 binding to its EP2 or EP4 receptor vasodilates the afferent arteriole whereas binding to EP1 or EP3 receptor results in vasoconstriction of the afferent arteriole (Nasrallah, 2014). The IP receptor, distributed throughout the renal cortex and medulla, binds PGI_2, and increases intracellular cAMP levels

(Kim, 2008). $PGF_2\alpha$ binds to its FP receptor which is coupled to G_q, mobilizing intracellular calcium and activating Protein Kinase C. At present, there is little evidence for DP receptor (PGD_2) expression in the kidney (Hao, 2008). Below is a summary of renal functions, possible signaling pathways, and renal distributions of prostaglandin receptors (Modified from Li, 2018). Because multiple prostaglandins can be synthesized from COX, and because these prostaglandins can interact with different receptors that can vary in their signaling pathways, the biological effects of COX-derived prostaglandins are diverse and complex (Hao, 2008).

Prostaglandin receptors	Renal function	Signaling pathways	Expression sites
EP1	Hemodynamics, transport, renin release	G_q-Ca^{2+}	CD, MG, P, PT, V
EP2	Hemodynamics, transport, renin release	G_s-cAMP	IC, MD, P, V, CD
EP3	Transport, vasoconstriction	G_i-cAMP	DT, CD, MD, V
EP4	Hemodynamics, transport, renin release, vasodilation	G_s-cAMP	P, CD, MG, MD, DT, PT, V
IP	Hemodynamics, vasodilation, transport, renin release	G_s-cAMP	MG, MD, DT, CD, PT, P, V
FP	Transport, hemodynamics	G_q-Ca^{2+}	CD, DT, P

Abbreviations: CD: Collecting duct, MG: Mesangial Cells, P: Podocytes, PT: Proximal Tubule, DT: Distal Tubule, V: Vasculature, IC: Interstitial Cells, MD: Macula Densa

Figure 3: Renal functions, possible signaling pathways, and renal distributions of prostaglandin receptors (Modified from Li, 2018)

Kidney basics - the anatomy and physiology of the kidneys

The kidneys, located on either side of the lower vertebral column near the posterior abdominal wall, are traditionally known for their role in excreting wastes from the body. While this is a vital function that they carry out, the kidneys also perform a wide array of other functions that are essential to our overall health. Some of those functions are, but not limited to, hormone secretion, blood pressure maintenance, and regulation of: water and electrolyte balance, plasma osmolality, acid-base balance, and red blood cell production (Eaton, 2013). These functions allow the kidney to produce diluted or concentrated urine, depending on the body's water needs. The functional units of the kidney, the nephron, consists of two parts: the renal corpuscle, which is involved in the ultrafiltration of the blood across the walls of the glomerular capillaries within, and the tubular system, which is lined by epithelial cells involved in the reabsorption and secretion process (Eaton, 2013).

Prostaglandins have been shown to impact several of these functions and are of high relevance to overall renal function. Prostaglandins have important physiological and pathophysiological effects on salt and water handling, intrarenal hemodynamics, renin release, inflammation and injury (Hao, 2008; Imig, 2015; Nasrallah, 2014).

Renal blood flow

Renal blood flow is about 1L/min, constituting roughly 20% of the resting cardiac output (Eaton, 2013). Blood enters the kidney through the renal artery. After several divisions into smaller arteries, a series of afferent arterioles are formed, leading to the glomerulus. Leaving the glomerulus is the efferent arteriole. The efferent arterioles branch several times, forming the peritubular capillaries. These capillaries intermingle with the tubular segments of the nephron, where reabsorption and secretion occurs. These capillaries feed into venules, which ultimately lead to the formation of the renal vein, which leaves the kidney. Not all efferent arterioles merge

to form peritubular capillaries; some form parallel arrangement vessels, called vasa recta, which carry out similar functions to peritubular capillaries. Various substances can leave the capillaries to be secreted into the tubular system or be reabsorbed from the tubular system and enter the capillaries. Renal blood flow follows the same hemodynamic principles as blood flow throughout the body. Renal blood flow is a ratio of the change in pressure between the renal artery and renal vein over the total vascular resistance (Eaton, 2013).

Renal corpuscle anatomy and function

The ultrafiltration of blood occurs across the walls of the glomerulus, a tuft of interconnected capillaries. The glomerulus is surrounded by a hollow sphere, the Bowman's capsule. These two structures collectively comprise the renal corpuscle. Two arterioles penetrate the Bowman's capsule at the vascular pole. The afferent arteriole brings blood to the glomerular capillaries. The efferent arteriole drains blood away from the glomerulus. As the blood passes through the glomerular capillaries, it undergoes filtration, wherein large proteins and cells are retained in the blood, whereas water and solutes freely pass into the Bowman's space, the space within the Bowman's capsule. Whatever contents enter the Bowman's space will eventually pass into the tubular system of the nephron (Eaton, 2013). The kidneys filter roughly 180 liters of blood each day in an average 70 kg human and the rate at which the kidney filters the blood is known as the glomerular filtration rate (Pitts, 1968).

Prostaglandins and their effects on renal blood flow and the ultrafiltration of blood

COX-derived prostaglandins play a critical role in modulating renal blood flow and glomerular filtration (Hao, 2008). PGI_2 and PGE_2 are primarily vasodilatory, preferentially vasodilating the afferent arteriole (Kim, 2008; Edwards, 1985). Vasodilation relaxes vascular smooth muscle cells, decreasing resistance to blood flow in the afferent arteriole, thus increasing renal blood flow and glomerular filtration rate under conditions associated with decreased

effective circulating volume (Kim, 2008). Examples of states associated with decreased circulating blood volume include congestive heart failure and cirrhosis (Hao, 2008). Afferent arteriole vasodilation is cAMP-dependent: both PGI_2 and PGE_2 bind to their receptors, IP2 and EP2/EP4 respectively, which stimulates intracellular cAMP accumulation (Kim, 2008; Nasrallah, 2014). cAMP inhibits myosin light chain kinase, resulting in vasodilation (Klabunde, 2012).

The vasodilatory role of these prostaglandins in healthy individuals has little importance in renal hemodynamics (Dibona, 1986). Thus, under conditions of decreased renal perfusion, the production of renal prostaglandins serves as an important compensatory mechanism (Kim, 2008).

Prostaglandins and renin release

Renal prostaglandins can stimulate or inhibit the release of renin. PGI_2 and PGE_2 stimulate the secretion of renin, while $PGF_2\alpha$ inhibits its release (Kim, 2008; Dunn, 1977). PGE_2 and PGI_2 increases in renin secretion is cAMP-mediated; cAMP activates Protein Kinase A, which phosphorylates renin vesicles, leading to the release of renin into the circulation. Renin release activates the renin-angiotensin-aldosterone system (RAAS). Through RAAS signaling, a potent systemic vasoconstrictor, angiotensin II, is formed. Angiotensin II constricts the glomerular efferent arteriole, increasing intraglomerular pressure, impacting renal blood flow and maintaining the glomerular filtration rate (Hao, 2008). In addition, through renin regulation, prostaglandins influence sodium and water reabsorption primarily in the distal segments of the nephron. Angiotensin II stimulates the release of aldosterone from the adrenal cortex which acts to increase sodium and water reabsorption and potassium secretion in the distal tubule.

Nephron tubule anatomy and function

As the blood is filtered at the glomerulus, water, solutes and other various substances pass the glomerular filtration barrier, and enter into Bowman's space. The now called filtrate then passes into the nephron tubule. Throughout different parts of the tubule, the filtrate will be

modified via two primary processes: reabsorption and secretion. Reabsorption is the process where water and various solutes are removed from the tubular fluid and are reabsorbed back into the bloodstream. In contrast, secretion is the movement of substances back into the tubular fluid from the epithelial cells of the tubule or from the blood.

The first part of the tubule is the proximal convoluted tubule. This is the site of the majority of water and solute reabsorption. As shown in Figure 4 below, aquaporin 1 is constitutively expressed in the epithelium of the proximal tubule as well as the thin descending limb of the loop of Henle which allows for water reabsorption in these segments (Tamma, 2011; Kwon, 2001).

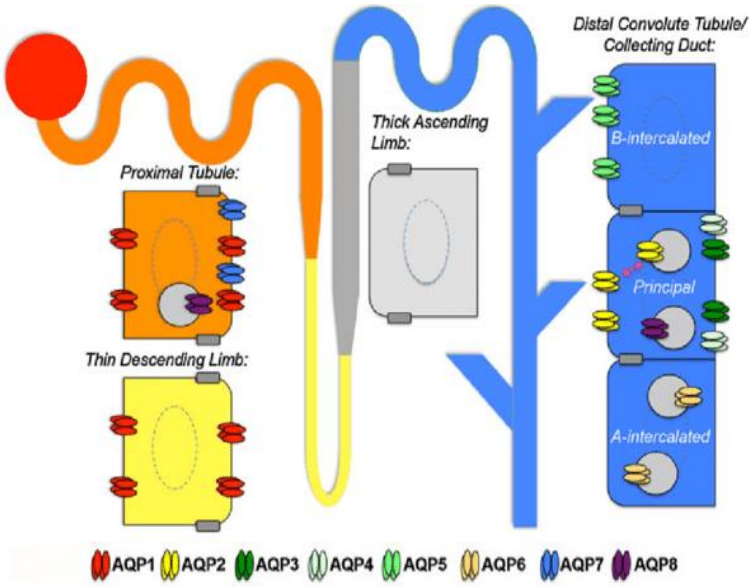

Figure 4: Aquaporin distribution within the nephron (Tamma, 2011)

After entering the proximal tubule, the filtrate enters the loop of Henle. The loop of Henle begins as a descending component, diving down into the medullary tissue of the kidney; it then makes a hairpin turn as the ascending limb and ascends back towards the cortex. The ascending limb typically has a thin and thick component. The descending limb is highly permeable to water due to the abundance of aquaporin 1 water channels (Kwon, 2001; Tamma,

2011), but impermeable to sodium due to a lack of sodium channels (Kokko, 1970). In contrast, the ascending limb is permeable to sodium, due to the presence of various sodium transporters such as the sodium-potassium-2 chloride cotransporter 2 (NKCC2) and the sodium-hydrogen exchanger (Greger, 1985). The primary function of the thick ascending limb is to reabsorb large amounts of remaining sodium chloride therefore diluting the tubular fluid just prior to entering the next segment, the distal convoluted tubule (Ares, 2011). The ascending limb is impermeable to water due to the absence of aquaporin water channels. The NKCC2 in the medullary thick ascending limb of the loop of Henle plays a role in countercurrent multiplication in the outer medulla, which is one of the two important factors in establishing the medullary osmotic gradient. The thick ascending limb reabsorbs 25-30% of the sodium chloride filtered by the glomeruli in a process mediated by NKCC2 (Ares, 2011). As a result, the medullary interstitium becomes hypertonic, whereas the tubular fluid becomes hypotonic. The hypertonic interstitium will then attract water, which the descending limb of the loop of Henle is permeable to (Chou, 1999). This is the basis of countercurrent multiplication within the loop of Henle.

