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Renal Epithelial Sodium Channel (ENaC) Regulation of Pregnancy Mediated Hemodynamic Adaptations: Mechanistic Insights

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Abstract

Pregnancy-mediated sodium (Na) retention is required to provide an increase in plasma volume for the growing fetus. The mechanisms responsible for this Na retention are not clear. In the first study, we generated a renal tubule protein profile indicating α ENaC as the only sodium transport protein upregulated in mid and late pregnancy. To determine the *in vivo* activity of ENaC we conducted *in vivo* studies in late pregnant rats (day 18-20) and virgin rats to measure the natriuretic response to ENaC blockade (with benzamil). The *in vivo* activity of ENaC ($U_{Na}V$ post benzamil - $U_{Na}V$ post vehicle) was markedly increased in late pregnancy and this difference was abolished by pretreatment with the mineralocorticoid receptor antagonist, eplerenone. These findings demonstrate that the increased α ENaC subunit of pregnancy is associated with a mineralocorticoid-dependent increase in ENaC activity. The plasma volume increased progressively during pregnancy with the greatest plasma volume being evident in late pregnancy. ENaC inhibition abolished the difference in plasma volume status between virgin and pregnant rats. This indicates ENaC activity is a major contributor of plasma volume status in late pregnancy.

Inadequate plasma volume expansion results in complicated pregnancy with growth restricted (GR) fetus and maternal/fetal death. The second study aimed to determine the importance of renal vs extrarenal ENaC in sodium retention and blood pressure regulation during pregnancy to do so we chronically blocked ENaC with either daily subcutaneous injections of benzamil (BZ) or intrarenal transfection of αENaC shRNA. Chronic ENaC blockade with benzamil prevented normal sodium retention in the pregnant rat. Prevention of sodium retention resulted in reduced maternal serum sodium concentration, blood pressure, body weight, and fetal growth restriction. However, chronic benzamil treatment had no effect on sodium retention, body weight, or BP in virgin rats. Intrarenal transfection of a shRNA targeting αENaC successfully decreased renal αENaC mRNA expression in late pregnant rats compared to controls transfected with scrambled sRNA. Intrarenal transfection of

αENaC shRNA reduced sodium retention maternal, ΔBW, and pup weight. These findings suggest that renal ENaC is necessary for maintenance of sodium balance, blood pressure regulation, and progression of a healthy pregnancy.

In the third study, we performed large-scale proteomic analysis on late pregnant and virgin principal collecting duct cells, isolated by laser capture microscopy. The primary aim of this project was to identify potential proteins or signaling pathways that could account for the sodium retention occurring in pregnancy. Large-scale liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed at the NIH which identified a total of 365 proteins in virgin and late pregnant collecting duct cells. We identified pregnancy associated abundance changes in six proteins related to the ubiquitin/proteasome degradation pathway. Since a major mechanism of ENaC regulation is through trafficking we focused on changes in this pathway and their implications for enhancing ENaC activity during pregnancy.

The final study aimed to apply these findings to the overall theme of pregnancy as a state of arterial underfilling. We performed a Na transporter profile in kidney cortex homogenates from animals treated chronically with vasodilators (nifidipine or sodium nitrate). We found that only the abundance of transporters in the distal nephron (NCC, ENaC, AQP2) was similar to pregnancy, however differences were seen in pre-macula densa transporters (NHE3, NKCC2). The similar changes observed in the distal convoluted tubule and renal collecting duct promote Na and water retention. The changes in these transporters may explain the similar plasma volume expansions previously described in pregnant and chronically vasodilated rats, thus supporting the underfill hypothesis of pregnancy.

Taken together, this project supports an important role for the collecting duct in the pregnancy mediated sodium and fluid reabsorption during pregnancy.

I: Literature Review

Introduction

A healthy pregnancy requires many maternal cardiovascular and renal hemodynamic adaptations. All of these adaptations support positive sodium balance and expansion of plasma volume. This plasma volume expansion drives the 50% increase in blood volume [Girling 2004]. Blood volume expansion is necessary for adequate perfusion of the uterus and fetus. Failure of volume expansion results in the compromised maternal states of intrauterine growth restriction (IUGR) and pre-eclampsia. IUGR afflicts ~5% of all pregnancies and increases perinatal morbidity and mortality [Brodusky 2004]. Further, IUGR has been associated with an increased risk for development of long-term complications such as impaired neurodevelopment, type II diabetes, and hypertension. Pre-eclampsia is the leading cause of maternal and fetal morbidity and mortality afflicting 3-5% of all pregnancies [WHO 2005]. Volume contraction occurs before all other clinical manifestations of this disease. Further, the severity of pre-eclampsia is directly correlated with the degree of volume contraction [Escher 2007]. In order to find potential therapeutic targets for volume depleted states of pregnancy we must first understand the mechanism of sodium retention and volume expansion in the normal pregnant state.

Blood Pressure Regulation

Although blood pressure can be described by a simple equation relating flow (cardiac output) and resistance its regulation involves the integration of many systems. The arterial baroreceptors, the renin-angiotensin-aldosterone system (RAAS), the cardiac-derived natriuretic peptide system, the kinin-kallikrein system, and the endothelium-derived vasoactive factors are some of the major systems involved in blood pressure regulation. [Lifton 2001]

Baroreceptor Reflex

Arterial baroreceptors are stretch-sensitive fibers located in the aortic arch and carotid sinuses. They sense acute changes in intravascular pressure and control sympathetic output to the heart and vascular smooth muscle [Kougias 2010, Thomas 2011]. At rest the arterial baroreceptors are actively engaged (loaded). A decrease in mean arterial pressure (MAP) unloads the baroreceptors and leads to increased sympathetic output, cardiac output, and vasoconstriction. Increased sympathetic output promotes renin release and renal sodium reabsorption [Thomas 2011]. Conversely, an increase in MAP stimulates the baroreceptors leading to a reduction in sympathetic outflow [Kougias 2010, Thomas 2011].

Renin-Angiotensin-Aldosterone-System

The RAAS is a hormone system that regulates vascular volume and tone. Renin release is regulated by baroreceptors of the afferent arteriole, tubuloglomerular feedback (TGF), and sympathetic stimulation. In response to low blood volume the kidneys secrete renin from the juxtaglomerular cells which stimulates the production of angiotensin II (AII) [Castrop 2010]. AII induces constriction of the arterioles, sodium reabsorption, release of vasopressin from the posterior pituitary, and secretion of aldosterone from the zona glomerulosa cells of the adrenal cortex. AII has direct effects via the AT1 receptor in the proximal tubule and distal nephron promoting sodium reabsorption. Vasopressin increases water reabsorption in the collecting ducts through the water channel aquapourin-2 (AQP2) and sodium reabsorption via epithelial sodium channel (ENaC). Aldosterone increases sodium reabsorption in the distal convoluted tubule and collecting duct through actions on sodium chloride cotransporter (NCC), sodium-potassium-ATPase (Na-K-ATPase) and ENaC. Increased net renal sodium reabsorption necessitates increased water reabsorption in order to maintain plasma sodium concentration. The resulting increased intravascular volume enhances venous blood return to the heart, raising cardiac output and elevating blood pressure. Tissue perfusion exceeding metabolic demand, leads to autoregulation of blood flow via increased vasoconstriction, this results in a steady-state

characterized by elevated blood pressure, increased systemic vascular resistance, and normal cardiac output. [Lifton 2001]

Cardiac-Derived Natriuretic Peptide System

Atrial and brain natriuretic peptides (ANP, BNP) are polypeptide hormones comprising the cardiac-derived natriuretic peptide system. ANP is synthesized in the atria, and BNP the ventricles [Kuwahara 2010, Maak 2006]. ANP is released by the atria in response to increased blood volume. In physiologic conditions ANP is the main circulating natriuretic peptide [Maak 2006]. Upon release, ANP acts at multiple sites to exert diuretic, natriuretic, and vasorelaxant effects through activation of guanylyl cyclase-A (GC-A) [Kuwahara 2010, Maak 2006]. In the kidney, ANP increases glomerular filtration rate (GFR) through dilation of the afferent and constriction of the efferent arteriole [Maak 2006]. It also acts in the distal nephron to inhibit sodium and fluid reabsorption [Kuwahara 2010, Maak 2006]. ANP antagonizes RAAS by decreasing renin secretion and inhibiting aldosterone synthesis directly through action on the adrenal zona glomerulosa cells. ANP also antagonizes vasoconstriction and sympathetic effects [Maak 2006].

Kinin-Kallikrein System

The kinin-kallikrein system is a peptide hormone system that influences vascular tone and renal sodium handling. The kinin family includes bradykinin, kallidin, and methionyl-lysyl-bradykinin. Kinins are derived from circulating precursosrs (kininogens) by the enzymatic action of kallikreins, serine proteases. Kinins are potent vasorelaxant peptides that can produce hypotension, diuresis, natriuresis, increased renal blood flow, and reduced total peripheral resistance. Kinins induce vasodilation by stimulating the release of nitric oxide (NO), prostacyclin (PGI2), and the endothelium derived hyperpolarizing factor (EDHF). [Dendorfer 1999, Sharma 1996]

Endothelium-Derived Vasoactive Factors

The vascular endothelium synthesizes and releases vasoactive substances that play a fundamental role in the basal and dynamic regulation of blood pressure. Endothelial derived factors induce vasodilation (NO, PGI2) or contraction (endothelin). NO is released from endothelial cells by receptor activation or shear stress. NO is synthesized from L-argenine by nitric oxide synthase (NOS). NO diffuses from the endothelium to the vascular smooth muscle cells. There it activates soluble guanylylcyclase (sGC) increasing intracellular cGMP leading to phosphorylation of the myosin light chain kinase and Ca^{2+}-ATPase, inducing relaxation of smooth muscle cells [Spieker 2000, Feletou 2010, Fels 2010]. NO is continuously released and determines the basal tone of peripheral blood vessels. NO is a potent natriuretic. Phosphodiesterase 5 (PDE5) is an enzyme that terminates the signaling pathway by degrading the second messenger cGMP. PGI_2 is another endothelium-derived relaxing factor. PGI_2 is synthesized from arachidonic acid by cyclooxygenase (COX) and released in response to shear stress. PGI_2 is a powerful diuretic and natriuretic with effects both in the proximal and distal nephron [Gullner 1980]. Endothelin-1(ET-1) is synthesized as Prepro-ET-1 which is processed by a series of proteolytic cleavages to form the immediate precursor, Big ET-1. Big ET-1 is cleaved by endothelin converting enzyme to become ET-1[Ohnaka 1993, Chiou 1994, Xu 1994]. ET-1 exerts its major vascular effects (vasoconstriction and cell proliferation) through activation of specific ET_A receptors on vascular smooth muscle cells. ET_B receptors mediate vasodilation via release of NO and PGI_2. Binding to ET_A leads to activation of phospholipase C and the formation of inositoltriphosphate (IP3) and diacylglycerol (DAG) resulting in increased intracellular calcium and vasoconstriction. ET-1 is also important in maintenance of basal vascular tone. [Spieker 2000, Feletou 2010] ET-1 through ET_B receptors produces diuresis and natiuresis via NOS1 [Nakano 2008]. ET-1 also has a direct inhibitory effect on ENaC decreasing open probability of the channel and reducing channel number in the apical membrane [Pavlov 2010].

Given the diversity of the aforementioned physiologic systems responsible for blood pressure regulation it is noteworthy that all of the identified mutations that cause Mendelian forms of hypertension/hypotension act through the same physiologic pathway in the kidney, altering net renal sodium reabsorption. [Lifton 2001]

Sodium Balance

In order to maintain sodium balance on a normal diet the kidneys reabsorb ~99.5% of the filtered sodium. Sodium reabsorption along the renal nephron is accomplished by an integrated system of ion channels, exchangers, and transporters. Sodium transport in the proximal tubule is due mainly to the type 3 Na/H exchanger (NHE3), in the thick ascending limb the bumetanide-sensitive cotransporter (NKCC2) and NHE3, in the distal convoluted tubule the thiazide-sensitive Na-Cl cotransporter (NCC), and in the connecting tubule and collecting duct the amiloride-sensitive epithelial sodium channel (ENaC). Located along the basolateral surface of all tubular segments is Na-K-ATPase [Knepper 2003]. The proximal tubule reabsorbs the majority of filtered sodium (60%), while thick ascending limb accounts for 30%, the distal convoluted tubule 7%, and the final 2% is reabsorbed in the collecting duct. While this last step accounts for only a small fraction of sodium reabsorption, this is the principal site at which net salt balance is normally determined, as the activity of this channel is highly regulated by the RAAS [Lifton 2001]. Decreased sodium chloride in the tubular fluid is sensed by the macula densa, specialized cells at the end of the TAL. This leads to increased secretion of renin by the juxtaglomerular cells, which line the afferent arteriole, in a process known as tubuloglomerular feedback (TGF) [Schnermann 1986]. Increased renin results in increased formation of AII which induces the secretion of aldosterone from the adrenal zona glomerulosa cells. Aldosterone binds to the mineralocorticoid receptor (MR) in the principle cells of the distal nephron (late distal convoluted tubule, connecting tubule, and collecting duct) increasing ENaC activity and sodium reabsorption. [Lifton 2001]

ENaC

Final regulation of sodium excretion occurs in the aldosterone-sensitive distal nephron and is crucial for the maintenance of extracellular salt and volume homeostasis and thus for blood pressure control [Butterworth 2009, Loffing 2009]. Aldosterone-sensitivity is conferred to this section by the mineralocorticoid receptor (MR) and the enzyme 11-beta hydroxysteroid dehydrogenase type 2 (11βHSD2) that protects the MR from activation by glucocorticoids by rapidly degrading them to inactive metabolites [Loffing 2009]. Sodium transport in this region is mediated by ENaC in the apical membrane of principle cells. ENaC is a heteromultimeric channel composed of three homologous subunits (α, β, γ) having 30% to 40% identity at the level of their amino acid sequence [Loffing 2009]. The rate limiting subunit for channel formation is αENaC [May 1994].

Aldosterone increases ENaC activity by inducing αENaC mRNA and protein, trafficking of channel to the apical membrane, and cleavage of the extracellular loop of γENaC [Masilamani 1999, Loffing 2001]. Aldosterone treatment rapidly activates transcription of SGK1 mRNA along the entire length of the aldosterone-sensitive distal nephron. SGK1 increases ENaC activity by phosphorylating Nedd4-2. Nedd4-2 is an ubiquitin ligase that interacts with and ubiquitinates ENaC. Phosphorylated Nedd4-2 binds preferentially to aldosterone inducible protein 14-3-3, disrupting Nedd4-2-dependent inactivation and degradation of the channel [Lee 2008, Loffing 2009]. Aldosterone also induces GILZ which prevents channel phosphorylation by ERK. ERK phosphorylation of C-termini of β- and γENaC reduces ENaC cell surface density and activity by increasing the affinity of Nedd4–2 to ENaC. Thus aldosterone regulates the Nedd4–2/ENaC interaction at two different levels: (1) by phosphorylation of Nedd4–2 via SGK1 and (2) by inhibition of ERK-dependent ENaC phosphorylation and subsequent Nedd4–2 binding via GILZ [Loffing 2009]. Also, SGK1 phosphorylation of αENaC increases channel activity presumably by opening silent channels [Diakov 2004]. Aldosterone also induces prostasin, a channel activating protein, involved in the cleavage of the extracellular loop of γENaC. Cleavage of

γENaC induces a conformational change that increases open probability of the channel [Loffing 2009]. Insulin stimulation of ENaC converges on SGK1 and AVP activates PKA which phosphorylates Nedd4-2 at sites involved in SGK1 regulation [Butterworth 2009]. Angiotensin II stimulates channel activity and protein expression of αENaC via AT1R-dependent pathway and diminishes the inhibitory effect of 11, 12-EET on ENaC [Beutler 2003, Sun 2010]. Estrogen increases activity and mRNA expression of αENaC [Helms 2005, Chang 2007].

Progesterone inhibits ENaC activity through activation of ERK and binding of Nedd4-2 [Michlig 2005]. It is also a competitive inhibitor of the MR. NO acts as a signaling molecule to activate guanylyl cyclase to produce cGMP and activate protein kinase G, which inhibits ENaC activity [Yu 2007]. Aldosterone protects ENaC from NO presumably through production of superoxide and phosphorylation/inactivation of NOS [Helms 2005, Yu 2007]. Aldosterone stimulates superoxide production through activation of NADPH oxidase [Miyata 2005]. Superoxide reacts rapidly with NO to form peroxynitrite, which has no effect on ENaC activity [Yu 2007]. Thus, aldosterone may relieve NO inhibition of ENaC by decreasing NO synthesis and concurrently increasing NO degradation to peroxynitrite [Yu 2007]. Another known inhibitor of ENaC is WNK4. WNK4 inhibits ENaC in a kinase independent fashion. Inhibition requires an intact C-terminus in β and γ ENaC subunits. These contain the PY motifs used to target ENaC for clearance by Nedd4-2. It is unclear whether WNK4 is acting through Nedd4-2 or through an independent pathway [Ring 2007]. SGK1 phosphorylates and inactivates WNK4 [Ring 2007].

Mendelian Forms of Blood Pressure Dysregulation

Molecular genetic studies have identified mutations in 8 genes that cause Mendelian forms of hypertension and 9 genes that cause Mendelian forms of hypotension in humans. Mutations that

augment renal sodium reabsorption increase blood pressure, and those that diminish sodium reabsorption decrease blood pressure [Lifton 2001]. These mutations affect the circulating mineralocorticoid hormones, the MR, or the renal ion transporters/channels. Mutations affecting the circulating mineralocorticoid hormones are seen in glucocorticoid-remediable aldosteronism (GRA), defective aldosterone synthesis, and syndrome of apparent mineralocorticoid excess (AME). Mutations affecting the MR are seen in autosomal dominant pseudohypoaldosteronism type 1 (PHA1) and hypertension exacerbated by pregnancy. Loss of function mutations in the gene encoding NCC generate Gitelman syndrome and NKCC2 produces Bartter syndrome. Mutations of ENaC are seen in Liddle's Syndrome (gain of function) and recessive PHA1 (loss of function). [Lifton 2001]

Cardiovascular Adaptations of Pregnancy

Marked plasma volume expansion (~40%) is required to provide adequate blood supply for the growing uterus and fetus. Factors that influence volume expansion are reduced total peripheral resistance, opening of the uteroplacental circulation, and increased sodium retention. The 50% reduction in systemic vascular tone by the end of term is the result of increased production of vasodilators. Those implicated are oestrogen, prostaglandins, nitric oxide, and relaxin [Girling 2004]. In addition to the increased production of vasodilators there is decreased vascular responsiveness to the potent vasoconstrictors angiotensin II and norepinephrine. This decreased vascular tone reduces afterload which triggers barorecptor activity increasing the heart rate, cardiac output, cardiac contractility, and movement of venous blood into arterial compartments [Ganzevoort 2004]. The arterial underfill hypothesis was put forward to explain the modifications of pregnancy. This hypothesis proposes a primary enlargement of the vascular compartment, initially due to vasodilation and later supported by opening of the uterine-placental circulation, with a secondary increase in sodium and water reabsorption [Schrier 1987, Schrier 2010]. Opening of the uteroplacental circulation acts as a low resistance shunt, aiding in the reduction of total peripheral resistance [Ganzevoort 2004].

Total peripheral resistance decreases to such an extent that even with the increases in cardiac output there is an overall reduction in the mean arterial blood pressure [Duvekot 1994]. Maternal baroreceptors are reset to operate nearer the lower arterial pressure of pregnancy. It is postulated that this new operating point may be close to the limit of the receptors' ability to respond to hypotension. This would explain the more rapid decline in blood pressure during hypotensive hemorrhage and the smaller increases in sympathetic nerve activity during hypotension in pregnancy [Hines 2000].

RAAS in pregnancy

The RAAS is stimulated in response to the peripherial vasodilation of pregnancy. In normal pregnancy, plasma renin activity and aldosterone levels are increased throughout gestation with levels being highest near term [Elsheikh 2001]. The early increase in circulating renin is due to ovarian secretion and decidual production [Pirani 1973]. Increased circulating estrogen produced by the growing placenta stimulates synthesis of angiotensinogen in the liver [Gallery 1979]. Elevated levels of angiotensin I and angiotensin II are reported for all stages of pregnancy [Langer 1998]. Angiotensin I and angiotensin II are both elevated in all stages of normal pregnancy [Langer 1998]. Yet, vascular responsiveness to angiotensin II is blunted in pregnancy [Ganzevoort 2004]. RAAS activation is necessary to "re-fill" the vasculature as inhibition with an angiotensin converting enzyme inhibitor lowers BP in the pregnant rat [Baylis 1986].

Renal Adaptations of Pregnancy

Avid sodium retention drives the plasma volume expansion of pregnancy. In normal pregnancy blood flow to the kidneys is increased as a result of renal dilation and increased cardiac output [Lees 1967, Robson 1989]. This increase in renal blood flow is reflected in the effective renal plasma flow (ERPF). Both ERPF and glomerular filtration rate (GFR) are elevated by up to 50% in pregnancy [Davison 1980]. In rats this increase above virgin control values occurs soon after mating and is maintained at a

high, steady level through mid-pregnancy, falling before parturition [Atherton 1981]. Since GFR and RBF are increased proportionally during pregnancy the filtration fraction remains constant [Cha 1993].

Increased GFR permits increased filtered sodium despite reduced plasma sodium concentration, a result of the 50% increase in total blood volume [Alexander 1980, Girling 2004]. Sodium retention is elevated in pregnant animals; this increased retention contributes to a progressive positive sodium balance that is maximal at late pregnancy [Alexander 1980, Kirksey 1962]. In the pregnant rat, sodium balance is increased as early as mid-pregnancy, continues to rise through gestation, and peaks on the day of delivery, at which time these rats are excreting only 48% of their ingested sodium [Churchill 1980]. On a normal sodium diet, non-pregnant urinary sodium excretion is ~92% of ingested sodium [Boemke 1994].

For there to be positive sodium balance in the presence of the increased natriuretics (progesterone, NO, GFR) there must be modifications of the tubules. Otherwise there would be rapid salt wasting and fluid depletion. Whole kidney sodium and fluid reabsorption are increased in pregnancy [Atherton 1988]. However, microperfusion and micropuncture studies report conflicting findings as to the tubular segments responsible for this sodium retention in pregnancy [Cha 1993, Atherton 1988, Garland 1982, Arthur 1986].

Few studies have examined the molecular regulation of the sodium transporters along the renal tubule in pregnancy and those existing reports are also in disagreement. It has been shown that in normal mid- and late pregnant rats the renal cortical Na-K-ATPase activity and abundance are downregulated, whereas renal medullary Na-K-ATPase activity and abundance are unchanged [Mahaney 1998, Khraibi 2005]. Cortical NHE3 protein expression was also found to be significantly lower in mid and late pregnant rats compared with non-pregnant rats [Khraibi 2005]. However, another group found

that cortical mRNA expression of NHE3 and NCC were both significantly increased at mid pregnancy, but reported no change in NKCC2. They also found that in hypertensive pregnancy mRNA expression of NCC is increased, NKCC2 is decreased, and NHE3 is unchanged as compared to normal pregnancy [Abreu 2008]. ENaC activity and regulation are unknown in pregnancy. Both factors that are known to stimulate (aldosterone, angiotensin II, estrogen, insulin) and inhibit (nitric oxide, progesterone) ENaC activity are elevated in pregnancy. The kidney tubules are known to be refractory to the effects of ANP during pregnancy [Masilamani 1994]. This protective mechanism is thought to be mediated by increased expression of inner medullary PDE5. PDE5 degrades cGMP, the second messenger of both ANP and NO, thereby protecting the inner medulla from their natriuretic effects [Sasser 2010, Ni 2004].

Pseudo-pregnancy

The mating of a female rat with a vasectomized male can induce pseudo-pregnancy. Pseudo-pregnancy is a condition that physiologically mimics the first half of gestation but lacks fetal-placental development. This condition mimics the increases in RPF and GFR that are observed during early pregnancy in rats [Atherton 1982, Baylis 1982]. Thus, maternal influences alone may be sufficient to initiate the changes in the renal circulation during pregnancy. Although, the mechanisms recruited in pseudo-pregnancy are not necessarily the same as those utilized during early pregnancy [Jeyabalan 2007]. It is for these reasons that virgins are conventionally used as controls for pregnancy studies.

Pregnancy Complicated by Volume Contraction

Failure of volume expansion results in the compromised maternal states of intrauterine growth restriction (IUGR) and preeclampsia. IUGR is defined as a fetal weight less than the 10^{th} percentile for gestational age. IUGR afflicts ~5% of all pregnancies and is a leading cause of perinatal morbidity and

mortality [Brodusky 2004]. Further, IUGR has been associated with an increased risk for development of long-term complications such as impaired neurodevelopment, type II diabetes, and hypertension.

Pre-eclampsia is the leading cause of maternal and fetal morbidity and mortality afflicting ~3-5% of all pregnancies [WHO 2005]. The course of pre-eclampsia begins in a clinically asymptomatic stage that is marked by abnormal placentation [Escher 2007]. This leads to the production of soluble factors that enter the maternal circulation and cause endothelial dysfunction. Then, after 20 weeks of gestation, the clinical manifestation of the disease presents with the triad of hypertension, proteinuria, and edema [Escher 2007]. Proteinuria and vascular leakage due to endothelial damage lead to a decrease in plasma and total blood volume, resulting in decreased central venous and ventricular filling pressures and cardiac output [Ganzevoort 2004].

In general preeclampsia is marked by a shift from a low resistance state to high resistance state. Compared to normal pregnant women there is a reduction in plasma volume expansion and cardiac output, in addition to an increase in total peripheral resistance and mean arterial blood pressure. Increases in peripheral resistance can be attributed to increased vascular sensitivity to angiotensin II as well as impaired production of vasodilating prostaglandins and inhibition of nitric oxide synthesis [Ganzevoort 2004, Elsheikh 2001]. Women with pre-eclampsia also have reduced levels of angiotensin I, angiotensin II, and aldosterone as compared to normal pregnant women in their third trimester and increased levels of ANP [Ganzevoort 2004, Langer 1998]. Alterations in these factors aid in the reduction of plasma volume expansion. Recently, there has been a report that in hypertensive pregnancy mRNA expression of NCC is increased, NKCC2 is decreased, and NHE3 is unchanged as compared to normal pregnancy [Abreu 2008].

Untreated pre-eclampsia can develop into the convulsive state, eclampsia, which is characterized by tonic-clonic seizures. Currently, the only effective treatment for pre-eclampsia is delivery of the placenta [Escher 2007]. In order to find potential therapeutic targets for pre-eclampsia and IUGR we must first understand the mechanism of sodium retention and volume expansion in the normal pregnant state.

