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Mechanisms of Regulation of Proximal Tubule Sodium Transporters in Obesity-Induced Hypertension

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ABSTRACT

MECHANISMS OF REGULATION OF PROXIMAL TUBULE SODIUM TRANSPORTERS IN OBESITY-INDUCED HYPERTENSION

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Hypertension is one of the common complications of obesity. Using a rat model of diet induced obesity and hypertension we investigated some of the mechanisms that are involved in regulation of blood pressure in obesity. The first aim of this study was to determine the role of proximal tubule transporters on the renal sodium handling in obese hypertensive (OP) and lean normotensive (OR) rats. An acute increase in renal perfusion pressure resulted in a blunted natriuretic response in OP vs. OR rats and indicated that increased sodium reabsorption in the proximal tubule is casual, at least in part, for hypertension in OP rats. Subsequently, protein expression and activity of Na,K-ATPase and NHE3 were increased in obese rats compared to lean rats. Moreover, in OP rats more NHE3 was associated with its active pool located in the microvillus region. Together, these results suggest that hypertension in obese rats is characterized by the impaired pressure-natriuresis and diuresis that can be explained by the increased activity of proximal tubule sodium transporters.

Previous results from our lab determined that peroxisome proliferator activated receptor γ (PPARγ) has reduced expression and activity in the kidney of

OP vs. OR rats. Therefore, in the second aim we investigated the effect of PPARγ ligand activation on expression and activity of proximal tubule Na^+ transporters in OP and OR rats. In addition, by employing in vitro studies using proximal tubule epithelial cells, we determined whether pioglitazone exerts its effect via direct PPARγ activation. Pioglitazone reduced systolic blood pressure in obese rats while having no effect in lean rats. However, it increased sodium retention in the lean group. Pioglitazone increased Na,K-ATPase activity in OP rats, while its protein expression was increased in both groups. In contrast, NHE3 activity was reduced in obese rats treated with pioglitazone and protein expression was decreased in both groups. Pioglitazone did not have an effect on NHE3 localization in obese rats, but in lean rats, it had tendency to redistribute NHE3 towards the more active membrane pool. In cells transiently transfected to overexpress or silence PPARγ, we demonstrated that pioglitazone reduced Na,K-ATPase and NHE3 abundance via PPARγ activation. Collectively, the results indicated that pioglitazone reduced blood pressure in the obese group most likely by decreasing activity of NHE3. However, other factors besides trafficking are involved in the transporter regulation. Pioglitazone did not reduce blood pressure in lean rats, suggesting that the metabolic milieu is an important determinant of the pioglitazone differential effect on the blood pressure and on the proximal tubule transporters.

Nitric oxide (NO) plays an important role in regulating pressure natriuresis and diuresis and its availability seems to be altered in obese animals and humans. The third aim was designed to examine the role of NO on blood

pressure, pressure natriuresis and expression of sodium transporters NHE3 and Na,K-ATPase in OP and OR rats. To determine the role of NO, we performed *in vivo* study using L-NAME for chronic NO inhibition. The NO inhibition did not change glomerular filtration rate in either of the groups. Natriuresis and diuresis was significantly decreased only in treated OR rats. Also, NHE3 protein expression and activity were significantly elevated in treated vs. non-treated OR rats, with no significant changes in OP rats. Moreover, L-NAME caused a shift of NHE3 to the active pool located in microvillus region in OR group only. In conclusion, normotensive OR rats are more susceptible to NO deficiency and the mechanism involves an increase in activity of NHE3 with the transporter redistribution playing a significant role. In addition, we investigated in cell culture whether hormones elevated in obesity can modulate Na,K-ATPase and NHE3 via cGMP production. *In vitro* experiments provided some evidence that angiotensin II and insulin interact with the NO signaling pathway at the level of cGMP. cGMP could affect transporter activity by phosphorylation which could account for the effects determined *in vivo*.

To my Family

For their understanding and their sacrifice,
for their unwavering faith in me, and for their love.

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INTRODUCTION AND BACKGROUND

1.1 Obesity

It is possible that millions of years ago, when food and water were not easily available, individuals developed a very efficient system to utilize and store energy, salt and water. Conversely, descendants of those same individuals, living in a new environment of plentitude, would develop obesity. Obesity can be defined as a disease in which an excessive accumulation of energy in the form of body fat may adversely affect the health of the individual. The most widely used and accepted metric for identifying obesity is having a Body Mass Index (BMI) greater than 30. However, growing evidence suggests that a central (abdominal) fat distribution pattern as reflected by waist circumference is also an important factor in assessing obesity (Wang *et al.* 2005). Obesity is not a single disorder but a part of a group of diseases called "complex diseases". The phenotype of complex diseases reflects the multifactorial effect of all contributing genes (polygenic) and all environmental factors (Motulsky 2006). There are 2 distinct genetic mechanisms involved in obesity. One is caused by the infrequent presence of certain genes, which produce rare syndromes associated with significant obesity such as Prader-Willi or Bardet-Biedl syndrome. However,

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obesity is much more commonly mediated by the presence of other "susceptibility" genes. More than 50 such genetic sites have been identified and in their presence obesity will develop only if there is a favorable environment (Rankinen *et al.* 2006). These genes control different processes, such as regulation of fat distribution, metabolic rate, response to exercise and diet, control of feeding, and food preferences, etc. But the striking rise in the incidence of obesity, which has happened in the last few decades, is most probably not because of changes in the genetic background of the human race, since these changes take thousands of years to evolve. This "epidemic" is mainly caused by rapid lifestyle changes involving eating habits and exercise (Ravussin *et al.* 1988, Rissanen *et al.* 1991, Blundell & Macdiarmid 1997).

Although obesity is not a recent phenomenon, the epidemic of obesity is and continues to escalate in the United States (Ogden *et al.* 2006) and in much of the developed (Berghofer *et al.* 2008) and developing world (Hossain *et al.* 2007). According to data from the 2003-2004 National Health and Nutrition Examination Survey (NHANES), ~73% of men and ~60% of women are overweight or obese, with prevalence varying among the three major racial/ethnic groups in the U.S. (Ogden *et al.* 2007) and increasing rapidly. All in all, an estimated 145 million adults in the United States are overweight or obese. As prevalence of obesity increases, the rising economic costs of obesity continue to be a major health burden for national health economies (Thorpe 2009). Recent estimates indicate that obesity accounted for 9% of total health care costs in the U.S. in 1998, with diseases such as coronary heart disease, type II diabetes and

osteoarthritis accounting for 93 billion dollars of total obesity-attributable medical costs in 2002 (Colditz 1999, Finkelstein *et al.* 2003).

1.1.1 Obesity and associated co-morbidities

Higher body weights are associated with several cardiovascular risk factors including hypertension (Field *et al.* 1999, Brown *et al.* 2000), dyslipidemia (Brown *et al.* 2000) and hyperinsulinemia (Despres 1993). These risk factors could represent intermediate steps in the pathway between obesity and coronary heart disease or obesity could be an independent risk factor for cardiovascular disease (CVD) (Eckel & Krauss 1998, Kim *et al.* 2000, Grundy 2002). Being overweight or obese substantially increases the risk of other chronic diseases beside CVD such as stroke, diabetes mellitus, gallblader disease, osteoarthritis, sleep apnea and respiratory problems, as well as cancers of the endometrium, breast, prostate and colon (National Heart Lung and Blood Institute: Obesity Guidelines).

Previously, adipose tissue was thought to be a passive depot for storing excess calories. Recently however, it was discovered that in addition to storing excess energy as fat, adipose tissue has much more complex and dynamic functions and acts as an endocrine organ secreting various factors into the blood, termed adipokines (Rajala & Scherer 2003). These factors include leptin, adiponectin, resistin, angiotensin II, prostoglandins, plasminogen activator inhibitor-1, tumor necrosis factor-α, macrophage migration inhibitory factor etc.

and the list of these factors is growing rapidly. Most of these factors have important roles in energy homeostasis, insulin sensitivity, immune function, inflammation, and even in tumor progression in the local oncogenic microenvironments (Rajala & Scherer 2003). These secretory and non-secretory components of the adipose tissue may impact on the complications related to obesity directly or may affect the above mentioned risk factors.

1.1.2 Obesity and hypertension

As described earlier, the effects of obesity on cardiovascular health and disease are many, one of the most profound of which is hypertension. It was estimated that after adjustment for other risk factors such as age, BMI, degree of weight cycling, physical activity, smoking, and alcohol consumption each kilogram increase in body weight increased the risk of developing hypertension by 4.4% (Field *et al.* 1999). And it came as no surprise that with the significant rise in obesity in this last decade occurred a corresponding increase in the prevalence of hypertension (Field *et al.* 1999). However, clinical studies demonstrated that weight loss can effectively lower blood pressure (Schotte & Stunkard 1990, Wassertheil-Smoller *et al.* 1992).

It is also important to recognize that long-duration obesity does not appear necessary to elevate BP, as shown in obese, normotensive children (Sorof & Daniels 2002). Nevertheless, association between obesity and hypertension is

widely recognized, but the mechanisms responsible for weight-related changes in blood pressure have not been entirely elucidated.

1.2 Hypertension

More than 1 billion individuals worldwide, including 50 million Americans, have high blood pressures warranting some form of treatment (WHO 2002, Hajjar & Kotchen 2003, Kearney *et al.* 2005). Higher-than-optimal blood pressure is the number one attributable risk for death throughout the world and approximately 7.1 million deaths per year are credited to uncontrolled hypertension (WHO 2002). As life expectancy increases, hypertension is becoming an even more important medical and public health issue as blood pressure rises with aging in industrialized countries. The most recent National Health and Nutrition Examination Survey (NHANES) survey conducted in 2005-2006 revealed that almost 29 percent of the population in the United States is hypertensive having a blood pressure (BP) greater than 140/90 or using hypertensive medications (Ostchega *et al.* 2008). Primary (essential) hypertension, which accounts for 95 percent of all cases of hypertension, has been traditionally defined as high blood pressure for which no obvious secondary cause (e.g., renovascular disease, aldosteronism, pheochromocytoma, or individual gene mutations) can be determined. Moreover, risk estimates from the Framingham Heart study suggest that approximately 78% of hypertension in men and 65% in women can be attributed directly to an overweight condition and

obesity (Garrison *et al.* 1987). Therefore, rather than a special case, obesity hypertension should be considered the most common form of essential hypertension.

1.2.1 Blood pressure regulation

The most important function of blood pressure is to provide the driving force that moves blood through the vascular system in order to supply the needs of the tissues. Consequently, the regulation of blood pressure is a complex physiologic function that depends on the integrated actions of multiple cardiovascular, renal, neural, endocrine, and local tissue control systems. Blood pressure regulation, as is true for most physiologic control systems, involves short-term and long-term mechanisms. Following a sudden change in blood pressure, acute control occurs within seconds as a result of: 1) the arterial baroreceptors, which detect changes in blood pressure and send appropriate autonomic reflex signals into the vasomotor center of the brain and then back to the heart and blood vessels to return the blood pressure toward normal; 2) the chemoreceptors, which detect changes in oxygen or carbon dioxide in the blood and similar to baroreceptors initiate autonomic feedback responses that influence blood pressure; and 3) the central nervous system, which responds within a few seconds to ischemia of the vasomotor centers in the medulla, especially when blood pressure falls below about 50 mm Hg. Each of these nervous control mechanisms works rapidly and can have potent effects on blood pressure but

they correct a blood pressure abnormality only in part but never restoring it completely to the normal values.

Within a few minutes or hours after a blood pressure disturbance, when the nervous mechanism usually become less and less effective, several additional control systems are activated, including 1) a shift of fluid from the interstitial spaces into the blood stream in response to decreased blood pressure (or a shift of fluid out of the blood into the interstitial spaces in response to increased blood pressure); 2) the renin-angiotensin system (RAS) which is activated when blood pressure falls too low and suppressed when blood pressure increases above normal (Fig.1); 3) multiple vasodilator systems that are suppressed when blood pressure decreases and stimulated when blood pressure rises above normal. However, the renal-body fluid system is a dominant mechanism for long-term arterial pressure control (Guyton & Hall 2006).

1.2.2 Pressure natriuresis and diuresis

Normally, an increase in blood pressure would raise sodium and water excretion, a phenomenon usually referred to as pressure natriuresis and diuresis (Fig. 2). Under most conditions this mechanism acts to stabilize blood pressure and the body fluid volumes. For example, when blood pressure is increased above the renal equilibrium point, because of increased total peripheral resistance or increased cardiac pumping ability, this also increases sodium and water excretion via pressure natriuresis if kidney function is not impaired.

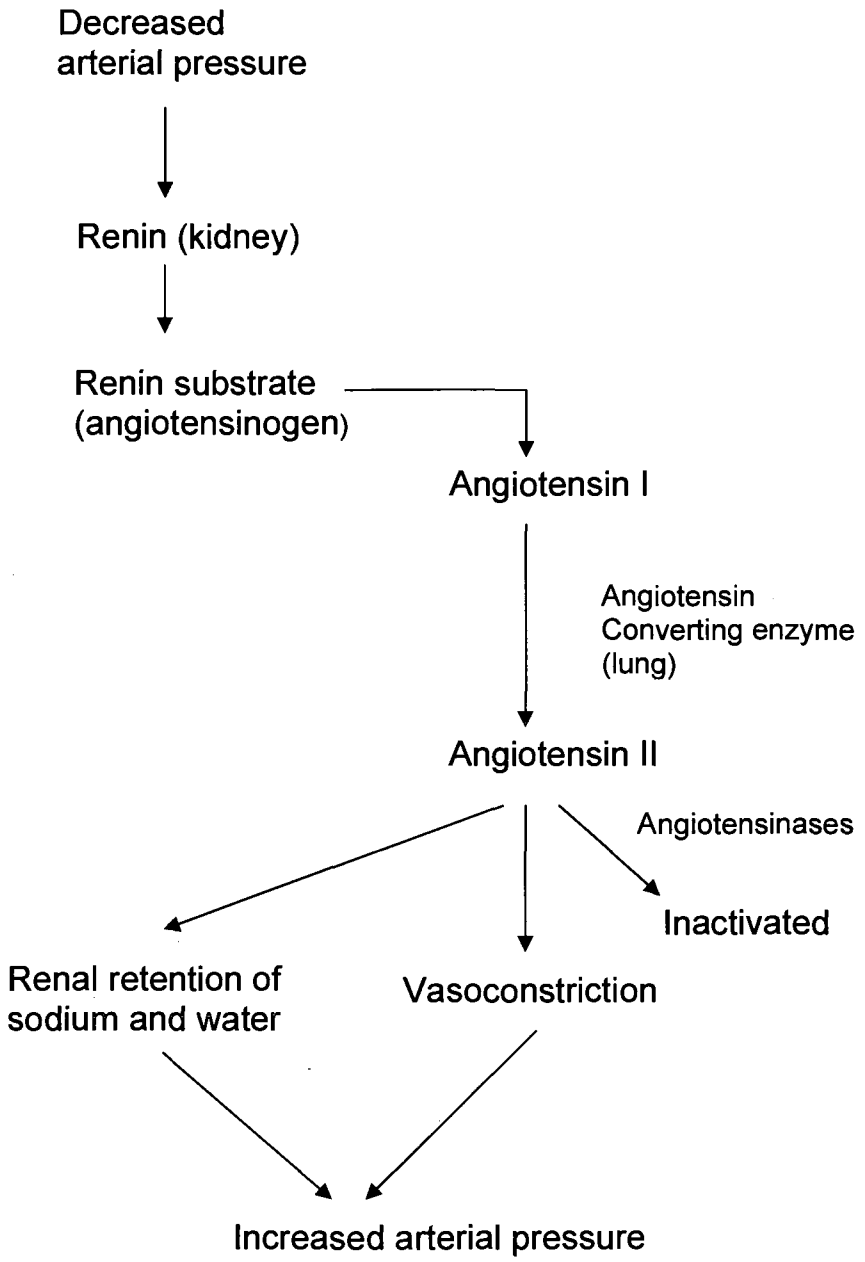

Figure 1 Components of the renin-angiotensin system.
A decrease in renal perfusion pressure causes the juxtaglomerular cells of the afferent arteriole to secrete renin. Renin catalyzes the conversion of angiotensinogen to angiotensin I in plasma. Angiotensin-converting enzyme (ACE) catalyzes the conversion of angiotensin I to angiotensin II, primarily in the lung. Angiotensin II is physiologically active and acts as a vasoconstrictor and also stimulates the synthesis and secretion of aldosterone by the adrenal cortex. Aldosterone increases Na^+ reabsorption. Angiotensin II is rapidly inactivated by angiotensinases.

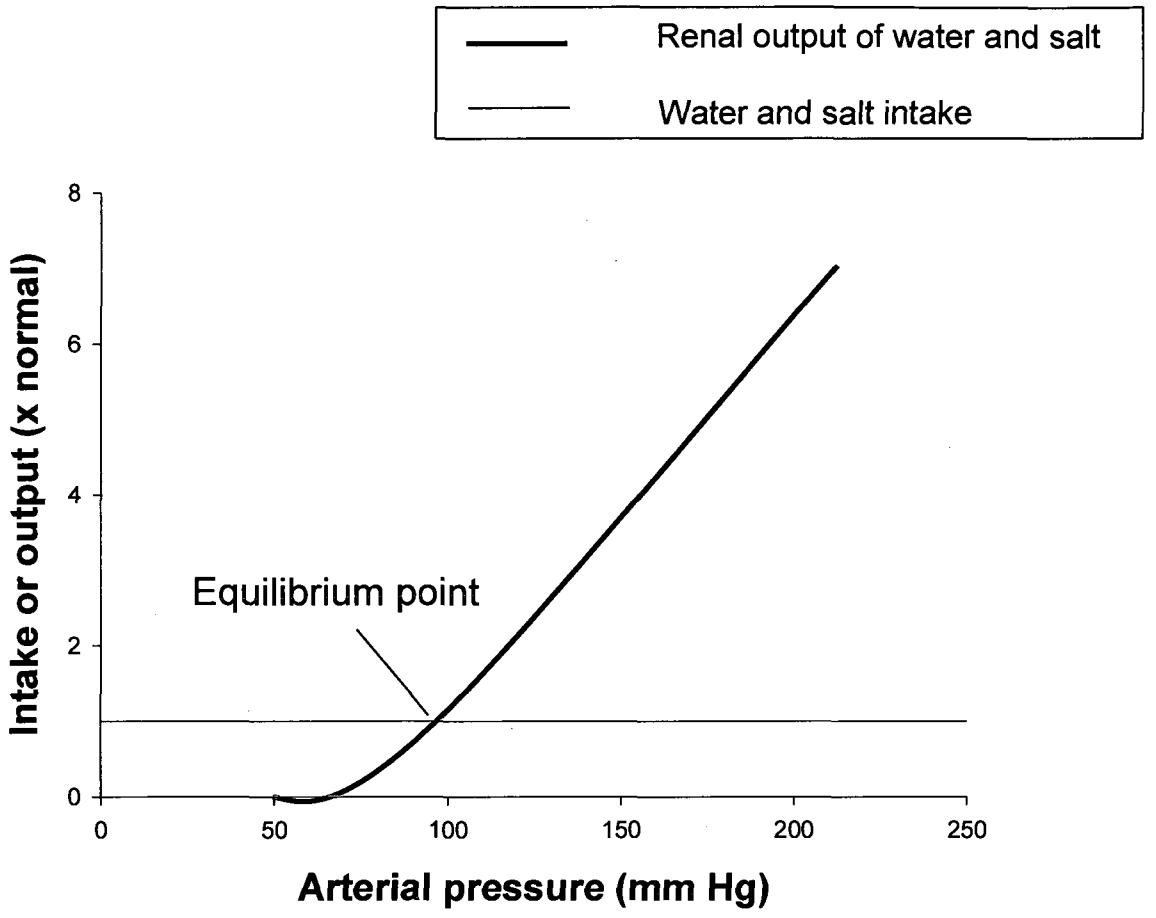

Figure 2 Graphical analysis of the infinite gain characteristic of the renal–body fluid feedback system on pressure control.
There are two separate curves that intersect each other: renal output curve (red) for water and salt in response to increasing arterial pressure and the line (black) which represents the net water and salt output. Redrawn from Textbook of Medical Physiology (Guyton & Hall 2006).

As long as fluid excretion exceeds fluid intake, extracellular fluid volume will continue to decrease, reducing venous return and cardiac output, until blood pressure returns to normal and fluid balance is reestablished. An important feature of pressure natriuresis is that various hormonal and neural control systems can greatly amplify or blunt the basic effects of blood pressure on sodium and water excretion. For example, during chronic increases in sodium intake only small changes in blood pressure are needed to maintain sodium balance in most people. One reason for this insensitivity of blood pressure to changes in salt intake is decreased formation of antinatriuretic hormones such as angiotensin II and aldosterone, which enhance the effectiveness of pressure natriuresis and allow sodium balance normally maintained with minimal increases in blood pressure. On the other hand, excessive activation of these antinatriuretic systems can reduce the effectiveness of pressure natriuresis, thereby necessitating greater increases in blood pressure to maintain sodium balance (Guyton & Hall 2006).

One important feature of pressure natriuresis is that it continues to operate until blood pressure returns to the original equilibrium point. In other words, it acts as part of an infinite gain feedback control system (Fig. 2) (Guyton 1990). It is the only infinite gain feedback system for blood pressure regulation in the body, so far known, and it is this property which makes it a dominant long-term controller of blood pressure (Fig. 2) (Guyton & Hall 2006). Moreover, in all forms of human or experimental hypertension studied thus far, there is a shift of

pressure natriuresis that appears to initiate and sustain the hypertension (Hall 2003).

1.2.3 Hypertension in obesity

It is well documented that blood pressure increases with weight gain and decreases with weight loss (Garrison *et al.* 1987, Jones *et al.* 1999, Stevens *et al.* 2001) . Some of the characteristics of primary hypertension caused by excess weight gain and obesity include: an expansion of extracellular fluid volume, as well as increased tissue blood flow in many tissues, including the heart, kidneys, gastrointestinal tract, and skeletal muscles and an increase in cardiac output (Hall *et al.* 1993, Carroll *et al.* 1995).

In addition, there is increasing evidence that obesity may provide the impetus for sympathetic nervous system (SNS) activation. This is especially important in the kidney since pharmacologic blockade of adrenergic activity lowers blood pressure to a greater extent in obese, compared to lean individuals. Also renal denervation markedly attenuates sodium retention and hypertension associated with a high-fat diet in experimental animals (Hall 2003). The mechanisms of sympathetic nervous system activation in obesity have not been fully elucidated, but one of the more promising candidates is hyperleptinemia (Hall *et al.* 2001). As mentioned before, leptin is produced by adipocytes and acts on the hypothalamus and other regions of the brain, such as the brainstem, to reduce appetite and increase sympathetic nervous system activity (Hall *et al.*

2001). In rodents, increasing plasma leptin concentration to levels comparable to those found in severe obesity not only increases sympathetic nervous system activity, but also raises blood pressure (Shek *et al.* 1998, Carlyle *et al.* 2002). Moreover, the hypertensive effects of leptin are enhanced when NO synthesis is inhibited (Kuo *et al.* 2001).

Obese individuals, especially those with visceral obesity, although having expansion of extracellular fluid volume, often have mild to moderate increases in plasma renin activity, angiotensinogen, angiotensinogen converting enzyme activity, angiotensin II, and aldosterone levels (Hall 2003). An important role for angiotensin II in stimulating renal sodium reabsorption and in mediating obesity hypertension is supported by studies in experimental animals demonstrating that angiotensin II receptor blockade or angiotensinogen converting enzyme activity (ACE) inhibition blunts sodium retention, volume expansion, and decreases blood pressure during the development of obesity (Robles *et al.* 1993, Boustany *et al.* 2005).

Most of all, in hypertension induced by obesity there is considerable evidence in humans that renal dysfunction, characterized by increased tubular sodium reabsorption and impairment of pressure natriuresis plays a key role in increasing blood pressure (Hall *et al.* 1993). The increased tubular reabsorption is closely related to the increase activity of the sympathetic nervous system and activation of the renin-angiotensin system in obesity. Other contributing factors are structural changes that cause the physical compression of the kidneys by fat accumulation within and around the kidneys and by the increased abdominal

pressure. Hyperinsulinemia, which occurs as a compensation for insulin resistance, is postulated to mediate increased blood pressure in essential hypertension via multiple mechanisms, such as stimulation of sympathetic nervous system activity and the renal tubular sodium reabsorption (Hall 1993). However, chronic hyperinsulinemia, in the absence of obesity, did not raise blood pressure in either dogs or humans and did not enhance the hypertensive effects of other pressor substances such as norepinephrine or angiotensin II (Hall *et al.* 1995). A study by Fujiwara and coworkers reported that altered pressure natriuresis and hypertension is associated with insulin resistance and impaired production of NO in cortex and medulla of the obese Zucker rats (Fujiwara *et al.* 1999).

Hall implied that a shift of pressure natriuresis and increased sodium reabsorption is caused by altered renal hemodynamics, increased sodium reabsorption, or both due to the mechanisms postulated above (Hall *et al.* 1993, Hall, J. E. 1994) such as increased sympathetic nerve activity, increased levels of angiotensin II, increased leptin and most likely increased levels of insulin. However, there was no renal vasoconstriction observed in obese dogs which could lead to a decrease in sodium filtered load. In fact, obese dogs were volume expanded and their glomerular filtration rate, filtered sodium load, and renal blood flow were elevated (Hall *et al.* 1993).

1.2.4 Animal models of obesity hypertension

Although association between obesity and hypertension is widely recognized, the mechanisms responsible for weight-related changes in blood pressure have not been elucidated. Animal models of obesity provided us with the opportunity to study the physiological and genetic basis of obesity and also to explore environmental aspects of the disease. There are a few models of single gene mutations available such as ob/ob and db/db mice and Zucker rats (fa/fa) with mutations in the leptin gene (ob/ob mouse) and leptin receptor (db/db; fa/fa rat). However, in humans, single gene mutations are of limited importance when considering the increasing obesity epidemic, as they account for less than 2% of obesity. In light of this, several polygenic models have been developed and characterized. Nevertheless, the cardiovascular and renal changes in most of these models have not been characterized. Among the ones that have been studied, most do not mimic the cardiovascular, renal, and neurohumoral changes found in obese humans (Kurtz *et al.* 1989).

As described before, one hypothesis for the rapidity of the obesity epidemic in humans is the possibility that our regulatory systems become overwhelmed by high-fat palatable foods which have become increasingly available (Blundell & Macdiarmid 1997). In addition, in contrast to genetic models of obesity, weight gain induced by long-term high-fat diet causes a reproducible rise in blood pressure in dogs, rabbits, and rats (Hall *et al.* 1993, Carroll *et al.* 1995, Rocchini *et al.* 1999). Dobrian *et al.* characterized a rat model of diet-

induced obesity that developed hypertension accompanied by vascular and renal changes similar to those observed in obese hypertensive humans (Dobrian *et al.* 2000). In this model which was used for the present study, Sprague-Dawley rats were fed a purified moderately high-fat (MHF) diet that contains 32% kcal fat, a value similar to the average Western diet, and showed a bimodal pattern in body weight gain. Approximately half of the rats gain weight rapidly- obesity prone (OP) and developed mild hypertension while the other half gain weight at a rate similar with chow-fed rats- obesity resistant (OR) group and were normotensive as described earlier. This model of diet-induced obesity shares a number of common features with human obesity. These include high blood pressure, activation of the renin-angiotensin system, and dyslipidemia (Dobrian *et al.* 2000), an increase in oxidative stress, a decrease in plasma and urine nitrate/nitrite content, suggesting a decreased NO production or bioavailability (Dobrian *et al.* 2001); hyperinsulinemia was also observed (Dobrian *et al.* 2004) as well as hyperleptinemia (Dobrian *et al.* 2003).

1.3 Sodium transport

Na^+ is the most abundant cation in extracellular fluid and since its salts account for over 90% of the osmotically active solute in the plasma and interstitial fluid, the amount of Na^+ in the body is the most important determinant of the extracellular fluid volume. Hence, reabsorption of Na^+ plays a major role in body electrolyte and water metabolism. In addition, Na^+ transport is coupled to the

movement of Cl^-, H^+, other electrolytes, glucose, amino acids, organic acids, phosphate, and other substances across the tubule walls. In the proximal tubules, the thick portion of the ascending limb of the loop of Henle, the distal tubules, and the collecting ducts, Na^+ moves by cotransport or exchange from the tubular lumen into the tubular epithelial cells down its concentration and electrical gradients and is actively pumped from these cells into the interstitial space. Thus, Na^+ is actively transported out of all parts of the renal tubule except the thin portions of the loop of Henle. Then, Na^+ is pumped into the interstitium by Na,K- ATPase. Normally about 60% of the filtered Na^+ is reabsorbed in the proximal tubule, primarily by the Na^+/H^+ exchange. Another 30% is absorbed via the Na^+-2Cl-K^+ cotransporter in the thick ascending limb of the loop of Henle, and about 7% is absorbed by Na^+-Cl^- cotransport in the distal convoluted tubule. The remainder of the filtered Na^+, about 3%, is absorbed via the ENaC channels in the collecting ducts. Thus, the kidneys reabsorb approximately 99.6% of the filtered Na^+ and it comes as no surprise that there are multiple regulatory mechanisms to control the excretion of this ion. The body regulates Na^+ transport using three major mechanisms: 1) glomerulotubular balance, 2) factors that increase Na^+ reabsorption such as the renin-angiotensin-aldosterone system, sympathetic nerve activation and antidiuretic hormone, and 3) factors that decrease Na^+ reabsorption such as atrial natriuretic peptide, prostaglandins, bradykinin and dopamine. Through the operation of these regulatory mechanisms, the amount of Na^+ excreted is adjusted to equal the amount ingested over a wide range of dietary intakes, and the individual stays in Na^+

balance. Therefore, the kidney ultimately governs the extracellular fluid volume and consequently is the principal agent in the long-term control of blood pressure (Boron & Boulpaep 2002).

1.3.1 Sodium/hydrogen exchanger type 3 (NHE3)

In the proximal tubule the essential components of absorption are luminal membrane Na^+/H^+ exchanger and basolateral Na,K-ATPase as mentioned earlier. Sodium is reabsorbed from the proximal tubule across the apical membrane via sodium-hydrogen exchangers, and it is actively pumped out of the cell by the basolateral sodium pump, Na,K-ATPase, which generates the gradient for Na^+ entry across the apical membrane. The inward gradient for Na^+ is used to drive several secondary active transporters. Quantitatively speaking, the most important Na^+-coupled transporter in proximal tubule is the Na^+/H^+ exchanger.

The sodium hydrogen exchangers (NHEs), also called antiporters, are widely expressed in the epithelial membrane and play an important role in salt and water reabsorption by virtue of the Na^+/H^+ exchange. NHEs belong to the gene family called SLC9A comprising nine isoform NHE1-9 (Donowitz & Li 2007). The first five isoforms are expressed largely at the plasma membrane, while the other isoforms (NHE6-9) have been shown to reside predominantly intracellularly (Donowitz & Li 2007). The NHE1 isoform is the 'housekeeping' isoform of the exchanger and is ubiquitously expressed in the plasma membrane of virtually all

tissues (Fliegel 2005). The NHE2–NHE5 isoforms are also localized to the plasma membrane, but have more restricted tissue distributions. NHE2 and NHE3 are predominantly located in the apical membrane of epithelia and are highly expressed in the kidney and the intestine (Biemesderfer *et al.* 1993, Hoogerwerf *et al.* 1996, Biemesderfer *et al.* 1997). In kidney, as found by immunohistochemistry analysis, NHE1 is expressed along the basolateral plasma membrane of most renal epithelial segments (Paillard 1997). At the same time NHE3 has been localized to the apical membrane of the proximal convoluted tubule in the cortex and to the thick ascending limb of the loop of the Henle in the medulla (Biemesderfer *et al.* 1993, Amemiya *et al.* 1995).

1.3.1.1 Structure and functional properties of NHEs

Structurally, human NHEs have between 645 and 898 amino acids with 12 putative encoded membrane-spanning domains (msd), the first being a cleaved signal peptide as demonstrated for NHE3 (Zizak *et al.* 2000, Zachos *et al.* 2005). The transmembrane N-terminus of about 500 aa is a transport domain that carries out electroneutral exchange of 1 Na^+ for 1 H^+. The long, hydrophilic, cytosolic C-terminus domain (~300aa) regulates activity of the amphipathic N-terminal domain is a target for phosphorylation by protein kinases and also participates in binding with regulatory proteins (Wakabayashi *et al.* 2000). The transport characteristics of NHEs include substrate specificity, ATP dependence, and their sensitivity to the diuretic amiloride (Kiela. *et al.* 2006), with NHE1 and

NHE2 isoforms being the most sensitive to amiloride inhibition (IC_{50}~1μM), and NHE3 being relatively amiloride resistant with IC_{50} >100μM (Kiela *et al.* 2006). Transport kinetics for NHE3, as well as all others NHEs, is consistent with simple, saturating, Michaelis-Menten kinetics.

1.3.1.2 Regulation of NHE3

Basic short-term regulatory mechanisms of NHE3 are: changes in turnover number and changes in trafficking, and recently described changes in endocytosis/exocytosis rates (Donowitz & Li 2007). NHE3 activity is rapidly stimulated by α-adrenergic activation (Gesek *et al.* 1989), angiotensin II (Morduchowicz *et al.* 1991), endothelin (Walter *et al.* 1995), and insulin (Gesek & Schoolwerth 1991) and inhibited by dopamine and parathyroid hormone (Gesek & Schoolwerth 1990). Those hormones are coupled to signaling pathways of various protein kinases which phosphorylate a number of serine and threonine residues that are located in the distal region of the C-terminal tail of NHE3 (Orlowski *et al.* 1992).

NHE3 is known to be phosphorylated under basal conditions (Donowitz & Li 2007). Its activity was inhibited by PKA and PKG by phosphorylation of Ser552 and Ser605 since mutations of those serines individually decreased and mutations of both simultaneously blocked NHE3 inhibition (Zhao *et al.* 1999). However, Kurashima *et al.* showed that only phosphorylation of Ser605 was essential for PKA-induced NHE3 inhibition (Kurashima *et al.* 1997), with Ser634

playing an additional role for the effect of cAMP, and Ser552 not being phosphorylated or functionally important. On the other hand, the effects of PKC activation by phorbol esters were quite controversial, showing that while phorbol ester induced NHE3 phosphorylation on identical serines on the cytoplasmic tail on all the clones studied in NHE-deficient fibroblasts transfected with NHE3, the final outcome was stimulation, inhibition or no effect on NHE3 activity (Wiederkehr *et al.* 1999). Moreover, Yip *et al.* demonstrated that when expressed in PS120 fibroblats NHE3 was inhibited by PKC stimulation without any changes in the phosphorylation (Yip *et al.* 1997), suggesting that regulation of NHE3 probably involves intermediate associated regulatory proteins such as Na^+/H^+ exchanger regulatory factors (NHERFs) which are often absent in those transfected cells. Several other kinases also either associate with or regulate NHE3 activity and those are: cGMK kinase II known to inhibit NHE3 by a phosphorylation process, phosphatidylinositol 3-kinase (PI3-K) known to stimulate NHE3 activity, and calmodulin (CaM) kinase II (Donowitz & Li 2007). From all those kinases mentioned, only the last one binds directly to NHE3 while others: PKA, PKC, and PKG associate with NHE3 by PDZ domains of NHERFs family (Donowitz & Li 2007). Besides protein kinase and NHE3 phosphorylation, protein phosphatases (PP) may also play a role in NHE3 regulation. PP1 and PP2A inhibitor okadaic acid was shown to stimulate NHE3 activity (Donowitz & Li 2007). In summary, while some regulation of NHE3 may involve changes in NHE3 phosphorylation, some other regulations may occur independently of NHE3 phosphorylation status but may require presence of additional factors

and/or complexes. However, regardless of the exact mechanism involved, the functional consequences of NHE3 phosphorylation was its reduction in turnover number and changes in trafficking described as an increase in endocytosis, and/or a decrease in exocytosis.

Study by Biemesderfer and coworkers demonstrated that in the renal proximal tubule brush border NHE3 was localized to two distinct pools: the microvillar and the intermicrovillar microdomains with nearly equal levels of NHE3 expression, and with the former domain containing the active form of NHE3 and the latter the inactive form (Biemesderfer *et al.* 2001). NHE3 remains in a state of dynamic equilibrium between cell surface and the intracellular compartment; it can undergo internalization via clathrin-coated vesicles (Chow W. *et al.* 1999) and exocytosis back to the apical membrane in a PI3-K dependent manner (Lee-Kwon *et al.* 2001). Action of PKC (Janecki *et al.* 1998), parathyroid hormone (Collazo *et al.* 2000), and dopamine (Hu *et al.* 2001) are associated with a decrease in NHE3 surface expression and inhibition of the transporter while growth factors (Donowitz *et al.* 2000) and endothelin (Peng *et al.* 2001) have been shown to stimulate NHE3 activity by increasing the surface protein pool in a PI3-K dependent manner. Collectively, these data support the notion that NHE3 redistribution is an effective means of transporter regulation. There are several models which analyzed changes in NHE3 trafficking. One of them is acute pressure-induced natriuresis and diuresis. Yang *et al.* provided evidence that acute hypertension was associated with redistribution of NHE3 protein from apical membranes to, at first, membranes enriched in intermicrovillar

cleft markers, and later to membranes containing markers of endosomes (Yang *et al.* 2002). Moreover, a study by Magyar illustrated that with the development of hypertension in spontaneously hypertensive rats (SHR) there was an internalization of apical NHE3-protein which partially mimicked changes observed during acute hypertension in normotensive Sprague-Dawley rats (Magyar *et al.* 2000).

There is evidence that intrinsic NHE3 activity can be modified without alteration in NHE3 abundance in transfected cells, renal cell lines and in renal cortex (Moe 1999). This regulation of NHE3 generally involves changes in NHE3 V_{max} (Donowitz & Li 2007) and is associated with NHE3 phosphorylation although the precise mechanism remains unknown. It is also possible that those changes can be explained by the presence of NHERFs.

The NHE3 C-terminus is necessary in all cases of NHE3 regulation discussed previously and contains stimulatory domains which respond to growth factors and okadaic acid, and inhibitory domain motifs for PKA, PKC and CaM. In addition, NHE3 can directly bind NHERF1, NHERF2, PDZK1, Hsc70, dipeptidyl peptidase IV (DPPIV), PP2A, megalin, and CaM kinase II (Donowitz & Li 2007). In the cell, NHE3 rarely exists as a monomer or a dimer (~90-180kDa) but forms complexes which range from 400kDa in the intracellular pool to ~1000kDa at the plasma membrane (Akhter, S. *et al.* 2002). These complexes are dynamic and are further changed by the stimuli participating in acute NHE3 regulation. The dynamic nature of NHE3 complexes allows association/dissociation from the cytoskeletal proteins, endocytosis/exocytosis and phosphorylation. These

mechanisms may act in concert to provide highly regulated turnover and activity of NHE3 (Donowitz & Li 2007).

NHE3 is regulated by binding to various PDZ domains containing proteins that are present in the brush border and act as scaffolds that connect the plasma membrane with members of the ezrin/radixin/moesin (ERM) family, thereby helping NHE3 link with the actin cytoskeleton. NHERFs (Na^+/H^+ exchanger regulatory factors) are PDZ domain proteins in or near the apical membrane and include NHERF1 and NHERF2 (both have 2 PDZ domains and an ERM binding domain), as well as NHERF3/PDZK1 and NHERF4/IKEPP (both have 4 PDZ domains) (Donowitz & Li 2007). NHE3 inhibition by cAMP in PS120 fibroblasts requires the presence of NHERF1 that links NHE3 to the actin cytoskeleton via binding to ezrin. In this case ezrin acts as a low-affinity protein kinase A anchoring protein (AKAP) (Yun *et al.* 1997, 1998). In the case of cGMP mediated inhibition of NHE3, NHERF2 acts as a G kinase-anchoring protein (GKAP) forming a complex between NHE3, NHERF2 and cGMP-activated protein kinase II (cGKII). This complex is anchored to the cellular cytoskeleton by the ezrin-binding domain of NHERF2 (Cha *et al.* 2005). Ca^{2+}-dependent regulation of NHE3 involves NHERF2, which binds α-actinin-4, a protein necessary for aggregation of the NHE3-containing plasma membrane complexes that occur after Ca^{2+} elevation and before NHE3 endocytosis (Kim *et al.* 2002). Thus, proteins like NHERFs are critical for NHE3 regulation, and their presence can explain a paradigm for multi-protein complexes required for regulation of cellular processes. NHE3 can also directly bind to the ERM proteins which is necessary

for basal trafficking, including basal exocytosis and delivery of newly synthesized NHE3 to plasma membrane (Donowitz & Li 2007). This association of NHE with the cytoskeleton seem to be yet another mechanism for the NHE3 regulation.

1.3.1.3 Long-term regulation of NHE3 and hypertension

In addition to the short-term NHE regulation that occurs in the kidney over minutes in response to drugs, changes in dietary intake of sodium, and changes in blood pressure/volume, these exchangers are also regulated over periods of hours to many days. The long-term regulation of NHEs generally mimic kidney disease related processes or responses to a disease or injury. The rat and human NHE3 promoters have GC rich regions and the former also contains atypical TATA and CCAAT boxes while the latter contains only TATA-like sequences. The rat NHE3 promoter includes putative cis-acting elements such as glucocorticoid and thyroid response elements, AP1, AP2 C/EBF, NF-1, Oct-1, PEA3, and Sp1 transcription-binding sites (Kandasamy & Orlowski 1996). The human NHE3 promoter contains transcription factor-binding sites for Sp1, AP-2, MZF-1, CdxA, Cdx-2, steroid and nonsteroid hormone receptor half sites, and a phorbol 12-myristate 13-acetate-response element (Malakooti *et al.* 2002). There are few studies focused on transcriptional regulation of NHE3. For example, glucocorticoid treatment significantly increased the luciferase activity of the chimeric NHE3 gene in renal epithelial OK cells and LLC-PK1 cells, thereby indicating that glucocorticoid regulation of NHE3 is mediated primarily by a

transcriptional mechanism (Kandasamy & Orlowski 1996). Interactions of multiple transcription factors and/or response elements are supposed to either up- or down-regulate NHE mRNA expression and thereby regulate the NHE3 gene followed by changes in protein levels (Zachos *et al.* 2005).

Altered expression and activity of NHE3 in primary hypertension have implied a potential role of this transporter in the pathogenesis of high blood pressure. Spontaneously hypertensive rats (SHR) have elevated NHE3 activity and abundance in isolated renal proximal tubules (LaPointe *et al.* 2002) suggesting that increased renal reabsorption of sodium may contribute to systemic sodium retention and development of hypertension. Studies in obese Zucker rats suggested that reduction in D1-like receptor binding sites, defective coupling with signaling pathway and reduced PKA activation may be responsible for the failure of dopamine to inhibit NHE3 in proximal tubules of obese rats and may contribute to their increase in sodium reabsorption and development of hypertension (Hussain *et al.* 2001). As mentioned earlier, in our model of diet-induced obesity we observed activation of the renin-angiotensin system, and elevated levels of insulin and leptin. Each one of those factors can have an effect on expression and/or activity of NHE3 in the proximal tubule. *In vivo*, intravenous angiotensin II was shown to stimulate bicarbonate absorption (presumably mediated by NHE3) in proximal tubule by reducing cAMP (Liu & Cogan 1989). Moreover, Xu *et al.* observed that in OK cells prolonged exposure to angiotensin II increased NHE3 mRNA and stimulated NHE3 activity by stimulating NHE3 promoter activity (Xu *et al.* 2006). Acutely, insulin has been shown to increase

NHE3 activity in OK cells and chronically, stimulation of NHE3 activity was accompanied by an increase in protein and transcript levels in the same cell line (Klisic *et al.* 2002). More recently, it was demonstrated by Fuster *et al.* that chronic insulin treatment activated NHE3 through phosphatidylinositol 3-kinase-serum and glucocorticoid dependent kinase 1 pathways (Fuster *et al.* 2007). Although sodium retention observed in essential hypertension apparently involves increased activity of NHE3 additional studies are necessary to elucidate its contribution to sodium absorption in obesity-induced hypertension.

1.3.2 The sodium-potassium activated adenosine 5'-triphosphate (Na,K-ATPase)

The most abundant ion pump in higher organisms is the sodium-potassium pump or Na,K-ATPase. It is found in the plasma membrane of practically every eukaryotic cell and is responsible for maintaining the low sodium and high potassium concentration in the cytoplasm using ATP hydrolysis. For every molecule of ATP hydrolyzed, three Na^+ ions from the intracellular space and two K^+ ions from the extracellular space are exchanged. Thus, Na,K-ATPase contributes substantially to the maintenance of the resting membrane potential of the cell and osmotic regulation of the cell volume. In addition, the electrochemical Na^+ gradient is the driving force for secondary transporter system such as transport of sodium into intestinal and renal epithelial cells, as well as the transport of other nutrients, such as glucose, amino acids, and ions, like Ca^{2+}. Na,K-ATPAse activity is irregularly distributed In the kidney: highest in

the outer medulla, intermediate in the cortex and lowest in the inner medulla and is restricted to the basolateral membrane of epithelial cells (Iannello *et al.* 2007).

1.3.2.1 Structure of Na,K-ATPase

The Na,K-ATPase belongs to the P-type ATPases, a family of enzymes that is phosphorylated by ATP (Horisberger 2004). This pump is a heterodimer of two major subunits: a α-subunit and a β-subunit. However, other proteins, such as members of the FXYD family of proteins, interact with this enzyme in some tissues, such as heart, kidney, and brain and are referred to as the γ-subunit.

The catalytic α-subunit has a molecular mass of 100-113 kDa, depending on the component isoforms α1, α2, α3, and α4 which are expressed in a tissue-specific manner. The α1 is present ubiquitously, while the α2 is detected mainly in skeletal muscle, heart, and certain neuronal cells (neurons and astrocytes). The α3 isoform is expressed mainly in neurons, and α4 is expressed in sperm (Blanco 2005). It is reasonable to believe that the tissue-specific distribution of the α-isoforms indicates that each isoform exhibits a particular function associated with the tissue in which it is expressed. The α-subunit has 10 transmembrane segments and both N- and C-termini on the cytoplasmic site; it transports the cations, hydrolyzes ATP and has binding sites for specific cardiac glycoside inhibitors such as ouabain, as well as several phosphorylation sites.

The β-subunit is a ~40 kDa glycoprotein. The mass of the protein moiety depends on the presence of β1, β2, or β3 isoforms. It crosses the membrane only

one time and the N-terminus is localized on the intracellular side of the membrane. The β-subunit acts a molecular chaperone for the correct membrane insertion and packing of the newly synthesized catalytic α-subunit, modulates the transport properties of mature Na,K-ATPase, and may play a role in processes related to cell polarity (Geering 2008).