Following the loop of Henle is the distal convoluted tubule, which is involved in sodium and chloride reabsorption via sodium-chloride cotransporters (McCormick, 2015). The final segment of the nephron tubule is the collecting duct. The collecting duct collects filtrate from multiple nearby nephron tubules and is the final site of regulation of the volume and composition of urine. The collecting duct will be further discussed in more detail later.

As indicated, substances such as ions, glucose, amino acids, water and urea can be reabsorbed or secreted along the various components of the nephron depending on the body's needs. The substances that are filtered and not reabsorbed through any segment of the nephron tubule, or that which is secreted and not later reabsorbed, will become a part of the final composition of urine which will be excreted from the body. Various hormones also play a role in the urinary concentrating mechanism and ultimately are critical to body fluid and electrolyte

homeostasis. The kidney's ability to reabsorb or excrete water to maintain body fluid osmolality is critical to the health of every cell in the body.

Prostaglandins and their effects on the nephron tubule

PGE_2 inhibits salt and water reabsorption through a variety of methods. One of those occurs at the thick ascending limb of the loop of Henle; PGE_2 decreases sodium reabsorption, thereby promoting salt excretion, via inhibition of the NKCC2 (Ares, 2011). EP3 receptor mRNA is expressed highly in the medullary thick ascending limb (Breyer, 1998). PGE_2 binds to the EP3 receptor, causing decreases in intracellular cAMP via G_i; through the cAMP regulatory element, expression of the transporter decreases (Shankar, 2003). 20-Hydroxyeicosatetraenoic acid (20-HETE), a cytochrome P450 derived metabolite of arachidonic acid, inhibits sodium reabsorption by the thick ascending limb. However, it is not known whether NKCC2 is the primary target since these lipid mediators may also inhibit Na^+-K^+-ATPase activity (Ares, 2011).

Collecting duct anatomy and function

The final segment of the nephron tubule is the collecting duct. The collecting duct receives filtrate from many surrounding nephrons. Any substance within the tubular fluid not reabsorbed by the collecting duct will be a part of the final urine composition. There are two main cell types present in the collecting duct: intercalated and principal cells. Intercalated cells help with acid-base regulation whereas the principal cells are involved in the final makeup of the urine volume and composition, and thus are important in water and salt regulation within the collecting duct (Eaton, 2013). The collecting duct mediates the final stages of regulation of urine volume and composition, and carries out the important role of maintaining a constant water balance for the body. Around 5% of the sodium filtered load is reabsorbed in the collecting duct (Garty, 1997). Water imbalances or urinary concentrating defects can have adverse effects on the body. Diabetes insipidus is a disorder that arises from a failure to maintain water balance, and the

patient produces large volumes of urine. These increases in dilute urine production can increase plasma osmolality, leading to a potentially fatal condition, hypernatremia (Moeller, 2012).

The renal cortical-medullary interstitial osmotic gradient and the regulation of water permeability of the collecting duct are key to forming a more concentrated or dilute urine as needed to control water balance. Water permeability of the collecting duct is regulated by vasopressin, secreted by the posterior pituitary gland, leading to increased water reabsorption. Principal cells contain multiple different isoforms of aquaporin water channels. Aquaporin 2 is the only aquaporin sensitive to vasopressin (Verkman, 2008). When released, vasopressin binds to its vasopressin-2 receptor within the collecting duct, promoting the shuttling of aquaporin 2 water channels from subapical vesicles into the apical membrane. The vasopressin 2 receptor is a G-protein coupled receptor and stimulates the increase of intracellular cAMP levels. Once phosphorylated by protein kinase A, the sub-apically aquaporin 2 vesicles are shuttled to the apical membrane, allowing for passive reabsorption of water. Water will pass transcellularly through the principal cell and exit the basolateral membrane via aquaporin 3 and 4 water channels. Water molecules are then able to move into the hyperosmotic interstitium, established by the medullary osmotic gradient, and enter into the bloodstream through nearby capillaries. Defective or dysregulated aquaporin 2 targeting and synthesis underlies a variety of clinical conditions such as nephrogenic diabetes insipidus, resulting in loss of body water, or the serious complication of water retention that can occur in heart disease (Moeller, 2012).

The formation of a concentrated urine requires the presence of the medullary interstitial osmotic gradient, and water reabsorption via aquaporin 2 in the collecting duct. Earlier, it was mentioned that there are two important factors in establishing the medullary osmotic gradient. Countercurrent multiplication in the outer medulla was previously discussed. The other factor is the accumulation of urea in the medullary interstitium by way of the collecting duct. Urea is passively reabsorbed via urea transporters within the inner medullary collecting duct and will

accumulate within the medullary interstitium. Urea reabsorption primarily occurs through the urea transporter A1 (UT-A1), which is expressed in inner medullary collecting duct epithelial cells (Chen, 2013).

Prostaglandins and their effects on the collecting duct

PGE_2 is known to influence the trafficking of aquaporin 2 in the principal cells of the collecting duct. It can do so by binding to one of its four receptors, EP 1-4, all of which are expressed in the collecting duct. EP2 is only expressed in the cortical segment of the collecting duct (Katsuyama, 1995), whereas EP1, 3, and 4 are expressed along the entire collecting duct (Nasrallah, 2014). As the EP receptors are coupled to different signal transduction pathways, they can evoke opposite effects on aquaporin 2 trafficking, depending on which receptor PGE_2 binds to.

The EP3 receptor is most recognized for its diuretic role opposing vasopressin (Nasrallah, 2014; Olesen, 2013); it mediates the inhibition of vasopressin-stimulated water permeability by PGE_2 in the collecting duct (Breyer, 1998). As shown below in Figure 7, within the cortical collecting duct, EP3 reduces intracellular cAMP levels, which reduces aquaporin 2 water channel vesicle transport to the apical membrane. EP1 binds G_q, and through the action of protein kinase C, increases the endocytosis of aquaporin 2. Both of these reduce water reabsorption, although the mechanisms are different. Within the inner medullary collecting duct, EP3 similarly reduces aquaporin 2 transport to the apical membrane (Olesen, 2013). PGE_2 may also exert a diuretic effect by binding to EP2 within the papillary interstitial cells. Binding to EP2 increases hyaluronan synthesis in the interstitial cells, which has a high capacity to bind water, thereby inhibiting water flow (Olesen, 2013; Rugheimer, 2009).

Nadler et al. (1992) also found that PGE_2 and agonists of EP1 and EP3 inhibit vasopressin-mediated water and urea permeability of the collecting duct in rat terminal inner

medullary collecting ducts. Rouch et al. (2000) discovered similar findings, in that PGE_2 inhibits water and urea permeability in the rat inner medullary collecting duct via post-cAMP dependent events.

Earlier it was discussed that PGE_2, through its EP3 receptor, inhibited NKCC2 in the medullary thick ascending limb of the loop of Henle. This is also shown in Figure 7 below. Sodium reabsorption in this part of the nephron is important in countercurrent multiplication, which helps establish the medullary interstitial osmotic gradient. With less sodium reabsorption, the medullary osmotic gradient is diminished, resulting in less drive for water reabsorption to occur in the collecting duct.

