THE RENAL BUMETANIDE-SENSITIVE NA-K-2CL COTRANSPORTER BSC-1/NKCC2 IN ESSENTIAL HYPERTENSION AND ITS REGULATION BY NOREPINEPHRINE

by

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The renal bumetanide-sensitive Na-K-2Cl cotransporter BSC-1/NKCC2 in essential hypertension and its regulation by norepinephrine

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The dissertation is based on the concept that pathogenesis of essential hypertension involves the kidney. In this regard, renal sodium ion transporters, responsible for sodium reabsorption and fluid balance, may be important candidates in hypertension. Many lines of evidence indicate that the sympathetic nervous system, *via* renal nerves, plays an important role in the pathogenesis of essential hypertension. The goals of the dissertation were to: *1)* identify whether renal sodium ion transporter expression is altered in an animal model of essential hypertension, the Spontaneously Hypertensive Rat (SHR) and if so, its physiological significance; *2)* determine the role of the sympathetic nervous system in regulation of renal sodium ion transporters and *3)* elucidate the underlying molecular mechanism.

Among the renal sodium transporters profiled in the SHR, the bumetanide-sensitive Na-K-2Cl cotransporter (BSC-1) of the thick ascending limb was found to be most elevated; suggesting that increase in BSC-1 abundance may contribute to altered tubular function in SHR. In support of this conclusion, our results demonstrate that the natriuretic response to furosemide is greater in SHR versus its normotensive counterpart the Wistar-Kyoto Rat (WKY), resulting in normalization of blood pressure. Additionally, progression from pre-hypertensive to hypertensive state in SHR is accompanied by an increase in steady state protein levels of BSC-1 and its distribution to plasma membrane. Thus our biochemical and pharmacological data are consistent with the hypothesis that BSC-1 is involved in the pathogenesis of hypertension in SHR.

Activation of renal sympathetic efferent nerves releases norepinephrine and, if chronic, increases arterial pressure. We hypothesize that long-term exposure of kidney to norepinephrine increases expression of renal sodium transport systems. Our results indicate that chronic 14-day norepinephrine infusion increased abundance of BSC-1 along with an increase in mean arterial blood pressure; an effect that could explain altered sodium handling associated with an over-active renal sympathetic system. Finally, studies in an immortalized thick ascending limb cell

line show that regulation of BSC-1 by norepinephrine involves post-transcriptional control mechanisms *via* the β-adrenoceptor-cAMP-PKA pathway, and involves in part MAP kinases and that the α-adrenoceptor negatively regulates BSC-1. Further elucidation of the mechanism would suggest new strategies to treat diseases associated with an over-active sympathetic nervous system such as essential hypertension.

FOREWARD

"Gurur brahmaa gurur vishnuh
gurur devo maheshvarah
gurur saakshaat parabrahma
tasmai shree gurave namah"

- Guru Gita, Skanda-Purana

Salutations to that beautiful and benevolent *Guru* (teacher) who is *Brahma* (the Creator), *Vishnu* (the Maintainer), and *Shiva* (the destroyer through whom all things return to their origin), as well as the direct experience of *Brahman*, the highest Divinity.

Dedicated to my parents
Anilkumar and Anuprita Sonalker

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LIST OF ABBREVIATIONS

SHR	Spontaneously Hypertensive Rat
WKY	Wistar-Kyoto Rat
DS	Dahl-salt sensitive Rat
MHS	Milan Hypertensive Strain
MABP	Mean Arterial Blood Pressure
HR	Heart Rate
SNS	Sympathetic nervous system
RAS	Renin-angiotensin system
ACE	Angiotensin converting enzyme
GFR	Glomerular filtration rate
TGF	Tubuloglomerular feedback
ECFV	Extracellular fluid volume
TAL	Thick ascending limb of loop of Henle
DCT	Distal convoluted tubule
PCT	Proximal convoluted tubule
CD	Collecting duct
BSC-1	Bumetanide-sensitive Na-K-2Cl cotransporter-1
NKCC2	Type-2 Na-K-2Cl cotransporter or BSC-1
TSC	Thiazide-sensitive Na-Cl cotransporter
ROMK-1	Type-1 Inwardly-rectifying K Channel
AQP-1	Aquaporin-1
AQP-2	Aquaporin-2
ENaC	Epithelial Na channel
NBC-1	Type-1 Na-HCO_3 cotransporter

NHE-3	Type-3 Na-H exchanger
Na-K-ATPase	Basolateral Na-K-ATPase pump
PM	Plasma membrane
IV	Intracellular vesicle
NE	Norepinephrine
Ang II	Angiotensin II
AVP	Arginine vasopressin
Aldo	Aldosterone
COX	Cyclooxygenase
WNK	With no lysine kinase
MAPK	Mitogen Activated Protein Kinase
PKA	Protein kinase A
PKC	Protein kinase C
DDAVP	1-desamino-[8-D-arginine]vasopressin
Phe	Phentolamine
Prop	Propranolol
Staur	Staurosporine

1.0 INTRODUCTION

1.1 GENERAL INTRODUCTION

Hypertension is defined as a systolic blood pressure of 140 mmHg or greater, or a diastolic blood pressure of 90 mmHg or greater. It affects 1 billion people worldwide and causes 7.1 million deaths per year, making it the third leading killer in the world(1). Importantly, hypertension plays a major etiologic role in the development of cerebrovascular disease, ischemic heart disease, cardiac and renal failure. In addition, hypertension often coexists with other cardiovascular risk factors such as diabetes, hyperlipidemia and obesity, which compound the cardiovascular risk attributable to hypertension, resulting in high morbidity and mortality. Treatment of hypertension has been associated with about a 40% reduction in the risk of stroke and about a 15% reduction in the risk of myocardial infarction and has been shown to extend and enhance life. In recent decades it has become increasingly clear that the risks of stroke, ischemic heart disease, renal failure and other disease are not confined to a subset of the population with particularly high levels (hypertension), but rather continue among those with average and even below-average blood pressure (> 115 mmHg). Thus, hypertension and hypertension-related cardiovascular diseases continue to be a global health concern.