The third subunit of Na,K-ATPase, the γ-subunit is a family of proteins which are tissue-specific auxillary subunits of Na,K-ATPase and are referred to as FXYD proteins (Geering 2008). They are not required for Na,K-ATPase normal activity, however, they can modify it. The seven members of the mammalian FXYD proteins family are small membrane proteins containing the FXYD motif and two conserved glycine residues in the transmembrane domain together with the serine residue. One of these proteins (FXYD2) has indeed been known as the γ-subunit of the Na,K-ATPase, but all members of this group have now been shown to be associated with the α/β-subunit of Na^+,K^+-ATPase and modulate some aspect of its function by changing its substrate affinity and by providing protection against thermal inactivation of Na,K-ATPase (Geering 2008). In the kidney, the main FXYD proteins expressed are: FXYD2 subunit and FXYD4 (or CHIF, corticosteroid hormone induced factor). FXYD2 is mainly expressed in proximal tubules and in the thick ascending limb of the loop of Henle which are the renal segments reabsorbing most of the filtered Na^+ load. FXYD4 is located in the outer and inner medullary collecting ducts and in the cortical collecting duct (Geering 2006). FXYD2 and FXYD4 have the most prominent, opposite effects on the Na^+ affinity of Na,K-ATPase. Whereas FXYD2

decreases, FXYD4 increases affinity of Na,K-ATPase for intracellular Na^+ which is consistent with the role of those proteins in the efficient reabsorption of sodium in the nephron segments with the high and low Na^+ load, respectively (Geering 2006).

1.3.2.2 Mechanism of Na,K-ATPase regulation

In general, Na,K-ATPase activation is tissue- and isoform-specific and in the rat kidney only α-1 and β-1 subunits are expressed (Therien *et al.* 1996). Regulation of the Na,K-ATPase activity can occur by different cellular mechanisms and can be achieved by modulation of the number of enzyme molecules present at the plasma membrane or by influencing the activity of the Na,K-ATPase already located at the cell surface. For long-term regulation, the amount of the enzyme at the plasma membrane can be modified by changes in the rate of synthesis or degradation of the individual Na,K-ATPase polypeptides while for short-term regulation this could be done by mobilization of Na pump molecules from the endosomal pools to the cell surface (Therien & Blostein 2000). Alternatively, the activity of the Na,K-ATPase at the cell surface can be directly regulated providing a rapid adjustment in its function. Several effectors have been implicated in this acute response; the primary one is the intracellular Na^+ concentration. Moreover, the activity of kidney tubule Na,K-ATPase is under control of hormones which can regulate natriuresis and Na^+ reabsorption in renal

cells such as catecholamines, peptide hormones and steroid hormones which have been shown to exert short- and long-term effects on Na,K-ATPase acivity.

Aldosterone, a steroid hormone synthesized and released by the adrenal cortex has long been known to promote Na^+ reabsorption and K^+ release in kidney epithelial cells. The long-term effect of aldosterone on Na,K-ATPase is to increase expression of transporter which is generally mediated by changes in mRNA/protein synthesis induced by direct interactions of receptor/corticosteroid complexes with the nuclear DNA. However, the short-term effects of aldosterone are thought to be mediated not by the nuclear receptor but instead by membrane-specific receptors (Therien & Blostein 2000) and is dependent on or independent of intracellular Na^+ concentration. An example of a Na^+-dependent effect is the translocation of Na,K-ATPase to the plasma membrane (Therien & Blostein 2000).

Among the catecholamines that affect Na,K-ATPase activity are dopamine and norepinephrine which often act antagonistically. Dopamine is a natriuretic factor synthesized in the kidney proximal tubule. Dopamine inhibits Na,K-ATPase activity in this segment of the nephron, through the dopamine receptors, DA_1 and DA_2, via a G protein-linked, PKC-dependent pathways (Bertorello & Aperia 1990). In the distal segments dopamine also inhibits Na,K-ATPase but acting mainly through DA_1 receptors and PKA-associated pathways (Satoh *et al.* 1993). It has to be noted, that in the rat isolated proximal tubule cells dopamine activated PKC and inhibited Na,K-ATPase activity by direct phosphorylation of Na,K-ATPase at a serine residue that induced transporter internalization via

clathrin-dependent endocytosis (Chibalin *et al.* 1999). On the other hand, the PKA-activated pathway of dopamine inhibition seems to involve phosphorylation of both the sodium pump and the so-called dopamine and cAMP-regulated phosphoprotein (DARPP-32), the latter being an inhibitor of a protein phosphatase (Aperia *et al.* 1991).

In contrast to dopamine, other catecholamines including norepinephrine, vasopressin, and angiotensin II stimulate Na,K-ATPase activity in various tissues including the kidney. In the kidney proximal tubules, stimulation of the sodium pump by α-adrenergic agents has been shown to involve protein phosphatase 2B (PP2B), a Ca^{2+}- and calmodulin-dependent phosphatase also called calcineurin. Because the actions of norepinephrine in the kidney appear to oppose the inhibitory effects of dopamine, it has been suggested that the Na,K-ATPase is regulated in this organ by the antagonizing actions of calcineurin, which would serve to keep the pump in an active, dephosphorylated state, and protein kinases, which would maintain the enzyme in an inactive, phosphorylated form (Therien & Blostein 2000). Efendiev *et al.* demonstrated that in a cell culture model of proximal tubule epithelial cells -LLC PK1, angiotensin II stimulated Na,K-ATPase activity by activating the PKC beta isoform followed by phosphorylation of α1-subunit at Ser11 (Efendiev, & Pedemonte 2006) and possibly its recruitment to the plasma membrane.

Insulin is a peptide hormone that can exert short- and long-term effects on Na,K-ATPase regulation. There are several mechanisms by which insulin can activate Na,K-ATPase. One example is an increase in intracellular Na^+

concentration; another is a change in the apparent affinity of the enzyme for Na^+ (Feraille *et al.* 1994). However, the mechanism of short-term regulation of Na,K-ATPase by insulin is not clear. The effect of insulin on Na,K-ATPase activity in fibroblasts depends on PI3-K, the zeta isoform of PKC, and p38MAP kinase (Sweeney & Klip 1998, Sweeney *et al.* 1998). Feraille and coworkers have shown that insulin-induced stimulation of Na,K-ATPase was associated with phosphorylation of Tyr10 residue on the α-subunit of Na,K-ATPase (Feraille *et al.* 1994). From the studies summarized above, it is evident that the effect of the hormones that regulate Na,K-ATPase are mediated by signaling intermediates targeting certain protein kinases and phosphatases.

Agents that increase cellular cAMP, as well as exogenous derivatives of cAMP, lead to Na,K-ATPase stimulation in the proximal convoluted tubules and to Na,K-ATPase inhibition in the medullary thick ascending limb of the loop of Henle and the cortical collecting duct (Therien & Blostein 2000). This response seems to be mediated, at least in part, by protein kinase A (PKA)-directed phosphorylation of Ser943 of the Na,K-ATPase α-subunit (Fisone *et al.* 1994). Moreover, cAMP-PKA induced the recruitment of active Na,K-ATPase units to the plasma membrane of the rat proximal tubule (Carranza *et al.* 1998). Phosphorylation of the Na pump α-subunit is reversible, as demonstrated by the decrease in Na,K-ATPase activity after activation of a dopamine- and cAMP-regulated phosphoprotein (DARPP-32), an endogenous inhibitor of protein phosphatase 1 (PP1) (Aperia *et al.* 1991). Nevertheless, DARPP is expressed at low levels in proximal tubules and this fact most probably precludes its role of

Na,K-ATPase regulator in this segment of the nephron. However, a phosphorylation/dephosphorylation event may dynamically regulate the activity of the Na,K-ATPase.

Some observations show that in the proximal nephron and in cultured canine kidney cells, Na,K-ATPase is inhibited by phorbol esters or diacylglycerol analogs, in a process that involves protein kinase C (PKC) activation (Bertorello 1992) and, possibly, the phosphorylation of the Na^+ pump α-subunit at Ser16 (Beguin *et al.* 1994). Ser23 has also been identified as a potential site for phosphorylation (Logvinenko. *et al.* 1996) together with Ser11 and Ser18 (Feschenko & Sweadner 1995). The general consensus is that PKC phosphorylation occurs primarily at the N-terminus of the α-subunit (Pedemonte *et al.* 1997). This region exhibits the most sequence divergence, both among species and isoforms. For example, Ser16 is present only in the α1-isoform but not on any other isoform, whereas Ser23 is present in the α1-subunit of the rat, and α1-subunit of human, pig, and mouse do not have Ser18 in their N-terminal region. In addition, the mammalian enzyme is phosphorylated at low levels at Ser16, and in the rat, at higher levels on Ser23 (Feschenko & Sweadner 1995, Efendiev & Pedemonte 2006). In contrast, phosphorylation of the α1-isoform by PKA takes place at Ser943 in a highly conserved cytoplasmic region between transmembrane segments 8 and 9. Thus the same residue might be involved in the phosphorylation of different isoforms by this protein kinase.

Recently, Efendiev *et al.* demonstrated that human-type Na,K-ATPase can be phosphorylated at the same serine residues by hormones affecting

enzyme activity in opposite ways (Efendiev & Pedemonte 2006). For instance, in LLC PK1 cells, dopamine inhibited while angiotensin II stimulated Na,K-ATPase activity and those opposite effects were mediated by the phosphorylation of Ser11 of α1-subunit of Na,K-ATPase by activation of PKC zeta and PKC beta, respectively. As mentioned before, the mechanism involved either endocytosis or exocytosis of phosphorylated enzyme from the plasma membrane or the intracellular compartment.

In the kidney, another kinase, protein kinase G (PKG), and the production of cGMP appear to mediate the loss of salt and water produced by acetylcholine, bradykinin, and atrial natriuretic peptide and to reduce Na,K-ATPase activity (Therien & Blostein 2000). It is not clear whether direct phosphorylation of the enzyme or secondary modulators are involved.

1.3.2.3 Na,K-ATPase in pathophysiological states

Na,K-ATPase activity is often altered in some models of obesity induced hypertension. Bickel and coauthors demonstrated that in 2 and 4 month old, hypertensive, insulin-resistant but non-diabetic, obese Zucker rats Na,K-ATPase abundance was significantly increased in the proximal tubule (Bickel *et al.* 2001) which was attributed to increased insulin levels. However, in slightly older (6 month old) obese Zucker rats, at early stages of diabetic nephropathy with insulin resistance progressing to diabetes, abundance of Na,K-ATPase was not changed at the proximal tubule while there was a decrease in the abundance of

apical transporters reflected in an almost 3-fold increase in the fractional excretion of sodium (Bickel *et al.* 2002). In another study young, obese Zucker rats which were hyperinsulinemic, hyperglycemic, and hypertensive displayed impairment in dopamine D_1 receptor function. This caused an ineffective coupling of the receptor with the G protein/effector enzyme complex signaling pathway leading to a lack of PKA activation, and subsequently to an increase in the activity of Na,K-ATPase (Hussain *et al.* 1999, 2001). In addition, it was reported that increased blood pressure in obese Zucker rats is accompanied by impaired pressure-natriuresis consistent with reduced dopamine-induced inhibition of sodium transporters and, subsequently, decreased sodium excretion.This could be provoked by hyperinsulinemia and/or other circulating factors associated with obesity since response to dopamine-induced inhibition of Na,K-ATPase of proximal tubule epithelial cells from both lean and obese Zucker rats expressing functional D_1 receptors was blunted when those cells were incubated with obese rat serum (Banday *et al.* 2004). Overall, further studies are necessary to address the mechanism of Na,K-ATPase regulation in obesity induced hypertension and how this relates to the impairment in pressure natriuresis.

1.4 Peroxisome proliferator-activated receptors (PPARs)

Peroxisome proliferator-activated receptors are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors. To date, three different PPAR subtypes have been cloned and characterized: PPAR-α,

PPAR-β/δ, and PPAR-γ.PPARs have been shown to be critical factors in regulating diverse biological processes, including lipid metabolism, adipogenesis, insulin sensitivity, immune response, cell growth and differentiation (Desvergne & Wahli 1999, Fajas *et al.* 2001, Guan & Breyer 2001, Willson *et al.* 2001). PPARs participate in the pathogenesis of a cluster of human diseases designated the metabolic syndrome, which includes insulin resistance, glucose intolerance, obesity, dyslipidemia, hypertension, atherosclerosis, and microalbuminuria (Ginsberg 2003, Gurnell *et al.* 2003, Scott 2003).

Like other nuclear receptors, PPARγ contains a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), and two transcription-activation function motifs (N-terminal ligand-independent AF-1 motif, C-terminal ligand-dependent AF-2 motif) (Rosen & Spiegelman 2001). PPARs regulate transcription of target genes by heterodimerizing with the retinoid X receptor (RXR) upon ligand activation and binding to PPAR response element (PPRE) of regulatory promoter regions of target genes (Tugwood *et al.* 1992, Gearing *et al.* 1993). This response element, generally of the direct repeat 1 (DR-1) type, is composed of two half-sites that occur as a direct repetition of the consensus sequence AGGTCA with a single nucleotide spacing between the two repeats (Michalik *et al.* 2006). Following binding of the PPAR/RXR complex to PPRE, different co-factors are recruited and may either induce (coactivators) or inhibit (corepressors) target gene transcription (Nolte *et al.* 1998). Various ligands engage different co-factors and result in different effects of the receptor on gene transcription. It has been also shown that nuclear receptor co-repressors (NCoR)

and the related factors, such as silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), bind to unliganded nuclear receptors and repress target gene expression until ligand triggers their dismissal accompanied by recruitment of coactivators (Glass & Rosenfeld 2000). However, selective action of a given PPAR isotype *in vivo* probably results from a complex interplay between expression levels of the PPAR and RXR isotypes, affinity for a specific promoter PPRE, ligands and cofactors availability, and possibly other transcription factor binding in the vicinity of the PPRE.

A wide range of compounds have been identified as PPAR ligands. PPARγ is activated by various metabolites derived from arachidonic acid through the lipoxygenase and cyclooxygenase pathways, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15-PGJ2) and 15-hydroxyeicosatetraenoic acid (15-HETE) (Willson & Wahli 1997, Nagy *et al.* 1998). FA-derived compounds from oxidized LDL (9-HODE, 13-HODE) are also natural PPARγ ligands (Shureiqi *et al.* 2003). In addition, the thiazolidinediones (TZDs) including rosiglitazone (Avandia) and pioglitazone (Actos) are synthetic, high-affinity ligands for PPARγ (Willson & Wahli 1997) and are insulin sensitizers used to treat hyperglycemia and type 2 diabetes.

PPARγ is expressed as two isoforms γ1 and γ2 in rodents and three isoforms γ1, γ2, and γ3 in humans (Fajas *et al.* 1997, 1998). Adipose tissue and large intestine have the highest levels of PPAR-γ mRNA; kidney, liver, and small intestine have intermediate levels (Fajas *et al.* 1997); PPAR-γ2 and γ3 are predominantly expressed in adipose tissue in both mouse and human (Fajas *et*

al. 1997, 1998). In rodents, Verreth *et al.* found that compared with lean mice, PPARγ expression was downregulated in obese double-knockout mice, and that diet restriction caused upregulation of PPARγ (Verreth *et al.* 2004). Dobrian *et al.* showed that PPARγ mRNA expression and activity in the renal cortex and medulla of obesity-prone rats were significantly lower compared with that in obesity-resistant rats (Dobrian *et al.* 2004). Vidal-Puig *et al.* reported that exposure to high fat diet increases adipose tissue expression of PPARγ in normal mice (Vidal-Puig *et al.* 1996). This implies that PPARγ expression might be stimulated by dietary fat until a determined threshold is reached and/or some other factors such as insulin may prevent further induction of PPARγ in obese animals.

1.4.1 PPARγ, obesity, insulin resistance, and type 2 diabetes

PPARγ has been implicated in the pathogenesis of obesity. PPARγ is expressed in various tissues with a higher level of expression in adipose tissue. Moreover, PPARγ target genes are generally involved in the lipogenic pathways and the storage of fatty acids in adipose tissue which is consistent with a role for PPARγ in differentiation of adipose tissue both *in vitro* and *in vivo* (Michalik *et al.* 2006). A missense mutation in the gene for PPARγ2 that resulted in the conversion of proline to glutamine at position 115 was associated with severe obesity in humans as well as accelerated adipocytes differentiation and greater

cellular accumulation of triglyceride compared to the wild-type PPARγ2 (Ristow *et al.* 1998). In contrast, a Pro12Ala substitution in PPARγ2 was associated with decreased binding affinity to the PPRE, reduced ability to transactivate responsive promoters, and with lower body mass index in middle-aged and elderly subjects (Deeb *et al.* 1998). In addition, treatment with TZDs resulted in lowering of triglyceride levels but also influenced weight gain and redistribution of body fat with an increase in subcutaneous depot (Guan & Breyer 2001). The mechanisms which contribute to the pathogenic role of PPARγ in obesity are not known but leptin was proposed as one of the modulators as well as TNF-α and uncoupling proteins (UCPs) (Guan & Breyer 2001). Nevertheless, these data demonstrate that PPARγ plays an important role in the pathogenesis of obesity.

In addition, PPARγ is involved in glucose metabolism. TZD treatment in patients with type 2 diabetes improves insulin resistance, hyperinsulinemia and hyperglycemia. Furthermore, Barroso *et al.* reported heterozygous mutations in the ligand-binding domain of PPARγ in subjects with severe insulin resistance, diabetes and hypertension thus providing genetic evidence for the role of this receptor in glucose metabolism and blood pressure control in humans (Barroso *et al.* 1999). Interestingly, this loss-of-function mutation was not associated with obesity. Also, Ristow *et al.* showed that patients with gain-of-function mutation of PPARγ are severely obese but have low levels of insulin and increased sensitivity to insulin (Ristow *et al.* 1998). These data suggest that excess PPARγ activity could contribute to obesity while reduced PPARγ activity might elicit insulin resistance. However, the study of animal models revealed that although PPARγ

is essential in regulating insulin sensitivity, the molecular mechanism is not well understood (Guan & Breyer 2001).

1.4.2 PPARγ and hypertension

PPARγ is also involved in blood pressure regulation. However, there are controversial data showing that PPARγ activation can have hypertensive or hypotensive effects. In a model with a generalized PPARγ ablation, the mice displayed severe lipodystrophy and insulin resistance and had surprisingly low blood pressure (Duan *et al.* 2007) associated with increased vascular relaxation and activation of the renin-angiotensin system. This is consistent with the recent findings by Todorov *et al.* showing that PPARγ agonist stimulates the renin gene in Calu-6 cells in culture (Todorov *et al.* 2007) suggesting that in same cells PPARγ leads to activation of the renin-angiotensin II system with relevance to blood pressure regulation. On the other hand, a study by Barroso *et al.* showed that patients with a dominant-negative mutation in PPARγ have extreme hypertension, severe insulin resistance and diabetes (Barroso *et al.* 1999). This serves as a proof of concept evidence for the role of this receptor in human metabolism and suggests a beneficial role for PPARγ activation in blood pressure reduction. Consistent with these data, TZDs, have been shown to lower blood pressure in hypertensive fatty Zucker rats (Yoshioka *et al.* 1993, Buckingham *et*

al. 1998), obese diabetic rats (Yoshimoto *et al.* 1997), diet-induced hypertensive rats (Buchanan *et al.* 1995, Kaufman *et al.* 1995, Uchida *et al.* 1997, Dobrian *et al.* 2004), obese insulin-resistant mice (Verreth *et al.* 2004), obese insulin-resistant humans (Nolan *et al.* 1994), and non-diabetic patients with arterial hypertension (Fullert *et al.* 2002). The mechanism by which blood pressure falls is not known, but in insulin-resistant animals, the blood pressure reduction may be at least partly due to increased insulin sensitivity (Uchida *et al.* 1997, Yoshimoto *et al.* 1997). Direct vascular effects of PPARγ may also play a role as PPARγ is expressed in endothelium and vascular muscle cells (Marx *et al.* 1999, Law *et al.* 2000). Furthermore, PPARγ activation possibly modulates release of endothelial vasoactive factors such as endothelin-1, prostacyclin, and nitric oxide (Ruan *et al.* 2008) and in this way indirectly affects blood pressure regulation. Recent data from Dobrian *et al.* support this by showing that treatment with TZDs prevented hypertension in Spraque-Dawley rats on high fat diet, reduced oxidative stress and increased renal formation of NO (Dobrian *et al.* 2004). Potentially, PPARγ may also directly reduce vascular tone by downregulating angiotensin II receptor 1 or by attenuation of sympathetic overactivity (Ruan *et al.* 2008). In summary, most of the data identify hypotensive role of PPARγ activation which can be attributed to multiple mechanisms.

The propensity for TZDs to cause fluid retention and pulmonary and peripheral edema has emerged as the most common, serious adverse drug reaction associated with these compounds (Dobrian 2006). The causes of edema and fluid retention with the use of TZDs are not known and are likely

multifactorial. However, increased renal sodium retention probably plays a role. This together with TZDs blood pressure lowering effect points to the direct role of PPARγ in regulating sodium and water reabsorption. Moreover, PPARγ and RXR have been found constitutively expressed in the inner medullary collecting ducts, proximal tubules, distal tubules, thick ascending limb of the loop of the Henle, glomerulus, and renal medullary microvascular endothelial cells in rats (Braissant *et al.* 1996, Yang *et al.* 1999, Nicholas *et al.* 2001), rabbits and humans (Guan *et al.* 1997, Guan & Breyer 2001) further suggesting their likely role in sodium and water reabsorption. Yang *et al.* demonstrated that PPARγ activation resulted in fluid retention and increased levels of nitric oxide but did not affect GFR or renal filtration fraction (RFF) in conscious rats indicating that the PPARγ effect on volume expansion was not related to changes in renal hemodynamics (Yang *et al.* 2003). Furthermore, a study by Zanchi and coworkers provided evidence that chronic treatment with a PPARγ agonist had no effect on the systemic and renal hemodynamic responses to salt in noninsulin-resistant, healthy, male volunteers. However, it reduced urinary sodium excretion and lithium clearance, suggesting increased sodium reabsorption at the proximal tubule and also significantly increased plasma renin activity. Body weights increased with pioglitazone treatment in most subjects (Zanchi *et al.* 2004). But not all data available support the above mentioned studies. Song *et al.* reported that three-day treatment with rosiglitazone produced reduction in creatinine clearance, an indirect measure of glomerular filtration rate, and also a reduction in sodium excretion and at the same time it lowered mean arterial pressure in normal Sprague-Dawley rats

(Song *et al.* 2004). Those discrepancies could be most likely attributed to different techniques used for GFR measurement or other experimental protocols. Moreover, duration of the treatments could also play a role in the final outcome of PPARγ activation.

Nevertheless, besides effecting hemodynamic parameters of the kidney, PPARγ may directly act on sodium transporters leading to reduction in blood pressure and/or fluid retention. Acute, 3 day treatment with rosiglitazone increased whole kidney protein abundance of the α-1 subunit of Na,K-ATPase, the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2), NHE3, the aquaporins 2 and 3, and endothelial nitric-oxide synthase (Song, J. *et al.* 2004) implying that blood pressure reduction observed with PPARγ agonists may occur at the level of the proximal tubule and the thick ascending limb of the loop of Henle. Further studies are needed to explain what mechanism leads to TZDs blood pressure lowering effect and whether it involves direct modulation of the sodium transporters.

1.5 Nitric Oxide

In the kidney, nitric oxide (NO) plays an important role in the control of renal hemodynamics (Romero *et al.* 1992), mediation of pressure natriuresis (Majid *et al.* 1993), blunting of tubuloglomerular feedback (Wilcox 1998), inhibition of tubular sodium reabsorption (Ortiz & Garvin 2002), and modulation of renal sympathetic nerve activity (Eppel *et al.* 2003). The net effect of NO in the

kidney is to promote natriuresis and diuresis (Lahera *et al.* 1991). Several investigators have shown that chronic inhibition of NO production by NO synthase inhibitors such as N^G-monomethyl-L-arginine acetate or N^G-nitro-L-arginine methyl ester (L-NAME) produces arterial hypertension in animals (Arnal *et al.* 1992, Ribeiro *et al.* 1992, Navarro *et al.* 1994, Fernandez-Rivas *et al.* 1995), a fall in glomerular filtration rate and an increase in filtration fraction (Ribeiro *et al.* 1992). The decreased NO production is associated with hypertension, ischemic heart disease, insulin resistance, and atherosclerotic disease (Moss *et al.* 2004, Zavaroni *et al.* 2004). NO is formed during oxidation of L-arginine to L-citrullin by the action of the enzyme NO synthase (NOS). There are three NOS isoforms designated as constitutive neuronal NOS (nNOS, NOS I), inducible NOS (iNOS, NOS II) and constitutive endothelial NOS (eNOS, NOS III) (Stuehr 1999).

While expression of all three isoforms was reported in the kidney, the most consistent documented sites of constitutive NOS expression in the kidney are the macula densa for nNOS expression (Wilcox 1998) and the renal vasculature for the expression of eNOS (Bachmann *et al.* 1995). It is controversial whether the proximal tubule produces NO under basal conditions. However, the proximal tubule constitutively expresses iNOS mRNA (Ahn *et al.* 1994). In addition to iNOS, eNOS mRNA was also detected by RT-PCR in some of the rat proximal tubule segments (Ujiie *et al.* 1994). NOS activity was detected in isolated rat proximal tubules, primary cultures of rat or human proximal tubule cells, and proximal tubule cell lines (McLay *et al.* 1994, Yu *et al.* 1994, Guzman *et al.*

1995). In contrast, no studies have shown the presence of NOS proteins in the proximal tubule under basal conditions, which makes questionable whether the proximal tubule is able to produce NO constitutively. However, recent study provided evidence that in diabetic rats 3-5 wk after streptozotocin injection eNOS and nNOS expression in proximal tubules increases (Baines & Ho 2002). Moreover, Jarry *et al.* described the presence of nNOS in most tubules of the human nephron including proximal tubule by several different approaches such as immunohistochemistry, RT-PCR analysis, western blotting, and measurement of NOS activity (Jarry *et al.* 2003). In addition, it was noted that epithelial cells expressing nNOS also express soluble guanylyl cyclase, indicating that these cells possess the machinery for an autocrine/paracrine effect of nitric oxide. Why the earlier studies were not able to demonstrate the presence of NO protein in proximal tubule using the same techniques is hard to explain. However, whether or not there is a significant level of NOS protein expression in the normal kidney under physiological conditions, evidence suggests that the proximal tubule is constantly exposed to NO that might include NO from non-proximal tubule sources such as the vasculature or other nephron segments (Amorena & Castro 1997, Linas & Repine 1999) .

1.5.1 Functions of NO in the kidney

Synthesis of NO appears to play a substantial role in mediating pressure-induced diuretic and natriuretic responses in the kidney (Majid *et al.* 1993). In

anesthetized dogs intrarenal infusion of a NOS inhibitor blunted the diuretic and natriuretic responses to the increases in renal perfusion pressure (RPP) without altering renal blood flow (RBF) or GFR. This resulted in higher blood pressure that was prevented by the infusion of the NO synthesis precursor L-arginine (Salom *et al.* 1992). Furthermore, with the elevation of renal arterial pressure a significant increase in urinary excretion of NO metabolites was observed and there was a positive correlation between changes in NO metabolites excretion rate and changes in RPP or urinary sodium excretion (UnaV) (Suzuki *et al.* 1992, Majid *et al.* 1995) prompting the authors to suggest that during acute changes in RPP, intrarenal changes in NO production rate may be responsible for the changes in sodium excretion. Finally, changes in renal tissue NO activities were assessed directly using an NO-selective microelectrode inserted into the cortical tissue of anesthetized dogs and it was demonstrated that reductions of renal arterial pressure lead to decreases in tissue NO activity, urinary nitrate/nitrite excretion, and urinary sodium excretion (Majid *et al.* 1998). The mechanisms by which RPP effects renal production of NO are not entirely known however the shear stress is a likely candidate. Likewise, how increased NO in the kidney promotes natriuresis and diuresis is not well understood but a direct effect on tubular transport seems to play an important role. More recently, a study by Jin *et al.* showed that renal interstitial cGMP, formed as a result of stimulation of soluble guanylyl cyclase (sGC) activity by NO, induced natriuresis via protein kinase G without changes in renal blood flow or GFR (Jin *et al.* 2001). Furthermore, the same group demonstrated that pressure-natriuresis was

mediated by activation of the cGMP/PKG pathway in renal proximal tubule cells (Jin *et al.* 2004). In addition, there is evidence that export of cGMP from its renal synthesizing cells into the extracellular renal interstitial compartment was necessary for NO-induced natriuresis. This effect was accompanied by no change in GFR and an increase in fractional excretion of sodium and lithium suggesting a major role for renal proximal tubule cells in this type of natriuresis (Ahmed *et al.* 2007).

Many *in vitro* and *in vivo* studies have demonstrated that NO induces natriuresis by directly inhibiting nephron transport especially at the level of proximal tubule (Lahera *et al.* 1991, Jin *et al.* 2004, Ahmed *et al.* 2007). For example, McKee observed that in the kidney NO through generation of cGMP and stimulation of PKG inhibited Na,K-ATPase (McKee *et al.* 1994). Guzman *et al.* have shown that in mouse proximal tubule epithelial cells Na,K-ATPase activity decreased after NO production was induced by LPS/IFN gamma; this inhibition of Na,K-ATPase activity was prevented by simultaneous incubation with N omega-nitro L-arginine and markedly blunted by removal of L-arginine from the medium. The NO donors sodium nitroprusside and SIN-1 also inhibited Na,K-ATPase activity to a similar extent as LPS/IFN gamma (Guzman *et al.* 1995). Linas and Repine reported that NO produced by endothelial cells, caused a decrease in sodium transport and inhibition of Na,K-ATPase as well as an increase in cGMP levels in primary cultures of rat proximal tubule cells (Linas & Repine 1999).

In addition to a decrease in Na,K-ATPase activity, several studies have shown that NO decreases activity of NHE3. Roczniak *et al.* have shown that NO stimulated soluble guanylyl cyclase in rabbit proximal tubule and caused inhibition of Na^+/H^+ exchange which was at least partly mediated by the generation of cGMP (Roczniak & Burns 1996). Furthermore, following chronic treatment with L-NAME in Sprague-Dawley rats, the renal expression of NHE3 as well as expression and activity of Na,K-ATPase were increased (Kim *et al.* 2006) suggesting an inhibitory effect of NO on the transporters. As in previously cited studies, L-NAME treatment also significantly increased blood pressure and decreased fractional excretion of sodium with no effect on GFR. Finally, Coon *et al.* reported that in rabbit intestinal villus cell brush border membrane inhibition of constitutively expressed NOS with L-N(G)-nitroarginine methylester (L-NAME) stimulated Na^+/H^+ exchange while a selective inhibitor of inducible NOS, did not affect Na^+/H^+ exchange. These findings indicate that under physiological conditions constitutive NO most likely maintains an inhibitory tone on NHE3 expression (Coon *et al.* 2007). Although there are also studies that indicate an opposite effect for the NO (De Nicola *et al.* 1992) most of the data confirms that in the proximal tubule NO inhibits activity of NHE3 and Na,K-ATPase (Roczniak & Burns 1996, Stoos & Garvin 1997, Ortiz & Garvin 2002).

The other mechanism by which NO can regulate proximal tubule reabsorption appears to involve modulation of the effects of the sympathetic nervous system in the kidney. It has been reported that renal denervation prevented a decrease in proximal tubule sodium reabsorption caused by NOS

inhibition (Gabbai *et al.* 1995) and at the same time NOS inhibition significantly enhanced the vasoconstrictor responses to sympathetic nerve stimulation (Reid & Rand 1992). However, Khraibi demonstrated that the natriuretic effect of NO production inhibition in Okamoto spontaneously hypertensive rats was changed by kidney denervation, while in Wistar-Kyoto rats it was independent of renal innervation (Khraibi 1995) suggesting different effects depending on the animal model used. Moreover, a study by Wu found that while NO has a direct inhibitory effect on proximal tubular reabsorption of sodium its presence is also required for the stimulatory effect of renal sympathetic nerves on proximal sodium transport (Wu & Johns 2002).

Yet another mechanism by which NO can modulate proximal tubule transport is its interaction with angiotensin II. Several studies suggested that NO might be a direct modulator of ACE activity (Higashi *et al.* 1995, Takemoto *et al.* 1997), may regulate angiotensin II receptors *in vitro* through a cGMP-independent mechanism (Cahill *et al.* 1995), and an exogenous NO donor was able to abolish the stimulatory effect of angiotensin II on proximal tubule transport (Eitle *et al.* 1998).

There is a general consensus that hypertension is associated with impaired endothelium dependent vasodilation due to reduced NO signaling (Schiffrin & Touyz 2004). Endothelial dysfunction may result from reduced generation of NO due to diminished expression or activation of endothelial NO synthase or an increase in oxidative stress that reduces bioavailability of NO (Schiffrin & Touyz 2004). High levels of superoxide anions, which rapidly react

with NO to form highly toxic peroxynitrite anion is the mechanism leading to reduced NO bioavailability (Beckman & Koppenol 1996). Dobrian *et al.* reported that in obese hypertensive animals on high fat diet there was a reduction in urinary NO metabolites while expression of eNOS and nNOS were increased in the renal cortex and medulla of those animals (Dobrian *et al.* 2001, 2004) implying that in obesity-induced hypertension NO bioavailability is reduced most likely due to increased oxidative stress. Decreased renal cortical nitric oxide production was observed in the kidney of obese Zucker rats (Erdely *et al.* 2004) and a recent study in obese young humans showed a negative correlation between BMI and NO metabolites and a positive correlation between arginine and BMI suggesting increased NO production but reduced NO bioavailability (Gruber *et al.* 2008) .

However, another important mechanism which regulates the biological activity of NO in the kidney is the sensitivity of NO signalling. For example, Ortiz demonstrated that even though a high salt diet did not change NO production, it increased sensitivity of the thick ascending limb to NO with regard to sodium reabsorption (Ortiz *et al.* 2003).

In summary, obesity induced hypertension is extremely common in the general population especially with the increase in number of overweight and obese people. As in all other forms of hypertension, it is associated with the impairment of pressure-natriuresis. However, the exact mechanisms leading to fluid retention are not known but some suggest that direct change in tubular reabsorption might be a cause. We propose that in obesity, activities of the

sodium transporters at the proximal tubule level are increased leading to increased sodium reabsorption. Therefore, we determined the responses of obese and lean rats fed a high fat diet to changes in renal perfusion pressure and analyzed the expression, activity and localization of the tubular transporters NHE3 and Na,K-ATPase.

Moreover, as PPARγ is a transcription factor involved in obesity and hypertension we examined the role of PPARγ activation in hypertension related to obesity with regard to proximal tubule sodium transport, by chronically treating our obese and lean rats with a PPARγ agonist. To determine the direct involvement of PPARγ during the course of agonist treatment we studied modifications of NHE3 and Na,K-ATPase expression in cultured proximal tubule cells.

As NO might play an important role in regulating pressure natriuresis in the kidney and its availability seems to be altered in obese animals and humans, we wanted to determine its role in hypertension induced by obesity by chronically blocking its production *in vivo*. Moreover, since this molecule appears to affect some other pathways in blood pressure regulation such as the renin-angiotensin system, leptin, and insulin which are all activated in obesity, we further studied its role on the sodium transporters *in vitro*.

SECTION 2

SPECIFIC AIMS

Specific Aim #1

Theoretical and experimental studies have shown that in all forms of hypertension, including obesity hypertension, there is an abnormality of kidney function characterized by a hypertensive shift in renal pressure natriuresis. When obesity is induced by feeding a high fat diet, there is marked sodium retention and expansion of extracellular fluid volume. Moreover, sodium retention and altered pressure natriuresis appears to be caused mainly by increased tubular sodium reabsorption.

Hypothesis: Development of hypertension in diet-induced obesity is due to the increase in Na^+ reabsorption by means of changes in activity of sodium transporters at the level of the proximal tubule.

Aim 1: Determine mechanism of sodium handling in obese hypertensive (OP) and lean normotensive (OR) rats on high fat diet.

Aim 1.A. Establish the renal hemodynamic parameters in OP, OR rats.

Aim 1.B. Determine the abundance, activity and localization of proximal tubule sodium transporters-Na,K-ATPase and NHE3.

Experiments: In the first part of the study, Sprague-Dawley rats were put on the high fat diet and systolic blood pressure (SBP) was measured by tail cuff method at weeks 8 and 12 on the diet in obese and lean animals. Results were in

accordance to previous data to show that OP rats had high blood pressure while OR rats were normotensive.

After 12 weeks on the diet, renal function was assessed in OP and OR rats during surgical procedures in which animals were challenged with a change in renal perfusion pressure. Experiments were designed to determine whether there is a difference in sodium excretion between the groups and which nephron segment is defective with regard to sodium reabsorption. At the end of the experiment, fat content in obese and lean rats was measured while body weights were recorded weekly for the duration of the study.

Next, protein expression of proximal sodium transporters- Na,K-ATPase and NHE3 was examined by western blotting in basolateral membrane (BLM) and brush border membrane vesicles (BBMV) preparation of kidney cortex, respectively. In subsequent experiments activity of above transporters was assessed in fresh BLM and BBMV fractions from obese and lean rats. Immunohistochemistry was employed on paraffin-embedded, and formalin fixed kidney cortexes from OP and OR rats to determine localization of NHE3 within the villus membrane.

Specific Aim #2

Peroxisome Proliferator-Activated Receptor γ (PPARγ) modulates transcription of genes involved both in obesity and hypertension (leptin, angiotensin II). PPAR γ is expressed both in the adipose tissue and kidney. Two heterozygous mutations in the PPAR γ ligand binding domain of the gene cause

severe insulin resistance and hypertension in humans. Treatment with PPAR γ ligands lowers blood pressure in both humans and rodent models.

Hypothesis: PPARγ activation lowers blood pressure by altering activity of sodium transporters Na,K-ATPase and NHE3 and thereby controlling tubular Na^+ reabsorption.

Aim 2.A. Investigate the effects of *in vivo* PPARγ activation on blood pressure, renal hemodynamics, and expression, localization and activity of proximal tubule sodium transporters in obese and lean rats.

Aim 2.B. Determine whether PPARγ ligands exert their action on sodium transporters dependent or independent of PPARγ using primary cultures of human renal proximal tubule epithelial cells (RPTEC).

Experiments: In this aim OP and OR rats were treated with 0.1% (w/w) pioglitazone (PPARγ agonist) for 4 weeks from week 8 to week 12 during dietary regimen. The effect of PPARγ activation on the systolic blood pressure (SBP) was determined. SBP was measured in obese and lean animals by the tail cuff method as described in Aim #1, at week 8, before starting the treatments, and at week 12, after treatment completion, on the day of the surgery. Body weights and food intake were measured weekly. Before starting treatment and after its completion, rats from all experimental groups were placed in metabolic cages for 24 hour urine collection and the assessment of water intake. Urine was analyzed for Na^+ content.

In the second part of the study, glomerular filtration rate, urinary sodium excretion, fractional excretion of sodium and lithium were analyzed. Rats were

anesthetized and catheters were implanted in the left carotid artery for the measurement of mean arterial pressure and blood sampling, in the left jugular vein for fluid infusions (saline solutions with inulin and lithium chloride), and in the bladder for urine collection.

Separate groups of animals were used for the following part of the study where we examined by western blotting protein expression of Na,K-ATPase and NHE3 in baso-lateral membranes and brush border membrane vesicles, respectively, isolated from renal cortexes. The same preparations were also used for activity measurements of the above transporters.

Finally, we estimated the effect of pioglitazone treatment on NHE3 distribution in the renal proximal tubule in obese and lean rats as described in Aim #1.

In vitro experiments were designed to demonstrate the effect of pharmacological and molecular stimulation and inhibition of PPARγ on Na,K-ATPase and NHE3 expression using primary culture of renal proximal tubule epithelial cells (RPTEC). At first, cells were incubated with PPARγ agonists and antagonists for 4 hours and expression of sodium transporters were measured by western blotting. Those experiments confirmed that PPARγ stimulation decreases and inhibition increases protein abundance of Na,K-ATPase and NHE3.

Afterwards, using the nucleofection technique RPTEC were transiently transfected with full length human PPARγ to overexpress PPARγ or with small interfering RNA technique to silence the latter, followed by 12 hours of

pioglitazone treatment. Altogether, *in vitro* experiments suggested that pioglitazone effect on Na^+ transporters requires activation of PPARγ.

Specific Aim #3

NO stimulates soluble guanylyl cyclase in rabbit proximal tubule and causes inhibition of activity of Na,K-ATPase and Na^+/H^+ exchange which is at least partly mediated by generation of cGMP. In carotid arteries of obese rats levels of cGMP are enhanced. Previous studies demonstrate that feeding rats and dogs with moderately high fat diet results in higher levels of renin activity in obese animals compared with lean animals, higher levels of leptin in animals as well as obese humans and that those changes are paralleled by changes in insulin levels.

Hypothesis: The tonic inhibition on sodium transporters in the absence of NO is achieved in obesity by increased levels of Angiotensin II, Insulin or Leptin. These hormones act to maintain low activity of sodium transporters in obesity via an increase in cGMP production.

Aim 3.A. To investigate the effects of chronic *in vivo* inhibition of nitric oxide (NO) production on blood pressure, renal hemodynamics, and expression, localization and activity of proximal tubule sodium transporters in obese and lean rats.

Aim 3.B. To determine whether angiotensin II, insulin, or leptin modulate the expression of Na,K-ATPase and NHE3 in RPTEC *in vitro* by utilizing cGMP as a messenger.

Experiments: Sprague-Dawley rats were put on the high fat diet for 12 weeks and treated with nitric oxide synthase inhibitor-L-NAME (N^G-nitro-L-arginine methyl ester) between weeks 8-12. Rats were placed in metabolic cages before and after the treatment for 24-hour urine collection and the assessment of water intake.

After 12 weeks on the diet, renal function was assessed in OP and OR rats treated or not with L-NAME, during acute manipulation of renal perfusion pressure. Rats were anesthetized and catheters were placed in the trachea, jugular vein and in the left carotid artery for mean arterial pressure (MAP) measurement. A catheter was also implanted in the left femoral artery and in the bladder. MAP was measured and recorded continuously during the procedure. Blood and urine were analyzed for electrolytes, lithium, and inulin. As a result we obtained pressure-natriuresis and diuresis curves for obese and lean animals with or without chronic NO inhibition. Body weights were recorded weekly for the duration of the study while fat content was measured at the conclusion of the procedure.

Protein expression and activity of Na,K-ATPase and NHE3 in obese and lean rats with or without L-NAME treatment were analyzed as described in Aim #1.

Also, immunohistochemistry was employed to provide information on effect of L-NAME on NHE3 distribution as described previously in Aim #1.

In vitro experiments were performed using primary cultures of human renal proximal tubule epithelial cells (RPTEC). At first, ability of cells to produce

intra- and extracellular cGMP in response to various NO donor concentrations at different time points was assessed. It was followed by the measurements of the effect of ODQ (inhibitor of soluble guanylyl cyclase) and probenecid (an organic anion transporter). Those experiments proved that RPTEC are capable of producing cGMP in response to an NO donor in a concentration-dependent manner.

Next, we evaluated the capability of angiotensin II (AII), insulin and leptin, to affect production of cGMP in the presence or absence of NO donor s-nitro-acetylpenicillamine (SNAP) and have chosen concentrations of hormones which significantly increased cGMP in the presence of SNAP in RPTEC for further study.

Finally, we measured protein expression of Na,K-ATPase and NHE3 by western blotting in membrane fractions of RPTEC stimulated with AII, insulin or leptin together with SNAP for 6, 12 and 24 hours. Since these data were not conclusive, phosphorylation levels of Ser16 and 552 for Na,K-ATPase and NHE3, respectively were assessed in membrane fractions of RPTEC treated with AII/insulin and an NO donor. From this study we concluded that there is interaction between NO and insulin which influence phosphorylation of Na,K-ATPase and NHE3, probably leading to changes in activity of both transporters.

SECTION 3

STUDIES FOR SPECIFIC AIM #1

3.1 Materials and Methods for Specific Aim #1

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Eastern Virginia Medical School. Male 3-month old Sprague-Dawley rats (Charles River, Wilmington, MA), weighting ~300 g were housed individually in a humidity- and temperature-controlled room with a 12 hr dark-light cycle and allowed ad libitum access to water and food throughout the experiment . Rats were fed either a moderately high fat diet (MHF; ~ 32% kcal as fat; Research Diets, New Brunswick, NJ) or low fat (~11.8% kcal as fat) calorie matched control diet (controls) for 8 weeks. Between weeks 6 and 7 they were assigned, based on the difference in the body weight (BW) gains to an obesity-prone (OP) and an obesity-resistant (OR) group as described previously (Dobrian *et al.* 2001) (Fig. 3). All the OP rats had body weights higher that the heaviest control rat and all the OR rats had body weights equal or lower than the heaviest control. The control animals were not used later in any of the experimental protocols. Separate groups, fed and treated exactly the same way, were used for physiological measurements and for preparation of membrane fractions, brush border membrane vesicles, and any subsequent experiments involving those preparations.