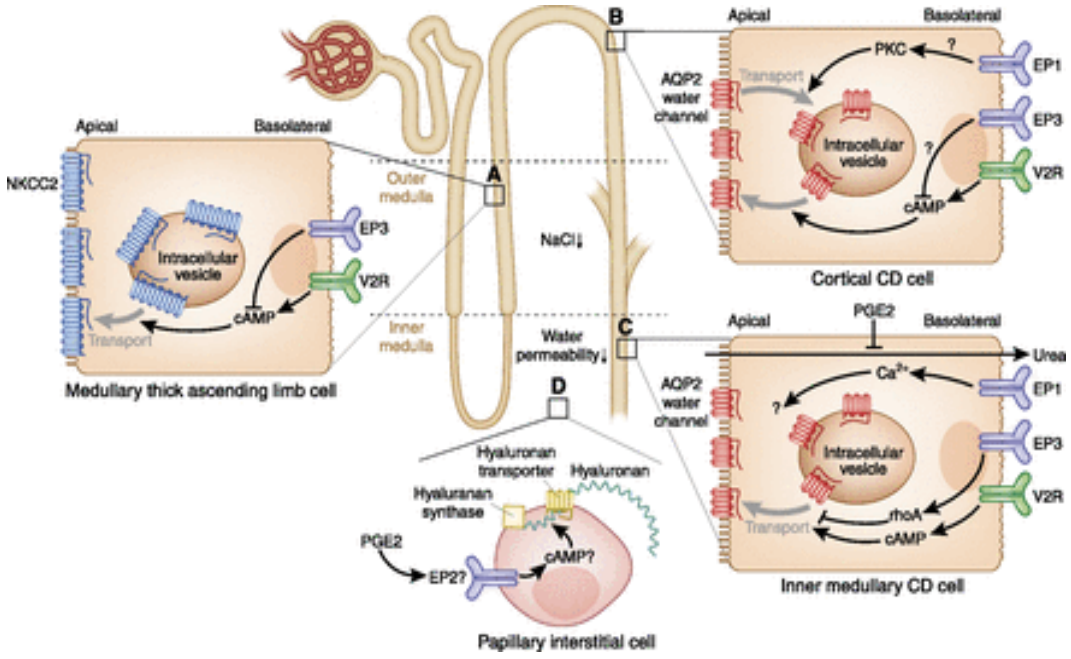

Figure 5: Proposed diuretic effects of PGE_2 along the kidney nephron and interstitium (Olesen, 2013)

In contrast, PGE_2 binding to EP2 or EP4 increases aquaporin 2 phosphorylation and transport to the apical membrane. The effects of EP2 are mediated through an increase in cAMP, however, the mechanism of aquaporin 2 transport to the apical membrane via the effects of EP4 are unknown (Olesen, 2013).

Differences between the cortical and inner medullary collecting duct

The collecting duct extends from the outermost parts of the renal cortex, down to the deepest parts of the renal medulla. Along the length of the collecting duct, there are differences in tubular fluid and surrounding interstitial tissue osmolarities. When forming a hypo-osmotic or more dilute urine, tubular fluid osmolarity can decrease to as low as 50 mOsm/L. Under the influence of vasopressin, in the formation of a hyper-osmotic or more concentrated urine, tubular fluid osmolarity can increase to as high as 1200 mOsm/L. Vasopressin increases the permeability of the collecting duct to water along the entire length of the collecting duct; it also increases the permeability to urea within the inner medullary collecting duct. Urea reabsorption contributes greatly to the high osmolarity within the inner medulla. As the collecting duct descends further down into the medulla, the magnitude of the medullary interstitial osmotic gradient increases, favoring even more water reabsorption. The medullary osmotic gradient is established by countercurrent multiplication in the loop of Henle and urea deposition within the medulla, and maintained by countercurrent exchange in the vasa recta. As evident, vasopressin regulation of water permeability in the collecting duct dictates the formation of a more dilute or heavily concentrated urine. Another important difference is vasopressin 2 receptor mRNA distribution; the distribution is of greater intensity in the renal medullary collecting duct compared to the cortical collecting duct (Mutig, 2007).

Cell lines

The cell lines, mpkCCD and mIMCD3 cells, utilized in this study arise from different parts of the collecting duct. The mpkCCD cells are mouse cortical collecting duct cells whereas the mIMCD3 cells are mouse inner medullary collecting duct cells, specifically being derived from the terminal one third of the IMCD (Valkova, 2006). Therefore, they experience different environments of tubular fluid and surrounding interstitial osmolarity. mpkCCD cells endogenously express aquaporin 2 water channels, and expression is upregulated in the presence

of vasopressin. mIMCD3 cells do not express aquaporin 2. The endogenous aquaporin 2 expression in mpkCCD cells shows vasopressin-regulated phosphorylation of aquaporin 2 vesicles, increasing intracellular trafficking to the apical membrane, hence, a widely used collecting duct cell model. mIMCD3 cells have been shown to be an effective cell line for studying cellular adaptation to osmotic stress. They are readily able to adapt to grow in hypertonic mediums up to 910 mOsmol/kg H_20, an environment that is lethal to most other cells (Rauchman, 1993). Hypertonic stress results in water escaping from the cell, and to compensate for the volume loss, they take up inorganic ions. In addition, they adapt to increases in osmotic pressure by accumulating organic osmolytes such as sorbitol and betaine (Valkova, 2006).

mpkCCD cells have been shown to produce prostaglandins with PGE_2 and $PGF_2\alpha$ being the major prostaglandins produced (Kortenoeven, 2011). EP1, EP4 and FP receptors could be detected by RT-PCR in the same study. In mIMCD3 cells, mRNA expression of each of the 4 EP receptors was determined using RT-PCR. EP1 was detected at relatively high levels, with lower levels of EP4 and EP3, and no detection of EP2 (Elberg, 2012). This is consistent with reports of EP2 only being present in the cortical collecting duct (Katsuyama, 1995). There are currently no reported studies comparing prostaglandin production in mIMCD3 cells.

Aim of the study and hypothesis

A comparison of PGE_2 and $PGF_2\alpha$ production between the two cell lines will be carried out, while studying different variables, such as cell growth and treatments with various substances, such as COX inhibitors or changes in osmolarity. Due to the differences in the location of these cells within the collecting duct, expression profile differences of vasopressin 2 receptor mRNA along the collecting duct, varying degrees of prostaglandin production and receptor expression, as well as extracellular and tubular fluid osmolarity differences that these cells are exposed to, I hypothesize that these two cell lines will respond differently in terms of

prostaglandin production in the presence of the different, and that cell growth time will be directly related to prostaglandin production.

CHAPTER 2: MATERIALS AND METHODS

Cell Culture

The mpkCCD (murine immortalized cortical collecting duct) cells, kindly provided by Dr. Knepper, NIH, and the mIMCD3 (mouse inner medullary collecting duct 3) cells, purchased from ATCC, were utilized in this study. The mpkCCD cells were cultured as previously described (Hasler, 2002) in modified DMEM/F-12 medium with the addition of the following supplements: 2 mM L-glutamine, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 50 nM dexamethasone, 10 ng/mL epidermal growth factor, 1 nM tri-iodothyronine, and 2% fetal bovine serum (FBS). mIMCD3 cells were cultured in DMEM/F-12 with 10% FBS. T-75 flasks and 6-well plates were seeded to grow the cells with each subsequent passage of cell collection. Cells were cultured at 37 °C, with 5% CO_2.

Time Course Experiments

A time course experiment was carried out to see how different growth times affected prostaglandin production. mIMCD3 and mpkCCD cells were seeded at 50,000 cells/well in 6-well plates and grew in their respective complete media. One plate from each cell line grew in complete medium for 24 hours that, at this point, was switched over to serum and hormone (SH) - free media (complete media minus dexamethasone, epidermal growth factor, tri-iodothyronine, and FBS for mpkCCD cells and DMEM/F12 without FBS for mIMCD3 cells). Another plate of cells grew in the medium for 72 hours, prior to switching to SH-free media. Following the time interval, the cells were washed with 2 mL DPBS, and then exactly 2 mL SH-free medium was added to each well. Cells were then incubated for a further 48 hours at 37 °C and then the media and cell lysates were collected, as described below.

Treatment Experiments

Various treatments to the cells were carried out to compare their effects on prostaglandin production by the two cell lines. Five 6-well plates of each cell line were seeded at 50,000 cells/well. Cells crew in cultured complete medium for 72 hours, at which point, the medium was exchanged for 2 mL SH-free medium, and the treatment was added. Cells remained in SH-free medium plus treatment or vehicle for 48 hours (except where indicated) and then media and cell lysates collected as described below. Treatments included: 1-deamino-8-D-arginine vasopressin (dDAVP, a vasopressin analog), SC-560 (a cell-permeable selective inhibitor of COX-1), NS-398 (a cell-permeable selective inhibitor of COX-2), and sodium chloride (NaCl). In each case, three wells received the experimental treatment while the other three wells received the corresponding vehicle. The final concentrations of treatments were determined through prior literature utilizing these treatments.

- dDAVP: 2 µL of stock solution, dissolved in 0.9% sterile saline (Sigma-Aldrich, St. Louis, MO) was added to each 2 mL of treated cell medium for a final concentration of 1 nM. 2 µL was re-added at 24 hours to replenish dDAVP (the media was not removed)
- SC-560 (a selective COX-1 inhibitor): SC-560 (Sigma-Aldrich, St. Louis, MO) was added to treated cells to give a final concentration of 100 nM (Flores, 2012). For the control wells, an equivalent volume of vehicle (5 µL DMSO) was added. SC-560 displays 700-fold selectivity for COX-1 over COX-2, and the IC_{50} for COX-1 is 9 nM, and 6.3 µM for COX-2.
- NS-398 (a selective COX-2 inhibitor): NS-398 (Sigma-Aldrich) was added to treated cells for a final concentration of 5 µM. An equivalent volume of vehicle was added (5 µL DMSO). The dose chosen was based on a study of mIMCD3 cells by Rocha, et al. (2001). The IC_{50} for COX-2 is 3.8 µM and COX-1 activity is unaffected up to concentrations of 100 µM.

- NaCl/Osmolarity: One plate of each cell line was seeded and collected at 48 hours of treatment. 4.5 mg of NaCl was added per 1 mL of SH-free media to increase media osmolality. A 0.22 μM syringe filter was used to sterilize the media. Exactly 2 mL of the sterilized SH-free media with NaCl was added to the treated wells. Exactly 2 mL of SH-free media was added to the control wells. The remainder of the sterilized media with NaCl and a 1 mL aliquot of SH free media was saved and the osmolality was determined using a vapor pressure osmometer (Wescor, Logan, UT).