Although the exact cause of hypertension is unknown, it is clear from epidemiological studies that hypertension arises from a complex interplay between genetic and environmental lifestyle exposures including dietary sodium intake, excess alcohol consumption and body weight. About 95% of hypertensive patients have high blood pressure due to an unknown cause, termed essential hypertension; and only a small percentage have an identifiable cause or secondary hypertension. Thus, a better understanding of the pathophysiology of essential hypertension would ultimately aid in the development of better therapies for the treatment of essential hypertension.

1.2 ESSENTIAL HYPERTENSION AND SHR

Various rodent forms of genetic hypertension have been established for the experimental investigation of hypertension genetics and pathophysiology, including spontaneously hypertensive rats of the Okamoto-Aoki strain (SHR), Dahl salt-sensitive rats, Milan hypertensive rats, Lyon hypertensive rats, and Prague hypertensive rats. Of these, the spontaneously hypertensive rat (SHR) has been one of the most extensively used models of human essential hypertension. Several pathophysiological features of this rat strain are important for the understanding of human hypertension and for preclinical development of antihypertensive drugs. The spontaneously hypertensive rat (SHR) of the Okamoto strain was developed by selective inbreeding of Wistar rats exhibiting elevated blood pressure(2) and exhibits spontaneous hypertension with many features in common with human essential hypertension(3). These include elevated peripheral resistance, increased cardiac output, elevated sympathetic activity and cardiovascular hypertrophy(4, 5). Furthermore, as in humans, its blood pressure is readily lowered with peripheral vasodilators, calcium channel antagonists and blockers of the renin–angiotensin system. Although factors responsible for increased blood pressure in the SHR are not fully understood, studies indicate both renal and non-renal mechanisms to be involved(6-8). In the SHR, blunting of the pressure-natriuresis curve is observed, such that greater perfusion pressures are required to achieve the same level of diuresis compared to its normotensive counterpart, the Wistar-Kyoto rat (WKY)(9).The pressure-natriuresis curve is altered even in very young SHR, indicating that the resetting of kidney function occurs very early and may be necessary for the development of hypertension in the SHR(10). In addition to intrarenal mechanisms, neuroendocrine factors may contribute to the development of arterial hypertension in the SHR. Sympathetic nerve activity is elevated in this strain, and neurohumoral reactivity to environmental stress is enhanced compared with normotensive rats. Brief angiotensin-converting enzyme inhibition in juvenile SHR, as well as neonatal interruption of peripheral sympathetic innervation, chronically reduces arterial pressure associated with a reduction in peripheral vascular resistance(11, 12). These effects may be at least in part due to interference with renal development and function.

Renal transplantation studies have been extremely helpful in understanding the renal contribution to the development of hypertension in the SHR. Transplantation of an SHR kidney

in WKY rats following removal of both native kidneys, causes the recipients to develop hypertension(13). However, transplantation of a WKY kidney into SHR rats does not induce hypertension suggesting that an intrinsic defect in the SHR kidney is responsible for the development of hypertension(14). Although no differences in daily water intake, plasma urea concentration, glomerular filtration rate, renal blood flow, and weight of transplanted kidneys have been observed between the two groups, renal sodium retention in recipients of an SHR kidney is higher compared with controls transplanted with a WKY kidney(6). Data on the renin-angiotensin system do not suggest that its activation is a major contributor to the development of renal post-transplantation hypertension in recipients of an SHR kidney(15) and sympathetic re-innervation of SHR kidney grafts does not contribute to renal post-transplantation hypertension(16). Thus, data on renal transplantation studies support the hypothesis that a renal factor(s) is involved in the development/maintenance of hypertension in the SHR.

1.3 SYMPATHETIC NERVOUS SYSTEM AND ESSENTIAL HYPERTENSION

Evidence drawn from a number of sources, utilizing both electrophysiologic and neurochemical techniques, provides compelling evidence that over-activity of the sympathetic nervous system is commonly present in younger patients with essential hypertension. In borderline and established hypertension, nerve firing rates in postganglionic sympathetic fibers passing to skeletal muscle blood vessels are increased. There is also increased spillover of the sympathetic neurotransmitter norepinephrine from the heart and kidneys, providing evidence of stimulated sympathetic outflow to these organs(17-20). The increased cardiac and renal sympathetic nerve firing provide a plausible mechanism for the development of hypertension, through the regulatory influence of the sympathetic nervous system on renin release, glomerular filtration rate, and renal tubular sodium reabsorption, and on cardiac growth and pump performance. Furthermore, normotensive offspring of hypertensive patients frequently show exaggerated increases in sympathetic nerve activity and plasma norepinephrine concentration in response to mental stress as well as increased sympathetic nerve activity and total body norepinephrine spillover in resting states(21). Because the activation occurs in prehypertensive or initial stages of hypertension, it is possible

that the sympathetic nerve activation has a causative relation with the development of essential hypertension in association with genetic backgrounds.