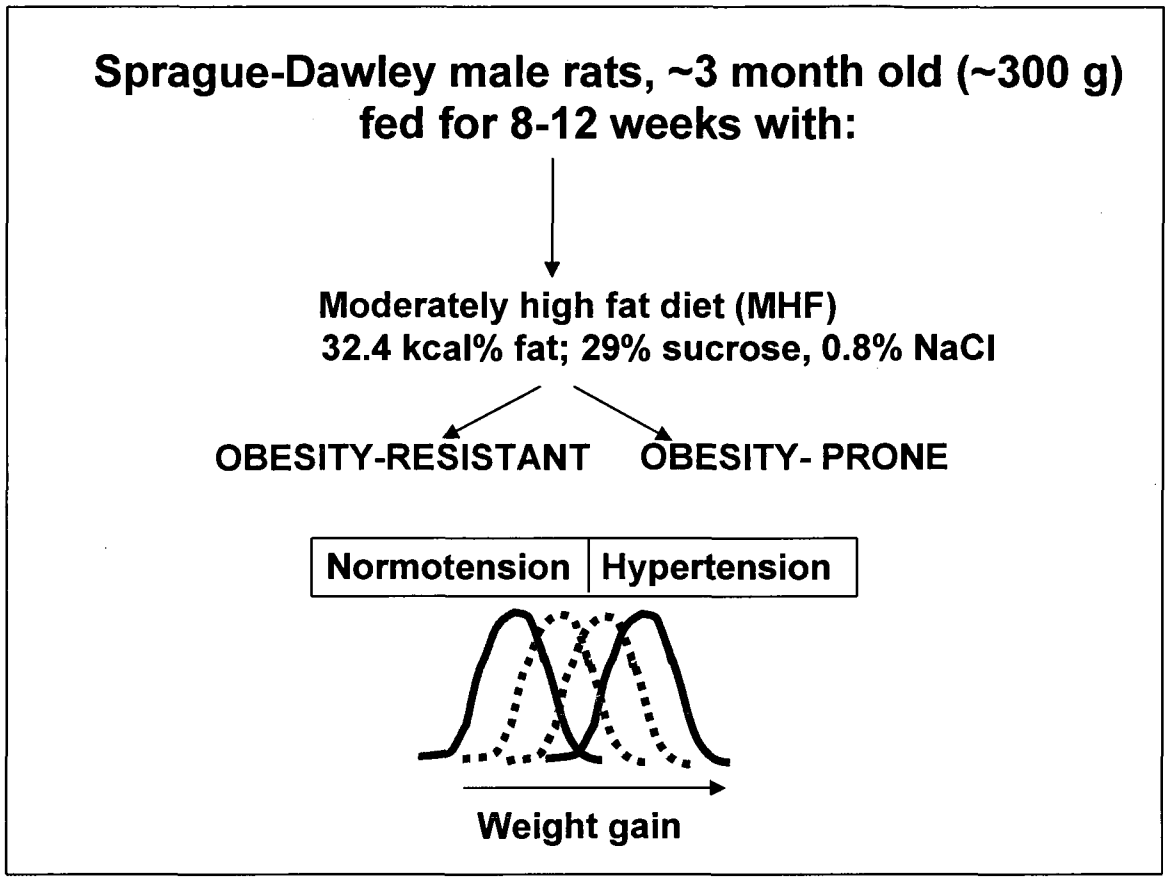

Figure 3 Rat model of diet-induced obesity and hypertension.
Male, 3 months old Sprague-Dawley rats were put on moderately high fat diet and stayed on this diet for 12-16 weeks. They diverged into two statistically different populations according to their body weights and were assigned to obesity-resistant (OR) and obesity-prone (OP) groups between weeks 6^{th} and 7^{th}. After being on the diet for 8-10 weeks OR rats were normotensive while OP rats developed high blood pressure.

Surgical procedure for acute pressure natriuresis experiments

The surgical procedure was performed as previously described (Khraibi 2000). On the day of the acute experiment, rats were anesthetized with Inactin (100 mg kg^{-1}), and catheters were placed in the trachea (PE-240) and left jugular vein (PE-50) for intravenous infusion of 0.75 ml per 100 g body wt^{-1} h^{-1} of saline with 6 mM lithium chloride (LiCl) and 0.75 ml per 100 g body wt^{-1} h^{-1} of a solution of 3% inulin and 6.25% bovine albumin in saline (which also contained 6 mM LiCl). A PE-50 catheter was implanted in the left carotid artery for mean arterial pressure (MAP) measurement and blood withdrawal. A PE-50 catheter was implanted in the left femoral artery for the measurement of renal perfusion pressure (RPP). A PE-90 catheter with a flared tip was placed in the bladder for urine collection. An adjustable clamp was placed around the abdominal aorta above both renal arteries and was used to control RPP. The rats were allowed 1 h to recover after completion of the surgical procedures. Then RPP was controlled at a lower level (~100 mmHg) by tightening the clamp around the aorta and reducing renal perfusion pressure. After 10 minutes a clearance period of 30 min was started; MAP was measured and recorded continuously. At the end of this period, ~1 ml of blood was withdrawn from the left carotid artery for plasma electrolytes, lithium, and inulin measurements. Urine was collected for 30 min. At the conclusion of the clearance period the aortic clamp was loosened to allow renal perfusion pressure to increase and then tightened again at the higher level (~140 mmHg). After 10 minutes the second clearance period started during which MAP was measured and recorded continuously. And again at the end of

this period, ~1 ml of blood was withdrawn from the left carotid artery for plasma electrolytes, lithium, and inulin measurements. Urine was collected continuously for 30 min. All rats were killed by an Inactin overdose (50 mg; 0.5 ml of a solution of 100 mg ml^{-1} of Inactin in saline) at the end of the experiment while still under deep anesthesia.

To avoid any manipulation of the kidneys, different groups of animals were used for basolateral membrane and brush border membrane vesicle fractions. At the completion of the diet protocol the rats were anesthetized with Inactin (100 mg kg^{-1}), kidneys were removed without any further handling, epididymal and perirenal fat were removed and frozen in liquid nitrogen, and finally rats were killed by an Inactin overdose as mentioned above.

Physiological parameters

Systolic blood pressure was assessed by tail-cuff method using Visitech Blood Pressure Analysis System (BP-2000-R; Visitech Systems, Apex, NC) on previously trained, conscious animals from groups used for physiological measurements. The average of 3-5 readings was recorded for each animal. *Glomerular filtration rate (GFR)* was calculated from the clearance of inulin, and inulin concentrations was measured by the anthrone method (Khraibi *et al.* 1989, Khraibi & Knox 1989). Lithium concentrations in plasma and urine were measured using flame photometry (model 943, Instrumentation Laboratory, Lexington, MA), phosphate by the method of Chen (Chen & Jorgensen 1956) and sodium concentration in plasma and urine were measured using EasyLyte Na/K

analyzer(Medica, Bedfrod, MA). *Fractional excretions of sodium and, lithium* were calculated as the ratio between their urinary excretion and the glomerular filtration rate. *Urinary sodium excretion* was expressed as the rate of sodium excretion per urine volume.

Baso-lateral membrane (BLM) and brush border membrane vesicle (BBMV) preparations

The kidneys were rapidly isolated after the last Inactin injection and placed on ice in the appropriate homogenizing buffers. Their capsules were removed; cortexes dissected out and then cut out into small pieces. One kidney was processed for baso-lateral membrane (BLM) preparation and the other for brush border membrane vesicles (BBMV) preparation.

For BLM preparation modified protocol from V. Scalera (Scalera *et al.* 1980) was used. Pieces of cortex were placed into 15 ml of the homogenizing buffer (sucrose buffer) which contained 10 mM Tris-HCl, 0.25 M sucrose, and 0.5 M phenylmethanesulphonyl fluoride (PMSF) at pH 7.6 and homogenized on ice with Polytron homogenizer at 20 000 rpm for 60-90 seconds. The crude homogenate was centrifuged at 3 000 ***g*** for 15 minutes to remove whole cells, and nuclei. The supernatant was then centrifuged at 30 000 ***g*** for 30 minutes and the fluffy, upper layer of the pellet was resuspended again in sucrose buffer, homogenized with Teflon glass homogenizer for 20 strokes and centrifuged at 30 000 ***g*** for 30 minutes. The pellet was resuspended in buffer that had 5 mM HEPES, 100 mM KCL, and 100 mM mannitol final concentration

at pH 7.2 (mannitol buffer) and centrifuged at 30 000 ***g*** for 30 minutes. The final pellet was dissolved in a small volume of mannitol buffer and the protein concentration for each lysate was determined on this freshly prepared sample by using a BCA protein assay kit (Pierce Chemical) with BSA as a standard. On the same day part of the lysate was used for Na,K-ATPase activity measurements and the rest was frozen at -80 °C for future western blotting.

For brush border membrane vesicles (BBMV) preparation protocol from J. Biber was used based on a different reactivity of the brush-border membrane compared to other cellular membranes with divalent cations, such as Mg^{2+} (Biber *et al.* 2007). Pieces of cortex were homogenized in the buffer which contained 12 mM Tris base, 5 mM EGTA, 300 mM D-mannitol, and 0.1 mM PMSF pH 7.1(buffer A) using a Polytron homogenizer (~20 000 rpm) for 90 sec, on ice. Ice-cold water was then added to this crude homogenate and mixed with it, followed by addition of magnesium chloride ($MgCl_2$) and subsequent incubation on ice for 15 min. Then homogenate was centrifuged at 3 000 ***g*** for 15 min and supernatant transferred to a new tube and centrifuged at 30 000 ***g*** for 30 minutes at 4 °C. The resulting pellet was resuspended in 1 ml of buffer containing 6 mM Tris base, 2.5 mM EGTA and 150 mM D-mannitol pH 7.1 (buffer B, which was prepared by diluting buffer A 1:1 with distilled water) by using a 27-gauge needle attached to a 1cc syringe. Then buffer B was added to final volume of 35 ml followed by addition of $MgCl_2$ and subsequent incubation on ice for 15 minutes. Next, the homogenate was centrifuged at

3 000 ***g*** for 15 minutes and the supernatant transferred into a clean tube and spun at 30 000 ***g*** for 30 minutes at 4 °C. The resulting pellet was resuspended in 1 ml of buffer B by using a 27-gauge needle attached to a 1cc syringe aspirating 5-7 times and buffer B was added to a final volume of 35 ml. The suspension was centrifuged for 30 minutes at 30 000 ***g*** and pellet consisting of brush border membrane vesicles was resuspended in 1 ml of buffer B. Protein for each lysate was determined in freshly prepared samples by using a BCA protein assay kit (Pierce Chemical) with BSA as a standard. NHE3 activity measurements were performed in the same day and the remainder of the lysate was frozen at -80 °C and used for western blotting.

Western Blotting on BLM and BBMV fractions

Na,K-ATPase and NHE3 protein expression were assessed in BLM and BBMV, respectively. The samples were diluted with a 1/5 vol of Laemmli buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% [wt/vol] SDS, 5% ß-mercaptoethanol, 10% [vol/vol] glycerol, and 0.001% bromophenol blue) and equal amounts of proteins (10-20 μg per lane) were subjected to SDS-polyacrylamide (7.5%) gel electrophoresis and blotted onto PVDF membranes. The membranes were treated with Odyssey Blocking solution diluted 1:1 with phosphate-buffered saline (PBS) to block nonspecific binding sites, for 1 hour RT, and incubated with either monoclonal anti-NHE3 antibody (1:500 dilution, Novus Biologicals, Littleton, CO), monoclonal anti-Na,K-ATPase antibody (Upstate , Lake Placid, NY,1:10 000 dilution), or monoclonal Villin (1:1 000 dilution, Sigma-Aldrich,

Saint Louis, MO) overnight. Villin served as loading control for NHE3, loading of Na,K-ATPase was assessed by Ponceau red staining. Antigen detection was performed using appropriate secondary antibodies conjugated to fluorescent tag (IRDye 680 and IRDye 800) at 1:15 000 dilution for 45 minutes at RT. Membranes were scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Semi-quantitative analyses of the specific bands were performed using Li-Cor Odyssey software. Results were expressed as relative fluorescence units (RFU).

Activity of Na,K-ATPase in BLM fractions from renal cortex

Na,K-ATPase activity was assayed by measuring the amount of inorganic phosphate (P_i) liberated from ATP during incubation with the fresh membrane fraction of renal cortex at 37 °C in the assay buffer. The assay buffer contained 120 mM NaCl, 20 mM KCl, 6.0 mM EGTA Na_2, 7.5 mM $MgCl_2$, 50 mM imidazole-HCl, 30 mM Tris-HCl (pH 7.5) and 50 μl of tissue fraction. Reaction was started with addition of 4 mM of Na_2ATP (Sigma-Aldrich, Saint Louis, MO), carried out for 15 minutes at 37 °C, and after that stopped by putting samples on ice and completed by adding 35 μl of ice-cold 72% trichloroacetic acid (TCA) solution (TCA, Sigma-Aldrich, Saint Louis, MO). Then samples were centrifuged at 5 800 ***g*** for 5 minutes and the supernatant used for further assay. To determine ouabain insensitive Na,K-ATPase activity, the same mixture as above was incubated with 5 mM ouabain (Sigma-Aldrich, Saint Louis, MO) dissolved directly into assay buffer, and final Na,K-ATPase

activity was calculated as the difference between the activities assayed in the absence of ouabain (total activity) and in the presence of 5 mM ouabain. P_i concentration was measured by the method of Chen (Chen *et al.* 1956). To 200 µl of the sample diluted 1:10 1.6 ml of working reagent was added and incubated for 90 minutes at 37 °C. Working reagent was prepared fresh daily by addition of 5 ml of 8 N H_2SO_4, 5 ml of 2.5% ammonium molybdate and 5 ml of 10% ascorbic acid added to 35 ml of water. After incubation absorbance was read at 820 nm. To correct for spontaneous ATP breakdown, the absorbance of the blank sample prepared as described above but with water instead of supernatant from the membrane fraction was read and subtracted from the absorbance of the test sample. The activity was expressed as mmol of P_i hydrolyzed by 1mg of protein during 1 minute of incubation time (mmol min^{-1} mg $^{-1}$ of protein). Each sample was assayed in duplicate.

Measurement of NHE3 activity in BBMV from renal cortex

BBMV were labeled with cell-permeable AM ester of the polar fluorescein derivative BCECF (Molecular Probes Inc., Eugene, OR) by incubation in a solution of BCECF-AM for 30 min in a buffer without Na^+ at pH 7.2. A sample containing 200 µg of protein was used for each measurement. The labeled sample was mixed with a buffer containing 150 mM NaCl, pH 9.2, using a stop-flow kinetic device (KinTek SF 2001, KinTek Corporation, Austin, TX); the change in pH response to a sodium load was recorded every 0.6 seconds for 1 minute for each preparation. To normalize for the potential differences in sample

protein the unlabeled samples were also measured in the same conditions and the readings were subtracted from the labeled samples. Dual-excitation ratio of 440/490 nm was used to transform the changes in fluorescence into changes in intracellular pH, according to the Henderson-Hasselbalch equation. The pKa of the indicator was determined by measuring the changes in fluorescence in response to different values of extracellular pH. That was done by mixing the labeled sample with buffers of known pH preincubated with nigericin (Sigma-Aldrich, Saint Louis, MO) - K^+/H^+ ionophore, which causes equilibration of intracellular and extracellular pH in the presence of a depolarizing concentration of extracellular K^+. The activity of NHE3 was calculated from the power of the first exponential curve and expressed as a rate of intracellular pH recovery vs. time in response to an extracellular sodium load. The specificity of the reaction for NHE3 was tested in several arbitrary samples in which 100 μM of 5-(N-Ethyl-N-isopropyl) amiloride (Sigma-Aldrich, Saint Louis, MO) was added to the membrane preparation 15 minutes before the beginning of the fluorescence recordings. At this concentration, the amiloride derivative is expected to inhibit virtually all NHE1 and NHE2 activity, with only minimal inhibition (~10%) of NHE3 activity.

Immunohistochemistry

Formalin-fixed, paraffin-embedded kidney sections (4 μm) were incubated at 55 °C for 1hr, deparaffinized, rinsed in water and processed for antigen retrieval with Dako Target Retrieval Solution (Dako Corporation,

Carpinteria, CA) for a total of 15 minutes. Then sections were blocked with 5% normal goat serum and 1% BSA in PBS for 1 hr at room temperature and simultaneously labeled with polyclonal NHE3 antibody (Catalog number AB3085, Lot: 23101099; Chemicon International, 1:200 dilution) and monoclonal Villin antibody (Catalog number 0258, Lot: 17; Immunotech, Chicago, IL; 1:50 dilution) in blocking solution , overnight, at 4 °C. Subsequently, sections were incubated with a mix of fluorophore-conjugated secondary antibodies-Alexa Fluor488 goat anti-rabbit for NHE3, 1:500 dilution; AlexaFluor594 goat anti-mouse for villin, 1:500 dilution (Molecular Probes, Inc., Eugene, OR) - for 45 min, at room temperature, followed by 1% Sudan black to block tissue autofluorescence. After final washes and mounting with VectaShield (VectaShield Mounting Medium, Vector Labs, Burlingame, CA) images were visualized and recorded by confocal fluorescence microscopy using a Zeiss LSM 510 laser scanning microscope (Carl Zeiss Microscopy, Germany). Some sections in which the primary antibodies were omitted were used as negative controls. Each tissue section was excited with two lasers, Argon (488 nm), and HeNe1 (543 nm) and scanned pixel by pixel using frame mode with line averaging. Emission signals were collected by using the 505-550 nm band pass and 560 nm long pass filters and quantified using a photomultiplier tube and the LSM5 software. The composite image consisted of green staining for NHE3, red staining for villin, and yellow staining for colocalization of the two. For each slide 6-10 z-stacks were taken from different areas of the tissue. Each z-stack contained from 12 to 20 XY images recorded

at intervals of 1 arbitrary unit apart with a zoom of 2X on a 40X oil immersion objective. Image analysis was performed using MetaMorph software vs.6.3 (Molecular Devices, Downingtown, PA).

Image analysis and data collection

Metamorph software separated each composite image into two images, one for each laser. Regions of interest were drawn around tubules which were round in shape and had an opened lumen. For each image a manual threshold was set to subtract background from true signal. The green fluorescent signal (NHE3 staining) as well as the overlapping of the green (NHE3) over the red (villin) fluorescent signals on each plane of the Z-stack were used in the final computation. A number of 12-20 planes were analyzed for each individual tubule. Colocalization was expressed as the percentage of the sum of green fluorescence overlapping the red fluorescence to the sum of green fluorescence only, in all the planes selected from an individual z-stack and was recorded using an Excel spreadsheet. Six to ten tubules were analyzed for each animal, 3-4 animals were analyzed for each experimental group, and the final percentage for each group was expressed as mean ± SD.

Statistical analysis

Results are presented as mean ± standard error of mean (SEM) or standard deviation (SD), as indicated for each experiment unless stated

otherwise. Statistical analysis was performed using Student's t test for paired and unpaired data. The null hypothesis was rejected for a p-value<0.05.

3.2 Results for Specific Aim #1

Body Weights, Fat content and Systolic Blood Pressure

When fed moderately high fat diet (MHF) normal male Sprague-Dawley (SD) rats spontaneously diverge into two groups: obesity-prone (OP) and obesity-resistant (OR) as previously described (Dobrian *et al.* 2001). Retrospectively, in our study SD rats segregated into distinct populations of obese and lean rats after being fed with MHF for 4 weeks and attained a significance in their body weights starting at week 6 (Fig. 4a). This difference was maintained until the end of the experiment at week 12. OP group was on average 12% heavier than OR group at week 6 and continuously obese animals were weighting more than lean until week 12 when OP rats weighted on average 22% more than OR rats (737.8 ± 15.4 g vs. 604.9 ± 14.8 g).

Those differences were paralleled by higher content of epididymal fat in OP rats compared to OR rats, 22.93 ± 1.33 g vs. 15.8 ± 1.64 g ($p<0.01$) but not reflected by differences in perirenal fat pads, 27.98 ± 4.49 g vs. 16.65 ± 2.03 g where it failed by a small margin to attain significant difference ($p=0.06$) (Fig. 4b). This could be explained by the small number of animals in the groups at the time of the surgeries ($n = 4$). Overall, total fat (sum of perirenal and epididymal fat) content was 56.85% higher in obese rats compare to lean rats, 50.90 ± 5.32 g vs. 32.45 ± 3.43 g (Fig. 4b) and statistically significant.

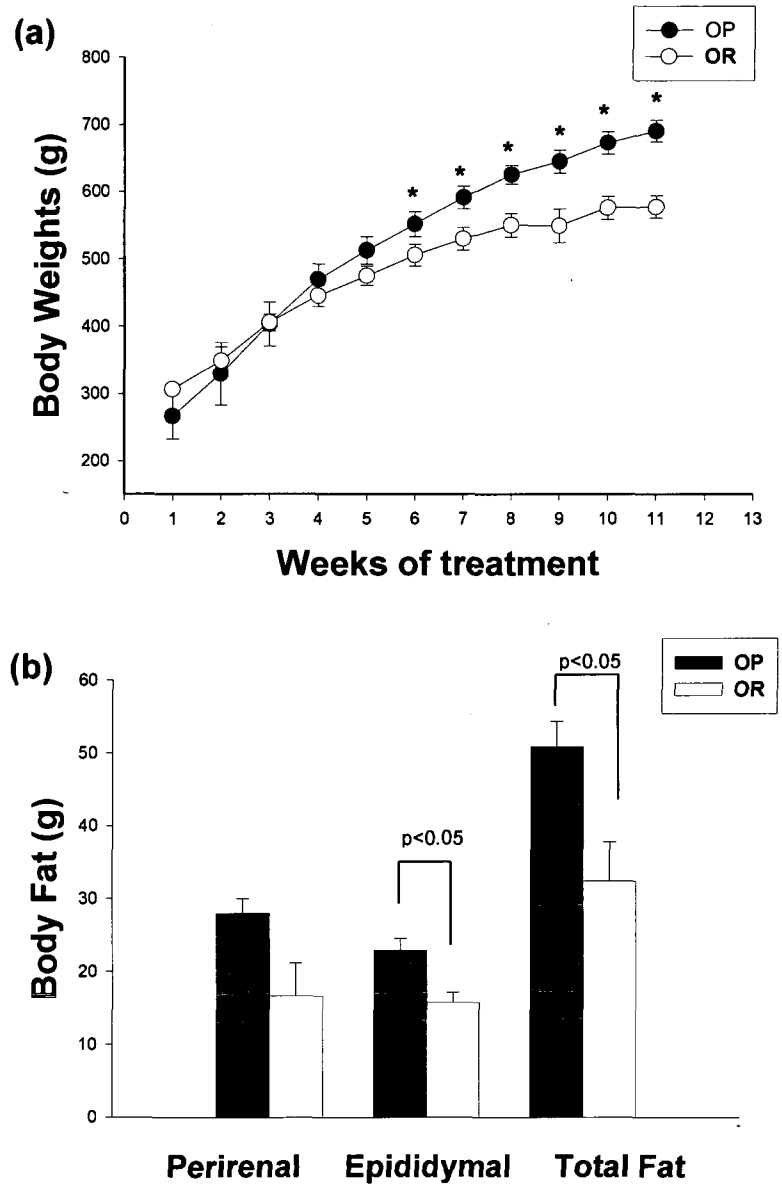

Figure 4 Representative graphs illustrating the typical body weights and fat content in OP and OR rats.
Panel (a): Body weights and food intake (not shown) were measured weekly. Starting at week 6 BWs between OP and OR groups were significantly different. Panel (b): Weights of perirenal and epididymal fat pads were measured at the completion of the experiments. Results are means ± SEM of n=4-5 rats/ group. Significance was determined using unpaired Student's t-test and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to lean counterparts.

In this study we measured systolic blood pressure (SBP) by the tail-cuff method on previously trained rats at week 8 and week 12 of the diet. At week 8 SBP was significantly higher in obese animals than in lean (153.7 ± 3.2 mmHg vs. 142.4 ± 2.6 mmHg) and remained higher at the completion of the study at week 12 (158.9 ± 3.5 mmHg vs. 143.5 ± 4.2 mmHg) (Fig. 5).

Acute pressure natriuresis and diuresis in OP and OR rats

As previously described by our lab, an increase in SBP in obese animals compared to lean rats is not reflected in 24 hour sodium excretion (Dobrian *et al.* 2001). 24 hour sodium excretion was not significantly different between OP and OR groups in that study, suggesting that after 16 weeks on the diet both groups reached steady state. However, in this study we aimed to determine differences in pressure natriuresis and diuresis in OP and OR rats when they were challenged with acute changes in renal perfusion pressure (RPP). Between low (101.25-102.75 mmHg) and high (134.0-135.63 mmHg) renal perfusion pressures (RPPs) urine flow rate in OP rats failed to achieved statistical significance (18.75 ± 2.81μl min^{-1} vs. 46.25 ± 16.79 μl min^{-1}), but in the OR group an increase in RPP was accompanied by an increase in urine flow rate (V) from 14.75 ± 1.75 μl min^{-1} to 53.25 ± 15.67 μl min^{-1} (Fig. 6a). This indicates blunted diuresis in obese rats compared to their lean counterparts. Urinary sodium excretion (UNaV) between low and high RPP were not different in the obese group (from 1.18 ± 0.59 μEQ min^{-1} to 7.01 ± 4.87 μEQ min^{-1}) but once more gained statistical significance in the lean group (from 1.03 ± 0.72 μEQ min^{-1} to

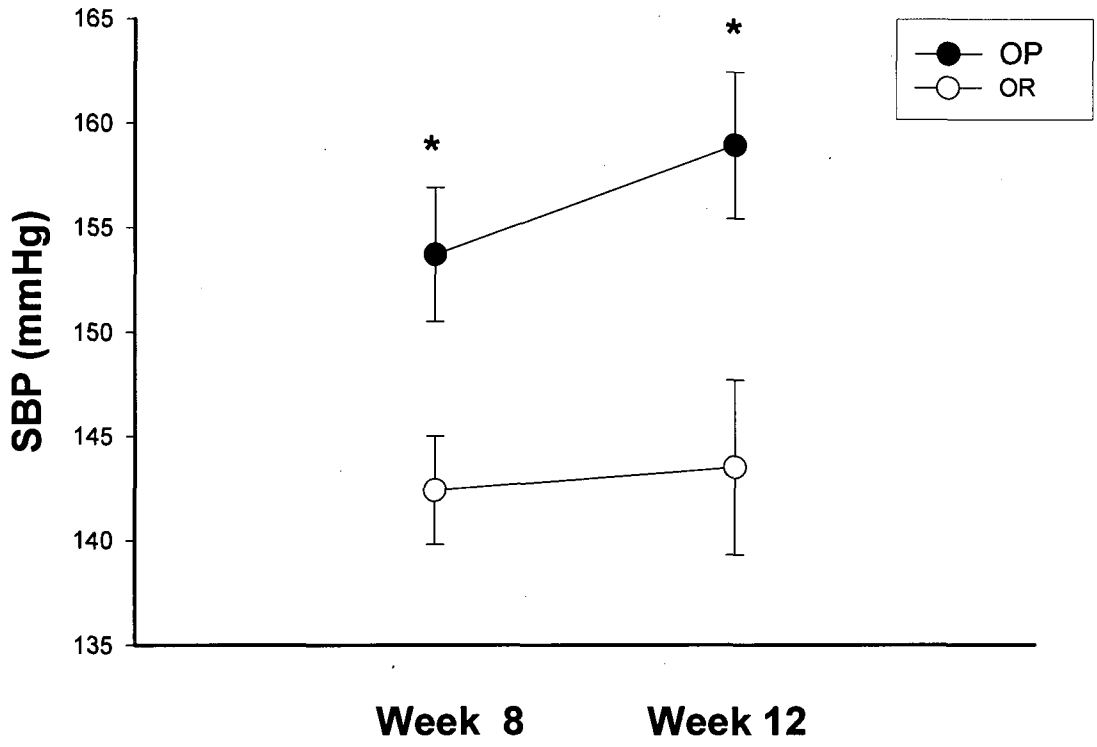

Figure 5 Systolic blood pressures in lean and obese Sprague-Dawley rats. SBP was assessed in previously trained rats by tail cuff method at week 8th and 12th of the diet, right before completion of the study. Obese rats had significantly increased blood pressures compared to lean. Values are means ± SEM of n=6 rats/group with 3-5 readings for each animal. Significance was determined using unpaired Student's t-test and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to lean rats.

9.97 ± 3.63 μEQ min^{-1}, p-value=0.05) (Fig. 6b). The same response was achieved when fractional excretion of sodium (FENa) was calculated: in OP rats there was no significant change in FENa between low and high RPP (from 0.13 ± 0.05% to 0.79 ± 0.57%). However, there was an increase in FENa in OR rats from 0.20 ± 0.14% to 1.34 ± 0.39% (Fig. 6c). The magnitude of change for UNaV and FENa amid low and high RPP were not significantly different in obese and lean rats. These results show that obese animals do not respond to the increase in pressure the same way lean animals do, suggesting that their pressure natriuresis and diuresis is altered compare to lean counterparts. However, despite those differences in UnaV and FENa among the groups glomerular filtration rate (GFR) remained the same: in OP rats: 5.66 ± 0.65 ml min^{-1} at low RPP and 5.6 ± 0.7 ml min^{-1} at high RPP; in OR rats: 3.95 ± 0.66 ml min^{-1} at low RPP and 4.9 ± 0.64 ml min^{-1} at high RPP (Fig. 6d) suggesting that autoregulatory capability of kidneys were preserved.

Finally, fractional excretion of lithium (FELi) was determined and used as an index of reabsorption in the proximal tubule. In the OP group a change in RPP from 101.2 ± 0.63 mmHg to 134.2 ± 2.58 mmHg caused a change in FELi from 5.61 ± 1.74% to 13.89 ± 3.09% which failed by a small margin to attain statistical significance (p-value=0.058) (Fig. 7). However, in the OR group an increase in RPP from 102.7 ± 1.1 mmHg to 135.6 ± 1.38 mmHg caused a significant increase in FELi from 8.74 ± 4.09% to 19.77 ± 1.37% (Fig. 7). This result suggests that OP's attenuated pressure natriuresis is most likely associated with a defect at the proximal tubule level.

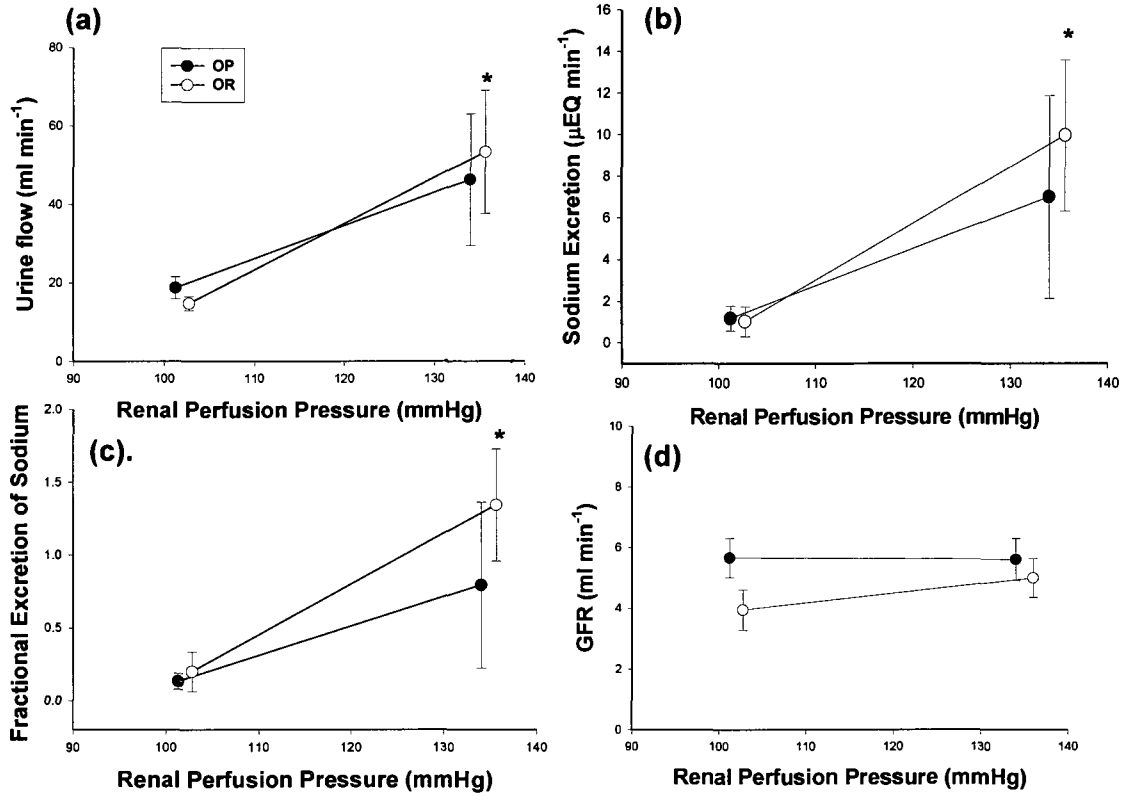

Figure 6 Acute pressure natriuresis and diuresis in obese (OP) and lean (OR) rats.

During surgical procedure an adjustable clamp was placed around aorta above renal arteries to control renal perfusion pressure (RPP). At first RPP was set ~100 mmHg and urine collected for 30 min. At the end of this period blood was drawn from carotid artery for plasma electrolytes, lithium and inulin measurements. Then RPP was set ~140 mmHg and all above procedures were repeated. Panel (a): Effect of RPP on urine flow in OP and OR rats. At high RPP lean rats had an increase in urine flow rate, obese animals had impaired diuresis. Panel (b): Renal perfusion pressure effect on urinary sodium excretion (UNaV) in obese and lean animals. Increasing RPP caused a significant increase in sodium excretion rate in OR group but not in OP. Panel (c): Fractional excretion of sodium (FENa) at low and high RPP in OP and OR groups. While lean rats had significantly higher FENa at higher RPP obese rats demonstrated no change manifesting impaired natriuresis. Panel (d): Relations between renal perfusion pressure and glomerular filtration rate (GFR), calculated as the rate of inulin clearance, in OP and OR rats. Both groups showed well preserved autoregulatory kidney function. Results are expressed as means ± SEM of n = 4-5 rats/ group. Significance was determined using paired or unpaired Student's t-test and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to low RPP in the same group of animals.

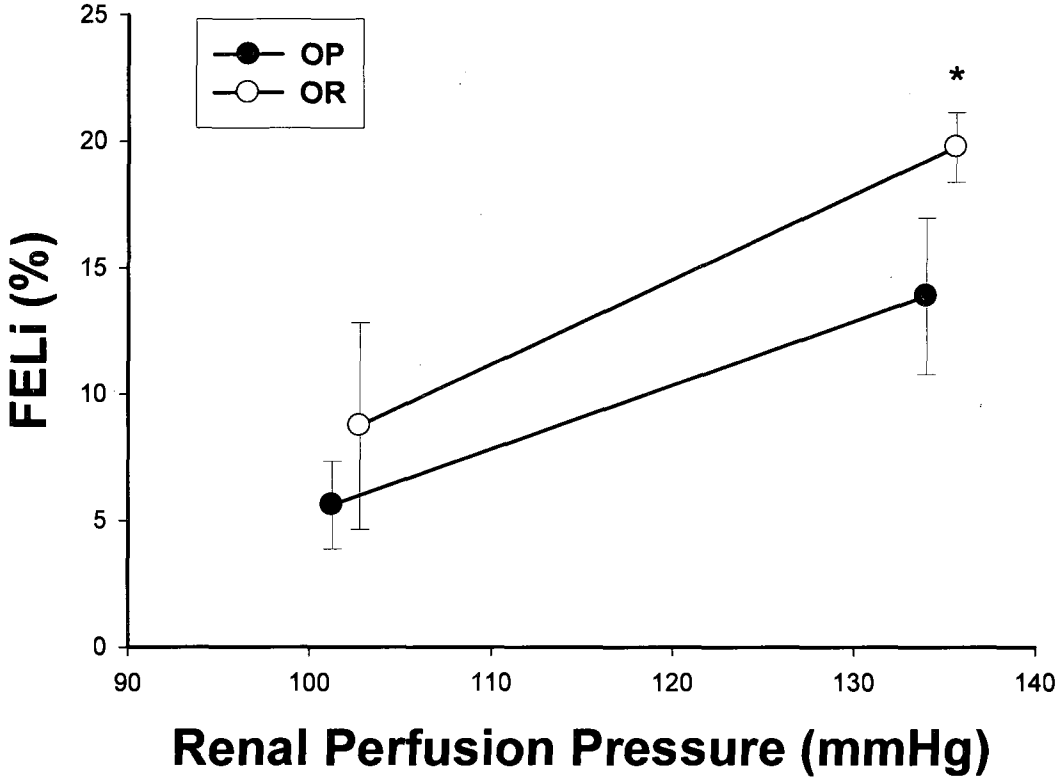

Figure 7 Fractional excretion of lithium (FELi) at low and high RPP in obese and lean Sprague-Dawley rats.
During surgical intervention rats were infused with lithium and after the experiment lithium concentrations in urine and plasma were assessed. FELi was calculated as the ratio between its urinary excretion and the amount filtered at low and high RPP and it was used as a marker for the proximal tubule function. High RPP significantly increased excretion of lithium in OR group but not in OP rats. Results are expressed as means ± SEM of n = 4-5 rats/ group. Significance was determined using paired Student's t-test for data within the group or unpaired t-test was used for group comparison. The null hypothesis was rejected for a p-value< 0.05. *= significant compared to low RPP in the same group of animals.

Expression, activity and localization of proximal tubule transporters: NHE3 and Na,K-ATPase

In a separate and similar experiment using different groups of rats we measured protein expression, activity and localization of quantitatively the most important transporter on the apical side of proximal tubule -the Na/H exchanger type 3 and on basolateral side, the Na,K-ATPase.

The protein expression of Na,K-ATPase assessed by western blotting in a membrane fraction enriched in baso-lateral membrane (BLM) was significantly higher in obese rats when compared to lean rats 54.39 ± 3.25 RFU vs. 30.69 ± 0.69 RFU, respectively (Fig. 8a and 8b). The membranes were stained with Ponceau Red to insure equal loading of the gel. Na,K-ATPase activity measured as a release of inorganic phosphate in the fresh preparations of BLM was not different between the groups (0.27 ± 0.04 mmolP_i mg^{-1} min^{-1} vs. 0.37 ± 0.04 mmol P_i mg^{-1} min^{-1}) (Fig. 9). This latter result could most probably be explained by impurity of the preparations and contaminations with other membranes; as well as the fact that in this ouabain-sensitive ATPase fractions the inhibition by ouabain was 30% and 37% for OP and OR groups respectively (data not shown). Again this suggests that our preparations were impure and contained other pumps insensitive to ouabain like H^+,K^+-ATPase from proximal tubule or cortical segment of collecting tubules. In addition, although Na,K-ATPase is an oligomeric protein, we measured expression levels of the α-subunit only, which may not directly correlate with the activity of the pump.

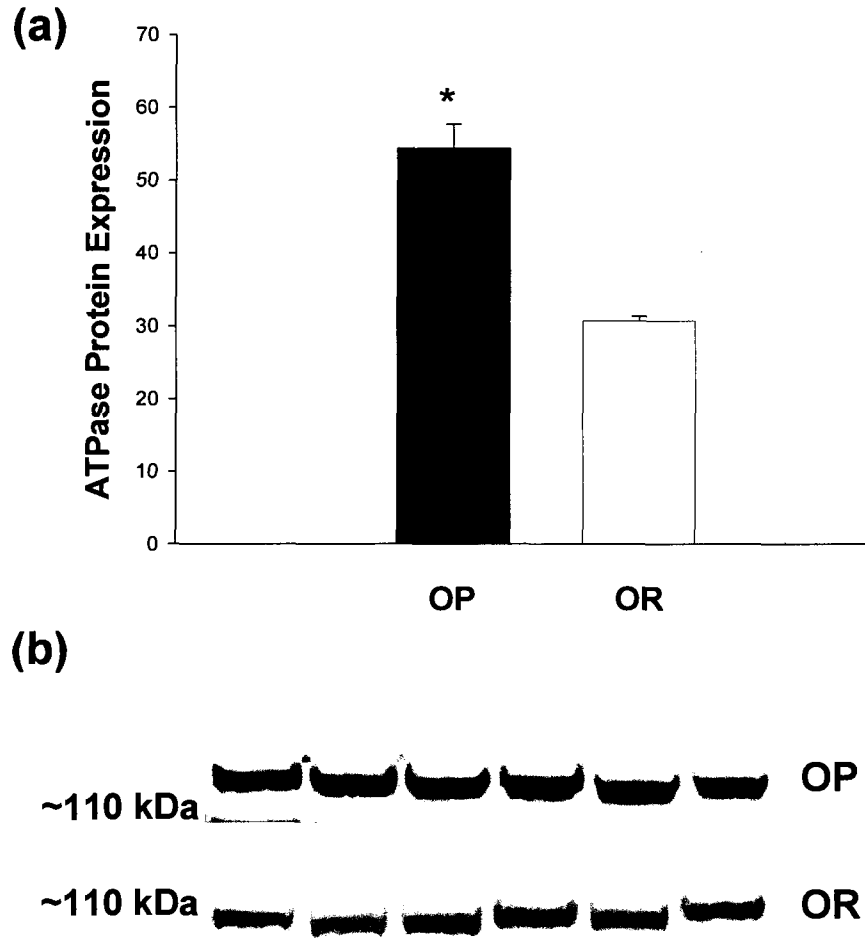

Figure 8 Na,K-ATPase protein expression in fractions of baso-lateral membranes (BLM) from OP and OR rats kidneys.
For this set of experiments different groups of animals were used to avoid any manipulations of kidney before harvesting. Those animals were kept at the same conditions, fed the same diet for the same amount of time as animals used for physiological measurements. Panel (a): Graph representation of Na,K-ATPase abundance, measured in arbitrary units, represented as means ± SEM of n = 6 rats/group. Blots were probed with Na,K-ATPase α1-subunit specific antibody. Semi-quantitative densitometry analyses were done by using Odyssey Infrared Imaging System software. Significance was determined using unpaired Student's t-test and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to lean rats. Panel (b): Immunoblots of BLM samples from OP and OR rats. Each lane represents a sample from an individual rat. Equal protein loading was ensured by staining each membrane with Ponceau red.

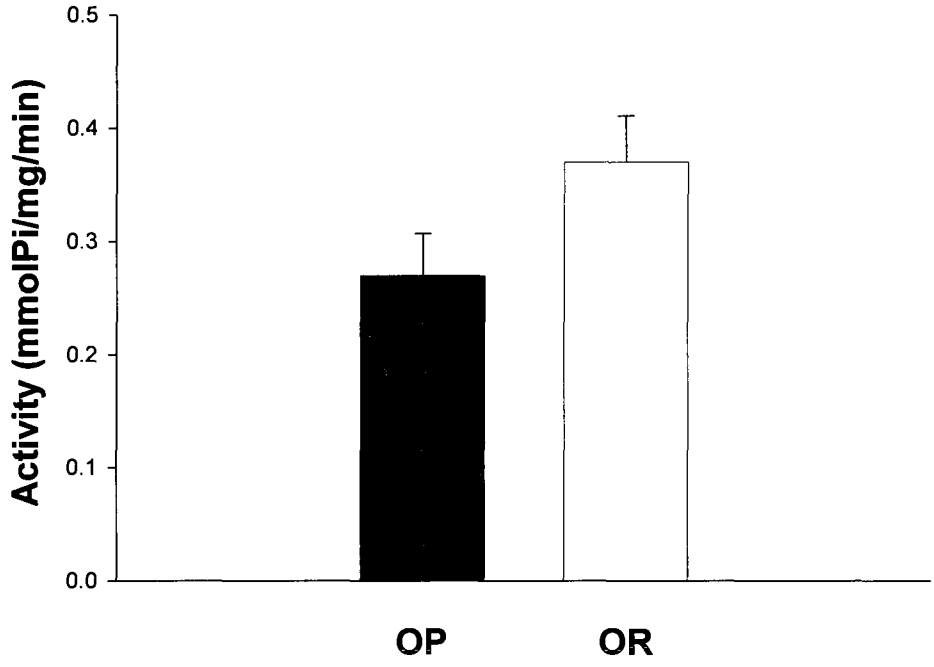

Figure 9 Na,K-ATPase activity in fractions of baso-lateral membranes (BLM) from OP and OR rats kidneys.
Na,K-ATPase activity was expressed as a mmol of inorganic phosphate (P_i) liberated per mg of protein per minute and performed on freshly prepared baso-lateral membrane (BLM) fractions. It was calculated as the difference between activities assayed in the absence of ouabain (total) activity and in the presence of 5 mM ouabain. Values are means ± SEM from 6 rats per group. Each sample was assayed in duplicate and background was subtracted from the mean. There was no significant difference between the groups. Significance was determined using unpaired Student's t-test and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to lean rats.

The protein expression of NHE3, the other transporter of interest was measured in brush border membrane vesicles (BBMV). Villin was used as a loading control. NHE3 expression was significantly higher in obese compared to lean rats (0.56 ± 0.04 RFU vs. 0.35 ± 0.06 RFU) (Fig. 10a and 10b). This was paralleled by higher activity of this transporter in OP animals then in OR rats (0.414 ± 0.06 $\Delta pH\ \Delta t^{-1}$ vs. 0.306 ± 0.09 $\Delta pH\ \Delta t^{-1}$) measured in fresh BBMV fractions as the rate of pH recovery in response to an external sodium load (Fig. 11). Randomly chosen samples were incubated with 100 μM 5-(N-ethyl-N-isopropyl) amiloride (EIPA) to insure that activity measured was of NHE3 since NHE1 isoform is 100 times more sensitive to inhibition by EIPA than NHE3 isoform. In our BBMV preparations NHE activity was resistant to EIPA (data not shown) suggesting that it contain mostly NHE3 isoform.

NHE3 is one of the most regulated transport proteins and its stimulation and inhibition are at least partially due to changes in trafficking. In the apical membrane of proximal tubule cells it exists in two pools: in the microvilli where it appears to be active and in the intervillus spaces where it appears to be less active or inactive (Biemesderfer *et al.* 2001). Using immunohistochemistry and fluorescent microscopy we have measured localization of NHE3 transporter with regard to villin which is localized in the microvilli of the brush border of the epithelial cells. The highest overlapping of the two suggests that more NHE3 is in the active form. Representative fluorescent pictures from OP and OR groups are shown in the Fig. 12a. There was a significantly higher colocalization of villin

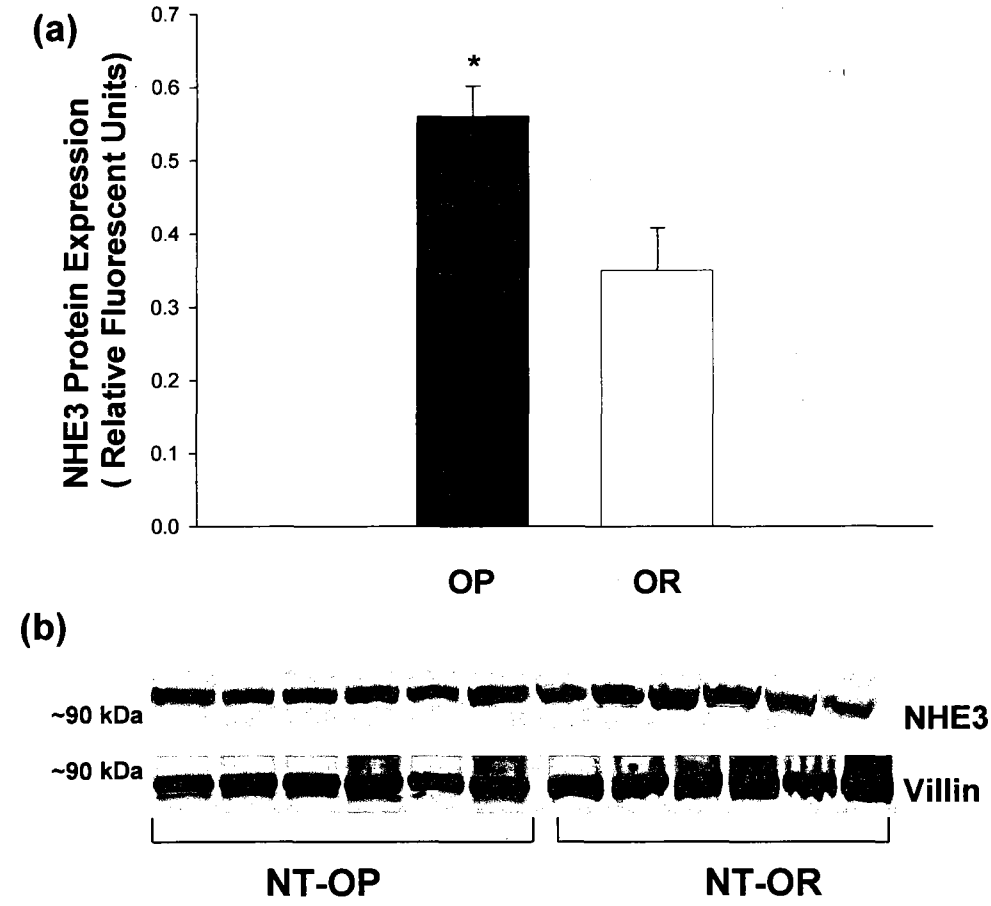

Figure 10 Na/H exchanger protein abundance in fractions of brush border membrane vesicles (BBMV) from OP and OR rats kidneys.
For this set of experiments different groups of animals were used to avoid any manipulations of kidney prior to harvesting. Animals were kept at the same conditions, on the same diet for the same time interval as the ones used for physiological measurements. Panel (a): Semi-quantitative densitometry analyses, measured in relative fluorescent units, represented as mean ± SEM of n = 6 rats/group. Blots were probed with anti-NHE3 and anti-villin antibodies. Semi-quantitative densitometry analyses were done by using Odyssey Infrared Imaging System (Li-Cor) software. Significance was determined using unpaired Student's t-test and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to lean group. Relative abundance of NHE3 was increased in OP group compared to OR group. Panel (b): Immunoblots of BBMV samples from OP and OR rats. For each blot, each lane was loaded with equal amount of protein from brush border membrane vesicles (BBMV) from a different rat. Equal protein loading was ensured by probing duplicates of each membrane with anti-villin antibody, and density of NHE3 bands was normalized to that of villin bands. Proteins were detected with Odyssey Infrared Imaging System (Li-Cor).