Sample Collection

The media in each well of the 6-well plates was collected, centrifuged at 10,000 g for 5 minutes at 4 °C to remove debris and the supernatant was stored at -80 °C for later analysis. Cells were then washed briefly in chilled DPBS, then with protease and phosphatase inhibitors added (1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 2 μM pepstatin, 0.1% aprotinin, 5 μL 2-mercaptoethanol (BME), and 50 μL phosphatase inhibitors) were subjected to lysis in 200 μL M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL). The cells were detached with a cell scraper and placed into Eppendorf tubes. The lysates were centrifuged at 10,000 g for 5 minutes at 4 °C and the supernatant was collected. A protein assay to measure protein concentration of the lysate was then done (Bradford method, Bio-Rad protein assay kit, Bio-Rad Laboratories, Hercules, CA). Lysates were stored at -80 °C for later analysis.

Measuring Prostaglandin E_2 and $F_2\alpha$, and Prostaglandin E Metabolite Production Utilizing Enzyme-linked Immunosorbent Assay (ELISA) kits

Mouse-specific ELISA kits (Cayman Chemicals, Ann Arbor, MI) were used to determine prostaglandin concentrations in cell culture media. Culture media samples were assayed for PGE_2 and $PGF_2\alpha$ without further purification according to the manufacturers' instructions. For PGEM determination, all samples and standards underwent derivatization for 20 hours at 37 °C

according to the manufacturers' instructions prior to assay. Results are calculated as total pg per 2 mL culture media, normalized to total cell protein per well, determined from all lysates via Bradford assay as described above. The average percentage changes in treatment vs. control groups were also calculated.

Statistical Analysis

All data are represented as mean ± SEM for n (indicating number of wells) as indicated. Statistical analysis was performed using Graph Prism (v.6). Results of the time course experiments were compared between the two cell lines by two-way analysis of variance (ANOVA), testing for main effects of growth time (P_{Growth}), cell line (P_{Line}), and the interaction between the two ($P_{Growth*Line}$). Results of the treatment experiments were compared between the two cell lines by two-way analysis of variance (ANOVA), testing for main effects of treatment ($P_{Treatment}$), cell line (P_{Line}), and the interaction between the two ($P_{Treatment*Line}$). The percentage changes in prostaglandin production with different treatments were compared between cell lines by Students' unpaired t-test. $P < 0.05$ was considered statistically significant, and was determined by post-hoc Bonferroni test

CHAPTER 3: RESULTS

Effect of cell growth on prostaglandin production

It has been previously reported that prostaglandin production is affected by cell growth time in culture. Hammarström (1977) determined maximal prostaglandin E_2 concentrations in fibroblast cells were obtained between 6 and 18 hours following a media change, and that prostaglandin concentrations decreased with later times. In addition, Jiang et al. (2004) reported that mechanically wounding endothelial cell monolayers stimulated rapid COX-2 activity, leading to increased prostaglandin production, and that COX-2 activity decreased in more confluent cells. Prostaglandin accumulation in SH-free media was determined over a 48 h period following growth in complete media of 24 h to reflect an early, sub-confluent time point and 72 h representing 80-90% confluency. Photographed images of cultured mIMCD3 and mpkCCD cells at a subconfluent and confluent growth were taken (Figure 6).

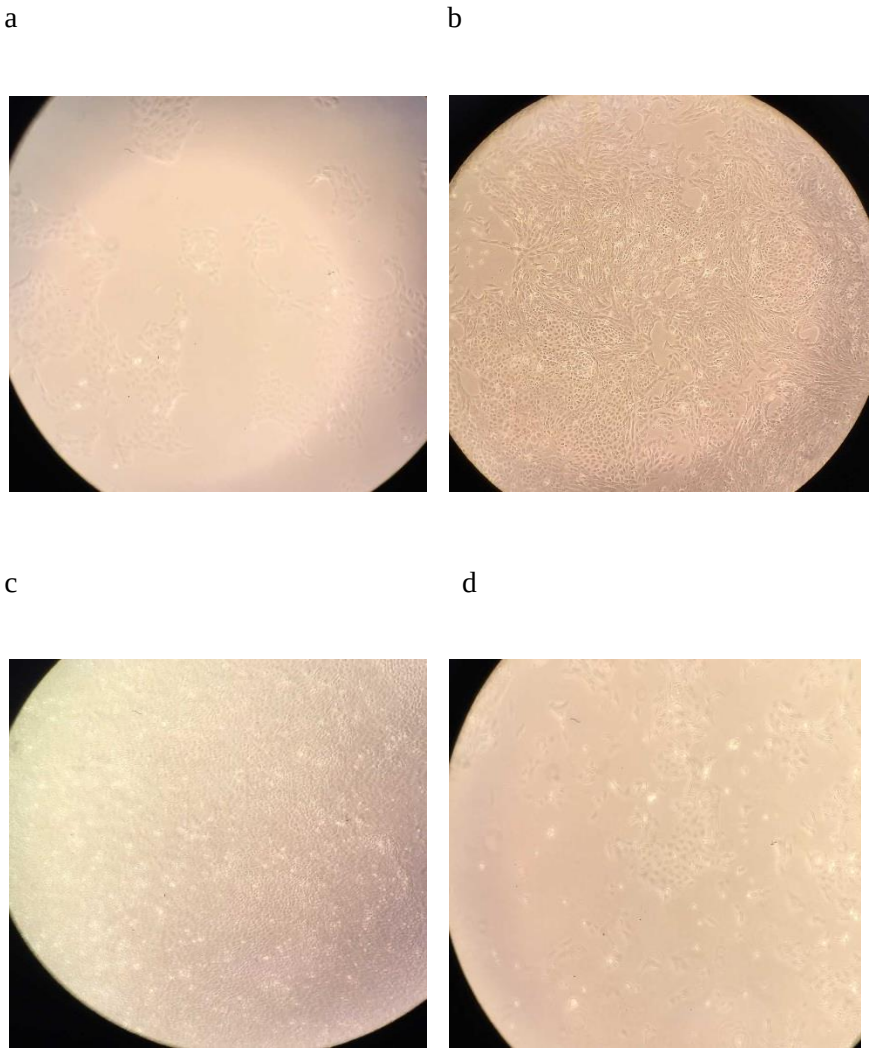

Figure 6: Photographed images of cultured mIMCD3 cells at a subconfluent (24 h) (a) and confluent (72 h) (b) growth; for mpkCCD cells at a subconfluent (c) and confluent (d) growth.

Figure 7a shows that PGE_2 accumulation was significantly affected by prior growth time independently of cell line ($P_{Growth} < 0.0001$), with both lines showing a reduction in accumulation with increased confluency. There was also a significant difference in PGE_2 accumulation between cell lines independently of growth time ($P_{Line} < 0.0001$), with higher accumulation observed in mIMCD3 compared to mpkCCD cells. The two cell lines also displayed differences in PGE_2 accumulation with different periods of growth ($P_{G*L} < 0.0001$), but mIMCD3 cells still displayed

increased PGE_2 accumulation compared to mpkCCD cells at both 24 and 72 h ($P < 0.05$ by Bonferroni post hoc test).

Similar to PGE_2, mIMCD3 cells displayed greater accumulation of PGEM in the media compared with mpkCCD cells ($P_{Line} < 0.0001$; Fig 7b) at both time points. There was no overall significant effect of growth time on PGEM accumulation ($P_{Growth} = 0.3$). However, the two cell lines displayed a difference in the effect of growth time ($P_{G*L} < 0.01$), with mIMCD3 cells showing an increase in PGEM accumulation with longer growth time ($P<0.05$), whereas there was no significant difference between 24 and 72 h in mpkCCD cells by post hoc testing.

$PGF_2\alpha$ accumulation significantly decreased with growth time independently of cell line ($P_{Growth} < 0.0001$; Fig 7c). The mpkCCD cells displayed increased $PGF_2\alpha$ accumulation compared to mIMCD3 cells overall ($P_{Line} < 0.0001$). However, the change in $PGF_2\alpha$ accumulation with growth time significantly differed between cell lines ($P_{G*L} = 0.0096$), with accumulation being significantly different between cell lines at 24 h ($P < 0.05$ by Bonferroni post-hoc test) but not at 72 h.