It has been largely unresolved whether the sympathetic nervous system plays a role in the long-term regulation of arterial blood pressure and hypertension. Guyton concluded from a quantitative mathematical systems analysis that increasing the total peripheral resistance without altering the kidney's capability to excrete salt and water will not cause sustained hypertension because of counter-regulatory pressure-natriuresis(7). The kidney itself, however, can be controlled by other factors such as nervous and humoral factors. The renal sympathetic nerves can promote antinatriuresis directly through renal tubular innervation and indirectly by neurally mediated increases in renin release and renal vascular resistance(22). Increased renal sympathetic nerve activity causes a parallel shift of the pressure-natriuresis relationship to higher renal perfusion pressures in dogs. Chronic renal sympathetic nerve stimulation and norepinephrine infusion into the renal artery produced sustained hypertension in dogs and rats(22). In sinoaortic denervated rabbits, NaCl feeding produced hypertension that was prevented by prior renal denervation(23). An increase in renal vascular resistance and a decrease in renal blood flow are the most consistent abnormalities in benign essential hypertension. In many patients with essential hypertension of recent onset, the increased renal vascular resistance represents a functional abnormality that is sympathetically mediated(24). Genetic and environmental (high salt diet and stress) factors interact to disturb neural control of renal function. Normotensive offspring of hypertensive parents were reported to respond to mental stress with enhanced decreases in renal blood flow and sodium excretion(21). In borderline hypertensive subjects, dietary salt loading produced greater decreases in renal blood flow, enhanced renal vasoconstriction, and enhanced water retention during sympathetic activation; and salt-sensitive hypertensive patients exhibited blunted decreases in plasma norepinephrine concentration during salt loading(21). Taken together with other extensive evidence, there is little doubt that increased activity of the sympathetic nervous system could be the cause of essential hypertension sustained by a sympathetic effect and later permanent renal changes to elevate the set point level of the kidney-body fluid pressure-regulating mechanism(25). Another mechanism by which the sympathetic nervous system may contribute to the long-term regulation of arterial pressure is a trophic effect on vascular smooth muscle, which promotes increasing vascular resistance and response to vasoconstrictor stimuli(21).

Thus, there is increasing evidence that essential hypertension, at least in early stages, is accompanied by increased sympathetic activation. The sympatho-renal interactions in conjunction with genetic and environmental factors may play roles in the long-term control of arterial blood pressure. The causes, however, of sympathetic activation and its precise mechanisms leading to the pathogenesis of essential hypertension remain to be determined.

1.4 IMPAIRED SODIUM EXCRETION AND HYPERTENSION

The kidney is usually histologically normal in the early stages of essential hypertension. Nevertheless, a wealth of data obtained from both humans and experimental models suggests that inadequate sodium excretion is a risk factor for essential hypertension.

A variety of approaches have found that an inability to excrete sodium leads to increased blood pressure in humans and experimental animals(26). On intravenous infusion of saline, renal sodium excretion is markedly blunted in patients with essential hypertension(27). In a subset of essential hypertensive patients, the "salt retention" is associated with impaired pressure natriuresis response(28) and numerous studies also point to a causal link between a chronically high salt intake and the development of hypertension when the kidneys have a reduced ability to excrete salt(29).

Cross-transplantation of kidneys between normotensives and hypertensives have provided strong evidence that the kidney plays a key role in primary hypertension(30). Studies in humans show a normalization of blood pressure in six hypertensive patients who, following bilateral nephrectomy, received kidney transplants from normotensive cadaver donors(31). These patients, in whom high blood pressure was resistant to a four-drug antihypertensive treatment, showed a prolonged (4 yr) lowering of MABP without the need for therapeutic intervention. Conversely, it is noted that the incidence of hypertension in transplant recipients correlated strongly with the familial incidence of hypertension in the donor's family(32).

Several independent groups performed rodent cross-transplantation studies in the 1970s. Dahl's original findings(33), confirmed later in a number of studies(34-36), found that on a 0.3% salt diet, blood pressure was "determined by the genotype of the donor kidney rather than by the genotype of the recipient." Interestingly, the insertion of a control kidney into a Dahl-salt

sensitive (DS) rat did not prevent blood pressure increases evoked by a high-salt diet (8%), indicating that extrarenal factors also exert a significant influence on MABP. One possible criticism of these experiments is that they demonstrate the effect of transplanting a kidney already damaged by exposure to sustained hypertension. This issue was addressed in young Milan hypertensive (MH) rats studied before the onset of hypertension. Insertion of a normotensive control kidney into a bilaterally nephrectomized MH rat prevented development of hypertension, whereas insertion of an MH kidney into a control rat induced chronically elevated MABP(37). Likewise, cross-transplantation of kidneys from spontaneously hypertensive (SHR) rats, given life-long antihypertensive therapy by angiotensin converting enzyme (ACE) inhibition, and never therefore exposed to high perfusion pressure, conferred hypertension on the genetically normotensive recipient(6).

