FLOW REGULATION OF ENDOTHELIN-1 PRODUCTION IN THE COLLECTING DUCT

by

Meghana Madhukar Pandit

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Pharmaceutics and Pharmaceutical Chemistry

The University of Utah

December 2015

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Meghana Madhukar Pandit**

has been approved by the following supervisory committee members:

Donald Kohan	, Chair	**02/13/2015**
		Date Approved
David Grainger	, Member	**02/13/2015**
		Date Approved
James Herron	, Member	**02/13/2015**
		Date Approved
Mingnan Chen	, Member	
		Date Approved
Yan-ting Shiu	, Member	**02/13/2015**
		Date Approved

and by **David Grainger**, Chair of

the Department of **Pharmaceutics and Pharmaceutical Chemistry**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

The collecting duct (CD) is the major site of endothelin-1 (ET-1) synthesis in the body. CD-derived ET-1 is an important autocrine inhibitor of CD Na^+ and water reabsorption; deficiency of CD ET-1 causes marked hypertension, Na^+ and water retention. Salt and water loading causes an expansion of extracellular fluid volume (ECFV), resulting in increased fluid flow in the CD. ECFV expansion augments CD ET-1 production, thus suggesting an association between volume loading, natriuresis and diuresis. In cultured cortical collecting duct cells, fluid flow stimulates ET-1 production in a Na^+-dependent manner via ENaC and subsequent activation of the mitochondrial Na^+/Ca^{2+} exchanger. In contrast, in mouse inner medullary CD cells, fluid flow regulates ET-1 production in a Na^+-independent manner involving Ca^{2+}-dependent signaling via primary cilia/polycystin/purinergic pathways. Finally, a high solute load augments inner medullary CD ET-1 production. Thus, fluid flow regulates ET-1 production differently in the cortical and inner medullary CD; Na^+ reabsorption takes place mainly in the cortical CD while water reabsorption occurs in the inner medullary CD. Taken together, the findings in this dissertation provide exciting, novel information about the existence of a complex pathway through which ECFV status controls CD-derived ET-1 production, thereby achieving blood pressure (BP) maintenance and volume homeostasis through acute and sustained regulation of natriuresis and diuresis.

CONTENTS

ABBREVIATIONS

AC	Adenylate cyclase
ADH	Anti-diuretic hormone
ADPKD	Autosomal dominant polycystic kidney disease
ATP	Adenosine triphosphate
AVP	Arginine vasopressin, also called ADH
BP	Blood pressure
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular calcium
CaM	Calmodulin
CaMK	Calmodulin kinase
cAMP	Cyclic adenosine monophosphate
CCD	Cortical collecting duct
CD	Collecting duct
cGMP	Cyclic guanosine monophosphate
Cn	Calcineurin
CO	Cardiac output
COX	Cyclooxygenase
DAG	Diacyglycerol
DCT	Distal convoluted tubule

eNOS	endothelial nitric oxide synthase
ECE	Endothelin converting enzymes
ECFV	Extracellular fluid volume
EET	Epoxyeicosatrienoic acid
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
ET-1	Endothelin-1
ETA	Endothelin receptor type A
ETB	Endothelin receptor type B
GPCR	G-protein coupled receptor
IMCD	Inner medullary collecting duct
KO	Knockout
MAPK	Mitogen-activated protein kinase
MDCK	Madine-Darby canine kidney
Na+	Sodium ion
NFAT	Nuclear transactivating factor
NFATc	cytoplasmic nuclear transactivating factor
NO	Nitric oxide
OM	Outer medullary
PAH	Pulmonary arterial hypertension
PC-1	Polycystin-1
PC-2	Polycystin-2
PCT	Proximal convoluted tubule
PGE2	Prostaglandin E2

PKC	Protein kinase C
PLC	Phospholipase C
Po	Open channel probability
PPAR	Peroxisome proliferator-activated receptors
RAS	Renin angiotensin system
RBF	Renal blood flow
RIHP	Renal interstitial hydrostatic pressure
ROS	Reactive oxygen species
TAL	Thick ascending limb
TonEBP	Tonicity-enhanced binding protein
TPR	Total peripheral resistance

ACKNOWLEDGEMENTS

My PhD journey was a great learning experience. Looking back, I want to take this as an opportunity to thank all those who supported me and helped me take this degree to completion. First and foremost, I would like to thank my PhD advisor, Dr. Donald Kohan. Don has always been a constant source of inspiration and motivation. He is one of the smartest people I have met and I am very proud to say that I had the best training possible in a very conducive environment. Along with Don, I would like to thank Dr. Kevin A. Strait and Dr. Alfred van Hoek. Kevin initially trained me when I first joined the lab and taught me all the basics of molecular biology along with introducing me to relevant techniques that later on proved to be very useful for the completion of my project. Alfred has not only been a mentor but also a very good friend. I would like to thank him for his help in the immunocytochemistry experiments and I greatly admire his technical ability with regards to fixing lab equipment. He has given me great advice both scientific and nonscientific, which I greatly value. I would also like to thank my labmates, Debbie, Niru and Shuping. Apart from helping me learn different techniques in the lab, I have always enjoyed having casual conversation with them. They have been the greatest stress-busters when I needed a break from experiments or writing.

I would like to thank my committee, Dr. David Grainger, Dr. Mingnan Chen, Dr. Herron and Dr. Yan-Ting Shiu. Their advice and constructive criticism has improved the way I present my data without losing sight of the “big” picture. I would especially like to

thank Dr. Grainger for letting me learn molecular biology techniques in his lab in the first year of my PhD along with Dr. Amanda Brooks. Amanda first got me interested in molecular biology – a subject that I was petrified of initially. She taught me the nitty-gritties of all the techniques along with the essentials of good experimental design. I would also like to thank Dr. Kishore for his encouraging suggestions with regards to my experiments.

My time in graduate school would have not been as enjoyable without my friends in Salt Lake City, Utah. SLC indeed has been my home away from home and I have found a second family here with my friends Anusua, Saurav, Niladrish, Protonu, Saurabh, and Shraddha. I can never forget the very enjoyable dinners, conversations and the closeness that I shared with each one of them. Also, my friends in the pharmaceutics department have made my entire journey very enjoyable- my classmates in the classes I took, my colleagues from when I served on SAC, gave me the needed respite from work whenever needed.

Finally, I would like to thank my best friend and husband, Arijit. I am grateful for his companionship and love in addition to bearing with me when I have been nothing but a nuisance due to the typical grad school stress. He has always believed in me, more than I have ever believed in myself.

Lastly, I would like to thank my most important support system, my family. I greatly admire my parents for their resilience and perspective towards life. I also cherish my brother's companionship and his ability to help people get through tough situations with his sense of humor. My PhD dissertation is a tribute to my family's unconditional love and encouragement towards me.

CHAPTER 1

BACKGROUND

1.1.Hypertension

Hypertension or high blood pressure (BP) is a medical condition characterized by a sustained elevation of resting systolic BP (> 140 mm Hg), diastolic BP (> 90 mm Hg), or both. In the US alone, approximately 75 million people have hypertension (1). There are two forms of hypertension: primary hypertension and secondary hypertension.

The most common form of hypertension, primary hypertension, may develop due to changes in hemodynamic and physiologic components, including plasma volume, activity of the renin-angiotensin system (RAS), malfunctioning of ion pumps on smooth muscle cell membranes and heredity. In the genetically susceptible younger population, environmental factors such as obesity, stress or dietary sodium (Na^+) contribute towards the development of hypertension. In patients above 65, high Na^+ intake is more likely to cause hypertension.

Secondary hypertension is usually due to primary aldosteronism, renal parenchymal disease (chronic glomerulonephritis or pyelonephritis, polycystic renal disease, connective tissue disorders, obstructive uropathy), renovascular disease, Cushing syndrome and hyperthyroidism. Excessive alcohol intake and use of drugs such as oral contraceptives, sympathomimetics, NSAIDS, corticosteroids, cocaine or licorice further

worsen BP control. While there is no cure for primary hypertension, secondary hypertension may be corrected (2).

High BP is a "silent killer" with no symptoms. If left untreated, uncontrolled BP can have dangerous consequences, namely heart and coronary artery damage, stroke, kidney damage, vision loss, erectile dysfunction, memory loss, fluid build-up in the lungs, angina and peripheral heart disease (3).

1.2.Kidneys and BP Maintenance

The kidneys play a crucial role in maintenance and development of normal BP. BP is a product of cardiac output (CO) and total peripheral resistance (TPR). TPR is dependent on extracellular fluid volume (ECFV). ECFV is determined by the amount of total Na^+ in the body. The kidneys help to maintain a balance between dietary Na^+ intake and Na^+ excretion by a variety of mechanisms. An inability of the kidneys to excrete excess Na^+ in order to maintain this balance leads to hypertension. (4). Thus, in the case of hypertension, volume regulation and the relationship between BP and Na^+ excretion becomes skewed. (5). Despite extensive research being carried out, the mechanisms responsible for normal control of BP and the development of essential or primary hypertension are poorly understood. However, Guyton and colleagues have established a strong link between long-term control of BP and the inability of kidneys to excrete Na^+ to maintain normal ECFV at normotensive arterial pressures (6, 7, 8, 9). They proposed the importance of the ability of the kidneys to regulate arterial pressure by excreting appropriate amounts of NaCl to maintain sodium balance and ECFV (Figure 1.1).

Not surprisingly, deviation from normal renal function is involved in the

pathogenesis of primary hypertension. Further, renal dysfunction is the most common cause of secondary hypertension. When left untreated, hypertension may lead to severe renal injury, ultimately resulting in a vicious cycle with more serious consequences such as stroke and death. In a normal person, increase in total body NaCl levels is compensated by different humoral, neural and paracrine mechanisms; these increase natriuresis via systemic and renal hemodynamic adjustments without increasing arterial pressure (8, 10). Renal dysfunction reduces the ability of the kidneys to excrete excess Na^+, leading to chronic hypertension. Increases in arterial pressure lead to an increase in renal Na^+ excretion, which restores Na^+ balance via the mechanism of pressure natriuresis. However, an elevation of arterial pressure is still observed.

Apart from renal dysfunction, several extrinsic factors can also lead to a reduction in the ability of kidneys to excrete excess Na^+. Different physiologic parameters such as CO, TPR and cardiovascular capacitance might be affected. Thus, hypertension can be caused under different conditions which may augment Na^+ reabsorption in the tubule, severely alter filtration capacity in the glomerulus or reduce the Na^+ excretion capacity of the kidneys (11). Hence, understanding the mechanisms affecting Na^+ homoeostasis is essential to developing treatment approaches for hypertension.

The fundamental unit of the kidney is the nephron (12). The nephron carries out almost all of the kidney's functions, namely reabsorption and secretion of ions, carbohydrates and amino acids. Every segment of the nephron has highly specialized functions. The nephron is composed of the proximal tubule, loop of Henle, distal tubule, and the collecting duct (CD).

1.3. The Collecting Duct System

The CD is the final segment of the tubule where the tubule fluid is "fine-tuned". The CD system begins in the renal cortex and continues into the medulla. In response to a variety of stimuli, it determines the final concentration of urine. The CD contains two distinct cell types – principal cells and intercalated cells. The function of the principal cells is to maintain Na^+ and K+ homeostasis via Na^+ and K^+ channels present on the apical membrane of the cells; intercalated cells are involved in acid-base balance maintenance. In humans, the CD is responsible for 4-5% of the total Na^+ reabsorption and 5% of the total water reabsorption taking place in the kidney. Both processes, Na^+ and water reabsorption, are performed under the influence of different hormones (13).

Further, the CD is divided into two major segments – the cortical collecting duct (CCD) and the inner medullary collecting duct (IMCD). In the CCD, the principal cells are responsible for Na^+ transport. The Na^+ channels present on the membrane may either be activated or inserted from subapical compartments in order to reabsorb Na^+.

The CD is inherently impermeable to water. However, both the CCD and IMCD can become water-permeable in a state of dehydration in the presence of the antidiuretic hormone (ADH). ADH allows aquaporins (AQP2), which are water channels, to insert into the apical membrane and reabsorb water as it passes through the CD. In the CD, Na^+ transport is carried out via the amiloride-sensitive Na^+ channel, epithelial Na^+ channel (ENaC) that is located on the apical membrane. Other reports demonstrate the involvement of additional mechanisms such as an electroneutral NaCl cotransporter. The Na^+-K^+-ATPase creates the driving force for transport of Na^+ and is located on the basolateral membrane.

1.4. The Endothelin System

In 1988, Yanagisawa and coworkers purified and cloned a 21 amino acid peptide, which they termed “endothelin” (14). In general, endothelins carry out a number of functions essential to hypertension, atherosclerosis, cardiac disorders, pulmonary disease and renal disease. (15) The ET system is important in systemic BP control and maintenance of Na^+ balance in the body. ET affects different hormonal systems such as the natriuretic peptides, aldosterone, catecholamines and angiotensin. Important physiologic parameters involved in the pathogenesis of hypertension, namely cardiac activity, renal Na^+ and water excretion, systemic vascular resistance and venous capacitance are also directly under the influence of ET. This regulation is complex and is dependent on the receptor subtype activated, the type of cells involved and other relevant factors (16).

1.4.1. The ET-1 Gene, Synthesis and Degradation

The mammalian ET gene family consists of 3 members: ET-1, ET-2 and ET-3 (16). The human ET-1 gene is located on chromosome 6 (17), consists of 5 exons distributed over 6838 bps and encodes a 2026-bp mRNA (18). All 3 peptides contain 21 amino acids and 2 intrachain disulfide linkages (16). ET-1 differs from ET-2 and ET-3 in two and six amino acid residues, respectively. The human ET-1 mRNA consists of a coding sequence or prepropeptide of 212 amino acids. Cleavage of the prepropeptide, upon removal of a short signal sequence by furin and endopeptidase cleavage, yields 38 amino acid big ET-1. The biologically inactive big ET-1 is cleaved by endothelin-converting enzymes (ECE) to produce mature ET-1 (16, 19, 14, 20) (16).

The ECEs belong to a family of M13 proteins, which contains type II integral membrane proteins with zinc metalloprotease activity (21, 22). Additionally, one report also suggests the existence of an ECE-1 independent pathway, which involves tissue chymases and non-ECE metalloproteases in the processing of big ET-1 (23).

The half-life of ET-1 mRNA is very short (~15 mins) due to the presence of 3 destabilizing AUUUA motifs in the 3'-untranslated region (UTR) (18). ET-1 is degraded partly by neutral endopeptidase, which has a neutral optimum pH (24). Additionally, deamidase or the lysosomal protective protein can proteolyze ET-1 at an optimum acid pH (25, 26). Both these enzymes are widely present throughout the body. ET-1 can be degraded by virtually all tissues, especially the kidney (16). In nephrectomized rats, ET-1 causes prolonged BP, thus suggesting that the kidney substantially degrades it (27).

1.4.2. ET Receptors

In mammals, ETs exert their actions through 2 receptor subtypes, ETA and ETB, which belong to the superfamily of G-protein coupled receptors (GPCRs) (28, 29, 30). Both receptor subtypes contain seven transmembrane domains of 22-26 hydrophobic amino acids (31). The ETA receptor is located on chromosome 4, contains 427 amino acids (32) and mediates vasoconstrictive and proliferative responses upon ET-1 binding (16). The ETB receptor is located on chromosome 13, contains 442 amino acids and has more widespread effects related to ET-1 clearance, endothelial cell survival, eNOS signaling, NO production, prostacyclin synthesis and ECE-1 inhibition (33, 34, 16). Almost every cell type expresses at least one of these receptor subtypes. While ETA receptors are predominantly found in vascular smooth muscles and myocytes, ETB

receptors are found in endothelial cells and renal tubules. ETA and ETB have opposite actions in the vasculature. While ETA activation causes vasoconstriction, ETB activation causes vasodilation. ETA stimulation also promotes cell proliferation and fibrosis which is opposite in effect to ETB stimulation (16). After binding, ET-1 can associate with ETA for up to 2 h after endocytosis, thus suggesting that it remains active even upon internalization (35). On the other hand, ETB binding is more short-lived (36). Apart from ETA and ETB, a third receptor subtype ETC has been identified in Xenopus laevis, which when stimulated causes NO release. However, this receptor subtype does not seem to exist in mammals (15, 16).

1.4.3. Endothelin-1 in the Kidney

The kidney is the largest source of ET-1 in the body (37). Cultured cells isolated from renal tubules, mesangium, glomerular endothelium and medullary interstitium synthesize and release ET-1 (38). The renal medulla produces larger amounts of ET-1 in comparison to the cortex in experimental animals and humans (as evidenced by immunohistochemical analysis and mRNA expression) (16, 37).

1.5. Physiological Role of ET-1 in the CD

Overall, ET-1 plays a pivotal role in the control of renal hemodynamics and Na^+ and water excretion. While its inhibitory effects on sodium and water reabsorption are mediated via ETB, its effects on renal hemodynamics are regulated via ETA receptors. These mechanisms are important in case of high dietary Na^+ and water since they help to eliminate excess amounts from the body through the pressure-natriuresis approach (11,

16). This is supported by the fact that ET-1 blunts the ability of AVP to increase water reabsorption by the CD (39, 40, 41). Also, ET-1 inhibits ENaC by decreasing the channel's open probability (P_o) in the isolated CD (42, 43).

Since administration of exogenous ET-1 produces hypertension (44, 45, 46, 47, 48, 49), one might make the assumption that ET-1 raises arterial pressure on account of its activity to maintain an elevated vascular resistance. However, this system is complex due to the opposing actions of ETA and ETB receptors to produce vasoconstriction and vasodilation, respectively. The ET system along with being of importance in the kidney is also active in the regions of the central and peripheral nervous system that are essential to the process of BP control (16).

1.5.1. Studies Using Genetic Models

Global knockout of the ET-1, ETA, and ETB genes in experimental animals results in lethal phenotypes. Knockout of the ET-1 gene causes malformations in the pharyngeal arch-derived structures and the heart. ET-1 null mice die shortly after birth (50). A similar consequence is observed in mice with ETA gene deletion (51). Global ETB gene knockout causes intestinal aganglionosis resulting in death at about 3 weeks of age. Gariepy and colleagues studied ETB deficiency in animal models and observed that genetic deficiency of ETB resulted in elevated BP and salt-sensitive hypertension (52, 53). This study was the first piece of in vivo evidence for ETB receptor-mediated control of BP and natriuresis. However, since mutation of the ETB receptor resulted in death in animals at the time of weaning, it was a poor model for studying hypertension.

Previously, our lab developed CD-specific ETA, ETB and ET-1 knockout mice

using the Cre-lox method. CD-specific knockout of ET-1 and ETB genes resulted in elevated basal BP levels that worsened on a high salt diet, while ETA knockout mice showed normal basal BP and were found to be resistant to the hypertensive effects of a high salt diet (54, 55). Interestingly, ETA/ETB double knockout mice exhibited a greater hypertensive response to a high salt diet than that observed in ETB knockout mice. These findings suggest that along with the ETB receptor, the ETA receptor in the CD might also have a role in BP control and natriuresis. Thus, ETA and ETB receptors seem to function synergistically in the CD (56). Further studies are required to elucidate how the two receptors interact.

1.5.2. Studies Using Pharmacological Agents

Along with genetic models, pharmacological agents have been extremely useful in the field of ET research. Development of highly selective and potent ET receptor antagonists has helped to discern the physiological role of ET-1 in the body. The ETA-selective antagonist BQ-123 has very little effect on renal and cardiovascular function although it does lower BP to some extent. In contrast to ETA, treatment with an ETB-selective antagonist produces severe salt-dependent hypertension quite similar to that observed in genetic models of the ETB receptor knockout (57). Similar to the observations in genetic models, the hypertensive response with ETB antagonism can be reversed with ETA receptor blockade, thus suggesting that the ETB receptor might render protection from ETA-dependent effects by removing ET-1 and promoting vasodilatation (56). Taken together, the pharmacologic and genetic studies suggest that a main physiological role of the ET system in the kidney is regulation of salt and water balance

and ultimately BP. Disturbances in the CD ET system can result in fluid-electrolyte imbalance and hypertension.

1.6. CD ET-1 Regulation of Na^+ Transport

One of the most significant effects of ET-1 in the CD is inhibition of Na^+ reabsorption via inhibition of ENaC activity. ENaC-dependent Na^+ reabsorption is greatly inhibited in isolated CD and CD cell lines when ET-1 is administered exogenously (39, 42, 58, 59, 60). CD-specific knockout of ET-1 in genetic models of experimental animals elevates systolic BP by ~15 mm Hg on a normal Na^+ diet and by ~35 mm Hg on a high Na^+ diet (54). In addition to these effects, an abnormal weight gain and reduced natriuresis was observed in CD ET-1 KO mice. These effects were mitigated by the administration of amiloride, an ENaC-specific inhibitor. Hence, based on these findings, CD ET-1 seems to function as an autocrine inhibitor of Na^+ reabsorption.

The above findings raise possibilities about the normal physiological role of CD ET-1. CD ET-1, owing to its ability to inhibit Na^+ reabsorption, helps to get rid of excess Na^+ in the body as a means to maintain normal ECFV status. Na^+ loading increases tubule fluid flow and Na^+ delivery to the CD which tends to increase CD Na^+ reabsorption; without the presence of the CD ET system, the increased Na^+ reabsorption could leading to worsening of hypertension (61). Hence, the CD ET-1 system may exist, at least in part, to sense increases in CD Na^+ delivery and reduce the tendency of ENaC to further reabsorb Na^+. Thus, the ET-1 system works along with other local factors to mitigate the activity of ENaC in case of salt loading or a high Na^+ diet (Figure 1.2).

1.6.1. Regulation of Na^+ Transport in the CD by ET Receptor Isoforms

In cultured cells and isolated CCD, ENaC inhibition by ET-1 appears to be ETB-receptor mediated (42, 58, 62). In CD-specific ETB KO mice on a normal salt diet, a ~8 mm Hg increase was observed while a ~20 mm Hg increase in systolic BP in ETB KO mice on a high salt diet was observed, which was associated with impaired natriuresis (55). Quite interestingly, the extent of hypertension in CD-specific ETB KO mice was only about half of that observed in CD-specific ET-1 KO mice, thus implying that along with CD ETB receptors there might be another factor contributing to the antihypertensive effects of CD ET-1. This raises the possibility of the involvement of ETA receptors. However, the data obtained on ETA-mediated inhibition of ENaC are complex.

Interestingly, in isolated split-open CCD studies, an effect of ETA blockade on ENaC activity was not observed (42, 62). Also, CD-specific ETA KO mice did not exhibit any changes in BP or natriuresis (63). However, CD-specific ETA/ETB combined KO mice showed a higher degree of hypertension on a normal as well as high Na^+ diet along with significantly reduced natriuresis compared to what was observed with only the CD ETB KO mice (64). These data bring into question the interaction between ETA and ETB receptors in the CD. Obviously, these receptors interact somehow but there have been little to no studies to demonstrate their interaction. A recent finding regarding ET-1 inhibition of transepithelial Na^+ flux in isolated perfused CCD and the effect of ETA or ETB blockade suggests that both the isoforms of the ET receptor may affect CD Na^+ transport (65). Also, ETA receptor nephron KO in experimental animals causes mild fluid volume retention (66). Clearly, this issue needs further investigation.

1.6.2. Mechanisms of ET Regulation of CD Na^+ Transport

Different signaling pathways are involved in the ET-1 regulation of CD Na^+ transport.

1.6.2.1. Src and MAP Kinases

Src and mitogen-activated protein kinases (MAPK) have been thought to be involved in the inhibition of ENaC. ENaC open probability (P_o) inhibition via ETB seems to be Src kinase-dependent in studies in cultured cells and isolated rat CCD (42, 59). In cells transfected with all isoforms of ENaC, ET-1 decreases ENaC activity through a Src kinase-dependent pathway although direct phosphorylation of any of the ENaC subunits was not observed. Rapid inhibition of ENaC Po and activation of Src kinase and MAPK 1/2 by ET-1 via ETB was observed in split-open rat CCDs (67). Upon blockade of Src and MAP kinases, inhibition of ENaC by ET-1 did not occur. Additionally, ET-1 stimulates MAPK activity in rat outer medullary CD and IMCD. Thus, inhibition of CD ENaC by ET-1 is mediated, at least in part, by stimulation of Src kinase by ETB which leads to activation of MAPK (42). Further, MAPK signaling results in phosphorylation of ENaC, thereby leading to its inhibition (reduction in ENaC P_o and channel number).

1.6.2.2. Nitric Oxide Signaling

ET-1 inhibition of CD Na^+ reabsorption is also likely to be partly mediated by NO. ET stimulates NO production in the CD. In cultured rat IMCD cells, NOS1-dependent NO production and cGMP accumulation is stimulated by ET-1 via ETB activation (68, 69). In m-IMCD3 cells (a mouse IMCD cell line), NOS1 expression is

augmented by high concentrations of ET-1 (50 nM). This effect may be ETA-mediated (69). Blockade of ETA or ETB significantly reduces NOS3 mRNA and protein expression in rat IMCDs. In CD ET-1 KO mice fed either a high or normal Na diet, reduction in urinary nitrate/nitrite excretion was observed as compared to normal mice (64). Additionally, CD ET-1 KO mice, as compared to control mice, had decreased augmentation of excretion of NO metabolites in the setting increased renal perfusion pressure. In the same study, inner medullary NOS1 and NOS3 activities were significantly lower in CD ET-1 KO mice as compared to controls. Further, NOS inhibition significantly increased BP in control mice as compared to a reduced elevation in BP in CD ET-1 KO mice. These data suggest that lack of NO is most involved in the high BP in CD ET-1 KO animals (70). Additionally, experimental evidence suggests that NO significantly lowers ENaC Po in renal and mouse CCD cell lines (71).

The above findings provide convincing evidence that inhibition of CD Na^+ transport by ET-1 is partly mediated by NO. However, further studies are needed in order to elucidate the mechanisms by which NO exerts this effect.

1.6.2.3. COX and COX Metabolites

Initial studies found that ET-1 stimulates accumulation of COX metabolies in the CD. Blockade of COX prevented the inhibitory effect of ET-1 on CD Na^+ transport (72). Additionally, PGE2 production in rabbit and mouse IMCDs by ET-1 is ETB-mediated. This effect is also COX2 dependent (72, 73). Interestingly, blockade of COX or COX2 did not affect Na^+ retention or BP in CD ET-1 KO mice (74). Thus, there is controversial evidence regarding COX-mediated natriuretic effects of ET in the CD.

1.7. CD ET-1 Regulation of Water Transport

ET-1 promotes diuresis due to inhibition of water reabsorption in the CD. AVP-stimulated water permeability in rat CCD and IMCD is inhibited by ET-1 in a dose-dependent manner (39, 41, 75, 76). CD ET-1 KO mice exhibit an impaired ability to excrete a water load as compared to control mice. Additionally, CD ET-1 KO mice also had reduced plasma AVP levels (77). These findings suggest that ET-1 KO in the CD increases sensitivity towards the urine concentrating effects of AVP and that the CD is regulated by ET-1 in an autocrine manner.

1.7.1. Regulation of Water Transport in the CD by ET Receptor Isoforms

The inhibitory effect of ET-1 on water transport is ETB-mediated. BQ123, a specific ETA antagonist did not prevent the activity of ET-1 in inhibiting AVP-induced cAMP accumulation in rat IMCDs (39, 73, 78). Additionally, CD ETA KO mice show elevated plasma AVP levels and an increased ability to excrete a water load. IMCD suspensions from these mice show reduced AVP and forskolin-stimulated cAMP accumulation (63). Thus, CD ETA may be antidiuretic in nature. Based on the above data, ETB may indeed be the main inhibitor of water transport in the CD. However, in order to draw definitive conclusions about the mechanism by which ET-1 inhibits water transport, it is necessary to perform similar studies in CD ETB KO and CD ETA/ETB KO animals.

1.7.2. Mechanisms of ET Regulation of CD Water Transport

1.7.2.1. Adenylyl Cyclase

The inhibitory effect of ET-1 on AVP-stimulated water transport in the CD is mediated via reduction of adenylyl cyclase (AC)-dependent cAMP accumulation. This inhibition of cAMP accumulation is essential to the inhibitory effect of ET-1 on water permeability. Studies in rat IMCDs show that ET-1 does not decrease dibutyryl cAMP-stimulated osmotic water permeability (68). Further, CD ET-1 KO mice exhibit increased AVP and forskolin-stimulated cAMP accumulation, thus suggesting that lack of ET-1 increases AC activity (73). Additionally, antibodies against ET-1 enhanced AVP-stimulated cAMP accumulation in IMCD cells (79). All these findings suggest that ET-1 inhibits AVP action in an autocrine fashion.

1.7.2.2. Calcium

Various studies demonstrate protein kinase C (PKC) as a regulator of the inhibitory effect of ET-1 on AVP-stimulated osmotic water permeability and cAMP accumulation in rat CD (73, 76, 78, 80, 81). ET peptides stimulate accumulation of inositol phosphates (IP) in rat IMCDs. PKC activation and IP accumulation may be related to ET-1 stimulated elevation in $[Ca^{2+}]_i$. In acutely isolated mouse tubules or cultured porcine CD cells, ET-1 increases cell Ca^{2+} in a biphasic manner. Further, studies in experimental animals suggest that the initial peak is transient and is mainly due to Ca^{2+} release from $[Ca^{2+}]_i$ stores, while the second sustained elevation is due to Ca^{2+}-entry through L-type Ca^{2+} channels (60, 76, 82, 83). This $[Ca^{2+}]_i$ increase inhibits AVP-stimulated AC activity in rat IMCDs via phospholipase C (PLC)-mediated activation of

PKC (84), thus suggesting that $[Ca^{2+}]_i$ may be involved in the ET-1 inhibition of water transport in the CD.

1.7.2.3. COX and NO

As mentioned previously, ET-1 augments production of PGE2 and NO. These mediators may potentially be involved in ET regulation of water transport in the CD. However, inhibition of COX does not prevent ET-1 inhibition of AVP-stimulated cAMP accumulation in cultured cells and isolated tubules of the CD (73, 78, 80). While NO inhibits AVP-induced water permeability and cAMP elevations in rat CCD, blockade of NOS had no effect on the same in suspensions of IMCD. While NO inhibitors reduced water permeability, NO donors did not alter the response in IMCD cells (68). Thus, NO might be regulating ET-1 inhibition of AC activity differently in the CCD and IMCD. However, neither COX nor NO have been unequivocally demonstrated to have a role in ET-1 regulated inhibition of water transport in the CD.

1.8. Fluid Flow Regulation of CD ET-1 Production

Under physiological conditions, the production of CD ET-1 is mediated by factors related to ECFV status (Figure 1.3). Na^+ and water loading in experimental animals raises ET-1 levels in the urine (54, 64, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94), mRNA in the renal medulla (54, 95) and acutely isolated tubules (96). In response to a high salt diet, mice deficient in the ET-1 gene show significantly reduced urinary ET-1 excretion (54).

Thus, Na^+ is important in the production of ET-1, thereby establishing a link between ECFV status and CD ET-1 synthesis. However, how ECFV expansion might

increase CD ET-1 is poorly understood. Hormones such as vasopressin and atrial natriuretic peptide do not conclusively affect CD ET-1 production. Although *in vitro*, aldosterone increases CD ET-1 levels, it cannot explain how volume expansion increases CD ET-1 levels (97, 98, 99). Medullary Na^+ concentration might also raise CD ET-1 levels. High interstitial tonicity elevates ET-1 production in the thick ascending limb (100). However, the data from the *in vitro* studies are conflicting. *In vitro*, increasing media NaCl concentration increases (98, 99, 101) as well as decreases (89, 99, 102) ET-1 levels. Also, an increase in tonicity does not elucidate how both Na^+ and water loading modulate CD ET-1 synthesis.