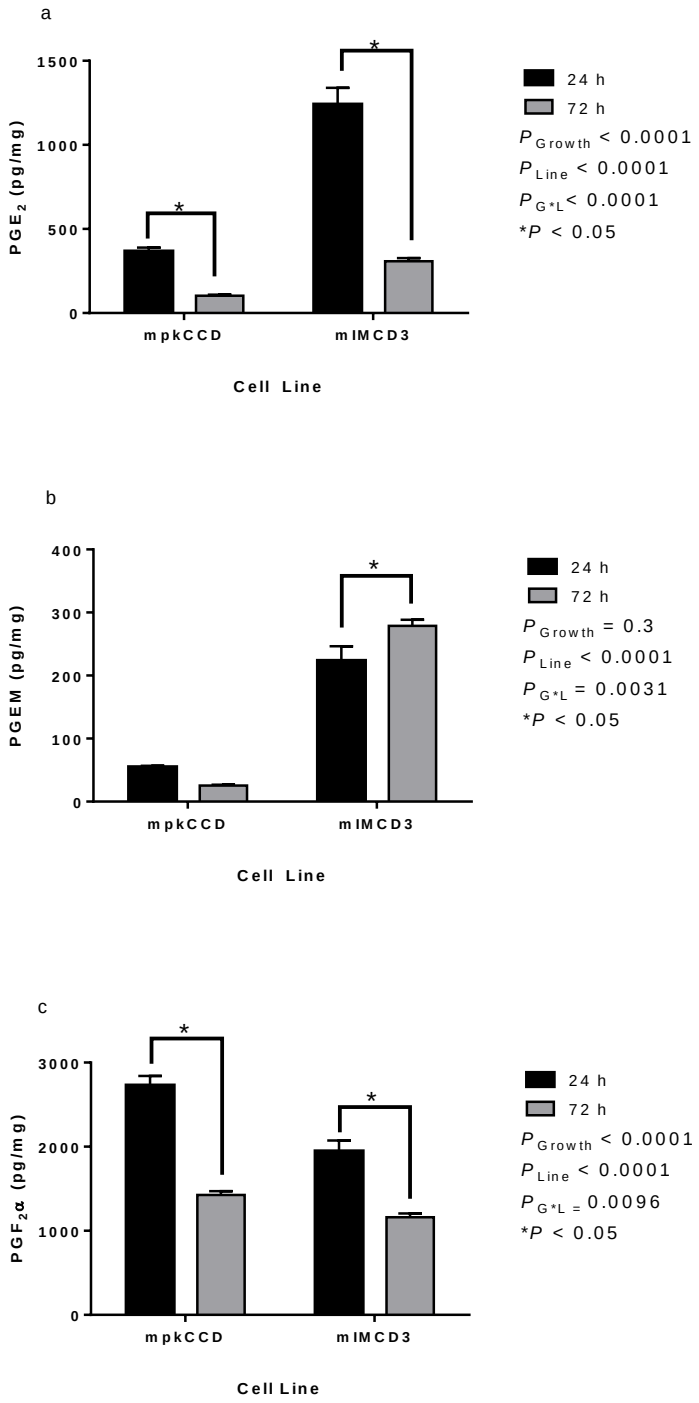

Figure 7: Effect of growth time on PGE_2 (a), PGEM (b), and $PGF_2\alpha$ (c) production. *P* values were determined by two-way ANOVA, testing for effects of cell line (P_{Line}), growth time (P_{Growth}), and the interaction between the two (P_{G*L}). $*P < 0.05$ determined by post-hoc Bonferroni test. Data presented are mean ± SEM for n=6 per cell line, per time.

Effect of dDAVP on prostaglandin production

It has been previously reported that vasopressin augments PGE_2 synthesis, resulting in a negative feedback loop to limit vasopressin's antidiuretic effect (Bisordi, 1980). Zusman et al. (1977) determined that vasopressin stimulates prostaglandin synthesis in a dose-dependent manner, while Kirschenbaum et al. (1982) and Beck et al. (1981) determined both a dDAVP and vasopressin dose-dependent increase in biosynthesis and release of all of the prostaglandins, with the major effect being on PGE_2. The effect of dDAVP, a vasopressin analog, on prostaglandin production was thus assessed.

mIMCD3 cells showed significantly greater PGE_2 accumulation compared to mpkCCD cells, independently of treatment ($P_{Line} < 0.001$; Fig 8a). While there was no significant effect of dDAVP treatment when taking both cell lines into account ($P_{Treatment} = 0.3$), the two lines responded differently ($P_{T*L} < 0.05$), with mIMCD3 cells showing an approximately 20% increase in PGE_2 accumulation in response to dDAVP ($P < 0.05$), whereas mpkCCD cells did not.

Likewise, mIMCD3 cells showed significantly greater PGEM accumulation compared to mpkCCD cells, independently of treatment ($P_{Line} < 0.0001$; Fig 8b). There was a significant effect of dDAVP treatment when taking both cell lines into account ($P_{Treatment} = 0.0025$); the two cell lines did respond differently ($P_{T*L} = 0.0027$), with mIMCD3 cells showing a statistically significant 15% increase in PGEM accumulation in response to dDAVP ($P < 0.05$), whereas mpkCCD cells did not.

dDAVP-treated cells showed increased $PGF_2\alpha$ compared to control cells in each cell line (Fig 8c). mIMCD3 cells showed significantly greater PGE_2 accumulation compared to mpkCCD cells, independently of treatment ($P_{Line} < 0.0001$). There was a significant effect of dDAVP treatment when taking both cell lines into account ($P_{Treatment} < 0.05$). The effect of dDAVP to increase $PGF_2\alpha$ production did not differ between cell lines ($P_{T*L} = 0.8$).

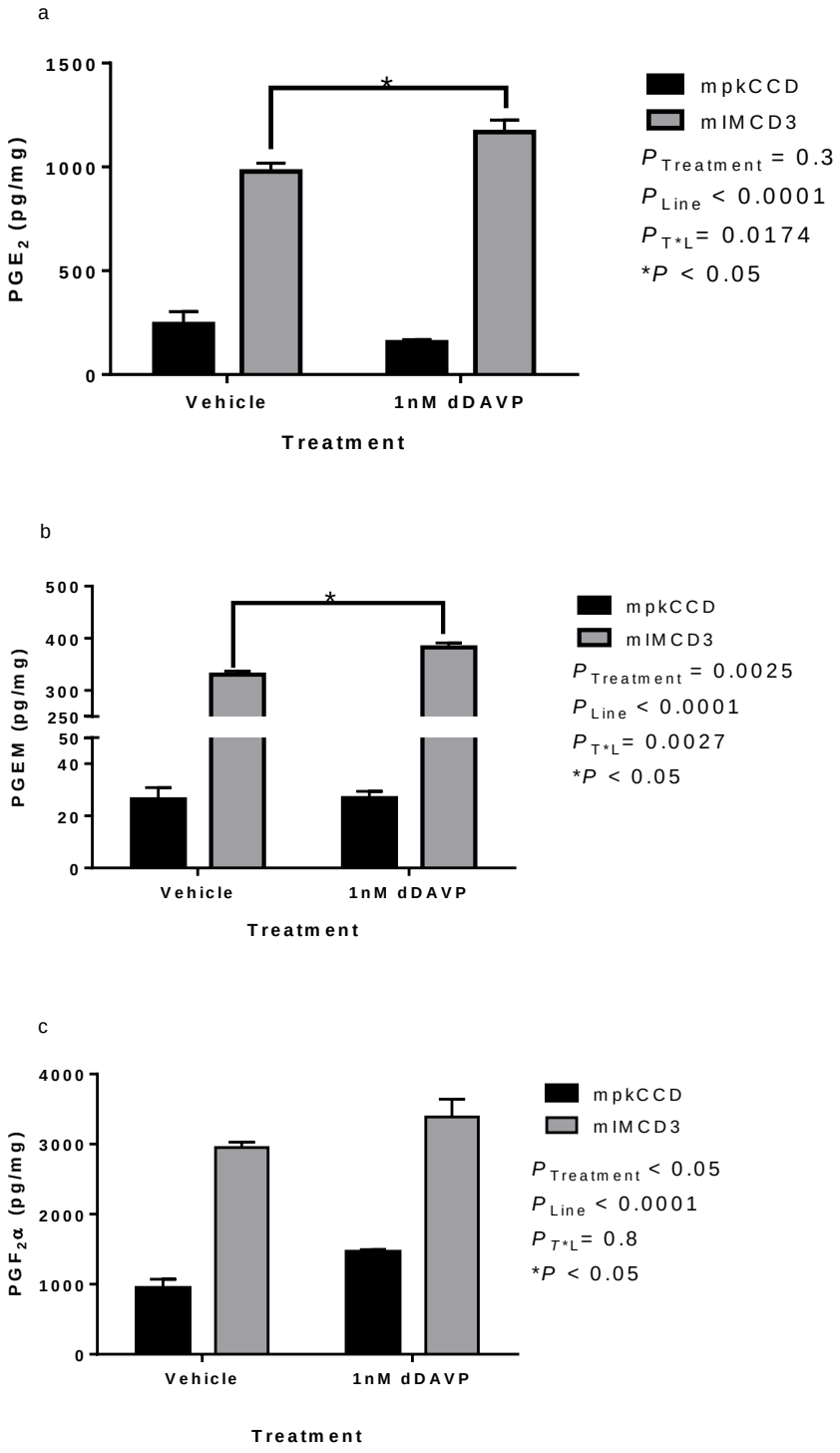

Figure 8: Effect of dDAVP on PGE_2 (a), PGEM (b), and $PGF_2\alpha$ (c) production. *P* values were determined by two-way ANOVA, testing for effects of cell line (P_{Line}), treatment ($P_{Treatment}$), and the interaction between the two (P_{T*L}). $*P < 0.05$ determined by post-hoc Bonferroni test. Data presented are mean ± SEM for n=3 per cell line, per treatment.

Effect of COX-1 (SC-560) and COX-2 (NS-398) inhibition on prostaglandin production

Ferguson et al. (1999) reports that COX-2 is the major contributor to the pool of PGE_2 synthesized by the cortical collecting duct. From the study, when COX enzyme activity was measured in murine M-1 cortical collecting duct cells, both indomethacin (a COX-1 and -2 inhibitor) and the specific COX-2 inhibitor NS-398 effectively blocked PGE_2 synthesis, demonstrating that COX-2 is the major contributor to PGE_2 synthesis. Other studies have concluded that hyperosmolar conditions, conditions such as what inner medullary collecting duct cells are exposed to, have led to increased COX-2 expression, while inhibiting COX abolished PGE_2 production (Zhang, 1995).

The individual effects of COX-1 and COX-2 inhibition on mpkCCD and mIMCD3 cell prostaglandin production was tested. The addition of a COX-1 or COX-2 inhibitor to both cell lines showed a statistically significant decrease of both PGE_2 and $PGF_2\alpha$ accumulation in the media ($P < 0.05$) compared to the control groups (Fig 9 a, c, d, f).

PGE_2 levels were higher in mIMCD3 cells compared to mpkCCD cells with a COX-1 inhibitor (Fig 9a). PGE_2 accumulation by SC-560-treated mpkCCD cells is suppressed to approximately 22% of that seen in vehicle control cells whereas mIMCD3 cells treated with SC-560 is suppressed to approximately 28% of that seen in vehicle control cells ($P_{Treatment}$ and $P_{Line} < 0.0001$). Similarly, PGE_2 accumulation was greater in both the treated and control groups of mIMCD3 cells with a COX-2 inhibitor (Fig. 9d). There was similar inhibition of PGE_2 accumulation in the media; NS-398-treated mpkCCD cells was suppressed to approximately 25% of that seen in vehicle control cells whereas mIMCD3 cells treated with NS-398 is suppressed to approximately 20% of that seen in vehicle control cells ($P_{Treatment}$ and $P_{Line} < 0.0001$).