The studies described above suggest that *1)* blood pressure can be set by the kidney and *2)* the renal defect is genetically determined. Congenic approaches have been used to localize the genomic region responsible for setting of blood pressure by the kidney. For example, congenic SHR rats carrying a segment on chromosome 1 from the normotensive Brown-Norway rat have markedly lower blood pressures than noncongenic SHR rats(38). Elegant cross-transplantation studies between progenitor SHR rats and the congenic strain revealed that the Brown-Norway fragment of chromosome 1 lowered blood pressure. It is important to note that the hypotensive effect was observed whether the fragments were present renally or extrarenally, indicating again that other factors exert powerful influences on MABP(39).

1.5 VASCULAR-DEPENDENT HYPERTENSION

It can be difficult to envisage a central role for the kidney in the onset of hypertension since gross renal abnormalities are mostly absent in the early stages of the disease. Moreover, volume expansion and increased cardiac output would be expected if blunted natriuretic capability plays a primary role in essential hypertension, but neither of these are cardinal features. Guyton's hypothesis argues that the period during which blood pressure is volume-dependent may only be transitory since elevation of MABP would increase renal salt excretion to restore sodium balance(7). Failure to return blood pressure to normal is attributed to autoregulatory

vasoconstriction in the peripheral vascular beds, triggered locally in response to prolonged exposure to high perfusion pressure. Despite the fact that chronic hypertension, under this model, is maintained by the vasculature, impaired renal sodium excretion remains the initiating event. Data in support of this hypothesis, show that prevention of volume expansion following salt loading in DS rats prevents the development of hypertension(40).

Nevertheless, other studies in salt-sensitive hypertensive models do not find volume expansion to be a key hypertensive event(41, 42). It is known, for example, that an increase in sympathetic nervous system (SNS) activity is often observed in the early stages of hypertension(43). It has been proposed that this increase in sympathetic drive is the initiating hypertensive event(44). These data suggest that repeated intermittent bouts of sympathetic hyperactivity cause renal vasoconstriction and promote subclinical changes to the renal structure, particularly the afferent arteriole, which in turn leads to altered salt handling(45). Impaired renal sodium excretion persists as a key feature for hypertension but is no longer the initiating event. Instead, the hypertension adheres to the Guytonian paradigm only after the kidney is subjected to repeated ischemic episodes following vasoconstriction and reduced renal plasma flow(46). Moreover, this may be a vicious circle in that small increases in plasma sodium concentration can exert a central pressor effect *via* activation of both the RAS and SNS(47).

1.6 REGULATION OF ARTERIAL BLOOD PRESSURE AND THE ROLE OF THE KIDNEY

Regulation of arterial blood pressure is a complex phenomenon, with many intervening genetic and environmental factors. Blood pressure is a function of cardiac output, which is influenced by extracellular fluid volume, and the kidneys play a major role in the long-term control of this volume by matching urinary sodium and water output to dietary intake. The second parameter that determines blood pressure is peripheral vascular resistance, which is continuously regulated by the arterioles to adjust blood flow to the metabolic needs of each tissue. Blood pressure is thus expressed as:

Blood Pressure= Cardiac Output X Total Peripheral Vascular Resistance

Finally, the functions of the kidneys, heart, and blood vessels are tightly coordinated by multiple regulatory systems acting *via* endocrine and paracrine pathways.

1.6.1 Short-term control of arterial blood pressure

The control of arterial blood pressure is a complex mixture of the long- and short-term influences of hormones, local vascular factors, and neural mechanisms. Short-term control of arterial blood pressure is mediated by three different nervous pressure control mechanisms: the baroreceptor feedback mechanism, the central nervous system ischemic mechanism (responds to diminished blood flow to the brain), and the chemoreceptor mechanism (responds to lack of oxygen)(48). Thus, the first line of defense against abnormal pressures is subserved by neural mechanisms. Of these, the baroreceptor reflex is the best known mechanism for short-term control of arterial blood pressure(48, 49). Baroreceptors are stretch-sensitive receptors located in the arterial wall of the carotid sinus, the aortic arch and the large vessels of the thorax, that buffer abrupt transients of blood pressure by providing the afferent input to a medullary circuit that controls sympathetic drive to the heart and peripheral vasculature. Baroreceptor activity is related directly to the level of arterial pressure and baroreceptor activation results in vasodilatation throughout the peripheral vasculature (*via* inhibition of the medullary vasoconstrictor center) and decreased heart rate and strength of contraction (*via* excitation of the vagal center). Thus, excitation of the baroreceptors by pressure in the arteries reflexively causes the arterial blood pressure to decrease and conversely, low pressure has the opposite effects, reflexively causing the pressure to rise back to normal. Arterial baroreceptors are therefore vitally important in the short term (seconds to minutes) control of mean arterial pressure (MAP), and provide a tonic inhibitory influence on sympathetic tone, controlling peripheral vasoconstriction and cardiac output. However, arterial baroreceptors are not involved in the long-term control of mean arterial blood pressure since: *1)* baroreceptors have little effect on the absolute level of MAP chronically; *2)* baroreceptors adapt to imposed changes in pressure and therefore cannot provide an error signal to drive a change in MAP; and *3)* the gain of the baroreceptor mechanism is insufficient to account for the long-term stability of MAP(50).

In addition to the rapidly acting nervous mechanisms for control of arterial pressure, there are at least three hormonal mechanisms that also provide either rapid or moderately rapid control

of arterial pressure: *1)* the norepinephrine-epinephrine vasoconstrictor mechanism, *2)* the renin-angiotensin vasoconstrictor mechanism and *3)* the vasopressin vasoconstrictor mechanism(48).