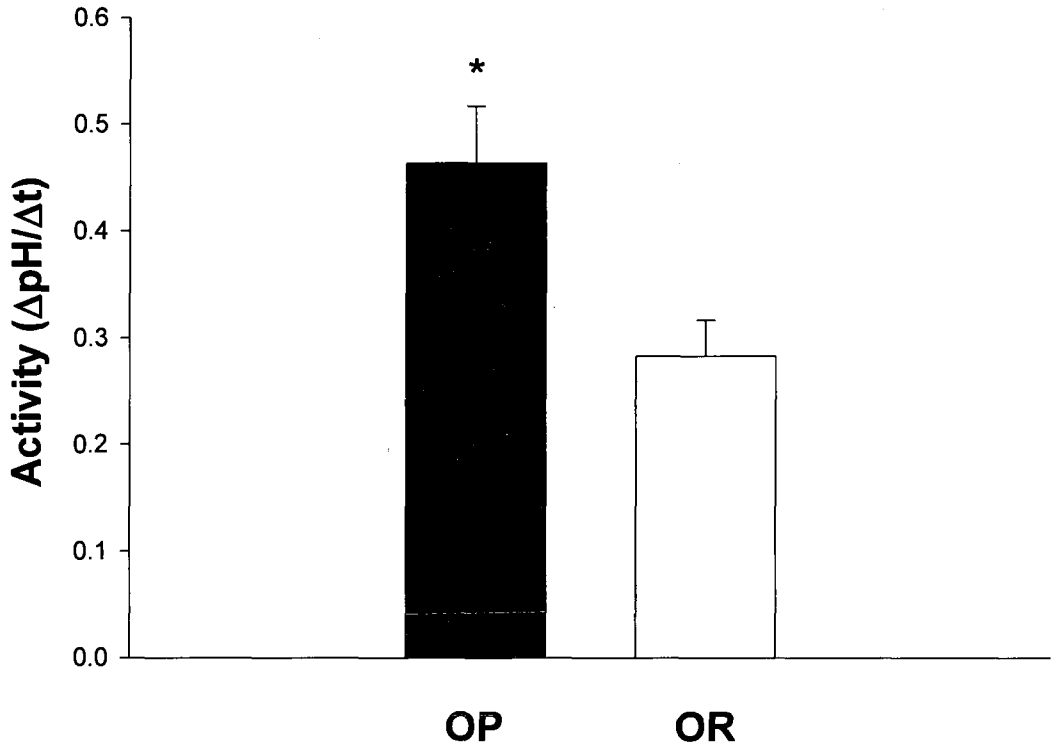

Figure 11 NHE3 activity in fractions of brush border membrane vesicles (BBMV) from OP and OR rats kidneys.
Na/H exchanger activity was measured in freshly obtained BBMVs. from OP and OR rats and it is expressed as a change in pH over time. Na^+/H^+ exchange activity was determined as the rate of Na^+-dependent intracellular pH recovery assessed using BCECF-AM in the presence and absence of external sodium. Some samples were incubated with NHE1 inhibitor amiloride to test the specificity of the reaction. NHE3 activity was greater in OP rats then in OR rats. Values are means ± SEM from 6 rats per group. Significance was determined using unpaired Student's t-test and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to lean counterparts.

(red) and NHE3 (green) in obese animals when compared to lean (21.5 ± 4.4% vs. 9.1 ± 3.7%) (Fig. 12b). This suggests that localization of NHE3 transporter as well as its protein levels could contribute to the higher activity measured in OP rats.

3.3 Discussion for Aim #1

In our study we aimed at understanding the mechanism responsible for the weight-related increase in blood pressure in a rat model of diet-induced obesity. We have used a moderately high fat diet containing 32% kcal fat which mimics the fat content in the Western type of human diet. Similarly to the human population, not all animals fed with this diet become obese. Additionally, our experimental model has many characteristics of obese humans (Kopelman 2000, Hall 2003) including hyperinsulinemia (Levin & Keesey 1998), hyperleptinemia (Levin *et al.* 2003), increased renin activity and high blood pressure (Dobrian *et al.* 2000).

After 12 weeks of high fat feeding obese rats weighted significantly more than lean rats. Body weight in the OP group was on average 17% higher than in the OR group. Associated with this increase in BW was a 10% increase in systolic blood pressure in the obese compared to lean rats. According to a study by Guyton (Guyton 1990) all forms of hypertension are linked to the impairment in pressure natriuresis. We also report that pressure natriuresis and diuresis are attenuated in obese compared to lean rats. In obesity induced hypertension,

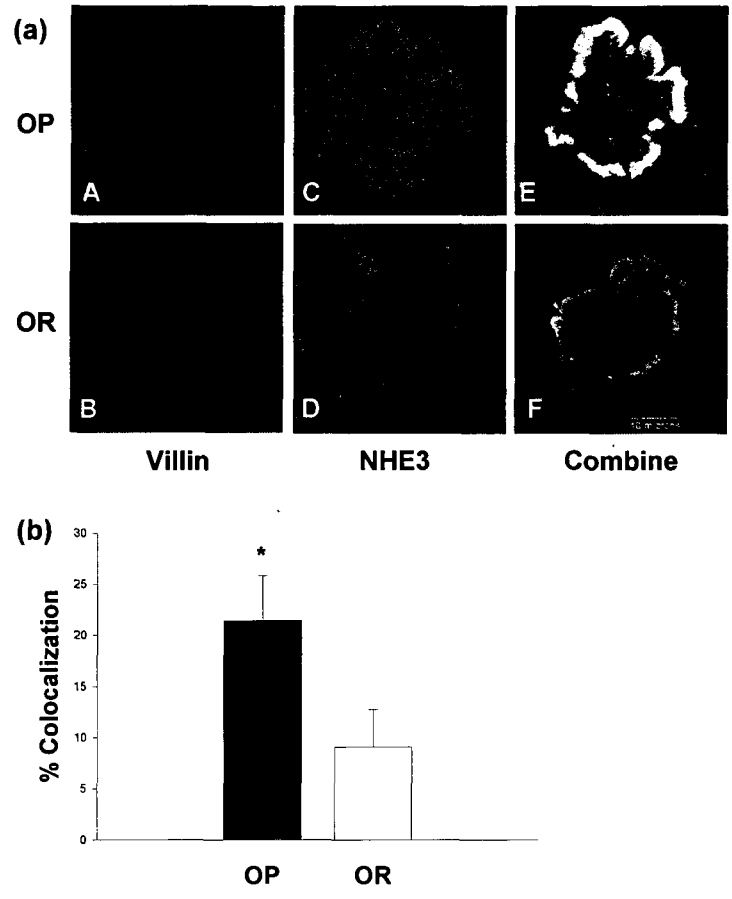

Figure 12 Confocal immunofluorescence analysis of NHE3 distribution in obese (OP) and lean (OR) Sprague-Dawley rats.
4 micron sections of paraffin embedded kidneys were double labeled with polyclonal NHE3 and monoclonal anti-villin antibodies followed by the corresponding anti-rabbit and anti-mouse secondary antibodies (AlexaFluor 488-green, AlexaFluo594- red). Panel (a): Representative images of cortical tubules from obese and lean animals double stained with NHE3 and villin. In OP group a larger fraction of NHE3 is localized at the apical brush border, illustrated by colocalization with villin (yellow). Panel (b): Semi-quantitative analysis of NHE3 and villin colocalization in kidney section from OP and OR animals. 6-10 z-stack images were taken with laser scanning confocal microscope from each animal (n = 3 rats per group). Images were analyzed using Metamorph software and colocalization was expressed as the percentage of the sum of the green staining overlapping the red staining to the sum of the green fluorescence only. OP group had higher colocalization of NHE3 and villin compared to OR group. Significance was determined using unpaired Student's t-test and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to lean rats. Bar= 10microns.

abnormal kidney function is initially associated with increased tubular sodium reabsorption, which leads to sodium retention and expansion of extracellular and blood volumes (Hall 2003). This increase in sodium reabsorption results in a rightward shift in the renal pressure-natriuresis curve and an increase in blood pressure. As a result, an obese individual has to have a higher blood pressure set point in order to maintain sodium and water balance. In our study, glomerular filtration rates were not different between anesthetized obese and lean animals under basal conditions or following an increase in renal perfusion pressure, suggesting that GFR is well autoregulated between renal perfusion pressure (RPP) values of 100 and 135 mmHg in both groups. These findings indicate that the difference in the sodium excretory responses to high RPP between obese and lean rats are most likely caused by abnormalities in renal tubular reabsorption of sodium. Although fractional excretion of lithium was similar between the two rat groups, there was a difference in response to an acute increase in RPP between lean and obese animals suggesting that the defect in sodium reabsorption may be at the level of the proximal tubule.

In our study we intended to define the cellular mechanisms which are responsible for sodium retention seen in obese rats. Sodium is reabsorbed from the proximal tubule across the apical membrane via sodium-hydrogen exchanger type 3 (NHE3), and actively pumped out of the cell by the basolateral sodium pump, Na,K-ATPase, which generates the gradient for Na^+ entry across the apical membrane. Protein expression of Na,K-ATPase and NHE3 were studied by immunoblots after preparation of baso-lateral membrane fractions (BLM) and

brush border vesicle membrane (BBMV), respectively. Protein expression and activity of the transporters in BLM and BBMV were measured and the subcellular distribution of NHE3 was studied by immunohistochemistry. We reported that expression of Na,K-ATPase was 77% higher in OP than the OR group but its activity was the same between the groups. However, the activity of Na,K-ATPase was reported to correlate to the number of pump units (Ewart & Klip 1995) and this suggests that in general Na,K-ATPase was more active in obese rats. This is in agreement with the study by Bickel *et al.* (Bickel *et al.* 2001) in which the increase in the abundance of Na,K-ATPase was reported in the kidneys of obese, hypertensive, hyperinsulinemic Zucker rats evaluated by semiquantitative immunoblotting. Not all reports on Na,K-ATPase abundance in obesity are in agreement, as a reduction of Na,K-ATPase activity was reported in liver and kidneys of obese animals (Bray & Yukimura 1978, York *et al.* 1978) and humans (Klimes *et al.* 1982). However, the different results most likely reflect cell-specific regulation of Na,K-ATPAse in liver, renal or blood tissue. Also, a number of studies indicate no change in Na,K-ATPase activity in obese humans (Simat *et al.* 1983, Klimes *et al.* 1984).

It is known that Na,K-ATPase is an oligomeric protein composed of a α subunit, a β subunit and a FXYD protein (γ subunit). Expression of the catalytic subunit α, the one which hydrolyzes ATP and transports the cations is most commonly measured and was also evaluated in our study. However, recent findings suggest that β subunits and FXYD proteins essentially contribute to the various physiological roles of Na,K-ATPase in different tissues and can modulate

its activity in a tissue-specific way (Geering 2008). It is not clear whether changes in expression and activity of the α subunit are paralleled by changes in the β and FXYD subunits and how they can affect the overall activity and expression of the transporter. Moreover, Na,K-ATPase is irregularly distributed along the whole length of the nephron and we can not exclude the fact that beside proximal tubules our membrane preparations could as well include other nephron segments such as the thick ascending limb of Henle's loop, distal convoluted tubule, and connecting tubule.

Beside the protein abundance, phosphorylation and subcellular distribution play a major part in the regulation of Na,K-ATPase enzyme activity. The catalytic α subunit of Na,K-ATPase can be phosphorylated by PKA at the C-terminus at Ser943 (Feschenko & Sweadner 1995), as well as by PKC at the N-terminal sites: Ser11, Ser18 and Ser23 (Logvinenko *et al.* 1996). Phosphorylation is generally associated with altered enzyme activity (Lal *et al.* 2000, Bertuccio *et al.* 2007), though phosphorylation status of Na,K-ATPase was not assessed at this point in our study. Previous studies demonstrated that in the proximal tubule the effect of angiotensin II on Na,K-ATPase is mediated by PKC (Rangel *et al.* 2002). The mechanism of regulation of Na,K-ATPase by insulin in the kidney is largely unknown. It has been shown that PKC may play a role in the insulin-mediated activation of Na,K-ATPase in cultured rat skeletal muscle cells (Sampson *et al.* 1994) . Sweeney and Klip have shown that in 3T3-L1 fibroblasts phosphatidylinositol 3-kinase and PKC-zeta appear to be involved in the signaling pathway of insulin effect on Na,K-ATPase activity (Sweeney *et al.*

1998). However, there are also data showing that stimulation of Na,K-ATPase activity by insulin in the proximal tubule is likely mediated by phosphorylation of Tyr10 (Feraille *et al.* 1999).

In addition, redistribution of a transporter between the plasma membrane and cytosolic compartments is another mechanism by which its activity could be regulated (Periyasamy *et al.* 2005). There is some evidence that trafficking can be associated with phosphorylation of Na,K-ATPase (Chibalin *et al.* 1999, Efendiev *et al.* 2003). These are all important regulatory mechanisms that deserve further investigation.

The other transporter of interest to us was sodium/hydrogen exchanger type 3 (NHE3) found in the kidneys almost exclusively in the apical side of the epithelium in the proximal tubule segment. We reported that obese rats had 60% higher expression of NHE3 when compared to lean rats (0.56 ± 0.04 RFU vs. 0.35 ± 0.06 RFU) and this was paralleled by 35% higher activity of the transporter in the OP group (0.414 ± 0.06 ΔpH Δt^{-1} vs. 0.306 ± 0.09 ΔpH Δt^{-1}). Also, we found a higher degree abundance of NHE3 in the villus tip in OP vs. OR group.

The role of NHE3 in hypertension is of potential importance. NHE3 has been studied in various models of obesity and hypertension but the data which links NHE3 and high blood pressure is not conclusive. In agreement with our study there are reports of increased activity of exchanger in spontaneously hypertensive rats (SHR) and Milan hypertensive rats (MHR) compared to their normotensive controls of the same age (Morduchowicz *et al.* 1989, Parenti *et al.*

1992, Hayashi *et al.* 1997). These results were supported by other studies in which both activity and abundance of Na/H exchanger was determined in freshly isolated or cultured proximal tubule cells and in renal cortical tubules from the spontaneously hypertensive rat (SHR) and WKY rats before and after the development of hypertension (Kelly *et al.* 1997, LaPointe *et al.* 2002). In contrast, there is as well evidence of decreased NHE3 activity or no change in NHE3 activity and protein expression in primary cultured renal cells from spontaneously hypertensive rats (Orlov *et al.* 1991), in induced acute or chronic hypertension in Sprague-Dawley and spontaneously hypertensive rats, respectively (Yip *et al.* 1998, Zhang *et al.* 1998, Magyar *et al.* 2000) , in hypertensive Dahl salt-sensitive rats on high-salt diet (Kobayashi *et al.* 2004) and in obese Zucker rats (Bickel *et al.* 2002). The reason for the discrepancy is unclear, although the background on which hypertension develops could modulate the final outcome of exchanger activity as much as the high blood pressure itself (Kobayashi *et al.* 2004). Furthermore, since NHE3 activity is the resultant of several factors besides protein abundance, such as interaction with NHE regulatory factors (NHERF), NHE3 phosphorylation, membrane trafficking and changes in turnover rate, any above mentioned changes can determine NHE3 activity. There is evidence for redistribution of proximal tubule NHE3 from the apical microvilli to the intermicrovillar region, as determined by both subcellular fractionation and confocal microscopy (Zhang *et al.* 1998, Yang *et al.* 2002). Interestingly, the distribution was observed with both acute and chronic hypertension (Yip *et al.* 1998) and was associated with decreased proximal tubule sodium transport

(Zhang *et al.* 1996, 1998). In our study we have observed significantly higher colocalization of NHE3 with villin in obese vs. lean groups (21.46 ± 4.38% vs. 9.10 ± 3.67%) and we concluded that in OP rats more NHE3 is distributed in the physiologically active membrane pool compared to the OR rats.

In summary, in our study we demonstrated possible cellular mechanisms responsible for the changes seen in hypertension related to obesity. Approximately half of SD rats fed high fat diet became obese and had high blood pressure while the rest stayed lean and normotensive. That was well correlated with higher protein expression and activity of NHE3 and higher overall activity of Na,K-ATPase in the renal proximal tubule. The mechanisms which contribute to those changes are multifactorial and can include phosphorylation of the transporters, activation/inhibition of their regulatory factors or transporters redistribution between different membrane pools. We provided evidence of differences in NHE3 abundance, activity and membrane distribution between lean and obese rats which could contribute to the increased sodium retention seen during acute pressure-induced natriuresis and diuresis.

SECTION 4

STUDIES FOR SPECIFIC AIM #2

4.1 Materials and Methods for Specific Aim #2

Animals

The animal husbandry and separation into groups was done as described in methods for specific Aim #1. The OP and OR groups were randomly divided into sub-groups (n = 6): 1) no treatment, diet only: NT-OP/NT-OR and 2) treatment with 0.1% (w/w) pioglitazone (Takeda Pharmaceuticals, Lincolnshire, IL): PIO-OP/PIO-OR. The rats were kept on respective diet/treatments for 4 additional weeks (Fig. 13). Pioglitazone was incorporated into the food pellets. Body weights and food intake were measured weekly as mentioned in section 4. Before and after the treatment rats were put into the metabolic cages and urine was collected for 24 hours for sodium measurements. Separate groups, fed and treated exactly the same way, were used for preparation of membrane fractions, brush border membrane vesicles, and any subsequent experiments involving those preparations.

Surgical procedure

The surgical procedure was performed as previously described (Khraibi *et al.* 2002). On the day of the acute experiment, rats were anesthetized with Inactin (100 mg kg^{-1}), and catheters were placed in the trachea (PE-240) and

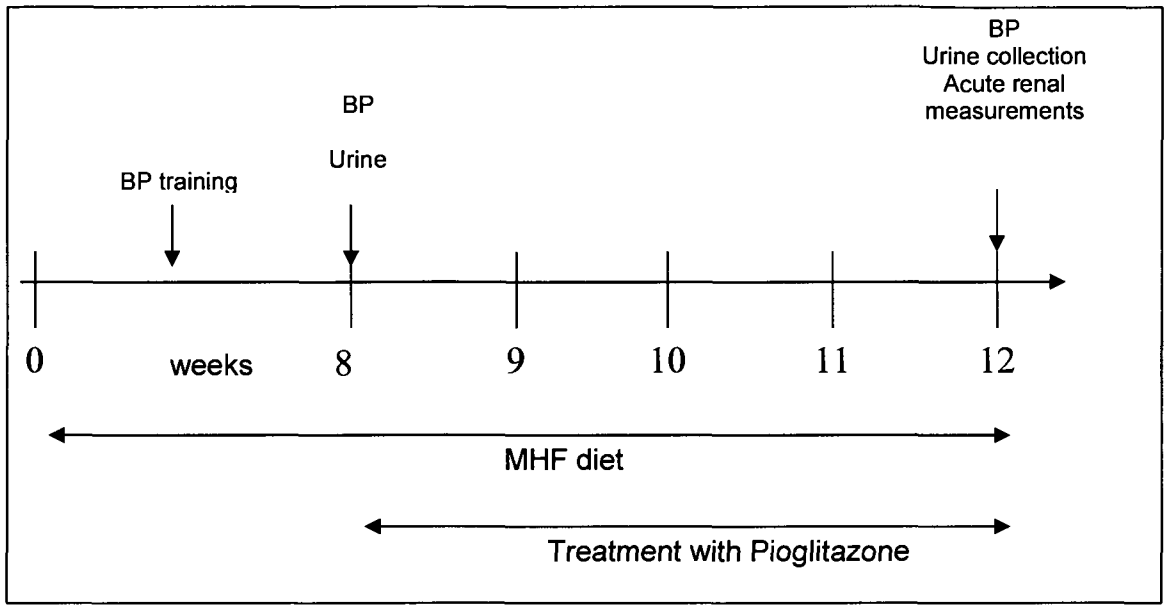

Figure 13 Research design for specific Aim #2.
Male, 3 months old Sprague-Dawley rats were started on moderately high fat diet (MHF) a week after arrival to the facility. At week 5, halfway through the diet, rats were trained for the tail-cuff blood pressure measurements by the blood pressure analysis system (BP-2000-R; Visitech Systems; Apex, NC). Blood pressure was measured at week 8, before starting the treatments, and at week 12, after treatment completion on the day of the surgery. Average of 3-5 readings were taken for each animal and recorded by the automated software. Before and after treatment, rats from all groups were placed in metabolic cages for 24-hour urine collection. Non-treated groups OP and OR were placed in metabolic cages only once, right before surgeries. Water intake and urine volume was recorded and urine was analyzed for Na^+ content. Starting with week 8th of the diet each group: OP and OR was further divided into 2 subgroups and was treated for additional 4 weeks with: 0.1% (w/w) pioglitazone incorporated in the food pellets or left untreated (diet only). After 4 weeks of treatments, and total of 12 weeks of the diet acute measurements of renal function were performed during surgical procedure.

left jugular vein (PE-50) for intravenous infusion of 0.75 ml per 100 g body wt^{-1} h^{-1} of saline with 6 mM lithium chloride (LiCl) and 0.75 ml per 100 g body wt^{-1} h^{-1} of a solution of 3% inulin and 6.25% bovine albumin in saline (with 6 mM LiCl). A PE-50 catheter was implanted in the left carotid artery for mean arterial pressure (MAP) measurement and blood withdrawal. PE-90 catheter with a flared tip was placed in the bladder for urine collection. The rats were allowed 1 hr to recover after completion of the surgical procedures. Then, a clearance period of 30 min was started; MAP was measured and recorded continuously. At the end of this period, ~1 ml of blood was withdrawn from the left carotid artery for plasma electrolytes, lithium, and inulin measurements. Urine was collected for 30 min. All rats were killed by an Inactin overdose (50 mg; 0.5 ml of a solution of 100 mg ml^{-1} of Inactin in saline) at the end of the experiment while still under deep anesthesia.

To avoid any manipulations of the kidneys different groups of animals were used for membrane and brush border membrane vesicle fractions. At the completion of the treatments rats were anesthetized with Inactin (100 mg kg^{-1}), kidneys were removed without any further handling and then rats were killed by an Inactin overdose as mentioned above.

Physiological parameters

All physiological parameters were assessed, measured and calculated as described in the Material and Methods section for Aim #1.

Baso-lateral membrane (BLM) and brush border membrane vesicles (BBMV) preparations

BLM and BBMV fractions of renal cortexes from nontreated animals and rats treated with pioglitazone were prepared using the same protocols as described in the Material and Methods paragraph for Aim #1.

Western Blotting on BLM and BBMV preparations

Na,K-ATPase and NHE3 protein expression were assessed by western blotting as described in the Material and Methods paragraph for Aim #1.

Activity of Na,K-ATPase and NHE3

Na,K-ATPase activity was measured in freshly obtained BLM preparation and NHE3 activity was measured in freshly obtained BBMV fraction as previously described in the Material and Methods paragraph for Aim #1.

Immunocytochemistry, image analysis and data collection

Colocalization of NHE3 (green) and Villin (red) were evaluated on paraffin-embedded sections of kidney from untreated and treated animals; images were recorded by confocal fluorescent microscopy and overlapping of both colors were analyzed by MetaMorph software as described in the Material and Methods paragraph for Aim #1.

Cell culture and treatments

Human renal proximal tubule epithelial cells (RPTEC) (primary cells) were purchased from Lonza, Inc (Walkersville, MD). Cells were grown in the media recommended by the supplier-REBM, containing 5% fetal bovine serum. RPTEC were seeded at density of 3.3×10^5 per 100 mm plate and 2.5×10^5-1×10^6 per well on 6-well plates for transfection purposes, grown to ~80% confluence and used between passages 3 and 6 in all experiments. They were kept in serum-free medium 24 hr before experiments. Quiescent cells were treated with various PPARγ agonists: pioglitazone (50 μM), rosiglitazone (100 mM), and PGJ2 (2 μM) or PPARγ antagonists BADGE (100 μM), and GW (2 μM) for 4 hrs, harvested and total homogenate or membrane fractions were prepared to assess protein expression of Na,K-ATPase and NHE3. The protein concentration for each lysate was determined by using a BCA protein assay kit (Pierce Chemical) with BSA as a standard. PPARγ agonists and antagonists were purchased from Cayman Chemicals (Ann Arbor, MI). Transiently transfected cells (to either overexpress or silence PPARγ) were seeded in 6-well plates and incubated for 12 hours, at 37 °C, in transfection media with serum, followed by additional 24 hour incubation in media without serum. Afterwards, the cells were incubated for 12 additional hours in fresh serum free medium supplemented with either pioglitazone (50 μM) or PGJ2 (2 μM), both PPARγ agonists, and subsequently harvested and processed for appropriate assays.

Plasmid preparation

GeneHogs strain of E.coli (GeneStorm clone ID # RG001382, Invitrogen Carlsbad, CA) was streaked from a glycerol stock onto a freshly prepared agar plate containing Zeocin as a selective antibiotic and placed at 37 °C. After 24 hrs 2-3 single colonies were picked from the plate and inoculated into 2 ml of LB medium again containing Zeocin and grown for 8 hours at 37 °C with vigorous shaking (250 rpm). 500 μl of the starter culture was then diluted into 150 ml of LB medium and further grown with vigorous shaking (~300 rpm) to saturation (~12-16 hours). The bacterial cells were then harvested by centrifugation at 6 000 ***g*** for 15 min at 4 °C in Beckman JA-25 centrifuge and cDNA was isolated from plasmid according to MidiPrep kit instructions (Qiagen, Valencia, CA). In the last step DNA was eluted in DNAase free water and the purity of plasmid was checked by measurements of the A260:A280 ratio. The integrity of plasmid was verified by Bgl II restriction enzyme digestion and visualized on 0.8% agarose gel.

Transient transfection and PPARγ overexpression

Full length PPAR-γ cloned in pcDNA3.1/GS vector already transformed in GeneHogs strain of E.coli (GeneStorm clone ID # RG001382) was purchased from Invitrogen (Carlsbad, CA). Transfection was done by Amaxa's Nucleofector technology following the basic nucleofection protocol for primary mammalian epithelial cells (Amaxa Biosystems, Gaithersburg, MD). After transfection, cells were left undisturbed at 37 °C for 36 hrs. Afterwards, the transfection medium

was replaced with the media supplemented with pioglitazone for 12 hrs. Control cells were transfected with empty vector in identical conditions. The efficiency of the transfection was verified by fluorescent microscopy of cells transfected with pcDNA3.1/GFP.

Transient transfection and small interfering RNA (siRNA) technique

One day prior to transfection, RPTEC were plated in REBM into 6-well plates, at density of 2.5 x 10^5 cells/well. For transfection with X-tremeGene siRNA Transfection Reagent (Roche Applied Science, Indianapolis, IN) expression vector and either a specific siRNA duplex or a control siRNA was diluted in a serum-free medium. In parallel, X-tremeGene siRNA Transfection Reagent (Roche Appied Science, Indianapolis, IN) was added to serum-free REBM, and the resulting mixture was combined immediately with the diluted nucleic acids. After 20 minutes incubation at room temperature the solution was added to the cells. Without further medium change the cells were assessed for viability by microscopic inspection. Next day additional media with serum was added to each well. Subsequently cells were washed and incubated for 12 hr with PPARγ ligands, then lysed in the homogenizing buffer. siRNA (sense and antisense) were designed to target a common sequence in both PPAR-γ1 and γ2 isoforms. The following sequences were used for PPAR-γ1, 2:

sense siRNA: 5'-GACUACAUUGGCUGGACCUTT-3';

antisense siRNA- 5'AGGUCCAGCCAAUGUAGUCTT-3';

control sense: 5'-GACUACAUUGGCUGGACCUTT-3'; control antisense: 5'AGGUCCAGCCAAUGUAGUCTT-3'.The efficiency of the transfection was verified by fluorescent microscopy of cells transfected with Cy3 luciferase-labeled oligonucleotides.

Protein preparation from RPTEC and Western blotting

Na,K-ATPase and NHE3 protein expression was assessed in total homogenates and in membrane fractions of RPTEC. The latter was prepared using Compartmental Protein Extraction Kit (Chemicon, Billerica, MA) according to the protocol from the manufacturer. Cell pellets were homogenized in RIPA buffer without detergents, with 5% sorbitol, 1mM PMSF, 1mM NaF, 1mM Na_3VO_4 final concentration, Protease Inhibitor Cocktail (Sigma Aldrich, Saint Louis, MO) at 1:1 000 dilution. Protein concentration for each lysate was determined using a BCA protein assay kit (Pierce Chemical) with BSA as a standard. The general western blot protocol was described in Materials and Methods paragraph for specific Aim #1. For RPTEC the following antibodies were used: polyclonal anti-NHE3 (Chemicon, Billerica, MA, 12 $\mu g\ ml^{-1}$) and monoclonal Na,K-ATPase (Upstate , Lake Placid, NY,1:10 000 dilution). Polyclonal anti-α-tubulin antibody (Abcam, Cambridge, MA, 1:500 dilution) or monoclonal anti-α- tubulin (Sigma, Saint Louis, MO, 1:1 000 dilution) served as a loading control. Antigen detection was performed using appropriate fluorescent secondary antibodies (IRDye 680 and IRDye 800, Li-Cor Biosciences, Lincoln, NE) at 1:15 000 dilution for 45 minutes at RT. Membranes

were scanned using Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Semi-quantitative analyses of the specific bands were performed using Li-Cor Odyssey software. Results were expressed as relative fluorescence units (RFU). For some blots, secondary HRP-antibodies were used at 1:7 500 dilution (Amersham, Piscataway, NJ, USA) and the antigen-antibody complexes were detected using enhanced chemiluminescence (ECL, Amersham, Piscat away, NJ, USA). The films were scanned and the intensity of the bands was measured by densitometry using SigmaGel software (Jandel Scientific).

Statistical analysis

Results are presented as mean ± standard error of mean (SEM) of the indicated number of experiments unless stated otherwise. Statistical analysis was performed using Student's t-test for unpaired data (treatment versus control), or by analysis of variance (ANOVA) and Holm-Sidak modification for multiple group comparisons, as appropriate using InStat software (San Diego, CA). The null hypothesis was rejected for a p-value<0.05.

4.2 Results for Specific Aim #2

In vivo experiments

Physiological parameters

The summary of the effect of 4 weeks of pioglitazone treatment on various physiological parameters in obese and lean rats is presented in Table 1 and Table 2. OP rats had significantly higher body weights (BW) than OR rats. Chronic treatment with pioglitazone significantly increased body weights in the obese group by ~10%, had no effect on body weights in the lean group and thus made the difference in BWs between treated OP and OR animals significant (Table1). Those changes were paralleled by a significant increase in total visceral fat after treatment with pioglitazone in obese rats with no effect in lean rats (Table1). Furthermore, average food intake (Table 1) during the 4 weeks of treatment was significantly increased in the pioglitazone treated OP group and was significantly higher compared to the treated OR group. Treatment did not have an effect on food intake in lean animals. Systolic blood pressure (SBP) was significantly higher in obese animals compared to lean; pioglitazone treatment lowered SBP in OP groups from 158.9 ± 3.5 mmHg to 140.3 ± 8.9 mmHg and had no significant effect in the OR rats (Table 1). Since it is known that sustaining a hypertensive state requires an increase in the blood volume which is achieved by sodium and water retention we have estimated chronic sodium excretion in all groups of animals placed in metabolic cages. Urine sodium excretion (UNaV) over the period of 24 hours was not different between obese and lean rats. Also, pioglitazone treatment did not significantly change 24 hours UNaV in OP and OR groups (Table 1). In addition, there was no significant difference in water intake and urine volume over 24 hours in obese and lean rats non-treated or treated

Table 1 Final measurements of the body weights (BW) and total fat, food and water intake, systolic blood pressure (SBP) and chronic urinary sodium excretion in obese (OP) and lean (OR) rats with or without pioglitazone treatment.

	OP (n = 6)	PIO OP (n = 6)	OR (n = 6)	PIO OR (n = 6)
BW (g)	639.7±26.6	702.5±22.4 *	563.6±4.2 †	566.6±19.6 §
Average food intake (g/week) over 4 weeks of Pio treatment	157.1±3.0	172.1±7.0	137.2±4.0 †	129.4±10.0 §
Total visceral fat (g)	27±3.6	42.7±4.5 *	26.4±4.4	21.6±2.5 §
SBP (mmHg)	158.9±3.5	140.3±8.9 *	143.5±4.2 †	149.7±6.9
Urine Na^+ (mmol 24 h^{-1})	2.12±0.27	2.75±0.23	2.03±0.10	2.27±0.19
Water intake (ml 24 h^{-1})	20.83±2.01	22.50±4.43	22.50±1.83	22.50±1.12
Urine volume (ml 24 h^{-1})	11.67±1.86	14.50±2.35	12.54±1.28	12.17±0.84

Body weights represent averages for each group from the last week of the diet before sacrifice. Food intake represents average over the 4 weeks of treatment. Significance remained the same when data of food intake was calculated over the entire period of the study. Total visceral fat represents sum of epididymal and retropritoneal fat. Systolic blood pressure corresponds to final readings of the experiment. Values for urine sodium, water intake and urine volume were obtained from metabolic cages over 24 hours period. Values are mean ± SEM from n = 6 rats/group. Significance was determined using one-way ANOVA with Holm-Sidak modification for multiple group comparisons and null hypothesis was rejected for a p-value <0.05. *=significant compared to non-treated; †=significant compared to OP; §= significant compared to PIO-OP.

with pioglitazone (Table 1). The lack of difference in sodium excretion could most probably be explained by the fact that after being fed with high fat diet for 8 weeks high blood pressure was already established in obese groups and those animals were in sodium balance; additional 4 weeks of treatment did not change that (Hall 2003). Next, we examined natriuresis and diuresis in obese and lean rats after acute saline loading (Table 2). No significant difference was found in glomerular filtration rate (GFR), urine flow (V) and fractional excretion of sodium (FENa) between four experimental groups. However, acute measurements showed sodium retention in OR group treated with pioglitazone compared to OP group (14.8 ± 3.2 μEq min^{-1} vs. 2.9 ± 1.2 μEq min^{-1}) with no effect on sodium excretion (UNaV) in non-treated groups. Fractional lithium excretion (FELi) which assesses proximal tubule function was not different between OP and OR rats after saline loading and was comparable after pioglitazone treatment. The limited natriuretic and diuretic response to acute volume expansion in our study could be explained by relatively fixed GFR in our experimental groups and different extend of volume expansion between obese and lean rats. Together, these data suggest that pioglitazone has different effects in obese and lean rats with respect to sodium reabsorption and regulation of blood pressure. Next, we examined the expression, activity and localization of the renal proximal tubule sodium transporters. This part of the study was conducted in different set of animals subjected to identical dietary and treatment protocols.

Table 2 Renal parameters measured during surgical procedure in obese (OP) and lean (OR) rats with or without pioglitazone treatment: glomerular filtration rate (GFR) and urinary sodium excretion (UNaV), fractional excretion of sodium (FENa), fractional excretion of lithium (FELi) and urine volume (V).

	OP	PIO OP	OR	PIO OR
	(*n* = 6)	(*n* = 6)	(*n* = 5)	(*n* = 5)
GFR (ml min^{-1})	4.08±0.7	5.75±0.8	3.45±0.4	3.23±0.8
UNaV (μEq min^{-1})	9.9±2.3	14.8±3.2	8.3±1.6	2.9±1.2 §
FENa (%)	2.6±1.3	1.9±0.4	1.8±0.4	0.6±0.2
FELi (%)	41.9±16.2	25.4±5.1	27±4.4	14.7±3.9
V (μl min^{-1})	71.3±13.1	81.3±14.0	56.7±7.0	28.2±16.4

Anesthetized rats were infused with lithium and inulin, urine was collected and blood withdrawn. Glomerular filtration rate was calculated from the clearance of inulin. Urinary sodium excretion was measured as the rate of sodium excretion per volume of urine per time unit. Fractional excretion of sodium and lithium was calculated as the ratio between the amount excreted in the urine versus the amount reabsorbed by the kidney. Values presented are mean ± SEM from n = 5-6 rats/group. Significance was determined using one-way ANOVA with Holm-Sidak modification and null hypothesis was rejected for a p-value <0.05. §= significant compared to PIO-OP.

Activity, protein expression and localization of sodium transporters

In this part of the study we investigated some of the mechanisms which were responsible for blood pressure lowering effect of pioglitazone in obese but not lean rats on high fat diet. As described in results paragraph of Aim #1 Na,K-ATPase and NHE3 are two proximal sodium transporters of interest to us. Their activity, as well as activity of any transporter, is the resultant of several factors including protein abundance, modifications of the function of single units of transporter and/or localization. We have measured protein expression of the α1-subunit of Na,K-ATPase by western blotting in baso-lateral enriched membrane (BLM) preparations from renal cortex (Fig. 14). Pioglitazone treatment significantly increased Na,K-ATPase expression by 21% in OP group from 54.4 ± 3.2 RFU to 68.3 ± 3.3 RFU and by 46% in OR group from 30.7 ± 0.7 RFU to 56.1 ± 1.7 RFU. The activity of Na,K-ATPase was also measured in BLM, on the day of preparation (Fig. 15). The 4 week treatment with pioglitazone almost doubled the activity of the pump in obese animals (0.27 ± 0.04 vs. 0.52 ± 0.04 mmol P_i mg^{-1} min^{-1}) while had a tendency to an increase in lean rats (0.37 ± 0.04 vs. 0.47 ± 0.04 mmol P_i mg^{-1} min^{-1}) although did not attain significance (p-value= 0.113).

Next, we examined NHE3 protein levels in brush border membrane vesicles (BBMV) prepared from renal cortex of obese and lean rats with or without pioglitazone treatment by western blotting (Fig.16). Villin served as loading control. NHE3 expression was dramatically reduced by treatment in both groups: in OP from 0.56 ± 0.04 to 0.07 ± 0.007 RFU and in OR from 0.36 ± 0.05

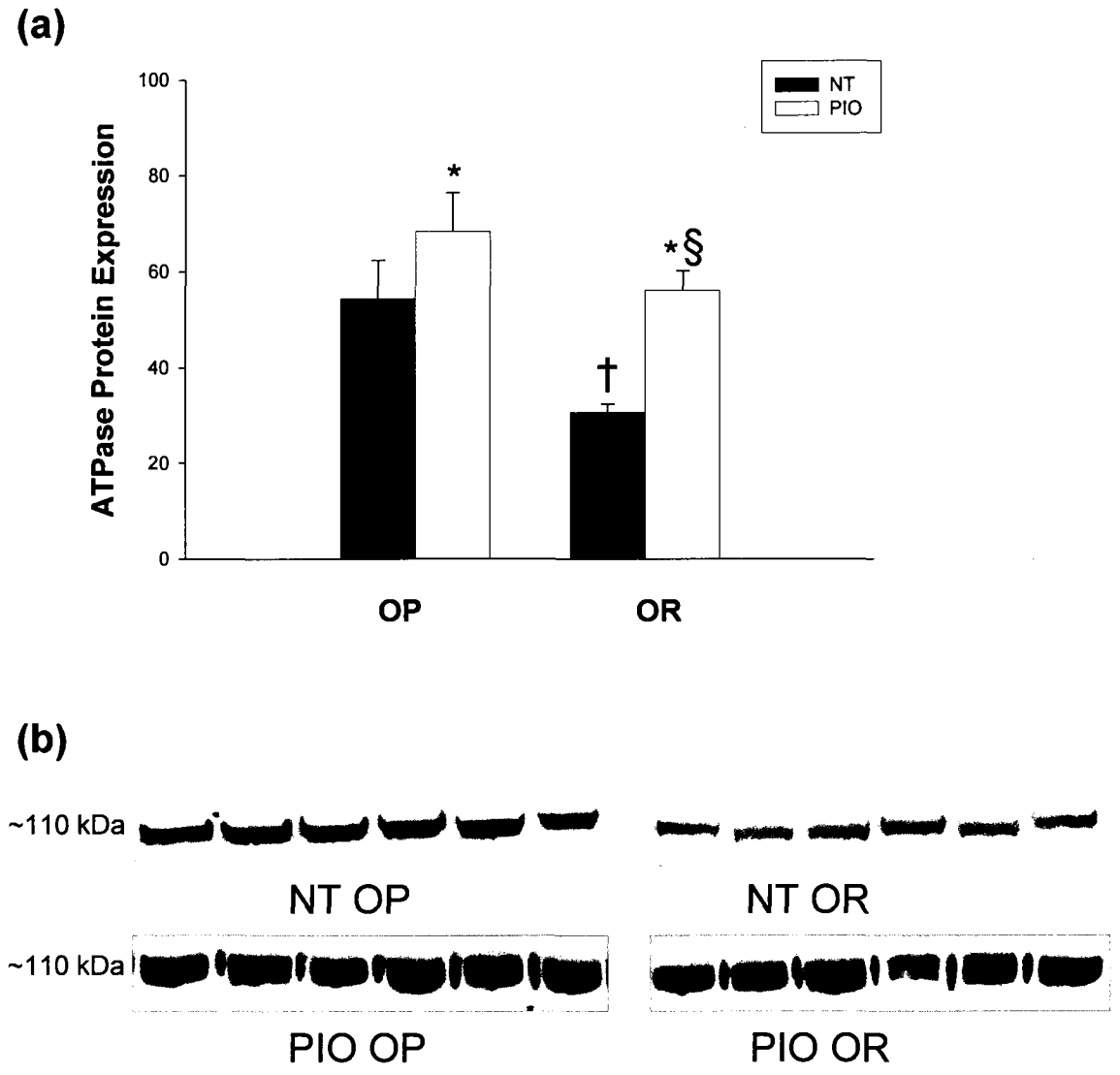

Figure 14 Na,K-ATPase protein expression measured in baso-lateral-enriched membranes isolated from renal cortex of obese (OP) and lean (OR) rats with (PIO) or without pioglitazone treatment (NT).
Panel (a): Graph representation of Na,K-ATPase abundance, measured in arbitrary units, represented as means ± SEM of n = 6 rats/group. Blots were probed with Na,K-ATPase $\alpha 1$ subunit specific antibody. Semi-quantitative densitometry analyses were done by using Odyssey Infrared Imaging System software. Significance was determined using one-way ANOVA and the null hypothesis was rejected for a p-value< 0.05. *=significant compared to non-treated; †=significant compared to OP; §= significant compared to PIO-OP. Panel (b): Immunoblots of BLM samples from non-treated and treated OP and OR rats. Each lane represents a sample from an individual rat. Equal protein loading was ensured by staining each membrane with Ponceau red.

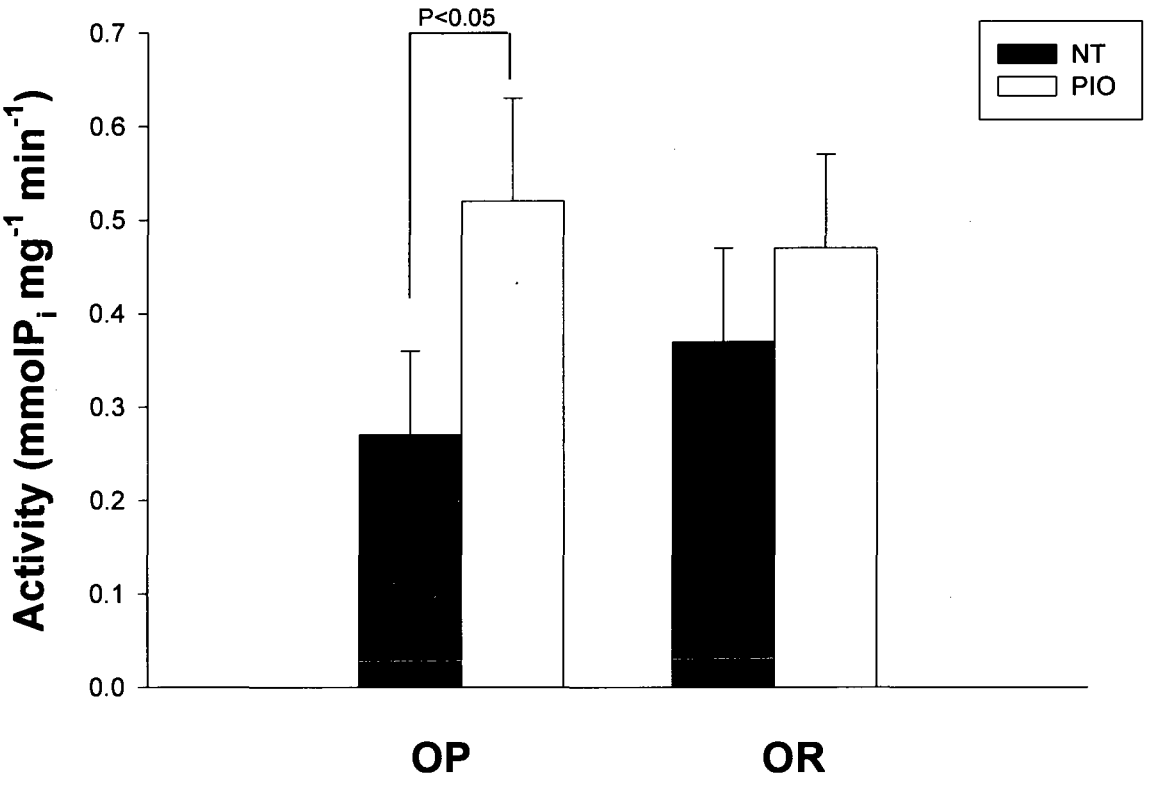

Figure 15 Effect of pioglitazone on Na,K-ATPase activity assessed in baso-lateral membranes prepared from renal cortexes of obese (OP) and lean (OR) animals.
Na,K-ATPase activity expressed as mmol of inorganic phosphate liberated per mg of protein per minute performed on freshly prepared baso lateral membrane (BLM) fractions. Na,K-ATPase activity was calculated as the difference between activities assayed in the absence of ouabain (total) activity and in the presence of 5 mM ouabain. Values are means ± SEM from 6 rats per group. Each sample was assayed in duplicate and background was subtracted from the mean. Pioglitazone treatment significantly increased activity of the pump in obese rats with no effect in lean group. Significance was determined using one-way ANOVA and the null hypothesis was rejected for a p-value< 0.05.