II: Purpose

Pregnancy is marked by increased salt appetite and significantly positive sodium balance. This retention is most pronounced in late pregnancy and the retained sodium is distributed in part to the products of conception and in part to the mother, particularly in expanding her extracellular fluid volume [Alexander 1980]. Although chronic volume expansion is present the absolute tubular sodium reabsorption is elevated. However, microperfusion and micropuncture studies report conflicting findings as to the tubular segments responsible for the positive sodium balance of pregnancy. Further, few studies have examined the molecular regulation of the sodium transporters along the renal tubule in pregnancy and those existing reports are also in disagreement. Chapter 2 addresses this gap in knowledge with a renal tubule protein profile of the major sodium transporters during mid and late pregnancy. This profile identified abundance changes in a single protein, αENaC. *In vivo* channel activity, contribution to the maintenance of plasma volume expansion, and general mechanism are also addressed. Chapter 3 investigates the contribution of renal ENaC to the positive sodium balance of pregnancy and maternal blood pressure homeostasis. Chapter 4 addresses possible mechanisms of pregnancy-mediated ENaC activation using large scale proteomics and bioinformatics approach. Chapter 5 compares of proteomics profiles of chapter 1 to chronically vasodilated virgin female rats to support the hypothesis of pregnancy as a state of arterial underfilling. Taken together, these findings will provide the field of women's health research with information regarding the required physiologic adaptations for a healthy pregnancy and thus will aid in determining treatments in pathologies of pregnancy.

III:

Increased Renal αEpithelial Sodium Channel (ENaC) Protein and Increased ENaC Activity in Normal Pregnancy

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Abstract

Pregnancy-mediated sodium (Na) retention is required to provide an increase in plasma volume for the growing fetus. The mechanisms responsible for this Na retention are not clear. We first used a targeted proteomics approach and found that there were no changes in the protein abundance compared to virgin rats of the β or γ ENaC, type 3 Na/H exchanger (NHE3), bumetanide-sensitive cotransporter (NKCC2) or NaCl cotransporter (NCC) in mid- or late- pregnancy. In contrast, we observed marked increases in the abundance of the α ENaC subunit. The plasma volume increased progressively during pregnancy with the greatest plasma volume being evident in late pregnancy. ENaC inhibition abolished the difference in plasma volume status between virgin and pregnant rats. To determine the *in vivo* activity of ENaC we conducted *in vivo* studies in late pregnant rats (day 18-20) and virgin rats to measure the natriuretic response to ENaC blockade (with benzamil). The *in vivo* activity of ENaC ($U_{Na}V$ post benzamil - $U_{Na}V$ post vehicle) was markedly increased in late pregnancy and this difference was abolished by pretreatment with the mineralocorticoid receptor antagonist, eplerenone. These findings demonstrate that the increased α ENaC subunit of pregnancy is associated with an mineralocorticoid-dependent increase in ENaC activity. Further, we show that ENaC activity is a major contributor of plasma volume status in late pregnancy. These changes are likely to contribute to the renal sodium retention and plasma volume expansion required for an optimal pregnancy.

Keywords: Collecting duct, plasma volume expansion, sodium retention

Introduction

In humans and rats, pregnancy is accompanied by marked changes in cardiovascular function, renal function and fluid homeostasis. These adaptations permit an increase in blood volume that will supply the growing uterus and fetus without the development of maternal hypertension. A normal healthy pregnancy is associated with a cumulative plasma volume expansion (PVE) (30-50%) and avid sodium retention (2; 26; 27). Failure of this adaptation is associated with maternal morbidity/mortality and intra uterine growth restriction (3; 27). Despite the progressive PVE, there is no increase in maternal blood pressure due to a marked decrease in total peripheral vascular resistance (2; 7).

Renal sodium excretion determines volume homeostasis and the progressive PVE of normal pregnancy must reflect net renal sodium retention. Alexander et al (1) performed balance studies in the pregnant rat that demonstrated cumulative sodium retention during pregnancy. The reabsorption of sodium along the renal tubule is determined by the regulation of the individual tubular (co)transporters and channels (16), such that a change in the activity of any transporter or channel can lead to altered volume status (16). The regulation of transepithelial sodium transport across the renal tubules is mediated by apical sodium transporters. The first aim of this study was to identify any changes that occur in the abundance of specific sodium transporters that would permit the required sodium retention for a healthy pregnancy. We performed renal tubule sodium transporter profiling in virgin, mid pregnant and late pregnant female Sprague Dawley rats to determine time-course adaptations of the sodium transporters/channels. The second aim of this study was to examine if increases in protein abundance of specific sodium transporters/channels correspond to an increase in *in vivo* activity and determine its contribution to the PV status of pregnancy.

Methods

Animals and Experiments. Animals were housed in the Virginia Commonwealth University animal facility in agreement with institutional guidelines. All animal protocols were approved by the Institutional Animal Care and Use Committee (VCU IACUC) and in accord with the NIH Guide for animal use. Female Sprague Dawley (SD) rats from Harlan (Indianapolis, Indiana) were used for these experiments. Timed pregnant and age-matched virgin rats were used for this study. A total of 61 rats, 3-4.5 months of age were used. Rats destined to become pregnant were placed with a fertile male and day 1 of pregnancy was designated as the day that sperm was present in vaginal smears. Rat gestation is ~ 21 days.

Group 1 rats were used to acquire baseline physiological data and determine Na retention over the course of pregnancy. A total of 22 rats were used. Metabolic cage studies were performed in virgin, early pregnant (day 6-8), mid pregnant (day 12-14), and late pregnant (day 19-20) rats and most rats were studied at 2 or more time points. Rats were given *ad libitum* access to a gelled diet containing water (62.3%), agar (0.3%), and rat chow (37.4%) (Harlan Teklad LM-485 Mouse/Rat Sterilizable Diet) for two days to get rats acclimated to the metabolic cages. On the third day a 24 hour collection was made from which gel food intake (g), Na intake (mmol/24 hours), urine output (V, ml/24 hour), urine sodium ((U_{Na}, mmol/L) fecal Na was not determined), sodium excretion ($U_{Na}V = V*U_{Na}$, mmol/24 hour) and Na retention (Na intake – $U_{Na}V$) were determined. Urine samples were analyzed for sodium by flame photometry.

Group 2 rats were used for Western blotting analysis of whole kidney homogenates in age-matched virgin (n=12), mid pregnant (day 11-13, n=4) and late pregnant (day18-20, n=7) rats, for a total of 23 rats. Kidneys were harvested and homogenized whole using a tissue homogenizer (Pro Scientific, Oxford, CT). All samples were homogenized in a chilled isolation solution containing 10 mM

triethanolamine, 250 mM sucrose (Mallinckrodt Baker Inc., Phillipsburg, NJ) and protease inhibitors, phenylmethyl sulfonyl fluoride (25mg/ml isopropyl alcohol) (Biochemical Corp, Lakewood, NJ) and leupeptin (1mg/ml dd H_2O) (Novabiochem/EMD Chemicals, Inc., Gibbstown, NJ). Protein concentrations of the homogenates were determined by the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). All samples were solubilized at 60 °F for 15 minutes in a Laemmli sample buffer and stored at -80 °C. SDS-PAGE was performed on Criterion precast 10% Tris-HCl gels (Bio-Rad Laboratories, Inc., Hercules, CA). An initial gel was stained with GelCode Blue Reagent (Pierce Biotechnology, Rockford, IL) as described previously to confirm equal loading (28).

Whole kidney homogenates were used for semiquantitative immunoblotting. Each sample was loaded into individual lanes of Criterion precast 10% Tris-HCl gels. The proteins were transferred electrophoretically from unstained gels to nitrocellulose membranes. The blots were blocked with 5 g/dl blotting grade blocker non-fat dry milk (Bio-Rad Laboratories, Hercules, CA) for 1 hour and probed with primary antibody over night. Blots were incubated with peroxidase-conjugated secondary antibodies (Pierce Biotechnology, Rockford, IL, no. 31458 & 31452), followed by an enhanced luminol reagent, Western Lightning Chemilumnescence Reagent Plus (Perkin Elmer LAS, Inc., Boston, MA). Blots were exposed to X-ray film and band densities were quantified by VersaDoc Imaging System (VersaDoc Bio-Rad, Bio-Rad Laboratories, Hercules, CA). To facilitate comparisons, we normalized the densitometry values, defining the mean for the virgin control group as 100%.

We used the following previously characterized rabbit polyclonal antibodies (a kind gift from Mark Knepper, NIH/LKEM Bethesda MD) summarized in the following. 1) The Na-H exchanger type 3 (NHE3) (1:1000 dilution) (7). 2) The bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2) (1:1000 dilution) (14)). 3) The thiazide-sensitive Na-Cl cotransporter (NCC) (1:1000 dilution) (15). 4) The

three subunits of the amiloride-sensitive epithelial sodium channel (α, β, and γ subunits of ENaC) (1:500 dilution) (21).

Group 3 rats (virgin and pregnant) were implanted with catheters for determination of plasma volume status using Evan's Blue Dye technique and examination of *in vivo* ENaC activity (net natriuretic response to ENaC blockade with benzamil). Five pregnant rats were studied at mid (day 12-14) and six at late pregnancy (day 17-21) and seven virgin rats were studied at 13-14 days after catheterization. Catheters were placed using full sterile technique under general anesthesia with 1-4% isoflourane (Baxter Healthcare Corp., Deerfield, IL). The left femoral artery and vein were cannulated with micro-renthane tubing (Braintree Scientific, Braintree MA), fed forward with the tip of cannulas placed in the abdominal aorta and abdominal vena cava. The catheters were threaded under the skin and exteriorized at the nape of the neck. The catheters were primed with a 1:1 solution of heparin (1,000 IU/ml) and dextrose (50%) and the ends were plugged with stainless steel pins. Pregnant rats were catheterized 6-8 days after conception and at least 4 days were permitted for complete recovery from the surgery before any experiments were carried out. Prior to experiments, rats had been handled and trained to sit quietly in restraining cages to accept the noises and activity of the laboratory. There was no effect of anesthesia/surgery performed on the total number of pups (non surgical rats (n=6) = 12±1; catheterized rats (n=7) = 14±1, NS) or individual pup weights (non surgical rats = 2.25±0.24; catheterized rats = 2.69±0.36 g/pup, NS).

Evan's Blue Dye Administration. Plasma volume was measured by the Evan's blue dye technique. Following a baseline blood collection Evan's blue dye (0.3mg/mL) was infused into the venous catheter (tip of catheter in the vena cava via the femoral vein). Blood collections were then taken from the arterial catheter (tip of catheter in the abdominal aorta via the femoral artery) at 5 and 10 minutes

post infusion. The plasma was collected following centrifugation of whole blood. Erythrocytes were reconstituted in 13.4% ficoll in saline and returned to rat (iv). In order to determine ENaC-mediated actions on plasma volume status, we measured plasma volume before and 8 hours after benzamil (0.7 mg/kg) administration.

Evan's Blue Dye Analysis. The analysis that was used was a modification of the method used by Blair and Mickelsen (5). Briefly, the standards were made with 1% plasma at 0, 1, 5, 10, and 20 ug/ml of Evan's Blue Dye. A100 µl plasma sample was read by ThermoSpectronic Helios γ spectrophotometer at 610 nm, to determine the observed concentration of the Evan's Blue Dye at baseline (before dye administration) as well as concentrations 5 minutes and 10 minute following dye administration. Background correction was made by subtracting the baseline observed concentration from the observed concentrations of the 5 and 10 minute samples. PV was then calculated by dividing the amount of Evan's Blue Dye given (75 ug) by the background corrected concentrations. Averages were then taken of the 5 and 10 minute samples, to give the average plasma volume.

*In Vivo*ENaC Activity. Rats received iv vehicle (0.33 g/kg ddH_20) injections via perfusion pump (0.008 ml/min, 90-120 µl total volume) and were placed in metabolism cages for 8 hours with drinking water *ad libitum*. Urine collections were taken at 0-3 and 3-8 hours post injection which was used for determination of baseline sodium excretion ($U_{Na}V$). Benzamil (0.7 mg/kg) was given *iv* and post-benzamil$U_{Na}V$were subtracted from the previous days post- vehicle $U_{Na}V$ to determine the net natriuretic response to ENaC blockade (benzamil) which is used as an index of *in vivo* ENaC activity.

Group 4 rats were used to study mineralocorticoid receptor inhibition (with Eplerenone) in late pregnancy (day 19-20). Late pregnant (day 16-17, n=4) rats were given *ad libitum* access to a gelled diet containing water (62.3%), agar (0.3%), and rat chow (37.4%) (Harlan Teklad LM-485 Mouse/Rat

Sterilizable Diet) for 2 days. Then, based on intake, ~60g of gelled chow, was given overnight with eplerenone to deliver a dose of 100mg/kg. Only rats that ate >90% of the gelled chow containing eplerenone were included. Day 18-19 rats received intra-venous vehicle (0.33 g/kg ddH_20) injections via perfusion pump and were placed in metabolism cages with water *ad libitum*. Urine collections were taken at 0-3 and 3-8 hours post injection and used to determine baseline $U_{Na}V$. Overnight rats were given 100mg/kg eplerenone in gelled rat chow and the next morning benzamil was given (0.7 mg/kg, iv). Urine collections were taken at 0-3 and 3-8 hours post injection. The net natriuretic response to ENaC blockade was determined as described above. Kidneys were harvested and processed for immunoblotting as described above.

Statistical Analysis. Data are given as mean ± standard error (SE). An unpaired t-test, a one-way analysis of variance (ANOVA) with Bonferroni post hoc, or two-way repeated measures ANOVA with Bonferroni post hoc was performed. The null hypothesis was rejected at $p<0.05$

Results

Physiological data. Some of the physiological adaptations of pregnancy are summarized in Table 1. Pregnant rats demonstrated a progressive increase in body weight (BW), reaching significance in mid (~8%) and late pregnancy (~25%) compared to age-matched virgin rats. Likewise, the intake of the gelled diet was significantly increased in early (~12.5%), mid (~18%) and late pregnant (~32%) rats compared to virgins. Urine output (V), urine sodium concentration and sodium excretion ($U_{Na}V$) were similar between pregnant and virgin rats. Because of the increased sodium intake and increased renal sodium retention, pregnant rats were in positive sodium balance (intake of sodium > sodium excretion) compared to virgins with the sodium retention increasing progressively during pregnancy (Table 1).

Semiquantitative Immunoblotting of tubular sodium transporters. Protein abundance of the three subunits of ENaC (α, β, γ subunits) as well as the other major apical sodium transporters along the renal tubule (NHE3, NKCC2, NCC) in pregnant vs. virgin rats are summarized in Figure 1 and 2. The only protein that increased in abundance in both mid (Figure 1A and B) and late pregnancy (Figure 2A and B) was α ENaC (mid pregnant=167±29%; late pregnant 168 ± 17% of virgin control, $p<0.05$). Since the α subunit of ENaC is the rate-limiting subunit for channel formation (22), this is consistent with an increased activity of ENaC due to an increase in channel number. There was no difference among groups in the protein abundance of β ENaC or of γ ENac (85 kD or 70 kD forms).

Pregnancy mediated plasma volume expansion: contribution of ENaC . Plasma volume was determined in virgin and pregnant (mid and late) rats before and after ENaC blockade with acute administration of benzamil. A progressive plasma volume expansion was seen with significant increases compared to virgins noted at late pregnancy (Figure 3). ENaC blockade abolished the significant increase in plasma volume in late pregnant rats.

In vivo ENaC activity (natriuretic response to benzamil). To determine if there is a functional increase in ENaC activity in late pregnant rats we examined the natriuretic response to ENaC blockade with acute administration of benzamil (0.7 mg/kg, ip), an index for *in vivo* ENaC activity. Most of this natriuretic response occurred within 3 hours of benzamil administration. As shown in Figure 4, the net natriuretic response to ENaC blockade was markedly increased in late pregnant compared to virgin rats at 0-3h post benzamil (virgin= 123 ± 21; late pregnant= 328 ± 39 μmol/hr, $p< 0.05$). There were no differences in the net natriuretic response to ENaC blockade at 3-8h between virgin and late pregnant rats (virgin= 32 ± 15; late pregnant= 19 ± 35 μmol/hr, NS).

Effect of Mineralocorticoid Receptor (MR) Blockade (eplerenone 100mg/kg 12 hours) on in vivo ENaC activity. Figure 4 demonstrates that in late pregnant rats, the natriuretic response to ENaC blockade was not elevated if the rats were pre-treated with eplerenone, indicating that the increased ENaC activity in late pregnancy is mediated by the MR.

Effect of Mineralocorticoid Receptor (MR) Blockade (eplerenone 100mg/kg 12 hours) on αENaC protein abundance. Eplerenone administration for 12 hours did not significantly change αENaC protein abundance in late pregnant rats (Figure 5).

Discussion

The main finding from this study is that the αENaC protein abundance, which is the rate-limiting subunit for channel formation, is increased in the mid- and late pregnant rat. In late pregnancy when the plasma volume expansion and therefore the renal sodium retention are maximal, we observed that *in vivo* ENaC activity was maximal and ENaC inhibition with benzamil abolished the expanded plasma volume state in late pregnancy. This increased ENaC activity in late pregnancy was prevented by mineralocorticoid receptor blockade with eplerenone, demonstrating that the increased ENaC activity was the result of the increased aldosterone levels of normal pregnancy (10).

The sodium retention that occurs over the course of pregnancy mediates the increase in plasma volume that is needed for the developing uterus and fetus. It is well established that at the whole kidney level, sodium is retained over the course of pregnancy reaching its peak in late pregnancy (1). Sodium balance is determined by the amount of sodium filtered by the glomerulus and the sodium reabsorbed by each of the renal tubule segments. Glomerular filtration rate (GFR) increases in pregnancy, peaking at mid pregnancy and returning toward nonpregnant levels near term (2). Thus, this enhanced GFR would favor an increase in sodium excretion. Additionally, pregnancy is associated with increases in

other natriuretic factors such as nitric oxide (NO), atrial natriuretic peptide (ANP) and progesterone (2, 10, 18). However, there is also activation of the sodium retaining "renin, angiotensin aldosterone" system (RAAS) in normal pregnancy (10).

Taken together, adaptations must occur in the renal tubules to permit sodium retention to predominate. Consistent with this, we and others have shown that the pregnant rat is refractory to the renal tubular response to ANP (18,24) and acute pressure-natriuresis which requires an intact renal NO system (12,19). Recent studies suggest that pregnancy is associated with a loss of responsiveness to cGMP (second messenger for both ANP and NO) in the inner medulla, due to increased cGMP breakdown by phosphodiesterase-5 (23). Overall, there is evidence that adaptations occur locally in the inner medullary collecting duct leading to a loss of responsiveness to natriuretic agents that act via cGMP. However, the details regarding possible pregnancy-mediated adaptations of the individual sodium transporters and channels are unknown and are addressed in the current study.

We found no change at any stage of pregnancy in the protein abundance of 1) The Na-H exchanger type 3 (NHE3), present in proximal tubule and thick ascending limb; 2) The Na-K-2Cl cotransporter (NKCC2) present in the thick ascending limb, and 3) The thiazide-sensitive Na-Cl cotransporter (NCC) present in the distal convoluted tubule. Although the differences in expression of other Na transporters were not different there is a possibility that their activity may be changed with pregnancy as increases in sodium delivery occur in the tubules. The only other study to report on apical sodium transporters in pregnancy observed a fall in the renal cortical NHE3 protein abundance in mid and late pregnancy (13). These workers used the same rat strain and vendor as used in the present study and the only apparent difference was that they looked at a homogenate of cortex, whereas in the present study the entire kidney was homogenized. Since NHE3 is present in cortex and medulla we may have

missed regional differences, although it is difficult to reconcile falls in NHE3 with net sodium retention.

In contrast, we observed a marked increase in the α ENaC subunit which is present in the collecting duct. Of the 3 ENaC subunits, the rate of α ENaC production is rate limiting for assembly of the functional ENaC complex (22). Sodium transport by ENaC can be controlled by complex mechanisms which include regulating channel number, trafficking to the apical membrane, channel open probability, rate of degradation or recycling (6, 11, 25). However, the goal of the present study was not to determine the molecular mechanisms of ENaC activity in pregnancy, but rather to establish whether the increased αENaC subunit protein abundance translated into increased ENaC activity in pregnancy.

In the present study we confirmed earlier work showing that plasma volume expansion increase in a cumulative manner during pregnancy (2, 7, 26, 27). Unexpectedly we found that ENaC inhibition resulted in an increase in plasma volume in virgin rats which we attribute to a rebound effect since this is the opposite of what would be expected with ENaC inhibition. We also found that a major component of the plasma volume status in late pregnancy is mediated by ENaC activity since benzamil administration abolished the differences in plasma volume status in virgin and pregnant rats (Figure 3) we therefore selected late pregnancy for our functional ENaC activity studies.

Using the selective ENaC inhibitor benzamil we found that a much greater natriuretic response was seen to ENaC inhibition over a 3h period, in the late pregnant compared to the virgin rat (Figure 4). This indicates that basal ENaC-dependent sodium retention was much greater in the late pregnant rat, supporting a functional effect of the increased α ENaC subunit protein abundance. There is marked activation of the RAAS in normal pregnancy (10) and we have previously reported that both

angiotensin II and aldosterone stimulate the αENaC subunit (4,20). In the present study we found that the mineralocorticoid receptor antagonist eplerenone reversed the enhanced, acute natriuretic response to benzamil. This demonstrates that the late pregnancy mediated increase in ENaC-dependent sodium retention is the result of increased stimulation of the MR. In pregnancy we did not observe an increase in NCC or the 70 kD form of γENaC which is associated with the protein profile of aldosterone mediated stimulation of the MR. This finding is not surprising since unlike the protein profile studies that we previously performed where only one variable was being changed, either placing rats on a low Na diet or aldosterone administration (20), pregnancy is a state where many hormone systems and hemodynamic factors (systemic vasodilation and increased glomerular filtration rate) which are known to influence sodium transport and sodium transporter profiles are being changed over the course of pregnancy. Thus, the protein profile at the stages of pregnancy examined in this study likely represents the culmination of these actions. Further, although we did see eplerenone administration reverse the increase in ENaC activity in late pregnancy, we did not see a decrease in the protein abundance of αENaC. We attribute this finding to the fact that administration of eplerenone over 12 hours was not adequate time for changes in protein abundance to be observed. We have previously shown that although ENaC activity can change in as little as 15 hours, αENaC protein abundance changes are not seen 1 day following increases in plasma aldosterone levels (9, 21).

In conclusion our findings show that αENaC protein abundance and ENaC activity is increased in the late pregnant rat thereby contributing to the positive sodium balance of pregnancy. Furthermore, this increased ENaC activity is due to the stimulation of the mineralocorticoid receptor in normal pregnancy. Thus in spite of pregnancy mediated increases in both natriuretic and antinatriuretic factors, the important modifications to renal sodium transport that occur in pregnancy and permit net renal sodium retention and volume expansion are localized to the collecting duct. Specifically enhanced mineralocorticoid receptor mediated, ENaC-dependent sodium reabsorption occurs in the

collecting duct in combination with blunted inner medullary collecting duct responsiveness to natriuretic stimuli.

Acknowledgements:

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Table 1. Summary of physiological data in pregnant and virgin rats.

	Virgin (n=6)	Early Pregnant (n=9)	Mid Pregnant (n=9)	Late Pregnant (n=6)
BW (g)	272 ± 6	272 ± 2 ‡ §	296 ± 3 * † §	362 ± 6 * † ‡
Gel Food Intake (g/24hr)	44.38 ± 1.24	54.36 ± 1.16 * §	58.15 ± 1.60 * §	69.77 ± 1.23 * † ‡
Na+ Intake (mmol/24 hr)	2.31 ± 0.06	2.83 ± 0.06 * §	3.03 ± 0.08 * §	3.63 ± 0.06 * † ‡
V (mL/24 hr)	17.69 ± 1.34	24.3 ± 1.7	25.8 ± 2.00 *	23.6 ± 2.4
Urine Na+ (mmol/L)	129 ± 11	106 ± 8	98 ± 8	111 ± 9
UNaV (mmol/24 hr)	2.21 ± 0.09	2.48 ± 0.07	2.44 ± 0.13	2.55 ± 0.17
Na+ Retention (mmol/24 hr)	0.10 ± 0.08	0.35 ± 0.05 §	0.59 ± 0.10 * §	1.08 ± 0.18 * † ‡

Summary of physiological data from metabolic cage analysis of virgin, early (day 6-8), mid (day 12-14) and late-pregnant rats (day 17-20). Body weight (BW), gel food intake, sodium intake (Na intake), urine output (V), urine sodium concentration (fecal sodium was not determined), and sodium excretion ($U_{Na}V$). A one-Way ANOVA with Bonferroni Post Hoc was performed. Mean ± standard error (SE), $p< 0.05$. Symbols (*, †, ‡, and §) are based on results from a Bonferonni test following a statistically significant finding using a one-way analysis of variance (ANOVA). Significance is denoted as follows: * vs virgin, † vs early pregnant, ‡ vs mid pregnant, § vs late pregnant.