1.6.1.1 Renin-Angiotensin-Aldosterone System (RAS)

The hormone *angiotensin II* is one of the most potent vasoconstrictors known. Whenever the arterial blood pressure falls to very low levels, large quantities of angiotensin II appear in the circulation, resulting from a special mechanism involving the kidneys and the release of the enzyme *renin* from the kidneys. Thus, when blood flow to the kidneys is decreased, the juxtaglomerular cells secrete renin into the blood, which acts on plasma proteins, called *renin substrate*, to release the decapeptide, *angiotensin I*. Renin persists in the blood for as long as 1 hour and continues to cause formation of angiotensin I during the entire time. Within a few seconds after formation of angiotensin I, two additional amino acids are split from it to form the octapeptide *angiotensin II*, which occurs almost entirely in the small vessels of the lungs, catalyzed by the enzyme *converting enzyme*. During its persistence in the blood, angiotensin II has several effects that can elevate arterial blood pressure. One of these occurs very rapidly- vasoconstriction of the arterioles and to a lesser extent of the veins, resulting in an increase in peripheral vascular resistance and thereby raising arterial blood pressure back to normal. The other effects of angiotensin II are related to body fluid volumes: *1)* angiotensin II has a direct effect on the kidneys to cause decreased excretion of salt and water; and *2)* angiotensin II stimulates the secretion of the hormone *aldosterone* by the adrenal cortex, which also acts on the kidneys to cause decreased excretion of both salt and water. Both these effects tend to elevate blood volume- an important factor in the long-term regulation of arterial blood pressure.

1.6.1.2 Sympathetic system and blood pressure regulation

It is widely recognized that the sympathetic nervous system is pivotal to the short-term regulation of blood pressure. Recently, studies have suggested that in addition to the role of the sympathetic nervous system in regulation of blood pressure in the short-term, the sympathetic system may play an important role in the long-term control of arterial blood pressure as well. The sympathetic nervous system contributes importantly to arterial pressure control under varying conditions by modifying cardiac output, peripheral vascular resistance and renal function. The system can exert powerful acute pressor actions and participates in the

pathophysiology of chronic arterial hypertension. The renal volume/pressure control system is regarded to dominate physiological long-term arterial pressure regulation because of its infinite capability to return altered arterial pressure to its original level by increasing or decreasing water and electrolyte excretion in response to elevated or reduced systemic arterial pressure(51). Activation of sympathetic nerves to the kidney increases tubular sodium reabsorption, renin release and renal vascular resistance(22). These actions contribute to long-term arterial pressure elevations by shifting the pressure-natriuresis curve to the right. Signals generated in renal sensory receptors and conducted *via* renal afferent nerves modify efferent sympathetic nerve activity with consequences for arterial pressure regulation.

1.6.1.3 Vasopressin and blood pressure regulation

When arterial blood pressure falls, the hypothalamus secretes large quantities of vasopressin by way of the posterior pituitary gland. Vasopressin in turn has a direct vasoconstrictor effect on blood vessels, thereby increasing both the total peripheral resistance and the mean circulatory filling pressure, raising the arterial pressure back to normal(48). Studies have established that vasopressin is an even more potent vascular constrictor than angiotensin, and plays a very important role to re-establish normal arterial pressure when pressure falls acutely to dangerously low levels(52).

Vasopressin also plays an indirect role in the long-term control of arterial pressure through its effect on the renal collecting duct to cause decreased excretion of urine *via* water channels (aquaporins)(53). Because of this effect, vasopressin is called *antidiuretic hormone*. Even when minute quantities of vasopressin are secreted, kidney excretion of water decreases to a minimal amount, an effect that helps to increase blood volume when arterial pressure falls too low. Thus, vasopressin plays an important role in both acute and long-term regulation of arterial pressure. However, when excess vasopressin is secreted for long periods of time, the acute effect to decrease urinary output is not sustained since other factors such as arterial pressure, colloidal osmotic pressure and concentrations of glomerular filtrate change-leading to a re-establishment of the balance between body fluid intake and output. Thus long-term secretion of excess vasopressin plays only a small role in the regulation of body fluid volume(48).

1.6.2 Long-term control of arterial blood pressure

The neural regulators of arterial pressure, though acting very rapidly and powerfully to correct acute abnormalities of arterial pressure, generally lose their power to control arterial pressure after a few hours to days. The neural mechanisms therefore do not play a major role in long-term regulation of arterial pressure. Long-term regulation of arterial blood pressure, instead, is regulated by a renal-body fluid-pressure control mechanism also called the *pressure-diuresis-natriuresis mechanism*(48). This mechanism is intimately associated with extracellular fluid volume (ECFV) homeostasis that in turn is determined by sodium content. Sodium balance, i.e., the equalizing of sodium intake by sodium output, is critical to ECFV, and the kidneys, as the principal route through which sodium is eliminated from the body, are therefore central to the long-term stability of mean arterial blood pressure (MABP). Guyton's "renal-body fluid feedback" hypothesis used a systems analysis approach to demonstrate the primary importance of the kidney. Kidney perfusion studies, exemplified in renal function curves (Fig.1), show that a rise in MABP (or renal perfusion pressure) is matched by increased renal excretion of sodium, or pressure natriuresis, which reduces ECFV and cardiac output, and returns MABP to normal (Fig.1, point A). In other words, the kidney strives to protect against perturbation from the equilibrium set point, and sodium balance is thus restored by a feedback system displaying infinite gain. Likewise, if MABP falls below the equilibrium point, the resulting antinatriuresis increases ECFV and MABP. Thus the two primary factors that determine the long-term level of arterial pressure are: *1)* the pressure range of the renal output curve, and *2)* the net rate of fluid intake.