Another important factor to consider that is very relevant to this dissertation is tubule fluid flow. Our group has previously shown that flow is an important regulator of CD ET-1 synthesis in mpkCCDc14 cells, a mouse cortical collecting duct cell line. In these cells, flow increases CD ET-1 mRNA at shear stresses between 2 and 10 dyne/cm^2. The ET-1 response to flow is mediated by $[Ca^{2+}]_i$ and Ca^{2+}-signaling pathways under both flow and stationary conditions. However, these studies were performed in CCD cells and they do not explain how flow might regulate ET-1 production in the IMCD (96). The CCD and IMCD are physiologically different. Hence, one might assume that ET-1 production might be differently regulated in these two segments. While Na^+ is reabsorbed mainly in the CCD, the IMCD is mainly involved in water reabsorption. Thus, Na^+ delivery might not necessarily contribute to elevated IMCD ET-1 production. Total body water excess also occurs in ECFV expansion and may contribute to increased tubule fluid flow in the CD. The findings in this dissertation highlight the differences between the CCD and IMCD physiologically in the context of flow, with Na^+ delivery being the

major factor responsible for flow-regulated ET-1 synthesis in the CCD, and water and solute delivery being the major players in IMCD ET-1 production. Thus, the pathways by which ET-1 is regulated in the CD are extremely complex and depend heavily on the segment of the CD in which it is being synthesized.

In agreement with the findings in our flow system, similar conclusions were drawn from primary cell culture. In primary rat IMCDs, Ca^{2+}-signaling molecules such as calmodulin, calmodulin kinase and calcineurin in addition to intracellular and extracellular calcium appear to regulate ET-1 synthesis (103). ET-1 synthesis occurs at the transcriptional level. Rat ET-1 promoter-reporter constructs have a calmodulin-sensitive region between 0.56 and 1.9 kb 5' to the transcriptional start site in the IMCD (103). In addition to this, two consensus NFATc binding sites were identified at -1263 and -1563 bp 5' to the transcriptional site as well. Upon using mobility shift assays, our group found that ET-1263 bound NFATc1 while ET-1563 bound to NFATc3. Site-directed mutagenesis of either of the two ET-1 sites prevented binding to NFATc(s) and ET-1 promoter activity was greatly reduced (104). In summary, Ca^{2+}, calmodulin, calmodulin kinase, calcineurin, NFATc and PKC (105) seem to regulate CD ET-1 gene synthesis at the transcriptional level. Our future studies will determine how these relate to the flow response and whether their involvement in ET-1 synthesis is dependent on the region of the CD where ET-1 is being synthesized.

1.9. Major Flow Sensors in the CCD and IMCD

1.9.1. ENaC

A large amount of NaCl (1.7 kg) and water (180 L) is reabsorbed by renal tubules. The CD controls the absorption of approximately 10 – 15% of the filtered water under the influence of AVP and about 5% of the filtered Na^+; the process is under the control of aldosterone and the natriuretic peptide. Salt reabsorption is carried out by the principal cells which reabsorb Na^+ and secrete K^+. The major channel through which Na^+ is reabsorbed is luminal ENaC; the driving force is generated by the basolaterally located Na^+-K^+-ATPase (107). ENaC regulates Na^+ entry into the principal cells apically; it also augments the process of K^+ secretion from K^+ channels such as ROMK and BK channels along with increased H^+ secretion (108). ENaC has three structurally related, distinct subunits (α, β and γ) (Figure 1.4) (109) and is highly selective for Na^+ over other monovalent ions and the diuretic amiloride (a selective and potent ENaC inhibitor) (110). The activity and expression of ENaC is regulated by a variety of factors (hormonal, nonhormonal, autocrine and paracrine) to achieve total body Na^+ levels that meet physiologic demands. ENaC is organized into a large multiprotein complex, the ENaC regulatory complex (ERC). Regulatory molecules of the ERC interact with the cytoplasmic domains of ENaC in order to form a stable complex. This requires an aldosterone-induced chaperone (GILZ1) and a scaffold protein (CNK3). Although the ERC is required for the stability of ENaC, proteolytic cleavage at specific sites with the extracellular loops is needed for its activity (112, 113). ENaC is under hormonal regulation by different systems, namely the renin-angiotension-aldosterone system, ANP, insulin and AVP. Additionally, ENaC is also regulated in an autocrine and paracrine

manner in the CD by adrenergic nerves, prostaglandins and cytochrome P450 metabolites, kinins, PPARs, adenosine, ATP, renin, and most importantly to this discussion, fluid flow and ET-1 (110). In natriuretic states, there is an increase in tubule fluid flow in the CD. In the case of salt loading, this occurs as a result of an increase in glomerular filration rate (GFR) and decreased solute reabsorption in the nephron prior to reaching the CD. Previous findings indicate that ENaC is sensitive to Na^+ loading and shear stress (114). Shear stress stimulates the production of ENaC inhibitory factors which include 11-EET (epoxyeicosatrienoic acid), 12-EET, ATP, NO, PGE2 and ET-1 (96, 115, 116, 117, 118). Since this dissertation focusses on ET-1, the following text puts emphasis on this particular aspect. CD-derived ET-1 acts on basolaterally present ET receptors in order to inhibit Na^+ transport. Activation of ETB receptors in the CD by ET-1 results in inhibition of Na^+ transport in the CCD, an effect which is mediated by factors such as NO (16, 62). In experimental animals, ET-1 or ET receptor knockout results in marked salt-sensitive hypertension (54, 119). Clinically, these findings are extremely relevant since a significant side-effect of using ET receptor antagonists for the treatment of pulmonary hypertension and diabetic nephropathy appears to be fluid retention. This might be caused due to blocking CD ET receptors. (120)

1.9.2. Primary Cilium

The kidneys are not only excretory but also “sensory” organs. Renal epithelial cells possess a solitary non-motile primary cilium that protrudes into the tubular lumen (Figure 1.5) (124). It is important to note that, within the CD, only principal cells express primary cilium. This organelle has sensory functions and acts as a mechanosensitive

urinary flow detector, thus getting involved in a variety of paracrine signaling events. 4 μm in length, although non-motile, it has limited flexibility that allows slight mechanical deformation. Otherwise similar in structure to its motile counterpart, it differs in the arrangement of its microtubules. The primary cilium has an inner skeleton called the axoneme that emerges from the mother centriole (121, 122, 123). Cross-sectional examination of the axoneme reveals a 9+0 arrangement of the microtubules as opposed to a 9+2 arrangement in case of motile cilia. The ciliary structure is further ensheathed in the plasma membrane that is derived from the apical membrane of the epithelial cell (121) (Figure 1.6) (129). Earlier, the primary cilium was thought to be a visceral organelle. However, in 1997, Samuel Bowser's group imaged primary cilia in cultured renal epithelial cells. They also observed bending of the cilium in response to fluid flow. The degree of bending was found to be proportional to the flow rate. In extension of these findings, researchers discovered that bending of the primary cilium of MDCK cells (a CD-like cell line) triggered a strong intracellular Ca^{2+} increase (125). This response was not observed in cells lacking primary cilium or in cells where the primary cilium was removed by chloral hydrate treatment. This was the first report of primary cilium being a flow sensor (126). Additionally, in mice with defective primary cilia as a result of a mutation in the Tg737 gene (which encodes for polaris, a protein necessary for ciliogenesis and ciliary assembly), a blunted Ca^{2+} response to flow was observed (127). Primary ciliary bending also involves distension of the renal tubule during elevated flow conditions (122, 127, 128). Hence, the mechanical stimulus due to flow is a complex process and definitely involves other pathways that are equally important for the flow response. These pathways and their effects will be described in subsequent sections.

1.9.3. Polycystins

The polycystin (PC) family comprises of two subfamilies - PC1-like and PC2-like proteins. PC1-like proteins consist of PC1 (130, 131), PC-REJ (132), PC-1L1 (133) and PC-1L2 (134) which function as GPCRs (135, 136). PC2-like molecules are ion channels and consist of PC2 (137), PC-L (138, 139, 140) and PC-2L2 (141). While the disease-causing potential of the rest is unknown, PC1 and PC2 mutations cause polycystic kidney disease (PKD), a leading cause of renal failure. This disease is characterized by large cysts filled with fluid in the kidney (142). PC1, encoded by the gene *pkd1*, is a 460 kDa membrane glycoprotein with a large extracellular amino terminus, 11 transmembrane domains and a small carboxy terminus (130, 131). It is known to function as a GPCR (135) and is found on the plasma membrane and cell-cell junctions of cells and tissues (143, 144, 145). PC2, encoded by the gene *pkd2*, is a 110 kDa membrane protein (137). Owing to its sequence homology to ion channels, it has a pore-forming ability. Mutations in either PC1 or PC2 gives rise to similar phenotypes and the two proteins interact with each other through coiled-coil domains *in vitro*, thus suggesting that together PC1 and PC2 form a functional complex (146, 147). PC2 is present in both the ER, the plasma membrane (149) and more relevantly, found in the basolateral membranes of renal tubules (150).

PC1 and PC2 are colocalized in the primary cilium and convert mechanical stimuli into Ca^{2+} signaling responses with the help of ryanodine receptors (Figure 1.7) (148). PC2 alone can mediate $[Ca^{2+}]_i$ increase; PC2 translocates to the plasma membrane in the presence of PC1. Zhou and colleagues discovered that in mouse embryonic cells, antibodies specifically against PC2 blocked Ca^{2+} entry into cells in the context of flow.

Their data further suggest that PC1 is the primary sensor of flow. The tight association between PC1 and PC2 results in Ca^{2+} influx, further triggering $[Ca^{2+}]_i$ release inside the cytoplasm through Ca^{2+}-induced Ca^{2+} release (151). These changes in $[Ca^{2+}]_i$ are important for a number of physiologically events, including modification of gene expression and most relevant to the context of this dissertation, ET-1 synthesis. Additionally, the important proteins polaris and cystin are localized to primary cilia (152, 153, 154). Cells with mutant PC1 do not respond to flow similar to cells lacking polaris (151). Thus, while PCs are essential, they together with primary cilia are critical for normal kidney cell structure and function. Defects either in PC system or primary cilia leads to tubule dilatation and cyst formation (151). In patients with polycystic kidneys, hypertension is observed very early on in the disease, even before PKD is diagnosed (155, 156). This observation is of clinical significance to the findings reported in this dissertation. In cells lacking PC1, PC2 or primary cilium, we observe no increase in ET-1 mRNA levels in the setting of flow (unpublished data). Thus, along with other physiologically relevant responses, the cilia together with PCs seem to be essential to the process of mechanosensation and salt and water homeostasis by being involved in the ET-1 flow response.

1.9.4. Purinergic Signaling and ATP

In the early 1900s, the biological effects of extracellular nucleotides was first discovered by Drury and colleagues (157). In the 1970s, Geoffrey Burnstock observed that extracellular ATP conducted nonadrenergic and noncholinergic neurotransmission in the gut and urinary bladder (158). ATP, a nucleoside, activates its receptors either in a

paracrine or autocrine manner, thus regulating a variety of cellular functions. ATP signaling in CD cells occurs via purinergic receptors. In response to flow, ATP is release upon ciliary bending (Figure 1.8) (159). Upon release, ATP activates purinergic receptors (P2) and thus modulates processes downstream of purinergic receptors (159). In the mammalian system, there are two subclasses of P2 receptors – P2X and P2Y. P2X receptors are a class of 7 ligand-gated channels (P2X1-7) while P2Y receptors are G protein-coupled receptors (P2Y1,2,4,6,11,12,13,14).

All these receptors are found in the kidney (160). These receptors are expressed in both principal and intercalated cells. In this context, it is important to note that while ATP response to flow is observed in the principal cells it is also observed in intercalated cells which lack primary cilia (128). Thus, there seems to exist non-ciliary dependent ATP release from these cells. Additionally, in flow experiments performed in renal tubules lacking P2Y2 receptors, a reduced response to flow (no increase in $[Ca^{2+}]_i$) was observed. Further experiments were performed using a non-specific P2 receptor antagonist suramin and apyrase, an ATP scavenger which also reduced the flow response significantly (121).

Taken together, these findings suggest that ATP is released in response to tubule fluid flow followed by P2 receptor activation. In ciliated cells, flow stimulates ATP release (161). In renal epithelial cells isolated from a patient with ADPKD with a defect in PC-1, flow-stimulated ATP release was significantly reduced (162). Also, genetic rescue of orpk mouse epithelial cells (lacking normal cilia) resulted in rescued ATP secretion by the re-expression of primary cilia when triggered by fluid flow (163). Hence, intact cilia appear to be important for flow-induced ATP release. Thus, the primary cilium acts as a fluid flow sensor, bending of which in response to fluid flow triggers

nucleotide (ATP) release which activates P2 receptors in either an autocrine or paracrine fashion, thus resulting into $[Ca^{2+}]_i$ increase. Since $[Ca^{2+}]_i$ increase is important for ET-1 synthesis, it reasonable to hypothesize that the above signaling events lead to increased ET-1 gene transcription.

Pharmacological activation of purinergic receptors inhibits salt and water reabsorption through autocrine and/or paracrine mechanisms. When P2 receptors are locally absent, an increase in Na^+ absorption is observed which can lead to hypertension (164). Thus, activation of P2 receptors by ATP (Figure 1.9) provides a clinically significant antihypertensive effect (165).

Primary cilia are also associated with vasopressin V2 receptors (166). These receptors are found in greatest abundance on the basolateral side of CD cells, but also are localized to the ciliary membrane. When V2 receptors are stimulated, cAMP and $[Ca^{2+}]_i$-dependent trafficking of AQP2 channels to the apical side occurs. In patients with defective cilia (for example, in the Bardet-Beidl syndrome), the apical AVP response is lost – cAMP production is blunted and AQP2 channels are not transported to the apical side (167). These data suggest that primary cilium is essential to the ability of AVP to regulate water permeability. It is quite interesting that AVP-induced stimulation of V2 receptors releases ATP in the TAL and the CCD (120). Thus, ATP in the kidney tubule plays a natriuretic and diuretic role thus facilitating excretion of excess Na^+ and water. ATP release occurs either via lytic or non-lytic pathways. However, it is important to define the molecular mechanism by which ATP is released. Extensive research has suggested two major pathways. ATP may secreted via a conductive channel or vesicular exocytosis (168). In a variety of cell types, pannexin-1, a plasma membrane protein. is

responsible for ATP release. However, in the renal epithelium the mechanism of ATP release is poorly understood. In CD intercalated cells, another conductive pathway involving connexin 30 has been found to be involved in ATP release (169). In the TAL, a basolateral hemichannel connexin 37 might be releasing ATP (170). However, further studies need to be carried out in order to clearly define the exact mechanism of ATP release in the CCD and IMCD. Collectively, all these systems- ciliary, ATP and the purinergic system appear to work in concert to facilitate natriuresis and diuresis (Figure 1.10) (142). Along with flow, the above systems potentially regulate CD ET-1, which further helps to maintain salt and water homeostasis. This dissertation focusses on different signaling pathways in the CD that are responsible for ET-1 synthesis, thereby leading to maintenance of BP (Figure 1.11).

1.10. ET-1 and Fluid Retention due to ET Antagonists

ET-1 has a number of physiological and pathophysiological effects. Overexpression of ET-1 is observed in different diseased states such as arterial hypertension, pulmonary arterial hypertension, atherosclerosis, myocardial infarction, cancer, kidney disease, etc. (171). In experimental models, ETA has been implicated as a pathophysiolgoical mediator of ET-1 actions, including induction of cell proliferation, hypertrophy, fibrosis, increased vascular resistance, insulin resistance and inflammation (172). Accordingly, clinical trials have been largely conducted using antagonists against the ETA receptor. While these were being studied for conditions such as congestive heart failure, arterial hypertension and kidney disease, their clinical utility has been limited due to their potential to cause fluid retention (Avosentan at Phase III) (120).

How ETA blockers cause fluid retention has been a highly clinically relevant question. Administration of ETA blockers to mice causes fluid retention; however, this is prevented in mice lacking ETA receptors in the whole nephron or just in the CD. Hence, it is apparent that CD ETA blockade might be the major reason behind ETA antagonists causing fluid retention (173). Although these studies are in mice, these findings are highly relevant to humans. ETA antagonist-induced fluid retention might be due to blockade of the action of ET-1 in the CD. Thus, further studies on CD ET-1 and its receptors will definitely help in gaining a better understanding of Na^+ and water handling by the CD and ultimately guide use of ET blockers in clinical conditions. This dissertation will provide insights into how the CD ET system operates which may ultimately lead to development of improved therapeutic strategies using ET receptor antagonists.

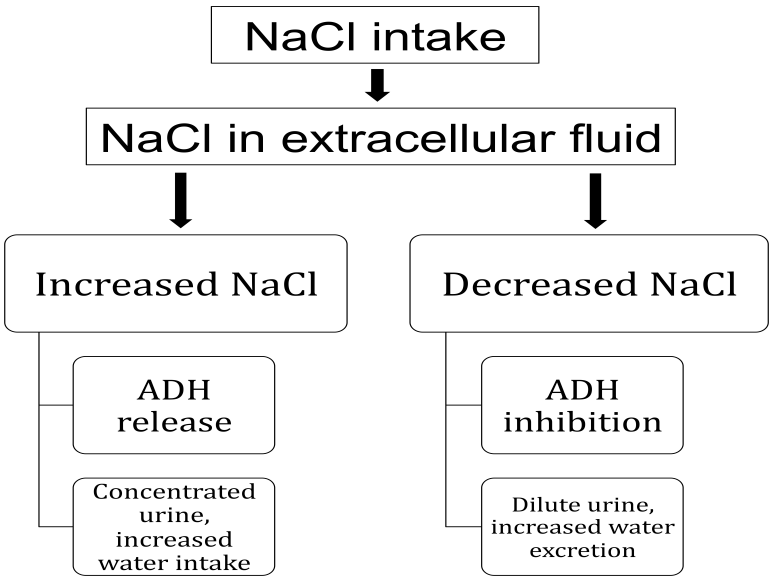

Figure 1.1. Schematic of the relationship between net sodium balance and extracellular fluid volume

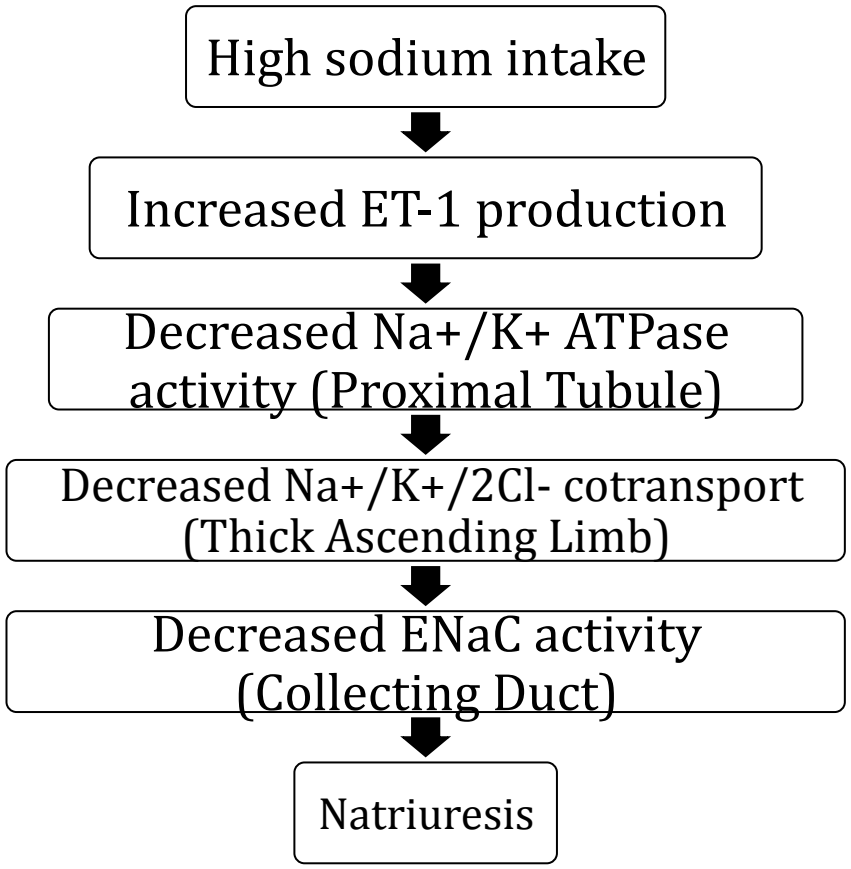

Figure 1.2. Schematic of the renal response of ET-1 to salt loading

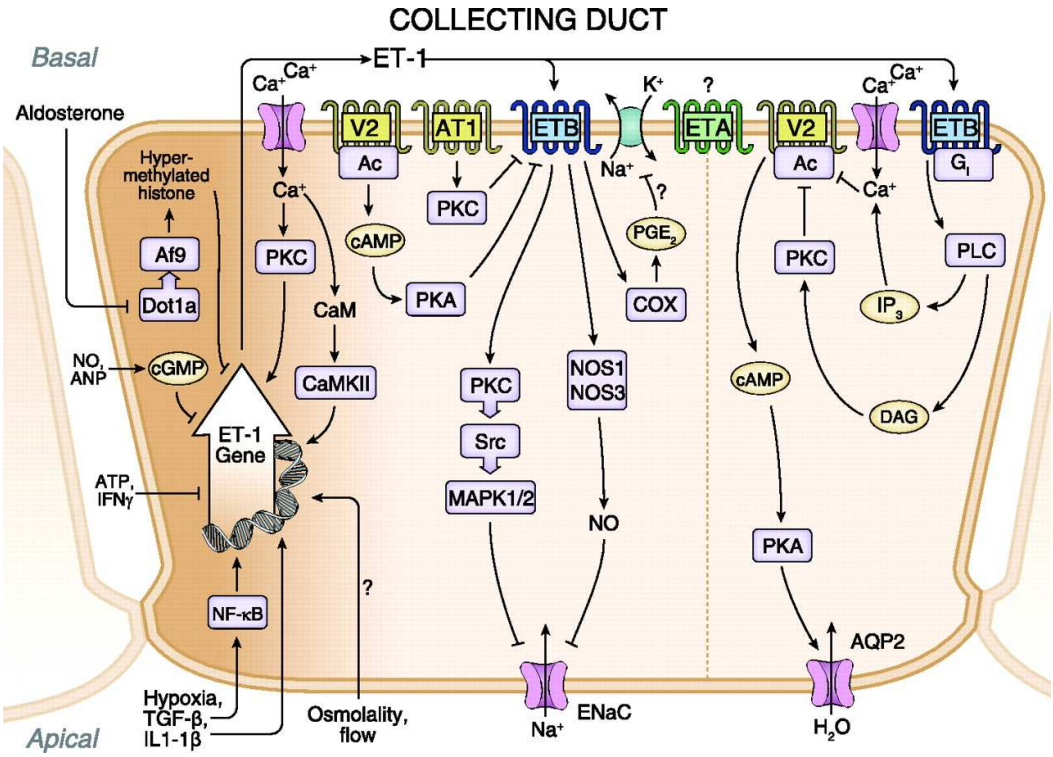

Figure 1.3. Synthesis and actions of ET-1 in the CD. Permission is not required for (16)

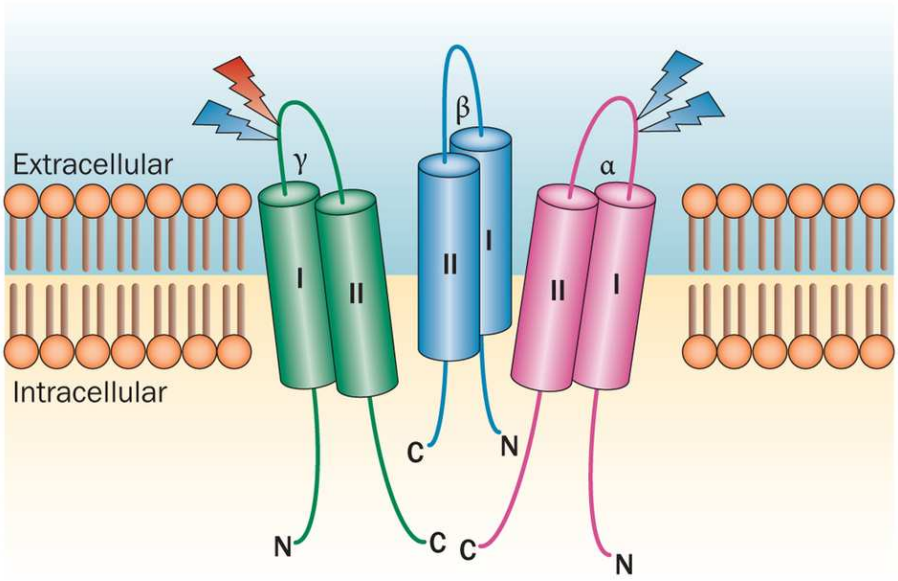

Figure 1.4. Structure of ENaC. Reprinted with permission from Nature Publishing Group (111)

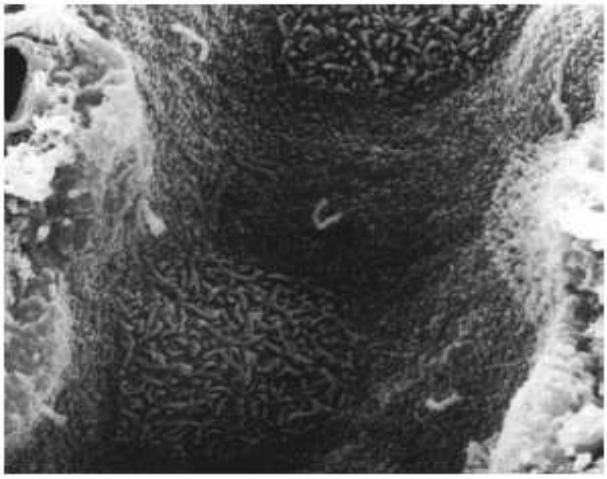

Figure 1.5. Ultramicroscopic view of the primary cilium in rat inner medulla. Reprinted with permission from Nature Publishing Group (124)

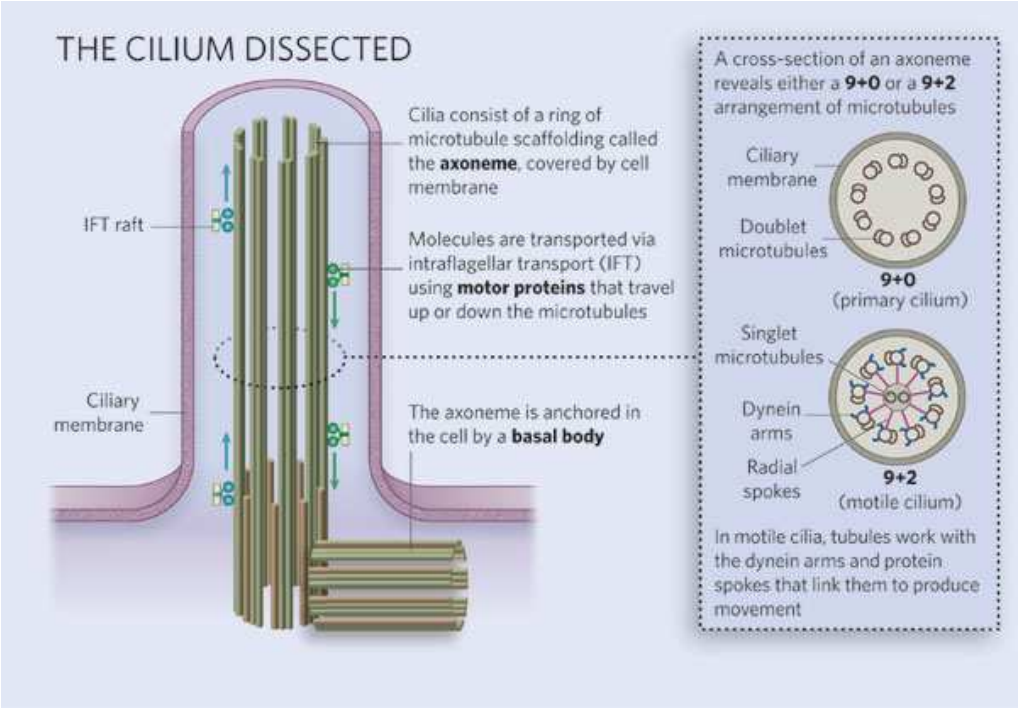

Figure 1.6. Structure of the primary cilium. Reprinted with permission from Nature Publishing Group (129)

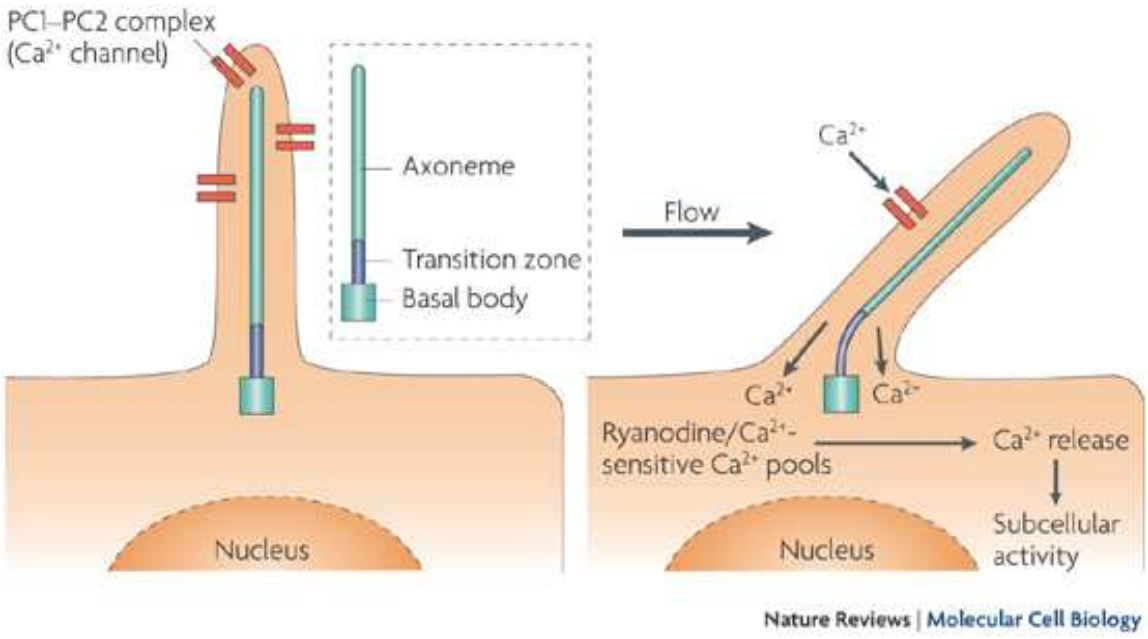

Figure 1.7. Polycystins mediate mechanosensation. Reprinted with permission from Nature Publishing Group (148)