Figure 1A

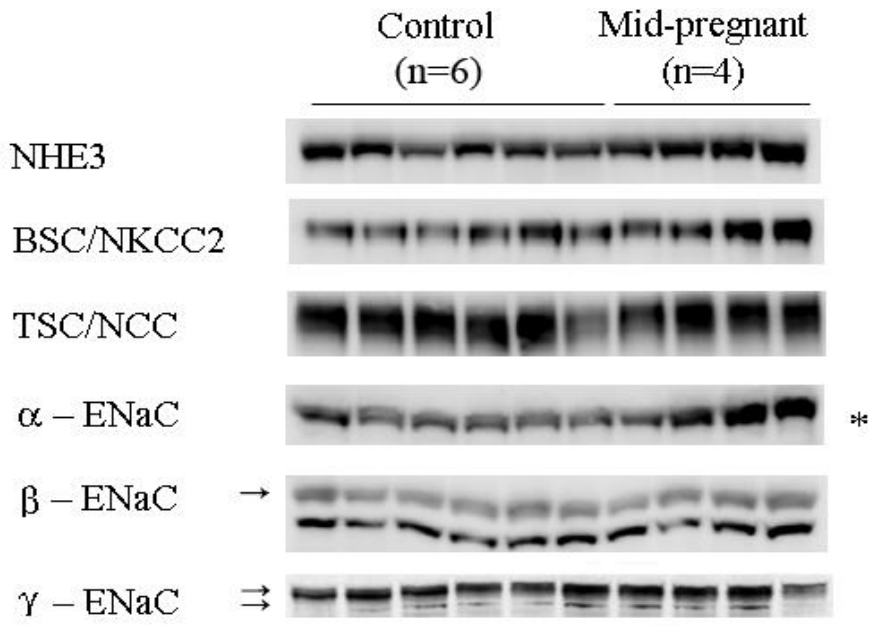


Figure 1B

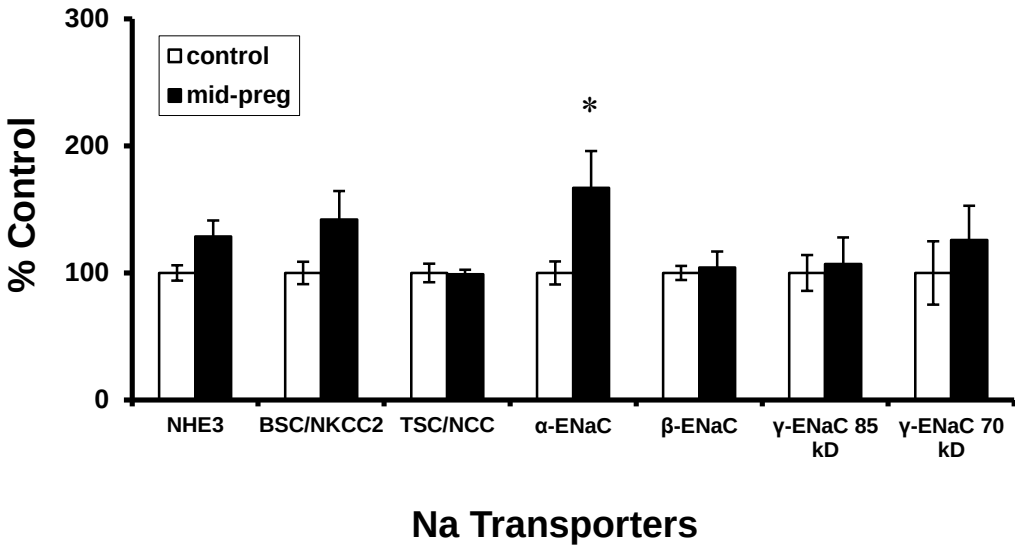
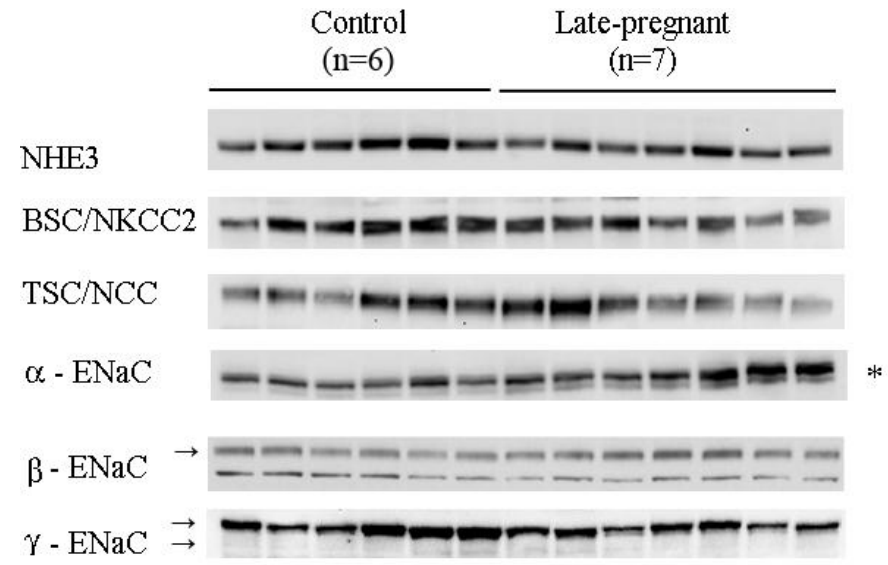


Figure 1

Semiquantitative immunoblots probed for the Na-H exchanger type 3 (NHE3), bumetanide-sensitive Na-K-2Cl cotransporter (NKCC), thiazide-sensitive cotransporter (NCC), and the three subunits of ENaC (α, β, and γ (70kD and 85kD forms)). A) Immunoblots compare whole kidney homogenates from six virgin and four mid pregnant rats (day 11-13 of pregnancy). B) Band densities were normalized to virgin controls with virgins set at 100% and summarized as bar graphs. An unpaired T-test was performed. Mean ± standard error (SE), * $p < 0.05$.

Figure



Figure

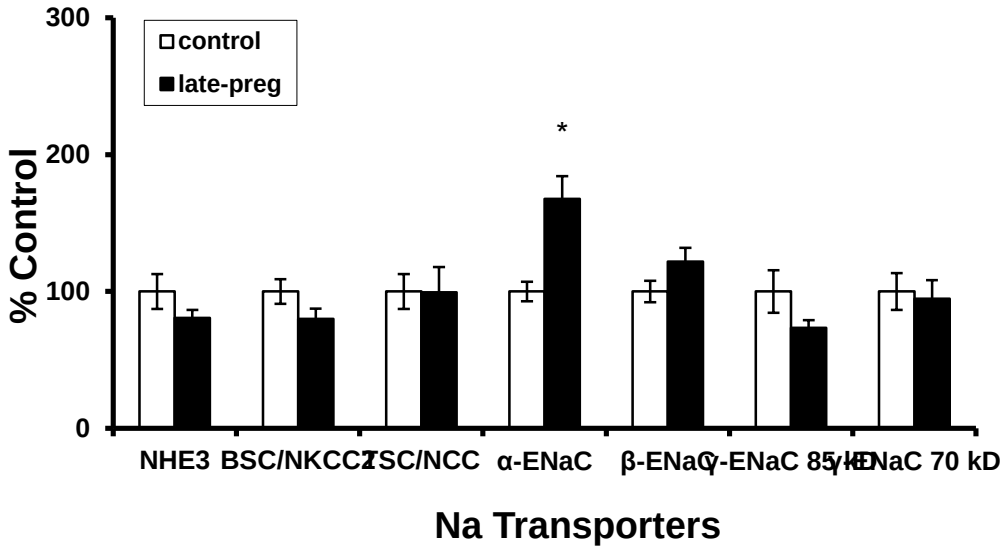


Figure 2

Semiquantitative immunoblots probed for the Na-H exchanger type 3 (NHE3), bumetanide-sensitive Na-K-2Cl cotransporter (NKCC), thiazide-sensitive cotransporter (NCC), and the three subunits of ENaC (α, β, and γ (70kD and 85kD forms)). A) Immunoblots compare whole kidney homogenates from six virgin and seven late-pregnant rats (day 18-20 of pregnancy). B) Band densities were normalized to virgin controls with virgins set at 100% and summarized as bar graphs. An unpaired T-test was performed. Mean ± standard error (SE), * $p < 0.05$.

Figure 3

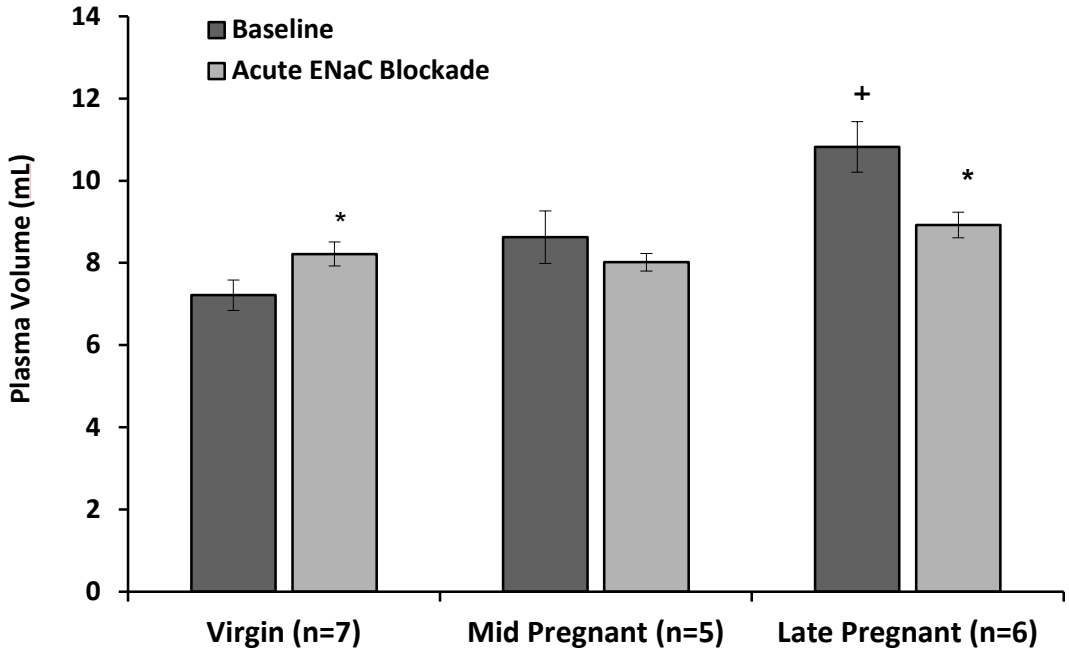


Figure 3

Plasma volume, measured as Evans blue dye space, in virgin (n=7), mid pregnant (n=5), and late pregnant (n=6) rats before and after ENaC blockade with benzamil (0.7 mg/kg). A two-way repeated measures ANOVA with Bonferroni Post Hoc was performed. Mean ± standard error (SE), $p < 0.05$. Significance is denoted as follows: * is significant versus corresponding baseline, † is significant versus virgin and mid pregnant baseline.

Figure 4

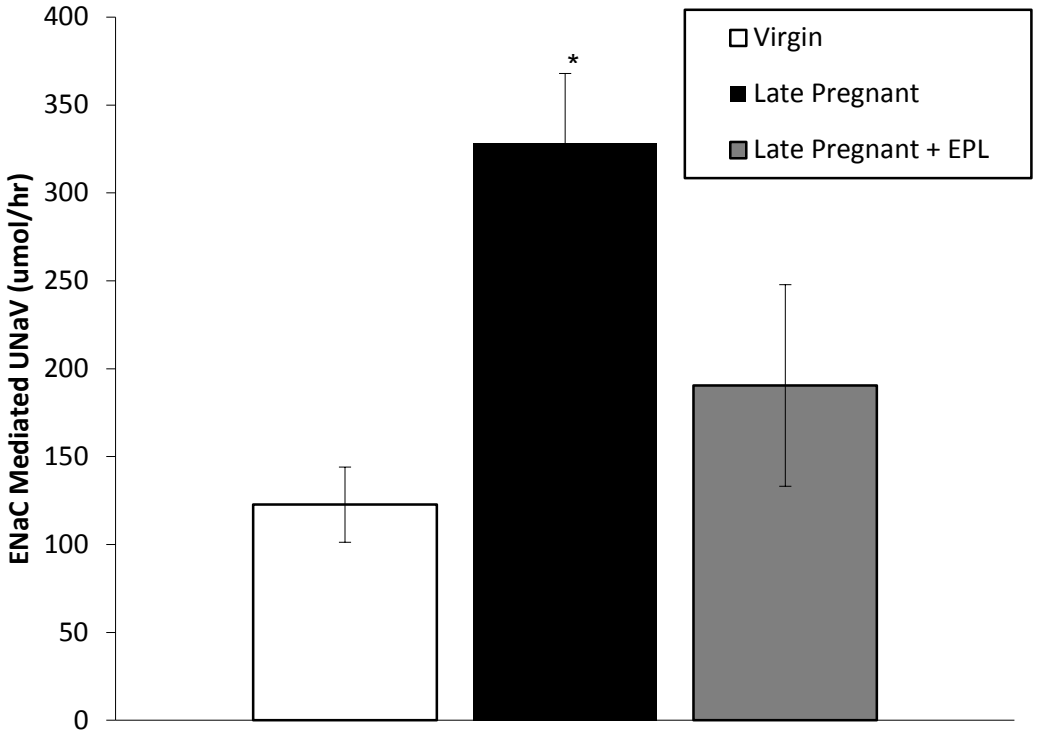


Figure 4

In vivo ENaC activity: Net natriuretic response to ENaC blockade with benzamil (0.7 mg/kg). Each rat had vehicle and benzamil injected intravenously (iv) on consecutive days. Five virgin, seven late pregnant and four late pregnant rats with eplerenone (100 mg/kg) were studied. Urine was collected 0-3 hours and 3-8 hours following injection for determination of sodium excretion ($U_{Na}V$). Data from the 0-3 hour period are shown here; there were no significant differences among groups in the natriuretic response 3-8 hours after ENaC blockade. Net $U_{Na}V$ was calculated as $U_{Na}V$ following benzamil - $U_{Na}V$ following vehicle. A One-Way ANOVA was performed. Mean ± standard error (SE), * $p < 0.05$ vs. Virgin.

Figure

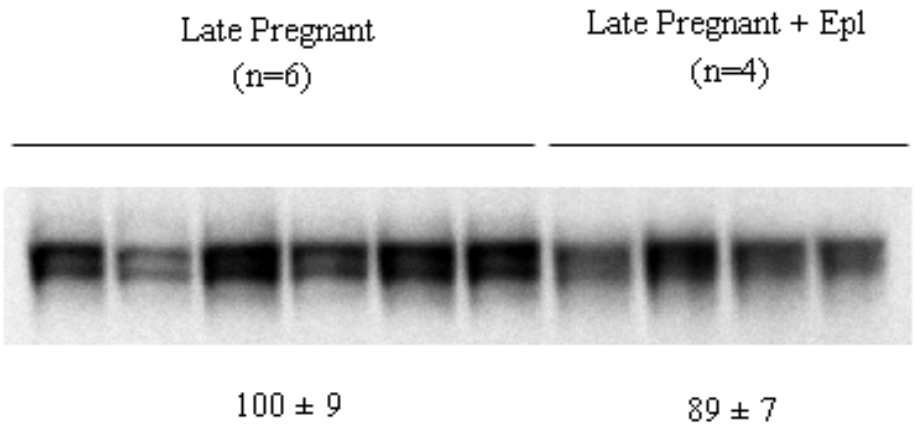


Figure 5

Semiquantitative immunoblots probed for the α subunit of ENaC. Immunoblots compare whole kidney homogenates from six late-pregnant rats and four late-pregnant rats treated with eplerenone (100 mg/kg). Band densities were normalized to late pregnant controls. An unpaired T-test was performed. Mean ± standard error (SE), values were not statistically significant.

References.

1) **Alexander EA, Churchill S and Bengele HH**. Renal hemodynamics and volume homeostasis during pregnancy in the rat. *Kidney Int* 18: 173-178, 1980.
2) **Baylis C**. Glomerular filtration and volume regulation in gravid animal models. *Baillieres Clin Obstet Gynaecol* 1: 789-813, 1987.
3) **Bedard S, Sicotte B, St-Louis J and Brochu M**. Modulation of body fluids and angiotensin II receptors in a rat model of intra-uterine growth restriction. *J Physiol* 562: 937-950, 2005.
4) **Beutler KT, Masilamani S, Turban S, Nielsen J, Brooks HL, Ageloff S, Fenton RA, Packer RK and Knepper MA**. Long-term regulation of ENaC expression in kidney by angiotensin II. *Hypertension* 41: 1143-1150, 2003.
5) **Blair ML and Mickelsen D**. Activation of lateral parabrachial nucleus neurons restores blood pressure and sympathetic vasomotor drive after hypotensive hemorrhage. *Am J Physiol* 291 (3): R742-R750, 2006.
6) **Butterworth MB, Edinger RS, Frizzell RA and Johnson JP**. Regulation of the epithelial sodium channel by membrane trafficking. *Am J Physiol Renal Physiol* 296: F10-F24, 2009.
7) **Dowell RT and Kauer CD**. Maternal hemodynamics and uteroplacental blood flow throughout gestation in conscious rats. *Methods Find Exp Clin Pharmacol* 19: 613-625, 1997.
8) **Fernandez-Llama P, Andrews P, Ecelbarger CA, Nielsen S and Knepper M**. Concentrating defect in experimental nephrotic syndrone: altered expression of aquaporins and thick ascending limb Na+ transporters. *Kidney Int* 54: 170-179, 1998.
9) **Frindt G, Masilamani S, Knepper MA, and Palmer LG.** Activation of epithelial Na channels during short-term Na deprivation. *Am J Physiol Renal Physiol* 280: F112-F118, 2001.
10) **Garland HO, Atherton JC, Baylis C, Morgan MR and Milne CM**. Hormone profiles for progesterone, oestradiol, prolactin, plasma renin activity, aldosterone and corticosterone during pregnancy and pseudopregnancy in two strains of rat: correlation with renal studies. *J Endocrinol* 113: 435-444, 1987.
11) **Garty H and Palmer LG**. Epithelial sodium channels: function, structure, and regulation. *Physiol Rev* 77: 359-396, 1997.
12) **Khraibi AA**. Renal interstitial hydrostatic pressure and pressure natriuresis in pregnant rats. *Am J Physiol Renal Physiol* 279: F353-F357, 2000.
13) **Khraibi AA, Dobrian AD, Yu T, Solhaug MJ and Billiar RB**. Role of RIHP and renal tubular sodium transporters in volume retention of pregnant rats. *Am J Hypertens* 18: 1375-1383, 2005.
14) **Kim GH, Ecelbarger CA, Mitchell C, Packer RK, Wade JB and Knepper MA**. Vasopressin increases Na-K-2Cl cotransporter expression in thick ascending limb of Henle's loop. *Am J Physiol* 276: F96-F103, 1999.
15) **Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB and Knepper MA**. The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. *Proc Natl Acad Sci U S A* 95: 14552-14557, 1998.
16) **Knepper MA, Kim GH and Masilamani S**. Renal tubule sodium transporter abundance profiling in rat kidney: response to aldosterone and variations in NaCl intake. *Ann N Y Acad Sci* 986: 562-569, 2003.
17) **Lifton RP, Gharavi AG and Geller DS**. Molecular mechanisms of human hypertension. *Cell* 104: 545-556, 2001.
18) **Masilamani S, Castro L and Baylis C**. Pregnant rats are refractory to the natriuretic actions of atrial natriuretic peptide. *Am J Physiol* 267: R1611-R1616, 1994.
19) **Masilamani S, Hobbs GR and Baylis C**. The acute pressure natriuresis response blunted and the blood pressure response reset in the normal pregnant rat. *Am J Obstet Gynecol* 179: 486-491, 1998.

20) **Masilamani S, Kim GH, Mitchell C, Wade JB and Knepper MA**. Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. *J Clin Invest* 104: R19-R23, 1999.
21) **Masilamani S, Wang X, Kim G-H, Brooks H, Nielsen J, Nielsen S, Nakamura K, Stokes JB, Knepper MA.** Time course of renal Na-K-ATPase, NHE3, NKCC2, NCC and ENaC abundance changes with dietary NaCl restriction. Am J Physiol Renal Physiol 2002 283: F648-F657.
22) **May A, Puoti A, Gaeggeler HP, Horisberger JD and Rossier BC**. Early effect of aldosterone on the rate of synthesis of the epithelial sodium channel alpha subunit in A6 renal cells. *J Am Soc Nephrol* 8: 1813-1822, 1997.
23) **Ni XN, Safai M, Rishi R, Baylis C, Humphreys MH**. Increased Activity of cGMP-Specific Phosphodiesterase (PDE5) Contributes to Renal Resistance to Atrial Natriuretic Peptide in the Pregnant Rat. *J Am Soc Nephrol*. 15: 1254 –1260, 2004
24) **Omer S, Mulay S, Cernacek P and Varma DR**. Attenuation of renal effects of atrial natriuretic factor during rat pregnancy. *Am J Physiol* 268: F416-F422, 1995.
25) **Rossier BC and Stutts MJ.** Activation of the Epithelial Sodium Channel (ENaC) by Serine Proteases. Annu. Rev. Physiol. 71:361-79, 2009.
26) **Salas SP, Giacaman A and Vio CP**. Renal and hormonal effects of water deprivation in late-term pregnant rats. *Hypertension* 44: 334-339, 2004.
27) **Salas SP, Marshall G, Gutierrez BL and Rosso P**. Time course of maternal plasma volume and hormonal changes in women with preeclampsia or fetal growth restriction. *Hypertension* 47: 203-208, 2006.
28) **Terris J, Ecelbarger CA, Nielsen S and Knepper MA**. Long-term regulation of four renal aquaporins in rats. *Am J Physiol* 271: F414-F422, 1996.

IV:

Increased activity of the epithelial sodium channel (ENaC) during pregnancy maintains pregnancy-mediated changes in sodium balance, volume status, and blood pressure.

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Abstract

Normal pregnancy is a state marked by avid Na retention and plasma volume expansion (PVE). Inadequate PVE results in complicated pregnancy with growth restricted (GR) fetus and maternal/fetal death. We have recently shown that renal *in vivo* ENaC activity in the late pregnant (LP) rat is increased. To determine the importance of renal vs extrarenal ENaC in sodium retention and blood pressure regulation during pregnancy, we have chronically blocked ENaC with either daily subcutaneous injections of benzamil (BZ) or intrarenal transfection of αENaC shRNA. Compared with untreated LP controls (n=6), LP rats with BZ (n=7) retain less sodium (LP = 1.88 ± 0.17, LP+BZ = -0.20 ± 0.13 mmol/day, $p<0.05$) and have reduced serum sodium concentrations (LP = 129 ± 2, LP+BZ = 114 ± 5 mmol/L, $p<0.05$). Further, chronic BZ reduced blood pressure (BP) in LP rats (LP=96±3, LP+BZ=85±2 mmHg, $p<0.05$). LP rats with BZ have lower maternal body weights (BW) (LP=401±11, LP+BZ =330 ±5g, $p<0.05$) and growth restricted pups (LP=5.1±0.1, LP+BZ =3.0±0.8g, $p<0.05$). However, chronic BZ treatment had no effect on sodium retention, body weight, or BP in virgin rats. Intrarenal transfection of αENaC shRNA (n=4) vs scrambled sRNA (n=5) successfully decreased renal αENaC mRNA expression in LP rats (LP+scramble=100±10, LP+shRNA=34±16%, $p<0.05$). Intrarenal transfection of αENaC shRNA reduced maternal sodium retention (LP+scramble=1.11±0.25, LP+shRNA=0.13±0.07 mmol/day, $p<0.05$) ΔBW (LP+scramble=94±9, LP+shRNA=16±24g, $p<0.05$) and pup weight (LP+scramble=5.1±0.1, LP+shRNA =2.2±0.6g, $p<0.05$). These findings suggest that renal ENaC is necessary for the progression of a healthy pregnancy.

Introduction

Normal pregnancy is characterized by many cardiovascular and renal hemodynamic adaptations. These include marked changes in blood pressure, sodium balance, and fluid regulation. Maternal blood pressure falls slightly during mid-pregnancy returning to non-pregnant values near term. This fall can be attributed to the significant reduction in total vascular resistance resulting from increased production of vasodilators and concomitant decreased vascular responsiveness to vasoconstrictors [Ganzevoort 2004, Girling 2004]. Later in gestation there is a further decrease in total peripheral resistance which can be ascribed to the opening of the utero-placental circulation. The reduced vascular tone stimulates the renin-angiotensin-aldosterone system which allows for increases in sodium and water reabsorption [Elsheikh 2001, Escher 2009, Ganzevoort 2004, Langer 1998]. Avid sodium retention during pregnancy drives the 50% blood volume expansion necessary for adequate perfusion of the uterus and fetus [Girling 2004]. Insufficient volume expansion results in the compromised maternal state of intrauterine growth restriction (IUGR) which afflicts ~5% of all pregnancies [Brodsky 2004].

We recently examined the protein profile of the major sodium transporters/channels along the renal tubule during pregnancy and found that only the protein consistently upregulated throughout gestation was the α subunit of the epithelial sodium channel (ENaC) [West 2010]. ENaC is a heteromultimeric channel composed of three homologous subunits (α, β, γ), with αENaC being the rate limiting subunit for channel formation. In the non-pregnant kidney ENaC is responsible for ~2% of sodium reabsorption. Although, ENaC is only responsible for a small fraction of sodium reabsorption, the collecting duct is the principal site for determination of net sodium balance, as ENaC expression and activity are highly regulated by aldosterone [Lifton 2001].

In the late pregnant (LP) rat the increased αENaC protein expression corresponds to increased *in vivo* channel activity via a mineralocorticoid receptor (MR) mediated pathway [West 2010]. Since sodium retention is a major determinant of blood volume and thus blood pressure the primary aim of this study was to determine if the increased ENaC activity in late pregnancy is necessary for maintenance of sodium balance and blood pressure. We used radio telemetry for monitoring blood pressure and metabolic cage studies for determining sodium balance in animals under chronic pharmacologic blockade with benzamil (Bz) and in animals intrarenally transfected with an shRNA targeting αENaC.

Methods

Animals

Animals were maintained in the Virginia Commonwealth University (VCU) animal facility in compliance with institutional guidelines and the National Institute of Health's (NIH's) Guide for Animal Use. Animal protocols were approved by VCU's Institutional Animal Care and Use Committee. Female Sprague-Dawley rats from Harlan (Indianapolis, IN) were used for all experiments. Female rats to become pregnant were mated with males and day 1 of pregnancy was determined as the day sperm appeared in the vaginal smear.

Pharmacologic Blockade

Pregnant rats received either chronic benzamil (0.7 mg/kg/day, SC) or vehicle injections beginning the evening of day 12. Day 15 the dose was increased to 1.05 mg/kg/day until harvest at day 20. The dosage was increased to compensate for the dilution of pregnancy mediated blood volume expansion. Age-matched virgin rats were given chronic benzamil or vehicle on the same dosing schedule as pregnant rats.

Genetic Blockade

Information on vector and shRNA sequence

Rat αENaC small interfering RNA sequences were designed and synthesized by Genscript. The target sequences were constructed into a pRNAT-CMV3.2/Neo vector (Genscript) to produce shRNA.

Sense siRNA Strand (5'→3'): GGAGCUGAACUAUAAAACUTT

Antisense siRNA Strand (5'→3'): AGUUUUAUAGUUCAGCUCCTT

The effective gene silencing of renal αENaC by shRNA *in vivo* was verified in preliminary experiments.

Intrarenal transfection of αENaC siRNA

Virgin rats nephrectomized 4 weeks prior to mating. On day 13 -15 of pregnancy left kidney is transfected with αENaC shRNA or scrambled sRNA. 50ug of the plasmid of interest is added to 8 uL transfection agent, in vivo jetPEI, in 600 uL 10% glucose solution (Polyplus-transfection SA, Illkirch, France). Renal artery and vein were isolated and tied off with 4-0 silk suture and the transfection mixture was injected into the renal artery and held in kidney for 4 min. A cellulose patch was then placed over the injection site and fixed with tissue glue. Artery and vein were untied and circulation was restored to kidney. Kidney specificity was confirmed with luciferase plasmid transfection using the above protocol. IVIS *in vivo* imaging system (Xenogen Corp., Alameda, CA) was used to non-invasively monitor and record the efficacy of the transfection within living rats chronically. Figure 1 illustrates *in vivo* bioluminescent imaging (5 days post transfection) of luciferase activity with lateral and ventral views of the same rat. This image demonstrates that genetic activity was apparent 5 days post-transfection and that the transfection was restricted to the left (transfected) kidney.