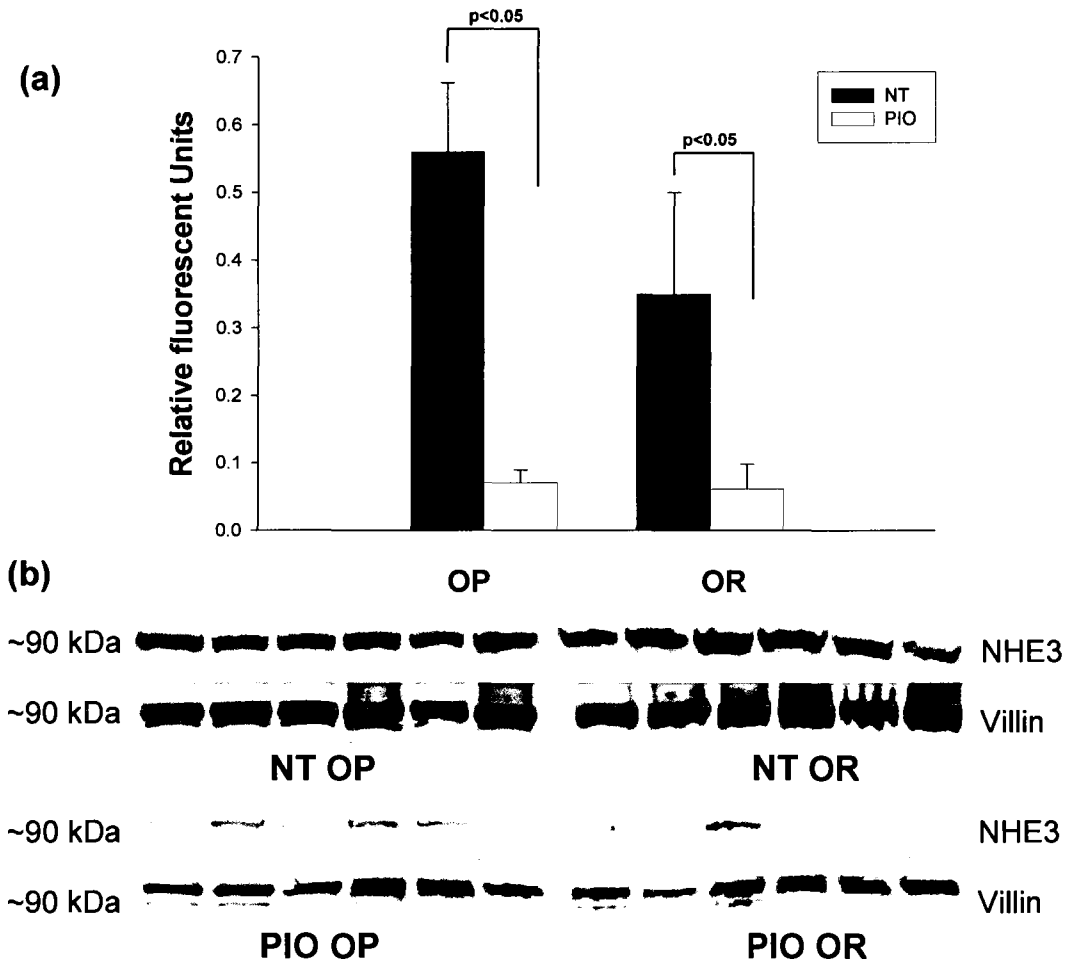

Figure 16 Na/H exchanger (NHE3) protein abundance after pioglitazone treatment in obese (OP) and lean (OR) rats.
NHE3 was assayed in brush border membrane vesicles (BBMV) fractions prepared from renal cortex of OP and OR groups treated or not with pioglitazone. Panel (a): Densitometric analysis of immunoblots shown below. NHE3 immunoreactivity is expressed as a ratio between NHE3 and Villin signals, in relative fluorescent units (RFU). Results represent average ± SEM of n = 6 rat/group. Significance was determined using one-way ANOVA and the null hypothesis was rejected for a p-value< 0.05. Pioglitazone treatment significantly decreased NHE3 protein expression in OP and OR groups. Panel (b): Immunoblots of BBMV samples from obese and lean rats. Equal amount of proteins were loaded into each gel and duplicates of membranes were probed with anti-villin antibody which served as a loading control. Antigen-antibody complexes were detected with Odyssey Infrared Imaging System (Li-Cor).

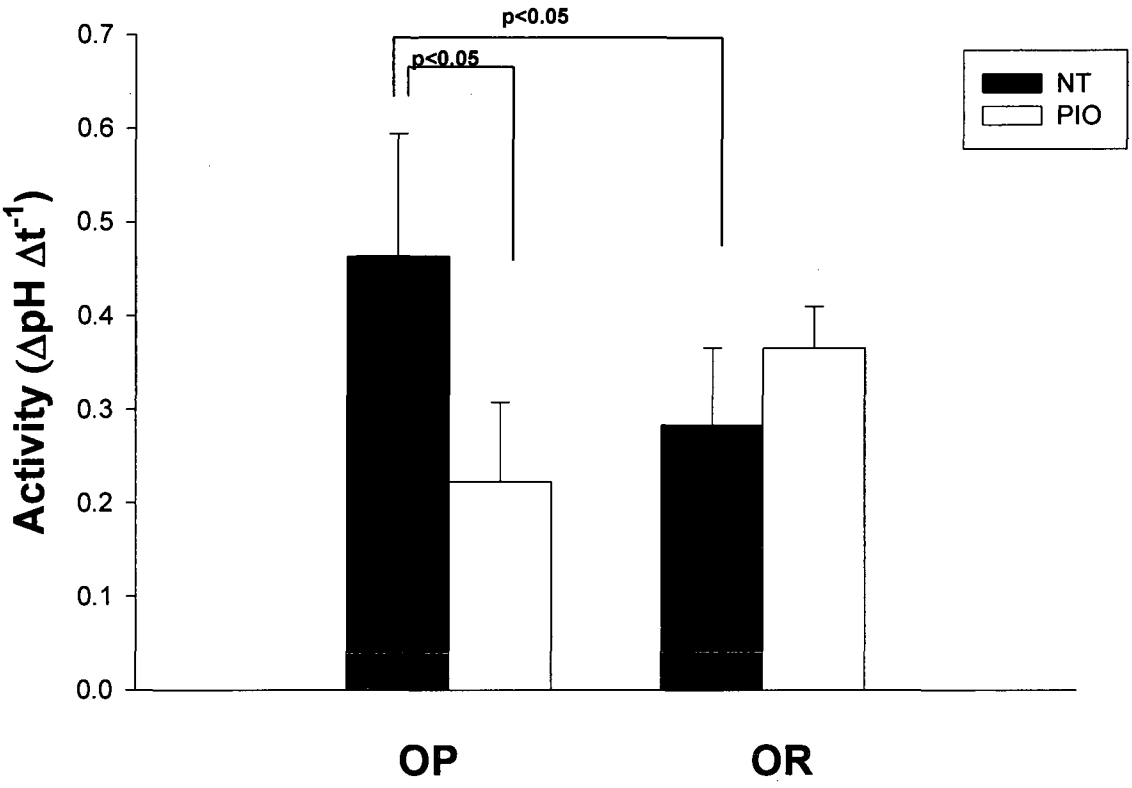

Figure 17 Na^+/H^+ antiporter activity in obese and lean rats non-treated (NT) or treated with pioglitazone (PIO).
Brush border membrane vesicles (BBMV) prepared from renal cortex were preloaded with pH indicator BCECF-AM in Na^+-free buffer, pH 7.2 and then mixed with the buffer solution containing 150 mmol l^{-1} NaCl, pH 9.2 for determination of pH recovery as described in methods paragraph for Aim #1. Equal amounts of protein were labeled and readings for unlabeled samples were subtracted from final values. NHE3 activity was expressed as a change in pH over time. Values are means ± SEM from 6 rats per group. Significance was determined using one-way ANOVA and the null hypothesis was rejected for a p-value< 0.05. Pioglitazone treatment significantly reduced activity of the exchanger in OP but not in OR group.

to 0.06 ± 0.015 RFU. To determine if reduced protein abundance was accompanied by modifications of sodium hydrogen exchanger activity, we measured the latter in freshly isolated BBMVs. and expressed it as the rate of pH recovery in response to extravesicular sodium load (Fig. 17). In the obese group, pioglitazone treatment significantly reduced activity of NHE3 from 0.46 ± 0.13 to 0.22 ± 0.09 $\Delta pH\ \Delta t^{-1}$, which corresponds well with the protein change whereas in lean group pioglitazone had no significant effect on the activity of the transporter. As described in result paragraph for Aim #1 to insure that the activity measured was specific for NHE3, randomly chosen samples of BBMV were incubated with 100 μM of 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) since it inhibits NHE1 isoform of the transporter (data not shown). This incubation resulted in less than 10% inhibition in the rate of fluorescence. To ensure the specificity of the NHE3 activity assay, randomly chosen samples of BBMV were incubated with a higher concentration of EIPA (500 μM), which was expected to block all NHE related activity. The inhibition found in our experiments was more than 95% in all assayed samples (data not shown). Next, we tested if localization of NHE3 plays a role in overall activity of the exchanger since it is possible that redistribution of NHE3 out of the apical microvilli is correlated with inhibition of the transporters. For this purpose we employed immunohistochemistry techniques and acquired images with confocal microscopy. Fig. 18a represents typical images from all four experimental groups. Sections of kidneys were simultaneously stained with NHE3 and villin where the latter was used as a marker of the top of the villi. Therefore, the greater the overlaying of colors the closer NHE3 was to the top of

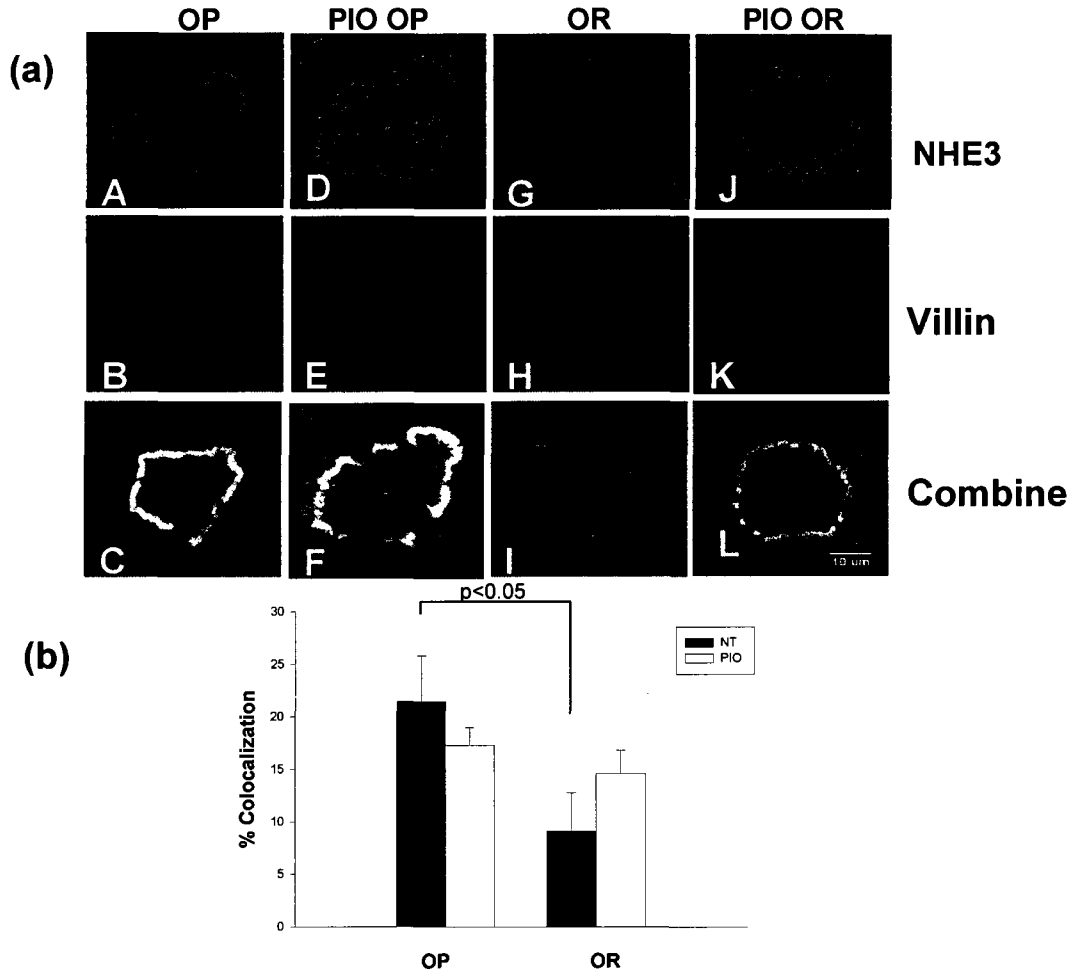

Figure 18 Confocal immunofluorescence analysis of NHE3 distribution in obese and lean rats with or without pioglitazone treatment.
4 micron sections of paraffin embedded kidneys were double stained with polyclonal NHE3 antibody and monoclonal anti-villin antibody followed by the corresponding anti-rabbit (AlexaFluor488-green) and anti-mouse (AlexaFluor594-red) secondary antibodies. Panel (a): Representative images of cortical tubules from all experimental groups double stained with NHE3 (A, D, G, J) and villin (B, E, H, and K). Overlapping (C, F, I and L) of NHE3 and Villin appears yellow. Treament with pioglitazone seemed to change this colocalization but not significantly. Panel (b): Semi-quantitative analysis of NHE3 and villin colocalization in kidney section. 6-10 z-stack images were taken with laser scanning confocal microscope from each animal (n = 3 rats per group). Images were analyzed using Metamorph software and colocalization was expressed as the percentage of the sum of the green staining overlapping the red staining to the sum of the green fluorescence only. Pioglitazone treatment did not produce significant NHE3 redistribution. Significance was determined using one-way ANOVA and the null hypothesis was rejected for a p-value< 0.05. Bar= 10microns.

apical brush border microvilli. Pioglitazone treatment did not have an effect on NHE3 localization in obese rats. However, in the OR group, it increased colocalization from 9.1 ± 2.1 to 14.6 ± 1.3% with borderline significance (p-value=0.09) (Fig. 18b). Therefore we concluded that since changes in protein expression of both transporters are paralleled to a certain extent by changes in activity, pioglitazone must exert its effect at least partially at the genomic level, although there are other factors which can influence general activity of transporters *in vivo*.

In vitro experiments

For our *in vitro* study we used primary cultures of human renal proximal tubule epithelial cells (RPTEC) and examined the effect of PPARγ modulation on NHE3 and Na,K-ATPase using pharmacological and molecular approach. Protein expression of both transporters was assessed by western blotting in cells treated with PPARγ antagonists (GW9682 and BADGE) and PPARγ agonists (PGJ2 and rosiglitazone). Rosiglitazone (Avandia) is a high-affinity ligand for PPARγ and belongs to the same group of anti-diabetic thiazolidinediones (TZD) as pioglitazone. 15-deoxy-Δ 12, 14-prostaglandin J2 (PGJ2) is a natural PPARγ ligand. Activation of PPARγ by PGJ2 and rosiglitazone caused a significant reduction in protein expression of Na,K-ATPase compared to control (C: 0.68 ± 0.04 vs. PGJ2: 0.34 ± 0.1 vs. Rosi: 0.42 ± 0.07 arbitrary units) while PPARγ antagonists: GW9682 and BADGE increased it by 70% and 30% respectively (Fig. 19). Activation and inhibition of PPARγ had

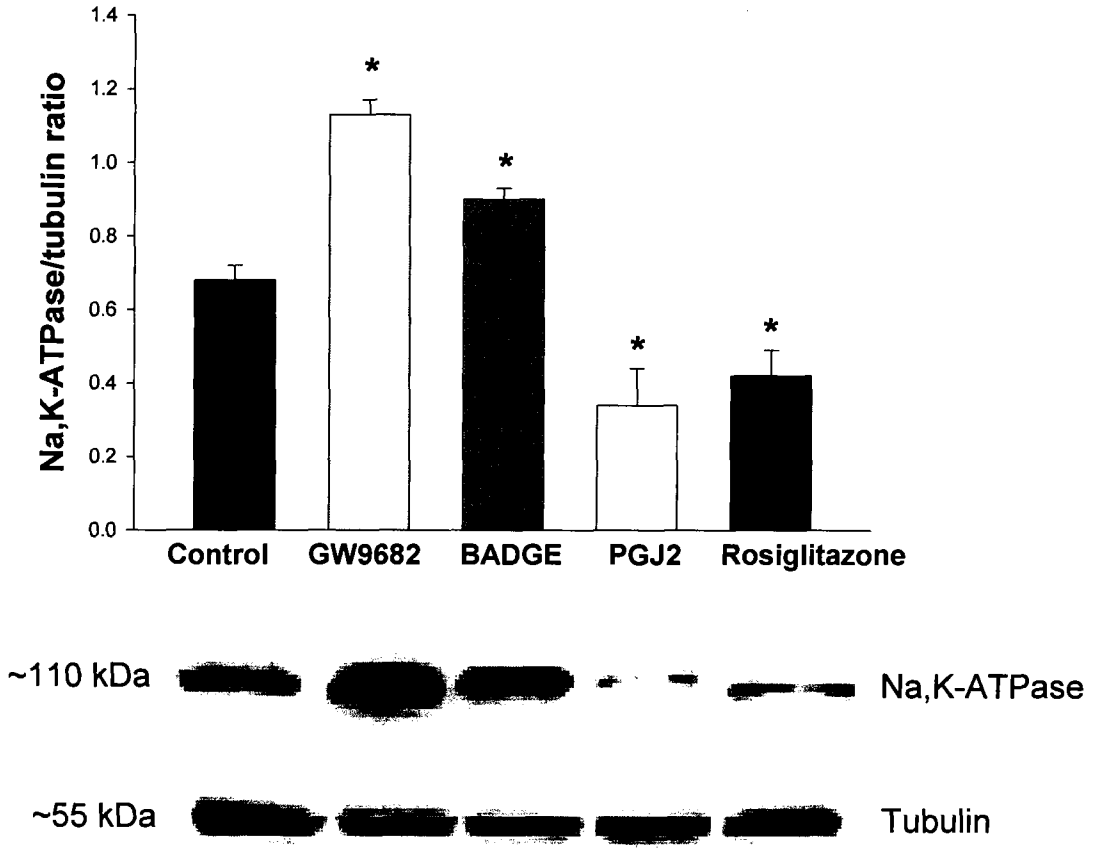

Figure 19 Effect of PPARγ activation or inhibition on Na,K -ATPase protein expression in human renal proximal tubule epithelial cells (RPTEC). RPTEC were incubated for 4 hours with PPARγ antagonists (GW9682 and BADGE) or PPARγ natural or synthetic ligands, PGJ2 and rosiglitazone, respectively. Top: Semi-quantitative analysis of western blots from membrane fractions of RPTEC. Results are expressed as a ratio of Na,K-ATPase to tubulin signal in arbitrary units and represent means ± SD from of 3-5 experiments. Significance was determined using one-way ANOVA and null hypothesis was rejected for a p-value <0.05. *=significant compared to control. PPARγ inhibition increased protein expression of Na,K-ATPase while PPARγ activation reduced it. Bottom: Representative western blots for Na,K-ATPase and tubulin. Equal amounts of protein were loaded into the SDS-PAGE gel and probed with a monoclonal antibody against Na,K-ATPase α1 subunit. Immunoreactive bands were visualized, after incubation with appropriate secondary antibody, using enhanced chemiluminescence. Then membranes were stripped and immunoblotted with anti-tubulin antibody as a protein loading control.

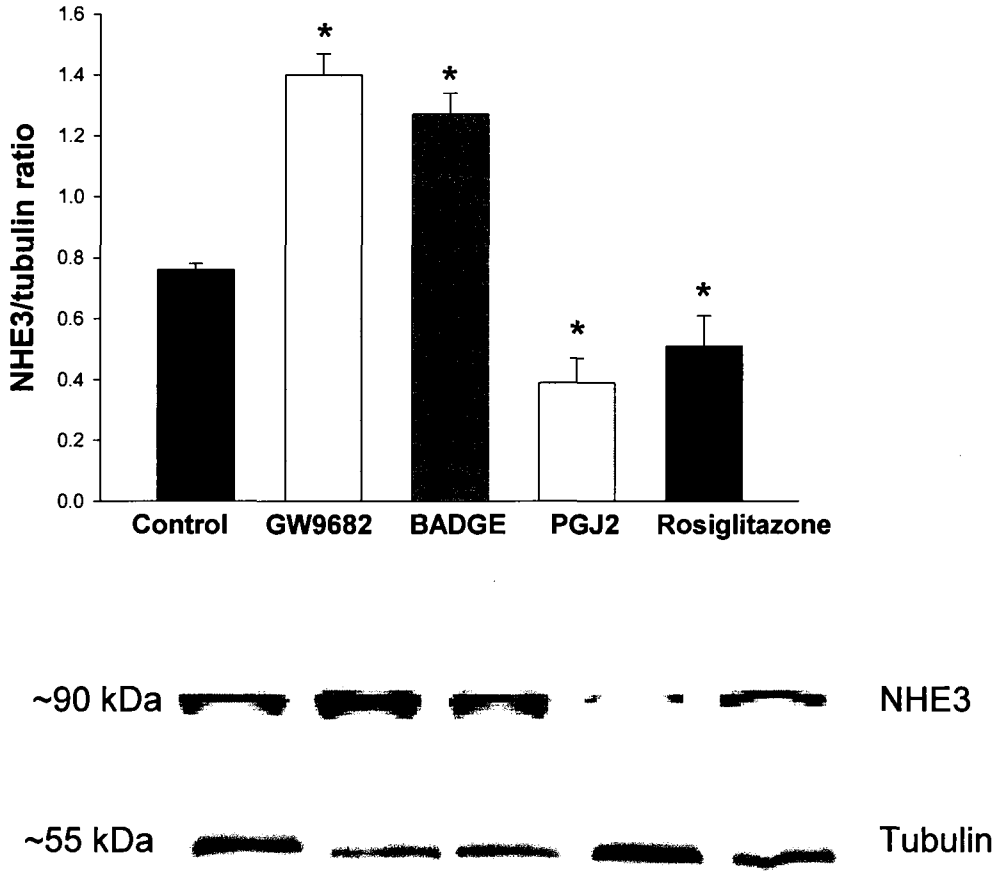

Figure 20 NHE3 protein expression in membrane fractions of human renal proximal tubule epithelial cells (RPTEC) treated with PPARγ agonist and antagonists.
RPTEC were incubated for 4 hours with PPARγ antagonists (GW9682 and bisphenol A diglycidyl ether-BADGE) or the PPARγ natural or synthetic ligands, PGJ2 and rosiglitazone, respectively. Top: Semi-quantitative analysis of western blots from membrane fractions of RPTEC. Results are expressed as a ratio of NHE3 to tubulin signal in arbitrary units and represent means ± SEM from of 3-5 experiments. Significance was determined using one-way ANOVA and null hypothesis was rejected for a p-value <0.05. *=significant compared to control. PPARγ inhibition increased the abundance of NHE3 while PPARγ activation reduced it. Bottom: Representative immunoblots for NHE3 and tubulin. Equal amounts of protein were loaded into the SDS-PAGE gel and probed with a monoclonal antibody against NHE3. Immunoreactive bands were visualized, after incubation with appropriate secondary antibody, using enhanced chemiluminescence. Then membranes were stripped and immunoblotted with anti-tubulin antibody as a protein loading control.

the same effect on NHE3 protein expression namely: GW9682 and Badge increased it from 0.76 ± 0.02 to 1.4 ± 0.07 arbitrary units (GW9682) and 1.27 ± 0.07 arbitrary units (Badge) while PGJ2 and rosiglitazone reduced it to 0.39 ± 0.08 and 0.51 ± 0.01 arbitrary units, respectively (Fig. 20). Those results suggest that PPARγ can effect protein expression of both sodium transporters in cultured renal proximal tubule epithelial cells.

To differentiate if the effects of agonists and antagonists are PPARγ dependent or independent we transiently over-expressed PPARγ using human full-length PPARγ cDNA, containing the V5 tag, cloned into a mammalian expression vector. The efficiency of transfection was verified in cells transfected with the same vector containing the green fluorescent protein gene and was ~55% (Fig. 21). Since PPARγ is a phosphoprotein whose activity is regulated by phosphorylation in addition to ligand binding (Chana *et al.* 2004), the levels of phosphorylated form of PPARγ were evaluated in transfected cells which expressed more phosphorylated PPARγ under basal conditions and upon pioglitazone treatment (Fig. 21).

To further test the involvement of PPARγ in response to pharmacological manipulation we used the small interfering RNA method. The efficiency of the transfection was verified by fluorescent microscopy of cells transfected with Cy3 luciferase-labeled oligonucleotides and was > 90%. Maximal inhibition of mRNA has been achieved after 24-48 hours whereas minimal protein levels were measured 48 to 72 hr after transfection. Based upon these data we performed the experiments 36 hours post-transfection, with an additional 12 hours of

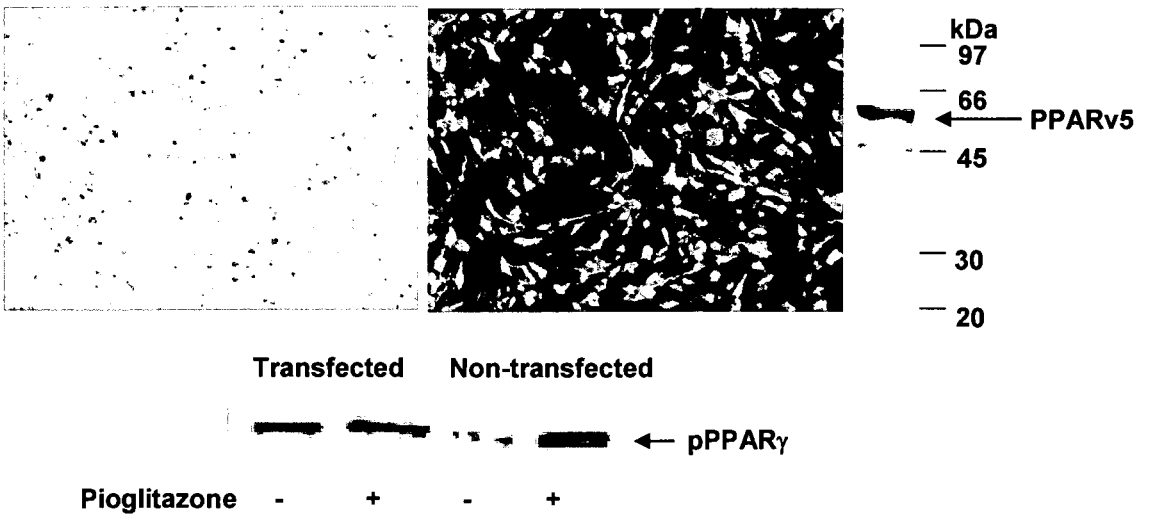

Figure 21 Efficiency of transient transfection for primary renal proximal tubule epithelial cells (RPTEC) by nucleofection method.
36 hours after cells were transfected with plasmid expressing green fluorescent protein (GFP) (pcDNA3.1/GFP) the efficiency of transfection was verified by microscopy. Top panel: Microscopic images of transfected cells taken with phase contrast (left) and fluorescence optics (right). On average, the efficiency of transfection was ~55%. The overexpressed PPARγ, which contains a C-terminal peptide encoding the V5 epitope, was detected by western blot using a anti-V5-HRP antibody (shown on far right). Bottom panel: Representative immunoblot of transfected and non-transfected RPTEC in basal conditions and with pioglitazone treatment probed with antibody against the phosphorylated form of PPARγ. Transfected cells expressed more phosphorylated PPARγ under both conditions.

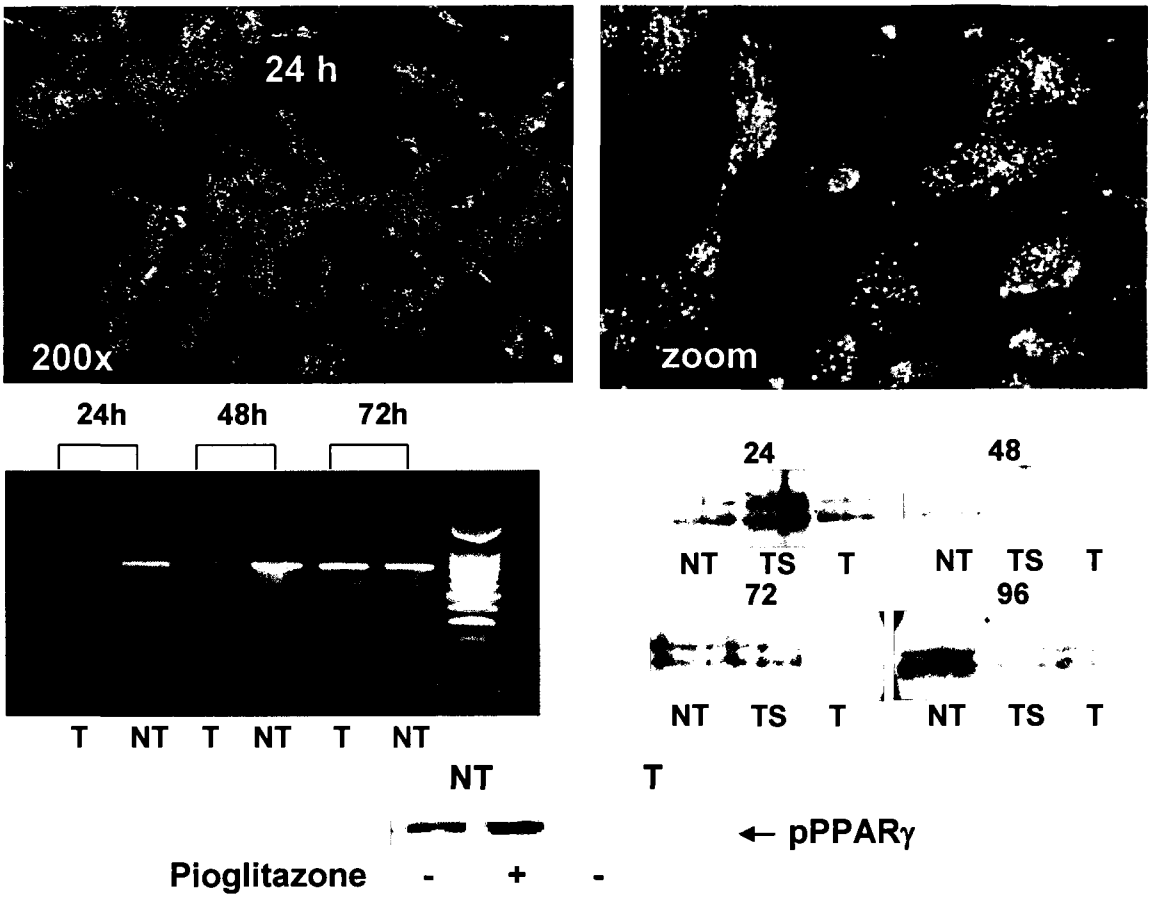

Figure 22 Efficiency of transient transfection with small interfering RNA (siRNA) for primary renal proximal tubule epithelial cells (RPTEC) using X-tremeGene siRNA Transfection Reagent.
Top panel: Representative results of transfection efficiency 24 hr after transfection of RPTEC with Cy3-Luciferase siRNA. The efficiency of the transfection was > 90%. Middle panel: Representative RT-PCR showing expression of PPARγ mRNA at 24, 48, 72 hours in RPTEC transfected (T) or non-transfected (NT) with siRNA (left). Transfection was most effective between 24 and 48 hours. Representative western blotting of protein expression of PPARγ at 24, 48, 72 and 96 hours post-transfection in cells non-transfected (NT), transfected (T) or transfected with scrambled oligonucleotides (TS) (right). The lowest protein expression was detected between 48-72 hours. Bottom panel: Representative western blotting of transfected and non-transfected RPTEC in basal conditions and with pioglitazone treatment probed with anti-phospho PPARγ. Expression of pPPARγ was virtually undetectable in RPTEC transfected with siRNA.

pharmacological stimulation with pioglitazone (Fig. 22). Na,K-ATPase protein expression was significantly reduced in wild type cells upon pioglitazone treatment to 50% of the control. Overexpression of PPARγ followed by pioglitazone treatment decreased Na,K-ATPase protein by 65% while silencing of the receptor abolished the inhibitory effect of the drug (Fig. 23). To diminish variability of the data from different blots controls are presented as 100% and treatment values are normalized to controls. Similar changes were estimated in protein expression of NHE3. In wild type cells pioglitazone significantly decreased NHE3 levels by 35%, simultaneous overexpression and activation of PPARγ caused reduction of exchanger by 54% while siRNA eliminated the effect of PPARγ activation (no change in protein levels) (Fig. 24). Together, the *in vitro* data indicate that pioglitazone exerts its action on human proximal tubule sodium transporters, Na,K-ATPase and NHE3, via PPARγ activation.

4.3 Discussion for Specific Aim #2

In this study we examined the effect of chronic pioglitazone treatment on systolic blood pressure (SBP) in obese and lean Sprague-Dawley rats on high fat diet and began to unravel cellular and molecular mechanisms underlying the observed effects. Pioglitazone (Actos) is an antidiabetic agent that acts primarily by decreasing insulin resistance and is used in the management of type 2 diabetes. Together with rosiglitazone it belongs to the thiazolidinedione (TZD) group of drugs which are high-affinity synthetic ligands for PPARγ. We reported

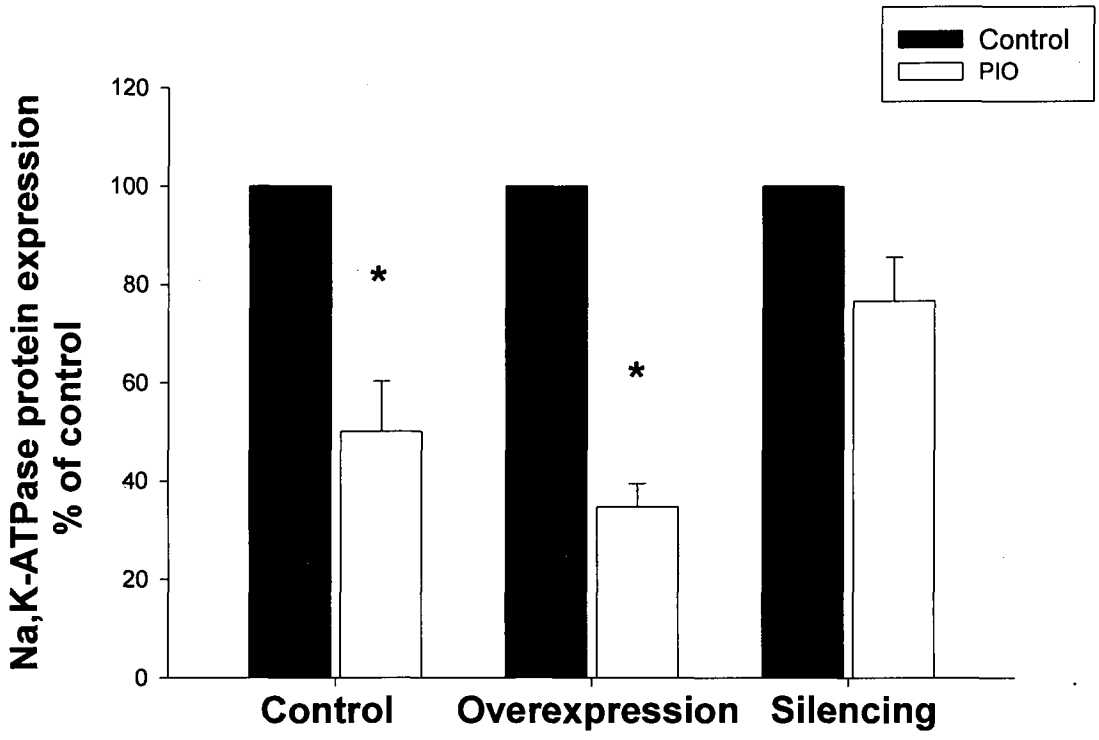

Figure 23 Na,K-ATPase expression levels in RPTEC following overexpression or silencing of PPARγ and pioglitazone stimulation.
Cells were transiently transfected with either a full length human PPARγ cDNA cloned in a pcDNA3.1/GS vector or with siRNA designed to target a common sequence in both PPAR-γ1 and γ2 isoforms. Equal amount of protein from total homogenates of cells were loaded into 7.5% polyacrylamide gel. Membranes were immunoblotted with monoclonal anti Na,K-ATPase α1 subunit antibody and detected by Odyssey Infrared Imaging System. Membranes were stripped and reprobed with anti-tubulin which served as a loading control. Results are expressed as a percentage of the controls of Na,K-ATPase/tubulin ratio to minimize variability between different blots. Values are mean ± SD from 3-5 experiments performed in duplicate. Significance was determined using unpaired two tail t-test and the null hypothesis was rejected for a p-value <0.05. *= significant compared to control.

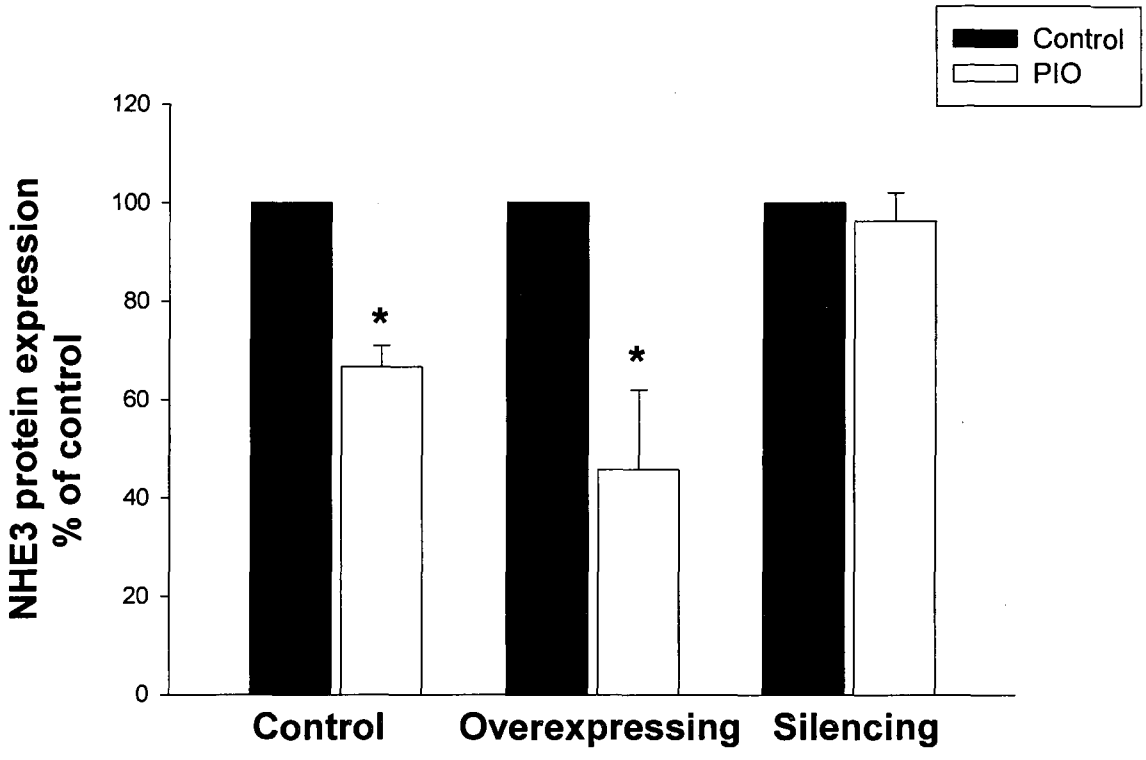

Figure 24 NHE3 expression levels in RPTEC following overexpression or silencing of PPARγ and pioglitazone stimulation.
Cells were transiently transfected with either a full length human PPARγ cDNA cloned in a pcDNA3.1/GS vector or with siRNA designed to target a common sequence in both PPAR-γ1 and γ2 isoforms. Equal amount of protein from total homogenates of cells were loaded into 7.5% polyacrylamide gel. Membranes were immunoblotted with polyclonal anti NHE3 antibody and detected by Odyssey Infrared Imaging System. Membranes were stripped and reprobed with anti-tubulin which served as a loading control. Results are expressed as a percentage of the controls of NHE3/tubulin ratio to minimize variability between different blots. Values are mean ± SD from 3-5 experiments performed in duplicate. Significance was determined using unpaired two tail t-test and the null hypothesis was rejected for a p-value <0.05. *= Significant compared to control.

that 4 week pioglitazone treatment lowered SBP by ~18 mmHg in obesity-prone (OP) rats while having no effect on blood pressure of obesity-resistant (OR) rats on a high fat diet. To date, there is extensive evidence that PPARγ agonists reduce blood pressure in a wide range of insulin-resistant (Yoshimoto *et al.* 1997, Walker *et al.* 1999) as well as in non-insulin-resistant animal models of hypertension (Zhang *et al.* 1994), and in human studies of diabetic or non-diabetic hypertensive subjects (Ogihara *et al.* 1995, Fullert *et al.* 2002). The effects of these PPARγ agonists on blood pressure in normotensive rats and humans are not very conclusive. Song *et al.* reported a decrease in blood pressure in normal rats after treatment with rosiglitazone (Song *et al.* 2004) while Tanimoto *et al.* reported no change in systemic blood pressure in diabetic KK/Ta mice treated with pioglitazone for 4 or 8 weeks (Tanimoto *et al.* 2004). Moreover, Zanchi and coworkers demonstrated that in healthy human subjects chronic administration of pioglitazone did not alter blood pressure (Zanchi *et al.* 2004). The discrepancy between the study by Song and our results could most likely be explained by differences in the selection of the animal model, the diet, the length of the treatments, or various TZDs used (rosiglitazone vs. pioglitazone).

Multiple mechanisms have been implicated in the anti-hypertensive effects of TZDs, including an increase in insulin sensitivity (Uchida *et al.* 1997, Walker *et al.* 1999), direct vascular effects (Diep *et al.* 2002, Ryan *et al.* 2004), modulation of endothelial vasoactive factors (Fujiwara *et al.* 1998) and direct renal action (Isshiki *et al.* 2000). The renal related effects of TZDs explained the fluid retention and edema, a serious side-effect, induced sometimes by TZD treatment

in both animals and humans (Yang *et al.* 2003, Zanchi *et al.* 2004). The present study was undertaken to elucidate the renal mechanisms of blood pressure lowering effects of TZDs. This could further facilitate our understanding of the complex renal effects of TZDs. We showed that sodium excretion was similar under chronic conditions in all the experimental groups. However, when challenged with acute volume expansion, lean rats treated with pioglitazone excreted significantly less sodium than obese rats. Glomerular filtration rate (GFR) remained the same in all the groups. In accordance with our data, studies by Yang *et al.* and Zanchi *et al.* showed no change in GFR after TZD treatment in animal and humans, respectively (Yang *et al.* 2003, Zanchi *et al.* 2004). Conversely, a study by Song *et al.* reported that acute, three-day administration of rosiglitazone, reduced the GFR, measured indirectly as creatinine clearance, (Song *et al.* 2004). It remains to be reconciled whether or not this discrepancy is related to the methods used to estimate GFR or differences in other experimental protocols such as the length of the treatment, the choice of the animal model or dietary regimen.

Our study demonstrated that in an animal model of obesity-induced hypertension pioglitazone lowers blood pressure without effecting renal parameters or sodium excretion while in normotensive lean rats it caused no difference in blood pressure even as it lead to sodium retention with no change in GFR. As we did not observe an alteration in renal hemodynamic parameters following treatment with pioglitazone we proceeded to evaluate the ability of TZDs to directly influence tubular reabsorption. We focused our work on the

proximal tubule since we showed in the previous aim that in our model of diet-induced hypertension the blunted pressure natriuresis and diuresis was mediated by increased sodium reabsorption in this segment of the nephron.

We evaluated expression, activity and distribution of two sodium transporters: Na,K-ATPase and Na^+/H^+ exchanger type 3 (NHE3) in obese and lean Sprague-Dawley rats chronically treated with pioglitazone. We reported that Na,K-ATPase protein expression was increased by pioglitazone in obese and lean rats while its activity was stimulated only in the OP group. This is difficult to reconcile with the observed reduction in blood pressure in obese rats treated with pioglitazone and a lack of changes in sodium excretion in this group. The reason for this is not clear but our less than pure basolateral membrane preparations could be one probable cause. Renal cortex is enriched in proximal tubules but it also contains other nephron segments such as the thick ascending limb of Henle's loop, distal convoluted tubule, and connecting tubule. All these segments express Na,K-ATPase in the epithelial cell basolateral membranes and may also contain other pumps which are ouabain sensitive such as H^+,K^+-ATPase (Beltowski & Wojcicka 2002). A contamination with intracellular membranes although unlikely, remains a possibility. A lower than expected ouabain inhibitable activity was found consistently in our membrane preparations. In OR rats treated with pioglitazone, an increase in Na,K-ATPase abundance correlated well with reported stimulation of sodium reabsorption in these animals but did not explain the lack of difference in their blood pressure after the treatment. The discordant data between activity and expression of Na,K-ATPase in this group

could be attributed to a redistribution of enzyme subunits to intracellular organelles (early endosomes) during Na,K-ATPase inhibition as suggested by Chibalin *et al.* in opposum kidney (OK) cells (Chibalin *et al.* 1998).