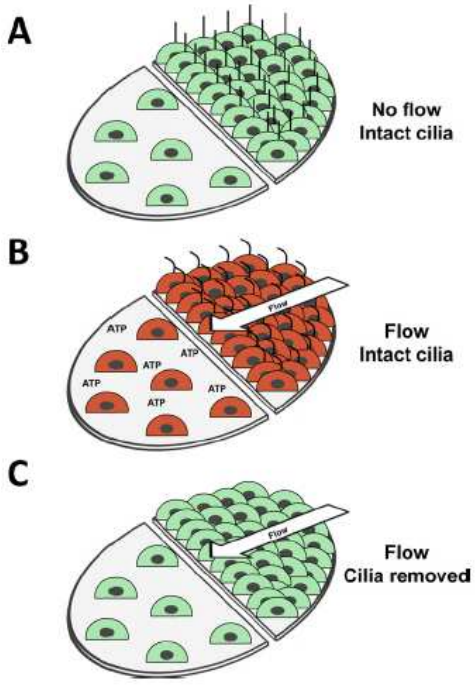

Figure 1.8. ATP release in response to flow - with and without cilia. Reprinted with permission from Elsevier Limited (159)

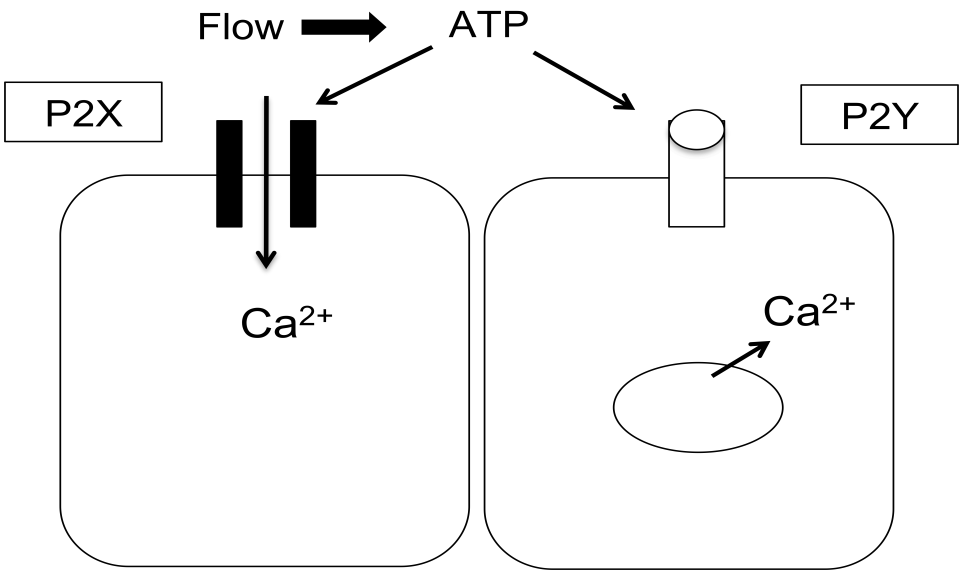

Figure 1.9. Flow-induced activation of P2 receptors

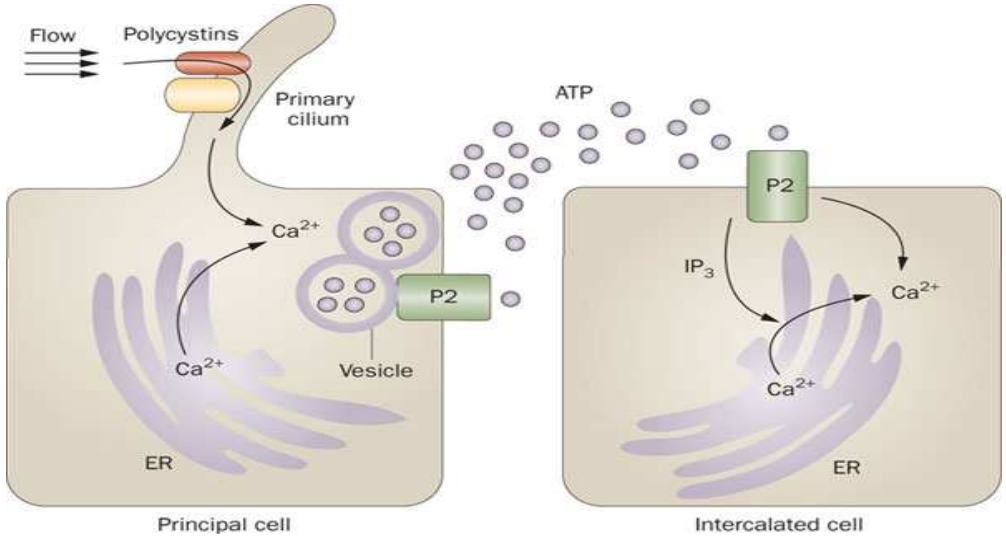

Figure 1.10. Flow sensing by ciliary and purinergic systems. Reprinted with permission obtained from Nature Publishing Group for (142)

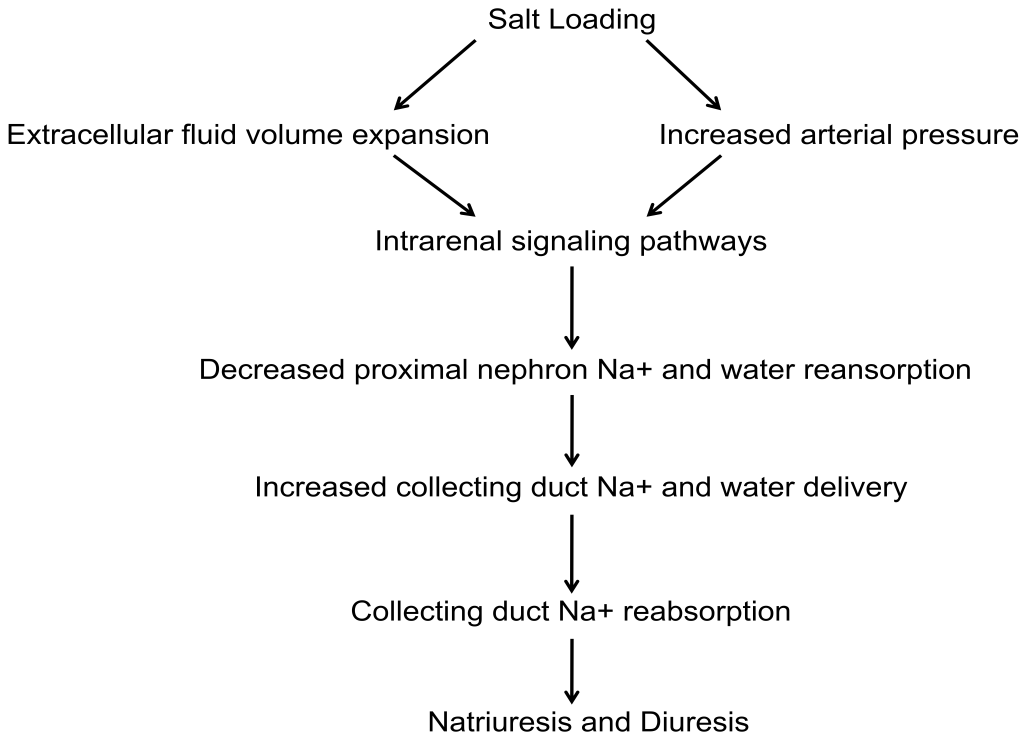

Figure 1.11. Summary of ET-1 regulation of renal salt and water handling. Adapted from and permission not required for (61)

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CHAPTER 2

INTRODUCTION

2.1 Overview of the Dissertation

CD-derived ET-1 is an autocrine inhibitor of sodium and water reabsorption (1, 2, 3, 4). The CD produces the largest amounts of ET-1 as compared to any other cell type in the body (5, 6, 7). A variety of factors are responsible for CD ET-1 production. Under physiological conditions, factors associated with ECFV status, namely, Na^+ (8, 9, 10, 11, 12) and/or water loading (13, 14), regulate the synthesis of CD ET-1. However, the mechanisms by which ECFV status regulates CD-derived ET-1 production are poorly understood. Na^+ loading appears to increase CD ET-1 accumulation in experimental animals (15). The role of hormones related to Na^+ excretion has been studied with regard to CD ET-1 synthesis. Aldosterone stimulates ET-1 synthesis but this does not explain how Na^+ loading might regulate CD ET-1 production (16). Other hormones such as atrial natriuretic peptide, angiotensin II and vasopressin (17, 18) also do not explain how Na^+ delivery to the CD and CD ET-1 production might be related. Hence, some other mechanism for coupling ECFV status to CD ET-1 must exist.

In addition to Na^+ loading, water loading increases urinary ET-1 excretion in experimental animals and humans; the mechanism is unknown. Since urinary ET-1 is derived largely from the collecting duct, this suggests that there exists a relationship

between ECFV status and CD ET-1 production.

Volume expansion causes more Na^+ and water to reach the CD; both increase flow in the nephron. Studies suggest that Na^+ and/or water loading increases urinary and medullary ET-1 in experimental animals. Thus, flow per se may be an important regulator of CD ET-1 production. However, the mechanisms by which this occurs are poorly understood.

Another interesting possibility is that flow regulates ET-1 production differently in different segments of the CD. The CD has two major segments – cortical collecting duct (CCD) and inner medullary collecting duct (IMCD). While, the CCD reabsorbs more Na^+, the IMCD is involved in water reabsorption. To date, very little is known about how flow regulates ET-1 synthesis in the CD. This dissertation elucidates complex signaling pathways involved in coupling ECFV status to CD ET-1 production, and defines differences in such regulation between the CCD and IMCD.

2.2 Aims and Scope of the Dissertation

This dissertation focuses on flow-regulated ET-1 production in two regions of the collecting duct, namely, the CCD and IMCD. In order to understand the mechanisms involved, three major aims were pursued.

Specific Aim 1: To determine the mechanisms responsible for flow-regulated ET-1 mRNA production in the CCD (Chapter 3).

Hypothesis: Tubule fluid flow augments CD ET-1 mRNA production in mpkCCDc14, a mouse CCD cell line.

Rationale: The CD ET-1 system perceives changes in ECFV status. Dietary Na^+

increases urinary ET-1 excretion and CD ET-1 production in experimental animals. Na^+ loading results in increased tubule fluid flow in the CD. However, while flow increases CD ET-1 production, the mechanisms by which Na^+ per se might increase ET-1 production are poorly understood. Thus, this aim explains the mechanisms by which Na^+ delivery might regulate ET-1 production in the CCD.

Specific Aim 2: To determine the mechanisms responsible for flow-regulated ET-1 mRNA production in the IMCD (Chapter 4).

Hypothesis: Tubule fluid flow augments ET-1 mRNA production in m-IMCD3, a mouse IMCD cell line.

Rationale: The IMCD is mainly responsible for water reabsorption. CD-derived ET-1 directly inhibits water reabsorption, thus promoting diuresis and maintenance of water balance in the body. Water loading causes an increase in tubule fluid flow in the kidney. Unlike in the CCD, ENaC, a major Na^+ channel, is much less abundantly expressed in the IMCD. Hence, flow-regulated ET-1 production in the IMCD may be Na^+ and ENaC-independent. Additionally, the IMCD produces significantly more ET-1 than the CCD (19), suggesting possibly unique regulatory mechanisms. Taken together, the above factors suggest that IMCD ET-1 may be differentially regulated as compared to CCD; this dissertation examines this question.

Specific Aim 3: To determine whether increased perfusate solute concentration augments ET-1 mRNA production in the IMCD (Chapter 5).

Hypothesis: Solute concentration increases flow-regulated ET-1 mRNA accumulation in m-IMCD3, a mouse IMCD cell line.

Rationale: ECFV expansion increases solute load in the CD. Since there is an

established link between ECFV status and CD ET-1 production, it is important to study whether solute load per se regulates flow-induced CD ET-1 mRNA accumulation.

2.3 Introduction to Methods

The flow chambers were purchased from Glycotech (Gaithersburg, MD). Cells were grown on plastic culture dishes and parallel plate perfusion chambers were attached onto the plates with the help of vacuum. For each experiment, the apparatus is assembled on a temperature-controlled slide moat as shown Figure 2.1.

Flow inside the chambers is laminar. HBSS enters the chamber from one manifold into an ante-chamber, flows over the cells into another ante-chamber on the other side and exits the chamber its corresponding manifold. The dimensions of the flow chamber are indicated in Figure 2.2 (Length: 5.9 cm; Width: 1 cm). The experiment was carried out according to the steps mentioned in Figure 2.3.

Flow rate through the pump was used to calculate the shear stress using the following equation:

$$\mathbf{Tw = \mu\gamma = 6\mu Q / a^2 b \quad OR \quad Q = (Tw)(a^2 b)/ 6\mu}$$

where, Tw = wall shear stress (dyne/cm^2)

Q = Volumetric flow rate (mL/sec)

μ = Apparent viscosity of the media (0.0076 P for HBSS at 37°C

a = Channel height or gasket thickness (0.0254 cm)

b = Channel width (1 cm)

ET-1 mRNA was quantified and normalized to GAPDH mRNA levels. Previously, ET-1 release was estimated from cells and perfusate using a Quantiglo ET-1 enzyme immunoassay (R&D Systems, Minneapolis, MN). Luminescence was determined using a luminometer (Molecular Devices LMax II). The data was analyzed using the Softmax Pro 4.7 data analysis program. Cells were dissolved in 0.1 N NaOH and the total protein content of the cells determined using the Bradford assay.

However, the levels of ET-1 were barely detectable despite using a highly sensitive ET-1 assay. ET-1 was undetectable possibly due to the small numbers of cells inside the perfusion chambers. Hence, all future experiments conducted for the purpose of this dissertation involved measuring ET-1 mRNA. The quantification of ET-1 mRNA over protein is justified owing to the following reasons: 1) ET-1 mRNA levels reflect ET-1 release in every condition under which this has been studied (20, 21) and 2) Further, the ET-1 mRNA has AUUUA sequences in its 3-UTR that destabilize the message conferring a very short half-life (~15 minutes).

For gene products largely regulated at the transcriptional level, this is typically observed (22, 23). As mentioned previously, GAPDH mRNA was used to normalize ET-1 mRNA since the GAPDH levels did not significantly differ under any of the conditions studied (varying flow and treatments), thus suggesting that GAPDH is a valid marker for the total RNA in a particular sample.

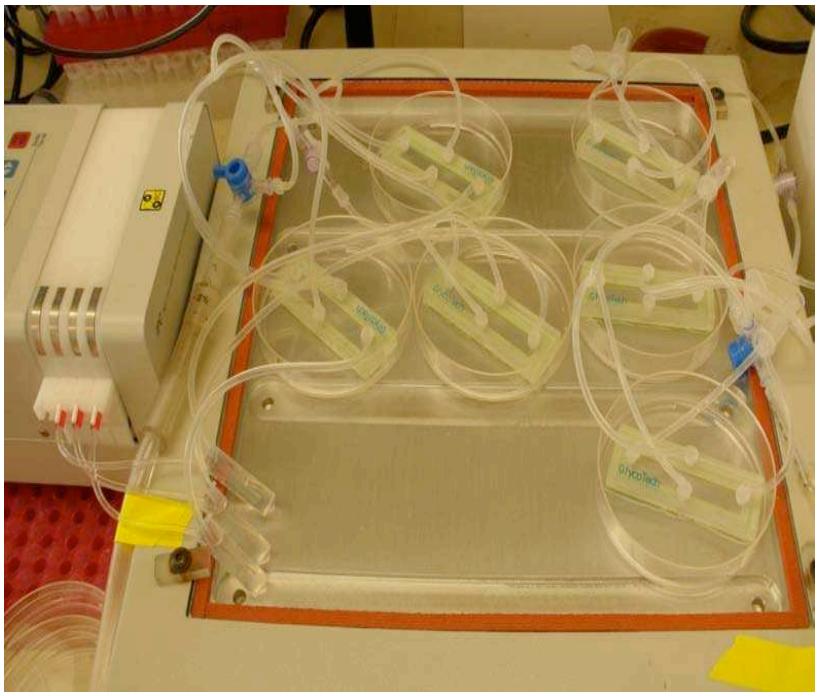

Figure 2.1. Flow set-up

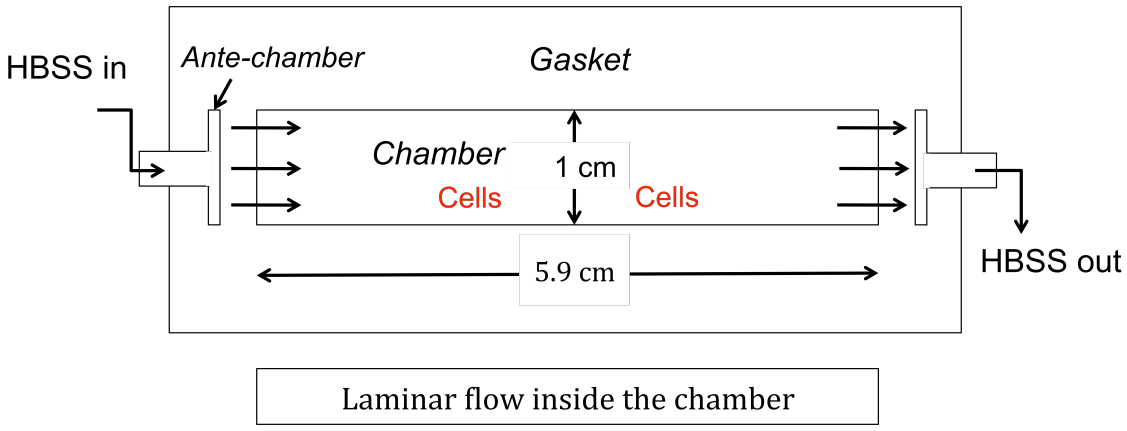

Figure 2.2. Depiction of flow inside the chamber and chamber dimensions

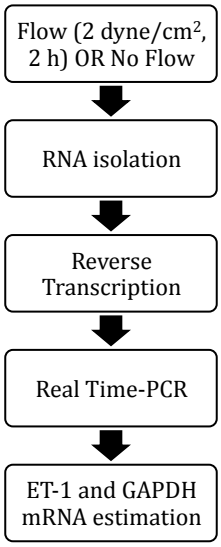

Figure 2.3. Flow chart of flow procedure

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CHAPTER 3

Na DELIVERY AND ENAC MEDIATE FLOW REGULATION OF COLLECTING DUCT ENDOTHELIN-1 PRODUCTION

Meghana M. Pandit[12], Kevin A. Strait[13], Toshio Matsuda[4], and Donald E. Kohan[123]

[1]Division of Nephrology, University of Utah Health Sciences Center, Salt Lake City, UT

[2]Department of Pharmaceutics and Pharmaceutical Chemistry, Salt Lake City, UT

[3]Salt Lake Veterans Affairs Medical Center, Salt Lake City, UT

[4]Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

Address for correspondence:

Donald E. Kohan, M.D., Ph.D.

Division of Nephrology, University of Utah Health Sciences Center

1900 East, 30 North, Salt Lake City, UT 84132

Tel: 801 581 2726

Fax: 801 581 4343

Email: donald.kohan@hsc.utah.edu

Permission for reprint not required for: Pandit MM, Strait KA, Matsuda T, Kohan DE. Na delivery and ENaC mediate flow regulation of collecting duct endothelin-1 production. Am J Physiol Renal Physiol 302: F1325-F1330, 2012.

3.1. Abstract

Collecting duct (CD) endothelin-1 (ET-1) is an important autocrine inhibitor of Na and water transport. CD ET-1 production is stimulated by extracellular fluid volume expansion and tubule fluid flow, suggesting a mechanism coupling CD Na delivery and ET-1 synthesis. A mouse cortical CD cell line, mpkCCDc14, was subjected to static or flow conditions for 2 hr at 2 dyne/cm^2, followed by determination of ET-1 mRNA content. Flow with 300 mOsm/L NaCl increased ET-1 mRNA to 65% above that observed under static conditions. Increasing perfusate osmolarity to 450 mOsm/L with NaCl or Na acetate increased ET-1 mRNA to about 184% compared to no flow, which was not observed when osmolarity was increased using mannitol or urea. Reducing Na concentration to 150 mOsm/L while maintaining total osmolarity at 300 mOsm/L with urea or mannitol decreased the flow response. Inhibition of ENaC with amiloride or benzamil abolished the flow response, suggesting involvement of ENaC in flow-regulated ET-1 synthesis. Aldosterone almost doubled the flow response. Since Ca^{2+} enhances CD ET-1 production, the involvement of plasma membrane and mitochondrial Na/Ca^{2+} exchangers (NCX) was assessed. SEA0400 and KB-R7943, plasma membrane NCX inhibitors, did not affect the flow response. However, CGP37157, a mitochondrial NCX inhibitor, abolished the response. In summary, the current study indicates that increased Na delivery, leading to ENaC-mediated Na entry and mitochondrial NCX activity, is involved in flow-stimulated CD ET-1 synthesis. This constitutes the first report of either ENaC or mitochondrial NCX regulation of an autocrine factor in any biologic system.

3.2.Introduction

Collecting duct (CD)-derived endothelin-1 (ET-1) is an important regulator of arterial pressure and urinary Na excretion. The CD is the predominant nephron site of ET-1 production and receptor expression (14). ET-1 binding to the CD results in reduction of epithelial Na channel (ENaC) activity through inhibition of channel open probability and apical membrane abundance (4, 27), as well as decreased Na/K ATPase activity (34). CD-specific deficiency of ET-1 causes marked salt-sensitive hypertension and impaired renal Na excretion (1). Hence, CD-derived ET-1, under normal physiologic conditions, exerts a natriuretic and hypotensive effect.

High Na intake enhances urinary ET-1 excretion (5, 6, 11, 19, 23) and CD ET-1 production (17), indicating that the CD ET-1 system perceives alterations in extracellular fluid volume (ECFV). However, the mechanism by which Na loading regulates CD ET-1 production is poorly understood. Circulating hormones that are modified by ECFV status do not explain Na loading-induced increases in CD ET-1 (8, 14). Na loading can increase medullary osmolarity leading to increased thick ascending limb ET-1 synthesis (9), however the effect of tonicity on CD ET-1 production is controversial – interstitial hypertonicity may even reduce CD ET-1 synthesis (13). Our group has recently shown that increasing fluid flow augments CD ET-1 production (17); since Na loading increases tubule fluid flow, this suggests a mechanism by which ECFV status regulates CD ET-1 production.

The mechanisms by which tubule flow increases CD ET-1 production are only beginning to be understood. Flow-induced increases in CD ET-1 synthesis are dependent upon Ca^{2+}, protein kinase C and phospholipase C (17). However, the mechanism by

which increases in tubule fluid flow results in activation of these intracellular signaling systems is unknown. While flow may cause mechanotransduced signals, an intriguing possibility is that Na delivery per se may activate signaling systems within the CD that lead to enhanced ET-1 production. Consequently, the current study was undertaken to investigate the relationship between Na delivery and CD ET-1 production, as well as to determine mechanisms by which Na delivery might regulate CD ET-1 production.

3.3. Materials and Methods

3.3.1. Materials

SEA0400 was synthesized by Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). KB-R7943 and CGP 37157 were obtained from Tocris Bioscience (Ellisville, MO). DMEM and F-12 were obtained from Invitrogen Life Technologies (Grand Island, NY). All other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

3.3.2. Cell Culture

The mouse cortical collecting duct cell line, mpkCCDc14, was generously provided by Professor Alain Vandewalle at INSERM, France. Cells were grown to confluence on 10 cm^2 plastic culture plates at 37°C in a 5% CO_2 incubator. 50:50 DMEM/F-12 supplemented with 2 μg/ml dexamethasone, insulin, transferrin, selenium, 400 nM triiodothyronine, 1 μg/ml epidermal growth factor, 2 mM glutamine, 1 mg/ml penicillin, 1 mg/ml streptomycin and 2% fetal bovine serum was used as a growth medium.

3.3.3. Flow Studies

Confluent cultures of mpkCCDc14 cells were rinsed with Hanks Balanced Salt Solution (HBSS) and flow chambers were attached to the plates. Rectangular parallel plate polycarbonate flow chambers (Cat. No. 31-010, Glycotech, Gaithersburg, MD) were sealed using vacuum and silastic gaskets on to individual culture plates to form a channel. The channel depth was 0.25 mm having a width of 1 cm and length of 5.9 cm. Thus, the surface area of cells exposed to flow was 5.9 cm^2. The flow chamber has two openings through which medium enters and exits the channel. The medium is re-circulated through the channel by a peristaltic pump (Ismatec, Glattbrug, Switzerland) at a shear stress of 2 dyne/cm^2 for 2 hours. The perfusing medium used was HBSS (pH 7.4) for control experiments and supplemented with drugs and/or chemicals for other experiments. After flow, RNA was isolated from the cells within the perfusion chamber. RNA from cells exposed to flow was compared to RNA from control cells studied on the same day. Culture dishes with the control cells were placed into the flow chambers and exposed to the same perfusate solution as in the cells undergoing flow, with the exception that the perfusate was static (no flow). Control cell RNA was isolated from cells within the area of the perfusion chamber.

3.3.4. ET-1 mRNA Measurement

RNA was extracted from cells exposed to perfusion using RNeasy Mini Kit, reverse transcribed using Omniscript RT Kit (Qiagen Incorporated, Valencia, CA), and cDNA levels were determined for ET-1 and GAPDH using real-time PCR (StepOne Plus, Applied Biosystems, Foster City, CA). PCR was performed according to instructions

provided by the manufacturer using Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA) with ET-1 (Cat. No. Mm00438656_m1) and GAPDH (Cat. No. Mm03302249_m1) primers.

3.3.5. Statistics

Data are represented as mean ± SEM. Differences between groups were determined using two-way analysis of variance. Bonferroni post-hoc tests were used to compare individual means. $p<0.05$ was taken as significant.

3.4. Results

3.4.1. Effect of Increased Na Concentration on Flow-stimulated ET-1 mRNA Production (Figure 3.1)

To determine the effect of increased Na delivery on CD ET-1 production, mpkCCDc14 cells were exposed to flow and static conditions for 2 hr at 2 dyne/cm2. Flow with 300 mOsm/L NaCl increased ET-1 mRNA content by 67% over that seen with cells exposed to 300 mOsm/L NaCl under static (no flow) conditions. Perfusing with 450 mOsm/L NaCl increased ET-1 mRNA by 184% as compared to static conditions; this was a significantly greater ET-1 flow response than seen with 300 mOsm/L NaCl. To determine the effect of increasing osmolarity per se, cells were perfused with 450 mOsm/L solution containing 300 mOsm/L NaCl and 150 mOsm/L mannitol; this yielded the same degree of increased ET-1 mRNA levels as seen with 300 mOsm/L NaCl, i.e., no augmentation of the flow response was observed by increasing perfusate osmolarity with mannitol. Increasing perfusate osmolarity to 450 mOsm/L using Na acetate (150 mOsm/L

Na acetate plus 300 mOsm/L NaCl) increased ET-1 mRNA by 205%, i.e., similar to that seen with 450 mOsm/L NaCl and greater than that with 300 mOsm/L NaCl. Finally, increasing perfusate osmolarity to 450 mOsm/L using choline chloride (150 mOsm/L choline chloride plus 300 mOsm/L NaCl) increased ET-1 mRNA by 70%, similar to that seen with 300 mOsm/L NaCl, but not as great as observed with increasing osmolarity with NaCl or Na acetate. Hence, increasing perfusate Na concentration, but not perfusate osmolarity or chloride concentration, increased the ET-1 mRNA response to flow.

3.4.2. Effect of Reduced Na Concentration on Flow-stimulated ET-1 mRNA Production

To determine the effect of reducing Na delivery on the ET-1 flow response, cells were exposed to flow conditions with 150 mOsm/L NaCl. Perfusate osmolarity was maintained at 300 mOsm/L by using either 150 mOsm/L urea, mannitol or choline chloride. The ET-1 flow response was decreased by 60%, 47% and 54% when using choline chloride, urea or mannitol, respectively. Thus, reducing perfusate NaCl concentration decreased flow-stimulated ET-1 mRNA content (Figure 3.2).

3.4.3. Role of ENaC in Flow-stimulated ET-1 mRNA Production

The above studies indicated that Na delivery is directly correlated with ET-1 mRNA accumulation. Since ENaC is the major channel through which Na enters collecting duct cells, we evaluated the role of ENaC in the ET-1 response to flow (Figure 3.3). When cells were treated with 1 μM amiloride, there was a complete loss of flow-stimulated ET-1 mRNA production. Similarly, 0.2 μM benzamil abolished the ET-1 flow

response. To further explore a potential role of ENaC, cells were treated with aldosterone (which has been shown to upregulate ENaC in mpkCCD cells (10)) for 48 hours (Figure 3.4). Aldosterone treatment markedly enhanced the ET-1 mRNA response to flow (190% increase in ET-1 mRNA as compared to no flow). This increase was substantially greater than flow-stimulated ET-1 mRNA accumulation in cells not treated with aldosterone (67% increase in ET-1 mRNA as compared to no flow). Addition of 1 μM amiloride for 30 minutes before flow experiments prevented the stimulatory effect of aldosterone on the ET-1 flow response. Taken together, these experiments indicate that ENaC is necessary for flow-induced ET-1 mRNA accumulation.

3.4.4. Role of Na/Ca^{2+} Exchange in Flow-stimulated ET-1 mRNA Production

Since Na delivery and ENaC-mediated Na entry into cells are necessary for flow-stimulated ET-1 mRNA production, and since Ca^{2+} is required for collecting duct ET-1 gene transcription (30), a potential relationship between Na and Ca^{2+} was investigated. The most apparent mechanism for such a relationship is the Na/Ca^{2+} exchanger (NCX) family. Since an increase in intracellular Na concentration ($[Na]_i$) can potentially increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through activation of plasma membrane NCX activity, the effect of inhibiting plasma membrane NCX was investigated. Treatment of cells with either 3 μM SEA0400, a highly selective inhibitor of plasma membrane NCX (20), as well as 10 μM KB-R7943, another plasma membrane NCX inhibitor, resulted in a flow-stimulated increase in the ET-1 mRNA content of 210% and 249%, respectively (Figure 3.5). Thus, plasma membrane NCX does not appear to be involved in the ET-1 flow response. In contrast, treatment with CGP-37157, the only known mitochondrial

NCX inhibitor, completely prevented the ET-1 flow response (Figure 3.6). While CGP-37157 is specific for the mitochondrial NCX (as opposed to plasma membrane NCX isoforms), one report suggested that it might also inhibit L-type Ca^{2+} channels (31). To determine if this mechanism was involved in CGP-37157 effect, cells were treated with 100 μM nifedipine. Nifedipine did not alter the ET-1 flow response (Figure 3.6), indicating that the CGP-37157 effect was not due to inhibition of L-type Ca^{2+} channels.

3.5. Discussion

Our previous study (17) demonstrated that flow increases CD ET-1 production; the goal of the current study was to explore mechanisms responsible for this effect. In the current study, we report that: 1) Na delivery increases ET-1 mRNA accumulation in CD cells, 2) ENaC is necessary for flow-stimulated ET-1 mRNA augmentation, and 3) mitochondrial NCX is necessary for flow-stimulated CD ET-1 mRNA accumulation. Thus, while flow or shear stress per se may exert effects on CD ET-1 production, our findings indicate that the magnitude of Na delivery is an important factor in determining CD ET-1 production. This observation is consistent with the known stimulatory effect of a high Na intake on CD ET-1 production *in vivo* (17). Since CD-derived ET-1 exerts an autocrine inhibitory effect on Na reabsorption (1), the above findings provide a rational system whereby increased Na intake leads to increased CD Na delivery, increased CD ET-1 production, ET-1 inhibition of Na transport, and ultimately enhanced natriuresis.