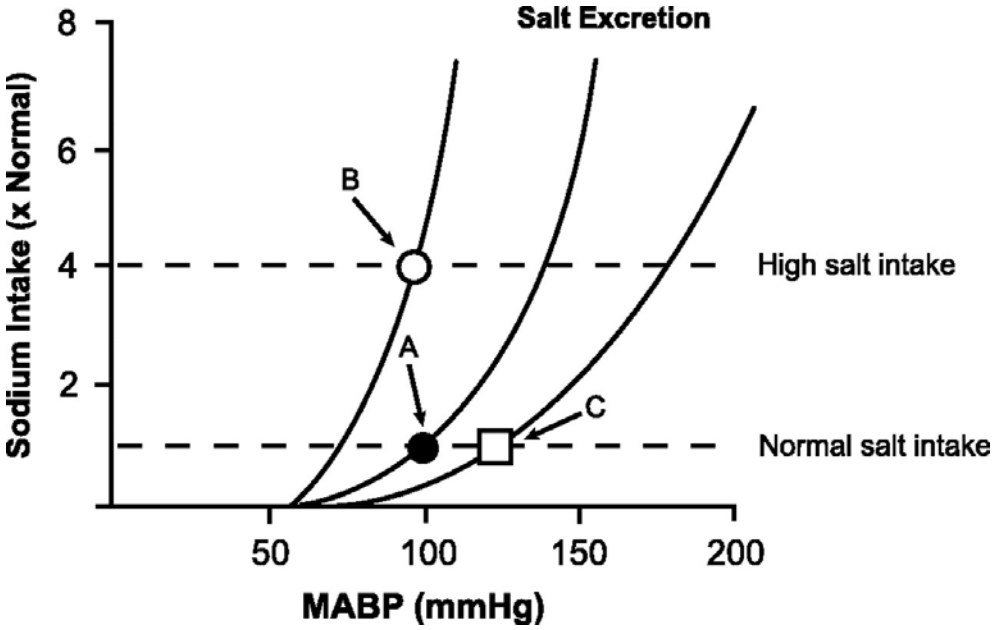

Figure 1. Pressure-Natriuresis curve

Renal function curve showing the effect of mean arterial blood pressure (MABP) on renal sodium excretion. *A*: the equilibrium pressure that is maintained through adjustment in sodium balance. *B*: on sustained increases in salt intake, function curve shifts to the left to give a higher level of excretion at any given pressure. *C*: if these adjustments fail, curve shifts to the right so that a higher equilibrium pressure is required to match sodium output to input(54)

In addition, factors that increase the effectiveness of the renal-body fluid system of pressure control include: *1)* the renin-angiotensin-aldosterone system, and *2)* the sympathetic nervous system.

Thus according to the Guyton hypothesis, hypertension results from either a failure to increase sodium output in response to an increase in intake (i.e., a failure to shift the renal function curve to the left to produce a higher level of excretion at any given pressure; Fig.1, point B) or a shift in the renal function curve to the right so that a higher equilibrium pressure is required to match sodium output to intake (Fig.1, point C). All forms of hypertension are predicted to be a consequence of abnormal pressure natriuresis responses(55); blood pressure homeostasis is sacrificed to preserve sodium balance, thus highlighting a crucial role for renal sodium ion transporters in the long-term control of arterial blood pressure.

1.7 RENAL SODIUM TRANSPORT

Sodium is freely filtered at the glomerulus, with 99% of the filtered load being reabsorbed along the nephron, by an integrated system of ion channels, ion exchangers and ion transporters (Fig. 2A). Sodium reabsorption across the nephron follows a general rule, i.e., Na^+ entry across the apical membrane is the primary determinant of the intracellular Na^+ concentration in epithelial cells. In turn, the intracellular Na^+ concentration directly controls the activity of the Na^+-K^+-ATPase responsible for Na^+ extrusion across the basolateral membrane(56). Therefore, apical Na^+ entry is limiting for transepithelial Na^+ and fluid transport, and any change in the quantity and/or activity of the proteins mediating this entry should affect the reabsorption rate. For this reason, fluid transport regulatory systems usually act primarily on these apical Na^+ transport proteins.

In the proximal convoluted tubule, 50% of filtered sodium is reabsorbed. Although there are 20 different sodium transporters in the apical membrane, most of these couple to "substrates" (such as amino acids and carbohydrates), and collectively they mediate only 10% of the proximal tubule sodium reabsorption. The sodium-hydrogen exchanger, NHE-3, mediates the majority of Na^+ reabsorption (Fig. 2B).

The loop of Henle as a whole reabsorbs considerable amounts of sodium (30–40% of the filtered load). It is a heterogeneous nephron segment, consisting of the straight portion of the proximal tubule (pars recta), the descending and ascending thin limbs, and the thick ascending limb (TAL). In the TAL, sodium is reabsorbed (20% of the filtered load) but water is not, thereby creating a steep osmotic gradient in the medullary interstitium, which permits vasopressin-dependent water reabsorption in the collecting duct. In the TAL, almost all sodium transport results directly or indirectly from Na^+-K^+-$2Cl^-$ cotransport(57). Efficient operating of this transporter (BSC-1/NKCC2) requires K^+ to recycle across the apical membrane through a K^+ channel (ROMK) and chloride to exit basolaterally through a chloride channel (CLCNKB; Fig. 2C). Potassium recycling creates an electrical potential difference, which drives the reabsorption of cations through the paracellular pathway.