Another regulatory mechanism for Na,K-ATPase activity involves the cytoplasmic sodium concentration (Soltoff & Mandel 1984) and the levels of circulating hormones such as dopamine (Meister & Aperia 1993) and insulin (Sweeney & Klip 1998). There are reports showing defects of dopamine receptors (D1) in proximal tubules of spontaneously hypertensive rats (SHR) (Kinoshita, S. *et al.* 1989) which lead to blunted pressure natriuresis (Felder *et al.* 1990). Similarly, dopamine was unable to inhibit the activity of Na,K-ATPase and NHE3 in proximal tubules of obese Zucker rats (Hussain *et al.* 1999). Umrani and co-workers reported that PPARγ activation restores renal dopamine receptor function in obese Zucker rats by lowering plasma insulin levels (Umrani *et al.* 2002). Although we did not measure dopamine receptors in our model, it is probable that the same effect was achieved in our study. In addition to dopamine, insulin exerts both short and long-term effects on Na,K-ATPase. Nevertheless, in diabetic experimental models, effects of PPARγ activation are rather controversial. An increase in whole kidney and cortical Na,K-ATPase activity was reported by Ng *et al.* in streptozotocin-induced diabetic rats (Ng *et al.* 1993) and by Bickel *et al.* in obese Zucker rats at 2 and 4 months of age (Ng *et al.* 1993, Bickel *et al.* 2001); no change in cortical Na,K-ATPase expression was found in 6-month old obese Zucker rats (Bickel *et al.* 2002) . Furthermore, Song *et al.* have demonstrated that a 3 day treatment with a PPARγ agonist increased the

renal abundance of Na,K-ATPase in normal Sprague-Dawley rats (Song *et al.* 2004). Riazi *et al.* reported that 12 week treatment with rosiglitazone also increased the whole kidney expression of Na,K-ATPase in obese Zucker rats (Riazi *et al.* 2006) whereas Umrani *et al.* showed that 4 week rosiglitazone treatment of obese Zucker rats attenuated the increased Na,K-ATPase activity (Umrani *et al.* 2002). In OP rats Dobrian *et al.* reported increased levels of insulin (Dobrian *et al.* 2004). This may account for differential regulation of Na,K-ATPase in different animal models. Moreover, the disagreement between different studies indicate that not only insulin but additional factors such as age and other hormones interacting together can influence Na,K-ATPase activity. Although TZDs effectively attenuate insulin resistance in hyperinsulinemic states they do not affect sodium transporters in an unequivocal way. This suggests that the effects of PPARγ stimulation on sodium transporters is dependent on the length of treatment, animal model used (normal versus obese versus lean treated high fat diet), the metabolic milieu of the animal, or type of TZD used.

We also examined the effect of PPARγ activation on the apical sodium/hydrogen exchanger type 3 (NHE3). NHE3 is one of the most regulated transport proteins. It can be modulated by changes in expression, alterations in intrinsic activity without changes in NHE3 protein abundance (Soleimani *et al.* 1995), phosphorylation status (Moe 1999), changes in trafficking (Biemesderfer *et al.* 2001), and presence or absence of its regulatory factors (NHERF) (Yun *et al.* 1997). Pioglitazone treatment decreased NHE3 protein expression in lean rats without a reduction in the activity of the transporter. We also found a

redistribution of NHE3 towards the top of the villus in the lean rats, which may account for the lack in activity change despite a reduction in protein abundance. In the obese rats we did not find a change in NHE3 membrane distribution and a reduction in both protein abundance and activity. These results suggest that both abundance and distribution of NHE3 are important for activity of the transporter. The results for both OP and OR groups correlate well with changes in sodium reabsorption reported in this Aim. However, this data further implies that ability to respond to TZDs can vary depending on the metabolic state of the subject (lean versus obese). Based on the reports by Hall (Hall 2003) and the results from this and the previous aims we may conclude that obese rats present with the expansion of extracellular fluid volume and most likely can not retain much fluid when challenged with acute saline loading. On the other hand, lean rats treated with pioglitazone react to acute volume expansion with the disproportionate expansion of the extracellular space which finally leads to fluid retention. Further studies are needed to address these observations.

In obese rats treated with pioglitazone, reduction in expression and activity of NHE3 at the proximal tubule level could explain a drop in systolic blood pressure. On the other hand, the lack of changes in sodium excretion in this group could possibly be justified by an upregulation of other transporters in this or other nephron segments. These transporters include, among others, the sodium phosphate cotransporter subtype II (NaPi-2), at proximal tubule level and the amiloride-sensitive sodium channel (ENaC) in the collecting duct (Knepper

2002). Circumstantial evidence provided by this study suggests that stimulation of NaPi-2 and ENaC represents a potential alternative.

Since our animal model of diet-induced obesity makes difficult the *in vivo* assessment of a direct effect of TZDs on PPARγ activation, we examined the latter *in vitro* in human renal proximal tubule epithelial cells (RPTEC). We modulated PPARγ pharmacologically or by gene overexpression or silencing. Protein expression of Na,K-ATPase was decreased upon activation of PPARγ by the natural and synthetic ligands PGJ2 and rosiglitazone, respectively and was increased by PPARγ inhibitors GW9682 and BADGE. Moreover, overexpression of the receptor followed by its activation led to reduction in Na,K-ATPase protein expression. This effect of pioglitazone was abolished in cells transfected with siRNA for PPARγ, providing evidence that pioglitazone requires the expression of peroxisome proliferator-activated receptor gamma in order to reduce Na,K-ATPase protein expression.

There are a few proposed mechanisms through which PPARγ activators can negatively regulate expression of other genes. The transcriptional suppression of Na,K-ATPase could be due to a direct binding of activated PPARγ to the PPAR-response element in the gene promoter, as previously described for the sex hormone-binding globulin gene (Selva & Hammond 2009) and for TZDs inhibition of B3-adrenergic receptors (Bakopanos & Silva 2000). Alternatively, inhibitory effects might occur independent of a PPAR binding site by a physical interaction with other transcription factors in a process called trans-repression. For instance, sumoylation of PPARγ ligand-binding domain leads to prevention of

the ubiquitylation machinery that normally mediates the signal-dependent removal of corepressor complexes required for gene activation as was noted for inducible nitric oxide synthase (iNOS) (Pascual *et al.* 2005). The trans-repression mechanism is indirectly supported by the lack of PPRE element in the promoter of human α1-subunit Na,K-ATPase (mapped data supplied by NCBI ID:NM_000701.6) as determined using Genomatix MatInspector software (Genomatix Software Inc., Ann Arbor, MI).

Nevertheless, it was surprising that the effects of PPARγ activation on sodium transporters expression *in vivo* and *in vitro* were contradictory. We observed an increase in Na,K-ATPase expression in the former experiments and a reduction in the latter after treatment with pioglitazone. This discrepancy could most likely be clarified by the fact that very different preparations were used for these experiments. As already discussed, baso-lateral fractions prepared from the rat kidney were not very pure and could contain some other segments of nephron as well as some intracellular membranes. On the other hand, for our *in vitro* measurements we used a pure preparation of renal proximal tubule epithelial cells. Furthermore, the metabolic milieu *in vivo* may have important influences on the transporters modulation as previously discussed. Finally, it is very likely that in our *in vivo* model of diet-induced obesity and *in vitro* cell culture model PPARγ expression and activity levels are different.

Similarly, we conducted *in vitro* experiments to investigate whether pioglitazone modulates NHE3 expression via a PPARγ related mechanism. The effects measured for Na,K-ATPase were mirrored by the results obtained for

NHE3. Protein expression of NHE3 was decreased upon activation of PPARγ by the natural or synthetic ligands PGJ2 and rosiglitazone, respectively and was increased by PPARγ inhibition by GW9682 and BADGE. Stimulation of PPARγ in cells overexpressing the receptor led to a reduction in NHE3 abundance while having no effect in cells transfected with PPARγ siRNA. Thus, we concluded that pioglitazone requires the presence of peroxisome proliferator-activated receptor gamma in order to exert its inhibitory effect on NHE3 protein expression. Studies by Oliver *et al.* showed that troglitazone inhibited NHE3 activity in proximal tubule-like LLC-PK1 cells (Oliver *et al.* 2005). Also, de Dios *et al.* showed that acute exposure to troglitazone but not rosiglitazone, inhibits Na/H exchange activity in cultured bovine endothelial cells (de Dios *et al.* 2001).

As discussed before for Na,K-ATPase, inhibition of NHE3 gene transcription by activated PPARγ can take place by binding to peroxisome proliferator responsive element (PPRE) in the gene promoter (Selva & Hammond 2009) or by trans-repression mechanisms (Pascual *et al.* 2005). Since human NHE3 does not contain a putative PPRE sequence in the promoter based on software analysis of the promoter sequence (Genomatix Software Inc., Ann Arbor, MI) it is unlikely to have a direct NHE3 transcriptional effect. However, in the rat NHE3 promoter (GeneBank # S833406) a putative PPRE sequence is reported (Genomatix MatInspector software; Genomatix Software Inc., Ann Arbor, MI) suggesting a possible direct transcriptional regulation of NHE3 gene and implying that the PPARγ signaling pathway may be species-specific.

In addition, some of the pleiotropic actions of PPARγ synthetic ligands on cellular function may involve pathways other than the classical PPARγ mediated pathway (Rangwala & Lazar. 2004). These involve Mitogen Activated Protein Kinase Kinase (MEKK) (Takeda. *et al.* 2001) and mitochondrial AMPK (AMP-activated protein kinase) activation (Feinstein *et al.* 2005). Turturro *et al.* demonstrated an inhibitory effect of troglitazone on NHE3 activity in proximal tubule-like LLC-PK1 cells and proposed that physiological responses to TZDs may reflect the interaction of more than one pathway (Turturro *et al.* 2007). This opens the possibility that in RPTEC PPARγ activation may use different pathways to exert effects on NHE3 expression and activity. However, further studies are warranted to examine the involvement of these different pathways.

In summary, the *in vitro* study demonstrated that pioglitazone can inhibit protein abundance of Na,K-ATPase and NHE3 by directly activating PPARγ. *In vivo* the reduction in blood pressure in obese animals was correlated with reduced NHE3 activity due, at least partially, to a reduction in protein expression without significant changes in transporter trafficking. Besides, pioglitazone stimulated basolateral Na,K-ATPase in the renal tubules. Also, blood pressure of lean rats was not changed following chronic pioglitazone treatment but sodium reabsorption was significantly increased.

Clearly, further *in vivo* studies are needed to determine the expression and activity of other sodium transporters along nephron length in obese and lean rats fed a high fat diet to complement our understanding of physiological processes underlying their diverse responses to PPARγ activation.

SECTION 5

STUDIES FOR SPECIFIC AIM #3

5.1 Materials and Methods for Specific Aim #3

Animals

The animal husbandry and separation into groups was done as described in methods for specific Aim #1. After 8 weeks on the diet the OP and OR groups were randomly divided into 2 sub-groups (each with n of 6): 1) no treatment, diet only: NT-OP/NT-OR and 2) treatment with 1.2 mg $kg^{-1}day^{-1}$ N^{G}-nitro-L-arginine methyl esther (L-NAME, Sigma-Aldrich, Saint Louis, MO) in drinking water for 4 additional weeks (L-OP/L-OR). This dose of L-NAME has been reported not to increase mean arterial pressure (Cadnapaphornchai *et al.* 2001). Body weights and food intake were measured weekly as mentioned in section 4, water intake was determined every other day during treatment with L-NAME (Fig. 25). For part of the study in which the effects of L-NAME on pressure natriuresis were evaluated, different groups of rats were used than for studies involving regualtion of sodium transporters. However, both groups followed the same dietary and treatment protocols.

Surgical procedure for acute pressure natriuresis experiments

The surgical procedure was modified from Khraibi *et al.* (Khraibi 2000) and performed as previously described in the Material and Methods part of specific Aim #1.

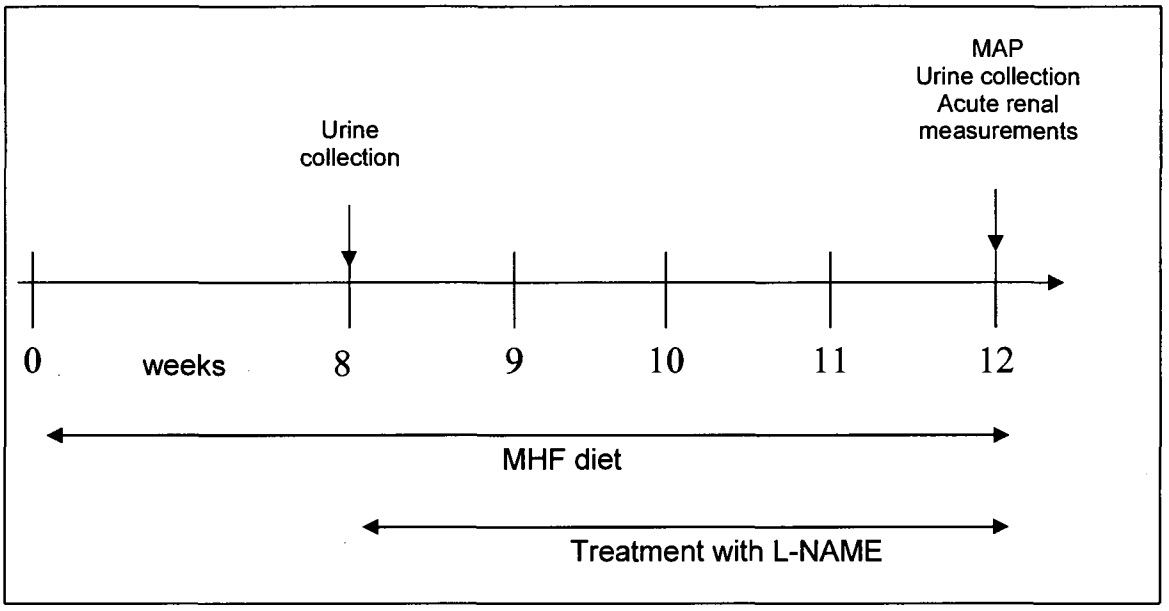

Figure 25 Research design for specific aim #3A.
Male, 3 months old Sprague-Dawley rats were started on moderately high fat diet (MHF) a week after arrival to the facility. Before and after treatment, rats from all groups were placed in metabolic cages for 24 hour urine collection. Non-treated groups OP and OR were placed in metabolic cages only once, right before surgeries. Water intake and urine volume was recorded and urine was analyzed for Na^+ content. Starting with week 8^{th} of the diet each group: OP and OR was further divided into 2 subgroups and was treated for additional 4 weeks with: 1.2 mg^{-1} kg^{-1} L-NAME in drinking water or left untreated (diet only). After 4 weeks of treatments, and total of 12 weeks of the diet, acute measurements of renal function were performed during surgical procedure.

Physiological parameters

All physiological parameters were assessed, measured and calculated as described in the Material and Methods section for Aim #1.

Baso-lateral membrane (BLM) and brush border membrane vesicles (BBMV) preparations

BLM and BBMV fractions of renal cortexes from nontreated animals and rats treated with pioglitazone were prepared using the same protocols as described in the Material and Methods paragraph for Aim #1.

Western Blotting on BLM and BBMV preparations

Na,K-ATPase and NHE3 protein expression were assessed by western blotting as described in the Material and Methods paragraph for Aim #1.

Activity of Na,K-ATPase and NHE3

Na,K-ATPase activity was measured in freshly obtained BLM preparations and NHE3 activity was measured in freshly obtained BBMV fractions as previously described in the Material and Methods paragraph for Aim #1.

Immunocytochemistry, image analysis and data collection

Colocalization of NHE3 (green) and villin (red) were evaluated on paraffin-embedded sections of kidney from untreated and treated animals; images were

recorded by confocal fluorescent microscopy and overlapping of both colors were analyzed by MetaMorph software as described in the Material and Methods paragraph for Aim #1.

Nitrite/nitrate Measurement

Total nitrite/nitrate levels were determined as a measure of NO production in urine collected from untreated and treated animals during a 24 hr period and in the supernatant from treated and control cells using an enzymatic kit (R&D Systems, Minneapolis, MN). This assay determines NO concentrations based on the enzymatic conversion of nitrate to nitrite by nitrate reductase, which is followed by colorimetric detection of nitrite. A set of standards was assayed in duplicates along with the samples. The background was subtracted from each reading, and the average optical density was calculated. The values were represented as micromole of nitrite/nitrate per milliliter.

Cell culture and treatments

The initial batch of human renal proximal tubule epithelial cells (RPTEC) was purchased from Lonza, Inc (Walkersville, MD) and was grown in the media recommended by the supplier-REBM, containing 5% fetal bovine serum. RPTEC were always used between passage 3 and 6. They were seeded at a density of 3.3×10^5 per 100 mm plate for western blotting and grown to ~80% confluence before the start of the experiments. For measurements of cGMP cells were seeded at 1×10^5 cells per well on 12-well plates 48 hours before experiments.

Cells were kept in serum and insulin-free medium 24 hrs before experiments. At first, RPTEC were treated with NO donor S-nitro-acetylpenicillamine (SNAP, Cayman Chemical, Ann Arbor, MI) at various concentrations (10^{-4} M, 10^{-5} M, 10^{-6} M) for 5 min, 30 min and 2 hrs in the presence of 1 mM 3-Isobutyl-1-methylxanthine (IBMX, Sigma, Saint Louis, MO). In the next step of the study, cells were preincubated with 1 mM IBMX and incubated with SNAP (10^{-5} M) and oxadiazolo-quinoxaline (ODQ, 10^{-4} M, Sigma, Saint Louis, MO), a guanylyl cyclase inhibitor or Probenecid (10^{-4} M, Sigma, Saint Louis, MO) which blocks an organic anion transporter (OAT). Next, RPTEC were preincubated with1 mM IBMX and incubated with different concentrations of Angiotensin II (10^{-6} M, 10^{-7} M, 10^{-8} M)(Sigma-Aldrich, Saint Louis, MO), Leptin (15 ng ml^{-1}, 30 ng ml^{-1}, 45 ng ml^{-1} and 60 ng ml^{-1}) (Calbiochem, San Diego, CA) and Insulin (10^{-7} M, 10^{-8} M, 10^{-9} M)(Sigma-Aldrich, Saint Louis, MO) for 2 hrs in the presence or absence of SNAP (10^{-6} M). Finally, cells preincubated with 1 mM IBMX were treated with the selected concentrations of Angiotensin II (10^{-8} M), Insulin (10^{-9} M), Leptin (45 ng ml^{-1}) with or without SNAP (10^{-5} M) in the absence or presence of Probenecid (10^{-4} M).

cGMP measurements

cGMP measurements were performed using a commercially available competitive enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor) following the instructions by the manufacturer. Levels of cGMP were initially assessed in the supernatant and cell lysates in the presence of 1 mM of IBMX

under basal conditions and upon treatments with different concentrations of SNAP at various time points. In the next step, levels of cGMP were assessed in the cells preincubated with 1 mM IBMX and incubated with SNAP (10^{-5} M) and ODQ (10^{-4} M) or Probenecid (10^{-4} M). Also, cGMP levels were tested in the supernatant and cell lysates of the cells preincubated with1 mM IBMX and incubated with different concentrations of angiotensin II, leptin, and insulin for 2 hrs in the presence or absence of SNAP (10^{-6} M). Afterwards cGMP levels were tested in supernatant or cell lysates preincubated with 1mM IBMX and incubated with angiotensin II (10^{-8} M), insulin (10^{-9} M), leptin (45 ng ml^{-1}) with or without SNAP (10^{-5} M) in the absence or presence of Probenecid (10^{-4} M). cGMP measurements were performed in the medium and cell lysates to test extra- and intracellular levels of cGMP, respectively. Medium from the wells were aspirated and cells were extracted according to the protocol provided and both preparations were stored at -80 °C for further tests. All samples were acetylated, diluted, and sampled in duplicates.

Protein preparation from RPTEC and Western blotting

Na,K-ATPase and NHE3 protein expression was assessed in membrane fractions of RPTEC which were prepared using Compartmental Protein Extraction Kit (Chemicon, Billerica, MA) according to the protocol from manufacturer. For assessing levels of phosphorylated forms of sodium transporters, membrane fractions were prepared during different steps of centrifugation. Cell pellets were homogenized in RIPA buffer without

detergents, with 5% sorbitol, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4 final concentration, Protease Inhibitor Cocktail (Sigma Aldrich, Saint Louis, MO) at 1:1 000 dilution and additionally 1:100 dilution of Phosphatase Inhibitor Cocktail (Sigma Aldrich, Saint Louis, MO) for phosphoantibodies. After incubation on ice, samples were centrifuged at 4 800 rpm for 10 min. Supernatants were transferred and centrifuged again at 38 000 rpm for 90 min. Pellets produced were reconstituted in RIPA buffer as described above. Protein concentrations for each lysate were determined by using a BCA protein assay kit (Pierce Chemical) with BSA as a standard. Western blot protocol was followed as described in Materials and Methods paragraph for specific Aim #1 with the exception of some antibodies used. The following antibodies were used: polyclonal anti-NHE3 (Chemicon, Billerica, MA, 12 $\mu g\ ml^{-1}$), monoclonal antibody against phosphoNHE3 [Ser 552] (Novus Biologicals, Littleton, CO; 12 $\mu g\ ml^{-1}$), monoclonal Na,K-ATPase (Upstate , Lake Placid, NY,1:10 000 dilution) and polyclonal phospho Na,K-ATPase [Ser 16] (Cell Signaling Technology, Danvers, MA; 1:500 dilution). Antigen detection was performed using appropriate secondary antibodies conjugated to fluorescent tag (IRDye 680 and IRDye 800) at 1:15 000 dilution for 45 minutes at RT. Membranes were scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Membranes probed with phospho antibodies were scanned and then stripped with Western Re-probe Buffer (Geno Technology, Inc, St. Louis, MO) according to the protocol provided and incubated with antibodies recognizing total form of transporters. Semi-quantitative analyses of the specific

bands were performed using Li-Cor Odyssey software. Results were expressed as the ratio of membrane to cytosolic fractions or phospho to total form in relative fluorescence units (RFU).

Statistical analysis

Results are presented as mean ± standard error of mean (SEM) of the indicated number of experiments unless stated otherwise. Statistical analysis was performed using Student's t test and non-parametric test for unpaired data (treatment versus control), or by analysis of variance (ANOVA) and Holm-Sidak or Student-Newman-Keuls post-hoc for multiple group comparisons, as appropriate using InStat software (San Diego, CA). The null hypothesis was rejected for a p-value<0.05.

5.2 Results for Specific Aim # 3

In vivo experiments

Physiological parameters

After 12 weeks of the moderately high fat diet (MHF) final body weights (BWs) of obese rats nontreated or treated with 1.2 mg^{-1} kg^{-1} day^{-1} L-NAME for 4 weeks were significantly higher then lean rats (OP-781.0 ± 33.4 g vs. OR-633.5 ± 2.8 g; L-OP-821.0 ± 10.6 g vs. L-OR-658.0 ± 13.6 g)(Fig. 26a). As in earlier studies, obesity prone rats weighted significantly more than obesity resistant rats and treatment with L-NAME did not affect BWs. Total fat content remained 45%

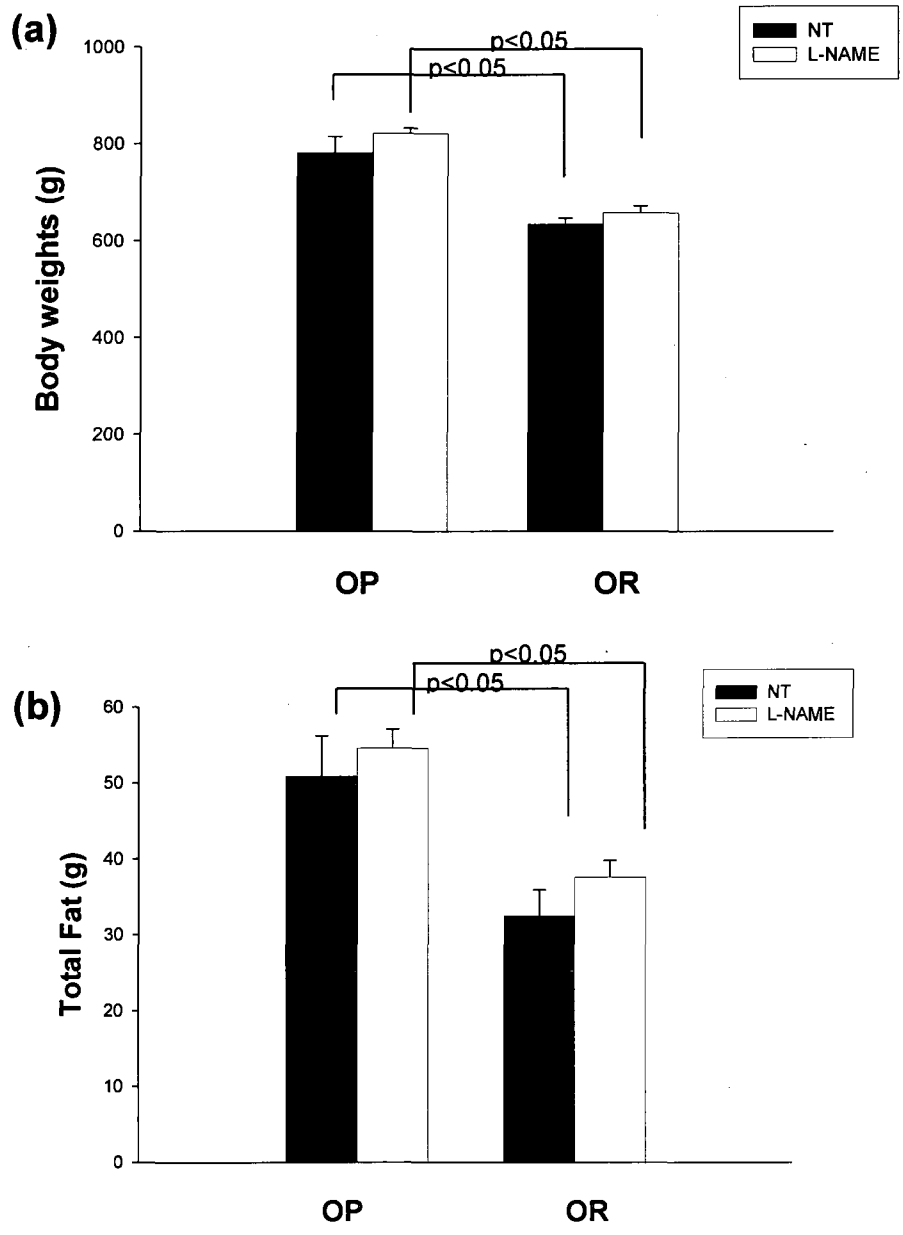

Figure 26 Final measurements of the body weights (BW) and total fat in obese (OP) and lean (OR) rats with or without L-NAME treatment.
Body weights represent averages for each group from the last week of the diet before sacrifice. Total visceral fat represents sum of epididymal and retroperitoneal fat. Values are mean ± SEM from n = 6 rats/group. Significance was determined using one-way ANOVA with Holm-Sidak modification for multiple group comparisons and null hypothesis was rejected for a p-value <0.05. Obese rats treated or not were significantly heavier then corresponding group of lean rats and those differences were paralleled by changes observed in the amount of total fat.

higher in the OP group treated with L-NAME compared to the OR group and paralleled changes observed in BWs (54.6 ± 2.5 g vs. 37.6 ± 2.2 g) (Fig. 26b). At the end of the dietary protocol, during the surgical procedure, mean arterial pressure (MAP) was measured (Fig. 27) and we found no significant differences in MAP between the experimental groups.

To ensure equal effectiveness of L-NAME treatment, metabolites of nitric oxide (NO) nitrate and nitrite were assessed in urine collected for 24 hours in metabolic cages. Levels of NOx in urine were not different between OP and OR groups (6.95 ± 1.0 μmol ml^{-1} vs. 6.61 ± 1.1 μmol ml^{-1}, respectively). Treatment with L-NAME caused a decrease in NO production to 4.2 ± 0.3 μmol ml^{-1} in obese rats and to 3.7 ± 0.4 μmol ml^{-1} in lean rats but failed to gain significance by an extremely small margin (p=0.052) in the former (Fig. 28). Daily water intake from 8th to 12th week of the diet was used to assess the L-NAME dose received since the drug was delivered in drinking water. Average daily water intake was increased in the OP group compared to the OR group: 42.3 ± 2.0 ml day^{-1} vs. 35.8 ± 1.6 ml day^{-1} (data not shown) suggesting that L-NAME levels were comparable between the groups when adjusted for differences in body weights.

Acute pressure natriuresis and diuresis in OP and OR rats treated with L-NAME

During the surgical procedure, animals from all experimental groups were challenged with an increase in renal perfusion pressure (RPP). At low RPP there were no differences in urine flow rate (V) between treated and nontreated obese

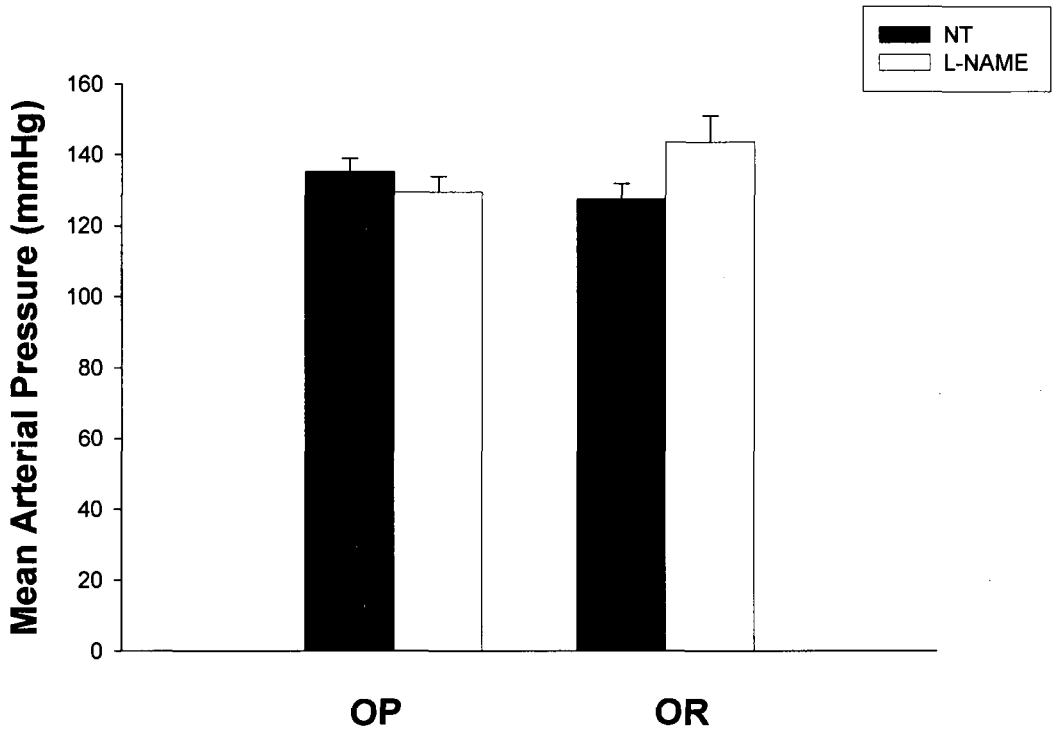

Figure 27 Mean arterial pressure (MAP) in OP and OR groups non-treated or treated with nitric oxide synthase inhibitor (L-NAME).
MAP was measured and recorded continuously during surgical procedure. L-NAME was administered in drinking water at dose which is known not to induce hypertension. There was no difference in MAP between the study groups. Values are mean ± SEM from n = 6 rats/group. Significance was determined using one-way ANOVA with Holm-Sidak modification for multiple group comparisons and null hypothesis was rejected for a p-value <0.05.

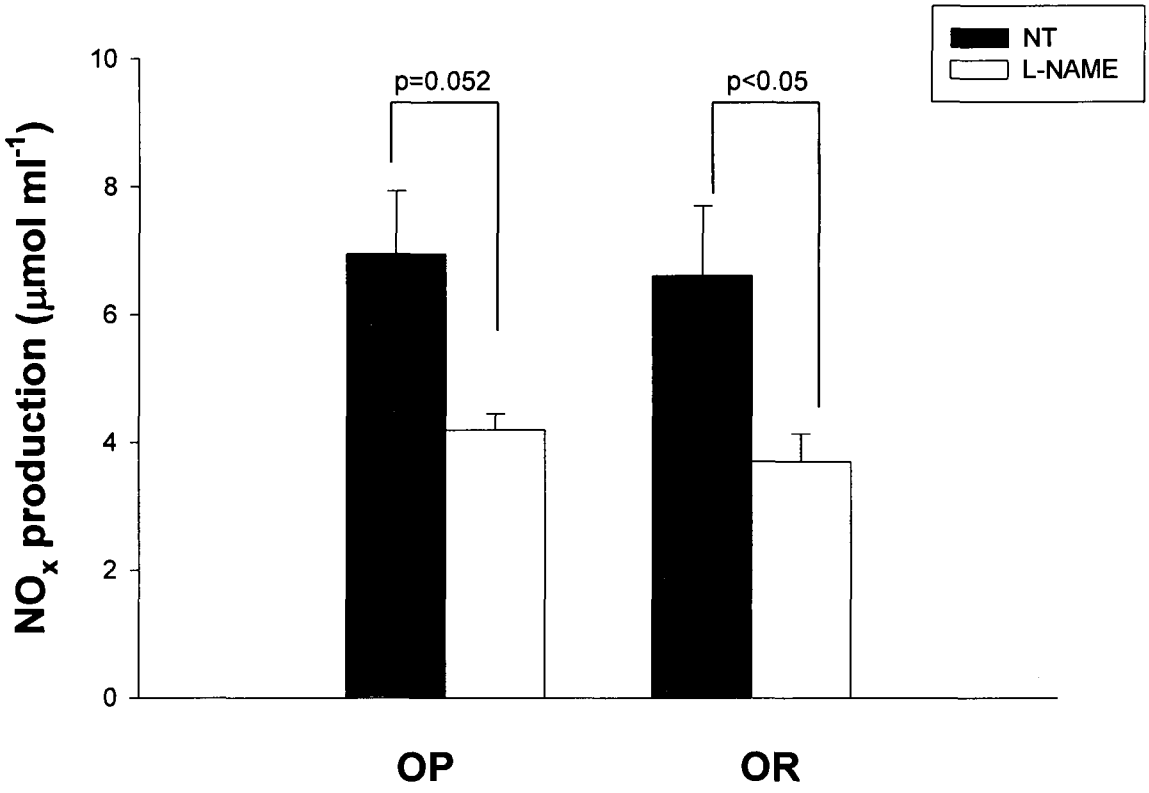

Figure 28 Final measurements of urinary nitrate and nitrite (NO_x) levels in OP and OR rats with or without L-NAME treatment.
Metabolites of nitric oxide were measured in urine collected over a period of 24 hours by using an enzymatic method and stored at -80 °C until used for an assay. Each sample was diluted in order to produce values within the dynamic range of the test and then assayed in duplicates. Background was subtracted from the average measurements and concentrations normalized to 24 hr urine. Values represent mean ± SEM from n = 6 rats/group. Significance was determined using one-way ANOVA with Student-Newman-Keuls method for multiple group comparisons and null hypothesis was rejected for a p-value <0.05.

and lean rats. In OR rats treated with L-NAME, elevation in RPP provoked a significantly lowered increase in urine flow rate (V) compared to nontreated animals (OR: 53.25 ± 15.7 μl ml^{-1}, L-OR: 23.5 ± 4.1 μl ml^{-1}) while having no significant effect on V in OP rats (Fig. 29a). This result suggests that L-NAME treatment reduced the diuretic response in lean rats whereas obese rats did not respond to NO inhibition in the same manner.

There were no differences in glomerular filtration rate (GFR) among the study groups with or without NOS inhibition at either low or high RPP suggesting that L-NAME treatment did not impair autoregulatory function of the kidney in the interval of RPP employed in the experiment (Fig. 29b).

Pressure natriuresis was assessed by urinary sodium excretion (UNaV) and fractional excretion of sodium (FENa) at two different RPP. Treatment with L-NAME caused a significant reduction in UNaV in OR group at higher RPP from 9.97 ± 3.63 μEq min^{-1} to 1.13 ± 0.45 μEq min^{-1} and did not change urinary sodium excretion in OP group (Fig. 30a). Change in FENa showed the same pattern: at high RPP lean rats treated with L-NAME had a significant decline in fractional excretion of sodium compared to their nontreated counterparts from 1.34 ± 0.39% in the latter to 0.27 ± 0.09% in the former while there was no change in FENa in the obese group (Fig. 30b). The above results indicate that L-NAME treatment while uniformly reducing NO production in both groups modifies pressure diuresis and natriuresis significantly only in the OR group.

Finally, we measured fractional excretion of lithium (FELi) in response to low and high RPP as a method of assessing proximal tubule function. The

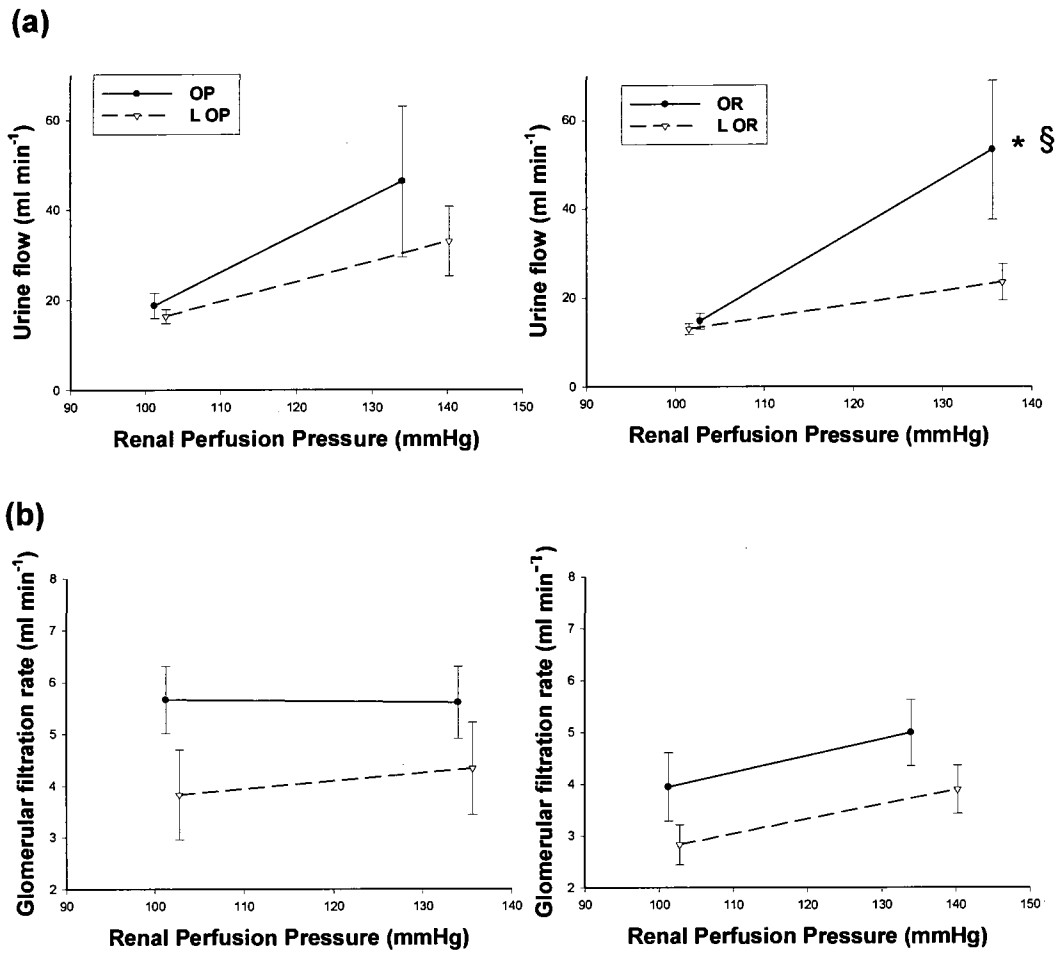

Figure 29 Relations between renal perfusion pressure (RPP) and urine flow (a) and glomerular filtration rate (b) in obese (OP) and lean (OR) rats with or without chronic inhibition of NOS.
During surgical procedure an adjustable clamp was placed around aorta above renal arteries to control renal perfusion pressure (RPP). At first RPP was set ~100 mmHg and urine collected for 30 min. At the end of this period blood was drawn from carotid artery for plasma electrolytes, lithium and inulin measurements. Then RPP was set ~140 mmHg and all above procedures were repeated. L-NAME treatment reduced response of lean rats to high RPP while having no effect on obese rats. Glomerular filtration rate, calculated as the rate of inulin clearance remained the same in non-treated and treated OP and OR rats suggesting that inhibition of NO production did not impair autoregulatory kidney function. Results are expressed as means ± SEM of n = 4-5 rats/ group. Significance was determined using one-way ANOVA with Student-Newman-Keuls method for multiple group comparisons and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to L-NAME treated at a similar perfusion pressure. §= significant compared to low RPP in the same group of animals.

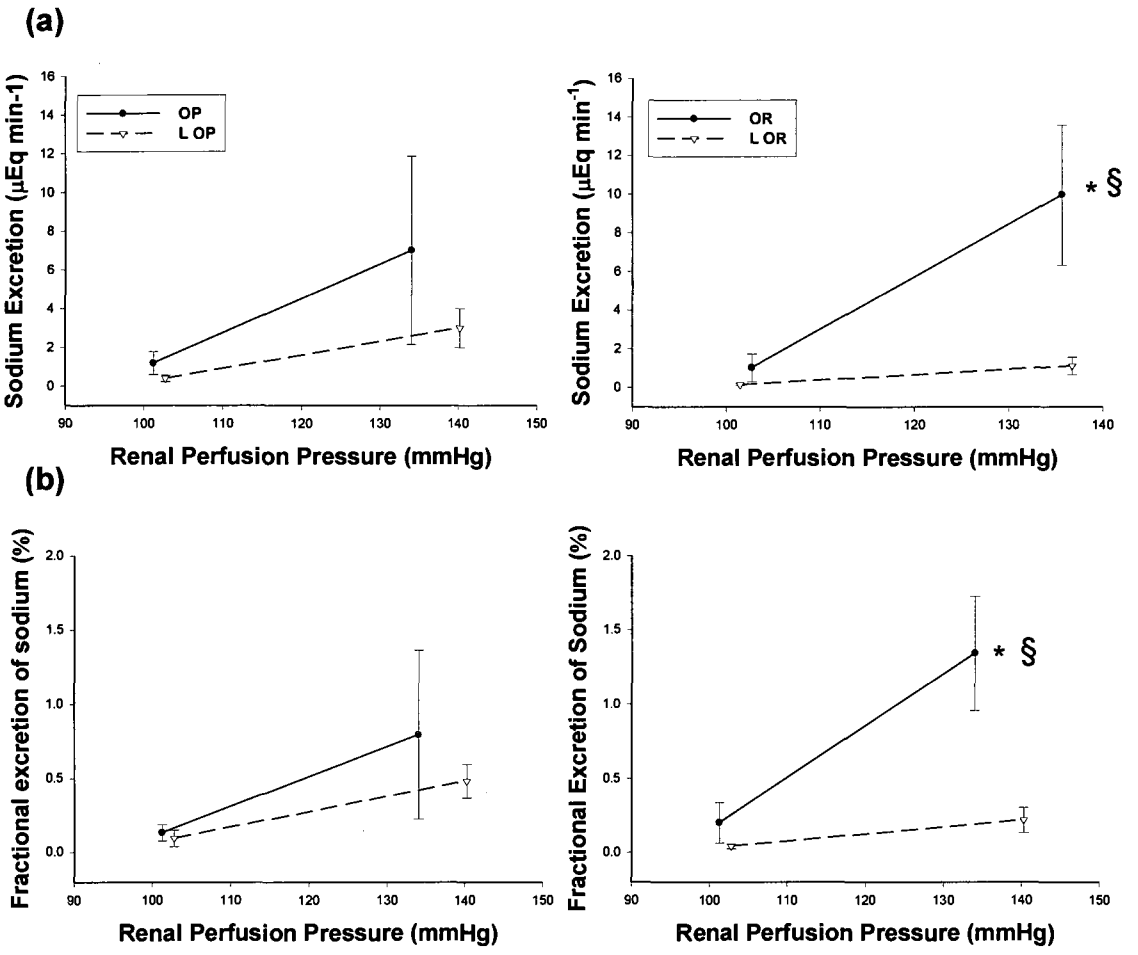

Figure 30 Pressure-natriuresis in obese and lean Sprague-Dawley rats treated or not with L-NAME.
In acute settings, as described in material and methods paragraph for Aim #1 and also in the previous figure, animals were challenged with the change in RPP from 98-105 mmHg to 128-145 mmHg. Panel (a): Relation between RPP and urinary sodium excretion (UNaV). When compared with that in non-treated lean rats, L-NAME treated OR animals excreted less sodium. Panel (b): Relation between RPP and fractional excretion of sodium. The changes mirrored those mentioned previously: L-NAME treated lean rats excreted less sodium than their non-treated counterparts. Results are expressed as means ± SEM of n = 4-5 rats/ group. Significance was determined using one-way ANOVA with Student-Newman-Keuls method for multiple group comparisons and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to L-NAME treated at a similar perfusion pressure. §= significant compared to low RPP in the same group of animals.

increase in the FELi in response to an elevation in RPP was reduced in the OR group treated with L-NAME compared to the untreated group from 19.8 ± 1.4% to 13.5 ± 5.4% while FELi did not change in the OP group treated with L-NAME vs. the untreated control (Fig. 31). This result suggests that inhibition of NO production impairs natriuresis and diuresis in the OR rats at least partially through a proximal tubule related mechanism. Moreover, in our previous aims we focused our efforts on this segment of the nephron. Therefore, we again examined the expression, activity and localization of two of proximal tubule transporters: Na,K-ATPase and NHE3 in the separate set of experiments as described in the methods.

Activity, protein expression and localization of Na,K-ATPase and NHE3 in OP and OR rats treated with L-NAME for 4 weeks

Activity of any transporter is the interplay of several factors which could act alone or in combination and include protein expression, membrane trafficking, interaction with regulatory factors or substrate affinity.