Our findings indicate that ENaC is necessary for flow-stimulated ET-1 production as evidenced by inhibition of the flow response by amiloride or benzamil. To our knowledge, this constitutes the first report of ENaC-mediated regulation of an autocrine

or paracrine factor. It is likely that ENaC-mediated increases in $[Na]_i$ are involved in flow-stimulated ET-1 production. Relatively little is known about $[Na]_i$ regulation of intracellular signaling processes in the CD. Increases in $[Na]_i$ may alter ENaC activity through modulation of G-proteins (15). In addition, increased $[Na]_i$ leads to augmented Na/K ATPase activity through modulation of salt-inducible kinase-1 (SIK-1) (29). Activation of SIK-1 is thought to be due to elevations in $[Na]_i$ driving increases in $[Ca^{2+}]_i$, which in turn activate calmodulin leading to activation of SIK-1 (29). This latter effect is relevant to possible mechanisms involved in ET-1 production; CD ET-1 synthesis is critically dependent upon $[Ca^{2+}]_I$ (30), while flow-stimulated CD ET-1 mRNA accumulation is dependent upon $[Ca^{2+}]_I$ and Ca^{2+}-regulated pathways, including protein kinase C and phospholipase C (17). An obvious mechanism coupling changes in $[Na]_i$ to changes in $[Ca^{2+}]_i$ involves NCX. Three plasma membrane isoforms of NCX have been described which can transport Ca^{2+} into cells in response to increases in $[Na]_i$ (18, 25). However, we found that pharmacologic blockade of all isoforms of plasma membrane NCX with either SEA0400 or KB-R7943, inhibitors of plasma membrane NCX (3, 16, 20) did not affect the ET-1 flow response. In addition to plasma membrane NCX, another NCX isoform, NCLX, is expressed exclusively in mitochondria and extrudes Ca^{2+} from mitochondria in response to increases in $[Na]_i$ (26). Blockade of NCLX with CGP-37157 (26) prevented flow-induced CD ET-1 mRNA accumulation, suggesting that mitochondrial NCLX-mediated increases in $[Ca^{2+}]_I$ are involved in flow stimulation of ET-1 production. Such a scenario of flow-stimulated mitochondrial Ca^{2+} release is not unprecedented; shear stress-induced increases in $[Ca^{2+}]_I$ in cardiac myocytes have been demonstrated to be dependent upon release of Ca^{2+} from

mitochondrial stores (2). Taken together, the above studies suggest that ENaC-mediated increases in $[Na]_i$ lead to increases in $[Ca^{2+}]_I$ through augmentation of mitochondrial NCLX activity; the increased $[Ca^{2+}]_I$ ultimately leads to stimulation of ET-1 production. It should be noted that we have not directly measured $[Ca^{2+}]_I$ or $[Na]_i$, however we believe that our data strongly supports the proposed changes in $[Ca^{2+}]_I$ and $[Na]_I$ without having directly measured their levels.

It is possible that other mechanisms are also involved in flow stimulation of CD ET-1 production. While ENaC is necessary for the flow response, the current studies do not demonstrate that it is sole mechanism involved. Other flow sensors exist in the apical membrane of the CD. Transient receptor potential (TRP) channels are expressed in the CD (including TRPV5 as well as TRPC1, 3 and 6 (7, 12)), can sense flow, and can function as Na as well as Ca^{2+} channels (33). In addition, P2X receptors are located in the CD, can sense flow, and can function as Na channels (24, 32). Finally, cilia in CD, which are well recognized flow sensors through activation of polycystins leading to augmented $[Ca^{2+}]_I$ (28), could be involved in the ET-1 flow response. Clearly, additional studies are needed to evaluate the role of these systems in mediating the ET-1 response to flow.

It should be noted that ET-1 mRNA, but not ET-1 protein, was measured in the current study. As previously reported (17), ET-1 protein, either released into the perfusate or within cells, was undetectable. Since ET-1 release parallels ET-1 mRNA levels in almost every condition under which this has been measured (14, 30), ET-1 mRNA was taken as an accurate index of ET-1 production. In addition, ET-1 mRNA is very short-lived (~15 minutes) due to the presence of destabilizing AUUUA sequences in the 3-UTR of ET-1 mRNA; this is very characteristic of gene products that are primarily

regulated at the transcriptional level (21, 22).

In summary, our findings suggest a link between Na delivery, ENaC, mitochondrial NCLX activity, and ET-1 mRNA accumulation. This system provides, at least teleologically, a link between Na intake and urinary Na excretion. In essence, high Na intake, leading to ECFV expansion and increased Na delivery to the CD, activates the above system to ultimately induce autocrine inhibition of Na reabsorption by the CD. Novel findings in the current study include ENaC-dependent regulation of a regulatory peptide as well as the dependency of ET-1 production upon mitochondrial NCLX activity. Further exploration of the pathways involved in flow stimulation of ET-1 production, as well as the potential roles of ENaC and mitochondrial NCLX in regulating CD function, is warranted.

Acknowledgements

The authors thank Professor Alain Vandewalle for generously providing the mpkCCDc14 cells.

Grants

This research was supported by NIH P01 HL095499 (D.E.K.).

Disclosures

None

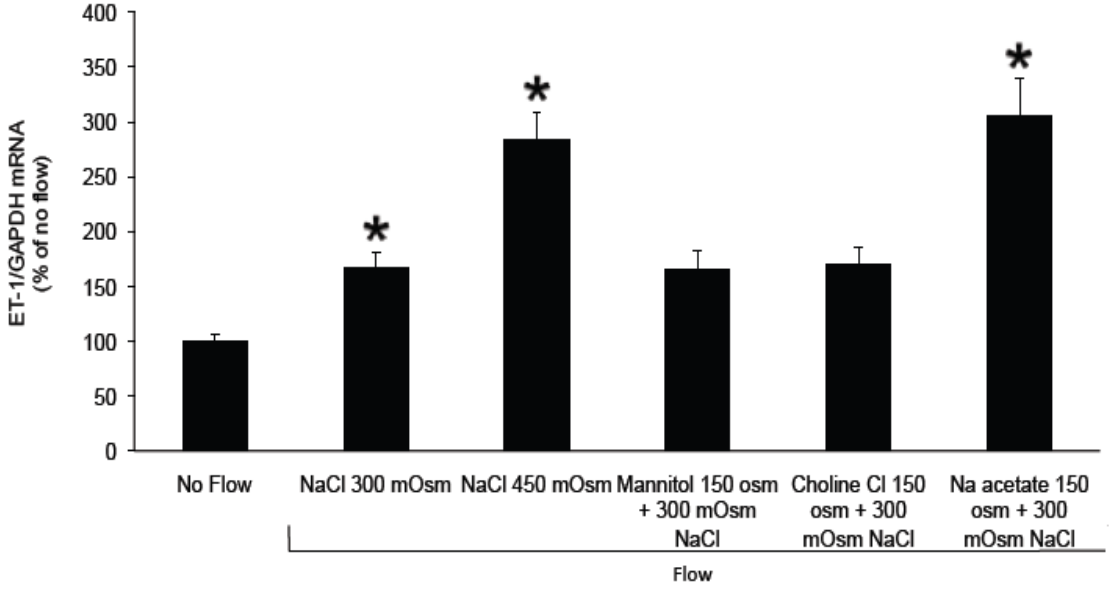

Figure 3.1. Effect of increased media osmolarity, Na or chloride concentration on flow-stimulated ET-1 mRNA content in mpkCCDc14 cells under static (no flow) and flow conditions. N=12 each data point. *$p<0.05$ vs. cells not exposed to flow.

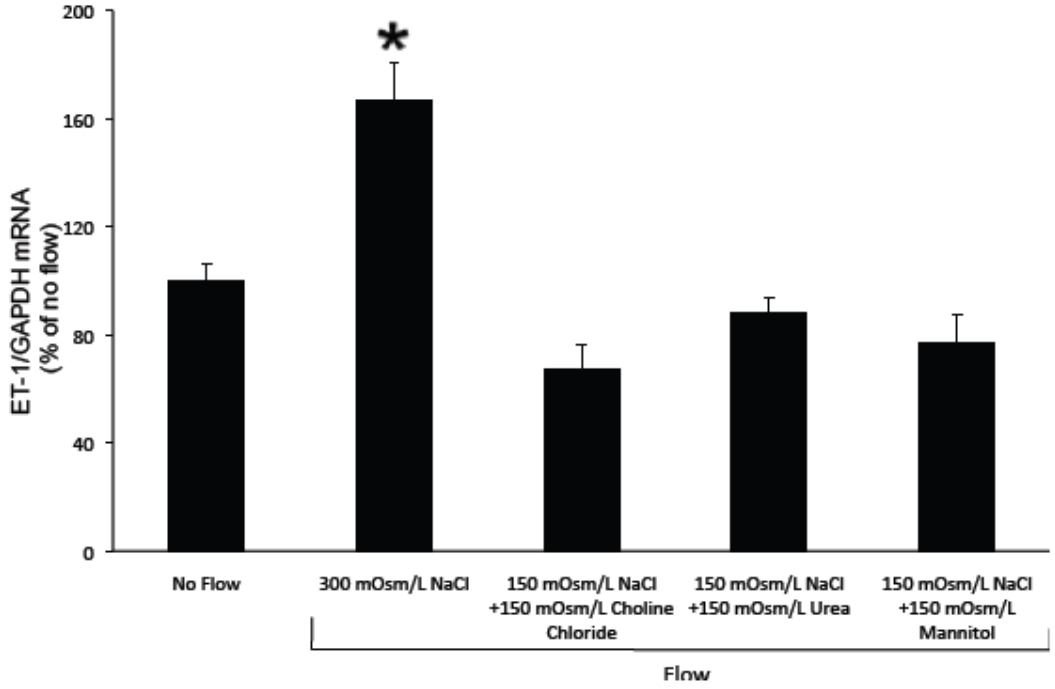

Figure 3.2. Effect of reduced Na concentration on flow-stimulated ET-1 mRNA content in mpkCCDc14 cells under static (no flow) and flow conditions. N=12 each data point. *$p<0.05$ vs. cells not exposed to flow.

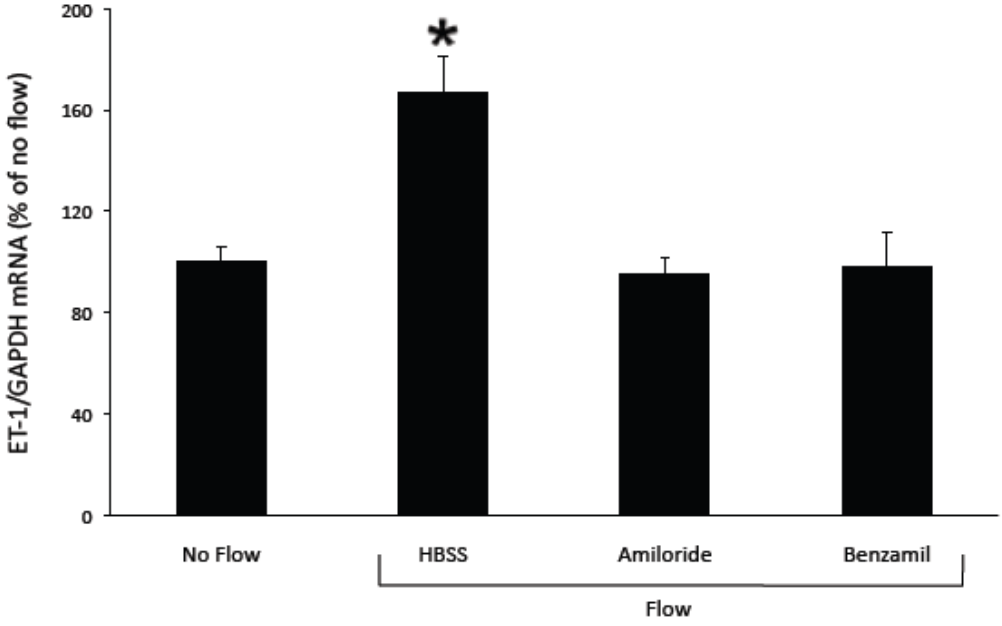

Figure 3.3. Effect of ENaC inhibition on flow-stimulated ET-1 mRNA content in mpkCCDc14 cells under static (no flow) and flow conditions. Cells were pretreated for 30 minutes with 1 μM amiloride or 0.2 μM benzamil. N=12 each data point. *$p<0.05$ vs. cells not exposed to flow.

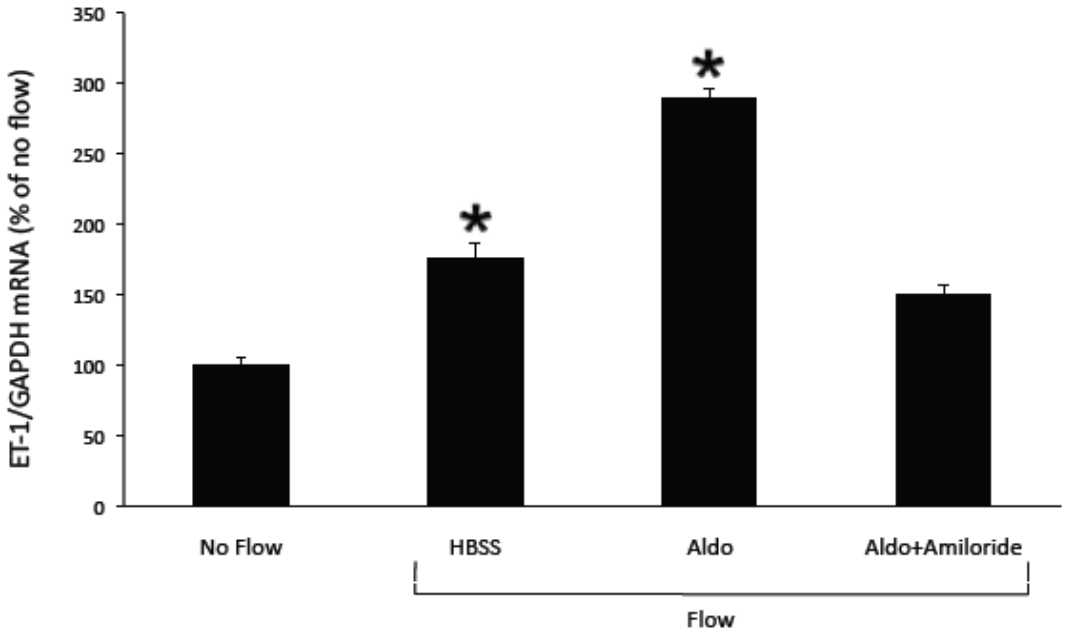

Figure 3.4. Effect of aldosterone ± amiloride on flow-stimulated ET-1 mRNA content in mpkCCDc14 cells under static (no flow) and flow conditions. Cells were pre-treated with 100 μM aldosterone for 2 days, and pretreated with 1 μM amiloride for 30 minutes. N=12 each data point. *$p<0.05$ vs. cells not exposed to flow.

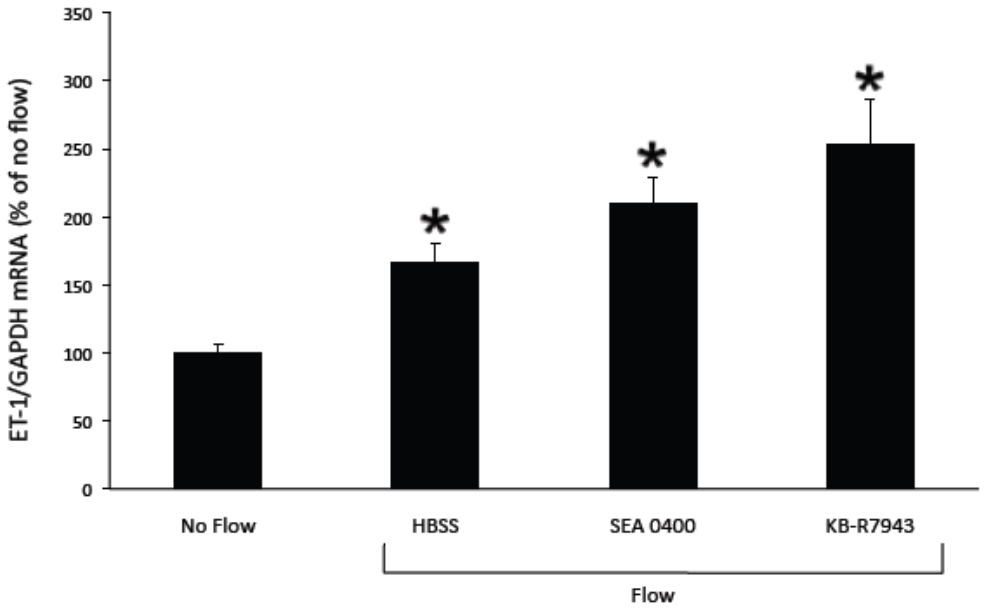

Figure 3.5. Effect of plasma membrane Na calcium exchanger inhibition on flow-stimulated ET-1 mRNA content in mpkCCDc14 cells under static (no flow) and flow conditions. Cells pretreated for 30 minutes with 3 μM SEA0400 (N=7) or 10 μM KB-R7943 (N=12). *$p<0.05$ vs. cells not exposed to flow.

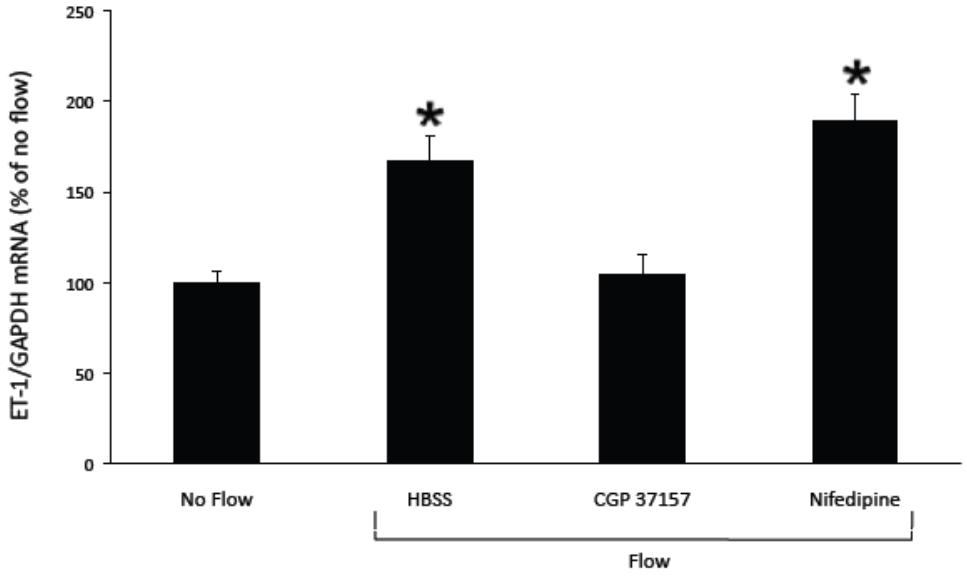

Figure 3.6. Effect of mitochondrial Na calcium exchanger inhibition on flow-stimulated ET-1 mRNA content in mpkCCDc14 cells under static (no flow) and flow conditions. Cells were pretreated for 30 minutes with 1.2 μM CGP-37157 (N=12). Cells were pretreated with 100 μM nifedipine (N=6) to control for an effect on L-type dihydropyridine Ca^{2+} channels. *$p<0.05$ vs. cells not exposed to flow.

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CHAPTER 4

FLOW REGULATION OF ENDOTHELIN-1 PRODUCTION IN THE INNER MEDULLARY COLLECTING DUCT

Meghana M. Pandit[12], Edward W. Inscho[3], Tsugio Seki[3], Rajeev Rohatgi[4], Luca Gusella[4], Jing Zhou[5], Shali Zhang[6], Bellamkonda Kishore[16] and Donald E. Kohan[126]

[1]Division of Nephrology, University of Utah Health Sciences Center, Salt Lake City, UT

[2]Department of Pharmaceutics and Pharmaceutical Chemistry, Salt Lake City, UT

[3]Georgia Regents University, Augusta, GA

[4]Department of Medicine, James J. Peter VAMC, Bronx, New York 10468 and Department of Medicine and Pediatrics, Icahn School of Medicine at Mount Sinai, New York, New York 10029.

[5]Brigham and Women's Hospital, Harvard Medical School, Boston, MA

[6]Salt Lake Veterans Affairs Medical Center, Salt Lake City, UT

4.1. Abstract

Collecting duct (CD)-derived endothelin-1 (ET-1) is an important autocrine inhibitor of sodium and water reabsorption; its deficiency causes marked hypertension and water retention. The CD is the major site of ET-1 synthesis in the body. Extracellular fluid volume (ECFV) expansion increases CD ET-1 production, thus establishing a link between volume loading, natriuresis and diuresis. In cultured cortical collecting duct cells, tubule fluid flow, which occurs in case of ECFV expansion, stimulates ET-1 production in a sodium-dependent manner; the mechanism in inner medullary collecting duct cells is sodium-independent. Mouse IMCD3 cells were subjected to static or flow conditions for 2 hr at 2 dyne/cm2, followed by determination of ET-1 mRNA. Absence of perfusate calcium Ca^{2+}, inhibition of intracellular calcium $[Ca^{2+}]_i$, calmodulin (CaM), calmodulin kinase (CaMK), calcineurin (Cn), protein kinase C (PKC) and phospholipase C (PLC) prevented the flow response, suggesting their role in the ET-1 response to flow. Flow failed to prevent ET-1 mRNA accumulation in polycystin-1/polycystin-2 deficient cells and cells subjected to cilia removal. Blockade of P2X7 and P2Y2 receptors completely prevented the flow response. Exogenous ATP failed to increase ET-1 mRNA production in polycystin-2 deficient cells, when compared to wild-type IMCD3 cells. Taken together, these findings provide exciting evidence that the cilia/polycystin/purinergic receptor/ET-1 system constitute a complex pathway through which the CD can detect changes in ECFV status and ultimately, achieve blood pressure control and volume homeostasis through immediate and sustained regulation of salt and water reabsorption.

4.2. Introduction

The collecting duct (CD) endothelin-1 (ET-1) system plays a key role in regulating arterial pressure and urinary Na^+ excretion. The CD produces relatively high amounts of ET-1 (possibly more than any other cell type in the body) (1) and expresses abundant ET receptors (1, 2). ET-1 directly inhibits CD Na^+ and water reabsorption (3, 4, 5, 6) raising the possibility that ET-1 functions as an autocrine, natriuretic and diuretic factor in the CD. This has been confirmed by studies wherein CD-specific knockout of ET-1 or ET receptors caused impaired urinary Na^+ and water excretion and salt-sensitive hypertension (7, 8). Notably, ET receptor antagonist-induced fluid retention, a major factor limiting the success of these agents in clinical trials (9, 10), is due in large part to blockade of CD ET receptors (11). Thus, the CD ET system has emerged as a major factor in controlling arterial pressure and body fluid volume (BFV).

Given that CD-derived ET-1 modulates body volume homeostasis, it is important to understand how this system is regulated by changes in body volume status. Numerous studies in experimental animals and humans have demonstrated that urinary ET-1 excretion is increased in response to salt (12, 13, 14, 15; 16) or water loading (17, 18). Since urinary ET-1 derives entirely from the kidney (19), and in large part from the CD (7), these findings suggest that BFV regulates CD ET-1 production. This notion is supported by studies showing that salt loading increases CD ET-1 mRNA content in rats (7). Thus, the natriuretic and diuretic response to BFV expansion appears to be mediated in part by enhanced CD ET-1 production.

The mechanism(s) by which BFV expansion enhances CD ET-1 synthesis are incompletely understood. Aldosterone paradoxically stimulates CD ET-1 production (20),

while atrial natriuretic peptide, angiotensin II and vasopression have minimal effects on the CD ET-1 system (21, 22), hence circulating hormones do not clearly explain BFV effects on CD ET-1. Increased interstitial NaCl concentration, as may occur in the renal cortex and outer medulla during salt loading, has been implicated in stimulating thick ascending limb ET-1 production (23). However, the effect of extracellular NaCl or osmolality on CD ET-1 production is controversial with conflicting findings being reported (17). Recent studies by our group suggest that tubule fluid flow may be involved - exposure of mpkCCD cells, a cortical CD cell line, to shear stress increased ET-1 mRNA (24, 25). Interestingly, this flow augmentation of mpkCCD ET-1 mRNA content was due to Na^+ delivery; increasing Na^+ delivery increased epithelial Na^+ channel (ENaC)-mediated Na^+ entry which led to enhanced mitochondrial Na^+/Ca^{2+} exchange (NCLX), increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), activation of Ca^{2+} signaling pathways, and ultimately increased ET-1 gene transcription (25). Thus, BFV expansion, leading to enhanced tubule fluid flow and Na^+ delivery, may be at least one mechanism by which CD ET-1 production is increased.

The above studies do not, however, explain how water loading increases CD ET-1 production. Furthermore, they are unlikely to explain how inner medullary CD (IMCD) ET-1 production is increased by BFV expansion since this nephron segment contains relatively little ENaC (26). This question is particularly relevant since the IMCD produces substantially more ET-1 and expresses a higher density of ET receptors than the CCD (27). Consequently, to address this question, the current study was undertaken to examine regulation of IMCD ET-1 production. We report flow-stimulated IMCD ET-1 production and, of greatest importance, describe two key findings: 1) an interaction

between the purinergic and ET systems that may combine to elicit a sustained diuretic and natriuretic response; and 2) a direct role for cilia and polycystins in this flow effect.

4.3. Materials and Methods

4.3.1. Reagents

Calcineurin inhibitory peptide, NFAT inhibitor or VIVIT peptide, Calphostin C, Pyr3, SKF-96365, 5-BDBD and A-438079 hydrochloride were obtained from Tocris Bioscience (Ellisville, MO). Diinosine pentaphosphate was obtained from Timtec LLC (Newark, DE). ARC-118925 was generously provided by Dr. Christa E. Müller, Pharmaceutical Institute, University of Bonn, Bonn, Germany. All other drugs and chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise.

4.3.2. Na and Water Loading Studies

Male Sprague-Dawley rats (200-250 g; Harlan Laboratories, Indianapolis, IN) and C57/BL6 mice (25 g) were handled in accordance with University of Utah IACUC requirements. For salt loading studies, all rats and mice were fed normal (0.25%) or high (8%) NaCl diets and given free access to drinking water for 3 days each. For water loading, rats and mice were given free access to a regular NaCl diet plus water or water containing 1% sucrose for 3 days each. IMCDs were isolated using previously described procedures (Ca paper). Briefly, renal inner medullas were minced and incubated in 0.1% collagenase (Type I; Worthington, Freehold, NJ) containing type I DNase in HBSS supplemented with 15 mM HEPES (pH 7.4) at 37°C. The digest was filtered through a 74-μm mesh screen to remove any residual tissue. The suspension was centrifuged for 5

min at 1500 rpm, the cell pellet resuspended in 10% bovine serum albumin in HBSS and further centrifuged and washed with HBSS. The final cell pellet was used for RNA analysis as described below.

4.3.3. RNA Analysis and Real-time PCR

RNA from acutely isolated and cultured cells was isolated using the RNeasy Mini Kit and reverse transcribed using Omniscript RT Kit (Qiagen, Valencia, CA). GAPDH and ET-1 mRNA levels were determined by real-time PCR (StepOne Plus, Applied Biosystems, Foster City, CA) using the Taqman Gene Expression Assay with ET-1 (Catalog number Mm00438656_m1) and GAPDH (Catalog number Mm03302249_m1) primers.

4.3.4. Cell Culture

The mouse inner medullary collecting duct cell line, IMCD3, was used for all studies unless specified otherwise. Polycystin-2 mutant IMCD3 were provided by Drs. Rajeev Rohatgi and Luca Gusella at the Icahn School of Medicine at Mt. Sinai University, NY (61). Cells derived from collecting ducts of Pkd1 null and wild-type littermate mice were obtained from Dr. Jing Zhou at Brigham and Women's Hospital, Harvard Medical School, Boston, MA (62). Cells were grown to confluence on 10-cm^2 plastic culture plates in a 5% CO_2 incubator at 37^oC; 50:50 DMEM/F-12 supplemented with 10% fetal bovine serum, 1 mg/mL penicillin and 1 mg/mL streptomycin was used as growth medium. For the control studies done under stationary conditions, cells were grown in 12-well plates under identical conditions.

4.3.5. Flow Studies

Rectangular parallel plate polycarbonate flow chambers (Catalog number 31-010, Glycotech, Gaithersburg, MD) were attached to individual 10-cm cell culture plates containing confluent IMCD3 cells using vacuum and silastic gaskets to form a channel. The channel has the following dimensions: 0.25 mm depth, 1 cm width and 5.9 cm in length, having a total surface area of 5.9 cm^2 for the cells exposed to flow. The flow chamber has two manifolds through which perfusate enters and exits the channel. The liquid is pumped through the channel by a peristaltic pump (Ismatec, Glattburg, Switzerland) to obtain specific shear stresses. HBSS (pH 7.4) was used as the perfusate for control experiments and was supplemented with drugs and/or chemicals for additional experiments. RNA was extracted from cells exposed to flow and from control cells. All the experiments were performed at 37^oC.

4.3.6. ATP Assay

Flow-medium was collected and 20 µl of the supernatant was analyzed for ATP content using the Enliten ATP Assay System Bioluminescence Detection Kit for ATP Measurement (Promega, Madison, WI). The luminometer (EG&G Berthold, Oak Ridge, TN) protocol used a 10-second RLU signal integration time following injection of 100 µl rL/L reagent.

4.3.7. Western Blot Analysis of P2 Receptor Expression

Mouse IMCD3 cells were lysed and prepared for western blot analysis as previously described (63). The protein concentration was determined using the Bradford

assay (Bio-Rad Laboratories, Hercules, CA). Samples were diluted with a sample buffer and denatured (10 min, 70°C) using a dry bath incubator. Equal amounts of protein from each sample (run in duplicate) were separated electrophoretically using 4-12% Bolt™ Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to Hybond ECL nitrocellulose blotting membranes (GE Healthcare Bio-Sciences, Piscataway NJ). The membranes were blocked in a solution of 5% nonfat dry milk and PBS + 0.1% Tween 20 (pH 7.4; 60 min), followed by overnight incubation (4°C) with rabbit polyclonal anti P2 receptor primary antibody ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$, P2Y1, $P2Y_2$, $P2Y_4$, P2Y $_6$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$;; Alomone Labs, Jerusalem, Israel). The blots were washed with PBS + 0.1% Tween 20 and incubated with secondary antibody (1:4,000 goat anti-rabbit IgG horseradish peroxidase conjugate, Cell Signaling Technology, Danvers, MA) at room temperature for 60 min. Immunoreactivity was detected by enhanced chemiluminescence (Clarity™ Western ECL Substrate, BIo-Rad) and images were developed after exposure to X-ray film (CLASSIC X-Ray Film, Research Products International Corp, Prospect, IL). The same membranes were stripped, washed and re-probed with monoclonal anti-β-actin antibody (1:10,000, Sigma) as a loading control.