Sodium reabsorption in the early distal tubule (DCT1 and DCT2) is mediated by the thiazide-sensitive NaCl cotransporter (TSC) (Fig. 2D) and also, to a lesser extent, by sodium-hydrogen exchange (NHE-2). The remaining reabsorption is achieved in the connecting tubule

and cortical collecting duct *via* ENaC, and it is this segment in which the fine-tuning of sodium reabsorption occurs, under the control of aldosterone (Fig. 2E).

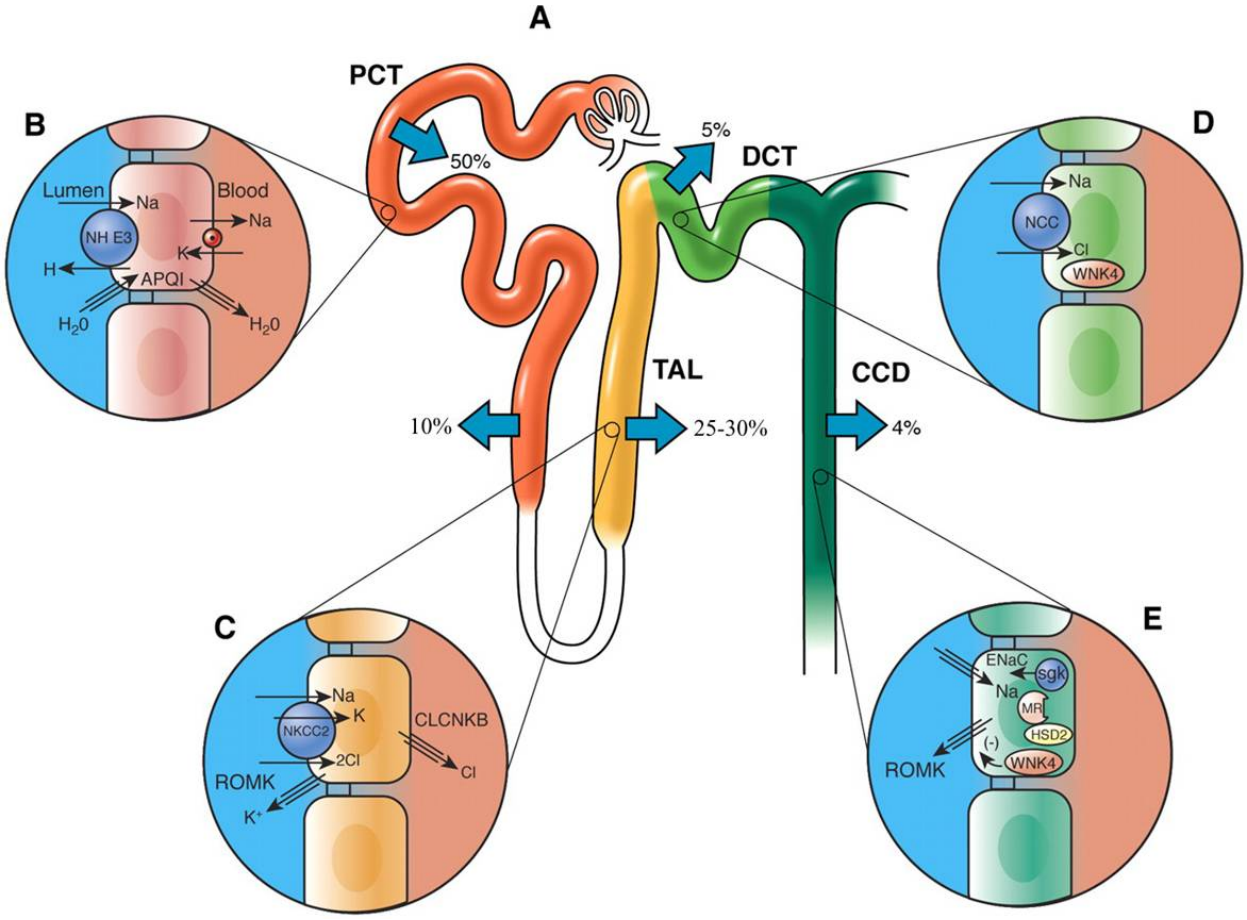

Figure 2. Renal sodium transport mechanisms

(A): percentage sodium reabsorption over the length of the nephron. Principal mechanisms of sodium reabsorption are shown in the proximal tubule (B), the thick ascending loop of Henle (C), the distal convoluted tubule (D), and the collecting duct (E). Adapted from Mullins LJ *et al.*(58)

1.7.1 Mutations in renal sodium transporters and associated disorders

Since apical Na^+ entry is limiting for transepithelial Na^+ and fluid transport, any change in the quantity and/or activity of the proteins mediating this entry affects the reabsorption rate, fluid transport regulatory systems usually act primarily on these apical Na^+ transport proteins.