In this aim we started to investigate some of the mechanisms that could lead to changes in natriuresis and diuresis in obese and lean rats chronically treated with nitric oxide synthase (NOS) inhibitor L-NAME observed in first part of the study. Protein expression of basolateral transporter Na,K-ATPase was measured by western blotting in basolateral membrane preparations (BLM) from renal cortex. The membranes were stained with Ponceau Red to insure equal loading of the gels. L-NAME treatment significantly elevated protein abundance

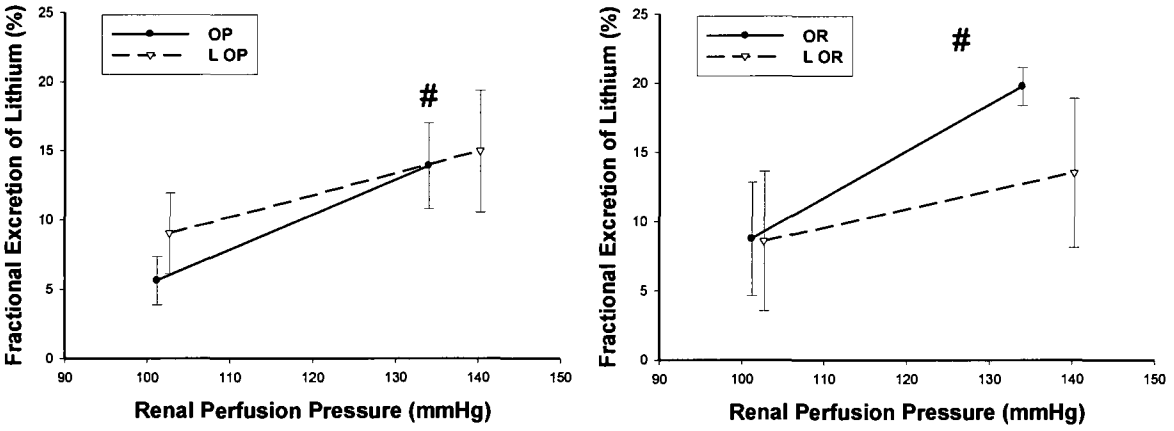

Figure 31 Fractional excretion of lithium (FELi) at low and high RPP in obese and lean Sprague-Dawley rats with or without chronic NO depletion. During surgical intervention rats were infused with lithium and after the experiment lithium concentrations in urine and plasma were measured. FELi was calculated as the ratio between its urinary excretion and the amount filtered and it was used as a marker for the proximal tubule function. NO depletion produced a reduction in response to high RPP in OR group but not in OP rats. Results are expressed as means ± SEM of n = 4-5 rats/ group. Significance was determined using one-way ANOVA with Student-Newman-Keuls method for multiple group comparisons and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to L-NAME treated at a similar perfusion pressure. §= significant compared to low RPP in the same group of animals. # = borderline significance (0.05<p-value<0.07) compared to the low perfusion pressure.

of Na,K-ATPase from 30.69 ± 0.69 RFU to 40.24 ± 2.43 RFU in lean rats and did not affect Na,K-ATPase expression in the OP group (Fig. 32). Induction of Na,K-ATPase protein expression could at least partially explain the decrease in urinary sodium excretion and fractional excretion of sodium observed after chronic administration of L-NAME in obesity resistant rats by association with pump activity. However, those changes were not reflected in our estimation of Na,K-ATPase activity measured as a release of inorganic phosphate from freshly prepared BLM. There was no difference in Na,K-ATPase activity between treated and nontreated groups of obese and lean animals (Fig. 33). This discrepancy between expression and activity could most probably be explained by different mechanisms of Na,K-ATPase activity regulation in normal and hyperinsulinemic states deprived of NO. Lack of correlation between activity and pressure diuresis and natriuresis in the lean group could be clarified by the approach used during basolateral membrane preparation and in a consequence partial contamination of basolateral membranes by apical membranes or different nephron segments. Moreover, ouabain-sensitive Na^+,K^+-pump activity accounted for about 38 and 36% of total activity of BLM fractions from obese and lean rats treated with L-NAME (data not shown), respectively; again suggesting that our preparations were impure and contained other pumps insensitive to ouabain like H^+,K^+-ATPase from proximal tubule or cortical segments of collecting tubules.

Next, we determined protein expression of the sodium/hydrogen exchanger type 3 (NHE3) by western blotting in brush border membrane vesicles

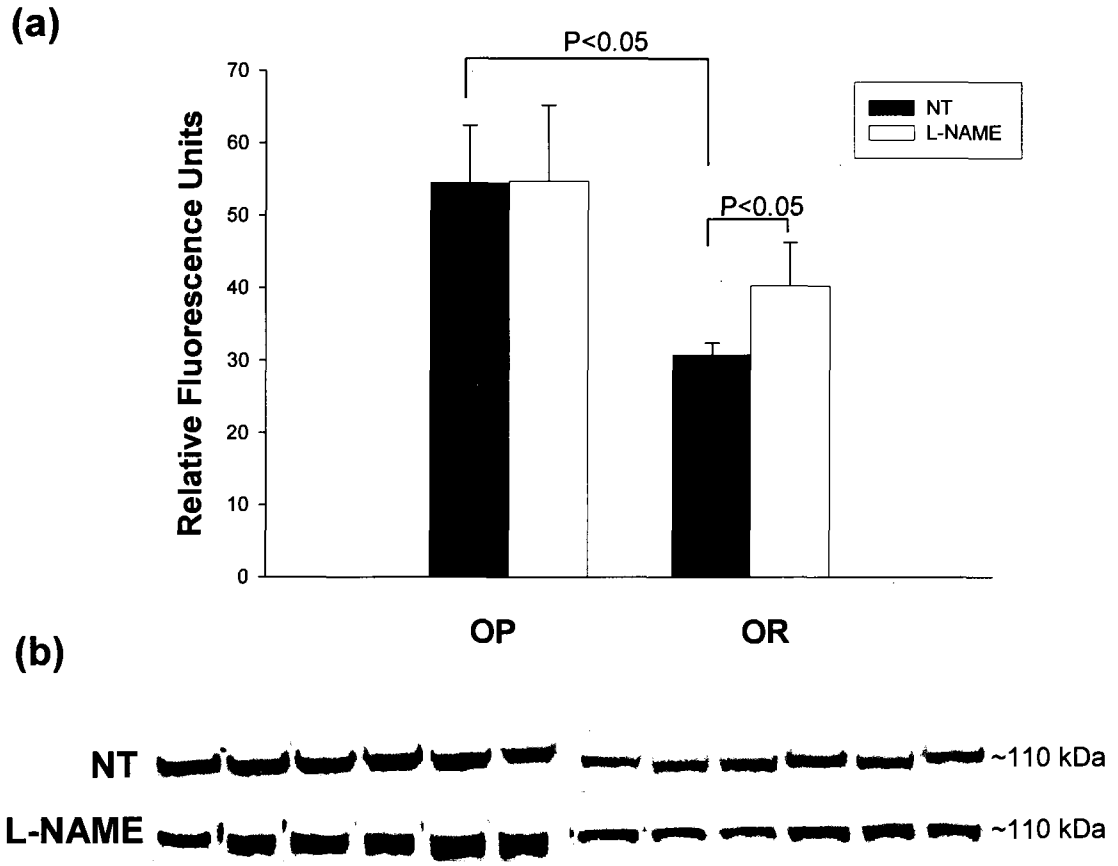

Figure 32 Na,K-ATPase protein expression measured in baso-lateral-enriched membranes isolated from renal cortex of obese (OP) and lean (OR) rats with or without L-NAME treatment.
Panel (a): Graph representation of Na,K-ATPase abundance, measured in arbitrary units, represented as means ± SEM of n = 6 rats/group. Blots were probed with Na,K-ATPase $\alpha 1$ subunit specific antibody. Semi-quantitative densitometry analyses were done by using Odyssey Infrared Imaging System software. Significance was determined using one-way ANOVA and the null hypothesis was rejected for a p-value< 0.05. Panel (b): Immunoblots of BLM samples from non-treated and treated OP and OR rats. Each lane represents a sample from an individual rat. Equal protein loading was ensured by staining each membrane with Ponceau red. Treatment with L-NAME significantly reduced expression of basolateral sodium pump in OP and OR rats.

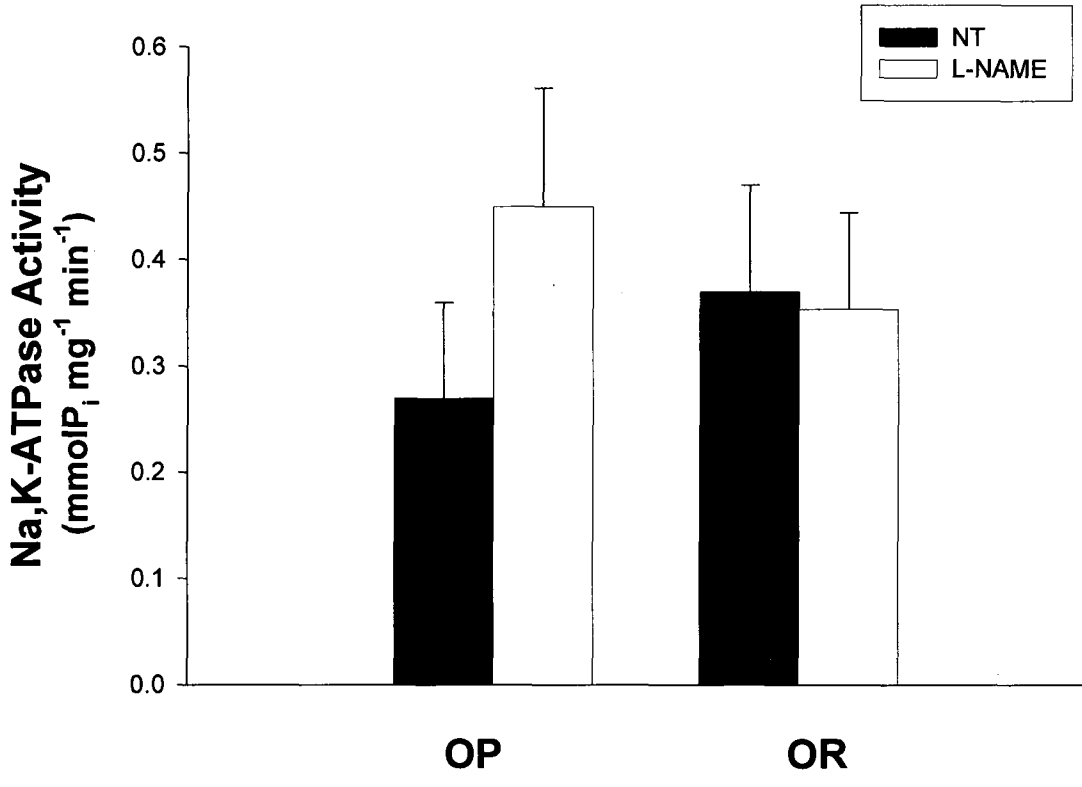

Figure 33 Effect of L-NAME on Na,K-ATPase activity assessed in baso-lateral membranes prepared from renal cortexes of obese(OP) and lean (OR) animals. Na,K-ATPase activity expressed as mmol of inorganic phosphate liberated per mg of protein per minute performed on freshly prepared basolateral membrane (BLM) enriched fractions. Na,K-ATPase activity was calculated as the difference between activities assayed in the absence of ouabain (total) activity and in the presence of 5 mM ouabain. Values are means ± SEM from 6 rats per group. Each sample was assayed in duplicate and background was subtracted from the mean. There was no difference in activities between study groups. Significance was determined using one-way ANOVA and the null hypothesis was rejected for a p-value< 0.05.

(BBMV) from renal cortex. Villin was used as a loading control. The purity of BBMV was estimated by measuring activity of leucine aminopeptidase N (LAP) for each sample and comparing it to activity of LAP in the initial homogenate. According to the protocol from Biber *et al.* (Biber *et al.* 2007) an average 18fold enrichment achieved for all samples provided us with high purity for brush border membrane vesicles. L-NAME treatment significantly increased expression of NHE3 in both groups: lean and obese compared to non-treated controls by ~36% and 42%, respectively (Fig. 34). Activity of NHE3 calculated in freshly obtained BBMV fractions corresponded only partially to the protein measurements. In obese rats reduction of NO did not change activity of the exchanger while in lean rats the 42% increase in protein levels was reflected in ~53% increase in activity of NHE3 (Fig. 35). As described earlier in the results paragraph of Aim #2, sensitivity and specificity of the NHE3 activity assay was assessed by incubation of randomly chosen samples with various concentrations of 5-(N-Ethyl-N-isopropyl) amiloride. We concluded that the assay was specific and sensitive for the NHE3 isoform.

Since changes observed in natriuresis and diuresis could most probably be elucidated by modifications in abundance and activity of NHE3 in lean rats treated with L-NAME but not in L-NAME treated obese rats, we pursued another mechanism of NHE3 activity regulation- membrane trafficking.

As described before, we used immumohistochemistry and image visualization techniques to quantify localization of NHE3 with regard to villin. Villin was used as a marker for the microvillus membrane, therefore the higher the degree of

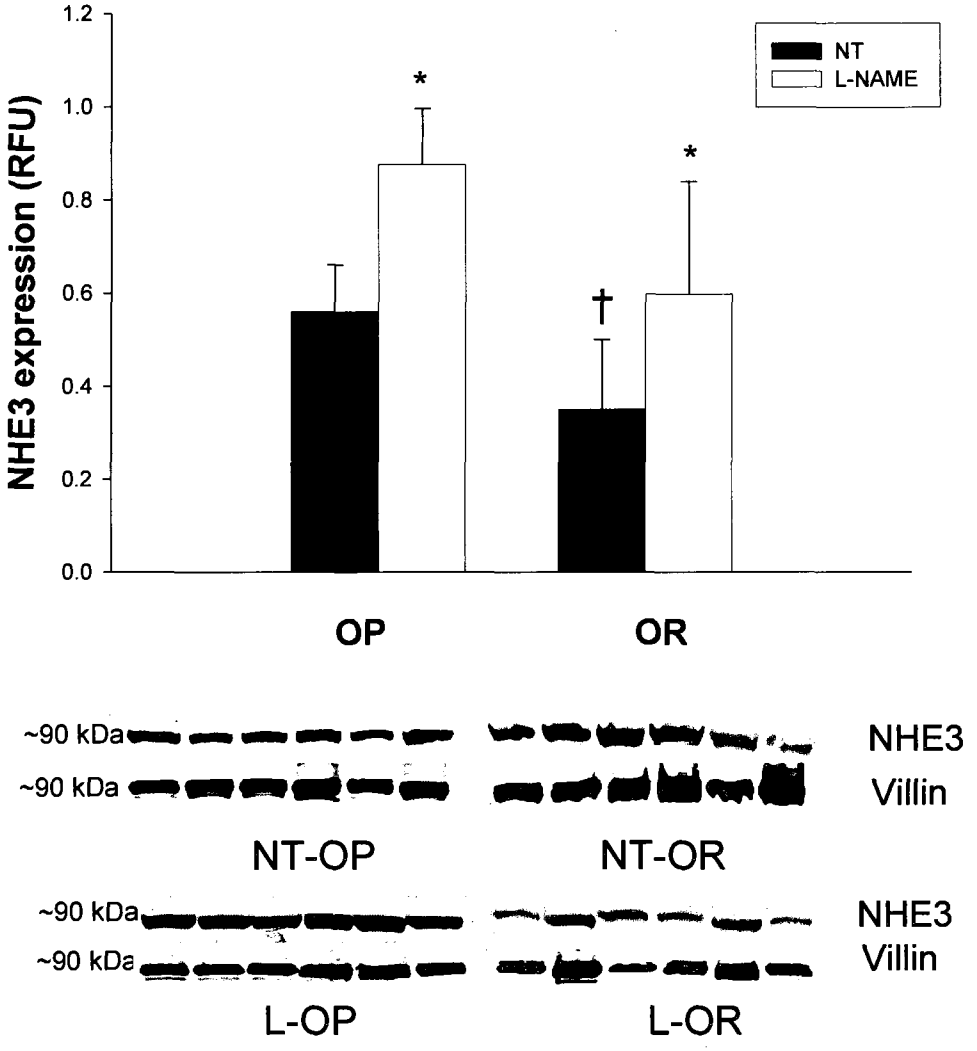

Figure 34 Na/H exchanger (NHE3) protein abundance after L-NAME treatment in obese (OP) and lean (OR) rats.
NHE3 was assayed in brush border membrane vesicles (BBMV) fractions prepared from renal cortex of OP and OR groups treated or not with L-NAME. Top panel: Densitometric analysis of immunoblots shown below. NHE3 immunoreactivity is expressed as a ratio between NHE3 and Villin signals, in relative fluorescent units (RFU). Results represent average ± SEM of n=6 rat/group. Significance was determined using one-way ANOVA and the null hypothesis was rejected for a p-value< 0.05. L-NAME treatment significantly increased NHE3 protein expression in OP and OR groups. Bottom panel: Immunoblots of BBMV samples from obese and lean rats. Equal amount of proteins were loaded into each gel and duplicates of membranes were probed with anti-villin antibody which served as a loading control. Antigen-antibody complexes were detected with Odyssey Infrared Imaging System (Li-Cor).

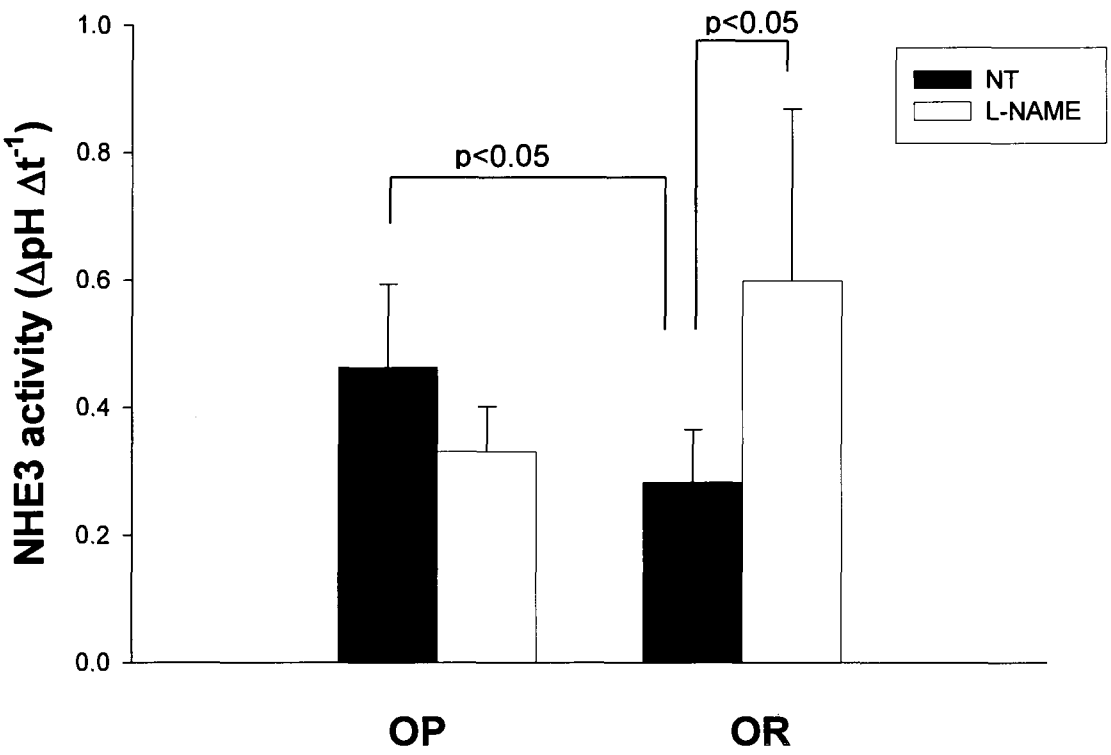

Figure 35 Na^+/H^+ antiporter activity in obese and lean rats non-treated (NT) or treated with L-NAME.
Brush border membrane vesicles (BBMV) prepared from renal cortex were preloaded with pH indicator BCECF-AM in Na^+-free buffer, pH 7.2 and then mixed with the buffer solution containing 150 mmol l^{-1} NaCl, pH 9.2 for determination of pH recovery as described in methods paragraph for Aim #1. Equal amounts of protein were labeled and readings for unlabeled samples were subtracted from final values. NHE3 activity was expressed as a change in pH over time. Values are means ± SEM from 6 rats per group. Significance was determined using one-way ANOVA and the null hypothesis was rejected for a p-value< 0.05. L-NAME treatment significantly elevated activity of exchanger in OR group while did not effect OP group.

colocalization between villin and NHE3, the more abundant is the transporter in the microvillus membrane (Fig. 36a). NO depletion had no effect on NHE3 distribution in the OP group (21.46 ± 4.38% vs. 23.29 ± 10.47%) though it significantly increased colocalization of NHE3 with villin in the OR group from 9.1 ± 3.67% to 20.50 ± 5.9% (Fig. 36b). As a result, alterations in NHE3 distribution may account for an increase in exchanger activity together with an increase in NHE3 protein expression in lean groups treated with nitric oxide synthase inhibitor.

In conclusion, similar depletion of NO in obese and lean animals lead to impairment in pressure natriuresis and diuresis supported by an increase in activity and expression of NHE3 and its redistribution to the tip of the villus in concert with elevation of Na,K-ATPase in obesity resistant rats only. Based on these results the following questions arose: 1) Why did obese and lean groups react differently to NO inhibition? 2) Are lean rats more sensitive to NO deprivation or are obese rats more resistant to it? 3) How can those differences be explained? 4) Does the metabolic state influence the effects of NO on sodium transport in the proximal tubule *in vivo*?

As this study did not provide definite information regarding the direct effects of NO on sodium transport in the proximal tubule in vivo, it seemed almost imperative to try to explain some of our questions using an *in vitro* system which is devoid of any other influences beside those imposed in the experiment.

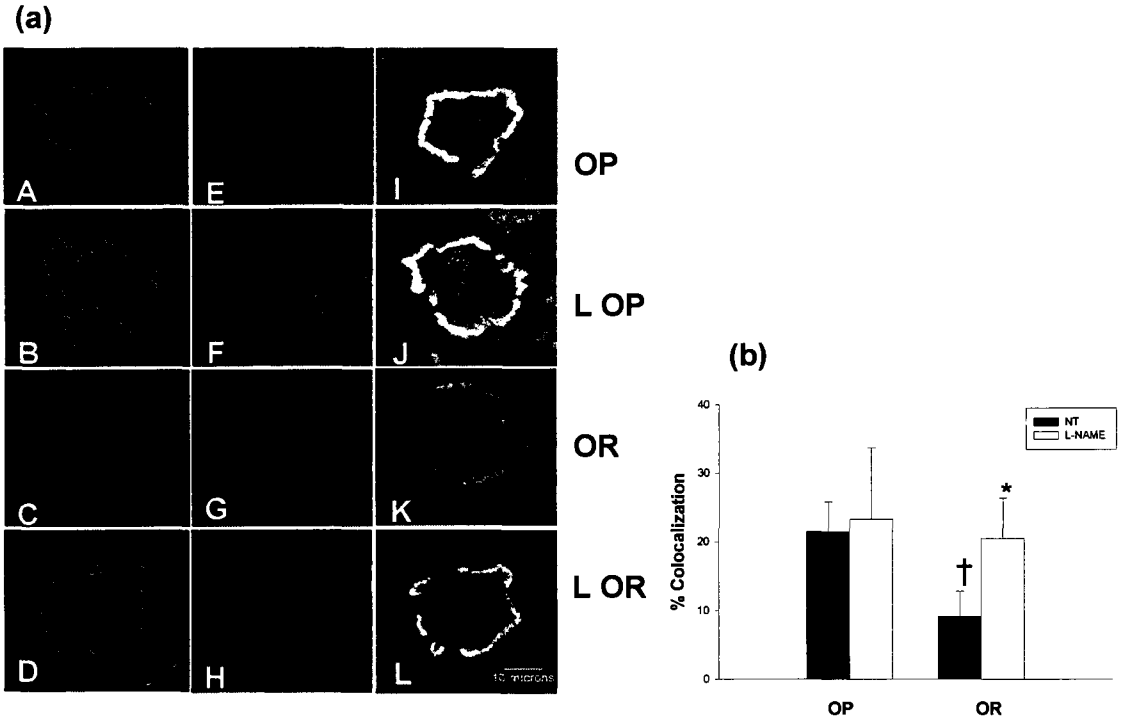

Figure 36 Changes in NHE3 distribution in obese and lean rats after chronic treatment with L-NAME.
4 micron sections of paraffin embedded kidneys were double stained with polyclonal NHE3 antibody and with monoclonal anti-villin antibody followed by the corresponding anti-rabbit (AlexaFluor488-green) and anti-mouse (AlexaFluor594- red) secondary antibodies. Panel (a): Representative images of cortical tubules from all experimental groups stained simultaneously with NHE3 (A, B, C, D) and villin (E, F, G, and H). Overlapping (I, J, K and L) of NHE3 and Villin appears yellow. Chronic L-NAME administration caused NHE3 redistribution in lean rats and did not have an effect in the obese group. Panel (b): Semi-quantitative analysis of NHE3 and villin colocalization in kidney section. 6-10 z-stack images were taken with laser scanning confocal microscope from each animal (n = 3 rats per group). Images were analyzed using Metamorph software and colocalization was expressed as the percentage of the sum of the green staining overlapping the red staining to the sum of the green fluorescence only. Significance was determined using one-way ANOVA and the null hypothesis was rejected for a p-value< 0.05. *= significant compared to non-treated group. †= significant compared to OP rats. Bar = 10microns.

In vitro experiments

cGMP levels, role of oxadiazolo-quinoxaline (ODQ) and probenecid in human renal proximal tubule epithelial cells (RPTEC) model

Since it is controversial whether renal proximal tubules cells produce NO under basal conditions, we determined the capability of primary cultures of RPTEC to generate guanosine cyclic 3', 5' monophosphate (cGMP) in response to direct stimulation of soluble guanylyl cyclase by the NO donor: S-nitroso-*N*-acetylpenicillamine (SNAP) (Fig. 37). In a concentration- and time-dependent manner SNAP caused significant stimulation of intracellular and extracellular cGMP. After 5 minutes of SNAP incubation intracellular cGMP production increased from a basal value of 0.14 ± 0.07 to 12.77 ± 4.5 pmol ml^{-1} at 10^{-5} M SNAP and to 104.32 ± 19.6 pmol ml^{-1}, at 10^{-4} M SNAP, while extracellular cGMP concentration did not change significantly at this time point (data not shown). After 30 minutes at a 10^{-6} M SNAP concentration, intracellular levels of cGMP remained elevated and extracellular levels began to increase from 0.03 ± 0.01 pmol ml^{-1} to 1.63 ± 0.24 pmol ml^{-1} (Fig. 38a). At the same time point, a concentration of 10^{-5} M SNAP significantly increased intracellular levels of cGMP from 0.15 ± 0.08 to 30.27 ± 10.9 pmol ml^{-1}. However, extracellular levels of cGMP did not reach statistical significance (from 0.03 ± 0.01 to 31.01 ± 20.94 pmol ml^{-1}), most probably due to the large variance and small number of experiments (Fig. 38a). As demonstrated in Fig. 38b, after 2 hours SNAP increased intracellular cGMP production and also extracellular cGMP levels in

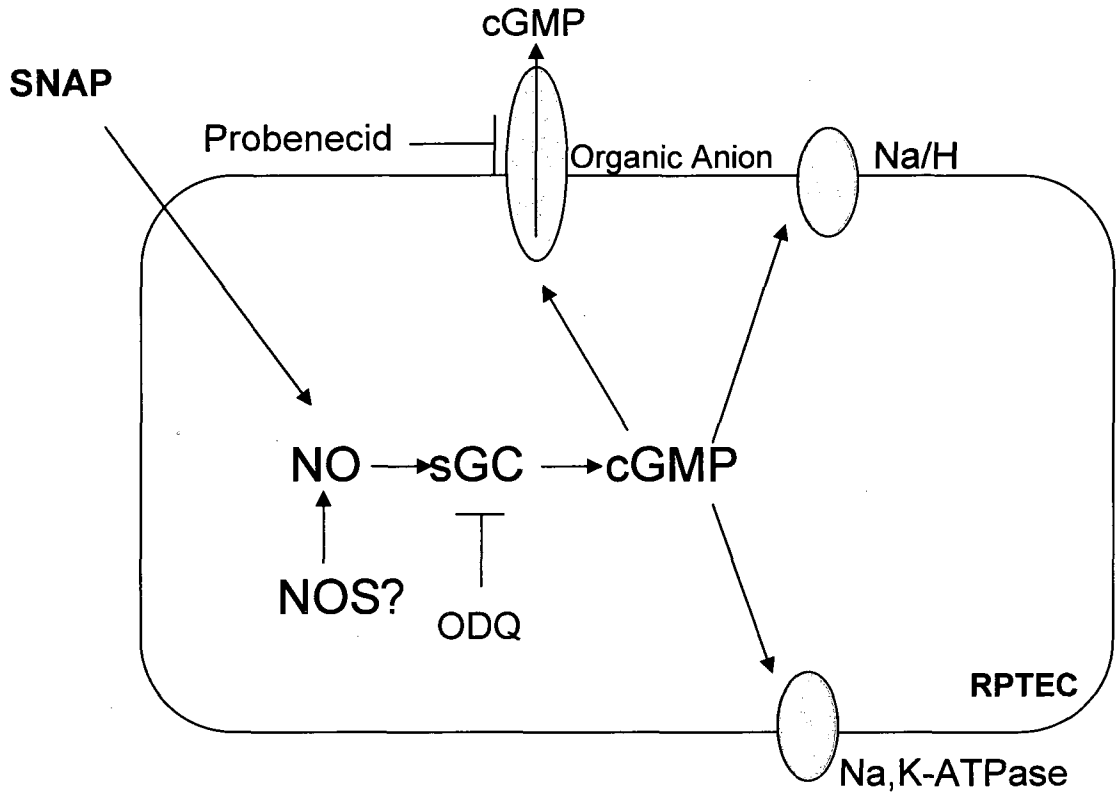

Figure 37 Schematic of experimental design for Aim # 3B.
As it is controversial whether renal proximal tubule epithelial cells (RPTEC) contain nitric oxide synthase (NOS), enzyme responsible for Nitric Oxide (NO) production, RPTEC were exposed to (NO) donor-SNAP (S-nitro-N-acetyl-DL-penicillamine) and intracellular and extracellular levels of cGMP (cyclic guanosine monophosphate) were measured. cGMP synthesis is catalyzed by guanylate cyclase (GC). Membrane-bound GC is activated by peptide hormones while soluble GC (sGC) is typically activated by NO to stimulate cGMP synthesis. ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), highly selective, irreversible, inhibitor of soluble guanylyl cyclase was used to test whether SNAP modifies levels of cGMP through its action on sGC. To investigate whether presence of cGMP in extracellular milieu is necessary for its action we used probenecid to block organic anion transporter through which cGMP is exported from the cell. The ultimate goal of this experiment was to measure protein expression of sodium transporters in RPTEC: apical sodium/hydrogen exchanger type 3 (NHE3) and basolateral Na,K-ATPase pump.

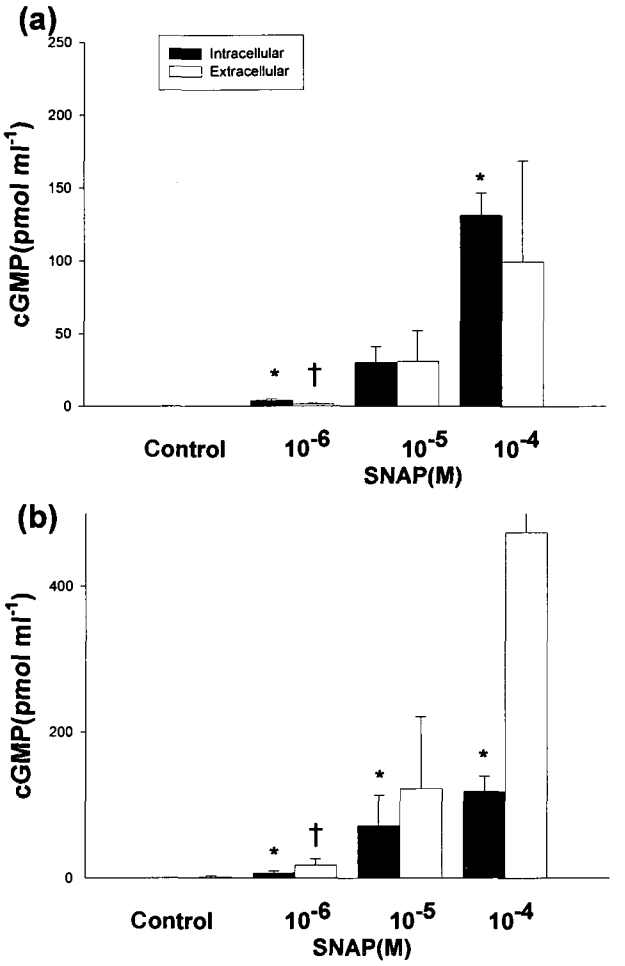

Figure 38 The effects of SNAP incubation on intracellular and extracellular cGMP levels in human renal proximal tubule epithelial cells (RPTEC) for 30 min (Panel a) and 2 hrs (Panel b).
Cells were seeded at 100 000 cells/well density in 12-well plates and kept in serum-free medium 24 hr before the experiments. RPTEC were incubated for 30 min or 2 hours with no SNAP (control) or with 10^{-6}, 10^{-5} and 10^{-4} M SNAP in the presence of 1 mM IBMX (isobutylmethylxanthine), a non-specific inhibitor of phosphodiesterases. The concentration of cGMP was quantified in cell lysates (intracellular) and in the medium (extracellular) using a commercially available EIA kit. The samples were acetylated and tested in duplicates. Dilutions were performed to insure that samples were in the range of the linear response. Values are mean ± SEM from 3 experiments performed in duplicates. Significance was determined using unpaired two tail t-test and the null hypothesis was rejected for a p-value <0.05. *= Significant compared to control for intracellular levels. †= Significant compared to controls for extracellular levels of cGMP. SNAP significantly in concentration-dependent manner increased cGMP levels intra- and extracellular.

a concentration-dependent manner. Based on those results we chose the 2 hour time point with a 10^{-6} M SNAP concentration to test the effects of different hormones.

To determine if the effect of SNAP was mediated by soluble guanylyl cyclase (sGC) we used a highly specific inhibitor of sGC, 1-H-[1,2,4] oxadiazolo – [4,2-alpha] quinoxalin-1-one (ODQ, 10^{-4} M). For those experiments we intentionally employed a higher concentration of SNAP then in previous experiments to show that ODQ and probenecid are capable of blocking cGMP generated by higher SNAP concentrations. ODQ significantly abolished the increase in intracellular and extracellular cGMP levels produced by SNAP (10^{-5} M) after 5 min (only intracellular; data not shown) and 30 minutes incubation (Fig. 39a). After 2 hours, the extracellular level of cGMP was significantly reduced, while intracellular cGMP levels were reduced with borderline significance (p-value=0.09) compared to levels of intracellular cGMP achieved after incubation with SNAP only. However, intra- and extracellular levels of cGMP measured in samples incubated with SNAP and ODQ for 2 hrs were not significantly different then control values of cGMP (without SNAP) (Fig. 39b).

In the next series of experiments we tested the ability of probenecid to prevent the export of cGMP in the extracellular compartment. As demonstrated in Fig. 39c, after 30 minutes probenecid (10^{-4} M) did not prevent the export of cGMP from the cells. After 2 hours, probenecid significantly decreased extracellular cGMP accumulation close to control levels from 79.4 ± 33.08 pmol ml^{-1} to 0.74 ± 0.33 pmol m^{-1} in the presence of SNAP (10^{-5} M)(Fig. 39d).

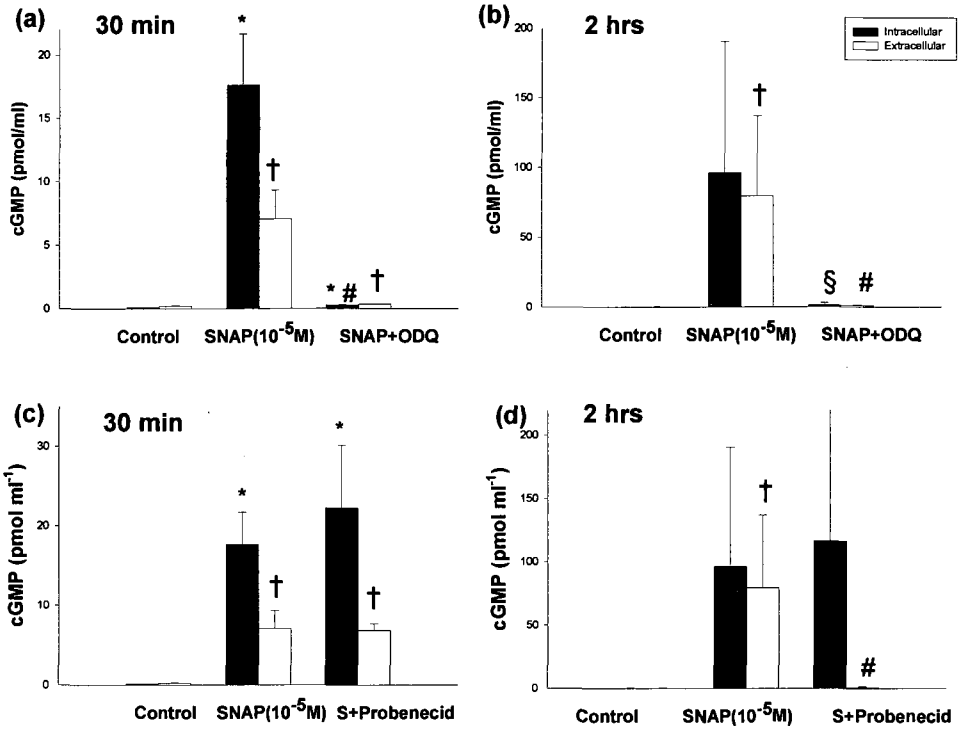

Figure 39 The effects of ODQ (a, b) and probenecid (c and d) on intracellular and extracellular cGMP responses to SNAP in RPTEC.
Cells were seeded at 100 000 cells/well density in 12-well plates and kept in serum-free medium 24 hr before the experiments. Panel (a) and (b): Effects of ODQ on intracellular cGMP following incubation with SNAP for 30 minutes (Panel a) or 2hrs (Panel b). To test whether the effect of SNAP was mediated by formation of cGMP, RPTEC were incubated without SNAP (control) or with SNAP at 10^{-5} M and with simultaneously with ODQ (10^{-4} M) plus SNAP at 10^{-5} M for 30 min or 2 hours in the presence of 1mM IBMX. ODQ significantly reduced intracellular cGMP levels after 30 minutes and 2 hours. Panel (c) and (d): Effects of probenecid on extracellular cGMP following incubation with SNAP for 30 minutes (Panel c) or 2 hrs (Panel d). Cells were incubated with probenecid and SNAP for 30 minutes or 2 hours. After 2 hr probenecid significantly abolished extracellular cGMP accumulation. Concentration of cGMP was quantified in cell lysates (intracellular) and in the medium (extracellular) using a competitive enzyme immunoassay. Samples were acetylated and dilutions performed to insure linearity of response. Values are mean ± SEM from 3 experiments performed in duplicates. Significance was determined using unpaired two tail t-test and non-parametric test and the null hypothesis was rejected for a p-value <0.05. *= Significant compared to control for intracellular levels. †= Significant compared to controls for extracellular levels of cGMP. #= Significant compared to SNAP treatment. §= Borderline significance (0.05<p-value<0.09) compared to SNAP treatment.

Accumulation of intracellular cGMP was also observed during this time course but it did not reach statistical significance, most probably due to large variability between the samples. In conclusion, at 2 hrs and 10^{-5} M SNAP concentration probenecid almost entirely blocks cGMP export.

Effect of angiotensin II, insulin and leptin on cGMP production in the absence or presence of NO

Angiotensin II and insulin at different concentrations significantly increased extracellular levels of cGMP after simultaneous incubation with 10^{-6} M SNAP for 2 hours (Fig. 40). Leptin however did not induce cGMP production at any of the concentrations tested in the experiment. The ability to modulate cGMP production was tested at 3-4 concentrations for the three hormones as follows: angiotensin II: 10^{-8}, 10^{-7}, and 10^{-6} M; insulin: 10^{-9}, 10^{-8}, and 10^{-7} M and leptin: 15, 30, 45 and 60 ng ml^{-1}, either in the presence or absence of SNAP (10^{-6} M) for two hours. This concentration of SNAP was chosen since as described earlier, it produced a significant increase in the levels of intracellular and extracellular cGMP at 30 min and 2 hrs.

In the absence of NO donor, angiotensin II, insulin or leptin had no effect on cGMP production (data not shown). Nevertheless, in the presence of SNAP extracellular cGMP was increased following treatments with 10^{-8} M angiotensin II (Fig. 40a) or 10^{-9} M insulin (p-value=0.05) (Fig. 40b). Leptin treatment at 45 ng ml^{-1} had increased cGMP production compared to control from 25.47±4.3 to 42.4±6.8 pmol ml^{-1} but failed to gain statistical significance (p-value=0.1)

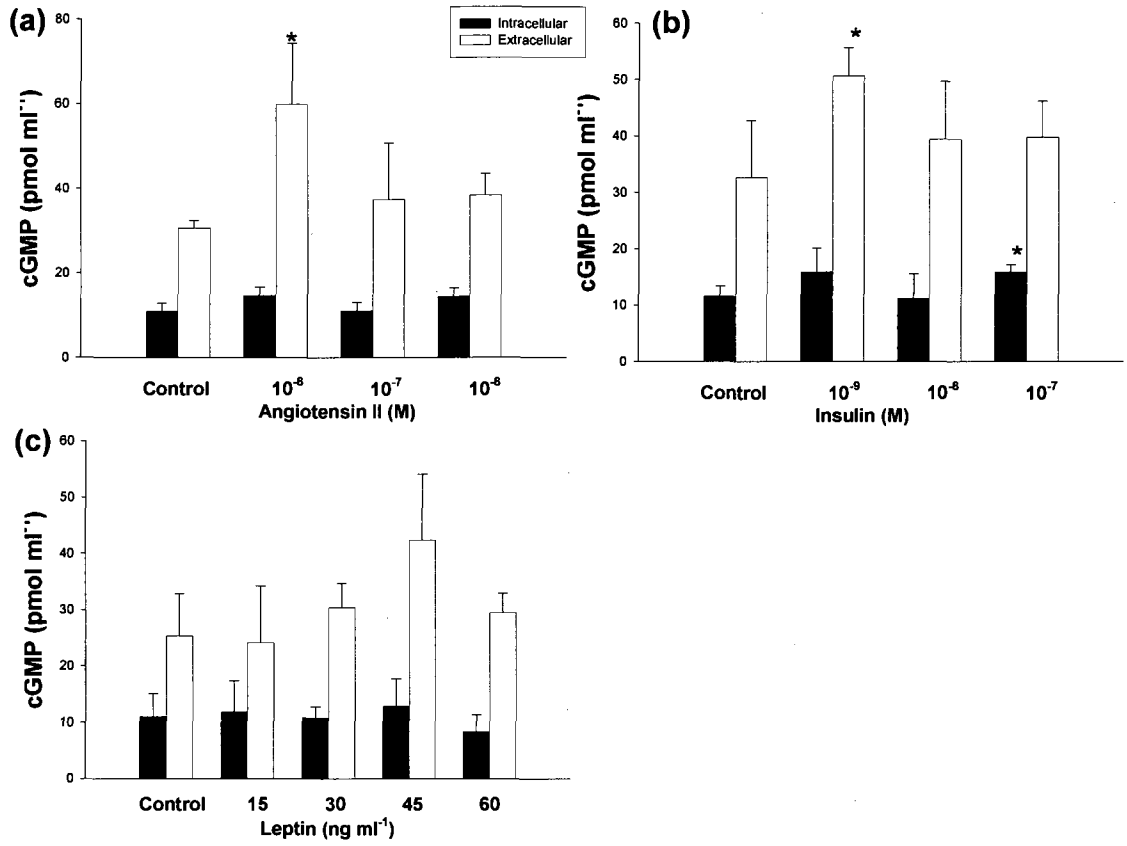

Figure 40 Angiotensin II, insulin and leptin effect on intra- and extracellular cGMP production in the presence of SNAP in RPTEC.
Cells were seeded at 100 000 cells/well density in 12-well plates and kept in serum-free and insulin-free medium 24 hr before the experiments. Panel (a): Effect of different angiotensin II concentrations on intracellular and extracellular cGMP production in the presence of SNAP. RPTEC were incubated simultaneously with SNAP (10^{-6} M) and angiotensin II (10^{-8}, 10^{-7}, and 10^{-6} M) for 2 hours in the presence of 1 mM IBMX. 10^{-8} M AII significantly increased extracellular cGMP over the control values after 2 hours incubation. Panel (b): Effect of different insulin concentrations on intracellular and extracellular cGMP production in the presence of SNAP. RPTEC were incubated simultaneously with SNAP (10^{-6} M) and insulin (10^{-9}, 10^{-8}, and 10^{-7} M) for 2 hours in the presence of 1mM IBMX. 10^{-9} M of Insulin significantly increased extracellular cGMP over the control values after 2 hours incubation. Panel (c): Effect of different leptin concentrations on intracellular and extracellular cGMP production in the presence of SNAP. RPTEC were incubated simultaneously with SNAP (10^{-6} M) and leptin (15, 30, 45 and 60 ng ml^{-1}) for 2 hours in the presence of 1mM IBMX. Leptin had no effect on cGMP production at any given concentration.

(Fig. 40C). However, we decided to proceed with all the above hormones because we considered that effect of leptin on cGMP production did not become statistically different because of the large assay variability as mentioned earlier.

Effect of NO and angiotensin II, insulin and leptin on Na,K-ATPase and NHE3 total protein expression

We investigated the effects of NO together with angiotensin II (AII), insulin and leptin on Na,K-ATPase and NHE3 on total protein expression in membrane fractions of RPTEC by western blotting. Tubulin was used as a loading control. Protein expression of both: Na,K-ATPase and NHE3 were not consistently changed at 6, 12 and 24 hour time course by any of the above mentioned hormone treatments concurrent with NO donor administration at 10^{-6} M (data not shown) or 10^{-5} M (Fig. 41 and 42) compared to controls (NO donor only). For this reason we did not pursue measurements of Na,K-ATPase and NHE3 protein abundance at those time points, however we continued to determine expression of phosphorylated forms of both transporters at shorter time periods. Also, we have decided to focus our efforts on treatments with angiotensin II and insulin only as leptin did not significantly increase cGMP production.