4.3.8. RT-PCR Analysis for Detection of P2 Receptor mRNA

P2X and P2Y receptor mRNA expression were identified in IMCD3 cell extracts using two-step RT-PCR. C57BL/6 mouse kidney and brain total RNA was used as positive controls. The extracted RNA was quantified by microplate spectrophotometer Take3 (Synergy™H1,Bio Tek® Instruments, Inc. Winooski, VT). P2X and P2Y receptor cDNA sequences were obtained from GenBank. Sense and antisense primers (Integrated

DNA Technologies, INC, Coralville, IA) for each gene (Table 4.1) were designed on different exons to avoid amplification of contaminating genomic DNA, except for $P2Y_4$ and $P2Y_{13}$ which contain a single coding exon. One microgram of total RNA was reverse-transcribed at 42°C for 30 min in a 20-µl reaction volume using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The cDNA template was added to a 50-µl PCR reaction including Taq DNA Polymerase (Taq PCR Core Kit QIAGEN Sciences), and PCR reactions were performed on the iCycler RT- PCR system (Bio-Rad). The amplified PCR products were run on 2% agarose gel containing 0.5 µg/ml ethidium bromide and visualized using the Fluorchem™ E system (Proteinsimple, Santa Clara, CA.). Product size was estimated with exACTGene 100-bp DNA ladder (Thermo Fisher Scientific, Waltham, MA,). Five primer sets were designed and tested for the $P2Y_{14}$ receptor gene, to detect all eight reported splice variants, and we found that the set included in Table 4.1 showed specific bands for IMCD3 cells.

4.3.9. Statistics

Data are represented as mean $\pm$ SE. One-way ANOVA was used to compare the differences between the groups. $P < 0.05$ was considered significant.

4.4. Results

4.4.1. Na and Water Loading in the IMCD

To determine the effect of salt or water loading on ET-1 mRNA production, isolated IMCD from mice or rats fed a high NaCl or water diet were evaluated for ET-1 mRNA content. In both mice (Figure 4.1A) and rats (Figure 4.1B), salt or water loading

increased ET-1 mRNA content. The data on NaCl loading has been previously published (7) but is included herein for purposes of comparison with water loading. It should be noted that ET-1 mRNA, as opposed to ET-1 protein, was measured in the current study (in both acutely isolated and cultured IMCD). ET-1 protein release or cell content is not detectable due to the relatively small numbers of cells involved. However, in essentially every condition in which ET-1 mRNA and protein have been measured, ET-1 mRNA reflects ET-1 protein levels (28, 29). This may be due, at least in part, to the fact that ET-1 mRNA is very short-lived (half life of ~15 min) due to the presence of destabilizing AUUUA sequences in the 3-UTR region of the ET-1 message (30, 31).

4.4.2. Effect of Flow on IMCD3 ET-1 mRNA production

In order to determine the parameters for the subsequent flow experiments, an empiric 2 h period was chosen for exposing the cells to flow at shear stresses from 0-7.5 dyne/cm^2. All levels of shear stress (0.25, 0.5, 1, 2, 5 and 7.5 dyne/cm^2) stimulated ET-1 mRNA; the degree of stimulation (~2-2.5-fold increase) was not significantly different between shear stress magnitudes (Figure 4.2A). A shear stress of 2 dyne/cm^2 was chosen for all further experiments. A time course was performed at a shear stress of 2 dyne/cm^2 for 15 min, 30 min, 1 h, 2h and 4 h. A stimulatory effect on ET-1 mRNA was first observed at 1 h and was maximum at 2 h (Figure 4.2B). Based on these findings, all subsequent experiments were carried out for 2 h at a shear stress of 2 dyne/cm^2.

4.4.3. Role of Calcium in Flow-stimulated IMCD3 ET-1 mRNA Production

Since shear stress-induced cell signaling may depend, at least in part, on changes in $[Ca^{2+}]_I$ (32, 33), the involvement of Ca^{2+} in flow-regulaTted ET-1 mRNA accumulation in IMCD3 cells was determined. Cells were exposed to no flow and flow conditions using Ca^{2+}-free HBSS. Absence of media Ca^{2+} prevented the flow-stimulated ET-1 mRNA increase. In order to look at the role of $[Ca^{2+}]_i$ in the ET-1 flow response, cells were pretreated for 30 min with BAPTA (an intercellular Ca^{2+} chelator) and then exposed to static and flow conditions. The ET-1 flow response was completely prevented by treatment with BAPTA (Figure 4.3A). Thus, both extracellular and intracellular Ca^{2+} are required for the ET-1 flow response in IMCD3 cells.

Please note that all data are presented as percent of no-flow control; in each case, the flow and no-flow control cells were exposed to the same perfusate and factors. So, for studies on absence of media Ca^{2+}, both flow and no-flow cells contained Ca^{2+}-free HBSS, while studies using BAPTA had the reagent added to the media of both flow and no-flow cells. This same experimental design was done for all ensuing studies in this manuscript.

4.4.4. Effect of Inhibition of Calcium Signaling Molecules on Flow-stimulated IMCD3 ET-1 mRNA Production

Given that Ca^{2+} is involved in the ET-1 flow response in IMCD3 cells, we next investigated the role of pathways mediating Ca^{2+} signaling. Inhibition of calmodulin (CaM) with calmidazolium chloride markedly reduced the ET-1 flow response (Figure 4.2B). KN-93, an inhibitor of CaM kinase (CaMK), also greatly inhibited the ET-1 flow response (Figure 4.3B). Since ET-1 in endothelial cells can be modulated by shear stress

via the Ca^{2+}-sensitive pathways, phospholipase C (PLC) and protein kinase C (PKC), we investigated their role in the ET-1 flow response. Inhibition of PKC (calphostin C) prevented the flow-stimulated ET-1 mRNA increase (Figure 4.3C). PLC inhibition (U-73122) also prevented flow-induced ET-1 mRNA accumulation in IMCD3 cells (Figure 4.3C). Since the above Ca^{2+} signaling pathways can modulate the calcineurin (Cn)/nuclear factor of activated T cells (NFAT pathway), the effect of inhibition of Cn and Ca^{2+}-dependent NFAT on the IMCD3 ET-1 flow response was evaluated. Inhibition of Cn (cyclosporine A or calcineurin inhibitory peptide) prevented the flow-stimulated ET-1 increase (Figure 4.3D). However, inhibition of Ca^{2+}-dependent NFAT (VIVIT peptide) did not reduce the flow response significantly (Figure 4.3D). Taken together, these data suggest the involvement of Ca/CaM/CaMK/PKC/PLC/Cn, but not Ca^{2+}-dependent NFAT, in flow-regulated IMCD ET-1 mRNA accumulation.

4.4.5. Role of Primary Cilia, Polycystin-1 and Polycystin-2 in Flow-regulated IMCD3 ET-1 mRNA Production

Since primary cilia and polycystins have been implicated in flow-stimulated increases in $[Ca^{2+}]_I$ (34), we examined their role in modulating the ET-1 flow response in IMCD3 cells. The presence of primary cilia in IMCD3 cells was confirmed by immunostaining for acetylated tubulin (data not shown). Primary cilia were chemically removed by chloral hydrate (48 h treatment) and cells were exposed to static and flow conditions; chloral hydrate completely prevented the flow response (Figure 4.4). To test for a role of polycystin-2, an IMCD3 cell line with deficient polycystin-2 expression was subjected to flow; no increase in ET-1 mRNA in response to flow was observed (Figure

4.4). To test for a role of polycystin-1, a mouse collecting duct cell line lacking polycystin-1 was exposed to flow; no increase in ET-1 mRNA in response to flow was noted (Figure 4.4). Taken together, these data suggest a role for primary cilia and polycystins-1 and -2 in flow-stimulated ET-1 mRNA accumulation in IMCD3 cells.

4.4.6. Transient Receptor Potential (TRP) Channels and Flow-stimulated IMCD3 ET-1 mRNA Production

While polycystin-2 mediates, at least in part, flow-stimulated IMCD3 ET-1 mRNA accumulation, it is possible that other transient receptor potential (TRP) channels that can transport Ca^{2+} are involved. Since TRPC3, TRPC6 and TRPV4 are known to be flow-sensitive and are expressed in collecting duct cells (35, 36), their role in the ET-1 flow response was determined. Treatment with inhibitors of TRPC3 (Pyr3), TRPC6 (SKF-96365) and TRPV4 (RN-1734) did not significantly alter flow-induced ET-1 mRNA accumulation (Figure 4.5). Thus, TRPC3, TRPC6 and TRPV4 do not appear to be involved in flow-stimulated IMCD3 ET-1 mRNA accumulation.

4.4.7. Purinergic Receptors and Flow-stimulated IMCD3 ET-1 mRNA Production

In addition to polycystin-2 and other TRP channels, Ca^{2+} can enter cells via purinergic P2X receptors (37). Furthermore, purinergic P2Y receptors can modify P2X receptor-mediated Ca^{2+} entry. To assess for a possible role of purinergic receptors, IMCD3 cells were first exposed to a nonspecific purinergic receptor antagonist (PPADS) and the effect on flow-stimulated ET-1 mRNA assessed. Treatment with PPADS abolished the ET-1 flow response (Figure 4.6). To further confirm the role of purinergic

receptors in the ET-1 flow response, IMCD3 cells were exposed to P2 receptor agonists in order to saturate the receptors and mitigate any additional purinergic signaling potentially elicited by flow. As shown in Figure 4.7, pre-incubation with γ-ATP (relatively P2Y-specific) and α,β-methylene-ATP (relatively P2X-specific) largely prevented additional flow stimulation of ET-1 mRNA content. Taken together, these data support the notion that purinergic receptors, and possibly both P2X and P2Y receptors, are involved in flow-regulation of IMCD3 ET-1 mRNA accumulation.

To begin to determine which P2 receptors may be involved in the ET-1 flow response, mRNA and protein for known P2 receptor isoforms were determined in IMCD3 cells. As shown in Figures 4.8 and 4.9, mRNA and protein for all known isoforms of P2X receptors were detected. In addition, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{13}$, but not $P2Y_{12}$ and $P2Y_{14}$, mRNA was found in IMCD3 cells (Figure 4.10). Protein for all known isoforms of P2Y receptors was detected in IMCD3 cells with the exception of $P2Y_{12}$ (Figure 4.11). Cells were then pretreated with a variety of specific P2 isoform antagonists and the effect on flow-stimulated ET-1 mRNA assessed. Please note at the outset that attempting to block each purinergic receptor isoform was not feasible due to lack of specific reagents and was also impractical. Consequently, efforts were focused on P2 receptors that are known to be expressed by IMCD in vivo and that had been implicated in possibly modifying CD salt and/or water transport. $P2Y_2$ inhibition with ARC-118925 completely prevented flow-stimulated ET-1 mRNA accumulation (Figure 4.6). Treatment with inhibitors of $P2X_1$, $P2X_3$ (0.1 μM and 10 μM diinosine pentaphosphate, respectively) or $P2X_4$ (5-BDBD) did not alter the ET-1 flow response (Figure 4.7). In contrast, treatment with the $P2X_7$ antagonist, A-438079, abolished the ET-1 flow

response (Figure 4.12). Thus, these data suggest that both $P2Y_2$ and $P2X_7$ mediate flow-stimulated IMCD ET-1 mRNA accumulation.

Given that purinergic receptors are involved in the ET-1 flow response, the question arises are to whether activation of cilia and subsequent involvement of polycystins might lead to ATP release which then activates P2 receptors, as has been reported (38, 39). To assess this, ATP release by normal and Pkd2-deficient IMCD3 cells was examined under stationary and flow conditions. Both normal and Pkd2-deficient cells had substantial flow-stimulated ATP release (Figure 4.13), suggesting that while ATP release may be involved in the ET-1 flow response, it does not explain the failure of Pkd2-deficient cells to increase ET-1 mRNA following flow exposure. To examine this issue in greater detail, normal and Pkd2-deficient IMCD3 cells were exposed to ATP under non-flow conditions for 2 h and the effect on ET-1 mRNA determined. As shown in Figure 4.14, addition of ATP markedly stimulated ET-1 mRNA in normal IMCD3 cells, but failed to enhanced ET-1 mRNA levels in Pkd2-deficient cells. Hence, there appears to be a coupling between polycystin-2 signaling and purinergic receptor signaling that is necessary for the ET-1 flow response.

4.4.8. Role of ENaC on Flow-stimulated IMCD ET-1 mRNA Production

As described in the introduction section, the ET-1 flow response in mpkCCD cells is mediated by Na^+ delivery via ENaC (25). To determine if this mechanism is operative in IMCD3 cells, the effect of specific ENaC inhibitors, amiloride and benzamil, on the ET-1 flow response was assessed. Neither ENaC inhibitor modified flow-stimulated ET-1 mRNA accumulation in IMCD3 cells (Figure 4.15). Finally, blockade of NCLX with

CGP371571 did not alter the ET-1 flow response. Thus, ENaC and NCLX are not involved in the ET-1 flow response in IMCD3 cells.

4.5. Discussion

The current study reports that flow increases ET-1 mRNA in IMCD cells and that this response is dependent upon: 1) extracellular and intracellular Ca^{2+}; 2) the CaM/CaMK/Cn and PLC/PKC pathways; 3) primary cilia and polycystin-1 and -2; and 4) activation of purinergic $P2Y_2$ and $P2X_7$ receptors. While these experiments were largely conducted in vitro, they raise a number of intriguing, albeit speculative, possibilities. First, they provide a possible explanation for how BFV expansion, at least in part, induces a natriuretic and diuretic response in the CD independent of circulating hormones: increased tubule fluid flow, by virtue of increased IMCD ET-1 production, could lead to autocrine inhibition of IMCD Na^+ and water transport. Second, these studies describe, for the first time, cilia and polycystin regulation of ET-1 production. Such a relationship may be relevant to renal salt and water excretion under normal physiological conditions as well as in the setting of impaired ciliary or polycystin function. The latter possibility is of particular interest given that hypertension commonly manifests in patients with polycystic kidney disease (PCKD) before apparent renal functional deterioration (40, 41). Third, the current studies report an interaction between the CD purinergic and ET systems. Given that the CD purinergic system elicits rapid and transient (minutes) (42) while the CD ET system causes delayed and sustained (hours), inhibition of Na^+ and water transport, these findings suggest the presence of a temporally integrated regulatory system in the CD that provides both rapid onset and sustained

inhibition of natriuresis and diuresis in response to BFV expansion. Taken together, our findings identify a novel system in the IMCD wherein flow, via cilia and polycystins, leads to purinergic receptor activation that, through Ca^{2+} signaling pathways, ultimately stimulates production of ET-1, a highly potent and long-acting inhibitor of CD Na^+ and water reabsorption.

Other key observations in the current study were that flow stimulation of IMCD ET-1 mRNA did not depend upon ENaC or mitochondrial NCLX. These findings are in contrast to those observed in mpkCCD cells, a mouse CCD cell line, wherein flow-stimulated ET-1 mRNA accumulation was prevented by blockade of ENaC or NCLX (25). Both mpkCCDc14 and IMCD3 cells express ENaC and exhibit amiloride inhibition of apical-to-basal Na^+ flux by amiloride (43); hence, the lack of ENaC dependence in IMCD3 is not due to absence of ENaC. However, given that the CCD contains substantially more ENaC than IMCD (26), it is tempting to speculate that CCD ET-1 production is primarily dependent upon Na^+ delivery, while IMCD ET-1 production is primarily regulated by fluid flow (which would be increased during both salt and water loading). In such a scenario, one could envision cortical and inner medullary CD-derived ET-1 serving different biologic roles wherein the former is primarily intended to respond to salt loads while the latter is primarily intended to respond to volume loads. In this regard, it is notable that in vivo IMCD ET-1 mRNA accumulation was stimulated by both salt and water loading (it is problematic to assess CCD ET-1 mRNA levels in response to changes in salt or water intake since accurate quantification of ET-1 mRNA content in CCD is very difficult due to the inability to isolate sufficient numbers of cells or tubules).

The current study reports that Ca^{2+} is essential for the ET-1 flow response in

IMCD3 cells. These findings are in agreement with previous studies showing that flow-stimulated ET-1 mRNA in mpkCCD cells is dependent upon intracellular and extracellular Ca^{2+} (24). In addition, ET-1 production by primary cultured rat IMCD cells under nonflow (stationary) conditions was Ca^{2+}-dependent (29). We also found that the ET-1 flow response is mediated by PLC and PKC; similar findings have been observed in flow-stimulated mpkCCD cells (24) and endothelial cells (29), as well as in stationary rat IMCD cells (29). Notably, PKC regulates ET-1 gene transcription in rat IMCD cells via a nonclassical AP-1-like site in the ET-1 promoter (44). Finally, the ET-1 flow response in IMCD3 cells was dependent upon CaM, CaMK and Cn in agreement with previous studies showing dependence of ET-1 production on these enzymes in stationary cultures of rat IMCD cells (29). In contrast, the ET-1 flow response in mpkCCD cells was not dependent on CaM-regulated pathways (24), suggesting a difference not only in the initial sensing component between CCD and IMCD, but also the Ca^{2+}-signaling pathways involved. Interestingly, inhibition of NFATc (Ca^{2+}-sensitive NFAT isoforms 1-4) did not alter the ET-1 flow response in IMCD3 cells, although NFATc isoforms are involved in maintaining basal IMCD ET-1 levels (45). These findings suggest that Cn is modulating IMCD flow-stimulated ET-1 production through non-NFATc dependent pathways, the nature of which remains to be determined. Taken together, the current studies indicate that Ca^{2+}/CaM/CaMK/Cn- and PLC/PKC-dependent pathways are required for flow-stimulated ET-1 mRNA accumulation in the IMCD.

Since extracellular Ca^{2+} is required for the ET-1 flow response in IMCD, we sought to determine the mechanisms responsible for presumed flow-stimulated Ca^{2+} entry. The IMCD contains several flow-regulated apical plasma membrane Ca^{2+}

channels, including TRPC3 (46), TRPC6 (47) and TRPV4 (48, 49); however, blockade of these channels did not alter the ET-1 flow response. In contrast, chemical removal of cilia or genetic deletion of polycystin-1 or -2 completely prevented the ET-1 flow response. Ciliary bending in response to flow is well known to increase $[Ca^{2+}]_I$ (34), an effect that is mediated by polycystin-1 activation of polycystin-2 (TRPP2), the latter being a Ca^{2+}-permeable cation channel (34). Importantly, in a dog distal nephron-derived cell line (MDCK), removal of primary cilia abolished the flow-regulated $[Ca^{2+}]_i$ increase (50). These findings represent the first observation in any biologic system of cilia and polycystins regulating ET-1 production. As stated earlier, one may speculate that this relationship has bearing on the early hypertension seen in polycystic kidney disease. The cause for this early hypertension has been related to cyst compression of normal renal structures and activation of the intrarenal renin-angiotensin system (51); however, a recent study found hypertension was present in a mouse model of Pkd1 deficiency when only minimal renal cysts were present (52). Notably, while renal ET-1 production is increased in cystic kidney disease after significant renal damage has occurred, there is no information on renal or CD ET-1 production in kidneys without functional polycystins or cilia, but before cysts form. Such studies were beyond the scope of the current analysis, but are clearly an important area for further investigation.

Another mechanism by which Ca^{2+} could enter cells in response to flow is via purinergic-gated P2X receptors. P2X receptors are expressed on IMCD cells in vivo (53); we found that IMCD3 cells express all known P2X isoform mRNA and protein. Blockade of $P2X_7$, but not $P2X_1$, $P2X_3$ or $P2X_4$, receptors prevented the ET-1 flow response. Notably, blockade of the $P2X_7$ receptors reduces ATP modulation of salt and water

transport in pronephric ducts (54, 55). Since P2Y receptors have been reported to interact with P2X receptors in elevating $[Ca^{2+}]_i$ levels (37), the role of P2Y receptors in modifying the ET-1 flow response was investigated. IMCD3 cells express mRNA and protein for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{13}$, a pattern similar to that seen in IMCD cells in vivo (53). Of these P2Y receptors, $P2Y_2$ receptor has been most extensively studied in the nephron; $P2Y_2$ receptor activation elicits an inhibitory effect on CD Na+ and water reabsorption (56, 57, 58), while $P2Y_2$ receptor knockout mice have reduced flow-stimulated increases in $[Ca^{2+}]_i$ (37). Blockade of $P2Y_2$ receptors prevented the ET-1 flow response in IMCD3 cells, indicating that both $P2X_7$ and $P2Y_2$ receptors are involved. How such an interaction occurs will require further analysis, including determination of their individual and combined effects on $[Ca^{2+}]_I$ and specific signaling pathways; such experiments were beyond the scope of the current study.

Given that cilia, polycystins and P2 receptors are involved in the ET-1 flow response in IMCD3 cells, the question arose as to whether and how these systems might interact. Mechanical stimulation of renal tubular epithelial cells, whether through ciliary bending or other forces, promotes the release of ATP (59). We detected no significant difference in flow-stimulated ATP release by normal or Pkd2-deficient IMCD3 cells; however, ATP-stimulated ET-1 mRNA accumulation was completely prevented in IMCD3 cells lacking polycystin-2. Thus, flow stimulates ATP release by these cells, at least in part, through nonpolycystin-dependent mechanisms; however, normal P2 receptor function requires polycystin-2. These findings are consistent with previous reports demonstrating shear stress-induced ATP release by both ciliated and nonciliated MDCK cells (60), as well as the observation that cilia are required for normal P2-receptor-

mediated Ca^{2+}-signaling (50). In regard to the latter, it is notable that $P2X_7$ receptor expression has been localized to primary cilia in human CD. We did not specifically test whether $P2X_7$ or P_2Y_2 receptor expression is altered in Pkd2-deficient cells nor did we evaluate whether these receptors co-localize with polycystins and/or cilia; however, these and other more in-depth analysis of cilia/polycystin/purinergic system interactions is clearly warranted.

In summary, the current study makes several novel and potentially important observations. First, we demonstrate that cilia and polycystins are necessary for flow-stimulated ET-1 synthesis in IMCD3 cells. Second, we report that $P2X_7$ or P_2Y_2 receptors are required for the flow response and that their activity depends upon polycystin-2. Third, these systems interact to regulate ET-1 mRNA accumulation through modulation of Ca^{2+}-dependent signaling pathways. While further studies are needed to validate this proposed system in vivo, we speculate that the cilia/polycystin/P2 receptor/ET-1 system constitutes a complex interactive pathway through which the CD can detect tubule fluid flow and ultimately achieve both immediate and sustained regulation of CD salt and water reabsorption. This system may be of physiologic relevance and has at least the theoretical potential to be involved in hypertensive states characterized by impaired CD ciliary, polycystin, purinergic or endothelin system dysfunction.

Acknowledgements

The authors thank Dr. Christa E. Müller, Pharmaceutical Institute, University of Bonn, Bonn, Germany for kindly providing ARC-118925. The authors also thank Shali Zhang for performing the western blotting and RT-PCR for the purinergic receptor screening in m-IMCD3 cells. The research was supported in part by NIH P01 HL095499 (to D.E.K. and E.W.I.), VA Merit Reviews (to R.R. and to B.K.), NIH DK044628 and HL098135 (to E.W.I.) and NIH RO1 DK51050 (to J.Z.).

Statement of conflicting interests

There are no conflicts of interests**.**

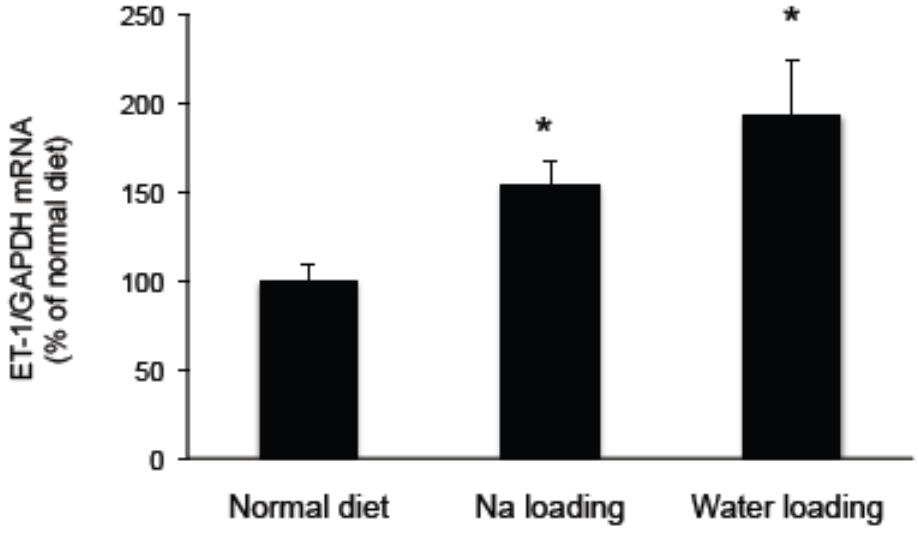
Table 4.1.

P2 Isoform	Sense Primer	Antisense Primer	Size (bp)
P2X1	5’-CGT CTG ATC CAG TTG GTG GTT CTG-3’	5’-TGC ACA ATG TCC TTG AGC CTG CTG-3’	255
P2X2	5’-CCA AGT TCA AGT TCT CCA AGG-3’	5’-AAG TCC AGG TCA CAG TTC C-3’	205
P2X3	5’-GGG AAA CCT CCT TCC TAA CCT CAC-3’	5’-GCC AGG GGA AAC ACT GCT TTT CTC-3’	268
P2X4	5’-TGC TCA TCC TGG CTT ACG-3’	5’-CCT TTG GCT TTG GTT GTC AC-3’	100
P2X5	5’-TGC CCA GTG GAG ACA AAG TCC ATG-3’	5’-TCA GGG CTA TGT CCT GGA AGT CAG-3’	247
P2X6	5’-GGG CAT CAG CAT TCA CTG GGA TTG-3’	5’-AGG TCA CAG AGG AAG GTG ACC ATG-3’	279
P2X7	5’-CCA GAC TTG GGA CCC TCA GTG TTC-3’	5’-CGA AGG ACT CAT CCG TGT TCT TGT C-3’	279
P2Y1	5’-AGG AAA GCT TCC AGG AGG AGT GAG-3’	5’-GTG GCA CAC ACT GGT CTT TTG GTC-3’	227

Table 4.1 continued

P2 Isoform	Sense Primer	Anti-sense Primer	Size (bp)
P2Y2	5’-AGG AAA GCT TCC AGG AGG AGT GAG-3’	5’-CTG CGT AGA GAG AGT CCG AAA CTG-3’	298
P2Y4	5’-AAG ATA GTC CTA CCT GTC AAG-3’	5’-GCG TCT ACT CCT GTT ACC-3’	368
P2Y6	5’-TCA GAC TGA GGA CGT CAG TGC TTC-3’	5’-AAA TCC TCA CGG TAG ACG CAG GTG-3’	282
P2Y12	5’-CAG AGG GCT TTG GGA ACT TAT-3’	5’-GAA CCT GGG TGA TCT TGT AGT C-3’	219
P2Y13	5’-GGT CCC TGA TGT TCT TCA TCT-3’	5’-CCT GCT GTC CTT ACT CCT AAA C-3’	239
P2Y14	5’-ATG CAG CAC TTC CCG CTT GTC AAC-3’	5’-GGG TCT GTG GTG GAG TTG TTC-3’	290

A.



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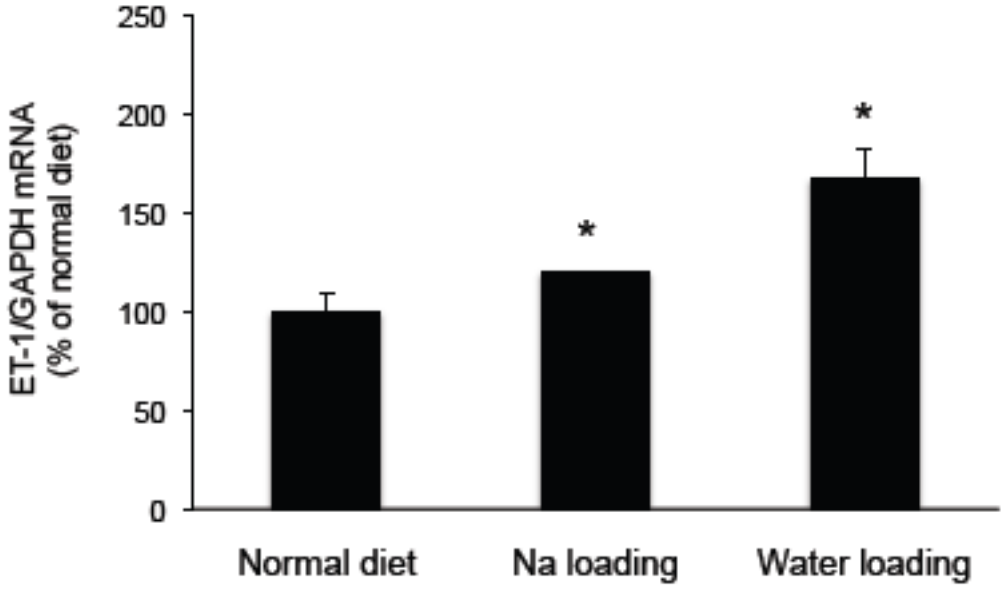
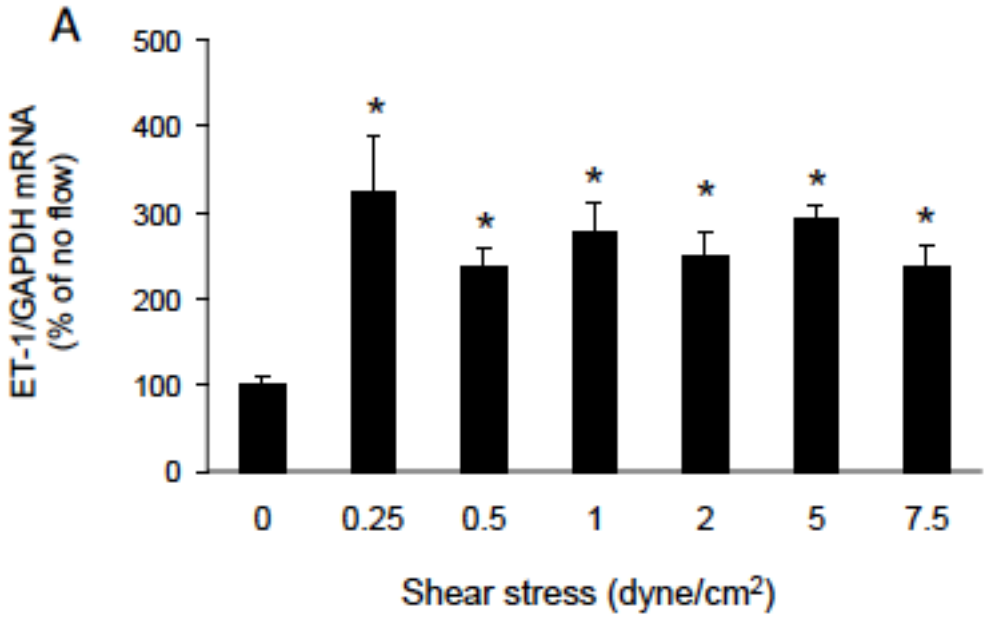


Figure 4.1. Effect of Na^+ and water loading on IMCD ET-1 mRNA content in rats (A) and mice (B). For Na^+ loading, rats and mice were fed a normal (0.25%) or high (8%) NaCl diet for 3 days. For water loading, rats and mice were fed 1% sucrose in normal drinking water for 3 days; n=3 each data point. *$p<0.05$ vs. animals fed a normal NaCl and water diet.

A.