Experiments

Sodium Retention

Metabolic cage studies were performed in virgin and pregnant rats (day 11-18) under chronic ENaC blockade and in control virgin and pregnant rats. Rats were acclimated to metabolic cages and gelled diet containing water (62.3%), agar (0.3%), and rat chow (37.4%) (Harlan Teklad LM-485 Mouse/Rat Sterilizable Diet) two days prior to measurements. On the following days 24 hour collections were made from which gel food intake (g), Na intake (mmol/24 hours), urine output (V, ml/24 hour), urine sodium (U_{Na}, mmol/L), sodium excretion ($U_{Na}V = V*U_{Na}$, mmol/24 hour) and Na retention (Na intake – $U_{Na}V$) were calculated. Urine sodium concentration was determined by flame photometry.

Blood Pressure and Heart Rate

Radio telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN) were implanted into the left femoral artery and fed into the abdominal aorta of day 6-8 pregnant rats and age-matched virgins using full sterile technique under general anesthesia with 1-4% isoflurane (Baxter Healthcare Corp., Deerfield, IL). MAP and HR were recorded in conscious unrestrained rats every 15 minutes for 15 seconds. Drift corrected data was then averaged excluding outliers in 12hr intervals 7:15AM to 7:00PM and 7:15PM to 7:00 AM.

Harvest

Blood was drawn from the vena cava at harvest, centrifuged and serum collected for determination of serum sodium. Serum sodium concentration was determined by flame photometry. Kidneys were collected and processed for protein or mRNA. Pups were removed from the placenta and weighed.

qPCR

The relative mRNA abundance of αENaC was determined by qPCR. Total RNA was extracted using TRIzol solution (Life Technologies, Inc. Rockville MD) and then reverse-transcribed (RT) (cDNA Synthesis Kit, Bio-Rad, Hercules, CA). The RT products were amplified using a TaqMan Gene Expression Assays kit (Applied Biosystems). The real-time quantitative PCR was performed using an iCycler iQ Real-Time PCR Detection System (Bio-Rad) according to manufacturer's manual**.** TaqMan probes were used for the fluorescent detection system. The primer pair used for the real-time PCR was forward primer: 5'-ACTGAACTGTGCTCAGGGATGA-3' and reverse primer: 5'-ATCTGCCTACCTGGTCCAAGTG-3'. The cycle threshold (Ct) values were used for calculation of gene expression in accordance with the ΔΔCt method. Relative mRNA levels were expressed by the values of $2^{-\Delta\Delta Ct}$.

Western Blotting

Western blotting analysis was performed on whole kidney homogenates from late pregnant siRNA (day 20 - 21, n=4) and scrambled (day 22, n=8) rats. Kidneys were collected at harvest and using a tissue homogenizer (Pro Scientific, Oxford, CT) were homogenized in a chilled isolation solution of 10 mM triethanolamine, 250 mM sucrose (Mallinckrodt Baker Inc., Phillipsburg, NJ), and the protease inhibitors: phenylmethyl sulfonyl fluoride (25mg/ml isopropyl alcohol) (Biochemical Corp, Lakewood, NJ) and leupeptin (1mg/ml dd H_2O) (Novabiochem/EMD Chemicals, Inc., Gibbstown, NJ). A BCA protein assay kit (Pierce Biotechnology, Rockford, IL) was used to determine the protein concentration of the homogenized samples. Samples were solubilized in a Laemmli sample buffer for 15 minutes at 60 °C prior to storage at -80 °C. SDS-PAGE was carried out using Criterion precast 10% Tris-HCl gels (Bio-Rad Laboratories, Inc., Hercules, CA). Equal loading of the gel was confirmed through staining with GelCode Blue Reagent (Pierce Biotechnology, Rockford, IL) (Musselman 2010).

Semiquantitative immunoblotting was performed on homogenized samples. Samples were loaded into the lanes of Criterion precast 10% Tris-HCl gels. The proteins were transferred from unstained gels to nitrocellulose membranes by electrophoresis. The blots were blocked for 2 hr with blotting grade blocker non-fat dry milk (5 g/dl) (Bio-Rad Laboratories, Hercules, CA) and probed with primary antibody for overnight. Peroxidase-conjugated secondary antibodies (Pierce Biotechnology, Rockford, IL, no. 31458 & 31452) were then incubated with the blots followed by Western Lightning Chemilumnescence Reagent Plus (Perkin Elmer LAS, Inc., Boston, MA). Blots were exposed to X-ray film and densitometry was completed using the VersaDoc Imaging System (VersaDoc Bio-Rad, Bio-Rad Laboratories, Hercules, CA). Densitometry was normalized to virgin controls with the mean for the control group being defined as 100%.

We used our own rabbit polyclonal antibodies against the three subunits of the amiloride-sensitive epithelial sodium channel (α, β, and γ subunits of ENaC) (1:1000 dilution) using the sequence and protocol previously described [Masilamani 1999].

Statistics

All data are presented as means $\pm$ standard error (SE). Analysis was competed using SPSS statistical program (SPSS 12.0, Chicago, Illinois). An unpaired t-test or a two-way analysis of variance (ANOVA) was performed with the null hypothesis being rejected at $p<0.05$

Results

Pharmacologic Blockade

Sodium retention

Sodium retention was tracked from day 11 to day 18 of pregnancy in rats treated with vehicle or benzamil beginning day 12 of pregnancy. Sodium retention was decreased in late pregnant rats under chronic ENaC blockade compared to late pregnant controls (Figure 2). Sodium retention is determined by intake – excretion (Figure 3). Serum sodium was decreased in pregnant animals treated with chronic benzamil compared to normal pregnant rats (LP = 129 ± 2, LP ChBz = 114 ± 5 mmol/L, $p<0.05$). However, there was no significant difference in sodium retention in virgin animals chronically treated with benzamil compared to virgin controls.

Blood Pressure and Heart Rate

Blood pressure was monitored using radio-telemetry from day 10 to day 20 of pregnancy in rats treated with vehicle or benzamil beginning day 12 of pregnancy. Mean arterial blood pressure was decreased in late pregnant rats under chronic ENaC blockade compared to normal pregnant rats (LP = 96 ± 3, LP ChBz = 85 ± 2 mmHg, $p<0.05$), with no significant difference between virgin rats (V = 105 ± 4, V ChBz = 102 ± 4 mmHg, NS) (Figure 4). There was no significant difference in heart rate between normal pregnant animals and pregnant animals under chronic ENaC blockade (LP = 401 ± 9, LP ChBz = 401 ± 5 BPM).

Maternal and Fetal Body Weights

Chronic benzamil prevented the normal increase in body weight associated with a healthy pregnancy (Figure 5). Pups from mothers treated with benzamil were significantly smaller than pups from untreated mothers (Figure 6).

Genetic Blockade

αENaC mRNA and Protein Expression

Pregnant rats transfected with αENaC shRNA had reduced αENaC mRNA expression compared to scrambled controls (Firgure 7A). Semiquantitative immunoblotting revealed the protein abundance of αENaC decreased with shRNA compared to scrambled (Figure 7B). Since the α subunit of ENaC is the rate-limiting subunit for channel formation [May 1997], this is consistent with decreased activity of ENaC due to reduced channel number. Renal specificity of knockdown was confirmed by measuring mRNA expression of distal colon αENaC (αENaC shRNA = 350 ± 215, scrambled sRNA = 100 ± 85, NS).

Sodium retention

Sodium retention was measured day 18-19 of pregnancy in rats transfected with αENaC shRNA or scrambled beginning day 14 of pregnancy. Sodium retention was decreased in late pregnant rats under genetic ENaC blockade compared to late pregnant controls (Figure 8). Serum sodium was decreased in pregnant animals transfected with αENaC shRNA compared to scrambled pregnant.

Maternal and Fetal Body Weights

Transfecting rats with αENaC siRNA prevented the normal increase in body weight associated with a healthy pregnancy (Figure 9). Pups from mothers transfected with αENaC shRNA were significantly smaller than pups from untreated mothers (Figure 10).

Discussion

The primary finding from this study is that chronic ENaC blockade (pharmacologic and genetic) prevented normal sodium retention in the pregnant rat. Prevention of sodium retention resulted in reduced maternal serum sodium concentration, blood pressure, body weight, and fetal growth

restriction. Virgin rats were unaffected by chronic ENaC blockade as demonstrated by their normal sodium retentions, blood pressures, and body weights. These results indicate that the detrimental effects of ENaC blockade are pregnancy specific. Taken together this study emphasizes the importance of increased ENaC activity during pregnancy in maintaining the pregnancy-mediated changes in sodium balance, volume status, and blood pressure.

Although, ENaC in known to play an essential role in the final adjustment of renal sodium, potassium, and water excretion and therefore the maintenance of extracellular salt and volume homeostasis and blood pressure control, there are many redundancies in pregnant physiology aimed at protecting volume expansion [Loffing 2005, Lifton 2001]. Therefore, it was unexpected to find that ENaC blockade caused complete loss of the normal positive sodium balance associated with pregnancy. Although whole kidney protein expression of the other renal sodium transporters (NHE3, NKCC2, and NCC) is unchanged during late pregnancy, this does not preclude changes in activity or trafficking. It is well established that the activity of NKCC2 and NCC are regulated by phosphorylation via WNK/SPAK/OSR1. This cascade induces increases in activity without altering whole cell expression levels. Further, trafficking of these transporters to the apical membrane is influenced by hormones that are known to be upregulated in pregnancy. Angiotensin II increases NHE3 and NCC trafficking [Flatman 2008].

Few have studied the effects of benzamil (or amiloride) during human pregnancy as the use of diuretics is controversial. In a previous study, amiloride (3.6 mg/kg/day) given to pregnant rats via drinking water from day 13-20 of pregnancy resulted in no difference in gestational body weight, serum sodium, or blood pressure compared to pregnant controls. However, they did observe increased reabsorptions, fetal deaths, and IUGR. They also noted a 20% fatality rate in pregnant mothers (day 16-18) receiving amiloride [Greenberg 1997]. Our pregnant animals under chronic blockade became

very sickly, not unlike the description given of the 20% of their animals which died. We believe that the differences between studies can be resolved by the specificity of the drug given, and the method of drug delivery. Benzamil is an amiloride analogue having a 10 fold greater affinity for ENaC [Frelin 1988]. The higher affinity would provide better ENaC inhibition allowing for increased sodium excretion. Rapid sodium excretion induces hyponatriemia which causes loss of appetite. Decreased food intake exacerbates salt wasting resulting in rapid loss of the normal positive sodium balance (Figure 11). Since sodium retention is driving volume expansion in pregnancy, volume depletion is expected. Although we have not directly measured plasma volume in the present study, maternal body weight has been shown to correlate significantly with plasma volume [Salas 1993, Rosso 1992].

Since we have prevented the normal weight gain and sodium retention associated with pregnancy we expect that these animals have diminished plasma volumes and that this is the cause of fetal growth restriction. The IUGR presenting in this study is likely due to dual insult of decreased ingestion and lack of compensation by the kidney resulting in reduced uterine perfusion pressure from decreased plasma volume. Diminished uterine blood flow would reduce delivery of nutrients and oxygen to the fetuses preventing them from reaching their growth potential.

It is well established that gestation in rats is associated with a decrease in blood pressure. This decrease was found to be larger in rats that are sodium restricted [Pike 1976]. We too found a significant reduction in blood pressure in late pregnant rats under chronic ENaC blockade compared to their normal pregnant controls. It is likely this fall can be attributed to the prevention of sodium retention and subsequent volume depletion.

In conclusion, this study has provided evidence for the importance of increased ENaC activity during pregnancy in maintaining the pregnancy-mediated changes in sodium retention, volume expansion, and

blood pressure regulation. This is highlighted by the fact that all of the other redundant systems upregulated during pregnancy in order to protect volume expansion were unable to compensate when ENaC activity was chronically inhibited.

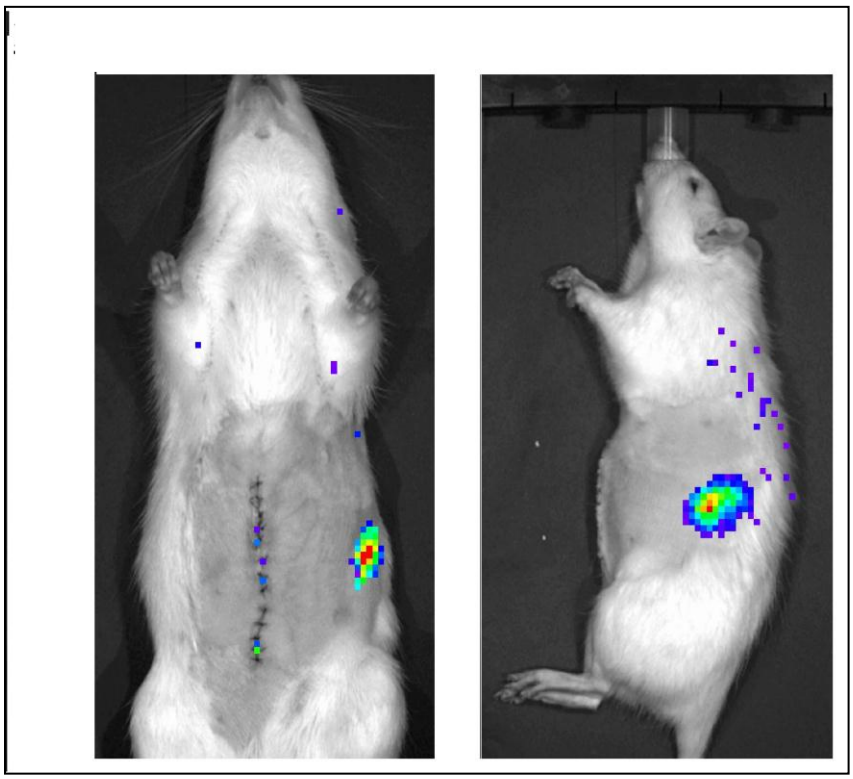

Figure 1. *In vivo* bioluminescent imaging (5 days post transfection) of luciferase activity (luciferase was cotransfected with siRNA) with lateral and ventral views of the same rat. This image demonstrates that genetic activity was readily apparent 5 days post-transfection.

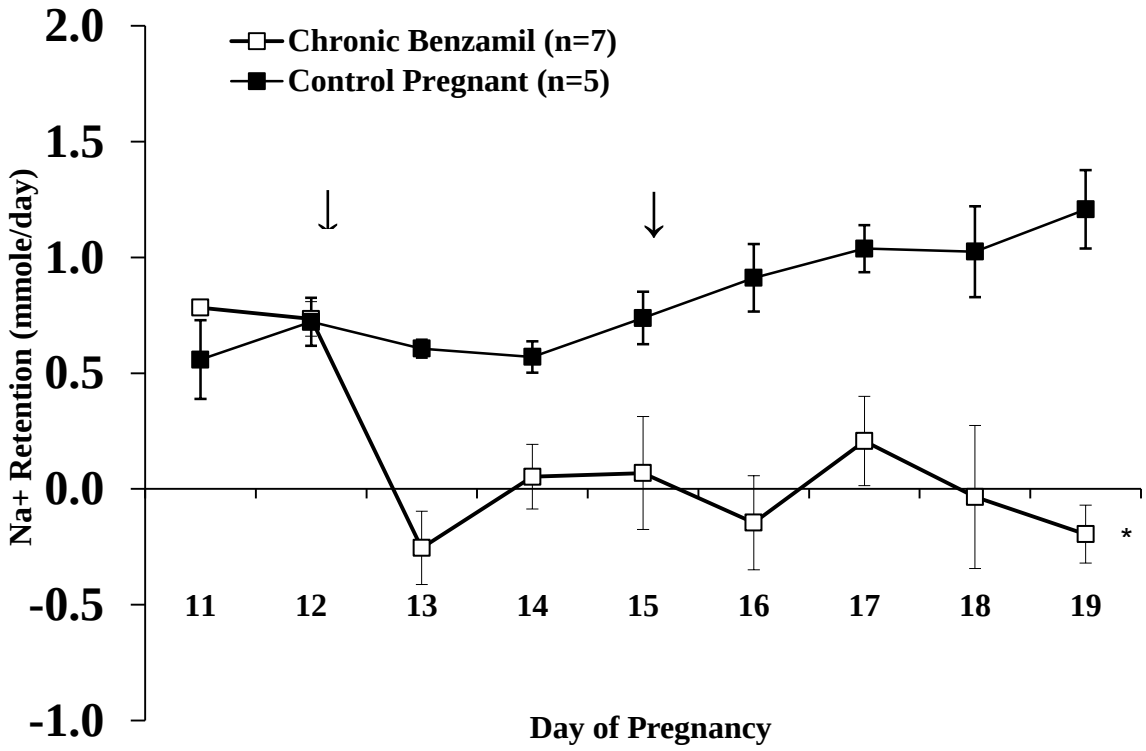

Figure 2. Effect of chronic ENaC blockade on Na+ retention in LP control = 5 and LP with Bz = 7. Na+ retention = Na+ Intake – UNaV. An unpaired T-test was performed on day 18, values are presented as mean ± standard error (SE), * $p < 0.05$ vs. control.

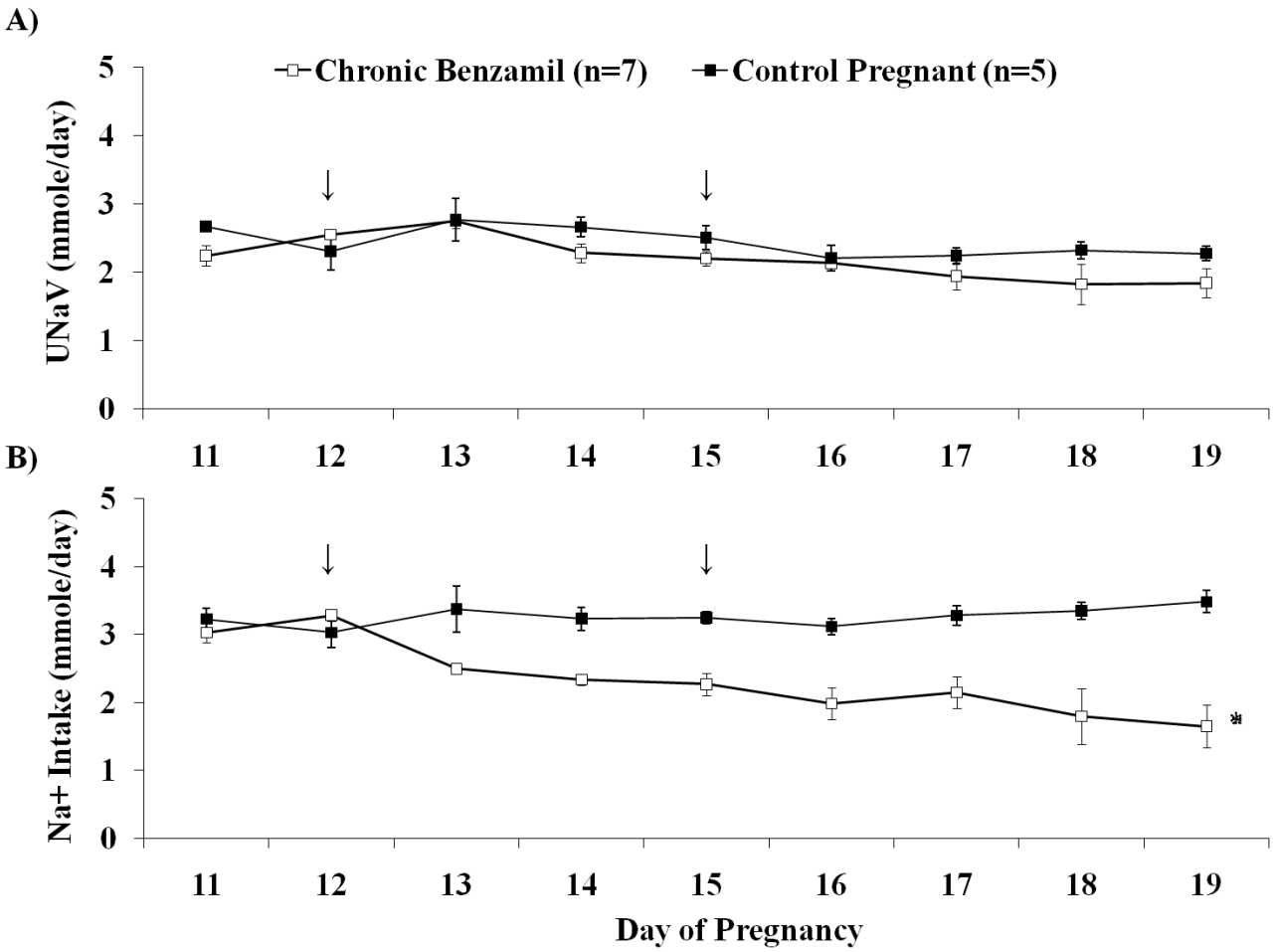

Figure 3. Effect of chronic ENaC blockade on (A) UNaV and (B) Na+ intake in LP control = 5 and LP with Bz = 7. An unpaired T-test was performed on day 18, values are presented as mean ± standard error (SE), * $p < 0.05$ vs. control.

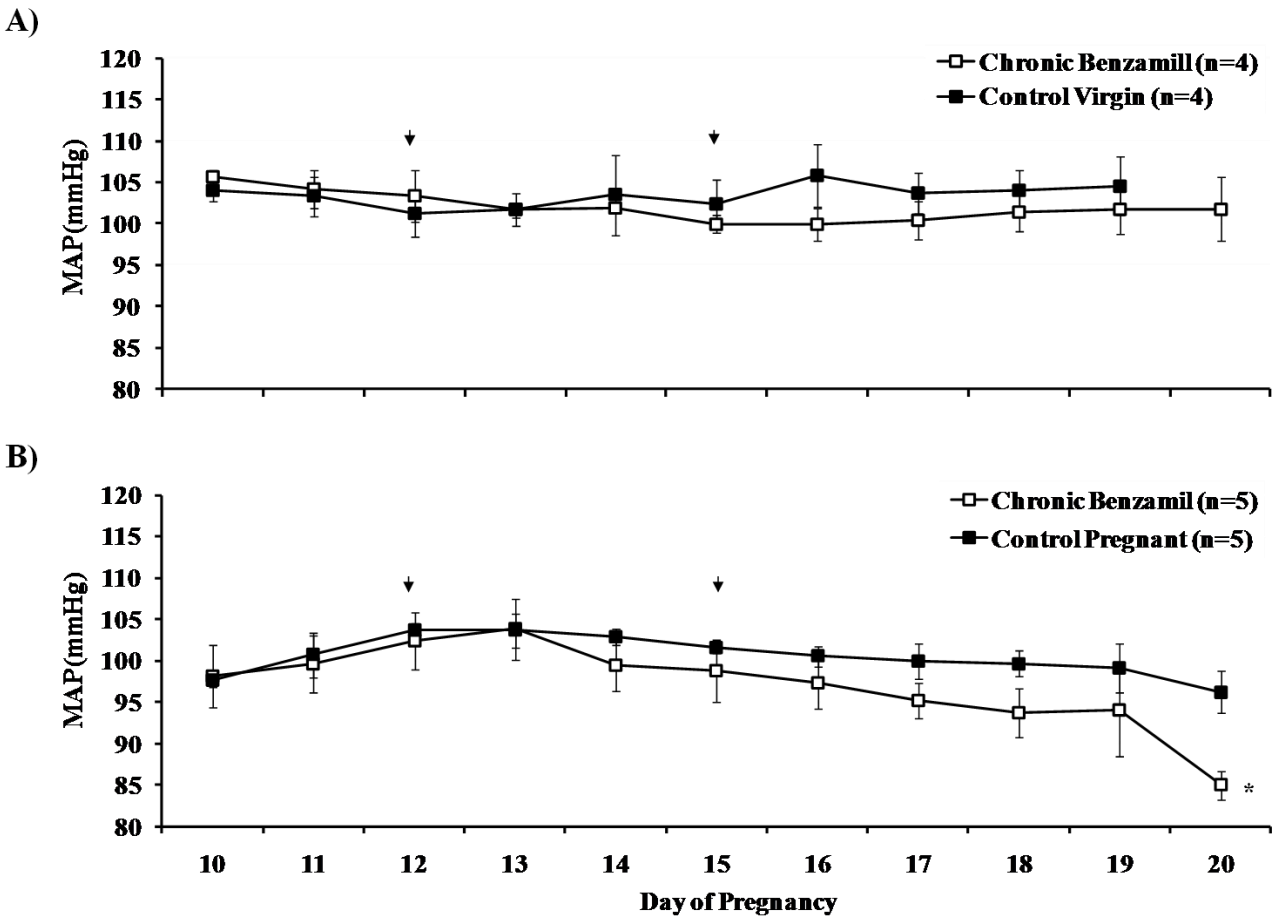

Figure 4. Effect of chronic ENaC blockade on blood pressure in A) virgin control (n=5) and virgin with Bz (n=9) and B) LP control n= 5 and LP with Bz n = 5. An unpaired T-test was performed on day 20, values are presented as mean ± standard error (SE), * $p < 0.05$ vs. control.