PGEM accumulation was significantly greater in the mIMCD3 cells compared to mpkCCD cells ($P_{Line} < 0.0001$), in both the treated and control cells, and in the presence of both a

COX-1 and COX-2 inhibitor (Fig. 9 b, e). Treated mIMCD3 cells paradoxically showed an increase in PGEM accumulation compared to untreated cells; there was a 28% increase in PGEM accumulation with a COX-1 inhibitor present and a 16% increase with a COX-2 inhibitor, both of which were statistically significant ($P < 0.05$).

As stated above, $PGF_2\alpha$ levels were significantly decreased with the addition of a COX-1 or COX-2 inhibitor in both cell lines (Fig. 9 c, f). $PGF_2\alpha$ levels in SC-560-treated mpkCCD cells is suppressed to approximately 10% of that seen in vehicle control cells whereas mIMCD3 cells treated with SC-560 is suppressed to approximately 34% of that seen in vehicle control cells ($P_{Treatment} < 0.0001$). There was not a statistically significant difference in $PGF_2\alpha$ production between the two cell lines ($P_{Line} = 0.1068$). Just as with a COX-1 inhibitor, mpkCCD cells treated with a COX-2 inhibitor showed a greater reduction in $PGF_2\alpha$ accumulation compared to mIMCD3 treated cells; NS-398-treated mpkCCD cells was suppressed to approximately 10% of that seen in vehicle control cells whereas mIMCD3 cells treated with NS-398 is suppressed to approximately 27% of that seen in vehicle control cells ($P_{Treatment} < 0.0001$; $P_{Line} = 0.0192$).

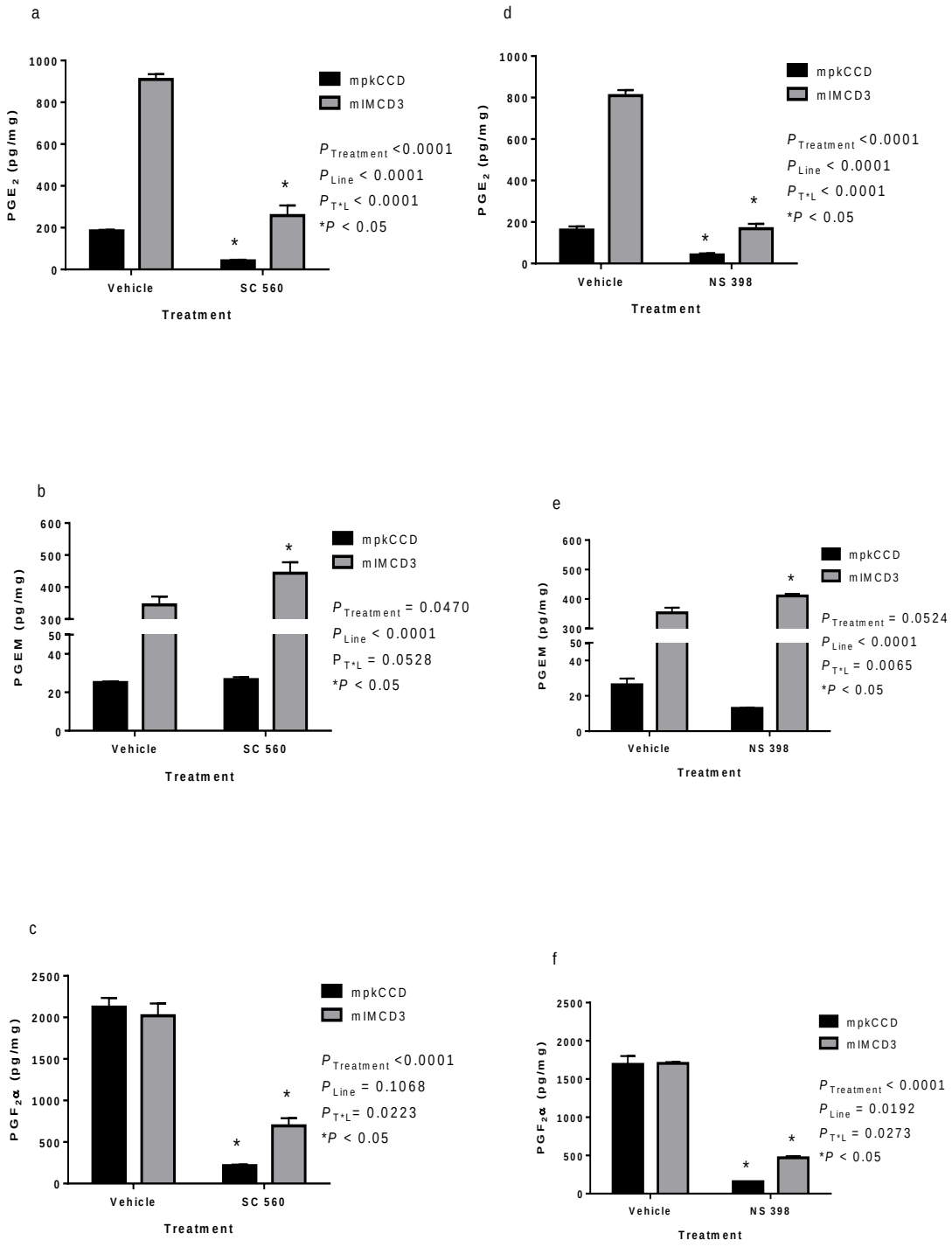

Figure 9: Effect of 0.1 µM SC-560 (COX-1) (a-c) and 5 µM NS-398 (COX-2) (d-f) inhibition on PGE_2 (a, d), PGEM (b, e), and $PGF_2\alpha$ (c, f) production. *P* values were determined by two-way ANOVA, testing for effects of cell line (P_{Line}), treatment ($P_{Treatment}$), and the interaction between the two (P_{T*L}). $*P < 0.05$ determined by post-hoc Bonferroni test, and indicates statistical significance compared to the vehicle treatment in the same cell line. Data presented are mean ± SEM for n=3 per cell line, per treatment.

Effect of extracellular osmolality on prostaglandin production

Several studies have confirmed hyperosmolarity-stimulated PGE_2 biosynthesis as a protective mechanism against hyperosmolarity-induced cell damage. Zusman et al. (1980) determined a hyperosmolar environment stimulated PGE_2 synthesis by increasing arachidonic acid release whereas Danon et al. (1978) concluded there was a concentration-dependent increase in PGE_2 and $PGF_2\alpha$ output by rat renal papillae when the NaCl concentration was increased above 300 mOsm/L.

To test the effect of osmolarity on prostaglandin production, culture media osmolality was increased by addition of 4.5 mg of NaCl per 1 mL of serum and hormone-free media, and the accumulation of prostaglandins compared to levels detected in cells cultured in parallel in normal serum and hormone-free media. The osmolality measured in mpkCCD normal culture media was 326 mOsmol/kg H_2O and 468 mOsmol/kg H_2O in the NaCl-supplemented media. The osmolality measured in mIMCD3 normal culture media was 291.5 mOsmol/kg H_2O and 435 mOsmol/kg H_2O in the NaCl-supplemented media.

Cells of both lines treated with NaCl showed a significant increase in PGE_2 production (Fig. 10a), with this effect not being significantly different between cell lines ($P_{T*L} = 0.28$; $P_{Treatment} < 0.05$). As seen in the other experiments presented, mpkCCD cells did not demonstrate much PGEM accumulation, whether they were treated with NaCl or not, while mIMCD3 cells showed significantly greater PGEM accumulation compared to mpkCCD cells, independently of treatment ($P_{Line} < 0.0001$ Fig. 10b), and showed a statistically significant increase in response to NaCl-supplementation ($P < 0.05$). $PGF_2\alpha$ was also increased by NaCl-supplementation in both mpkCCD and mIMCD3 cells, however the concentrations produced were too high to accurately measure when media was assayed undiluted (data not shown).