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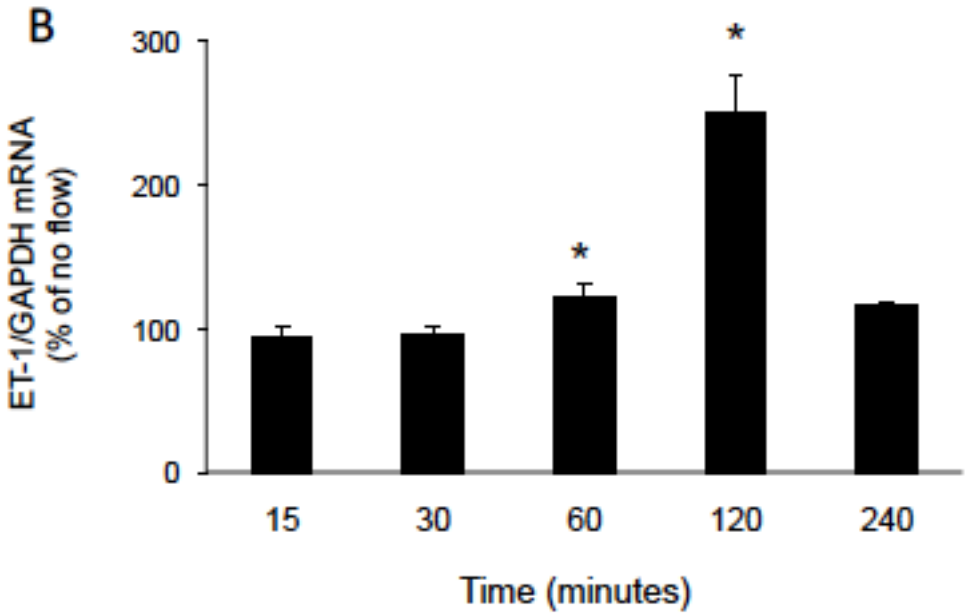
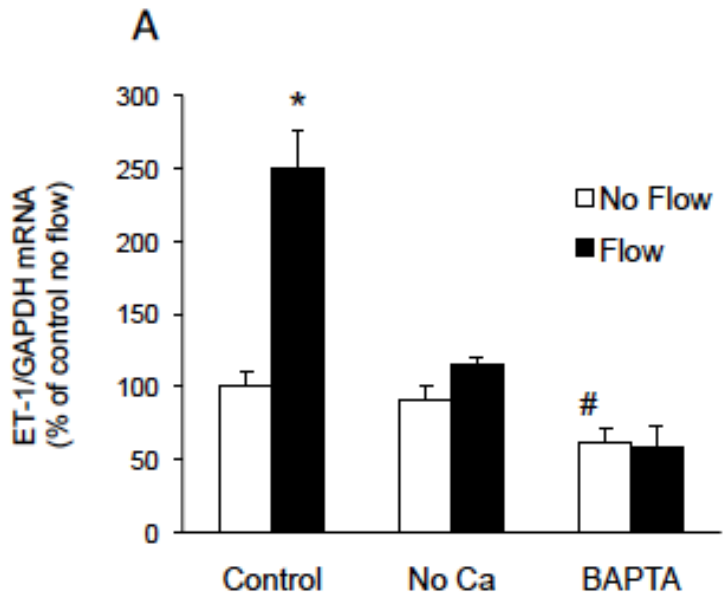


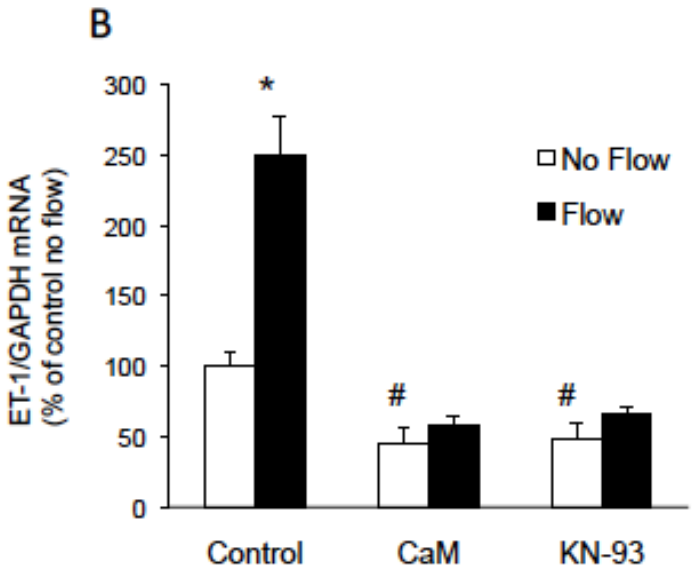
Figure 4.2. Dose response (A) and time course (B) of flow effect on ET-1 mRNA levels in IMCD3 cells. For the dose response, cells were subjected to different shear stress for 2 h; for the time course (B), cells were exposed to a shear stress of 2 dyne/cm^2 for different lengths of time; n=12 each data point. *$p<0.05$ vs. cells not exposed to flow.

Figure 4.3. Effect of Ca^{2+} and inhibitors of Ca^{2+} signaling molecules on flow-regulated ET-1 mRNA in IMCD3 cells. Cells were pre-incubated with perfusate HBSS, [Ca^{2+}]-free media, 50 μM BAPTA (intercellular Ca chelator) (A), 20 μM calmidazolium chloride (inhibitor of calmodulin), 10 μM KN-93 (calmodulin kinase inhibitor) (B), 0.1 μM calphostin C (CalC, PKC inhibitor), 2 μM U-71322 (PLC inhibitor) (C), 3 μg/mL cyclosporine A (CyA), 10 μM calcineurin inhibitory peptide (CiP) (both inhibitors of calcineurin), or 0.2 μM VIVIT peptide (NFAT inhibitor) (D) for 30 minutes and then subjected to flow for 2 h at 2 dyne/cm^2 followed by determination of ET-1/GAPDH mRNA levels; n=12 each data point. *$p<0.05$ vs. cells treated identically, but not exposed to flow.

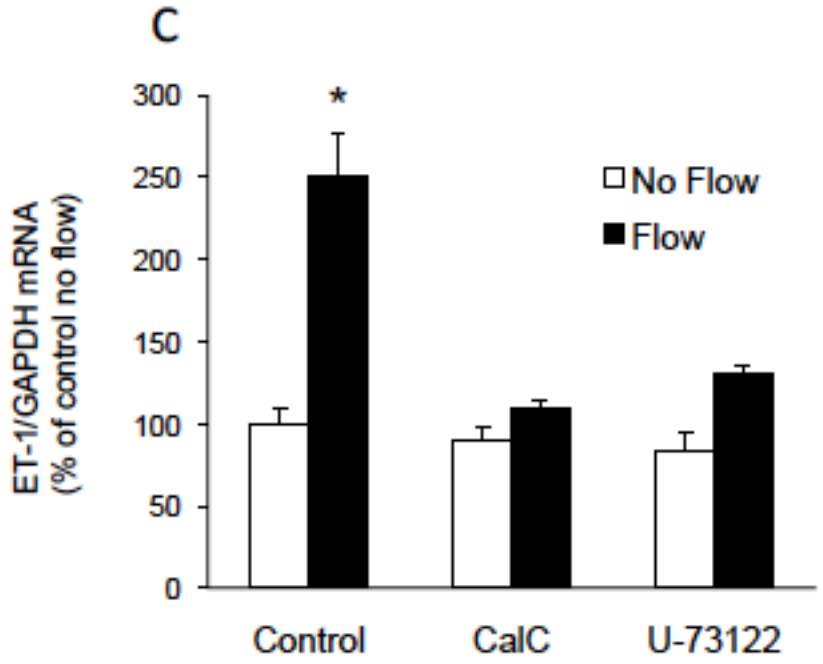
A.


A
ET-1/GAPDH mRNA
(% of control no flow)
300
250
200
150
100
50
0
*
#
No Flow
Flow
Control
No Ca
BAPTA

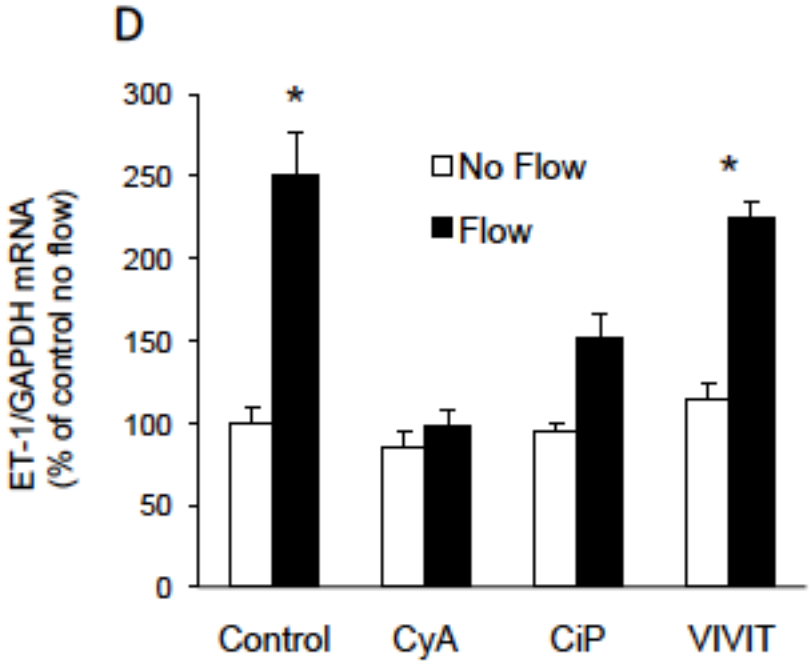
B.


B
ET-1/GAPDH mRNA
(% of control no flow)
300
250
200
150
100
50
0
*
#
#
No Flow
Flow
Control
CaM
KN-93

C.



D.



(Figure 4.3 continued)

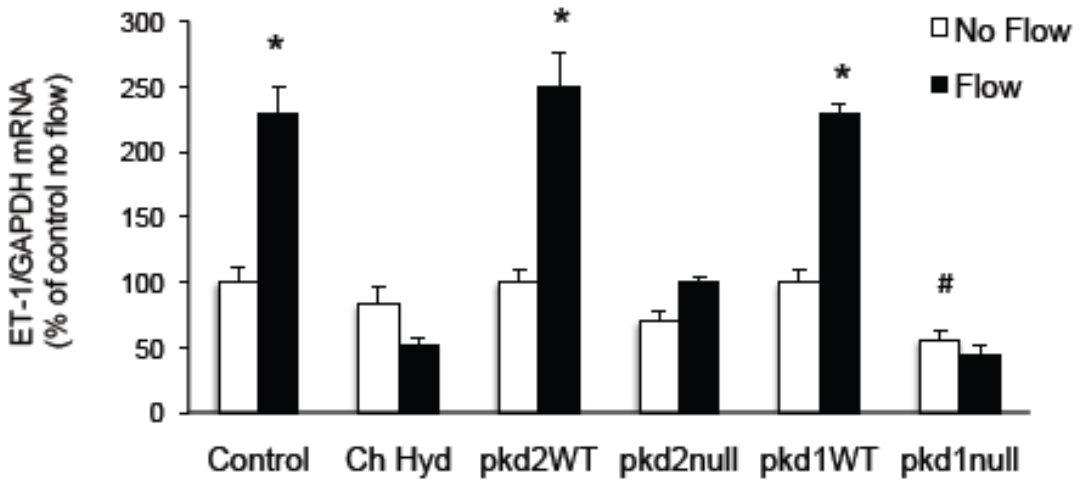


Figure 4.4. Effect of inhibition of primary cilia and polycystins on flow-regulated ET-1 mRNA in IMCD3 cells. For cilia removal, cells were pre-treated for 48 h with 4 mM chloral hydrate (Ch Hyd). To study the role of polycystin-2, IMCD3 cells lacking (Pkd2 null) or with (Pkd2 WT) polycystin-2 were used. To determine the role of polycystin-1, mouse collecting duct cells lacking (pkd1 null) or with (pkd1 WT) polycystin-1 were used. Cells were exposed to static or flow conditions (2 h at 2 dyne/cm^2), followed by determination of ET-1/GAPDH mRNA content; n=12 each data point. *$p<0.05$ vs. cells treated identically, but not exposed to flow.

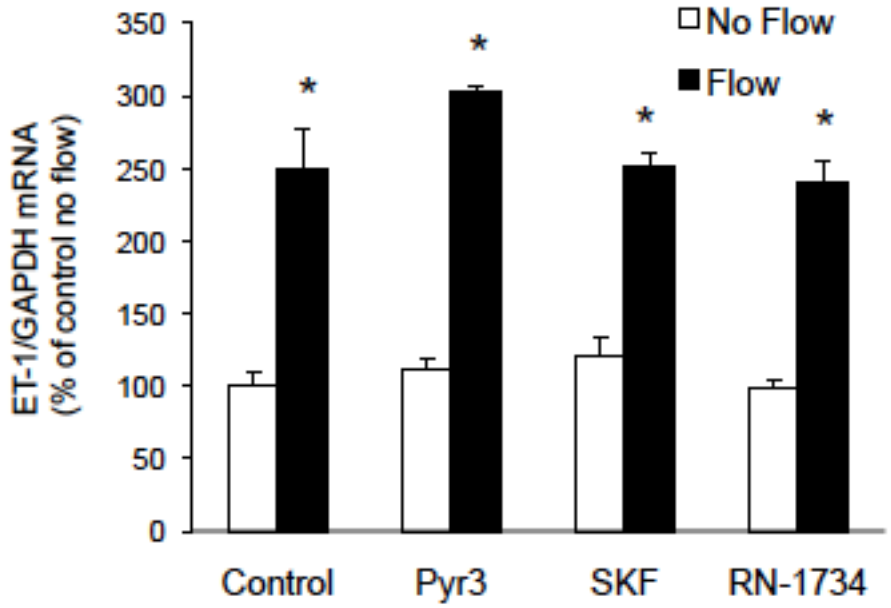


Figure 4.5. Effect of TRP channel inhibition on flow-stimulated ET-1 mRNA levels in IMCD3 cells. Cells were pretreated for 30 min with 10 μM Pyr3 (TRPC3 inhibitor), 20 μM SKF-96365 (SKF, TRPC6 inhibitor) or 30 μM RN-1734 (TRPV4 inhibitor), exposed to static and flow conditions for 2 h at 2 dyne/cm^2 followed by determination of ET-1/GAPDH mRNA levels; n=12 each data point. *$p<0.05$ vs. cells treated identically, but not exposed to flow.

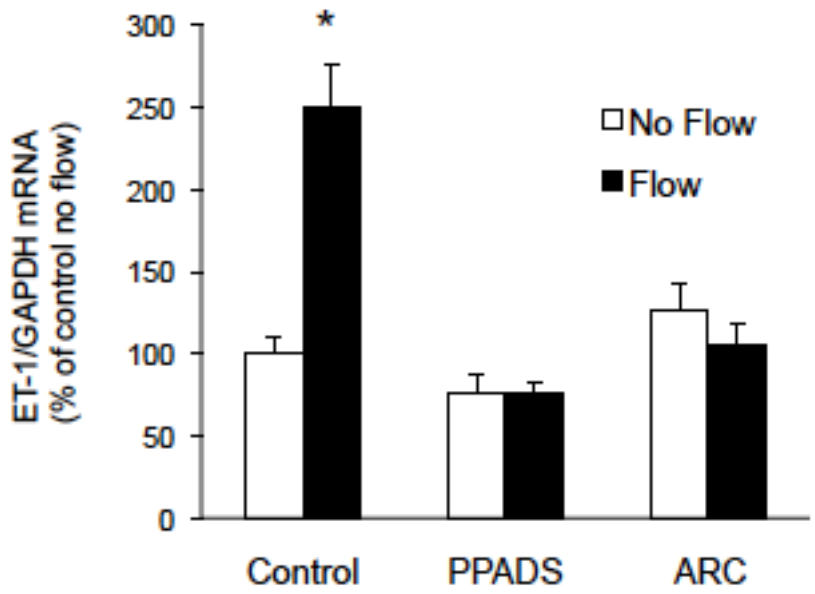


Figure 4.6. Effect of P2X or P2Y2 inhibition on flow-stimulated ET-1 mRNA levels in IMCD3 cells. Cells were pretreated for 30 min with 30 μM PPADS (P2X inhibitor) or 10 μM ARC-118925 (P2Y2 inhibitor), exposed to static and flow conditions for 2 h at 2 dyne/cm^2 followed by determination of ET-1/GAPDH mRNA levels; n=10 each data point. *$p<0.05$ vs. cells treated identically, but not exposed to flow.

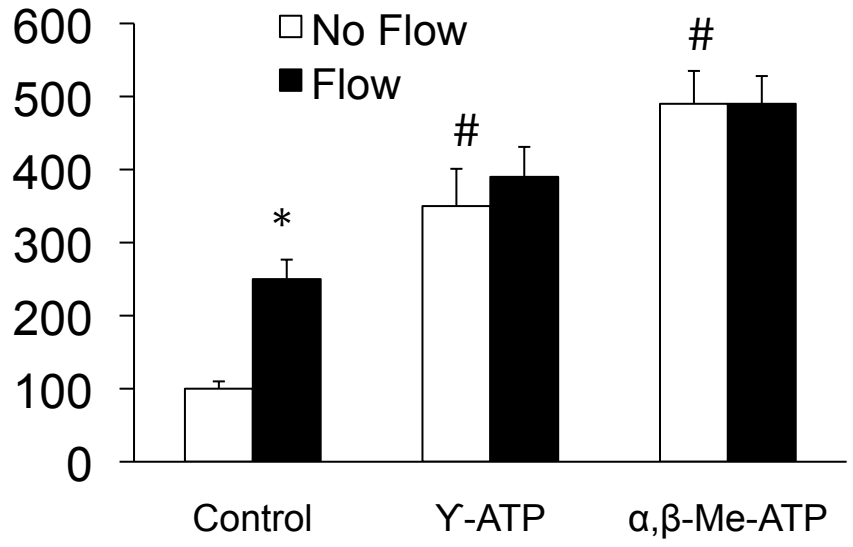


Figure 4.7. Effect of purinergic agonists on flow-stimulated ET-1 mRNA levels in IMCD3 cells. Cells were pretreated for 30 min with 30 μM Y-ATP (purinergic receptor agonist more specific for P2Y receptors) or α, β-methylene ATP (purinergic receptor agonist more specific for P2X receptors), exposed to flow and static conditions followed by ET-1/GAPDH mRNA levels; n=10 each data point.*$p<0.05$ vs. cells treated identically, but not exposed to flow.

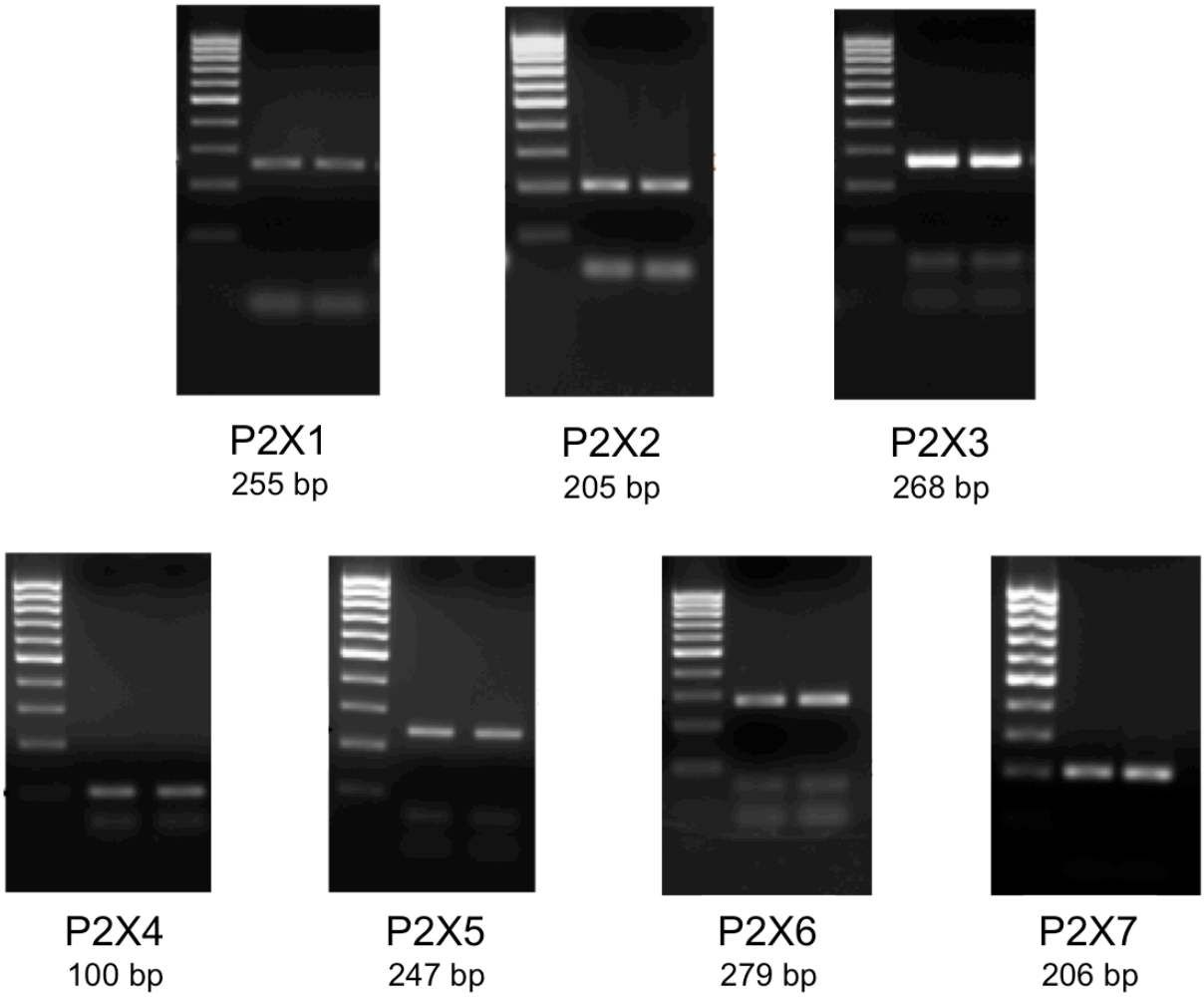


Figure 4.8. RT-PCR analysis of P2X receptor mRNA expression in IMCD3 cells. Results from two separate samples are shown. Primer sequences used for each receptor are shown in Table 4.1.

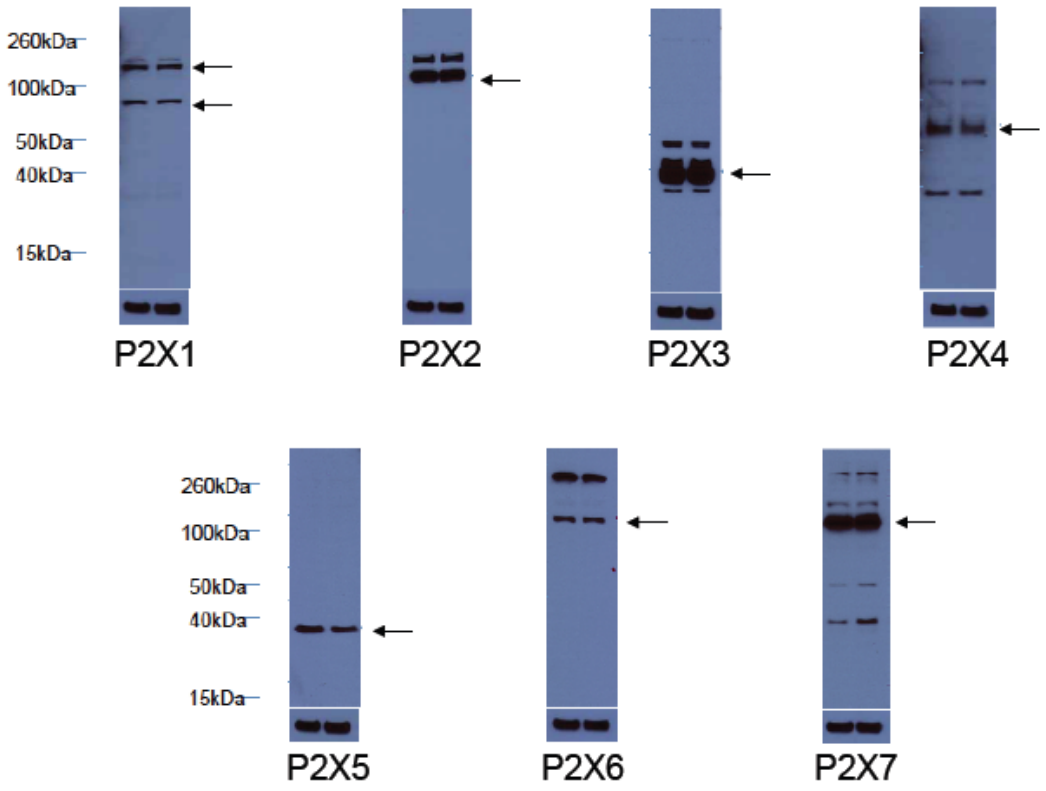


Figure 4.9. Western blot analysis of P2X receptor expression in IMCD3 cells. Results are shown in duplicate for each receptor subtype along with the β-actin loading controls. Lanes were loaded with IMCD3 cell lysates (20 µg protein/lane).

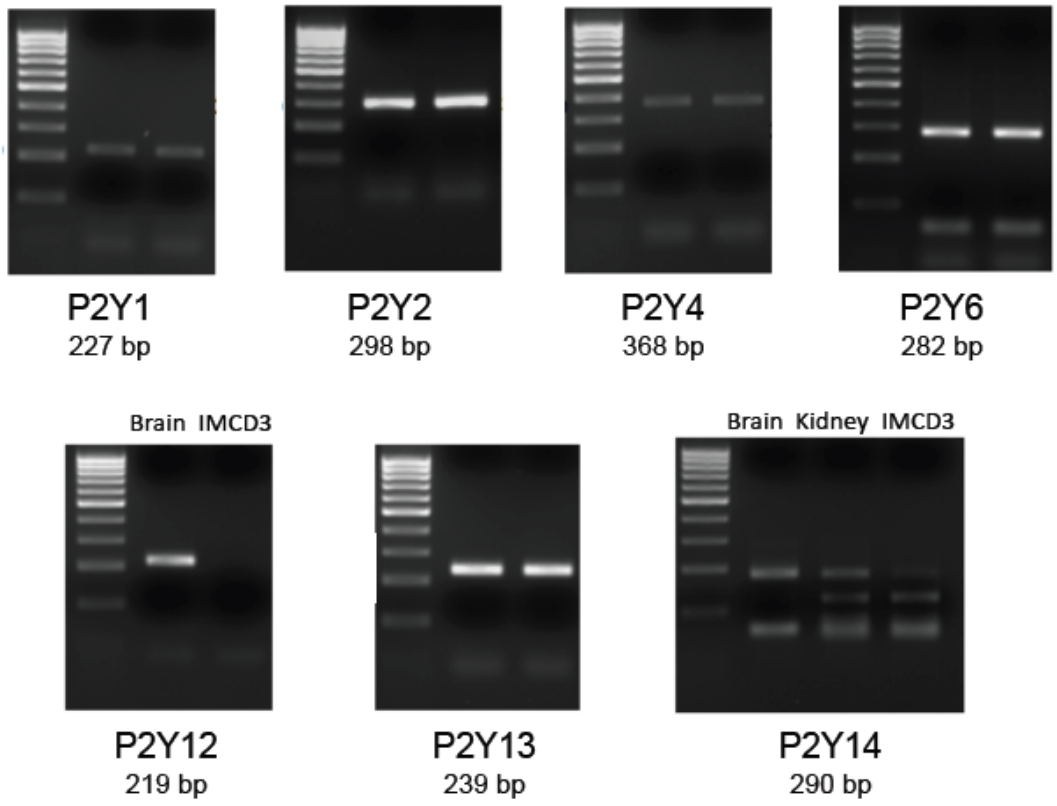


Figure 4.10. RT-PCR analysis of P2Y receptor mRNA expression in IMCD3 cells. Results from two separate IMCD3 samples are shown for all blots except $P2Y_{12}$ and $p2Y_{14}$. C57BL/6 mouse brain and kidney total RNA were used as a positive controls for $P2Y_{12}$ and/or $p2Y_{14}$. Primer sequences used for each receptor are shown in Table 4.1.

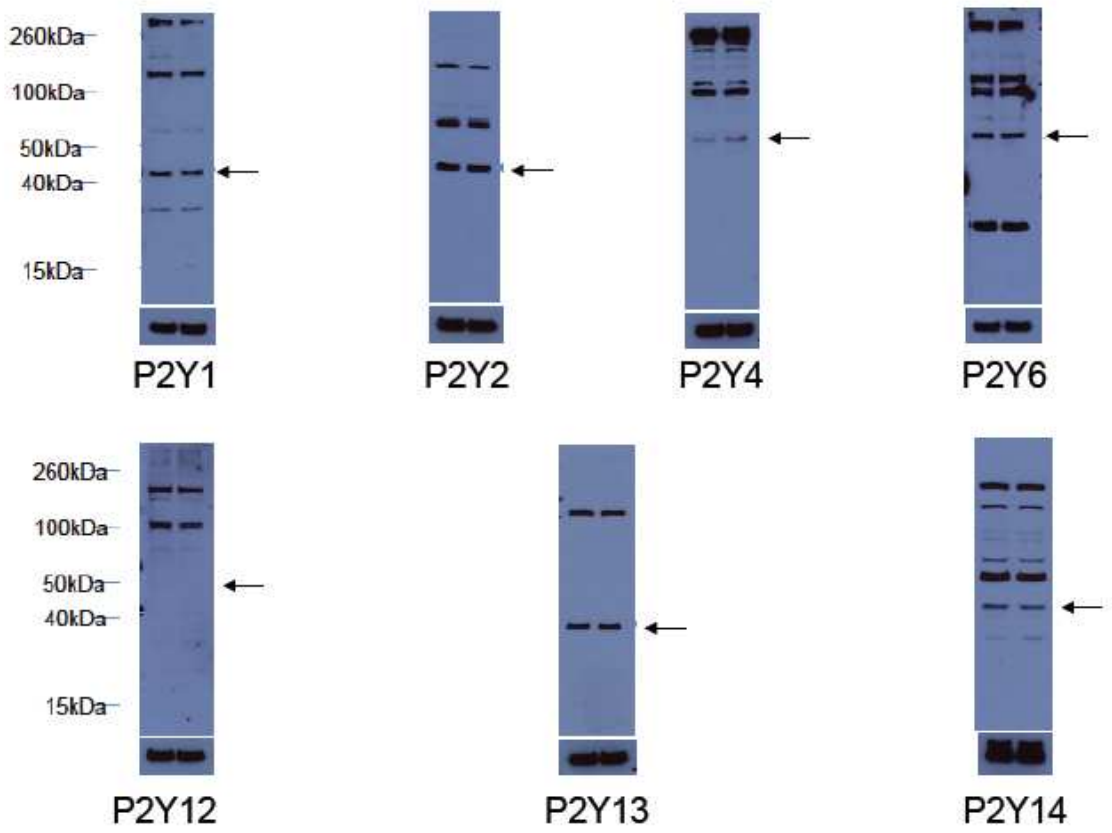


Figure 4.11. Western blot analysis of P2Y receptor expression in IMCD3 cells. Results are shown in duplicate for each receptor subtype along with the β-actin loading controls. Lanes were loaded with IMCD3 cell lysates (20 µg protein/lane).