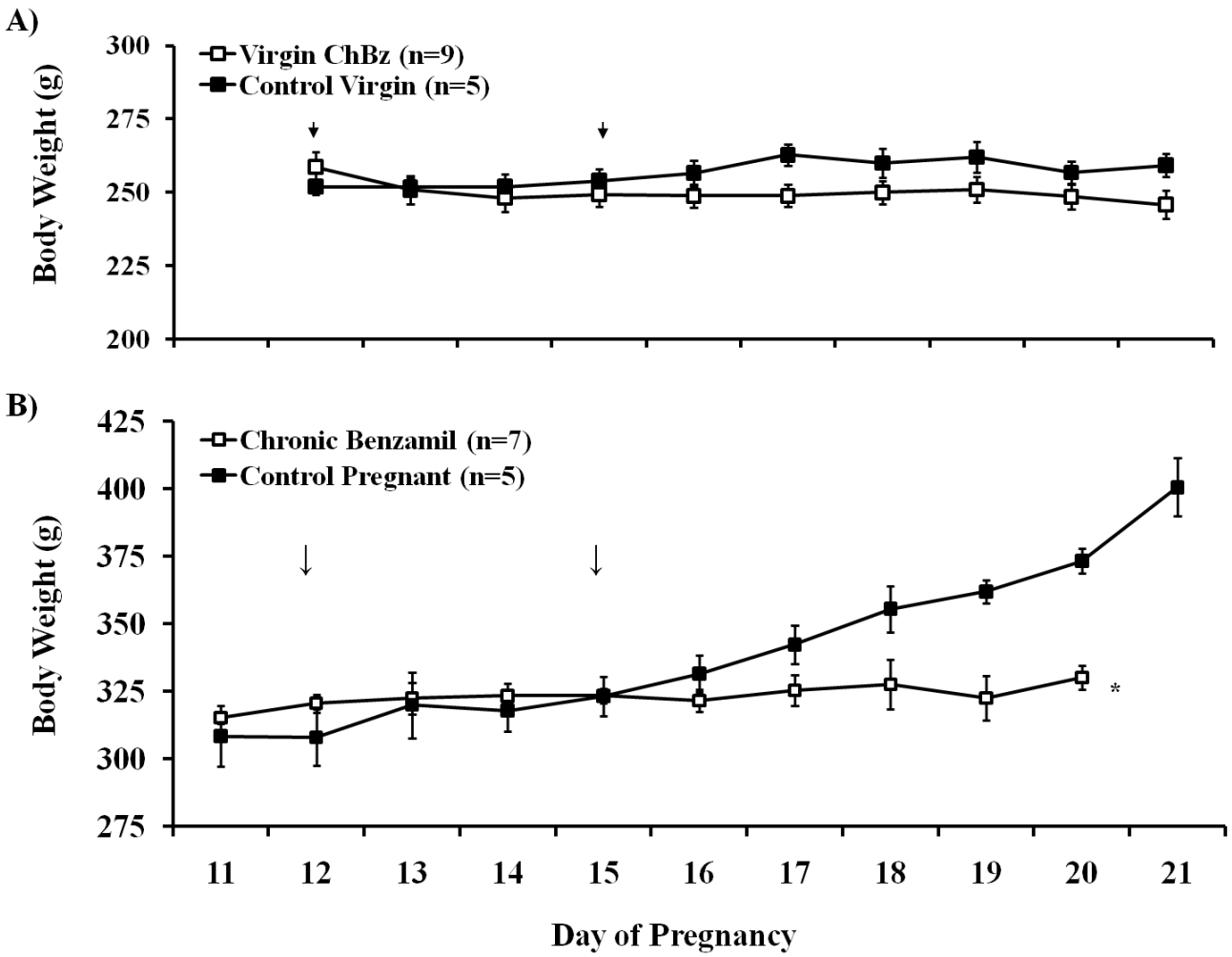

Figure 5. Effect of chronic ENaC blockade on maternal body weight in A) virgin control n=5 and virgin with Bz n=9 and B) LP control n= 5 and LP with Bz n =7. An unpaired T-test was performed on day 20, values are presented as mean ± standard error (SE), * $p < 0.05$ vs. control.

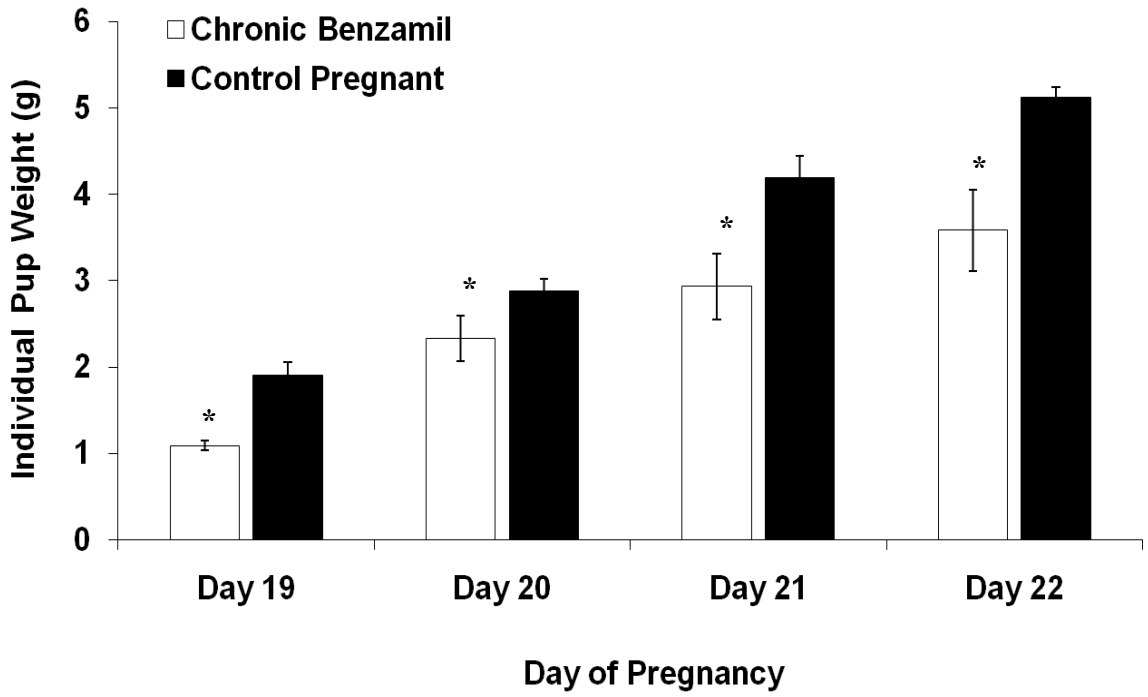

Figure 6. Effect of chronic ENaC blockade on fetal weights A 2-Way ANOVA with Bonferroni Post Hoc was performed, mean ± standard error (SE), * $p < 0.05$ vs. control late pregnant animals.

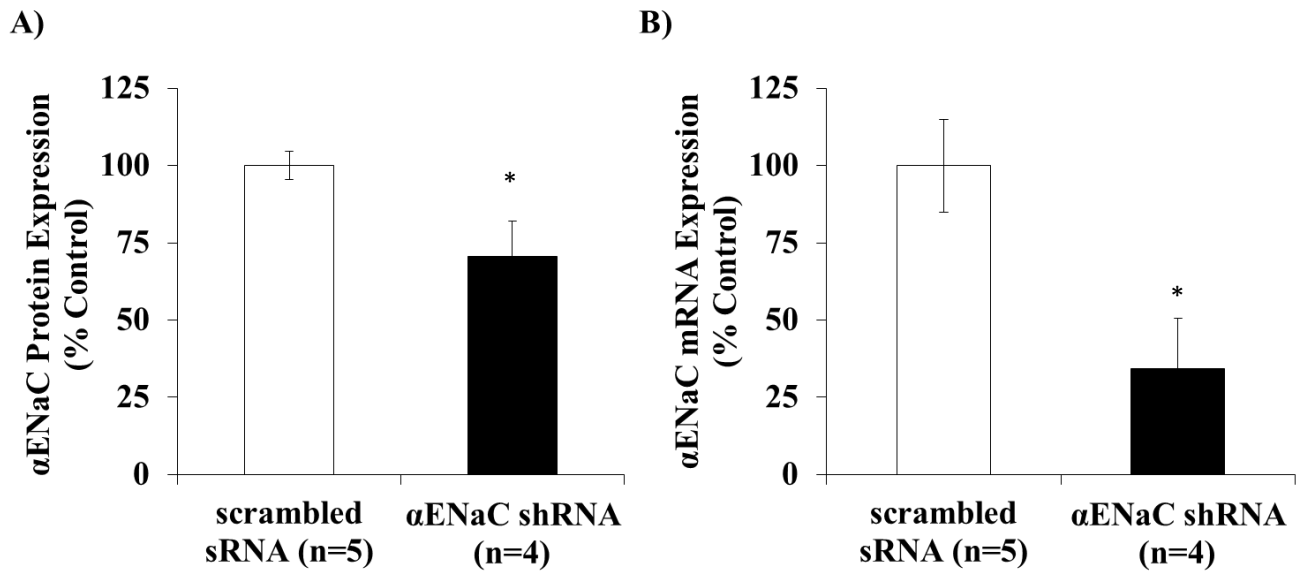

Figure 7. A) αENaC whole kidney protein abundance normalized to scrambled pregnant rats with controls set at 100% and B) αENaC mRNA expression normalized to scrambled pregnant rats with controls set at 100% summarized as bar graphs. An unpaired T-test was performed. Mean ± standard error (SE), * $p < 0.05$.

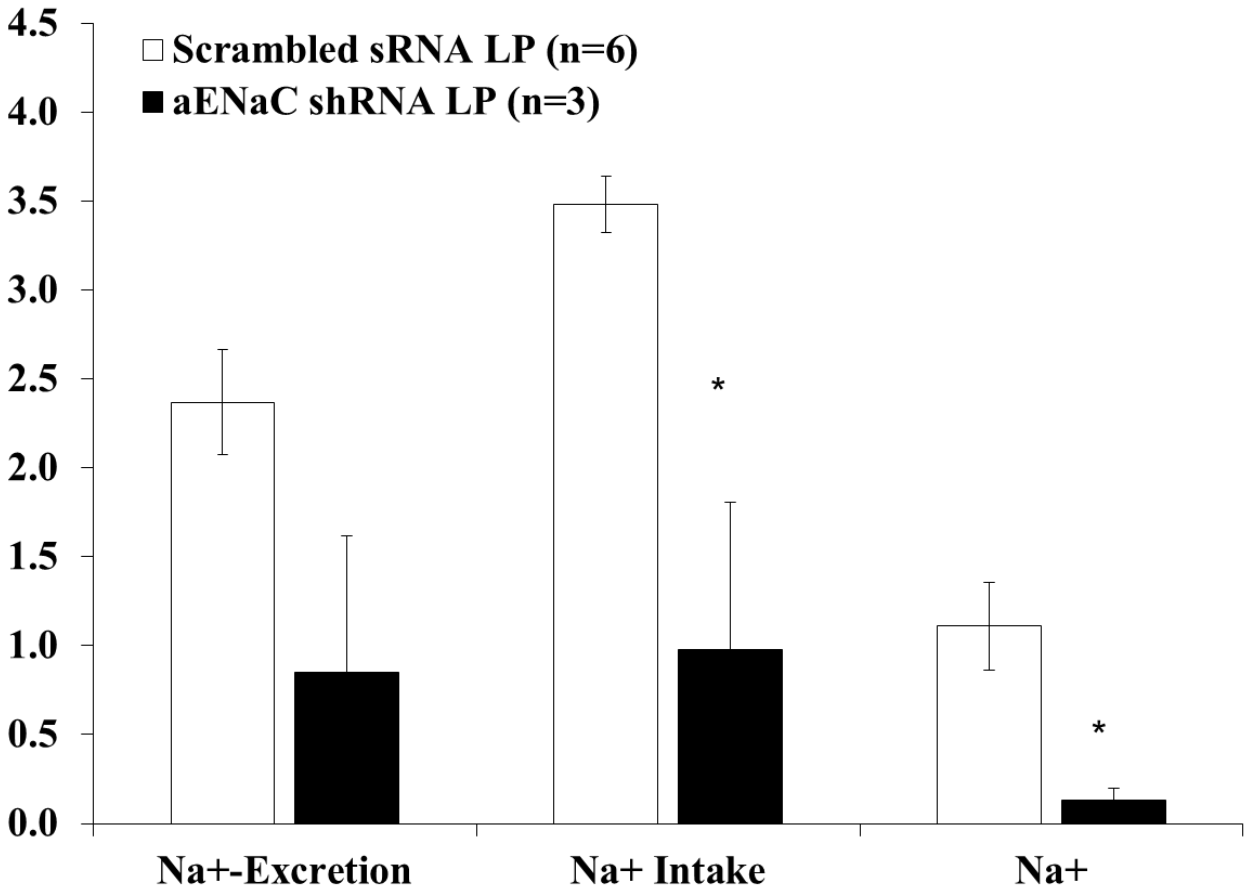

Figure 8. Effect of intrarenal transfection of αENaC shRNA on Na+ retention in late pregnancy (day 19-20), LP with scrambeld sRNA = 6 and LP with αENaC shRNA = 4. Na+ retention = Na+ Intake – UNaV. An unpaired T-test was performed, values are presented as mean ± standard error (SE), * p< 0.05 vs. control.

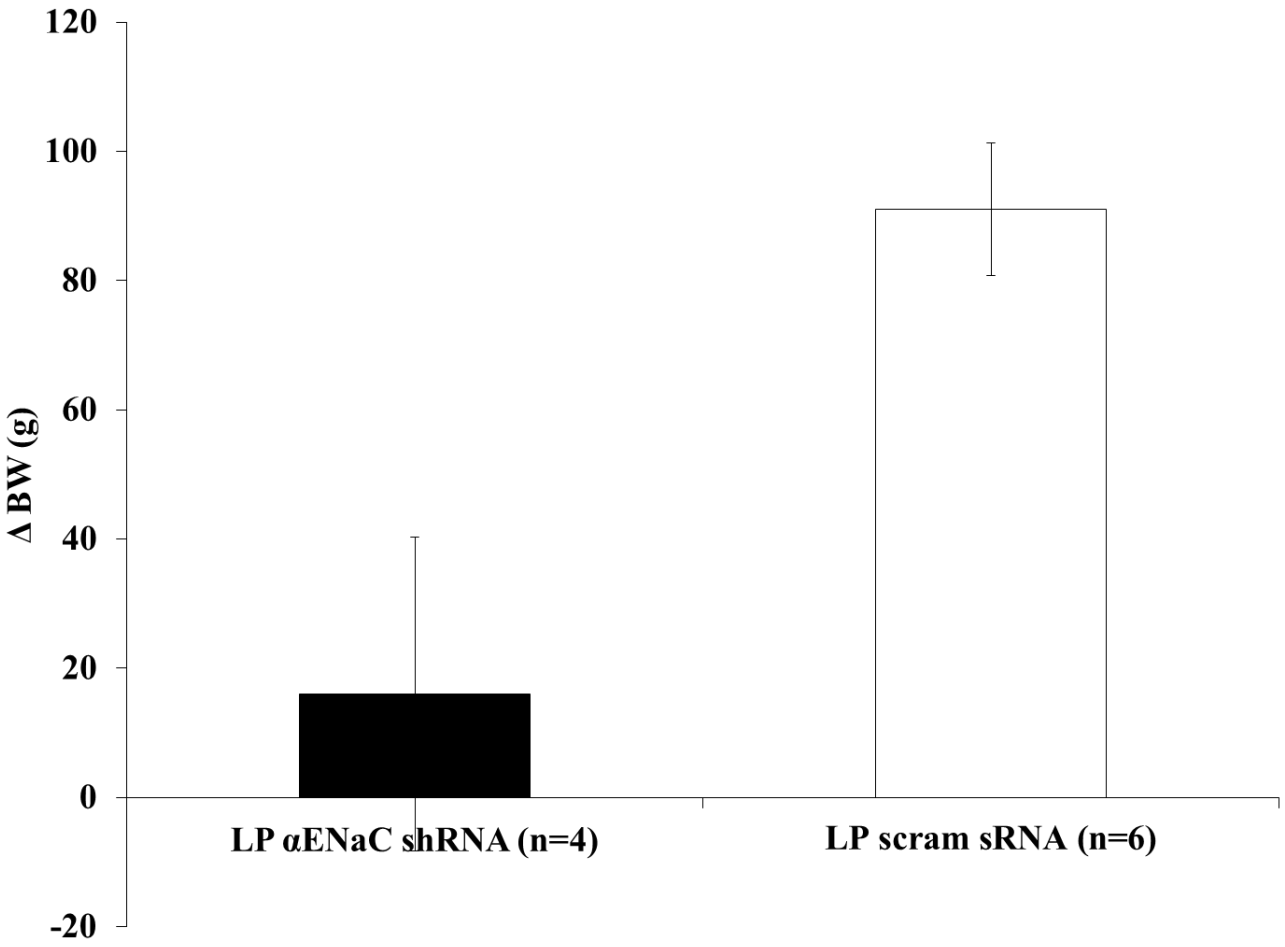

Figure 9. Effect of siRNA on maternal body weight in late pregnancy (day 19-20) LP scrambled n= 6 and LP αENaC n =4. An unpaired T-test was performed, values are presented as mean ± standard error (SE), * p< 0.05 vs. control.

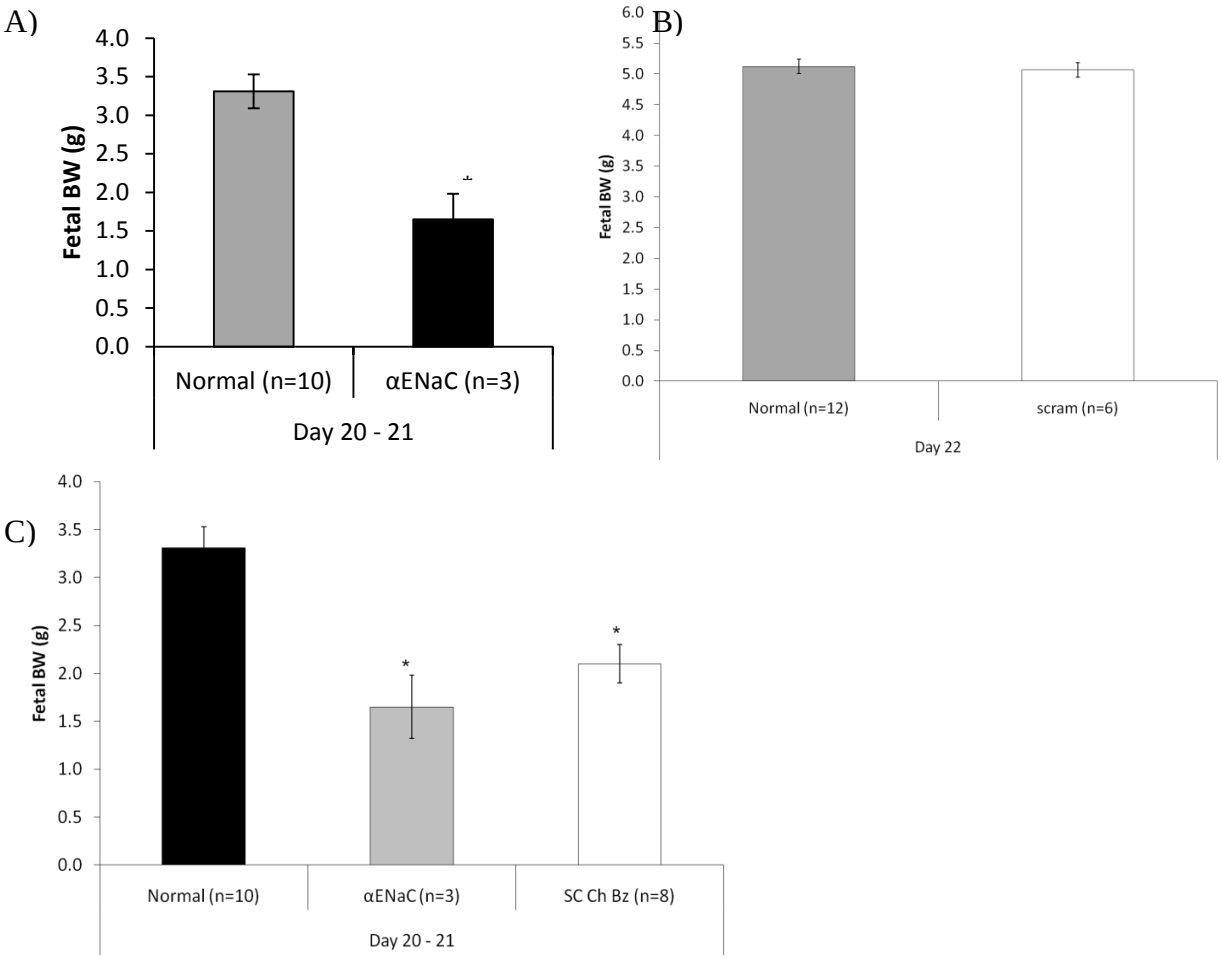

Figure 10. (A) Fetal body weights of rats transfected with αENaC shRNA (n=3) compared to normal pregnant rats (n=10), (B) scrambled sRNA (n=6) compared to normal pregnant rats (n=12) and (C) comparison between αENaC and chronic benzamil. For A and B, an unpaired T-test was performed, values are presented as mean ± standard error (SE), * $p < 0.05$ vs. control. For panel C a One-Way ANOVA with Bonferroni Post Hoc was performed, mean ± standard error (SE), * $p < 0.05$ vs. control late pregnant animals.

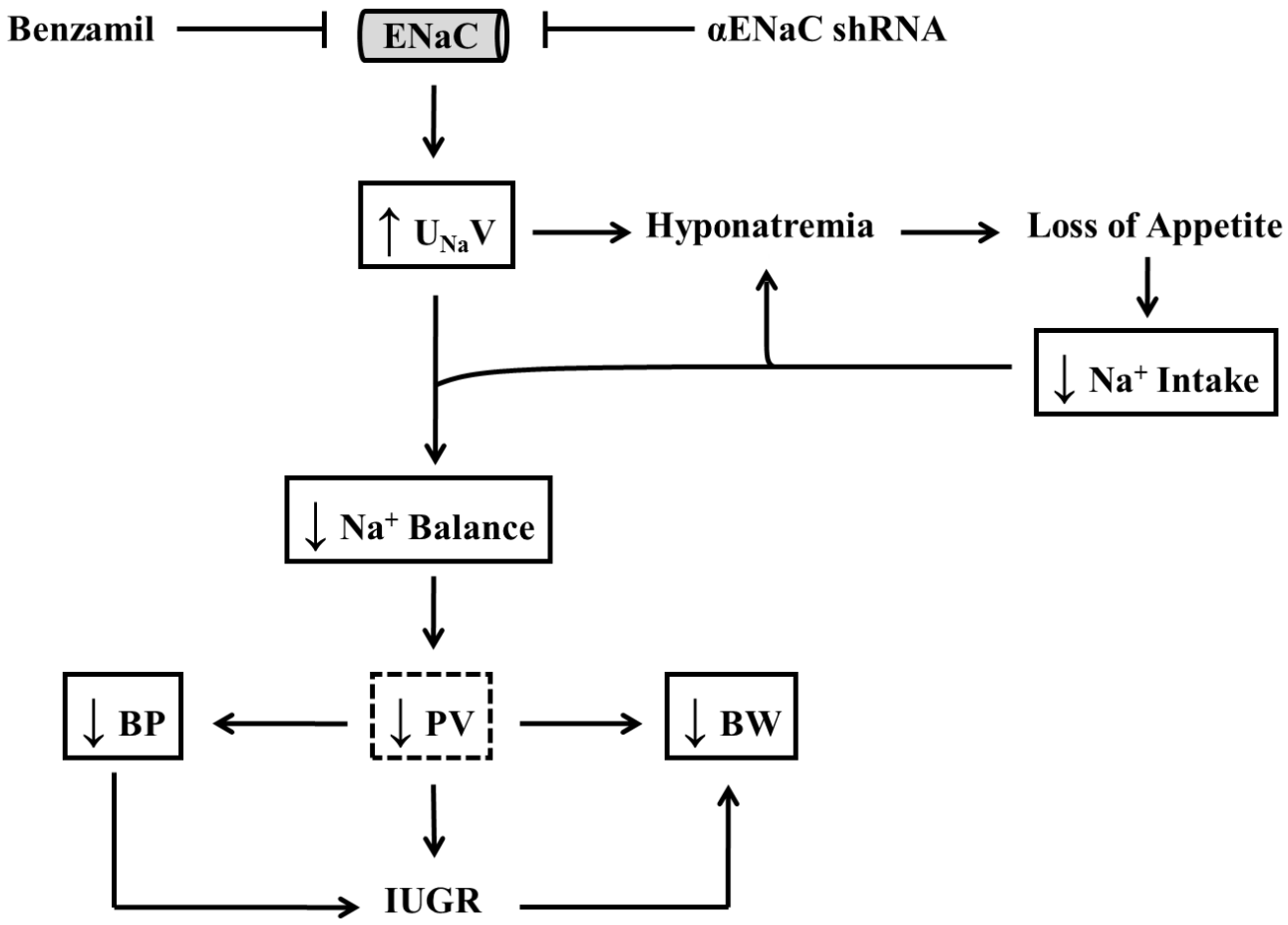

Figure 11. Summary effects of chronic ENaC blockade during pregnancy. ENaC = epithelial sodium channel, UNaV = sodium excretion, BP = blood pressure, PV = plasma volume, BW = body weight, IUGR = intrauterine growth restriction.

References

1) **Arthur SK and Green R.** Fluid reabsorption by the proximal convoluted tubule of the kidney in lactating rats. *J Physiol* 371: 267-275, 1986.
2) **Atherton JC, Bielinska A, Davison JM, Haddon I, Kay C, and Samuels R.** Sodium and water reabsorption in the proximal and distal nephron in conscious pregnant rats and third trimester women. *J Physiol* 396: 457-470, 1988.
3) **Brodsky D and Christou H.** Current Concepts in Intrauterine Growth Restriction. *J Intensive Care Med* 19: 307-319, 2004.
4) **Cha SC, Aberdeen GW, Mukaddam-Daher S, Quillen EW, Nuwayhid BS.** Tubular handling of fluid and electrolytes during ovine pregnancy. *Am J Physiol* 265: F278-F284, 1993.
5) **Elsheikh A, Creatsas G, Mastorakos G, Milingos S, Loutradis D, and Michalas S**. The renin-aldosterone system during normal and hypertensive pregnancy. *Arch Gynecol Obstet* 264: 182-5, 2001.
6) **Escher G.** Hyperaldosteronism in pregnancy. *Ther Adv Cardiovasc Dis* 3: 123-132, 2009.
7) **Flatman P.** Cotransporters, WNKs and hypertension: an update. *Curr Opin Nephrol Hypertens* 17: 186–192, 2008.
8) **Frelin C, Barbry P, Vigne P, Chassande O, Cragoe E, and Lazdunski M.** Amiloride and its analogs as tools to inhibit Na^+ transport via the Na^+ channel, the Na^+/H^+ antiport and the Na^+/Ca^{2+} exchanger. *Biochimie* 70: 1285-1290, 1988.
9) **Frindt G and Palmer LG.** Na channels in the rat connecting tubule. *Am J Physiol Renal Physiol* 286: F669–F674, 2004.
10) **Ganzevoort W, Rep A, Bonsel GJ, de Vries JI, and Wolf H.** Plasma volume and blood pressure regulation in hypertensive pregnancy. *Journal of Hypertension* 22: 1235–1242, 2004.
11) **Garland HO and Green R.** Micropuncture study of changes in glomerular filtration and ion and water handling by the rat kidney during pregnancy. *J Physiol* 329: 389-409, 1982.
12) **Girling JC.** Physiology of pregnancy. *Anesthesia and Intensive Care Medicine* 215-218, 2004.
13) **Greenberg S, Lancaster J, Xie J, Sarphie T, Zhao X, Hua L, Freeman T, Kapusta D, Giles T, and Powers D.** Effects of NO synthase inhibitors, arginine-deficient diet, and amiloride in pregnant rats. *Am J Physiol Regul Integr Comp Physiol* 273: R1031-1045, 1997.
14) **Langer B, Grima M, Coquard C, Bader AM, Schlaeder G, and Imbs JL.** Plasma active renin, angiotensin I, and angiotensin II during pregnancy and in preeclampsia. *Obstet Gynecol* 91:196-202, 1998.
15) **Lifton R, Gharavi A, and Geller D.** Molecular mechanisms of human hypertension. *Cell* 104: 545-556, 2001.
16) **Loffing J and Schild L.** Functional Domains of the Epithelial Sodium Channel. *J Am Soc Nephrol* 16: 3175-3181, 2005.
17) **Masilamani S, Kim GH, Mitchell C, Wade JB and Knepper MA**. Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. *J Clin Invest* 104: R19-R23, 1999.
18) **Musselman TM, Zhang Z, and Masilamani SM.** Differential regulation of the bumetanide-sensitive cotransporter (NKCC2) by ovarian hormones. *Steroids* 75: 760-765, 2010.
19) **Pike RL.** Sodium requirement of the rat during pregnancy. In: Lindheimer M, Katz A, Zuspan F, editors. Hypertension in pregnancy. New York: Wiley; 207-216, 1976.
20) **West C, Zhang Z, Ecker G, and Masilamani S**. Increased Renal α Epithelial Sodium Channel (ENaC) Protein and Increased ENaC Activity in Normal Pregnancy. *Am J Physiol Regul Integr Comp Physiol* 299: R1326-32, 2010.