Effect of NO, angiotensin II, and insulin on phosporylation status of Na,K-ATPase and NHE3

There is evidence that serine-threonine phosphorylation of the catalytic α-subunit by PKA or PKC is a key event in the short-term regulation of Na,K-ATPase

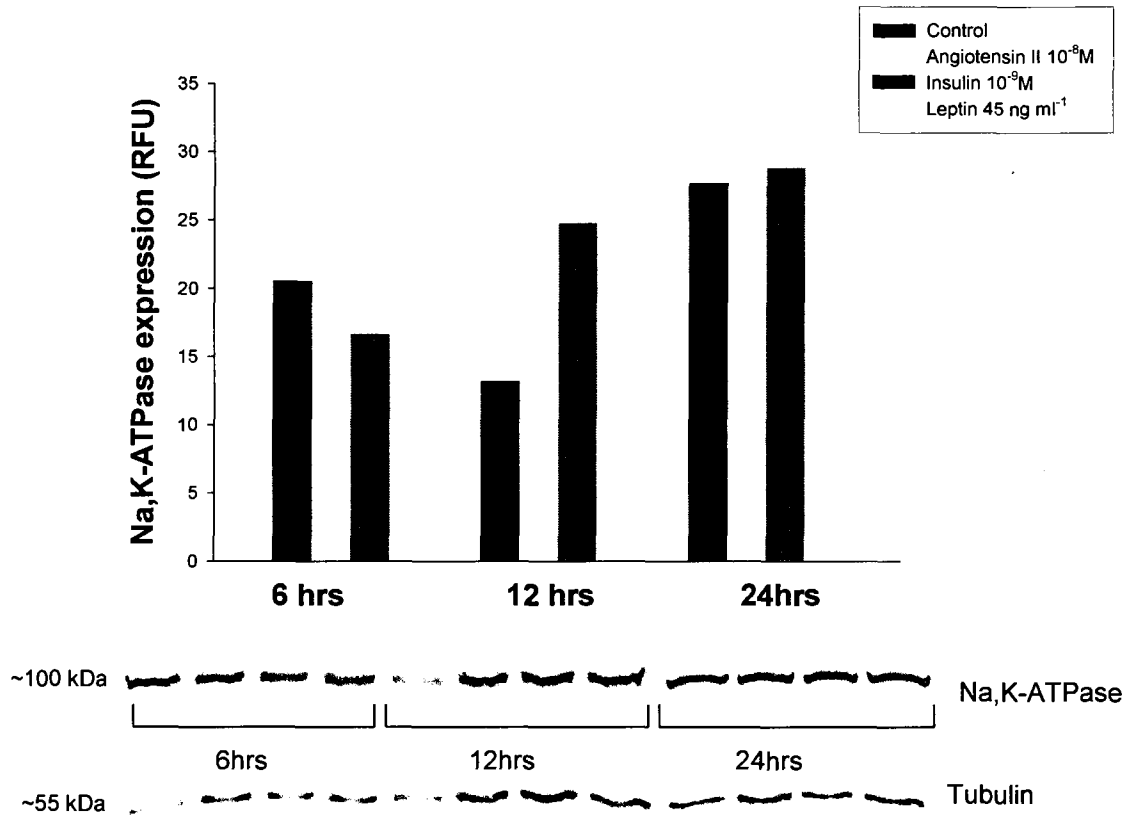

Figure 41 Protein levels of Na,K-ATPase α1 subunit in RPTEC stimulated with NO and treated with angiotensin II, insulin and leptin for 6, 12 or 24 hours. Cells were plated on 100 mm dishes at 330 000 cells/plate density and grown until they reached confluency; then kept in serum-free medium 24 hr before the experiments. SNAP, 10^{-5} M was added to all dishes followed by no treatment (control) or angiotensin II (10^{-8} M), insulin (10^{-9} M), leptin (45 ng ml^{-1}) for 6, 12 or 24 hours. Top: Values represent a ratio between Na,K-ATPase and tubulin signal from 1 experiment in relative fluorescent unit (RFU). Bottom: Western blots of Na, K-ATPase and tubulin which served as a loading control. Equal amount of proteins were loaded into SDS-PAGE gel and membrane were simultaneously probed with a monoclonal antibody against Na,K-ATPase and a polyclonal antibody against tubulin. Bands were visualized by Odyssey Infrared Imaging System.

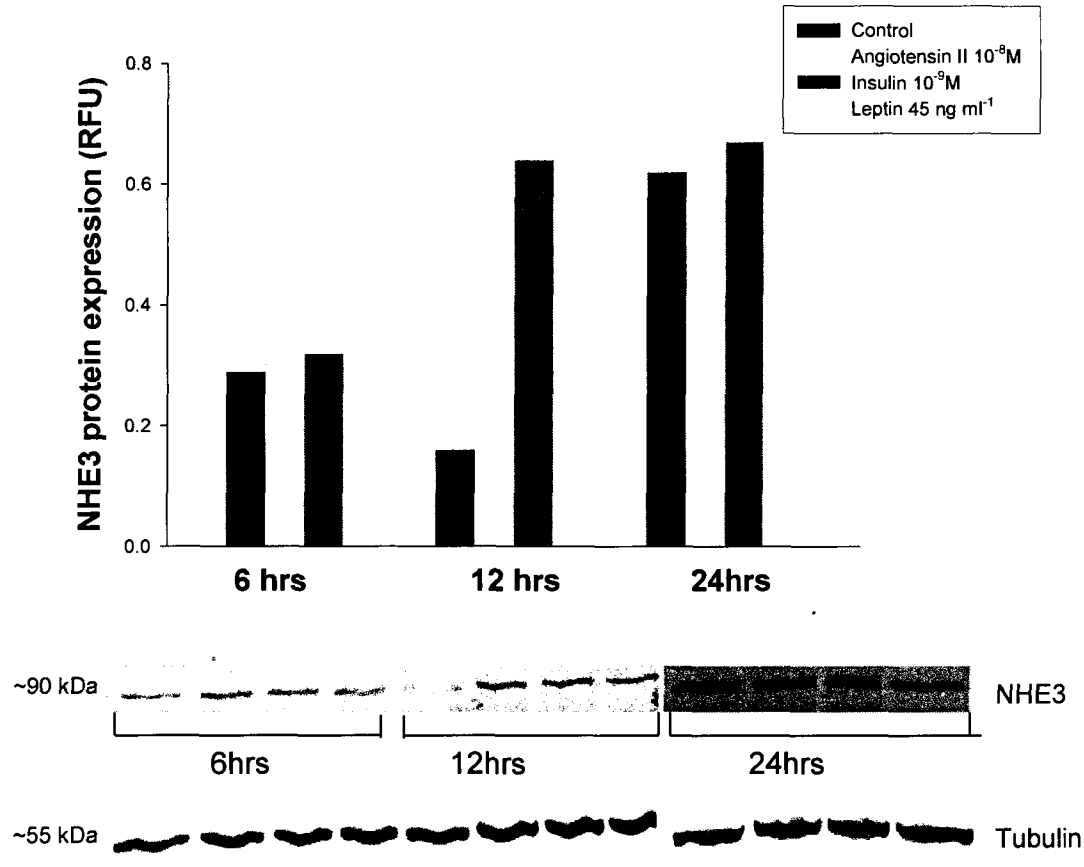

Figure 42 Protein levels of NHE3 in RPTEC stimulated with NO and treated with angiotensin II, insulin and leptin for 6, 12 or 24 hours measured by western blotting.
Cells were plated on 100 mm dishes at 330 000 cells/plate density and grown until they reached confluency; then kept in serum-free medium 24 hr before the experiments. SNAP, 10^{-5} M was added to all dishes followed by no treatment (control) or angiotensin II (10^{-8} M), insulin (10^{-9} M), leptin (45 ng ml^{-1}) for 6, 12 or 24 hours. Top: Values represent a ratio between NHE3 and tubulin signal from 1 experiment in relative fluorescent unit (RFU). Bottom: Western blots of NHE3 and tubulin which served as a loading control. Equal amount of protein were loaded into SDS-PAGE gel and membrane were simultaneously probed with a polyclonal antibody against NHE3 and a monoclonal antibody against tubulin. Bands were visualized by Odyssey Infrared Imaging System.

(Fisone *et al.* 1994, Belusa *et al.* 1997, Pedemonte *et al.* 1997, Chibalin *et al.* 1999). In contrast, phosphorylation of NHE3 is not always necessary for its acute regulation. The most definitive studies showed that cAMP effected *in vivo* phosphorylation of rat NHE3 Ser605 and Ser552 in a concentration-dependent manner (Moe 1999). Given all the above, we investigated whether AII and insulin could change the phosphorylation status of proximal tubule transporters and if this mechanism involves the NO pathway. RPTEC were incubated with 10^{-8} M angiotensin II and 10^{-9} M insulin in the presence or absence of 10^{-5} M SNAP for 30 minutes. Protein phosphorylation was expressed as the ratio between protein phosphorylated on Ser16 and Ser552 to total protein expression.

The phosphorylation status of Na,K-ATPase was increased by incubation with angiotensin II and insulin 3fold and 5fold, respectively, compared to control levels. Conversely, SNAP alone decreased phophorylation of Na,K-ATPase by 200fold. However, simultaneous administration of SNAP with AII further elevated phophorylation levels of Ser16 of Na,K-ATPase over the control with SNAP only (Fig. 43). At the same time, SNAP prevented the effect achieved by insulin alone (as percent of control: insulin, 534%; SNAP, 0.4%; insulin+SNAP, 56%) (Fig. 43). These results suggest that phosphorylation of the α1-subunit of Na,K-ATPase at Ser16 can be stimulated by angiotensin II and insulin independent of cGMP production since neither angiotensin II or insulin could change cGMP production in the absence of the NO donor. However, only insulin effect on the phosphorylation status of Na,K-ATPase was modulated in the presence of NO implying that insulin signaling pathway can also be influenced by the NO pathway

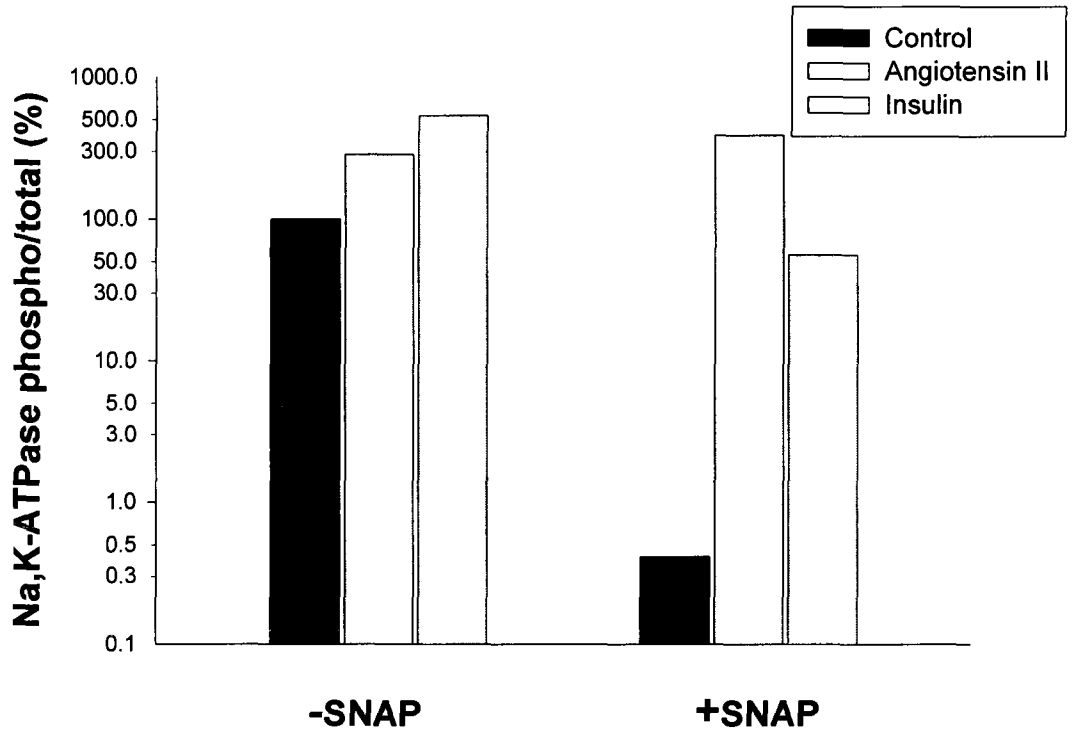

Figure 43 Serine 16 phosphorylation of Na,K-ATPase α1 subunit in RPTEC treated simultaneously with NO and angiotensin II (AII) or insulin.
Cells were plated on 100 mm dishes at a density of 330 000 cells/plate, grown to near confluency and then kept in serum-free, insulin-free medium 24 hr before the experiments. Cells were treated with10^{-5} M SNAP in the absence (control) or presence of angiotensin II (10^{-8} M) or insulin (10^{-9} M) for 30 min. In all conditions, phosphodiesterase inhibitor (IBMX) was used. Values represent a ratio between phospho- and total Na,K-ATPase signal from 1 experiment. Control without SNAP was set to 100% and treatments were expressed relative to the control level. Phosphorylation levels of Na,K-ATPase were greatly reduced by NO and increased by AII and insulin. NO together with AII further increased phosphorylation of Ser16 while reversed insulin response in RPTEC.

in renal proximal tubule epithelial cells. Whether cGMP is a share messenger of those pathways needs additional testing where ODQ compound should be used.

As demonstrated in Fig. 44, the phosphorylation level of Ser552 of Na/H exchanger 3 was slightly elevated by angiotensin II and remained unchanged after incubation with insulin when compared to control levels. SNAP alone decreased phosphorylation of NHE3 by 35% and it prevented the effect of angiotensin II (as percent of control: angiotensin II, 120%; SNAP, 65%; angiotensin II+SNAP, 88%). Simultaneous incubation of RPTEC with SNAP and insulin increased posphorylation of NHE3 to 117% from 97% with insulin alone. These results suggest that angiotensin II and insulin may not play a role in phosphorylation of NHE3 at Ser552. Nevertheless, in the presence of NO angiotensin II and insulin modulated the phosphorylation status of NHE3 differently, pointing to the fact that their signaling pathways could be influenced by NO pathway in RPTEC. Further experiments are required to establish whether cGMP plays a role.

Insulin cross-talk with NO at the level of cGMP production and the resultant production of cGMP could in turn induce phosphorylation of the two transporters. This may account at least in part for different levels of activity of those transporters determined *in vivo*. However, further studies are required to determine the precise mechanism(s) involved.

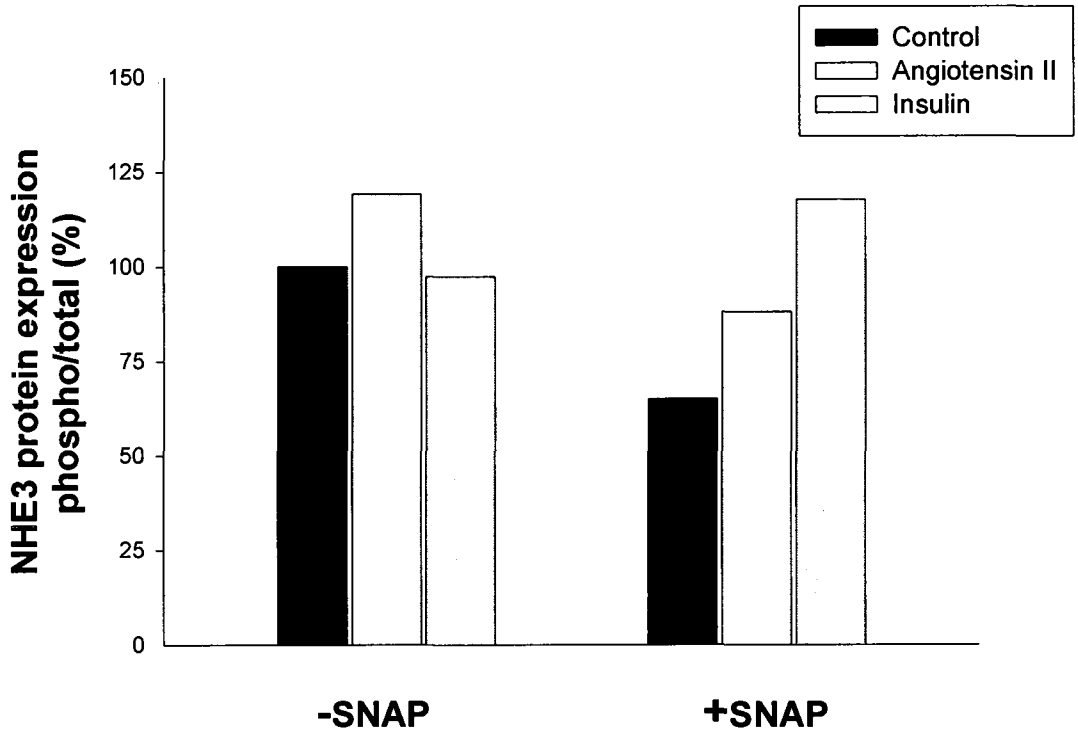

Figure 44 Serine 552 phosphorylation of NHE3 in RPTEC treated simultaneously with NO and angiotensin II (AII) or insulin.
Cells were plated on 100 mm dishes at a density of 330 000 cells/plate, grown to near confluency and then kept in serum-free, insulin-free medium 24 hr before the experiments. Cells were treated with SNAP 10^{-5} M in the absence (control) or presence of angiotensin II (10^{-8} M) or insulin (10^{-9} M) for 30 min. In all conditions phosphodiesterase inhibitor (IBMX) was used. Values represent a ratio between phospho- and total NHE3 signal from 1 experiment. Control without SNAP was set to 100% and treatments were expressed relative to the control level. NO decreased, AII slightly increased and insulin did not change NHE3 phophorylation levels. In the presence of NO, AII decreased and insulin increased phosphorylation of Ser552 of NHE3 in RPTEC.

5.3 Discussion for Specific Aim # 3

In the previous Aim we described the effects of pioglitazone on sodium transport and renal excretory function. Earlier data published by our group showed that pioglitazone treatment resulted in an increase of NOx as well as eNOS and nNOS expression in the kidney (Dobrian *et al.* 2004). Since several studies showed that NO can modulate renal sodium handling, we further investigated the effect of *in vivo* chronic NO blockade on expression and activity of Na,K-ATPase and NHE3.

The results of our present study demonstrated that inhibition of NO synthesis by chronic treatment with L-NAME lead to a marked attenuation of the urine flow rate and sodium excretion in response to changes in renal perfusion pressure in lean, but not obese, rats on a moderately high fat diet. The dose of L-NAME was chosen to inhibit NO without changes in blood pressure. This approach allowed us to determine if the changes in renal hemodynamics and sodium excretion were related to changes in NO production and not simply a consequence of the increase in blood pressure. Indeed, L-NAME reduced NO production in a comparable manner in both groups while having no significant effect on MAP. In obese rats, treatment with L-NAME did not produce any changes in pressure natriuresis and diuresis. In contrast, in lean rats NO inhibition reduced natriuresis and diuresis. In both groups, GFR was not affected by the treatment and remained the same in treated and non-treated animals. In agreement with our results from the lean animals, several studies have indicated that inhibition of renal NO synthesis blunted the diuretic and natriuretic

responses without altering renal blood flow or glomerular filtration rate (Salom *et al.* 1992, Majid *et al.* 1993). Moreover, there is evidence suggesting that excretion of urinary NO metabolites was increased with renal perfusion pressure (Suzuki *et al.* 1992, Majid *et al.* 1995). Finally, direct measures of renal cortical NO activity in the dog with an NO-sensitive microelectrode showed that it decreased linearly with the reduction in renal perfusion pressure within the autoregulatory range (Majid *et al.* 1998). However, these data do not explain the differences in natriuretic responses between the lean and obese rats. In animal models of obesity and in obese humans there are reports of elevated renal sympathetic nerve activity (Vaz *et al.* 1997, Hall 2003) and activation of the renin-angiotensin system (Sharma 2004). Interaction between NO and renal sympathetic nerves is an important factor in the regulation of renal hemodynamics and sodium homeostasis. Basal NO synthesis has been reported to blunt the vasoconstrictive effect of sympathetic nerve stimulation in the isolated perfused rat kidney (Reid & Rand 1992). In addition, there is also considerable evidence concerning NO interaction with the renin-angiotensin system. NO synthesis inhibition and angiotensin II stimulation are quite similar in terms of regulation of vascular tone. For example, inhibition of nitric oxide production stimulates endothelial angiotensin-converting enzyme (ACE) activity and generation of angiotensin II (Takemoto *et al.* 1997, Katoh *et al.* 1998). However, it is not clear the extent to which the vasoconstrictor response to NO blockade results from withdrawal of an NO vasodilatory stimulus or is related to the amplification of vasoconstrictor systems. Moreover, obesity is usually

associated with expansion of extracellular fluid volume (Carroll *et al.* 1995, Hall *et al.* 2002). Therefore, we propose that most probably lack of changes in excretion of water and sodium in obese Sprague-Dawley rats fed a high fat diet and treated chronically with L-NAME to inhibit NO production is a result of an imbalance between NO and other vasoconstrictor signaling pathways which leads to their defective mutual regulation.

Exactly how increased renal NO synthesis or inhibition of NO production cause changes in natriuresis and diuresis are not fully understood. Since NO did not alter renal autoregulatory function under the experimental conditions we used, inhibition of tubular sodium transport appears to be important in our model. In an attempt to elucidate whether overall actions of NO in the kidney can be attributed to a direct tubular effect we analyzed the outcome of chronic L-NAME treatment on proximal tubule transporters Na,K-ATPase and NHE3 expression and activity. We have observed in obese rats treated with L-NAME an increase in abundance of NHE3 but no changes in its activity. Also, we did not determine changes in abundance and activity of Na,K-ATPase. On the other hand, after NO inhibition in lean rats we reported an increase in protein expression of Na,K-ATPase without changes in its activity and an increase in NHE3 abundance and activity, as well as redistribution of this transporter to the more active membrane pool.

The effect of NO on tubular sodium transport varies in different segments of the nephron. In most cases, however, NO inhibits sodium transport which is consistent with its natriuretic and diuretic action (Ortiz & Garvin 2002). In the

proximal tubule, which is responsible for reabsorbing 50-60% of filtered sodium and water, the effects of NO are not fully understood. As reviewed by Ortiz and Garvin, there are reports showing that NO inhibits proximal tubule transport, while others suggest that NO stimulates proximal tubule transport (Ortiz & Garvin 2002). Wu *et al.* proposed that while NO itself has a direct inhibitory effect on proximal tubule sodium transport, the presence of NO is necessary to ensure that renal nerves can stimulate fluid reabsorption by the proximal tubules (Wu & Johns 2002).

However, the direct inhibitory role of NO on proximal tubule sodium transport seems to be due to decreased apical Na/H exchange (Roczniak & Burns 1996) and reduced Na,K-ATPase activity (Liang & Knox 1999a) which is in agreement with results we obtained from lean rats. The NO inhibition did not significantly change proximal Na^+ transport in obese rats. Ortiz and Stoos proposed that effects of NO in the kidney can be modulated by changes in the sensitivity to NO signaling (Ortiz *et al.* 2003). Differences in NO signaling pathway are possible between OP and OR rats and could play a role in final responses to NO production blockage. An interesting observation suggesting differences in NO sensitivity between obese and lean animals was reported by Jebelovszki *et al.* in coronary arterioles of rats. They reported that coronary arteriolar dilations to acetylcholine (ACh) were preserved and not significantly affected by NOS inhibition, whereas the dilations to NO donors were significantly enhanced in obese rats compared to lean. In contrast, the inhibition of NO synthesis with L-NAME decreased ACh-induced dilation in coronary arterioles

isolated from lean animals only. In addition, the authors found that NO donor-stimulated vascular cGMP immunoreactivity and cGMP levels were increased in obese rats compared to lean rats. The authors suggested that in high fat diet-induced obesity, due to the increased soluble guanylyl cyclase activity, the NO sensitivity of coronary arterioles was enhanced despite the impaired NO bioavailability (Jebelovszki *et al.* 2008).

Besides possible variations in NO sensitivity in OP vs. OR rats, a difference in the metabolic milieu of the obese and lean animals is another possibility which may lead to different responses to NO depletion. It is known that the NO signaling cascade is influenced by other systems. Previous studies demonstrated that feeding rats and dogs a moderately high fat diet results in higher levels of renin activity in obese animals compared with lean animals (Dobrian *et al.* 2000, Henegar *et al.* 2001, Boustany *et al.* 2004). Higher levels of leptin in animals as well as obese humans were observed in diet-induced obesity (Hirose *et al.* 1998, Lauterio *et al.* 1999, Levin *et al.* 2003, Ricci & Levin 2003). Moreover, those changes are paralleled by changes in insulin levels (Hall *et al.* 1998, Lauterio *et al.* 1999, Henegar *et al.* 2001, Levin *et al.* 2003). For example, angiotensin II stimulated NO production via both AT1 in cultured endothelial cells (Saito *et al.* 1996) and via AT2 receptors in SHR rats (Saito *et al.* 1996, Gohlke *et al.* 1998) while it decreased NO bioavailability by stimulating superoxide production in Wistar rats (Mollnau *et al.* 2002) as well as blocking NO signal transduction in animals (Kim *et al.* 2001, Mollnau *et al.* 2002). Experimental evidence also suggests that NO is involved in the pathogenesis of diabetes and

insulin resistance. NADPH oxidases in the vascular wall are activated in diabetes mellitus, leading to enhanced degradation of NO and the production of reactive oxygen species (Guzik *et al.* 2002). Furthermore, uncoupling of eNOS has been demonstrated in animal models of diabetes (Elrod *et al.* 2006). In the kidney, leptin interferes with NO synthesis and this effect depends on the exposure time to the hormone as well as its concentrations. For instance, long-term hyperleptinemia decreased natriuresis and urinary excretion of NO metabolites and cGMP (Beltowski *et al.* 2004) by increasing levels of systemic and intrarenal oxidative stress, leading to NO deficiency (Beltowski *et al.* 2004). However, in an acute setting leptin stimulated systemic NO release (Beltowski *et al.* 2002) while chronic inhibition of NO synthesis impaired the acute leptin-mediated natriuretic effect in normotensive lean rats (Villarreal *et al.* 2004). Interestingly, the short-term leptin-induced natriuretic effect observed in lean rats was attenuated in obese rats and was blunted in spontaneously hypertensive rats (SHR) (Villarreal *et al.* 1998, Beltowski *et al.* 2002). It was suggested that while leptin may be a potential salt-excretory factor in normal rats, in obesity and hypertension its function may be impaired. Overall, these data imply that in hypertension induced by obesity there are factors which could tip the already existing imbalance between NO and systems that antagonize its action, in the favor of the latter. Therefore, further NO inhibition in obese rats is unlikely to induce additional significant changes.

As mentioned before, to investigate whether the differences in natriuretic responses could be attributed to differences in sodium transport we examined

expression and activity of Na,K-ATPase and NHE3 in obese and lean rats treated with L-NAME. In the lean rats we reported an increase in protein expression of Na,K-ATPase and an increase in NHE3 abundance and activity as well as redistribution of this transporter. In obese rats we did not observe a significant change in either Na,K-ATPase or NHE3 activities following chronic L-NAME treatment. Little is known about the molecular mechanism of NHE3 regulation by NO. As described earlier, Roczniak and Burns showed that NO stimulated soluble guanylyl cyclase in both freshly isolated proximal tubule segments and in primary cultures of proximal tubule cells, and caused inhibition of Na^+/H^+ exchange. It was concluded that this effect was at least partly mediated by generation of cGMP (Roczniak & Burns 1996). Using the Caco-2 cell line, a human colonic carcinoma cell line that has been used to study regulation of electrolyte uptake by various hormones and growth factors, Gill *et al.* reported that NHE3 activity was decreased through the activation of soluble guanylyl cyclase, resulting in increased production of intracellular cGMP and activation of protein kinase G (PKG); there was no involvement of PKC- or protein kinase A (PKA)-mediated pathways in this process (Gill *et al.* 2002). Further downstream events in the NO signaling pathway which lead to NHE3 inhibition have not been fully deciphered although a study by Cha *et al.* demonstrated that cGMP inhibition of NHE3 required NHE3 regulatory factor 2 (NHERF2). NHERF2 binds cGMP-dependent protein kinase type II (cGKII), and then anchors this complex to the brush border membrane (Cha *et al.* 2005). It was also suggested that

cGMP could stimulate phosphorylation of NHE3 while additional signaling molecules still need to be identified.

Na,K-ATPase is also subjected to regulation by NO and various hormones that are increased in obesity and hypertension. Na,K-ATPase activity was found to be inhibited by NO in an opossum kidney (OK) proximal tubule cell line (Liang & Knox 1999a), in the mouse proximal tubule epithelial cells (Guzman *et al.* 1995, Seven *et al.* 2005)) and in guinea pig kidney (Seven *et al.* 2005). Moreover, it was noted that in primary culture of rat proximal tubule cells in the presence of an NHE3 inhibitor, NO still decreased Na,K-ATPase activity implying that this inhibition is independent of intracellular Na^+ concentration (Linas & Repine 1999). Recently, it was reported by Shahidullah *et al.* that various nitric oxide donors decreased Na,K-ATPase activity by activation of soluble guanylyl cyclase, generation of cGMP and activation of PKG in epithelial cells from porcine eyes (Shahidullah & Delamere 2006). Therefore, it is conceivable to believe that a similar mechanism might be responsible for the NO effect on Na,K-ATPase in the kidney as well. However, whether PKG regulates Na,K-ATPase through secondary modulators or by direct phosphorylation should be further investigated. We propose that the lack of responses from obese animals treated with L-NAME could be explained, as in the case of apical NHE3, by a difference in the metabolic and hormonal milieu in the obese vs. lean rats.

Our results did not provide definitive information regarding direct effects of NO on the activity of proximal tubule sodium transporters *in vivo* thus we designed *in vitro* experiments on sodium reabsorption in primary cultures of

human renal proximal tubule epithelial cells (RPTEC). We analyzed expression of Na,K-ATPase and NHE3 in the presence of angiotensin II, insulin or leptin and examined the role of cGMP as a potential mediator of their responses. We demonstrated that in response to an NO donor, RPTEC stimulated cGMP production by activation of soluble guanylyl cyclase (sGC) since the selective sCG inhibitor, ODQ, was effective in blocking this increase. However, it is controversial whether the proximal tubule produces NO under basal conditions. Nevertheless, evidence suggests that the proximal tubule is constantly exposed to NO that might include NO from nonproximal tubule sources such as the vasculature or other nephron segments (Amorena & Castro 1997, Linas & Repine 1999). Also, proximal tubule cells from humans contain sGC (Sasaki *et al.* 2004) therefore are being able to signal through NO, regardless of the source.

Next, we reported that in the presence of an NO donor, angiotensin II and insulin but not leptin increased cGMP levels in renal proximal tubule epithelial cells. To our knowledge, there are no reports about the effect of insulin and leptin on cGMP production in RPTEC. As already mentioned before, infusion of angiotensin II into rats significantly decreased expression of both subunits of sGC in blood vessels (Mollnau *et al.* 2002). Moreover, in isolated proximal tubules Zhang and Mayeux showed that angiotensin II induced a rise in cGMP production which was mediated by AT1 receptors (Zhang & Mayeux 1998).

As we did not observe any changes in total abundance of Na,K-ATPase and NHE3 after 6, 12 and 24 hours incubation of RPTEC with angiotensin II and insulin in the presence of an NO donor, we decided to determine changes in

phosphorylation status of Na,K-ATPase and NHE3 in the same conditions but in shorter periods of time. We reported that phosphorylation of the α1-subunit of Na,K-ATPase at Ser16 was stimulated by angiotensin II and insulin in the absence of NO while phosphorylation status of NHE3 at Ser552 was not changed. However, in the presence of an NO donor only insulin affected the phosphorylation state of Na,K-ATPase but both: angiotensin II and insulin modulated the phosphorylation status of NHE3. As already discussed in aim # 1, it has been shown that in the proximal tubule the α-subunit of Na,K-ATPase can be phosphorylated by PKA in the C-terminal at Ser943 (Feschenko & Sweadner 1995) as well as by PKC in the N-terminal at Ser11, Ser18 and Ser23 (Logvinenko *et al.* 1996). Modification of Na,K-ATPase by phosphorylation is generally associated with altered enzyme activity (Bertuccio *et al.* 2007) and changes in subcellular Na,K-ATPase distribution . For example, angiotensin II stimulated Na,K-ATPase in the proximal tubule by PKC activation (Rangel *et al.* 2002) very rapidly, by a direct mechanism that could involve changes in phosphorylation (Yingst *et al.* 2004). In the rat, angiotensin II induced the phosphorylation of both Ser11 and Ser18 of the Na,K-ATPase α-subunit by protein kinase C beta. This resulted in the recruitment of Na,K-ATPase molecules to the plasma membrane and an increased capacity to transport sodium ions (Efendiev *et al.* 1999, 2000). However, in human type Na,K-ATPase Ser11 was essential for the hormonal regulation of Na,K-ATPase activity and phosphorylation of this residue either lead to stimulation or inhibition of the enzyme with the subsequent recruitment or rectraction of Na,K-ATPase to or

from plasma membrane, respectively (Efendiev & Pedemonte 2006). Our data are supported by the above mentioned findings since incubation of RPTEC with angiotensin II lead to the increase in the phosphorylation status of Ser16 of the α1-subunit although we do not have evidence yet to indicate that this could be translated into changes in Na,K-ATPase activity.

The mechanisms by which insulin regulates Na,K-ATPase in the kidney is largely unknown. There are reports demonstrating that insulin can stimulate Na,K-ATPase (Sweeney *et al.* 1998) by tyrosine phosphorylation in kidney proximal tubule cells (Feraille *et al.* 1999). However, in the present study, we have observed that phosphorylation of Ser16 was stimulated following the insulin administration. To ensure that the effects of angiotensin II and insulin were in fact achieved by activation of protein kinase C further testing with PKC inhibitors is required.

NO was shown to inhibit Na,K-ATPase activity acting through the cGMP pathway and by activating protein kinase C-α (Liang & Knox 1999b), however, no phosphorylation sites were described. It is possible that as mentioned before, Ser11, Ser18 and Ser23 are involved. Our data suggest that an NO donor decreased the phosphorylation status of Ser16. However, in the presence of NO, angiotensin II at a concentration which increased cGMP levels, did not change the phosphorylation status of Ser16 of Na,K-ATPase while insulin did. To the best of our knowledge, reports about interaction of angiotensin II and NO/cGMP pathways in the proximal tubule and their effects on sodium transporters are missing. Zhang and Mayeux demonstrated that angiotensin II had a biphasic

effect on Na,K-ATPase activity in the rat proximal tubule and that activation of NOS opposed the stimulatory effects of angiotensin II on Na,K-ATPase activity (Zhang & Mayeux 2001). Also, there are no reports on simultaneous effects of NO and insulin on Na,K-ATPase and NHE3. To better understand the influence of the NO/cGMP signaling cascade on angiotensin II and insulin pathways additional studies are needed.

NHE3 is subjected to both short- and long-term regulation. Long-term regulation is associated with an increase in protein abundance, whereas short-term regulation is associated with transporter trafficking as well as post-translational modifications of the proteins. We examined one of the acute regulatory mechanisms of NHE3 *in vitro*. NHE3 was phosphorylated in intact tissue or in cell culture models by PKA and PKC (Wiederkehr *et al.* 1999, Zhao *et al.* 1999). While an increase in NHE3-phosphorylation was paralleled, in general, by a decrease in NHE3 activity (Fan *et al.* 1999, Zhao *et al.* 1999), PKC activation lead to either stimulation or inhibition of NHE3 activity (Wiederkehr *et al.* 1999). The result was dependent on the system used and suggested that phosphorylation of NHE3 per se is necessary but not sufficient to regulate activity. The study by Zhao *et al.* demonstrated that Ser552 and Ser605 are involved in PKA-inhibition of NHE3 (Zhao *et al.* 1999). Kocinsky and coworkers showed that phosphorylation of NHE3 at serines 552 and 605 by PKA did not alter NHE3 activity *in vivo*. Furthermore, in a proximal tubule cell model PKA activation reduced NHE3 activity in a time-dependent manner (Kocinsky *et al.* 2007). The authors implied that the lack of direct association between NHE3

phosphorylation and NHE3 activity demonstrates that NHE3 phosphorylation at serines 552 and 605 per se does not directly inhibit transport. Nonetheless, we assessed the phosphorylation state of NHE3 Ser552 RPTEC after stimulation with angiotensin II and insulin in the presence or absence of NO. In the absence of an NO donor, neither angiotensin II nor insulin modulated Ser552 phosphorylation of NHE3. Angiotensin II is known to stimulate NHE3 by its action on AT1 receptors through pathways that are dependent on phospholipase C, metabolism of arachidonic acid, phosphatidyl inositol 3 kinase and Akt in human intestinal epithelial Caco2BBE cells (Musch *et al.* 2009). Studies by Becker *et al.* revealed that angiotensin II stimulated NHE3 activity in the proximal tubule, but the stimulatory response was markedly greater in obese Zucker than in lean Zucker rats; in addition, angiotensin II caused greater inhibition in cAMP accumulation in the proximal tubule of obese compared to lean rats (Becker *et al.* 2003). Some in vivo results suggested that angiotensin II stimulated Na^+/H^+ exchange in the proximal tubule by a depression in intracellular cAMP (Liu & Cogan 1989). Although, no correlations have been made to the phosphorylation of NHE3 at Ser552, it is tempting to suggest that angiotensin II may stimulate NHE3 by reducing cAMP levels and by further decreasing the phosphorylation status of NHE3 at the Ser552 residue.

How insulin signals NHE3 stimulation is not known. In opossum kidney (OK) cells, insulin increased NHE3 activity in a time- and concentration-dependent manner (Klisic *et al.* 2002). While the chronic effect of insulin on NHE3 appeared to proceed through the phosphatidylinositol 3-kinase-serum- and

glucocorticoid-dependent kinase 1 pathway, the signaling cascade for the acute effect is not known; in addition, in clear contradistinction to hormones coupled to protein kinase A, insulin acutely alters NHE3 activity without changes in its phosphorylation (Fuster *et al.* 2007).

Our data suggest that in the presence of an NO donor, signaling cascades of angiotensin II and insulin may be altered and differently modulate Ser552 phosphorylation of NHE3. Further studies are necessary to evaluate whether those changes are cGMP dependent by using a soluble guanylyl cyclase inhibitor, such as ODQ. Moreover, it is imperative to measure how the phosphorylation status of Na,K-ATPase and NHE3 is changing when angiotensin II and insulin effects are combined in the presence of NO to more closely mimic the *in vivo* environment.

In summary, we demonstrated that inhibition of NO production by chronic L-NAME treatment impaired pressure-induced natriuresis and diuresis in lean, normotensive but not in obese, hypertensive animals. In the lean rats, impaired pressure natriuresis was paralleled by alterations in the abundance, expression and distribution of apical NHE3 and the basolateral Na,K-ATPase in the proximal tubule. The mechanism responsible for diverse regulation of both transporters in obese and lean animals could evolve most likely from different metabolic states of obese vs. lean rats. It could also involve an imbalance among the NO signaling pathway, angiotensin II and insulin and eventually lead to differential regulation of sodium transporters in both the acute and the long-term settings.

SECTION 6

SUMMARY AND CONCLUSIONS

Excess weight contributes to increased blood pressure in a large proportion of essential hypertensive patients. Epidemiological studies have shown that hypertension is more prevalent in obese than in nonobese individuals and that blood pressure is correlated to body weight, even in normotensive subjects. There are many animal models of obesity available but not all of them mimic closely enough the cardiovascular, renal, and neurohumoral changes found in obese humans. In this study we used a rodent model previously characterized by Dobrian *et al.* This rat model of diet-induced obesity develops hypertension and shows vascular and renal changes similar to those observed in obese hypertensive humans. Sprague-Dawley rats were fed a purified moderately high-fat (MHF) diet that contains 32% kcal as fat, a value similar to the average Western diet, and showed a bimodal pattern in body weight gain. Approximately half of the rats gain weight rapidly (obesity-prone (OP)) and develop mild hypertension while the other half (obesity-resistant (OR) group) gain weight at a rate similar with chow-fed rats- and are normotensive.

Theoretical and experimental studies have shown that in all forms of hypertension, including obesity hypertension, there is an abnormality of kidney function characterized by a hypertensive shift in renal pressure natriuresis. In addition, when obesity is induced by feeding a high fat diet, there is marked sodium retention and expansion of extracellular fluid volume. Moreover, sodium retention and altered pressure natriuresis appears to be caused mainly by

increased tubular sodium reabsorption. Based on this evidence we hypothesized that development of hypertension in diet-induced obesity is due to the increase in Na^+ reabsorption by means of changes in activity of proximal tubule sodium transporters. In summary, our study demonstrated that pressure-induced natriuresis and diuresis were attenuated in obese rats compared to lean rats fed high fat diet. This can explain the increased sodium reabsorption in the obese rats, at least in part due to impaired proximal tubule function. These findings were well correlated with higher protein expression and activity of the apical NHE3 and the higher overall activity of basolateral Na,K-ATPase in the renal proximal tubule. The mechanisms which contribute to these changes are likely multifactorial. We provided evidence on the differences in NHE3 distribution between lean and obese rats which could account for the differences in NHE3 activity and may explain the increased sodium retention seen in obese rats during acute pressure natriuresis and diuresis. For future studies, it would be of interest to examine other sodium transporters along the renal tubule as well as to investigate the mechanism of sodium handling and transporters activity in the obese and lean rats before the development of hypertension.

PPARγ is implicated in the pathogenesis of obesity and is also involved in blood pressure regulation. PPARγ are molecular targets for TZDs (pioglitazone, rosiglitazone) used in clinical practice to treat diabetes. Besides reducing blood glucose levels these agents increase insulin sensitivity, lower blood pressure, but can also cause sodium retention by effecting tubular reabsorption. While the water retention induced by the TZDs is explained by a distal mechanism, the

blood pressure lowering effects have no clear molecular explanation. Thus, we hypothesized that PPARγ activation lowers blood pressure by altering activity of sodium transporters Na,K-ATPase and NHE3 and by modulating tubular sodium reabsorption. Consequently, to address this hypothesis we examined the effects of chronic pioglitazone administration on blood pressure, sodium handling, and activity of proximal tubule transporters in obese and lean rats fed with a high fat diet. Also, using an in vitro model of proximal tubule epithelial cells we tested whether the pioglitazone effect on sodium transporters is mediated by peroxisome proliferator-activated receptor gamma. In summary, in an animal model of obesity-induced hypertension pioglitazone lowered blood pressure with a strong tendency to increase sodium excretion. In normotensive lean rats, pioglitazone had no effect on renal hemodynamics and blood pressure and at the same time caused sodium and water retention. Na,K-ATPase protein expression and activity were increased while NHE3 protein expression and activity were decreased by pioglitazone in obese rats. In lean animals Na,K-ATPase abundance was elevated while NHE3 protein expression was reduced. Moreover, in lean group after pioglitazone treatment NHE3 had a tendency to be redistributed to the active pool localized in the microvilli region. We concluded that simultaneous reduction in NHE3 abundance and activity could be responsible for blood pressure lowering effect of pioglitazone. Since we do not have a good candidate responsible for increased sodium and water reabsorption observed in lean rats we propose, that additional studies to analyze expression and activity of other transporters are needed to account for this effect. Our *in vitro*

experiments provided evidence that pioglitazone requires the expression of peroxisome proliferator-activated receptor gamma in order to act on Na,K-ATPase and NHE3. Furthermore, additional *in vitro* studies will provide further mechanistic understanding on PPARγ effect on sodium transporters.

It has been shown that chronic inhibition of NO production produces arterial hypertension. In the kidney NO plays an important role in the control of renal hemodynamics and its net effect is to promote natriuresis and diuresis. This mechanism is not well understood, but a direct effect on tubular transport seems to be involved. Many *in vitro* and *in vivo* studies have demonstrated that at proximal tubule level NO induces natriuresis by directly inhibiting activity of NHE3 and Na,K-ATPase through generation of cGMP and stimulation of PKG. There are reports that in obesity-induced hypertension NO bioavailability and sensitivity are altered. Moreover, NO is known to interact with pathways which are activated in obesity such as renin-angiotensin system, renal sympathetic nerves and leptin. Therefore, we hypothesized that NO may have different effects on Na^+ handling in obese and lean animals and its interaction with hormones such as angiotensin II, insulin and leptin is relevant for the blood pressure control. We measured GFR, fractional excretion of sodium and lithium at two different perfusion pressures, and expression and activity of Na,K-ATPase and NHE3 in basolateral or brush border membranes from renal cortex, respectively, *in vivo*. Moreover, we analyzed changes in phosphorylation status of Na,K-ATPase and NHE3 after treatment with angiotensin II or insulin in the presence of NO *in vitro*, in cell culture. In summary, L-NAME treatment did not change MAP in either group. The

changes in GFR were also not significantly different between groups. L-NAME attenuated the natriuretic response to increases in RPP in OR while it had no effect on pressure natriuresis and diuresis in the OP group. We have observed after NO inhibition in lean rats an increase in protein expression of Na,K-ATPase without changes in its activity and an increase in NHE3 abundance and activity as well as redistribution of this transporter to the more active membrane pool. On the other hand, in obese rats treated with L-NAME we report an increase in abundance of NHE3 but no changes in its activity or in abundance and activity of Na,K-ATPase. In conclusion, inhibition of NO production blunted pressure-induced natriuresis and diuresis in lean rats fed high fat diet most likely through releasing tonic inhibitory effect of NO on the proximal tubule sodium transporters. However, L-NAME treatment did not have any effect on renal hemodynamics and kidney excretory function in obese animals, leading us to believe that metabolic and hormonal differences between those animals are responsible for their diverse responses to NO blockage.

Our *in vitro* studies demonstrated that phosphorylation of the α1-subunit of Na,K-ATPase at Ser16 was stimulated by angiotensin II and insulin in the absence of NO while phosphorylation status of NHE3 at Ser552 was not changed. However, in the presence of an NO donor only insulin effected phosphorylation state of Na,K-ATPase but both: angiotensin II and insulin modulated the phosphorylation status of NHE3. Available data suggest that phosphorylation of Na,K-ATPase is usually associated with altered enzyme activity while phosphorylation of NHE3 is necessary but not sufficient to regulate

its activity. Our study provides evidence that Na,K-ATPase and NHE3 phosphorylation status is changed after treatment with hormones in the presence or absence of an NO donor but further studies are needed to extrapolate these findings to changes in the transporters' activity.

Moreover, additional *in vitro* studies are necessary to evaluate whether the changes are cGMP dependent by using a soluble guanylyl cyclase inhibitor, such as ODQ. It is also imperative to measure how the phosphorylation status of Na,K-ATPase and NHE3 is changing when angiotensin II and insulin effects are combined in the presence of NO to more closely mimic *in vivo* environment.

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