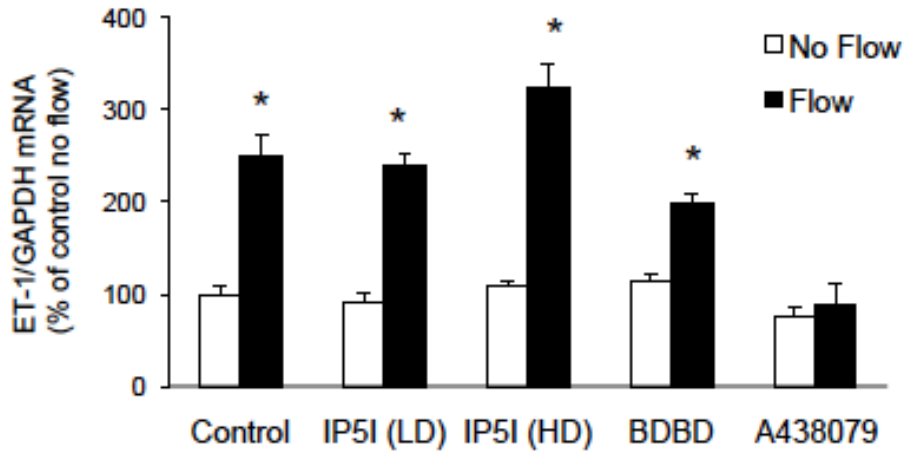


Figure 4.12. Effect of P2X receptor isoform inhibition on flow-stimulated ET-1 mRNA levels in IMCD3 cells. Cells were pretreated for 30 min with 0.1 μM diinosine pentaphosphate ($P2X_1$ inhibitor), 10 μM diinosine pentaphosphate ($P2X_3$ inhibitor), 15 μM 5-BDBD ($P2X_4$ inhibitor) or 10 μM A438079 ($P2X_7$ inhibitor), exposed to static and flow conditions for 2 h at 2 dyne/cm^2 followed by determination of ET-1/GAPDH mRNA levels; n=10 each data point. *$p<0.05$ vs. cells treated identically, but not exposed to flow.

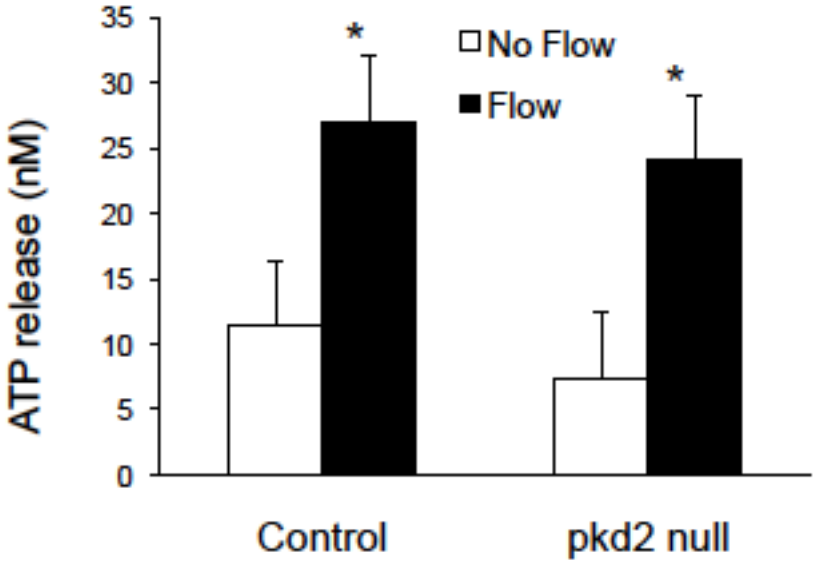


Figure 4.13. ATP release by normal IMCD3 cells and IMCD3 cells lacking polycystin-2. Cells were exposed to static or flow conditions at 2 dyne/cm^2 and ATP release into the supernatant after 2 h of exposure to flow or no flow determined; n=10 each data point. *p<0.05 vs. cells treated identically, but not exposed to flow.

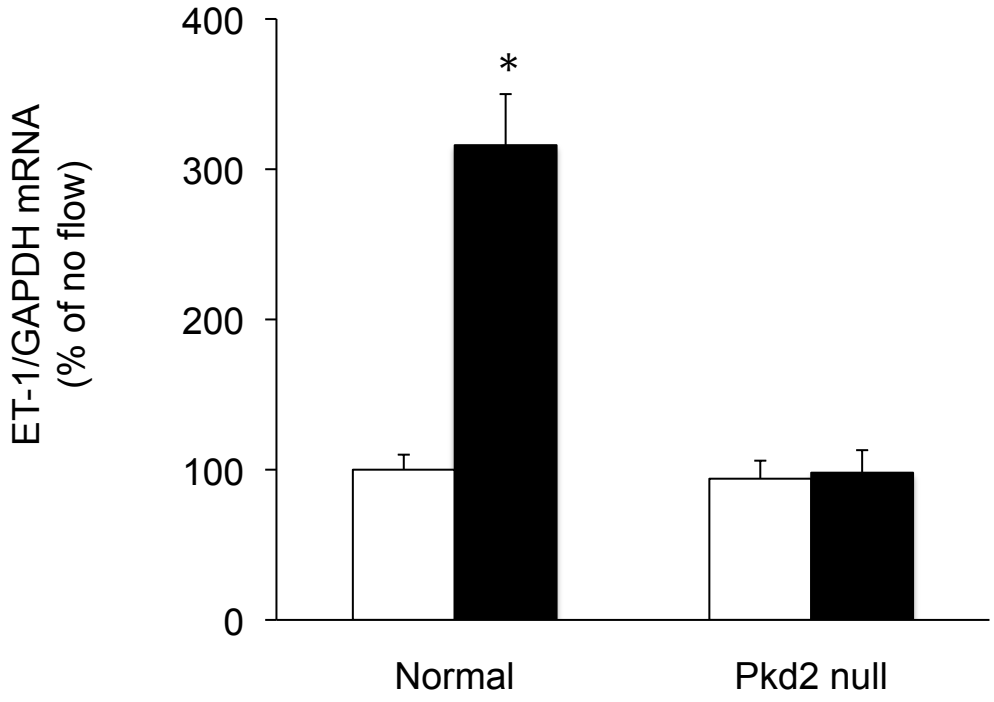


Figure 4.14. Effect of exogenous ATP on ET-1 mRNA levels in IMCD3 cells under nonflow conditions. N= 12 each data point. *$p<0.05$ vs. no flow in cells of same genotype.

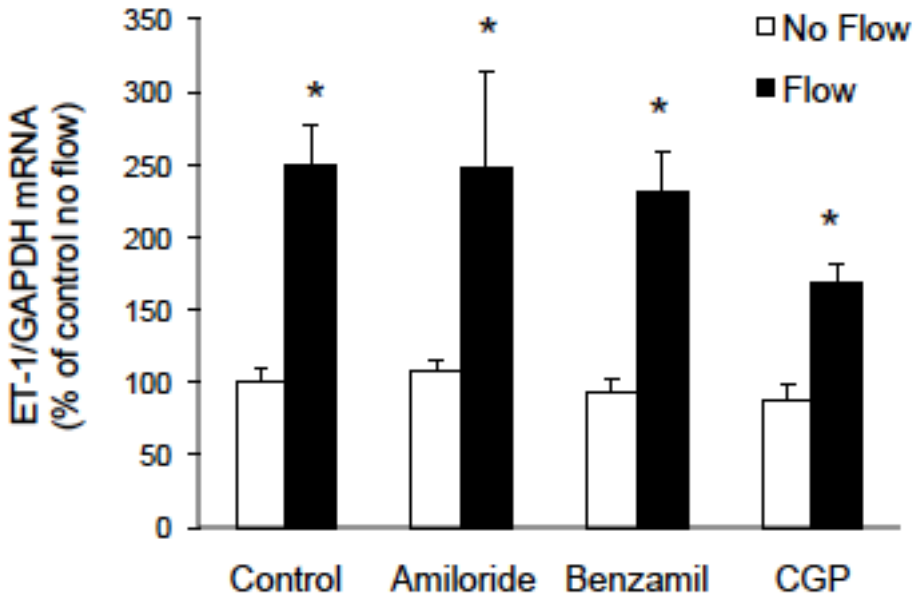


Figure 4.15. Effect of ENaC inhibition on flow-stimulated ET-1 mRNA levels in IMCD3 cells. Cells were pretreated for 30 min with either 1 μM amiloride, 0.2 μM benzamil or 1.2 μM CGP-37157, exposed to static or flow conditions (2 h at 2 dyne/cm^2) followed by determination of ET-1/GAPDH mRNA levels; n=12 each data point. *$p<0.05$ vs. cells treated identically, but not exposed to flow.

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CHAPTER 5

INCREASED PERFUSATE SOLUTE CONCENTRATION MODULATES FLOW-STIMULATED ET-1 PRODUCTION IN THE INNER MEDULLARY COLLECTING DUCT

(Work in Progress)

Meghana M. Pandit[12] and Donald E. Kohan[123]

[1]Division of Nephrology, University of Utah Health Sciences Center, Salt Lake City, UT

[2]Department of Pharmaceutics and Pharmaceutical Chemistry, Salt Lake City, UT

[3]Salt Lake Veterans Affairs Medical Center, Salt Lake City, UT

5.1. Abstract

Endothelin-1 (ET-1) is an important autocrine inhibitor of salt and water reabsorption. Collecting duct (CD)-specific knockout of ET-1 causes marked hypertension and impaired natriuresis and diuresis. Extracellular fluid volume (ECFV) expansion augments CD ET-1 production, thereby promoting excretion of excess salt and water via the urine. However, the coupling mechanism between volume status and CD ET-1 synthesis is poorly understood. Volume expansion increases fluid flow, thus causing more solutes and water to reach the CD. We have previously published that fluid flow augments ET-1 mRNA levels (~2.3 fold) in cultured m-IMCD3 cells and in cortical collecting duct (CCD) cells. While only Na^+ delivery augmented the ET-1 flow response in CCD cells, Na^+ along with other solutes such as mannitol or urea doubled flow-stimulated ET-1 mRNA production in inner medullary collecting duct (IMCD cells). Hence, increased solute delivery seems to augment ET-1 mRNA accumulation in m-IMCD3 cells. Ca^{2+}-signaling molecules and polycystin-2 do not mediate the effect of increased perfusate concentration on flow-stimulated IMCD ET-1 mRNA production as opposed to their effect on flow under isoosmolar conditions. However, inhibition of the tonicity-enhancer binding protein (TonEBP) prevented the ET-1 flow response under both isoosmolar and solute-rich conditions. Based on these findings, we propose that increase in osmolarity due to presence of excess solutes may elevate flow-regulated IMCD ET-1 production and this effect is mediated via TonEBP. Taken together, these studies suggest the existence of a novel pathway by which ECFV status might be maintained in the body, ultimately achieving blood pressure control.

5.2. Introduction

Endothelin-1 (ET-1) is an important regulator of Na^+ and water transport and arterial pressure (1). Various studies demonstrate that ET-1 inhibits Na^+ and water transport in an autocrine manner in the collecting duct (CD) (2, 3, 4, 5). The CD produces and binds more ET-1 than any other cell type in the body (6). Thus, CD-derived ET-1 is produced locally and exerts its actions via binding to high density, high affinity receptors resulting in and natriuresis (6, 7, 8), thereby combating fluid retention.

Several factors modulate the production of inner medullary collecting duct (IMCD) ET-1 (9). Previously, we reported that fluid flow stimulates ET-1 production in the CD. Na^+ delivery, via the epithelial Na^+ channel (ENaC) and subsequent activation of the mitochondrial Na^+/Ca^{2+} exchanger (NCLX), regulates flow-stimulated ET-1 production in the cortical collecting duct (CCD) (10). Interestingly, we found that flow-stimulated ET-1 production is regulated via an entirely different mechanism in the IMCD. Our studies demonstrate that Ca^{2+} signaling via purinergic and ciliary interaction mediates flow-stimulated IMCD ET-1 mRNA accumulation (unpublished data). Thus, the CCD studies demonstrate how ET-1 promotes natriuresis while the IMCD studies indicate how water reabsorption might be inhibited, both processes by which extracellular fluid volume (ECFV) status and ET-1 production can be related.

Additionally, ECFV expansion causes more solutes and water to reach the CD. An important function of the CD is to get rid of the excess solutes in the body. However, how the CD might be handling this excess of solutes is incompletely understood.

We report that increasing perfusate solute concentration, irrespective of the solute used, augments flow-stimulated ET-1 mRNA production along with the existence of a

pathway by which the solute effect might be mediated in the IMCD.

5.3. Materials and Methods

5.3.1. Reagents

NFAT inhibitor or VIVIT peptide and cyclosporin A were obtained from Tocris Bioscience (Ellisville, MO). All other drugs and chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise.

5.3.2. RNA Analysis and Real-time PCR

RNA from cultured m-IMCD3 cells was isolated using the RNeasy Mini Kit and reverse transcribed using Omniscript RT Kit (Qiagen, Valencia, CA). GAPDH and ET-1 mRNA levels were determined by real-time PCR (StepOne Plus, Applied Biosystems, Foster City, CA) using the Taqman Gene Expression Assay with ET-1 (Catalog number Mm00438656_m1), GAPDH (Catalog number Mm03302249_m1) and NFAT5 primers (Catalog number Mm00467257_m1).

5.3.3. Cell Culture

The mouse inner medullary collecting duct cell line, m-IMCD3, was used for all studies unless specified otherwise. Polycystin-2 mutant m-IMCD3 cells were provided by Dr. Rajeev Rohatgi at the Icahn School of Medicine at Mt. Sinai University, NY. Cells were grown to confluence on 10-cm^2 plastic culture plates in a 5% CO_2 incubator at 37^oC; 50:50 DMEM/F-12 supplemented with 10% fetal bovine serum, 1 mg/mL penicillin and 1 mg/mL streptomycin was used as growth medium. For the control studies

performed under stationary conditions, cells were grown in 24-well plates under identical conditions.

5.3.4. Flow Studies

Rectangular parallel plate polycarbonate flow chambers (Catalog number 31-010, Glycotech, Gaithersburg, MD) were attached to individual 10-cm^2 cell culture plates containing confluent m-IMCD3 cells using vacuum and silastic gaskets to form a channel. The channel has the following dimensions: 0.25 mm depth, 1 cm width and 5.9 cm in length, having a total surface area of 5.9 cm^2 for the cells exposed to flow. The flow chamber has two manifolds through which perfusate enters and exits the channel. The liquid is pumped through the channel by a peristaltic pump (Ismatec, Glattburg, Switzerland) at specific flow rates. HBSS (pH 7.4) was used as the perfusate for control experiments and was supplemented with drugs and/or chemicals for additional experiments. RNA was extracted from cells exposed to flow and from control cells. All the experiments were performed at 37^oC.

5.3.5. Transfection Experiments

Mouse NFAT5 siRNA (Catalog number MSS225643) and negative controls (scrambled siRNA sequences) of medium GC content were purchased from Invitrogen Corporation. Cells were grown on 25 mm glass coverslips (Warner Instruments, Hamden, CT) and flow studies were performed in polycarbonate flow chambers (Catalog number 64-1860, Warner instruments, Hamden CT) according to conditions described previously. After flow, RNA was extracted from cells exposed to flow and no flow conditions (as

described previously). All experiments were performed at 37°C.

5.3.6. Statistics

Data are represented as mean ± SE. One-way ANOVA was used to compare the differences between the groups. $P < 0.05$ was considered significant.

5.4. Results

5.4.1. Increased Perfusate Solute Concentration Augments Flow-stimulated ET-1 mRNA in m-IMCD3 Cells

Since volume expansion increases solute delivery to the CD, the effect of increased perfusate solute concentration on flow-stimulated ET-1 mRNA was evaluated. Since flow increases ET-1 mRNA accumulation in the CCD and the flow response is Na^+-dependent, we determined whether an increase in perfusate Na^+ concentration elevated flow-stimulated ET-1 mRNA levels in m-IMCD3 cells. We observed that an increase in Na^+ concentration augmented m-IMCD3 flow-regulated ET-1 mRNA content. In order to determine whether this augmentation was indeed due to increasing perfusate Na^+ concentration, the effect of other solutes such as mannitol or urea on the ET-1 flow response was also evaluated. It must be noted that in static conditions also, augmentation of ET-1 mRNA was observed in response to an increase in perfusate solute concentration (Figure 5.1); however the presence of flow further augmented the effect of increasing perfusate solute concentration. Irrespective of the solute employed, a doubling of the ET-1 flow response in m-IMD3 cells was observed (Figure 5.2). Thus, along with flow, increasing perfusate solute concentration to the IMCD elevates ET-1 mRNA

accumulation in m-IMCD3 cells.

It should be noted that all the data presented in this manuscript is represented as percent of no-flow control. In every experiment, the flow and no-flow cells were exposed to the same treatments and conditions.

5.4.2. ENaC and $[Ca^{2+}]_i$ are not involved in the Solute Effect on Flow-stimulated ET-1 mRNA in m-IMCD3 Cells

In order to further rule out the possibility of the solute effect being mediated by Na^+, cells were pretreated with 0.2 μM benzamil, a selective and potent inhibitor of ENaC. Cells were then exposed to static or flow conditions, the latter involving perfusion with isotonic HBSS (300 mOsm/L) or HBSS with mannitol (450 mOsm/L). We observed that ENaC inhibition failed to prevent the ET-1 flow response in m-IMCD3 cells and it also failed to prevent the flow response in case of increased perfusate solute concentration (Figure 4.3), thus suggesting that the effect of increased solute on flow-regulated IMCD ET-1 mRNA augmentation is not mediated via ENaC.

Previously, we observed that flow-regulated IMCD ET-1 mRNA accumulation requires an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (data not published). In order to study the role of Ca^{2+} in the ET-1 flow response mediated via increased perfusate concentration, cells were pretreated with 50 μM BAPTA, a $[Ca^{2+}]_i$ chelator, and exposed to static or flow conditions in an isotonic or solute-rich environment. We observed that while chelation of $[Ca^{2+}]_i$ prevented flow-stimulated ET-1 mRNA accumulation in m-IMCD3 cells, it failed to prevent the ET-1 flow response in case of increased perfusate concentration, thus suggesting that $[Ca^{2+}]_i$ may not be involved (Figure 4.3). Taken

together, ENaC and $[Ca^{2+}]_i$ do not mediate the effect of increased solute on flow-stimulated ET-1 mRNA augmentation in m-IMCD3 cells.

5.4.3. Cn/NFATc Pathway is not involved in the Effect of Increased Perfusate Concentration on Flow-stimulated ET-1 mRNA in m-IMCD3 Cells

An important signaling pathway that may be triggered by increased solute delivery to the CD is the Cn/NFATc pathway. Findings from one study suggest that hypertonicity as a result of increased solute delivery to the CD can increase $[Ca^{2+}]_i$ and activate the Cn/NFATc pathway to enhance aquaporin-2 (AQP2) expression (11). In order to investigate the role of Cn in the effect of solute delivery on the ET-1 flow response, cells were pretreated with 3 μg/mL cyclosporine A, followed by exposure to static and flow conditions in isotonic or hypertonic perfusate. While inhibition of Cn prevented the ET-1 flow response under isotonic conditions, it failed to do so in case of increased perfusate concentration (Figure 5.4A). In case of NFATc, 0.1 μM VIVIT peptide was used to pretreat cells followed by a similar treatment. NFATc inhibition failed to prevent the ET-1 response to flow in isotonic and perfusate-rich conditions (Figure 5.4B). Taken together, these data indicate that the Cn/NFATc pathway is not involved in the solute effect on the ET-1 flow response.

5.4.4. Polycystin-2 is not involved in the Effect of Solute Loading on Flow-stimulated ET-1 mRNA in m-IMCD3 Cells

Polycystin-2 (PC-2) encoded by the gene *pkd2*, is a major Ca^{2+} channel located at the base of primary cilia (12). Previously, we observed the role of PC-2 in the ET-1 flow

response and hence, the effect of increased perfusate concentration and flow was evaluated in normal m-IMCD3 cells and m-IMCD3 cells lacking the *pkd2* gene. Under isotonic conditions, the PC-2 deficiency prevent flow-stimulated ET-1 mRNA augmentation. In contrast, loss of PC-2 in m-IMCD3 cells did not affect the ET-1 response to flow after increased solute delivery when compared to the basal flow response (in normal m-IMCD3 cells) (Figure 5.5). Thus, PC-2 does not seem to be involved in the solute effect on the ET-1 flow response.

5.4.5. Increased Perfusate Solute Concentration Modulates Flow-stimulated ET-1 mRNA Accumulation via NFAT5/TonEBP in m-IMCD3 Cells

Increased solute delivery might increase tonicity in the CD, leading to activation of nuclear factor of activated T-cells (NFAT5) or the tonicity-response enhancer binding protein (TonEBP) that results in synthesis of a variety of genes that have an osmoprotective effect on IMCD cells. In order to determine whether NFAT5 was involved in the effect of increased solute delivery on the ET-1 flow response, NFAT5 was inhibited (65% NFAT5 mRNA knockdown; data not shown) and flow-regulated ET-1 mRNA in m-IMCD3 cells was determined. Pharmacological inhibition by 10 μM rottlerin (Figure 5.6A) or siRNA knockdown (Figure 5.6B) of NFAT5 prevented the ET-1 flow response in both isotonic and solute-rich conditions. Thus, NFAT5/TonEBP appears to mediate the effect of increased perfusate concentration on flow-regulated ET-1 mRNA accumulation in m-IMCD3 cells.

5.5. Discussion

In the current study, we report that: 1) increased perfusate solute concentration augments flow-stimulated ET-1 mRNA production in m-IMCD3 cells and 2) NFAT5/TonEBP mediates the effect of increased solute delivery on flow-stimulated m-IMCD3 ET-1 mRNA production.

These studies for the first time describe how increased solute delivery as a result of ECFV expansion might be balanced by ET-1 production in the IMCD. We propose that increased fluid flow as a result of body fluid volume (BFV) expansion brings more solute to the CD, elevates ET-1 production which further leads to autocrine inhibition of solute transport, thereby promoting its excretion via the urine. These findings are in complete agreement with previously published studies that report a direct link between urine flow and urinary ET-1 excretion (13, 14, 15, 16).

While increased perfusate solute concentration regulates CD ET-1 production, the mechanism is unknown. One key observation of this study was that inhibition of ENaC did not prevent the ET-1 response to flow in normal or solute-rich environments. This result is not unexpected since the IMCD expresses relatively low levels of ENaC (17).

Inhibition of $[Ca^{2+}]_i$ does not prevent the ET-1 flow response under solute-rich conditions. Further, Ca^{2+} signaling molecules Cn and NFATc do not seem to be involved in the effect of increased perfusate on the flow response. Interestingly, under normal conditions, Ca^{2+} is an important regulator of CD ET-1 production under stationary (18) and flow conditions (unpublished data). $[Ca^{2+}]_i$ is increased as a result of flow due to bending of primary cilia (19). Polycystin-2 (PC-2), a major Ca^{2+} channel present at the base of the cilium forms an integral part of the mechanosensory polycystin-1 (PC1)/PC2

complex (20). When PC2 null cells were exposed to flow in a solute-rich condition, ET-1 mRNA augmentation due to increased solute delivery was still observed. Taken together, Ca^{2+} signaling and primary cilia do not seem to be involved in the solute effect. Hence, another mechanism exists that senses the increased perfusate concentration and augments ET-1 production in the CD.

Hypertonicity augments $[Ca^{2+}]_i$ (21) and may increase ET-1 mRNA accumulation in CD cells. Hypertonicity activates NFAT5 (or TonEBP), a transcription factor, in order to increase the transcription of a variety of osmoprotective genes. TonEBP or NFAT5, a tonicity-responsive enhancer binding protein that belongs to the Rel family of transcription factors, plays a key role in protecting cells from shock in a hypertonic environment. Kidney cells adapt to this hypertonic environment by accumulating osmolytes in the cells. This protein is partially active under isotonic conditions but its activity is upregulated by hypertonicity via increased nuclear localization and transactivation (22, 23). Our findings suggest that pharmacological inhibition of NFAT5 with rottlerin prevents the ET-1 response under normal conditions as well as in case of increased perfusate solute concentration. However, rottlerin may be nonspecific and may inhibit PKCδ (24, 25, 26) and oxygen radical formation (27). Due to its nonspecificity, siRNA was used to transiently knockdown NFAT5 gene expression. The knockdown resulted in complete prevention of the ET-1 flow response. Thus, our findings demonstrate that the effect of increased perfusate concentration on flow-regulated IMCD ET-1 mRNA production may be mediated by NFAT5. In-depth analysis of additional pathways as well as further investigation of how NFAT5 mediates the ET-1 flow response is needed.

In summary, for the first time, we demonstrate that increased solute delivery to the IMCD modulates flow-stimulated ET-1 mRNA, thus further confirming that there is an established relationship between ECFV status and CD ET-1 production. Further, we propose a role for NFAT5 in this regulation. Further studies are needed defining how solute delivery, in the context of flow, stimulates CD ET-1 production.

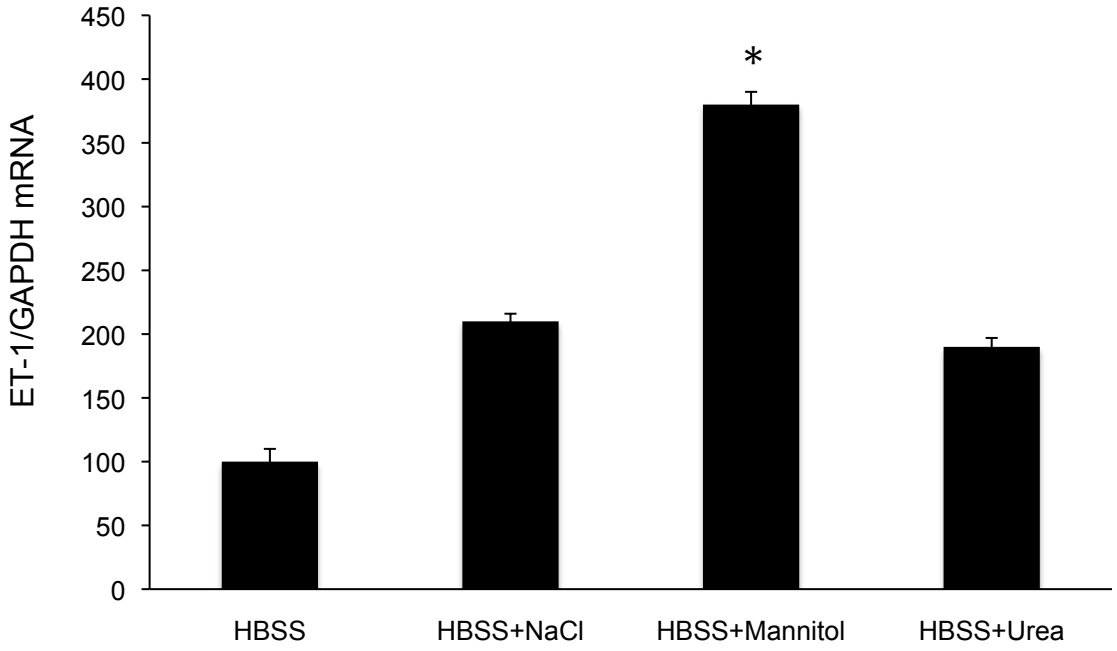


Figure 5.1. Effect of increased solute concentration on IMCD ET-1 mRNA content in stationary m-IMCD3 cells. Cells were exposed to static conditions for 2 hr with perfusate HBSS (300 mOsm/L), HBSS with 150 mOsm/L NaCl, HBSS with 150 mOsm/L mannitol or HBSS with 150 mOsm/L urea followed by determination of ET-1/GAPDH mRNA levels; n = 10 each data point. *$p<0.05$ vs. cells exposed to HBSS alone (300 mOsm/L NaCl)

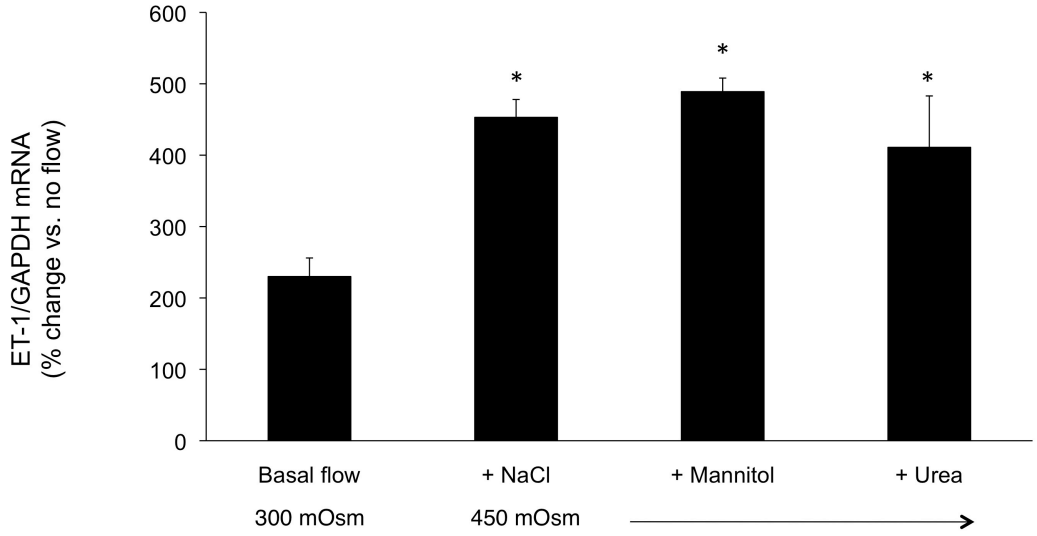


Figure 5.2. Effect of increased solute concentration on IMCD ET-1 mRNA content in m-IMCD3 cells. Cells were exposed to static or flow conditions for 2 hrs at a shear stress of 2 dyne/cm^2 with perfusate HBSS (300 mOsm/L), HBSS with 150 mOsm/L NaCl, HBSS with 150 mOsm/L mannitol or HBSS with 150 mOsm/L urea followed by determination of ET-1/GAPDH mRNA levels; n = 10 each data point. *$p<0.05$ vs. baseline flow (300 mOsm/L NaCl)

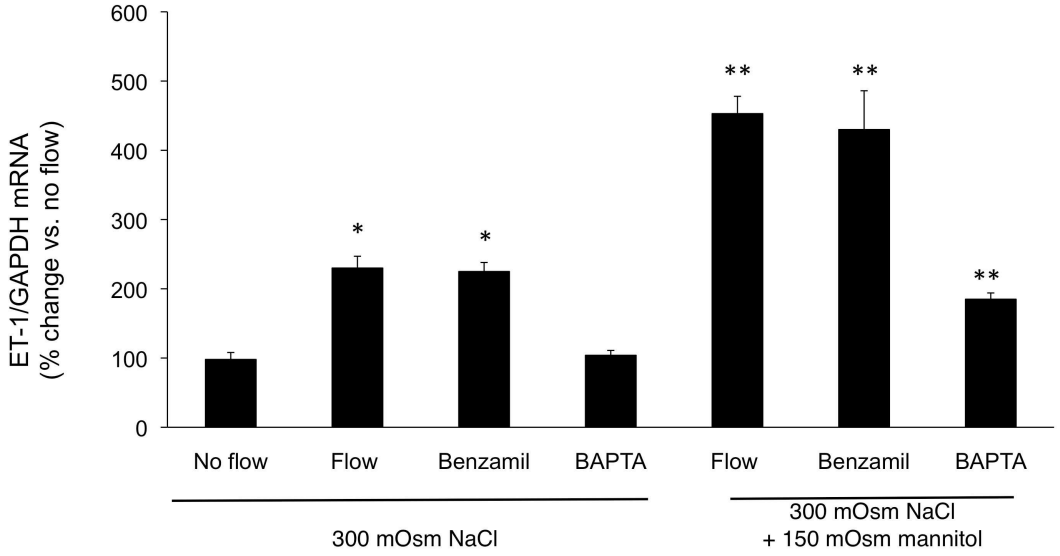
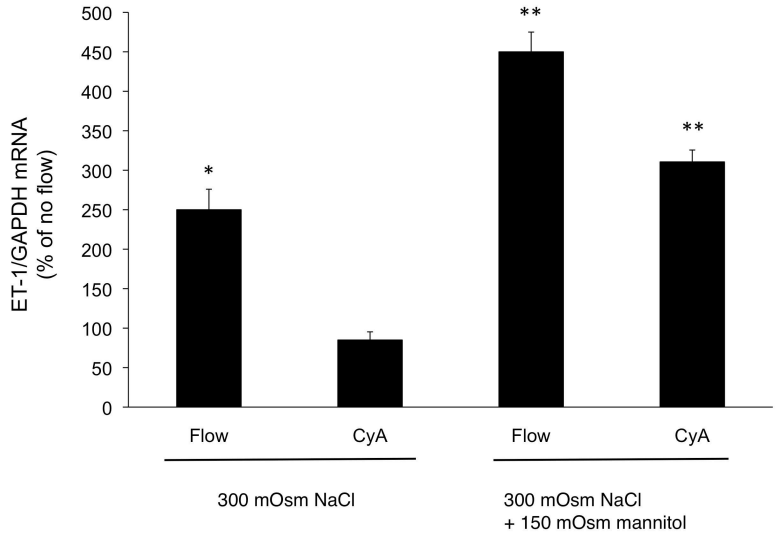


Figure 5.3. Effect of solute loading and ENaC or $[Ca^{2+}]_i$ inhibition on flow-regulated m-IMCD3 ET-1 mRNA content. Cells were preincubated with 0.2 μM benzamil or 50 μM BAPTA before the experiment. Cells were exposed to static or flow conditions at 300 mOsm/L or 450 mOsm/L (HBSS + mannitol) for 2 hr at a shear stress of 2 dyne/cm^2 followed by determination of ET-1/GAPDH mRNA levels; n = 10 each data point. *p<0.05 vs. cells not exposed to flow; **p<0.05 vs. cells exposed to 300 mOsm/L NaCl flow

Figure 5.4. Effect of solute loading and inhibition of calcium signaling molecules on flow-regulated ET-1 mRNA production. A) Effect of solute loading and calcineurin inhibition on flow-regulated m-IMCD3 ET-1 mRNA content. Cells were pre-incubated with 3 μg/mL cyclosporine A followed by exposure to static or flow conditions at 300 mOsm/L or 450 mOsm/L (HBSS + mannitol) for 2 hr at a shear stress of 2 dyne/cm^2 followed by determination of ET-1/GAPDH mRNA levels; n = 10 each data point. *$p<0.05$ vs. cells not exposed to flow; **$p<0.05$ vs. cells exposed to 300 mOsm/L NaCl flow. B) Effect of solute loading and NFATc inhibition on flow-regulated m-IMCD3 ET-1 mRNA content. Cells were pre-incubated with 0.2 μM VIVIT peptide followed by exposure to static or flow conditions at 300 mOsm/L or 450 mOsm/L (HBSS + mannitol) for 2 hr at a shear stress of 2 dyne/cm^2 followed by determination of ET-1/GAPDH mRNA levels; n = 10 each data point. *$p<0.05$ vs. cells not exposed to flow; **$p<0.05$ vs. cells exposed to 300 mOsm/L NaCl flow.