V:

Large scale proteomic analysis of renal collecting duct cells in pregnancy, insight into ENaC mediated mechanism of sodium retention.

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Abstract

Avid sodium retention drives the ~40% plasma volume expansion of normal pregnancy, which is necessary to support the growing uterus and fetus. The mechanisms responsible for this sodium retention are largely unknown. We performed large-scale proteomic analysis on late pregnant and virgin collecting duct cells to identify potential proteins or signaling pathways that can account for the sodium retention occurring in pregnancy. Large-scale liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed at the NIH which identified a total of 365 proteins in virgin and late pregnant collecting duct cells. Eighteen proteins were identified unique to late pregnancy, 21 unique to virgin controls, and 326 shared. Of the shared proteins 52 were upregulated in pregnancy, 59 downregulated, and 215 were unchanged. We identified pregnancy associated abundance changes in six proteins related to the ubiquitin/proteasome degradation pathway. Since a major mechanism of ENaC regulation is through trafficking we focused changes in this pathway and their implications for enhancing ENaC activity during pregnancy.

Introduction

Avid sodium retention drives the ~40% plasma volume expansion of normal pregnancy, which is necessary to support the growing uterus and fetus [Girling 2004]. The renal tubule protein profile revealed the only sodium transport protein upregulated at all three stages of pregnancy to be the α-subunit of the epithelial sodium channel (ENaC) [West 2010]. ENaC is located in the aldosterone sensitive distal nephron and is composed of three homologous subunits, α-, β-, and γENaC, with αENaC being the rate limiting subunit for channel formation [Loffing 2009, May 1997]. Each subunit consists of two transmembrane regions, a large extracellular domain and cytosolic N- and C-termini including proline-rich regions in each C-terminus [Staub 1997]. ENaC activity is regulated either by altering the channel open probability or the number of functional ENaC molecules in the plasma membrane. Changes in the number of channels will result from an imbalance between the synthesis and degradation of ENaC proteins [Malik 2001].

The primary aim of this study was to perform large-scale proteomic analysis on late pregnant and virgin collecting duct cells to identify potential proteins or signaling pathways that can account for the sodium retention occurring in pregnancy. Large-scale liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed at the NIH which identified a total of 365 proteins in virgin and late pregnant collecting duct cells.

Methods

Kidneys were perfused with ice cold PBS, removed rapidly, placed in holder with Tissue Freezing Medium (Triangle Biomedical Sciences Durham, NC) and stored at -80 C till sectioned. Frozen kidney sections were made (3-5 um thick). Sections were stained for Aquaporin 2 (primary antibody a kind gift from Dr. Mark Knepper) using a quick stain peroxidase protocol (Histostain Plus Broad Spectrum (DAB), Zymed Laboratories Inc). Positive labeled Aquaporin 2 principal cells were

captured by LCM and affixed to transfer film (CapSure TF-100; Arcturus Engineering Inc.) by brief laser pulses. Principal cells from 3 virgin and 3 late pregnant (20 days) rats were dissected by LCM and pooled. Total protein was extracted from samples attached to the LCM transfer film using 2M urea and 2% SDS. Samples were homogenized and solubilized in Laemmli sample buffer. A 1D protein gel was stained by gelcode blue and cut into slices. Then an in-gel protein digestion was performed using trypsin. The resulting peptides were then identified by LC-MS/MS using an LTQ-Orbitrap system. MS spectra were searched using the search algorithm, SEQUEST. Searches were conducted against the most recent Rattus norvegicus RefSeq Database (National Center for Biotechnology Information) using the target-decoy approach with filters adjusted to limit the false discovery rate to <2%. Quantification of relative abundance (area under MS1 time-course curve or extracted ion chromatogram elution profile) will be implemented using QUOIL, an in-house software program designed for quantification of label-free peptides by LC-MS.

Results & Discussion

Eighteen proteins were identified unique to late pregnancy, 21 unique to virgin controls, and 326 shared. Of the shared proteins 52 were upregulated in pregnancy, 59 downregulated, and 215 were unchanged. Pregnancy associated changes in abundance of proteins involved in steroid hormone regulation and ubiquitin/proteasome degradation are summarized in Tables 1 and 2.

Steroid Hormone Regulation

Abundance changes were identified in two proteins that are known to be involved in regulation of steroid hormones. Aldosterone sensitivity is conferred to the distal nephron by the presence of the MR and the protein 11-beta hydroxysteroid dehydrogenase type 2 (11βHSD2) [Loffing 2009]. 11βHSD2 was found to be elevated in pregnancy. This enzyme protects the MR from activation by glucocorticoids. It does so by catalyzing the conversion of cortisol to the inactive metabolite cortisone.

This protective mechanism is necessary as cortisol has equal binding affinity for the MR, yet it circulates at a much higher concentration than aldosterone. Therefore, cortisol out-competes aldosterone in cells lacking 11βHSD2. Since, late pregnancy is associated with elevated levels of cortisol, increased expression of 11βHSD2 in the collecting duct may be necessary to maintain aldosterone sensitivity [Burke 1970].

The protein 17-beta hydroxysteroid dehydrogenase type 10 (17βHSD10) was found to be decreased in pregnancy. This enzyme inactivates estradiol to estrone [Mindnich 2004]. Estrogen increases mRNA expression of αENaC and channel activity [Gambling 2004, Chang 2007]. Since, we found increases in both during pregnancy it reasons that the downregulation of this enzyme could aid in this mechanism.

Ubiquitination

A major point of regulation for ENaC is through ubiquitination. Ubiquitination of cellular proteins usually serves to tag them from rapid degradation. It involves the covalent attachment of ubiquitin or a polubiquitin tree onto lysine residues in target proteins. Several enzymes are involved in this process, including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein ligase (E3). In most studies described to date, ubiquitinated proteins are degraded by the 26S proteasome, a cytosolic threonine protease complex [Staub 1997].

We found expression changes in three proteins involved in ubiquitination, ubiquitin-like modifier activating enzyme 1 (UBA1), cullin associated neddylation dissociated 1(CAND1), and ubiquitination factor E4A (UBE4A). Attachment of ubiquitin-like proteins (UBLs) to their targets via multienzyme cascades (E1-E2-E3) is a central mechanism through which protein functions are modulated [Schulman 2009]. The ubiquitination process is initiated by a family of mechanistically and

structurally-related E1 enzymes [Schulman 2009]. UBA1 is an E1ubiquitin activating enzyme. E1 enzymes catalyze the initial step in all ubiquitin-dependent processes [Schulman 2009]. Since, UBA1 is at the apex of this cascade, downregulation of this E1 could relieve E3 ligase activation. ENaC is known to be regulated by E3 ubiquitin ligases. Thus, downregulation of UBA1 provides a potential mechanism for increased ENaC activity through channel retention in the apical membrane.

Cullin-1 has been show to interact with Rictor to form a functional E3 ubiquitin ligase. This complex causes SGK1 ubiquitination and destruction [Gao 2010a, Gao 2010b]. Futher, a recent abstract has shown that a cullin 1-dependent E3 ubiquitin ligase interacts with αENaC. Inhibition of this cullin-dependent E3 ligase activity with a chemical compound, MLN4924, results in increased ENaC activity [Kumar 2011]. We found increases in CAND1 during pregnancy. CAND1 inhibits cullin ubiquitin ligases by binding unneddylated cullins. The CAND1 N-terminus blocks the cullin neddylation site, whereas the C-terminus inhibits cullin adaptor interaction [Helmstaedt 2010]. Therefore, increases in this protein during late pregnancy could serve to increase ENaC activity by two mechanisms, preventing the ubiquitination and degradation of (1) SGK1 and (2) αENaC.

The UBE4A protein was found unique to late pregnancy. UBE4A is an E4 conjugation factor necessary for efficient multiubiquitination that is needed for proteasomal targeting. E4 binds to the ubiquitin moieties of these conjugates and, in conjunction with the E1, E2, and E3 enzymes, drives multiubiquitin chain assembly, yielding long chains [Koegl 1999]. In the absence of E4, ubiquitination of a model substrate is initiated, but only a few ubiquitin molecules are ligated to the substrate protein that are insufficient for proteasomal degradation *in vivo* [Koegl 1999]. Thus induction of UBE4A during pregnancy could serve to target ubiquitinated proteins preferentially for proteasomal degredation.

Proteasomal Degradation

Membrane proteins are frequently degraded in lysosomes but there are examples of transport proteins being degraded by the 26S ubiquitin-proteosome system. Protein degradation by the ubiquitin-proteosome pathway is initiaited when a target protein is conjugated to ubiquitin which leads to its recognition and degradation by the 26S proteosome. Conjugation of ubiquitin to the substrate protein is ATP-dependent through formation of an isopeptidic covalent linkage between the terminal glycine of ubiquitin and a lysine in the target protein. (Malik 2001)

We found decreases in three proteasome subunits (PSMA3, PSMD2, and PSME2) during pregnancy. ENaC is a short lived protein, the half-life of α and γ is ~1hr, βENaC is longer ~3hr. The half-life of α- and γENaC subunits is prolonged (2.1 and 1.7 fold respectively) when cells are treated with lactacystin, a proteosome inhibitor, thus indicating that the proteasome is involved in ENaC breakdown. (Staub 1997) Inhibition of proteosome activity increases the steady state levels of ENaC subunits and increases the number of functional ENaC molecules in the apical plasma membrane of A6 cells. Further, proteosome inhibition with MG-132 increases trans-epithelial current by 2.5 – 3-fold. (Malik 2001) Thus, downregulation of these proteasomal proteins during pregnancy could account in part for increases in ENaC activity.

Conclusion

Increased ENaC activity during pregnancy could be a result of increased channel retention in the apical membrane associated with a general downregulation of the ubiquitination/proteasomal degradation pathway.

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Table 1. Renal CD Principal Cell Proteins Increased in Pregnancy

GI #	Symbol	Entrez Gene Name	Fold Change
8393573	HSD11B2	11β hydroxysteroid dehydrogenase type 2	2.024
16758920	CAND1	cullin-associated and neddylation-dissociated 1	1.518
46485190	UBE4A	ubiquitination factor E4A	Unique LP

Table 2. Renal CD Principal Cell Proteins Decreased in Pregnancy

GI #	Symbol	Entrez Gene Name	Fold Change
13994225	HSD17B10	17β hydroxysteroid dehydrogenase type 10	−2.470
76573881	PSMA3	proteasome subunit, α type, 3	−2.964
72255509	PSMD2	proteasome 26S subunit, non-ATPase, 2	−3.458
8394091	PSME2	proteasome activator subunit 2 (PA28 β)	−2.306
62078893	UBA1	ubiquitin-like modifier activating enzyme 1	−1.365

References

1. **Burke C and Roulet F.** Increased exposure of tissues to cortisol in late pregnancy. *British Medical Journal* 1: 657-659, 1970.
2. **Chang C, Sun C, Pong C, Chen Y, Lin G, Chang T, and Wu M.** Interaction of estrogen and progesterone in the regulation of sodium channels in collecting tubular cells. *Chang Gung Med J* 30: 305-312, 2007.
3. **Gambling L, Dunford S, Wilson C, McArdle H, and Baines D.** Estrogen and progesterone regulate α, β, and γENaC subunit mRNA levels in female rat kidney. *Kidney Int* 65: 1774-1781, 2004.
4. **Gao D, Wan L, and Wei W.** Phosphorylation of Rictor at Thr1135 impairs the Rictor/Cullin-1 complex to ubiquitinate SGK1. *Protein Cell* 1: 881-885, 2010.
5. **Gao D, Wan L, Inuzuka H, Berg A, Tseng A, Zhai B, Shaik S, Bennett E, Tron A, Gasser J, Lau A, Gygi S, Harper J, DeCaprio J, Toker A, and Wei W.** Rictor forms a complex with Cullin-1 to promote SGK1 ubiquitination and destruction. *Molecular Cell* 39: 797-808, 2010.
6. **Girling J.** Physiology of pregnancy. *Anesthesia and Intensive Care Medicine* 215-218, 2004.
7. **Helmstaedt K, Schwier K, Christmann M, Nahlik K, Westermann M, Harting R, Grond S, Busch S, and Braus G.** Recruitment of the inhibitor Cand1 to the cullin substrate adaptor site mediates interaction to the neddylation site. *Molecular Biology of the Cell* 22: 153-164, 2011.
8. **Koegl M, Hoppe T, Schlenker S, Ulrich H, Mayer T, and Jentsch S.** A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* 96: 635-644, 1999.
9. **Kumar A, Goodson P and Helms M.** ROS regulates surface expression of epithelial sodium channel via attenuation of cullin-dependent signaling. *FASEB J.* 25: 1039.36, 2011.
10. **Loffing J and Korbmacher C.** Regulated sodium trasportt in the renal connecting tubule (CNT) via the epithelail sodium channel (ENaC). *Eur J PPhysiol* 458: 111-135, 2009.
11. **Malik B, Schlanger L, Al-Khalili O, Bao H, Yue G, Price S, Mitch W, and Eaton D.** ENaC degradation in A6 cells by the ubiquitin-proteosome proteolytic pathway. *J Biol Chem* 276: 12903-12910, 2001.
12. **May A, Puoti A, Gaeggeler HP, Horisberger JD and Rossier BC**. Early effect of aldosterone on the rate of synthesis of the epithelial sodium channel alpha subunit in A6 renal cells. *J Am Soc Nephrol* 8: 1813-1822, 1997.
13. **Mindnich R, Moller G, and Adamski J.** The role of 17 beta-hydroxysteroid dehydrogenases. *Molecular and Cellular Endocrinology* 218: 7-20, 2004.
14. **Schulman B and Harper J.** Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signaling pathways. *Nat Rev Mol Cell Biol* 10: 319-331, 2009.
15. **Staub O, Gautschi I, Ishikawa T, Breitschopf K, Ciechanover A, Schild L and Rotin D.** Regulation of stability and function of the epithelial Na+ channel (ENaC) by ubiquitination. *EMBO J* 16: 6325-6336, 1997.
16. **West C, Zhang Z, Ecker G, and Masilamani S**. Increased Renal α Epithelial Sodium Channel (ENaC) Protein and Increased ENaC Activity in Normal Pregnancy. *Am J Physiol Regul Integr Comp Physiol* 299: R1326-32, 2010.

VI:

Chronic Vasodilation Mimics Renal Sodium Transporter Profile Seen In Pregnancy.

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Abstract

A healthy pregnancy is associated with marked plasma volume expansion (PVE) mediated primarily by Na and water retention. This study tests the Underfill Hypothesis of pregnancy which proposes systemic vasodilation initiates PVE. Chronic vasodilation in virgin rats was induced by 14 days administration of NaNO2 or nifedipine (NIF) that led to sustained falls in BP of 4-6 mmHg. We performed a Na transporter profile in kidney cortex homogenates. The protein abundance of the Na-H-exchanger type 3 (NHE3) (219±21% and 333±11% of control respectively, $p<0.05$), Na-K-2Cl cotransporter (NKCC2) (160±5% and 167±11% of control respectively, $p<0.05$), and the α subunit of the epithelial Na channel (ENaC) (204+/-13% and 203+/-11% of control respectively) increased with NaNO2 and NIF. No change was seen in the Na-2Cl cotransporter (NCC), β ENaC or γ ENaC. We also noted an increase in the abundance of aquaporin 2 water channel (AQP2) (128+/-4% and 141+/-9% of control respectively).

We previously conducted Na transporter profiles of whole kidney homogenates in early (day 6-9), mid (day 13-15) and late (day 18-20) pregnant rats. Chronic vasodilation mimics the profiles seen in mid and late pregnancy where only α ENaC was upregulated (167+/-29% and 168+/-17% of control respectively). Others have shown increases in AQP2 in pregnancy. This suggests that 14 days chronic vasodilation mimics renal collecting duct adaptations, which enhance Na and water retention, seen in mid and late pregnancy thus supporting the Underfill Hypothesis of pregnancy.

Introduction

A healthy pregnancy requires several cardiovascular and renal hemodynamic adaptations. These changes include decreases in total peripheral resistance and blood pressure, as well as increases in cardiac output and glomerular filtration rate (GFR) [Chapman 1998]. These modifications support avid sodium and water reabsorption necessary for the ~40% increase in plasma volume [Girling 2004]. However, the mechanisms allowing sodium retention during pregnancy are unclear. The arterial underfill hypothesis was put forward to explain the modifications of pregnancy. This hypothesis proposes a primary enlargement of the vascular compartment, initially due to vasodilation and later supported by opening of the uterine-placental circulation, with a secondary increase in sodium and water reabsorption [Schrier 1987, Schrier 2010]. Schrier first suggested pregnancy as a state of arterial underfilling over 20 years ago, since then there have been several lines of support for this hypothesis.

The early peripheral vasodilation, lower systemic blood pressure, increased activation of the renin-angiotensin-aldosterone system, non-osmotic release of vasopressin, thirst stimulation, and hyponatremia all favor the arterial underfill hypothesis [Schrier 1987]. An early point of contention in this model was the 30-50% increase in GFR during pregnancy, as all other states of primary peripheral vasodilation are characterized by normal or decreased GFR [Schrier 1991]. It was later found that chronic nitric oxide synthase (NOS) inhibition in pregnant rats reverses systemic vasodilation and decreases cardiac output, glomerular filtration rate and renal plasma flow to non-pregnant levels [Schrier 2001]. This study supported a role for NO mediating the peripheral vasodilation and indicated that renal vasodilation could account for the increased renal plasma flow and GFR during pregnancy.

A recent study involving chronic vasodilation in virgin female rats provided further support for pregnancy as a state of arterial underfilling. Chronic vasodilation with either sodium nitrite (NO-donor) or nifedipine (calcium channel blocker) resulted in plasma volume expansion, hemodilution,

decreased plasma osmolarity, and reduced plasma sodium [Baylis 2011]. All of which were consistent with hemodynamic changes associated with pregnancy. This same study also observed increased expression of medullary phosphodiesterase 5 (PDE5), similar to pregnancy [Baylis 2011]. PDE5 is an enzyme that degrades cGMP, the second messenger of both ANP and NO, thereby protecting the inner medulla from their natriuretic effects [Sasser 2010, Ni 2004]. Another modification of the collecting duct during pregnancy is the increased abundance of the α-subunit of the epithelial sodium channel (ENaC). We found that αENaC, the rate-limiting subunit for channel formation, was the only sodium transport protein consistently upregulated throughout pregnancy and this increase corresponded to elevated *in vivo* channel activity [West 2010, May 1997]. Since, modifications are known to occur in the collecting duct during pregnancy and chronic vasodilation alone was enough to mimic pregnancy mediated changes in PDE5 expression, the primary aim of this work was to further test the underfill hypothesis by determining whether chronic vasodilation with nifedipine or sodium nitrite result in collecting duct protein profile changes similar to pregnancy.

Methods

Animal Experiments. Animal experiments were carried out at the University of Florida in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and in agreement with the University of Florida Institutional Animal Care and Use Committee; for detailed method see reference [Baylis 2011]. In brief, chronic vasodilation was produced by treating female Sprague-Dawley rats (Harlan Laboratories, Dublin, OH) for 14 days with either nifedipine (NIF; calcium channel blocker, 10 $mg \cdot kg^{-1} \cdot day^{-1}$ via diet; Sigma) or sodium nitrite (NaNO2; 70 $mg \cdot kg^{-1} \cdot day^{-1}$ via drinking water; Sigma). Rats were given access to a gelled diet containing water (52.3%), agar (1.1%) (Becton Dickenson), and normal rat chow (46.5%) (Harlan). All rats received water *ad libitum* in addition to the water in the gelled diet. On day 14, rats were euthanized and kidneys dissected, frozen in liquid nitrogen, and stored at -80C.

Western Blotting. SDS-PAGE was carried out using Criterion precast 10% Tris-HCl gels (Bio-Rad Laboratories, Inc., Hercules, CA). Equal loading of the gel was confirmed through staining with GelCode Blue Reagent (Pierce Biotechnology, Rockford, IL) (Musselman 2010). Cortical kidney homogenates were used for semiquantitative immunoblotting. Samples were loaded in lanes of Criterion precast 10% Tris-HCl gels. The proteins were transferred electrophoretically from unstained gels to nitrocellulose membranes. The blots were blocked for 2 hours with 5 g/dl blotting grade non-fat dry milk (Bio-Rad Laboratories, Hercules, CA) and probed with primary antibody over night. The next day blots were incubated for 1 hour with peroxidase-conjugated secondary antibodies (Pierce Biotechnology, Rockford, IL, no. 31458 & 31452), followed by Western Lightning Chemilumnescence Reagent Plus (Perkin Elmer LAS, Inc., Boston, MA). Blots were exposed to X-ray film and band densities were quantified by VersaDoc Imaging System and Quantity One Analysis Software (Bio-Rad Laboratories, Hercules, CA). Densitometry values were normalized defining the mean for the virgin control group as 100%.

We used the following previously characterized rabbit polyclonal antibodies (a kind gift from Mark Knepper, NIH/LKEM Bethesda MD) summarized in the following. 1) The Na-H exchanger type 3 (NHE3) (1:1000 dilution) (Fernandez 1998). 2) The bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2) (1:1000 dilution) (Kim 1999). 3) The thiazide-sensitive Na-Cl cotransporter (NCC) (1:1000 dilution) (Kim 1998). 4) The aquaporin 2 water channel (AQP2) (1:1000 dilution) [Nielsen 1995]. We used our own rabbit polyclonal antibodies against the three subunits of the amiloride-sensitive epithelial sodium channel (α, β, and γ subunits of ENaC) (1:1000 dilution) using the sequence and protocol previously described [Masilamani 1999].

Statistical Analysis. Data are given as mean ± standard error (SE). SPSS statistical program was used for all analysis (SPSS 12.0, Chicago, Illinois). A one-way analysis of variance (ANOVA) with Bonferroni post hoc was performed. The null hypothesis was rejected at $p<0.05$

Results

Abundance of NHE3 is increased in chronically vasodilated rats. The changes in abundance of the proximal tubule sodium transporter NHE3 were analyzed in control females, rats treated with NIF, and rats treated with NaNO2. As shown in Figure 1 and Table 1, densitometric analysis revealed a marked increase in cortical NHE3 abundance with NaNO2 and NIF (219±21% and 333±11% of control respectively, $p<0.05$).

Abundance of NKCC2 is increased in chronically vasodilated rats. Semiquantitative immunolotting from control and chronically vasodilated rats revealed changes in expression of the thick ascending limb sodium transporter NKCC2. As shown in Figure 2 and Table 1 the protein abundance of NKCC2 increased in both rats treated with NaNO2 and NIF (160±5% and 167±11% of control respectively, $p<0.05$).

Increased αENaC and unchanged NCC expression in response to chronic vasodilation. The changes in abundance of the distal convoluted tubule sodium transporter NCC and the three subunits of the collecting duct sodium channel ENaC were analyzed in control females, rats treated with NIF, and rats treated with NaNO2. As shown in Figure 3 and Table 1, the protein abundance of αENaC increased with NaNO2 and NIF (204±13% and 202±11% of control respectively, $p<0.05$). Since the α subunit of ENaC is the rate-limiting subunit for channel formation [May 1997], this is consistent with an increased activity of ENaC due to an increase in channel number. There were no differences seen with

chronic vasodilation in the protein abundance of NCC, βENaC, or γENaC (85 kDa or the cleaved 70 kDa form).

AQP2 abundance is increased in nifedipine treated rats. The anti-AQP2 antibody recognizes the nonglycosylated (25 kDa) and the glycosylated (37 to 45 kDa) bands of AQP2. As shown in Figure 4 and Table 1, semiquantitative immunolotting from control females, rats treated with NIF, and rats treated with NaNO2 revealed an increase in the glycosylated band of AQP2 in NIF rats only (141±9 % control, $p<0.05$). No changes were observed with chronic vasodilation in the nonglycosylated band.

Discussion

The primary finding from this study is that the transporter profile in the distal nephron of rats treated with nifedipine or sodium nitrite is similar to that found in pregnant rats. As such this study provides further support for pregnancy as a state of arterial underfilling. Specifically, we found increased abundance of αENaC and AQP2 with no change in NCC, βENaC, or γENaC. This profile matches closely with that previously reported for mid and late pregnancy [Schrier 1998, West 2010]. NO is a known inhibitor of ENaC and PDE5 is elevated in the collecting duct, it reasons that PDE5 may be supporting sodium retention by protecting ENaC from NO during pregnancy. The increased abundance of αENaC and AQP2 promotes sodium and water retention in the collecting duct. During pregnancy the plasma volume is expanded by ~40%, likewise, female rats chronically vasodilated for 14 days were found to have expanded plasma volume, similar to that observed in pregnancy [Baylis 2011]. This suggests that 14 days chronic vasodilation mimics renal collecting duct adaptations, which enhance Na and water retention, seen in mid and late pregnancy thus supporting the underfill hypothesis of pregnancy.