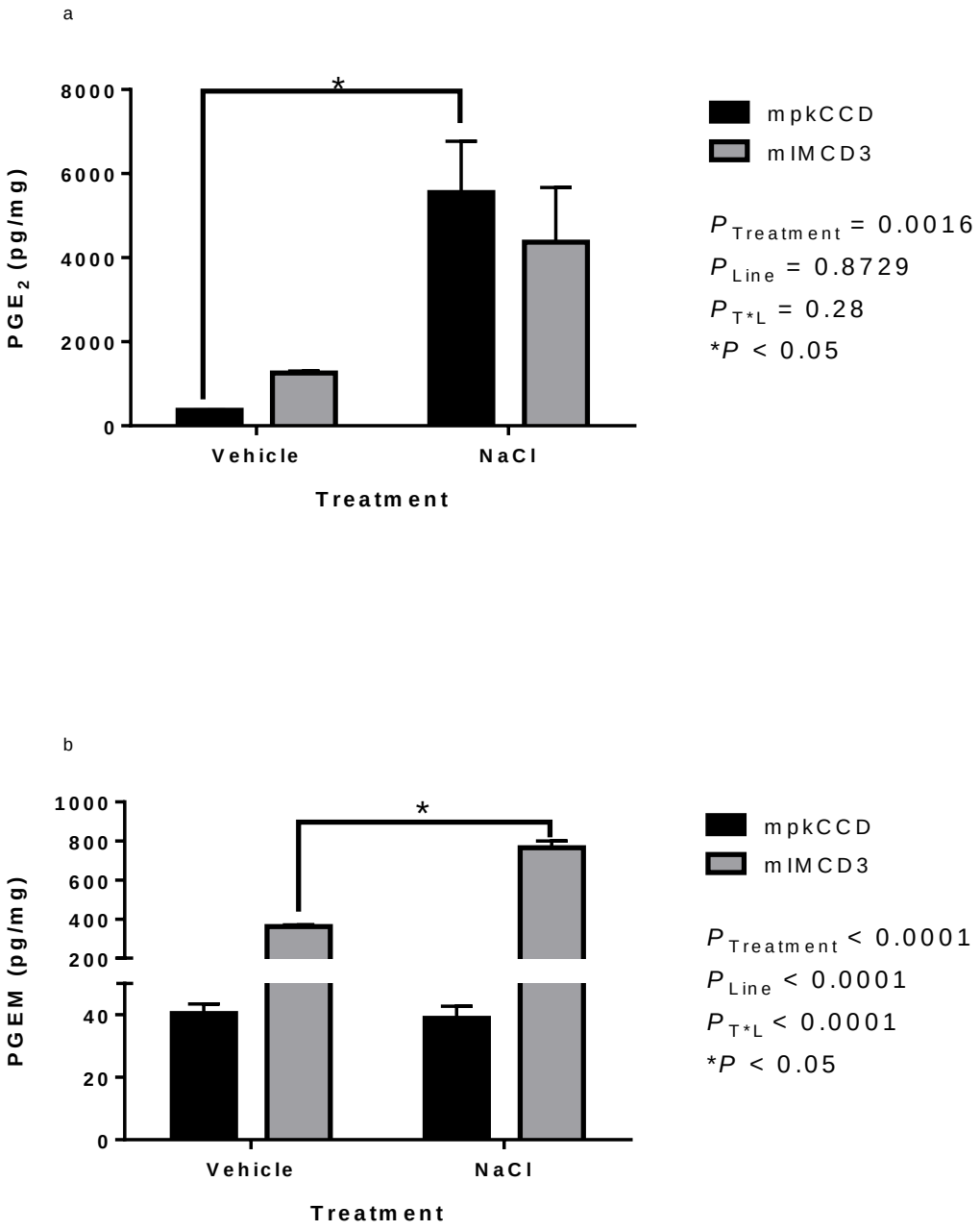

Figure 10: Effect of NaCl on PGE_2 (a) and PGEM (b) production. *P* values were determined by two-way ANOVA, testing for effects of cell line (P_{Line}), treatment ($P_{Treatment}$), and the interaction between the two (P_{T*L}). $*P < 0.05$ determined by post-hoc Bonferroni test. Data presented are mean ± SEM for n=3 per cell line, per treatment.

CHAPTER 4: DISCUSSION

The collecting duct is the terminal segment of the nephron tubule that is involved in the fine-tuning of urine volume and composition, creating a more dilute or more concentrated urine, based on the body's water needs. The collecting duct extends from the outer renal cortex, down to the deepest parts of the renal medulla. The water permeability of the collecting duct is regulated by the hormone vasopressin. In the short term, vasopressin stimulates the shuttling of aquaporin 2 water channels to the apical membrane, allowing passive water movement to move from the nephron tubular lumen and into the surrounding interstitium; long term, vasopressin enhances aquaporin 2 gene expression (Kuo, 2018). Prostaglandins, primarily PGE_2, is a known negative regulator of collecting duct water and urea permeability. Through various signaling pathways, depending on which particular receptor PGE_2 binds to, collecting duct water permeability can be decreased either by increased endocytosis of aquaporin 2 vesicles, or a decrease in aquaporin 2 trafficking to the apical membrane as a result of decreased intracellular cAMP levels.

The cell lines utilized come from different areas of the collecting duct. mpkCCD cells are cortical collecting duct cells whereas mIMCD3 cells are inner medullary collecting duct cells. These cells are exposed to different extracellular osmolarities. As demonstrated, they also differ in prostaglandin production, both as a result of different cell growth confluency and various treatment agents. There were general findings of the study. A general finding was that mIMCD3 cells made more PGE_2 than mpkCCD cells, but the amount of $PGF_2\alpha$ was more similar between the two cell lines. Additionally, as a general trend, PGEM levels did not change all that much between vehicle and treated cells of each cell line and between subconfluent and confluent cells for each cell line. Finally, subconfluent cells showed more prostaglandin production compared to confluent cells in both cell lines.

PGE_2 and $PGF_2\alpha$ accumulation decreased as cells approached confluency

Based on the experimental results, PGE_2 and $PGF_2\alpha$ production was relatively high while cell density was low, with production declining as cultures approached confluency at 72 h. It is likely that as the cells reach confluency, they reach a stable, basal level of prostaglandin synthesis. The confluent growth time is likely more reflective of normal collecting duct epithelium as a large sheet of in-contact, monolayer cells. A decrease in prostaglandin production with increased growth time is consistent with the findings of Hammarström (1977). In this study, maximal prostaglandin production occurred prior to 24 hours and that prostaglandin concentration decreased with later times. However, fibroblasts were utilized in that study, rather than epithelial cells from the kidney. Contrary to this, the subconfluent growth time is likely more reflective of injury and repair of epithelium. Previous reports have indicated increased COX-2 activity with mechanically wounded cells, leading to increased prostaglandin production (Jiang, 2004). While the cells in this study were not mechanically wounded, the loss of a solid monolayer and direct contact with one another may have similarly stimulated COX-2 expression, leading to the very high prostaglandin concentrations seen at 24 hours.

When comparing the cell lines, PGE_2 accumulation was much lower in the mpkCCD cells at both 24 and 72 hours, compared to mIMCD3 cells. PGE_2 is the major cyclooxygenated metabolite of arachidonic acid produced in the kidney and is synthesized at high rates throughout the nephron and collecting duct system (Li, 2018). This result is likely due to the fact that its main sites of synthesis include the medullary collecting duct and, to a lesser extent, the cortical collecting duct (Bonvalet, 1987). $PGF_2\alpha$ has roughly the same distribution along the collecting duct, however, the rate of synthesis is nearly 20-fold lower compared to PGE_2 (Bonvalet, 1987). Interestingly, $PGF_2\alpha$ accumulation was significantly greater than PGE_2 accumulation in each cell line, at both 24 h and 72 h. One plausible reason for this is that some of the PGE_2 was probably converted to $PGF_2\alpha$ as it can be synthesized directly from PGE_2 via a reductase reaction (Dozier, 2008).

PGEM was measured to see if the observed decrease in prostaglandin production at a confluent time growth was a result of metabolism. While there was an increase in PGEM from 24 to 72 hours in mIMCD3 cells, the increase does not account for the huge reduction seen in PGE_2 seen at 72 hours compared to 24 hours (approximately 25% of what was produced by cells allowed to grow for 24 hours; Fig. 7a). Most cultured cells that produce prostaglandin E_2 generally do not contain the enzymes required for PGE_2 metabolism, likely explaining this result in that most PGE_2 was not metabolized into its metabolites. There wasn't low PGEM observed at 24h growth and high PGEM at 72h growth, suggesting that production of PGE_2 is truly lower in confluent cells rather than the decrease reflecting increased metabolism. An assay to measure $PGF_2\alpha M$ should be included as a future step to observe the effects that cell confluency has on $PGF_2\alpha M$, and see how that compares in the two cell lines.

PGE_2 accumulation increased with dDAVP treatment in mIMCD3 cells while not having a significant effect in mpkCCD cells. $PGF_2\alpha$ accumulation increased with dDAVP treatment in both cell lines

Vasopressin has important physiological roles. In vivo, vasopressin is released in response to an increase in blood osmolarity or a decrease in blood volume. In the collecting duct, vasopressin binds to its vasopressin 2 receptor (V2R), which initiates a G_s-cAMP-Protein Kinase A mediated trafficking of aquaporin 2 water channels to insert into the apical membrane. As a result, water passively flows through the water channels, and enters the surrounding interstitium via aquaporin 3 and 4, where it is returned to the blood. PGE_2 is known for its diuretic role, opposing vasopressin-mediated water permeability of the collecting duct. This occurs through PGE_2 binding to its EP3 receptor, which decreases intracellular cAMP levels, or binding to its EP1 receptor, which activates G_q and Protein Kinase C, which increases the endocytosis of aquaporin 2 vesicles. $PGF_2\alpha$ primarily signals through G_q-Protein Kinase C, and therefore likely impacts aquaporin 2 trafficking in a similar manner as PGE_2 and EP1.

A V2R agonist, dDAVP, was used to compare the responses of the two cell lines to vasopressin stimulation. Based on the experimental results, there was an increased accumulation of prostaglandins and PGEM in the dDAVP-treated mIMCD3 cells, while dDAVP did not have a significant effect on PGE_2 accumulation in mpkCCD cells. However, $PGF_2\alpha$ similarly increased with dDAVP treatment in the mpkCCD cells.