A.


ET-1/GAPDH mRNA
(% of no flow)
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200
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100
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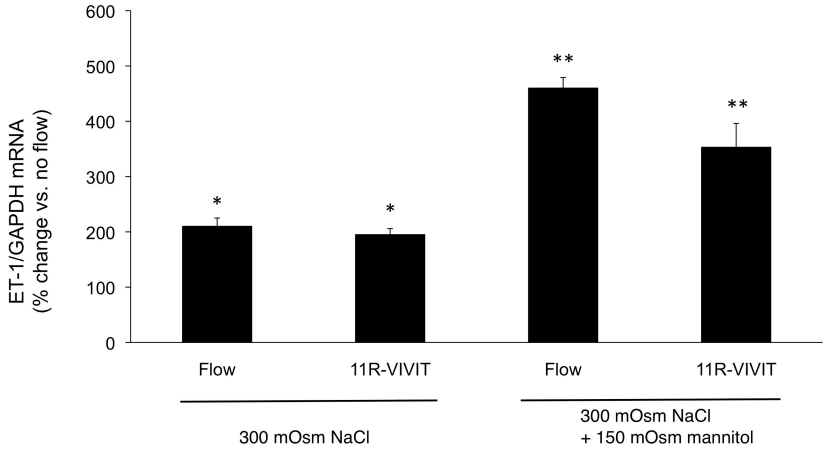
Flow
CyA
Flow
CyA
300 mOsm NaCl
300 mOsm NaCl
+ 150 mOsm mannitol

B.


ET-1/GAPDH mRNA
(% change vs. no flow)
600
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+ 150 mOsm mannitol

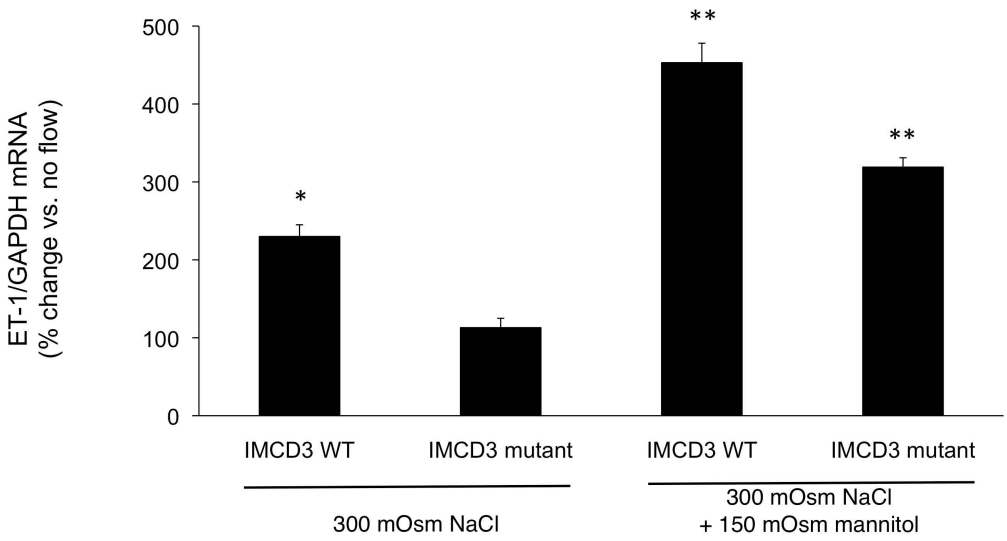
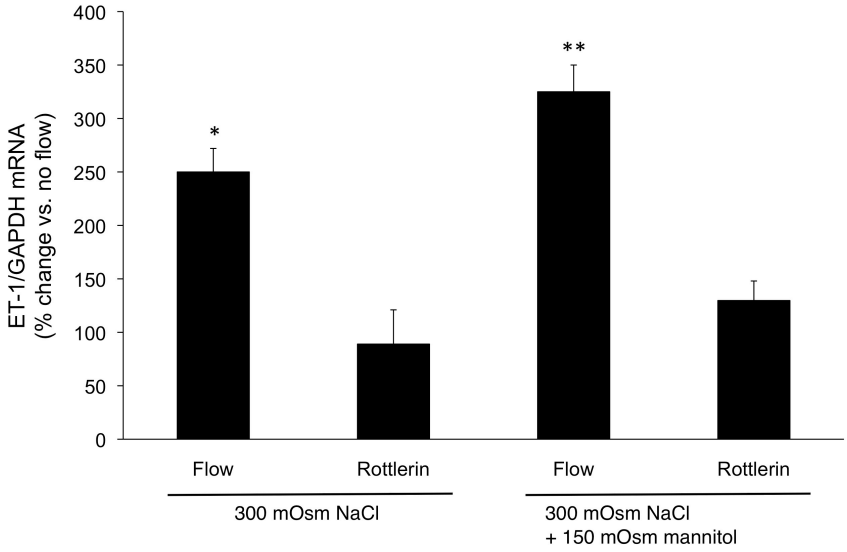


Figure 5.5. Effect of solute loading and polycystin-2 inhibition on flow-regulated m-IMCD3 ET-1 mRNA content. Cells were exposed to static or flow conditions at 300 mOsm/L or 450 mOsm/L (HBSS + mannitol) for 2 hr at a shear stress of 2 dyne/cm^2 followed by determination of ET-1/GAPDH mRNA levels; n = 10 each data point. *$p<0.05$ vs. cells not exposed to flow; **$p<0.05$ vs. cells exposed to 300 mOsm/L NaCl flow.

Figure 5.6. Effect of solute loading and TonEBP/NFAT5 inhibition on flow-regulated m-IMCD3 ET-1 mRNA content. A) Cells were pre-incubated with 10 μM rottlerin followed by exposure to static or flow conditions at 300 mOsm/L or 450 mOsm/L (HBSS + mannitol) for 2 hr at a shear stress of 2 dyne/cm^2 followed by determination of ET-1/GAPDH mRNA levels; n = 10 each data point. *$p<0.05$ vs. cells not exposed to flow; *$p<0.05$ vs. cells exposed to 300 mOsm/L NaCl flow. B) Cells were transfected for 48 hr with siRNA against NFAT5 followed by exposure to static or flow conditions at 300 mOsm/L or 450 mOsm/L (HBSS + mannitol) for 2 hr at a shear stress of 2 dyne/cm^2 followed by determination of ET-1/GAPDH mRNA levels; n = 10 each data point. *$p<0.05$ vs. cells not exposed to flow.

A.


400
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ET-1/GAPDH mRNA
(% change vs. no flow)
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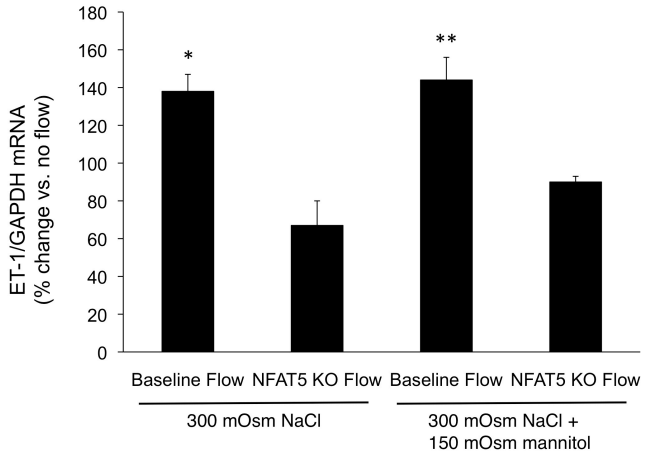
Flow
Rottlerin
Flow
Rottlerin
300 mOsm NaCl
300 mOsm NaCl
+ 150 mOsm mannitol

B.


180
160
140
120
100
80
60
40
20
0
ET-1/GAPDH mRNA
(% change vs. no flow)
*
**
Baseline Flow
NFAT5 KO Flow
Baseline Flow
NFAT5 KO Flow
300 mOsm NaCl
300 mOsm NaCl +
150 mOsm mannitol

5.7. References

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1. Conclusions

In this dissertation, the following points are conclusively demonstrated: 1) Tubule fluid flow increases ET-1 mRNA in both, the CCD and the IMCD; 2) The mechanisms of flow-regulated ET-1 production are differently mediated in the CCD and IMCD; 3) In a mouse CCD cell line, flow-stimulated ET-1 mRNA accumulation appears to occur as a result of Na^+ delivery via ENaC, Na^+/Ca^{2+} exchange, leading to increased $[Ca^{2+}]_i$ concentration and subsequent activation of Ca^{2+}-signaling pathways; 4) In a mouse IMCD cell line, flow-regulated ET-1 mRNA augmentation is intracellular and extracellular Ca^{2+}-dependent with the involvement of CaM/CaMK/Cn/PLC/PKC pathways, and a role for primary cilia, polycystin-1 and polycystin-2 along with activation of purinergic P2Y2 and P2X7 receptors and; 5) perfusate solute concentration augments the ET1 flow response in IMCD cells but not CCD cells.

The above findings suggest that fluid flow that is increased in case of ECFV expansion appears to have an effect on CD ET-1 production. The intracellular pathways through which CD ET-1 synthesis is mediated are certainly complex. Also, physiologically the IMCD and CCD being different, ET-1 production is regulated distinctly in the two segments, thereby helping in the overall maintenance of BP. Thus,

this system might exist in order to ameliorate flow-induced augmentation of CD Na^+ and water reabsorption.

In this dissertation, Chapter 3 explains how flow regulation of ET-1 occurs in the CCD. In the CCD, the magnitude of Na^+ delivery governs CD ET-1 production. This is in accordance with the fact that a high Na^+ diet stimulates CD ET-1 synthesis in vivo. CD ET-1, in turn, inhibits Na^+ reabsorption, thus providing a physiologic system whereby elevated Na^+ intake leads to increased delivery of Na^+ to the CD further increases CD ET-1 synthesis. ET-1 then inhibits Na^+ transport, thereby facilitating natriuresis. Thus, Chapter 3, for the first time, describes the ENaC-mediated regulation of ET-1 and the subsequent coupling of changes in intracellular Na^+ to changes in $[Ca^{2+}]_i$ (Figure 6.1).

Another key finding of this dissertation (Chapter 4) is that this is the first report of ET-1 regulation by primary cilia and polycystins. In experimental animals, a loss of PC-1 or PC-2 results in failure to increase $[Ca^{2+}]_i$ in response to fluid flow. These data, together with our findings, suggest the role of primary cilia and polycystins as mechanosensors. This observation is extremely relevant to renal salt and water handling under normal physiological conditions and in diseased states as a result of impaired ciliary and/or polycystic function. Interestingly, in patients with polycystic kidney disease even before renal function deterioration manifests itself, a hypertensive phenotype is observed.

It is important to note that while both CCD and IMCD ET-1 mRNA production is stimulated by fluid flow and both cell lines express ENaC, the CCD contains a significant excess of ENaC as compared to the IMCD. Thus, while the CCD ET-1 production is mainly Na^+-dependent, IMCD ET-1 production is regulated by fluid flow, thereby collectively taking care of salt and water loading, respectively.

Additional studies were undertaken to investigate Ca^{2+} entry into the cells. This dissertation comprises of reports of preliminary screening of all the different Ca^{2+} channels expressed in the CD that may be involved in the flow regulation of ET-1. Most importantly, P2X7 blockade resulted in a significant decrease of flow-stimulated ET-1 mRNA production. It is interesting to note that P2X7 blockade also reduces ATP regulation of salt and water transport in pronephric ducts. P2Y receptors were also studied since they interact with P2X receptors in order to increase $[Ca^{2+}]_i$. We observed that P2Y2 receptors, when blocked, significantly reduce flow-stimulated ET-1 mRNA levels. These data are in agreement with various reports published earlier. Activation of P2Y2 has an inhibitory effect on CD salt and water reabsorption. Additionally, mice deficient in the P2Y2 receptor show reduced flow-induced $[Ca^{2+}]_i$ increase.

Based on the findings that cilia, polycystins and the purinergic system are essential to the ET-1 flow response in the IMCD, one might ask how these systems interact. Previously published literature reports that bending of primary cilia involves the release of ATP. In our studies, we observed no flow-regulated ET-1 mRNA accumulation in PC-2 deficient cells. However, we found that ATP was released in normal as well as PC-2 deficient m-IMCD3 cells. These findings agree with previous reports, which state that shear stress promotes ATP release in ciliated as well as nonciliated cells, and ATP release might be partly mediated via non-PC-dependent pathways. Also in humans, P2X7 is colocalized in the cilia. Together, these studies suggest that all these systems interact and flow seems to increase ET-1 synthesis as a result of, at least in part, these systems interacting with each other. Based on our findings in Chapter 4, we conclude that ciliary bending as a consequence of flow is associated with an increase in $[Ca^{2+}]_i$ via PC-2 and

release of ATP. Further, ATP activates P2X7 and P2Y2 receptors to cause an additional increase in $[Ca^{2+}]_i$; both these events leading to augmented ET-1 mRNA accumulation via Ca^{2+}- dependent pathways (Figure 6.2). Although detailed studies about the ciliary and purinergic interaction are required, this dissertation, for the first time, introduces findings with great potential, about how the CD might detect mechanical stimuli and achieve both acute and prolonged regulation of salt and water reabsorption.

Thus, there exist complex pathways in both CCD and IMCD that sense tubule fluid flow (or ECFV changes) and help to achieve salt and water homeostasis. Since CD ET-1 is a potent anti-hypertensive system, this dissertation concretely establishes that flow-stimulated ET-1 production is highly physiologically relevant in normal as well as hypertensive states characterized by renal dysfunction.

Lastly, in Chapter 5, we looked at the effect of increased perfusate solute concentration on flow-regulated IMCD ET-1 mRNA production. ECFV expansion increases solute load to the CD. Our findings demonstrate that solute load affects flow-regulated ET-1 production only in the IMCD but not the CCD. Further, we observed that the effect of solute delivery was mediated via TonEBP. Although these findings are significant, a close look at the mechanisms by which this regulation occurs is of utmost importance.

In conclusion, this dissertation explains the existence of a system that couples ECFV expansion and CD ET-1 production, thereby maintaining salt and water homeostasis.

6.2. Future Directions

Overall, this dissertation explains flow-regulated CD ET-1 production in the CCD and IMCD in an in vitro cell-based model. While mimicking laminar shear stress at physiologic rates (2 – 20 dyne/cm^2), this system does not mimic the physiological environment that is under the influence of hormones and related factors. Thus, while it has its advantages, further evaluations in animal models are necessary. In order to simulate flow in experimental animals, in vivo studies using the isolated tubule technique may be important. However, this technique is difficult while our approach is simplistic. Also, the tubule might not be viable for a 2-hour period required for ET-1 gene transcription. However, pharmacological approaches (inhibitors) can be used in order to look at intracellular Ca^{2+} changes by fluorescence imaging by exposing the isolated tubule to flow for very short time periods (15- 60 seconds). Previously, our group studied urinary ET-1 and Na^+ excretion upon volume loading. In order to increase tubule fluid flow and observe its effect on ET-1 production in the distal nephron, furosemide was administered to rats and they were further examined. However, this study was performed under the influence of an anesthetic that might have impacted our results. Apart from this, this experiment did not explain whether flow directly augments ET-1 synthesis in the CD.

Secondly, ET-1 exerts its effects in the CD by binding to ET receptors. Flow might also regulate ET receptors. We did not study how flow might affect receptor expression since our experiments were conducted only for 2 hours. Also, we did not measure ET receptor mRNA levels. One might wonder whether ET receptor expression is regulated in response to flow. In the future, studying the effect of flow on receptor expression will be important in order to determine the mechanism of ET-1 in the context

of flow more conclusively. This can be studied by determining ETA or ETB mRNA levels in response to flow after 2 hours at 2 dyne/cm2. In order to establish the receptor subtype through which flow-regulated ET-1 exerts its effects, using mouse knockout models (established in the our lab) of ETA or ETB receptors will be essential. After furosemide infusion, which will increase flow in the tubule, urinary ET-1 excretion can be measured from either ETA or ETB knockout mice.

Additionally, while we defined a Ca^{2+}-dependent pathway in the CCD (previous studies performed by our group), we did not study the Ca^{2+} signaling in great detail. While PKC was found to be essential to Ca^{2+}-mediated flow regulation of ET-1 in the CD, we did not study the specific DAG-regulated PKC isoforms through which this regulation might occur. Use of different specific pharmacological inhibitors for PKC isoforms will determine the specific PKC isoform by which flow-regulated ET-1 synthesis might be regulated. Also, we observed that calcineurin (Cn) is important for the ET-1 flow response. However, using pharmacological inhibitors against NFATc, we could not establish the relationship between Cn and NFATc in the regulation of the flow response. These findings open up 2 major questions: 1) the pharmacological inhibitor using for our studies, the VIVIT peptide may not have blocked NFATc at the concentrations used or, 2) Cn modulates IMCD flow-stimulated ET-1 mRNA accumulation through non-NFATc dependent pathways. To answer these questions, transient knockdown of NFATc (1, 2, 3, 4) using siRNA technology followed by determination of flow-regulated ET-1 mRNA levels will explain whether NFATc is involved and whether Cn modulates IMCD ET-1 production via NFATc. Thus, additional studies need to be performed in order to elucidate the mechanism of Cn in regulating the

ET-1 response to flow in the CD.

Largely, Ca^{2+} appears to be a major player in the ET-1 flow response in mpkCCDc14 cells (previous studies by our group) and m-IMCD3 cells. However, we did not directly quantify $[Ca^{2+}]_i$ changes in these cells although our data strongly supports changes in $[Ca^{2+}]_i$. Future studies in order to demonstrate changes in $[Ca^{2+}]_i$ in our system will be useful in order to reconfirm our results. Intracellular Ca2+ imaging in response to flow and/or treatment with pharmacological inhibitors will directly quantify [Ca2+]i changes in the cells. This technique involves loading the cells with a fluorescent dye (Fura-2AM or Fluo 4) followed by exposure of cells to flow and subsequent determination of fluorescence in the cells using confocal miscroscopy in real time.

Additionally, using pharmacological inhibitors, we determined the role of TRP channels (TRPV4, TRPC3 and TRPC6) in flow-stimulated IMCD ET-1 production. We found that blockade of the channels had no effect on the ET-1 flow response. Previously published literature indicates that these channels are important flow sensors in the CD (1). One might ask why pharmacological blockade in m-IMCD3 cells did not block the flow response. Thus, it is important to first verify whether these TRP channels are expressed in m-IMCD3 cells using western blotting (protein) and real-time PCR (mRNA). Also, more robust techniques such as siRNA knockdown of the mentioned TRP channels followed by determination of the effect of knockdown on flow-regulated ET-1 mRNA levels need to be employed in order to reassess our findings. Further, we observed the role for primary cilia in the ET-1 flow response in m-IMCD3 cells. In our studies, we abolished primary cilia using chloral hydrate. However, chloral hydrate is highly toxic. Using siRNA technology to knockdown ciliary assembly proteins such as polaris

followed by determination of flow-regulated ET-1 mRNA levels will directly establish the relationship between primary cilium and the ET-1 flow response. These experiments may be performed in both mpkCCDc14 and m-IMCD3 cells. Dr. Bradley Yoder and colleagues have developed a polaris deficient cortical collecting duct cell line. This cell line may be used for further experiments in order to closely observe the role of primary cilium in the ET-1 flow response in the CCD. However, for studies in the IMCD, siRNA technology is a better approach since there is no primary cilium deficient cell line that has been developed.

Additionally, in order to assess the important of PC1 in the ET-1 flow response, a mouse epithelial kidney cell line lacking the *pkd1* gene was employed for our studies. However, *pkd1* siRNA knockdown in m-IMCD3 cells followed by determination of the ET-1 flow response needs to be performed for our findings to be more relevant to the performed studies. It will also be interesting to study the effect of primary cilia and polycystin knockdown in mpkCCDc14 cells since these cells are principal cells and express cilia and polycystins as well.

After studying knockdown of primary cilia and polycystins in mpkCCDc14 and m-IMCD3 cells, a closer look at ATP release after modulating the ciliary and polycystin pathways will help to determine the role of ATP in the ET-1 response to flow in the two cell lines. Our results also indicate that activation of P2X7 and P2Y2 receptors by ATP is essential to the ET-1 flow response. Thus, these receptors might be closely associated with each other and with the primary cilium. It will be interesting to study whether these receptors colocalize in the cilia or are closely associated with it by immunohistochemical/confocal microscopy-related techniques. Further, it will be of

utmost relevance to study P2X7 and P2Y2 receptor expression in *pkd2*-deficient cells or polycystic kidney disease models using western blotting and real-time PCR. These studies will help to establish their role in normal and diseased states.

Lastly, in Chapter 5, we report solute delivery (via NFAT5) as an important factor that modulates flow-regulated CD ET-1 production in m-IMCD3 cells. Further elucidation of related pathways is needed in m-IMCD3 cells. The fate of NFAT5 needs to be studied in detail in response to increased perfusate solute concentration and flow – whether it translocates to the nucleus after activation and the mechanism by which it regulates ET-1 gene transcription thereafter. Determination of NFAT5 localization can be carried out by preparing nuclear and cytoplasmic extracts followed by flow under ioosmolar and hyperosmolar conditions. Western blotting of the extracts will help to determine whether NFAT5 has translocated from the cytoplasm to the nucleus after exposure to hypertonicity and flow. Ultimately, these studies will further elucidate pathways responsible for handling water loading in the IMCD.

Taken together, this dissertation opens up a wide range of questions which when answered will lead to a clear understanding of how ECFV expansion (salt and water loading) in case of fluid retentive states might regulate CD ET-1 production, thus helping in the process of renal salt and water handling and maintenance of BP. These studies will be of great clinical relevance since better understanding of these pathway will ultimately help to design better antagonists against ET receptors used clinically, with less serious side effects.

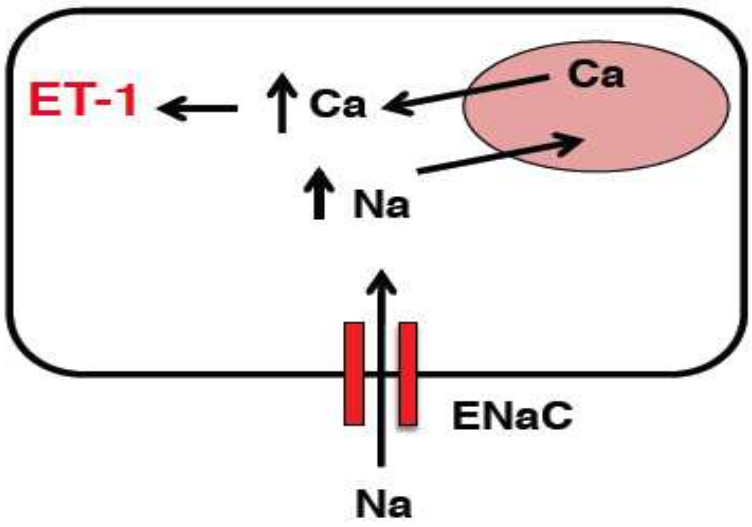

Figure 6.1. Mechanism of flow-regulated CD ET-1 production in the CCD

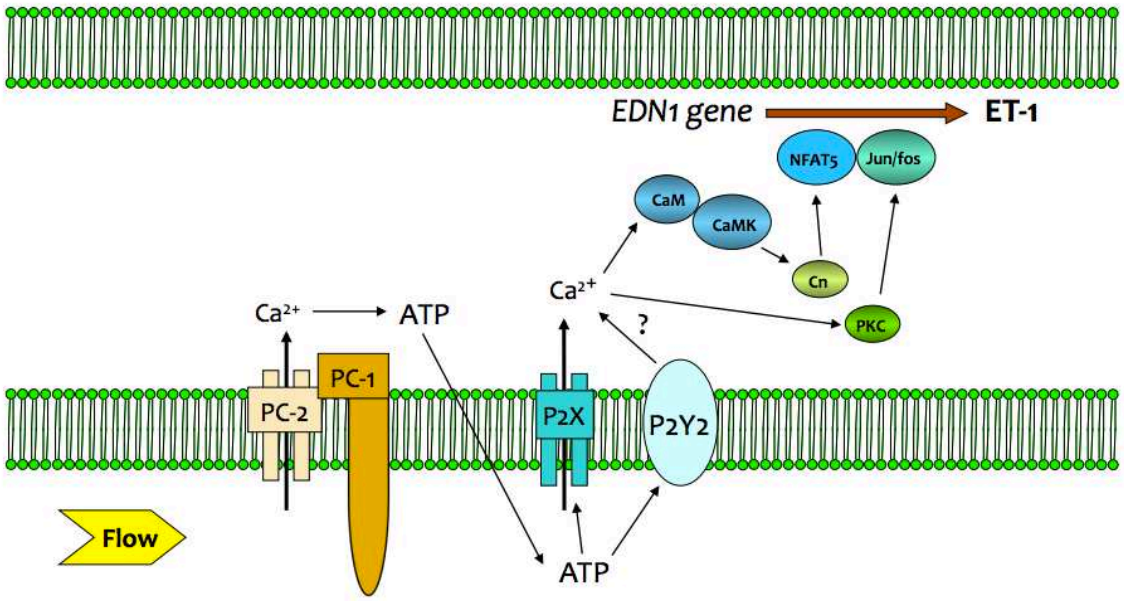

Figure 6.2. Mechanism of flow-regulated ET-1 mRNA production in the IMCD

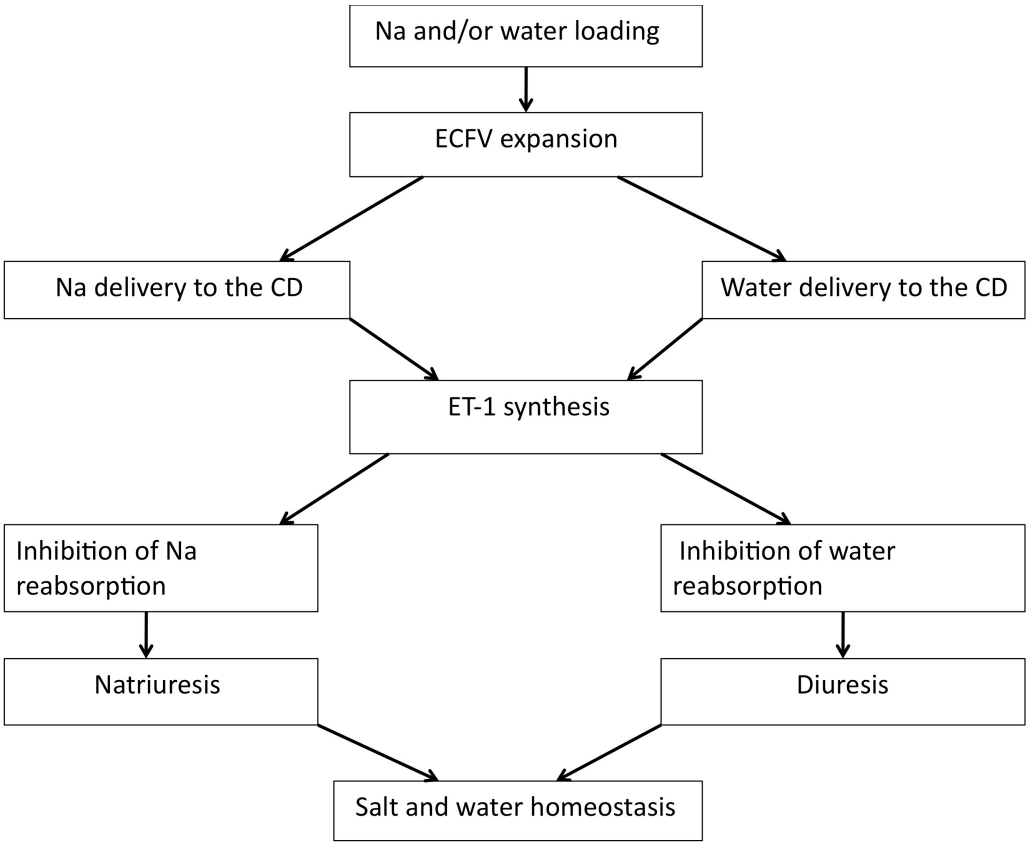

Figure 6.3. Summary of findings. ECFV expansion increases CD ET-1 synthesis thereby resulting into salt and water homeostasis.

5.3. References

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