Differences do exist between the protein profiles of normal pregnant and chronically vasodilated rats in the premacula densa segments of the nephron. Chronic vasodilation increased abundance of NHE3 and NKCC2 protein, whereas these transporters are unchanged during mid and late pregnancy. NO has been indicated in increasing NHE3 and NKCC2 abundance [Turban 2003]. $NaNO_2$ is a NO-donor and nifedipine is known to modulate endothelial NO availability by increasing eNOS activity and decreasing reactive oxygen species [Baumer 2007, Berkels 2001]. Although pregnancy is also associated with elevated NO, it is likely that the pharmacologic intervention ($NaNO_2$, nifedipine) produces much higher levels than seen in the physiologic state of pregnancy.

Others have observed a different renal profile after 19 days of nifedipine treatment [Wang 2001]. This study found no change in NKCC2, increased NCC abundance, and decreased NHE3 and AQP2. Differences may be resolved by protocol as they used a much higher dose of nifedipine (700mg/kg), a longer time course, and a different strain of rat than the present study.

In conclusion, the findings from the present study taken together with the previous report that chronic vasodilation induced collecting duct PDE5, emphasizes the importance of the collecting duct in contributing to the plasma volume expansion required for a healthy pregnancy.

Figure 1.

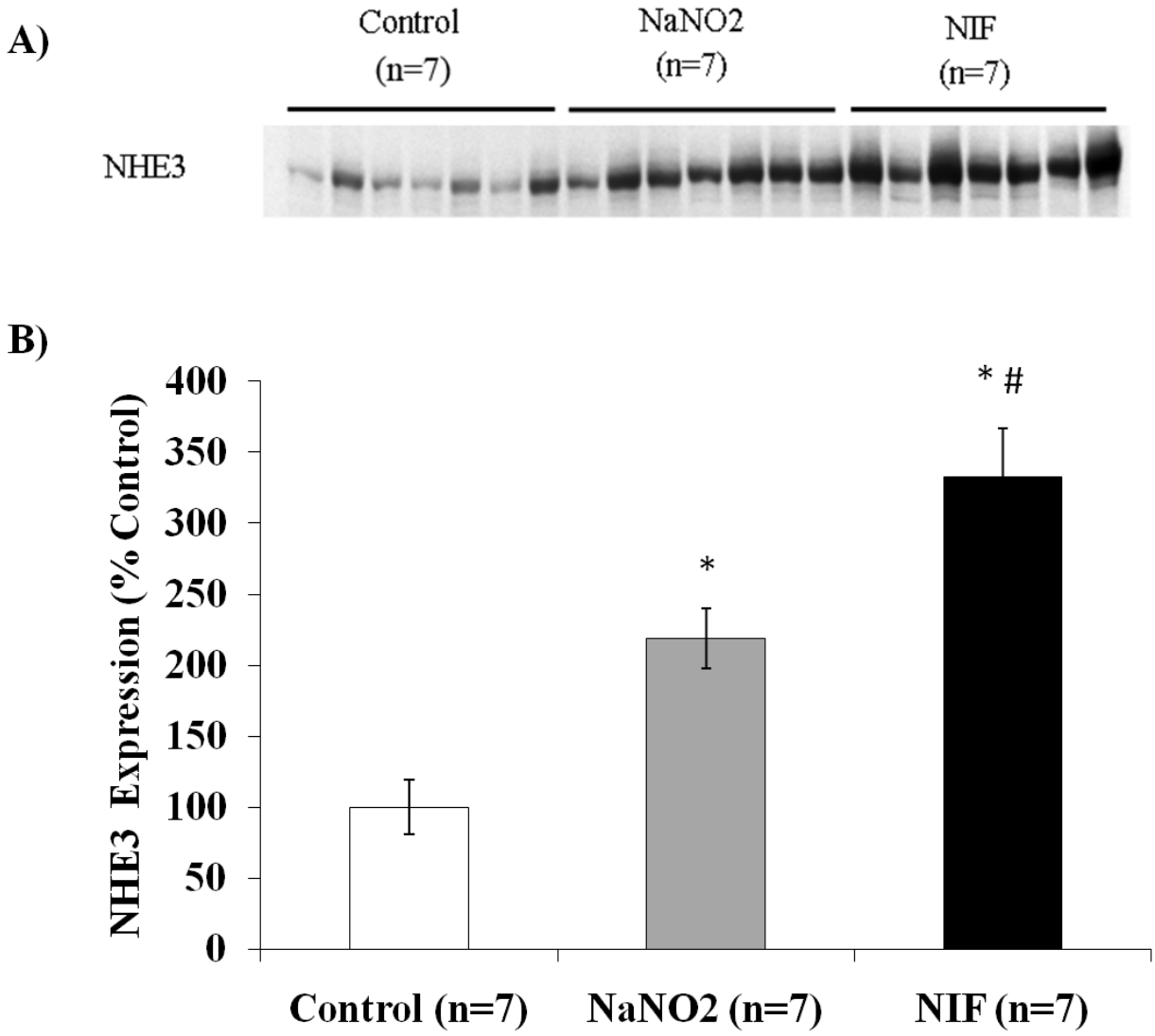

Figure 1: Semiquantitative immunoblots probed for the Na-H exchanger type 3 (NHE3). A) Immunoblots compare cortical kidney homogenates from seven control, seven nifedipine treated, and seven sodium nitrite treated rats. B) Band densities were normalized to virgin female controls with controls set at 100% and summarized as bar graphs. An ANOVA with bonferroni post-hoc was performed. Mean ± standard error (SE), * vs control, # vs. NaNO2, $p<0.05$.

Figure 2.

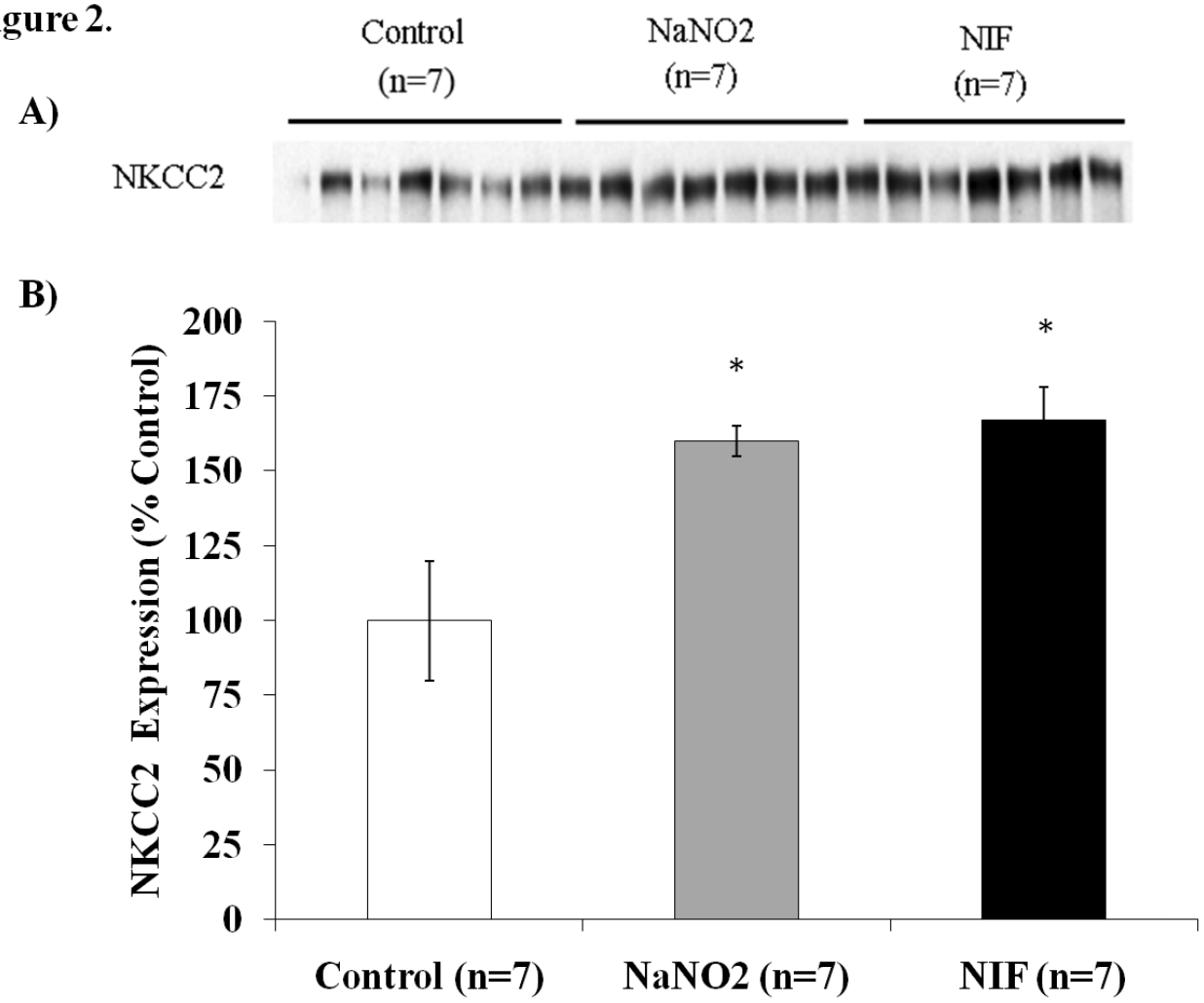

Figure 2: Semiquantitative immunoblots probed for the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2). A) Immunoblots compare cortical kidney homogenates from seven control, seven nifedipine treated, and seven sodium nitrite treated rats. B) Band densities were normalized to virgin female controls with controls set at 100% and summarized as bar graphs. An ANOVA with bonferroni post-hoc was performed. Mean ± standard error (SE), * vs control, $p<0.05$.

Figure 3.

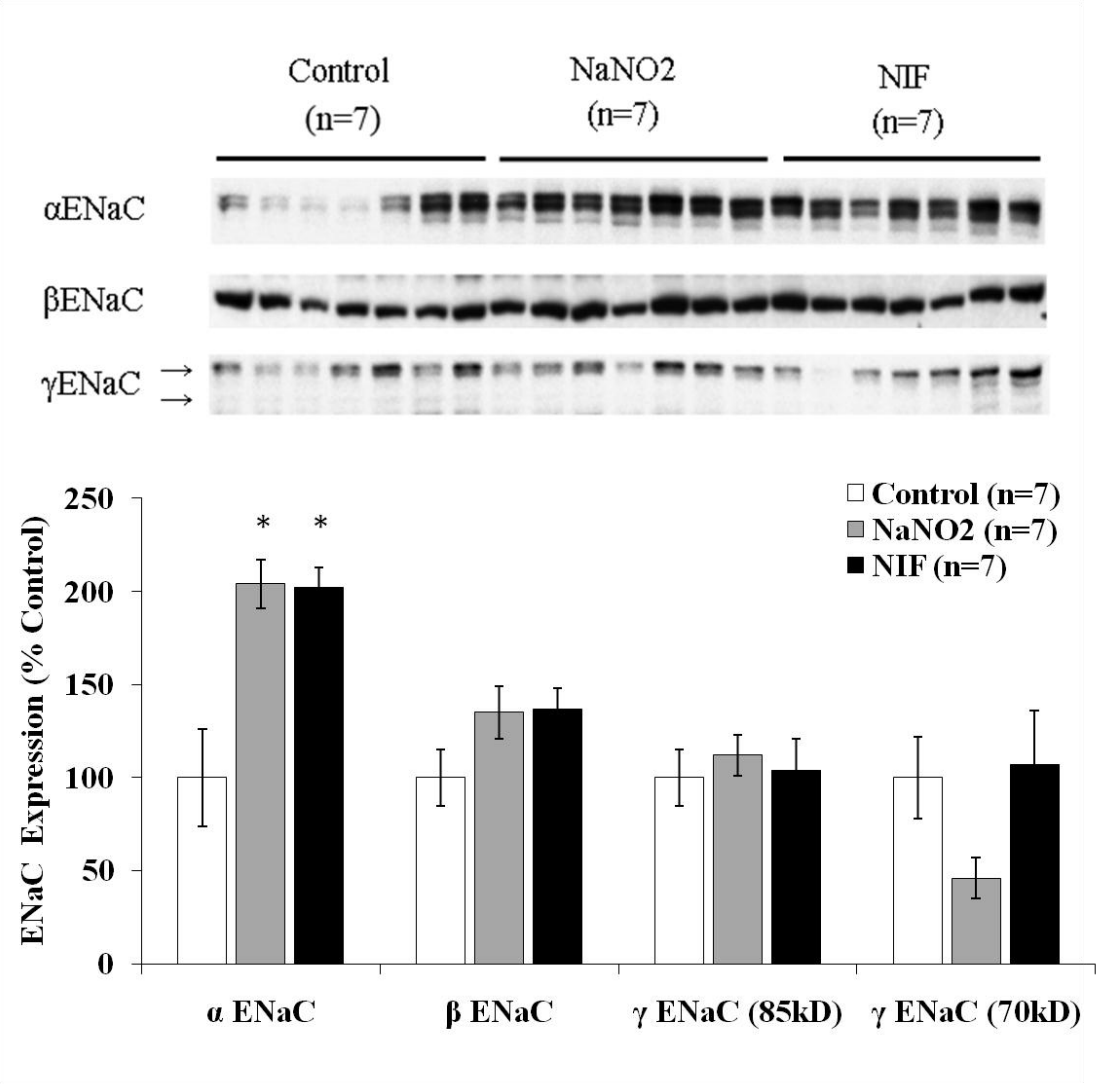

Figure 3: Semiquantitative immunoblots probed for the three subunits of ENaC (α, β, and γ). A) Immunoblots compare cortical kidney homogenates from seven control, seven nifedipine treated, and seven sodium nitrite treated rats. B) Band densities were normalized to virgin female controls with controls set at 100% and summarized as bar graphs. An ANOVA with bonferroni post-hoc was performed. Mean ± standard error (SE), * vs control, $p<0.05$.

Figure 4.

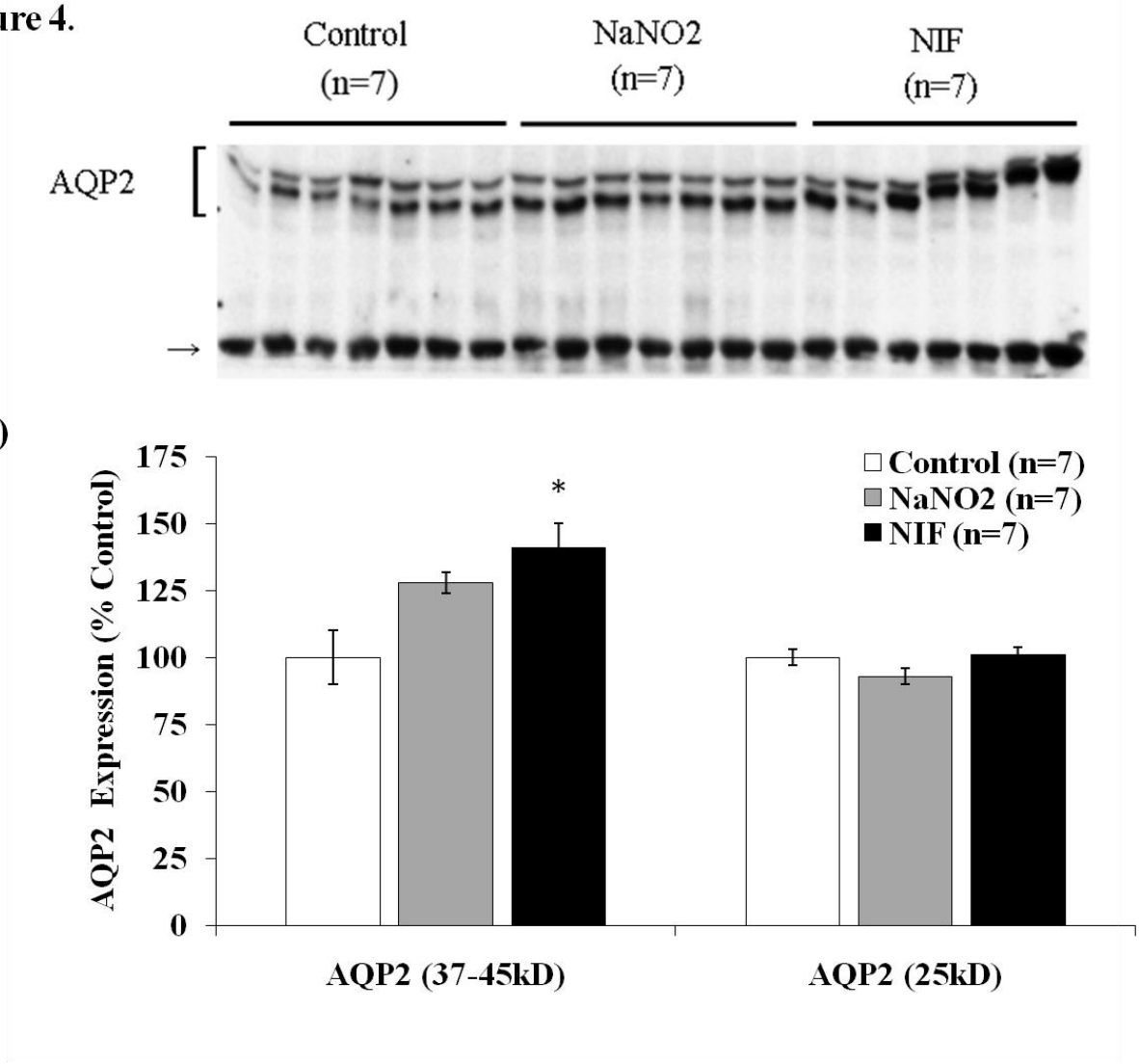

Figure 4: Semiquantitative immunoblots probed for the water channel aquapourin 2 (AQP2). A) Immunoblots compare cortical kidney homogenates from seven control, seven nifedipine treated, and seven sodium nitrite treated rats. B) Band densities were normalized to virgin female controls with controls set at 100% and summarized as bar graphs. An ANOVA with bonferroni post-hoc was performed. Mean ± standard error (SE), * vs control, $p<0.05$.

Table 1. Protein Abundance of Renal Sodium and Water Transporters

	Control	NaNO2	NIF
NHE3	**100 ± 19**	**219 ± 21 ***	**333 ± 34 *#**
NKCC2	**100 ± 20**	**160 ± 5 ***	**167 ± 11 ***
NCC	**100 ± 7**	**87 ± 4**	**84 ± 9**
αENaC	**100 ± 26**	**204 ± 13 ***	**202 ± 11 ***
βENaC	**100 ± 15**	**135 ± 14**	**137 ± 11**
γ ENaC (85kD)	**100 ± 15**	**112 ± 11**	**104 ± 17**
γ ENaC (70kD)	**100 ± 22**	**46 ± 11**	**107 ± 29**
AQP2 glycosylated (37-45kD)	**100 ± 10**	**128 ± 4**	**141 ± 9 ***
AQP2 (25kD)	**100 ± 3**	**93 ± 3**	**101 ± 3**

Values are means ± SE from nifedipine (NIF) treated, sodium nitrite (NaNO2) treated and control rats (n=7 for all groups). Type 3 sodium-hydrogen exchanger (NHE3); bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2); thiazide-sensitive Na-Cl cotransporter (NCC); three subunits of the amiloride sensitive epithelial sodium channel (α-, β-, γ-, ENaC); aquaporin 2 water channel (AQP2). Band densities were normalized to virgin female controls with controls set at 100%. * vs control, # vs. NaNO2, $p<0.05$.

References

1. **Baumer A, Kruger C, Falkenberg J, Freyhaus H, Rosen R, Fink K, and Rosenkranz S**. The NAD(P)H oxidase inhibitor apocynin improves endothelial NO/superoxide balance and lowers effectively blood pressure in spontaneously hypertensive rats: comparison to calcium channel blockade. *Clinical and Experimental Hypertension*, 29:287–299, 2007
2. **Berkels R, Egink G, Marsen T, Bartels H, Roesen R, and Klaus W**. Nifedipine increases endothelial nitric oxide bioavailability by antioxidative mechanisms. *Hypertension* 37: 240-245, 2001.
3. **Cadnapaphornchai M, Ohara M, Morris K, Knotek M, Rogachev B, Ladtkow T, Carter E, and Schrier R.** Chronic NOS inhibition reverses systemic vasodilation and glomerular hyperfiltration in pregnancy. *Am J Physiol Renal Physiol* 280: F592–F598, 2001.
4. **Chapman A, Abraham W, Zamudio S, Coffin C, Merouani A, Young D, Johnson A, Osorio F, Goldberg C, Moore L, Dahms T, and Schrier R.** Temporal relationships between hormonal and hemodynamic changes in early human pregnancy. *Kidney Int* 54: 2056-2063, 1998.
5. **Fekete A, Sasser J, and Baylis C.** Chronic vasodilation produces plasma volume expansion and hemodilution in rats: consequences of decreased effective arterial blood volume. *Am J Physiol Renal Physiol* 300: F113–F118, 2011.
6. **Fernandez-Llama P, Andrews P, Ecelbarger CA, Nielsen S and Knepper M**. Concentrating defect in experimental nephrotic syndrone: altered expression of aquaporins and thick ascending limb Na+ transporters. *Kidney Int* 54: 170-179, 1998.
7. **Girling JC.** Physiology of pregnancy. *Anesthesia and Intensive Care Medicine* 215-218, 2004.
8. **Kim GH, Ecelbarger CA, Mitchell C, Packer RK, Wade JB and Knepper MA**. Vasopressin increases Na-K-2Cl cotransporter expression in thick ascending limb of Henle's loop. *Am J Physiol* 276: F96-F103, 1999.
9. **Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB and Knepper MA**. The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. *Proc Natl Acad Sci U S A* 95: 14552-14557, 1998.
10. **Masilamani S, Kim GH, Mitchell C, Wade JB and Knepper MA**. Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. *J Clin Invest* 104: R19-R23, 1999.
11. **May A, Puoti A, Gaeggeler HP, Horisberger JD, and Rossier BC**. Early effect of aldosterone on the rate of synthesis of the epithelial sodium channel alpha subunit in A6 renal cells. *J Am Soc Nephrol* 8:1813-1822, 1997.
12. **Musselman TM, Zhang Z, and Masilamani SM.** Differential regulation of the bumetanide-sensitive cotransporter (NKCC2) by ovarian hormones. *Steroids* 75: 760-765, 2010.
13. **Ni X, Safai M, Rishi R, Baylis C, and Humphreys M**. Increased activity of cGMP-specific phosphodiesterase (PDE5) contributes to resistance to atrial natriuretic peptide natriuresis in the pregnant rat. *J Am Soc Nephrol* 15: 1254-1260, 2004.
14. **Nielsen S, Chou CL, Marples D, Christensen EI, Kishore BK, and Knepper MA.** Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. *Proc Natl Acad Sci USA* 92: 1013–1017, 1995.
15. **Ohara M, Martin P, Xu D, St. John J, Pattison T, Kim J, and Schrier R.** Upregulation of aquaporin 2 water channel expression in pregnant rats. *J Clin Invest* 101: 1076-1083, 1998.
16. **Sasser J, Ni X, Humphreys M, and Baylis C.** Increased renal phosphodiesterase-5 activity mediates the blunted natriuretic response to a nitric oxide donor in the pregnant rat. *Am J Physiol Renal Physiol* 299: F810-F814, 2010.
17. **Schrier R.** Systemic arterial vasodilation, vasopressin, and vasopressinase in pregnancy. *J Am Soc Nephrol* 21: 570-572, 2010.

18. **Schrier R and Briner V.** Peripheral arterial vasodilation hypothesis of sodium and water retention in pregnancy: Implications for pathogensis of preeclampsia-eclampsia. *Obstet Gynecol* 77: 632-639, 1991.
19. **Schrier R and Durr J.** Pregnancy: an overfill or underfill state. *Am J Kidney Dis* 9: 284-289, 1987.
20. **Turban S, Wang X, and Knepper MA.** Regulation of NHE3, NKCC2, and NCC abundance in kidney during aldosterone escape phenomenon: role of NO. *Am J Physiol Renal Physiol* 285: F843-F851, 2003.
21. **Wang W, Kwon T, Li C, Flyvbjerg A, Knepper M, Frokiaer J, and Nielsen S.** Altered expression of renal aquaporins and Na^+ transporters in rats treated with L-type calcium blocker. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R1632–R1641, 2001.
22. **West C, Zhang Z, Ecker G, and Masilamani S**. Increased Renal α Epithelial Sodium Channel (ENaC) Protein and Increased ENaC Activity in Normal Pregnancy. *Am J Physiol Regul Integr Comp Physiol* 299: R1326-32, 2010.

VII: Discussion

During pregnancy, renal sodium and water retention produces a 30– 50% expansion of extracellular fluid. The renal water retention is slightly greater than the sodium retention in pregnancy, resulting in decreased plasma sodium and osmolality. Plasma volume expansion is necessary for the adequate perfusion of the uterus and fetus. Failure of volume expansion results in poor pregnancy outcomes. Several pregnancy-mediated modifications of the collecting duct promote the accumulation of sodium and water. This review will examine the known adaptations.

Aquaporin-2 (AQP2)

The AQP2 water channel is located in the principal cells of the renal collecting duct, the site of vasopressin-mediated water reabsorption. The systemic arterial vasodilation of pregnancy is associated with thirst stimulation and nonosmotic release of arginine vasopressin (AVP). This results in a decrease in plasma osmolality by approximately10 mOsm/kg [Schrier 2010]. The hypotonicity seen in pregnant rats, which is similar to that observed in human pregnancy, is associated with increased plasma and hypothalamic vasopressin. In spite of the presence of hypoosmolality plasma AVP is not suppressed and the renin-angiotensin-aldosterone axis is activated early in pregnancy [Schrier 1991]. AQP2 expression is increased by day 7 of rat gestation and remains elevated through gestation. Also trafficking of this water channel to the apical membrane is increased. These effects on AQP2 during pregnancy are reversed by aV2 vasopressin receptor antagonist [Schrier 1998].

The binding of AVP to its V2 receptor on the basolateral membrane of the collecting duct produces a short-term translocation of AQP2 from cytosolic storage vesicles to the apical membrane through cAMP-mediated pathway. This trafficking process increases the water permeability of the apical membrane of the collecting duct cells, thereby promoting water retention. Long-term AVP controls AQP2 gene expression through a cAMP response element on the AQP2 promoter [Schrier 1998].

Such regulation determines AQP2 channel abundance. Moreover, urinary AQP2 has been shown to increase in human pregnancy as compared to the nonpregnant state. Thus, the modest lowering of the osmotic threshold for plasma vasopressin in pregnancy seems most likely to be secondary to systemic arterial vasodilation, leading to the nonosmotic stimulation of vasopressin and upregulation of AQP2, and promoting increased water retention [Schrier 1998, Schrier 2010].