It is generally known that vasopressin augments PGE_2 synthesis and that prostaglandins antagonize the hydro-osmotic effects of vasopressin in the collecting duct (Bonvalet, 1987), and that the inhibition of endogenous prostaglandin synthesis by COX inhibitors enhances the response to vasopressin (Bisordi, 1980). Several studies have demonstrated this finding: Zusman et al. (1977) proposed that vasopressin stimulates prostaglandin synthesis in a dose-dependent manner by activating phospholipase enzymes, thus increasing the amount of arachidonic acid availability in the toad urinary bladder. Kirschenbaum et al. (1982) and Beck et al. (1981 determined a dDAVP and vasopressin dose-dependent increase in biosynthesis and release of all of the prostaglandins, with the major effect being on PGE_2 in rabbit cortical collecting tubular cells. From these studies, it is concluded that, since prostaglandins antagonize the effect of vasopressin-mediated water permeability, by stimulating prostaglandin production, a negative feedback loop is completed, thereby limiting vasopressin's antidiuretic effect (Bisordi, 1980).

dDAVP had a much greater impact on PGE_2 production in mIMCD3 cells compared to mpkCCD cells. It is likely that these cells are more responsive to vasopressin - vasopressin 2 receptor mRNA increases in intensity in the renal medullary collecting duct compared to the cortical collecting duct (Mutig, 2007). Due to decreased vasopressin 2 receptor mRNA expression, mpkCCD cells are possibly less responsive to vasopressin. As a result, there is less prostaglandin production via the negative feedback loop to suppress vasopressin's antidiuretic effect.

Another possible explanation that dDAVP had little effect on PGE_2 production was that the concentration of dDAVP was not high enough. However Kortenoeven et al. (2011) utilized the same concentration of 1 nM dDAVP to maximally induce aquaporin 2 expression in their study. They determined that dDAVP-treated mpkCCD cells did stimulate both sufficient levels of PGE_2 and $PGF_2\alpha$ production compared to control mpkCCD cells. However, they utilized different culturing and incubation times, culturing the cells for 8 days and treating the cells with 1nM dDAVP for 96 hours to maximally induce aquaporin 2 expression. Their model also more accurately reflects in vivo conditions, using a semipermeable filter to create an apical and basolateral surface. Repeating this experiment with higher concentrations of dDAVP to see if this would result in increased PGE_2 accumulation in the mpkCCD cells or not is a potential next step, as well as utilizing their model of mpkCCD cell incubation with dDAVP.

PGE_2 and PGF_2a accumulation significantly decreased with COX-1 and COX-2 inhibitor treatment in both mIMCD3 and mpkCCD cells.

The biosynthetic pathway of prostaglandins involves the conversion of arachidonic acid to prostaglandin H_2, catalyzed by cyclooxygenase-1 and -2 (COX-1 and COX-2) enzymes. This is the rate-limiting step in prostaglandin synthesis. Therefore, the presence of COX-1 and COX-2 inhibitors would expect to greatly reduce prostaglandin production. In the kidney, COX-1 is constitutively expressed in the collecting duct, localized to both the cortical and medullary segments (Hao, 2008). COX-1 has also been determined to be present in the medullary interstitial cells (Nasrallah, 2014). The expression of COX-2 is much more unclear and conflicting in most studies. It has been reported to have varying amounts of expression within the collecting duct. Kortenoeven et al. (2011) report strong COX-2 expression in the collecting duct in three studies, and absence of it was suggested in three other studies. They also report that increased COX-2 levels have been detected in collecting ducts of animals that were dehydrated or subjected to a

chronic NaCl load, noting its inducible expression under stress conditions. It is, however, highly expressed in renal medullary interstitial cells (Hao, 2008; Kortenoeven, 2011).

Based on the experimental results, as expected, mpkCCD and mIMCD3 cells treated with either a COX-1 or COX-2 inhibitor greatly reduced prostaglandin accumulation. Both COX-1 (SC-560) and COX-2 (NS-398) inhibition resulted in similar decreases of PGE_2 accumulation in both cell lines. In mIMCD3 cells, PGE_2 accumulation was more reduced in the presence of a COX-2 inhibitor compared to a COX-1 inhibitor (28% vs. 20%). In mpkCCD cells, the presence of a COX-1 inhibitor reduced PGE_2 accumulation to 22% of that compared to vehicle control cells, and 25% with a COX-2 inhibitor. Due to the similarity in reduction of PGE_2 accumulation in the media with a COX-1 and COX-2 inhibitor in both cells, it is difficult to report what enzyme contributes more to PGE_2 synthesis in these cells. Ferguson et al. (1999) reports that COX-2 is the major contributor to the pool of PGE_2 synthesized by the cortical collecting duct. From the study, when COX enzyme activity was measured in their murine M-1 cortical collecting duct cells, both indomethacin (COX-1 and -2 inhibitor) and the specific COX-2 inhibitor NS-398 effectively blocked PGE_2 synthesis, demonstrating that COX-2 is the major contributor to the pool of PGE_2 synthesized by the cortical collecting duct. However, they did utilize a different cortical cell line, which may contribute to their results.

For $PGF_2\alpha$, while both COX-1 and COX-2 inhibitors reduced accumulation in the media in both cell lines, the reduction was greater in the mpkCCD cells, for both COX-1 and COX-2 inhibition. SC-560 and NS-398 treated mpkCCD cells suppressed $PGF_2\alpha$ accumulation to 10% of that seen in the vehicle control cells, whereas treated mIMCD3 cells with SC-560 and NS-398 suppressed $PGF_2\alpha$ accumulation to 34% and 27% of that seen in vehicle treated cells, respectively. Again, these percentages are too close to confidently say which enzyme contributes more to $PGF_2\alpha$ production, and this would need to be run again to determine which enzyme has a greater effect on prostaglandin production. Interestingly, there was a paradoxical increase in

PGEM accumulation in the mIMCD3 cells, in both the COX-1 and COX-2 inhibitor treated cells; however, there was very little PGEM accumulation in the mpkCCD cells, with both COX-1 and COX-2 inhibition as well as in the control cells.

PGE_2 and $PGF_2\alpha$ accumulation significantly increased with NaCl treatment in both mIMCD3 and mpkCCD cells.

The osmolarity of the tubular fluid as well as the surrounding interstitium varies along the length of the collecting duct. These cell lines are located in different regions of the collecting duct, and are thus exposed to the different osmolarities. In the formation of a concentrated urine, under the influence of vasopressin, the deepest parts of the interstitial medullary region as well as the tubular fluid within the nephron, can be exposed to osmolarities up to 1200 mOsm/L. Thus, the cells in this area must be able to function with significant increases in osmolarity. In a state of dehydration, the interstitial osmolality can increase several fold, and if prolonged, lead to cellular distress (Price, 2011). In the formation of a dilute urine, osmolarities of the tubular fluid can be as low as 50 mOsm/L, with a medullary interstitium osmolarity of 600 mOsm/L. The cortical collecting duct tubular fluid and surrounding interstitium usually has an osmolarity around 300 mOsm/L.

Based on the experimental results, PGE_2 and $PGF_2\alpha$ levels were significantly increased in NaCl-treated cells of both lines. $PGF_2\alpha$ concentration levels produced were too high to accurately measure when media was assayed undiluted in both cell lines. The result of a surge in prostaglandin production in the presence of NaCl is most likely a stress-protective mechanism, which is explained below. When comparing the two cell lines, PGE_2 was much greater in the mpkCCD-treated cells. This could be due to the idea that these cells are not as adaptable to such increases in osmolarity as the mIMCD3 cells are, and this protective mechanism was further enhanced. Levels of $PGF_2\alpha$ as a result of the addition of NaCl is something that needs to be determined.

Several studies have confirmed hyperosmolarity stimulated PGE_2 biosynthesis as a protective mechanism against hyperosmolarity-induced cell damage. In align with this, a well-noted phenomenon in clinical medicine is that certain COX-inhibitory drugs can cause necrosis of the renal papilla (Delmas, 1995; Eknoyan, 1994). Zusman et al. (1980) determined a hyperosmolar environment stimulated PGE_2 biosynthesis by increasing arachidonic acid release in rabbit medullary interstitial cells. Via a similar mechanism of increasing arachidonic acid mobilization from tissue lipids, Danon et al. (1978) concluded that there was a concentration-dependent increase in PGE_2 and $PGF_2\alpha$ output by rat renal papillae when the NaCl concentration was increased above 300 mOsm/L. Outside of the kidney, PGE_2 production by lipopolysaccharide-stimulated rat Kupffer cells was increased if exposed to a medium osmolarity above 305 mOsm/L, whereas a hypo-osmotic medium (255 mOsm/L) diminished PGE_2 formation. In this study involving the rat liver macrophages, the hyperosmolarity lead to COX-2 induction and inhibiting COX with indomethacin and specifically COX-2 with NS-398, abolished PGE_2 production (Zhang, 1995). In another relevant study, it was determined that hypertonicity increased COX-2 activity, leading to PGE_2-mediated inactivation of the pro-apoptotic protein, BAD, conferring a hyperosmolarity-induced prostaglandin production protective mechanism (Price, 2011). Several other studies have conferred a cytoprotective role of COX-2 in the osmotic response (Yang, 2000).

There are obvious limitations to this study. First and foremost, due to the lack of time, these experiments were only completed once. To verify these results and to ensure the results were not by random chance, these exact experiments should be run again, possibly three times, to gather stronger and more firm conclusions. In addition, the sample size utilized was low, which reduces the power of a study and increases the margin of error. With such a low sample size, it is difficult to discern whether these are the expected results in all of the experiments, or if the results would differ with a larger sample size. In alignment with the lack of available time, some of the

prostaglandins accumulation data needs to be repeated with diluted samples, as the concentration levels produced were too high to be accurately measured when media was assayed undiluted. With that said, this leads us to some potential future directions. Re-running these experiments is also a future direction, as well as trying different treatment concentrations, to see if there is a dose-dependent response. Another future direction would be to look at other prostaglandins, as the kidneys synthesize more than just PGE_2 and $PGF_2\alpha$. In addition, comparing expression levels of various enzymes, such as COX, between the two cell lines, by western blot is warranted.

Overall, the models used to study the collecting duct were appropriate for this study. There are many differences between the two cell lines that have been mentioned. These differences are primarily due to their differences in their location within the collecting duct. As a result, the two cell lines responded differently in prostaglandin production in response to various treatments.

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