Epithelial Sodium Channel (ENaC)

ENaC is composed of three homologous subunits (α-, β-, γ-) and is located in the aldosterone sensitive distal nephron (late distal convoluted tubule, connecting tubule, collecting duct). Although, ENaC is only responsible for a small portion of total sodium reabsorption, it is the primary determinate of net balance, as it is highly regulated by the renin-angiotensin-aldosterone system [Lifton 2001]. The α-subunit of ENaC is the only sodium transport protein consistently upregulated throughout gestation. The increased protein expression was found to be associated with increased *in vivo* channel activity, via a mineralocorticoid receptor (MR)-mediated mechanism. Aldosterone is known to selectively increase α ENaC transcription [Masilamani 1999]. Although, individual ENaC subunits can traffic from the ER to the cell surface, this process is inefficient. Efficient trafficking of ENaC out of the ER requires the assembly of α-, β-, and γ-subunits into a complex [Snyder 2005]. Under basal conditions in the kidney, when the rate of sodium transport is low, αENaC is transcribed to a lesser extent than β- or γENaC. Excess β - and γ-subunits are targeted for degradation in the proteasome [Snyder 2005]. Thus, ENaC trafficking out of the ER is limited by the number of available α -subunits. This bottleneck forms an important site for ENaC regulation [Snyder 2005]. Both the selective upregulation of α ENaC and the reduced *in vivo* activity with eplerenone treatment supports an aldosterone-mediated mechanism for increased ENaC expression and activity during pregnancy. Aldosterone is elevated throughout pregnancy and is decreased in preeclampsia, a volume depleted state.

Further, we have provided evidence that ENaC activity is important in mediating the positive sodium balance of pregnancy, plasma volume expansion, and blood pressure maintenance. Chronic ENaC inhibition studies revealed ENaC activity was necessary for positive sodium balance characteristic of pregnancy. These animals had greater reductions in blood pressure and serum sodium than normal pregnant animals. Inhibition of angiotensin II as well as low sodium diets during pregnancy also results in reduced plasma volume expansions and lower blood pressures. This implicates ENaC as an important target of RAAS mediated sodium retention during pregnancy.

11-β-Hydroxysteroid dehydrogenase type 2 (11βHSD2)

Aldosterone sensitivity is conferred to the distal nephron by the presence of the MR and the enzyme11βHSD2 [Loffing 2009]. 11βHSD2 was found to be elevated in principle cells during late pregnancy. This enzyme protects the MR from activation by glucocorticoids. It does so by catalyzing the conversion of cortisol to the inactive metabolite cortisone. This protective mechanism is necessary as cortisol has equal binding affinity for the MR, yet it circulates at a much higher concentration than aldosterone. Therefore, cortisol out-competes aldosterone in cells lacking 11βHSD2. Since, late pregnancy is associated with elevated levels of cortisol, increased expression of 11βHSD2 may be necessary for maintenance of aldosterone sensitivity in the collecting duct [Burke 1970].

Nitric Oxide Syntahse (NOS) & Phosphodiesterase-5 (PDE5)

Nitric oxide (NO) is a powerful vasodilator and natriuretic. NO is synthesized from L-argenine by NOS. NO acts as a signaling molecule to activate guanylyl cyclase to produce cGMP and activate protein kinase G. No changes were observed in endothelial NOS (eNOS) or neuronal NOS (nNOS) protein abundance in the kidney medulla during mid or late pregnancy in the rat [Smith 2010]. No change in NOS activity, determined by conversion of L-arginine to L-citrulline in the soluble fraction

of the medulla [Smith 2010]. Cortical NOS activity and nNOS expression were elevated in mid pregnant rats, when GFR is maximal, but by late pregnancy had returned to virgin levels [Smith 2010].

PDE5 is an enzyme that degrades cGMP, the second messenger of both ANP and NO, thereby protecting the inner medulla from their natriuretic effects [Sasser 2010, Ni 2004]. Inner medullary PDE5 expression is increased in pregnancy. Differential expression and regulation of nNOS and PDE5 during pregnancy could allow the cortex to vasodilate to NO while protecting the collecting duct from natiuresis [Fekete 2011]. Also, NO via cGMP and PKG is an inhibitor of ENaC activity, thus PDE5 could also be protecting ENaC mediated sodium retention.

Conclusion

Elevated levels of AVP and aldosterone during pregnancy induce increases in AQP2 and ENaC in the collecting duct, for the common goal of sodium and water retention. Protection of this mechanism is conferred in part by increased expression of 11βHSD2 and PDE5. PDE5 guards against high circulating levels of NO during pregnancy. It also contributes to the kidney being refractory to ANP. Thus the coordination of these factors aids sodium and water retention in the collecting duct during pregnancy. Taken together, this project supports an important role for the collecting duct (in general) and ENaC (in particular) in the pregnancy mediated sodium and fluid reabsorption during pregnancy.

VIII: References

1. Abreu N, Tardin JC, Boim MA, Campos RR, Bergamaschi CT, and Schor N. Hemodynamic parameters during normal and hypertensive pregnancy in rats: evaluation of renal salt and water transporters. *Hypertens Pregnancy* 27:49-63, 2008.
2. Alexander EA, Churchill S and Bengele HH. Renal hemodynamics and volume homeostasis during pregnancy in the rat. *Kidney Int* 18: 173-178, 1980.
3. Atherton JC, Bielinska A, Davison JM, Haddon I, Kay C, and Samuels R. Sodium and water reabsorption in the proximal and distal nephron in conscious pregnant rats and third trimester women. *J Physiol* 396: 457-470, 1988.
4. Atherton J, Bu'lock D, and Pirie S. The effect of pseudopregnancy on glomerular filtration rate and salt and water reabsorption in the rat. *J Physiol* 324: 11-20, 1982.
5. Atherton JC and Pirie SC. The effect of pregnancy on glomerular filtration rate and salt and water reabsorption in the rat. *J Physiol* 319:153-164, 1981.
6. Arthur SK, Green R. Fluid reabsorption by the proximal convoluted tubule of the kidney in lactating rats. *J Physiol*. 371:267-75, 1986.
7. Baylis C. Glomerular ultrafiltration in the pseudopregnant rat. *Am J Physiol* 243: F300-F305, 1982.
8. Baylis C and Collins RC. Angiotensin II inhibition on blood pressure and renal hemodynamics in pregnant rats. *Am J Physiol* 250: F308–F314, 1986.
9. Beutler KT, Masilamani S, Turban S, Nielsen J, Brooks HL, Ageloff S, Fenton RA, Packer RK and Knepper MA. Long-term regulation of ENaC expression in kidney by angiotensin II. *Hypertension* 41: 1143-1150, 2003.
10. Boemke W, Palm U, Corea M, Seelinger E, and Reinhardt W. Endogenous variations and sodium intake-dependent components of diurnal sodium excretion patterns in dogs. *Journal of Physiology* 476: 547-552, 1994.
11. Butterworth MB, Edinger RS, Frizzell RA and Johnson JP. Regulation of the epithelial sodium channel by membrane trafficking. *Am J Physiol Renal Physiol* 296: F10-F24, 2009.
12. Castrop H, Hocherl K, Kurtz A, Schweda F, Todorov V, and Wagner C. Physiology of kidney renin. *Physiol Rev* 90: 607-673, 2010.
13. Cha SC, Aberdeen GW, Mukaddam-Daher S, Quillen EW, Nuwayhid BS. Tubular handling of fluid and electrolytes during ovine pregnancy. *Am J Physiol* 265: F278-F284, 1993.
14. Chang CT, Sun CY, Pong CY, Chen YC, Lin GP, Chang TC, and Wu MS. Interaction of estrogen and progesterone in the regulation of sodium channels in collecting tubular cells. *Chang Gung Med J* 30:305-12, 2007
15. Chiou W, Shiosaki K, Tasker A, and Wu-Wong J. Characterization of two endothelin converting enzymes and their preference for big endothelin-1 and -2 as substrates. *Life Sci* 54: 1613-1619, 1994.
16. Churchill SE, Bengele HH, and Alexander EA. Sodium balance during pregnancy in the rat. *Am J Physiol* 239: R143-8, 1980.
17. Davison JM and Dunlop W. Renal hemodynamics and tubular function normal human pregnancy. *Kidney Int* 18: 152-61, 1980.
18. Del Mónaco S, Assef Y, and Kotsias BA. Epithelial sodium channel in a human trophoblast cell line (BeWo). *J Membr Biol* 223:127–139, 2008.
19. Del Mónaco SM, Marino GI, Assef YA, Damiano AE, and Kotsias BA. Cell migration in BeWo cells and the role of epithelial sodium channels. *J Membr Biol* 232:1-13, 2009.
20. Dendorfer A, Wolfrum S, and Dominiak P. Pharmacology and Cardiovascular Implications of the Kinin-Kallikrein System. *Jpn J Pharmaco.* 79: 403-426, 1999.

21. Diakov A, Korbmacher C. A novel pathway of epithelial sodium channel activation involves a serum- and glucocorticoid-inducible kinase consensus motif in the C terminus of the channel's alpha-subunit. *J Biol Chem* 279: 38134-42. 2004
22. Driver PM, Rauz S, Walker EA, Hewison M, Kilby MD, and Stewart PM. Characterization of human trophoblast as a mineralocorticoid target tissue. *Mol Hum Reprod* 9: 793-798, 2003.
23. Duvekot J and Peeters L. Maternal cardiovascular hemodynamic adaptation to pregnancy. *Obstet Gynecol Surv* 49:S1-14, 1994.
24. Elsheikh A, Creatsas G, Mastorakos G, Milingos S, Loutradis D, and Michalas S. The renin-aldosterone system during normal and hypertensive pregnancy. *Arch Gynecol Obstet* 264: 182-5, 2001.
25. Escher G and Mohaupt M. Role of aldosterone availability in preeclampsia. *Mol Aspects Med* 28:245-54, 2007.
26. Félétou M, Köhler R, Vanhoutte PM. Endothelium-derived vasoactive factors and hypertension: possible roles in pathogenesis and as treatment targets. *Curr Hypertens Rep* 12: 267-75, 2010.
27. Fels J, Oberleithner H, and Kusche-Vihrog K. Ménage à trois: Aldosterone, sodium and nitric oxide in vascular endothelium. *Biochimica et Biophysica Acta* 1802: 1193–1202, 2010.
28. Fekete A, Sasser J, and Baylis C. Chronic vasodilation produces plasma volume expansion and hemodilution in rats: consequences of decreased effective arterial blood volume. *Am J Physiol Renal Physiol* 300: F113–F118, 2011.
29. Frederiksen MC. Physiologic changes in pregnancy and their effect on drug disposition. *Semin Perinatol* 25:120-123, 2001.
30. Gallery E, Hunyor S, and Gyrory A. Plasma volume contraction: a significant factor in both pregnancy-associated hypertension (pre-eclampsia) and chronic hypertension in pregnancy, *J Med* 48: 593–602, 1979.
31. Ganzevoort W, Rep A, Bonsel GJ, de Vries JI, and Wolf H. Plasma volume and blood pressure regulation in hypertensive pregnancy. *Journal of Hypertension* 22: 1235–1242, 2004.
32. Garland HO and Green R. Micropuncture study of changes in glomerular filtration and ion and water handling by the rat kidney during pregnancy. *J Physiol* 329: 389-409, 1982.
33. Girling JC. Physiology of pregnancy. *Anesthesia and Intensive Care Medicine* 215-218, 2004.
34. Gullner H, Nicolaou K, and Bartter F. Prostacyclin has effects on proximal and distal tubular function in the dog. *Prostaglandins Med* 4: 141-146, 1980.
35. Helms, M. et al. Role of SGK1 in nitric oxide inhibition of ENaC in Na-transporting epithelia. *Am J Physiol Cell Physiol* 289: C717–C726, 2005.
36. Hines, T. Baroreceptor afferent discharge in the pregnant rat. *Am J Physiol Regulatory Integrative Comp Physiol* 278: R1433–R1440, 2000.
37. Huang B, Amin S, and Leenen F. The central role of the brain in salt-sensitive hypertension. *Curr Opin Cardiol* 21: 295–304, 2006.
38. Huang T, Stähler F. Effects of dietary Na^+ deprivation on epithelial Na^+ channel (ENaC), BDNF, and TrkB mRNA expression in the rat tongue. *BMC Neuroscience* 10, 2009.
39. Jain L, Chen X, Brown L, and Eaton D. Nitric oxide inhibits lung sodium transport through a cGMP-mediated inhibition of epithelial cation channels. *Am J Physiol Lung Cell Mol Physiol* 274: 475-484, 1998.
40. Jeyabalan A and Conrad K. Renal function during normal pregnancy and preeclampsia. *Frontiers in Bioscience* 12: 2425-2437, 2007.
41. Khraibi AA, Dobrian AD, Yu T, Solhaug MJ, Billiar RB. Role of RIHP and renal tubular sodium transporters in volume retention of pregnant rats. *Am J Hypertens* 18:1375-1383, 2005.
42. Kirksey A and Pike RL. Some effects of high and low sodium intakes during pregnancy in the rat. I. Food consumption, weight gain, reproductive performance, electrolyte balances, plasma total protein and protein fractions in normal pregnancy. *J Nutr* 77: 33-42, 1962.

43. Knepper MA, Kim GH, Masilamani S. Renal tubule sodium transporter abundance profiling in rat kidney: response to aldosterone and variations in NaCl intake. *Ann N Y Acad Sci* 986:562-569, 2003.
44. Kougias P, Weakley S, Yao Q, Lin P, and Chen C. Arterial baroreceptors in the management of systemic hypertension. *Med Sci Monit* 16: RA1–RA8, 2010.
45. Kunzelmann K and Mall M. Electrolyte Transport in the Mammalian Colon: Mechanisms and Implications for Disease. *Physiol Rev* 82: 245–289, 2002.
46. Kuwahara K and Nakao K. Regulation and significance of atrial and brain natriuretic peptides as cardiac hormones. *Endocrine Journal* 57: 555-565, 2010.
47. Langer B, Grima M, Coquard C, Bader AM, Schlaeder G, and Imbs JL. Plasma active renin, angiotensin I, and angiotensin II during pregnancy and in preeclampsia. *Obstet Gynecol* 91:196-202, 1998.
48. Lee, I. et al. Regulation of epithelial Na+ channels by aldosterone: role of Sgk1. *Clinical and Experimental Pharmacology and Physiology* 35: 235–241, 2008.
49. Leenen F. The central role of the brain aldosterone–"ouabain" pathway in salt-sensitive hypertension. *Biochimica et Biophysica Acta* 1802: 1132–1139, 2010.
50. Lees MM, Taylor SH, Scott DM, et al: A study of cardiac output at rest throughout pregnancy. *J Obstet Gynaecol Br Commonw* 74:319-328, 1967.
51. Lifton R, Gharavi A, and Geller D. Molecular Mechanisms of Human Hypertension. *Cell* 104: 545–556, 2001.
52. Lin W, Finger TE, Rossier BC, Kinnamon S. Epithelial Na1 Channel Subunits in Rat Taste Cells: Localization and Regulation by Aldosterone. *The Journal of Comparative Neurology* 405: 406–420, 1999.
53. Loffing J. et al. Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. *Am J Physiol Renal Physiol* 280: F675-F682, 2001.
54. Loffing J and Korbmacher C. Regulated sodium trasportt in the renal connecting tubule (CNT) via the epithelail sodium channel (ENaC). *Eur J PPhysiol* 458: 111-135, 2009.
55. Maak, T. The Broad Homeostatic Role of Natriuretic Peptides. *Arq Bras Endocrinol Metab* 50: 198-207, 2006.
56. Mahaney J, Felton C, Taylor D, Fleming W, Kong JQ, and Baylis C. Renal cortical Na+-K+-ATPase activity and abundance is decreased in normal pregnant rats. *Am J Physiol* 275:F812-817, 1998.
57. Masilamani S, Castro L, and Baylis C. Pregnant rats are refractory to the natriuretic actions of atrial natriuretic peptide. *Am J Physiol Regul Integr Comp Physiol* 267: R1611–R1616, 1994.
58. Masilamani S, Kim GH, Mitchell C, Wade JB and Knepper MA. Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. *J Clin Invest* 104: R19-R23, 1999.
59. Michlig, S., M. Harris, J. Loffing, B. Rossier, and D. Firsov. Progesterone Down-regulates the Open Probability of the Amiloride-sensitive Epithelial Sodium Channel via a Nedd4-2-dependent Mechanism. The Journal of Biological Chemistry Vol. 280, No. 46, pp. 38264–38270, November 18, 2005.
60. Miyata K, Rahman M, Shokoji T, Nagai Y, Zhang GX, Sun GP, Kimura S, Yukimura T, Kiyomoto H, Kohno M, Abe Y, Nishiyama A. Aldosterone stimulates reactive oxygen species production through activation of NADPH oxidase in rat mesangial cells. *J Am Soc Nephrol* 16: 2906–2912, 2005.
61. Nakano D, Pollock J, and Pollock D. Renal medullary ETB receptors produce diuresis and natiuresis via NOS1. *Am J Physiol Renal Physiol* 294: F1205-F1211, 2008.
62. Ni X, Safai M, Rishi R, Baylis C, and Humphreys M. Increased activity of cGMP-specific phosphodiesterase (PDE5) contributes to resistance to atrial natriuretic peptide natriuresis in the pregnant rat. *J Am Soc Nephrol* 15: 1254-1260, 2004.

63. Ohara M, Martin P, Xu D, St. John J, Pattison T, Kim J, and Schrier R. Upregulation of aquaporin 2 water channel expression in pregnant rats. *J Clin Invest* 101: 1076-1083, 1998.
64. Ohnaka K, Takayanagi R, Nishikawa M, Haji M, and Nawata H. Purification and characterization of phosphoramidon-sensitive endothelin-converting enzyme in porcine aortic endothelium. *J Biol Chem* 268: 26759-26766, 1993.
65. Page KR, Ashworth CJ, McArdle HJ, Finch AM, and Nwagwu MO. Sodium transport across the chorioallantoic membrane of porcine placenta involves the epithelial sodium channel (ENaC). *J Physiol* 547: 849-857, 2003.
66. Pavlov T, Chahdi A, Ilatovskaya D, Levchenko V, Vandewalle A, Pochynyuk O, Sorokin A, and Staruschenko A. Endothelin-1 inhibits the epithelial Na+ channel through betaPix/14-3-3/Nedd4-2. *J Am Soc Nephrol* 21: 833-843, 2010.
67. Pinto FM, Cintado CG, Mérida A, Hidalgo A, and Candenas ML. Differential expression of amiloride-sensitive Na+ channel subunits messenger RNA in the rat uterus. *Life Sci* 66: PL313-317, 2000.
68. Pirani B, Campbell D, and MacGillivray I. Plasma volume in normal first pregnancy, *J Obstet Gynaecol Br Commonw* 80: 884–887, 1973.
69. Ring AM, Leng Q, Rinehart J, Wilson FH, Kahle KT, Hebert SC, Lifton RP. An SGK1 site in WNK4 regulates Na+ channel and K+ channel activity and has implications for aldosterone signaling and K+ homeostasis. *Proc Natl Acad Sci U S A*104: 4025–4029, 2007
70. Ring, A. et al. WNK4 regulates activity of the epithelial Na channel in vitro and in vivo. *Proc Natl Acad Sci U S A*104: 4020–4024, 2007.
71. Robson SC, Hunter S, Boys RJ, et al: Serial study of factors influencing changes in cardiac output during human pregnancy. *Am J Physiol* 256:H1060-1065, 1989.
72. Sasser J, Ni X, Humphreys M, and Baylis C. Increased renal phosphodiesterase-5 activity mediates the blunted natriuretic response to a nitric oxide donor in the pregnant rat. *Am J Physiol Renal Physiol* 299: F810-F814, 2010
73. Schnermann J and Briggs J. Role of the renin-angiotensin system in tubuloglomerular feedback. *Fed Proc* 45: 1426-1430, 1986.
74. Schrier R. Systemic arterial vasodilation, vasopressin, and vasopressinase in pregnancy. *J Am Soc Nephrol* 21: 570-572, 2010.
75. Schrier R and Briner V. Peripheral arterial vasodilation hypothesis of sodium and water retention in pregnancy: Implications for pathogensis of preeclampsia-eclampsia. *Obstet Gynecol* 77: 632-639, 1991.
76. Schrier R and Durr J**.** Pregnancy: an overfill or underfill state. *Am J Kidney Dis* 9: 284-289, 1987.
77. Scifres C and Nelson D. Intrauterine growth restriction, human placental development and trophoblast cell death. *J Physiol* 587: 3453–3458, 2009.
78. Sharma JN, Urea K, Noor AR, and Rahman ARA. Blood Pressure Regulation By The Kallikrein-Kinin System. *Gen Pharmac* 27: 55-63, 1996.
79. Smith C, Santymire B, Erdely A, Venkat V, Losonczy G, and Baylis C. Renal nitric oxide production in rat pregnancy: role of constitutive nitric oxide synthases. *Am J Physiol Renal Physiol* 299: F830–F836, 2010.
80. Snyder P. Minireview: Regulation of Epithelial Na+ Channel Trafficking. *Endocrinology* 146: 5079-5085, 2005.
81. Spieker LE, Noll G, Ruschitzka FT, Maier W, and Luscher TF. Working under pressure: the vascular endothelium in arterial hypertension. *Journal of Human Hypertension* 14: 617–630, 2000.
82. Sun P, Lin D, Yue P, Jiang H, Gotlinger K, Schwartzman M, Falck J, Goli M, and Wang W. High postassium intake enhances the inhibitory effect of 11,12-EET on ENaC. *J Am Soc Nephrol* 21: 1667-1677, 2010.

83. Sweezey N, Tchepichev S, Gagnon S, Fertuck K, and O'Brodovich H. Female gender hormones regulate mRNA levels and function of the rat lung epithelial Na channel. *Am J Physiol Cell Physiol* 274: 379-386, 1998.
84. Thomas G. Neural control of the circulation. *Adv Phyiol Educ* 35: 28-32, 2011.
85. Thornton SN, Omouessi ST, and Falconetti C. Mineralocorticoid modulation of central angiotensin-induced neuronal activity, water intake and sodium appetite. *Brazilian Journal of Medical and Biological Research* 40: 699-705, 2007
86. Tsang LL, Chan LN, Wang XF, So SC, Yuen JPS, Fiscus RR, and Chan HC. Enhanced epithelial Na+ channel (ENaC) activity in mouse endometrial epithelium by upregulation of γENaC subunit. *Japanese Journal of Physiology* 51: 539-543, 2001.
87. WHO. World health report: make every mother and child count. Geneva; WHO p.63, 2005.
88. Williams D. Renal disease in pregnancy. *Obstetrics, Gynecology, and Reproductive Medicine* 17: 147-53, 2007.
89. Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D and Yanagisawa M. ECE-1: A membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* 78: 473-485, 1994.
90. Yu L, Bao HF, Self JL, Eaton DC, Helms MN. Aldosterone-induced increases in superoxide production counters nitric oxide inhibition of epithelial Na channel activity in A6 distal nephron cells. *Am J Physiol Renal Physiol*. 293:F1666-77, 2007.

IX.

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1. **Crystal A West**, Zheng Zhang, and Shyama ME Masilamani. Pregnancy-Mediated Adaptations of the Renal Epithelial Sodium Channel (ENaC). 5th Annual Women's Health Research Day, Richmond, VA, Wednesday, April 15, 2009.
2. **Crystal A West**, Ningjun Li, and Shyama ME Masilamani. Renal epithelial sodium channel (ENaC) is required for blood pressure (BP) maintenance in pregnant rat. Experimental Biology, Anaheim, CA, Wednesday, April 28, 2010.
3. **Crystal A West** and Shyama ME Masilamani. Chronic Blockade of the Epithelial Sodium Channel (ENaC) Reduces Blood Pressure and Induces Fetal Growth Restriction in the Late

Pregnant (LP) Rat. American Society of Nephrology 43rd Annual Meeting, Denver, CO, Thursday, November 18, 2010.

4. **Crystal West**, Jennifer Sasser, Andrea Fekete, Chris Baylis, Shyama Masilamani. Chronic Vasodilation Mimics Renal Sodium Transporter Profile Seen In Pregnancy. Experimental Biology, Washington, DC, Monday, April 11, 2011.
5. **Crystal A West** and Shyama ME Masilamani. A new role of extrarenal ENaC in the progression of a healthy pregnancy. Experimental Biology, Washington, DC, Monday, April 11, 2011.

ABSTRACTS:

1 **Crystal West**, Zheng Zhang, Goeffrey Ecker, and Shyama Masilamani. Epithelial sodium channel (ENaC) and pregnancy. *FASEB J.* 23: LB175 (2009).
2. **Crystal West**, Zheng Zhang, and Shyama Masilamani. Pregnancy-Mediated Adaptations of the Renal Epithelial Sodium Channel (ENaC). 5th Annual Women's Health Research Day at Virginia Commonwealth University (2009).
3. **Crystal West** and Shyama Masilamani. Renal epithelial sodium channel (ENaC) is required for blood pressure (BP) maintenance in pregnant rat. *FASEB J.* 24: 1041.12 (2010).
4. Helena Carvalho and **Crystal West**. Voluntary Participation in Active Learning Activity Leads to a Better Understanding in Physiology. *FASEB J.* 24: 633.11 (2010).
5. Helena Carvalho, **Crystal West**, and Kristin Simmons. Hands-on Exercise in Physiology: a Tool for Students Engagement. *FASEB J.* 25: 481.15 (2011).
6. **Crystal West**, Jennifer Sasser, Andrea Fekete, Chris Baylis, Shyama Masilamani. Chronic Vasodilation Mimics Renal Sodium Transporter Profile Seen In Pregnancy. *FASEB J.* 25: 836.4 (2011).
7. **Crystal West** and Shyama Masilamani. A new role of extrarenal ENaC in the progression of a healthy pregnancy. *FASEB J.* 25: 836.3 (2011).

PAPERS:

1. **Crystal West**, Zheng Zhang, Geoffrey Ecker, and Shyama Masilamani. Increased Renal α Epithelial Sodium Channel (ENaC) Protein and Increased ENaC Activity in Normal Pregnancy. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology.* 2010 Nov; 299(5):R1326-32.
2. Helena Carvalho and **Crystal West**. Voluntary Participation in an Active Learning Exercise Leads to a Better Understanding of Physiology. *Advances in Physiology Education.* 35: 53–58, 2011.
3. **Crystal West,** Weiqing Han, Ningjun Li, and Shyama Masilamani. Increased activity of the epithelial sodium channel (ENaC) during pregnancy maintains pregnancy-mediated changes in sodium balance, volume status, and blood pressure. (in Preparation).
4. Lori Kang, **Crystal West**, Shyama Masilamani, and Matthew Boegehold. Juvenile Growth Reduces the Influence of Epithelial Sodium Channels on Vascular Tone in Skeletal Muscle Arterioles. (in Preparation).