NHE-3: The Na^+-H^+ exchanger, NHE3, is the major pathway for sodium reabsorption in the proximal tubule (Fig. 2B) and accounts for about 60% of sodium reabsorption. As such, knockout of the exchanger would be expected to impair tubule function and result in renal salt

loss. However, despite a significant (50–60%) reduction in proximal tubule fluid reabsorption, NHE-3 knockouts have only mild volume depletion and hypotension(59, 60).

BSC-1/NKCC2: BSC-1/NKCC2 reabsorbs approximately 30% of the filtered Na^+ load in the thick ascending limb of Henle's loop and is selectively inhibited by diuretic agents such as bumetanide and furosemide(61, 62). Mutations in the gene encoding NKCC2 or any of the genes encoding ion channels required for its operation (ROMK, CLCNKB) in human subjects have been shown to cause Bartter's syndrome, presumably by inducing a loss of function of the transporter(63). Patients with Bartter's syndrome exhibit, at an early age, severe urinary Na^+ and water-wasting associated with extracellular fluid volume depletion, hypokalemic metabolic alkalosis, and increased urinary Ca^{2+} excretion(64). The importance of BSC-1 is demonstrated by the fact that homozygous knockout mice die within two weeks of birth from severe volume depletion(65). Indomethacin (a potent nonselective COX inhibitor), administered from birth, rescues the phenotype, implicating prostaglandins in the regulation of renal salt excretion(65). Surviving adults exhibit all the features of Bartter's syndrome and develop severe hydronephrosis.

TSC: Sodium reabsorption in the distal convoluted tubule (DCT) occurs *via* the apical thiazide-sensitive NaCl cotransporter (TSC/NCC), mutations of which cause Gitleman's disease (Figure 3). Patients with Gitelman's syndrome often present at adolescence with hypokalemia, metabolic alkalosis, and mild hypotension(66). In contrast to Bartter's syndrome, hypocalciuria is observed as a consequence of an increased driving force for Ca^{2+} reabsorption in the DCT. Mice lacking TSC have no overt salt wasting phenotype unless sodium restricted(67).

Patients with Gordon's syndrome (pseudohypoaldosteronism type II, a rare autosomal dominant condition) exhibit low-renin low-aldosterone hypertension, hyperkalemia, and metabolic acidosis(68). The syndrome can be corrected with thiazide diuretics, suggesting increased TSC activity as the underlying cause. There is, however, no significant linkage between Gordon's syndrome and the TSC gene locus(68). The clinical features, in a subset of these patients, arise from independent mutations in two members of a serine-threonine kinase family, WNK4(69), and WNK1(70), which regulate sodium and potassium transport proteins in the distal nephron. The hypertension stems from both impaired retrieval of NCC from the apical membrane of the DCT cell(71) and increased paracellular chloride flux(72). The hyperkalemia arises from a gain-of-function mutation in WNK4 (independent of its kinase activity), which

increases the inhibition of K^+ secretion *via* endocytotic retrieval of ROMK(73). Thus WNK4 has a dual role in controlling renal sodium and potassium excretion(74). The association between a single-nucleotide polymorphisim near the WNK1 promoter and severity of hypertension suggests that increased WNK1 expression might also contribute to increased blood pressure(70).

ENaC: ENaC is the primary target of diuretic agents such as amiloride and its derivatives, which selectively inhibit channel activity in the micromolar range. The channel is composed of three different subunits α, β and γ, which likely form a tetrameric pore with a stoichiometry of 2α:1β:1γ(75), although other stoichiometries have been proposed(76-81). β and γ subunit mutations associated with functional defects of the channel have been identified in human subjects with pseudohypoaldosteronism type 1 and Liddle's syndrome(63). The autosomal recessive form of human pseudohypoaldosteronism type I is caused by loss-of-function mutations in any of the three ENaC subunits (α, β and γ; Fig. 4). It is characterized by salt wasting, hyperkalemia, and high mortality immediately after birth(82, 83). Unlike patients with the autosomal dominant form of pseudohypoaldosteronism type 1, patients fail to improve with age and require massive salt supplementation. Mice with knockout mutations in the Scnn1b or Scnn1g genes encoding the β or γ subunits of the sodium channel die shortly after birth from dehydration and hyperkalemia(84, 85).

Liddle's syndrome is characterized by early-onset hypertension, hypokalemic alkalosis, suppressed plasma renin activity, and low plasma aldosterone levels. The autosomal dominant syndrome is caused by mutations at the conserved PY motif in either the β or the γ subunit of ENaC, which delete or modify their cytoplasmic COOH termini, resulting in increased ENaC activity(86), and increased water and salt reabsorption in the renal collecting tubules. The number of channels in the membrane is effectively increased due to their reduced clearance from the cell surface. Normally, a ubiquitin-protein ligase, Nedd4, binds to the PY motif of ENaC subunits leading to ubiquitination and degradation(87). In cells derived from the mouse collecting duct, it has been shown that Nedd4–2 is the isoform responsible for binding to the ENaC complex and negatively regulating it(88, 89). No knockout models of Nedd4l have as yet been published, but *in-vitro* analysis has shown that siRNA against Nedd4–2 specifically increases amiloride-sensitive Na+ current, while the mutation associated with Liddle's syndrome (β_{R566X}) abolishes the effect of the siRNA(90).

Thus, in summary, loss of function of sodium transporters tends to have severe consequences; the more distally the loss occurs, despite the fact that, in absolute terms, the distal transporters account for a small proportion of overall sodium reabsorption. This may reflect the loss of compensatory capacity; there are no more lines of defense for the body to fall back on(91).

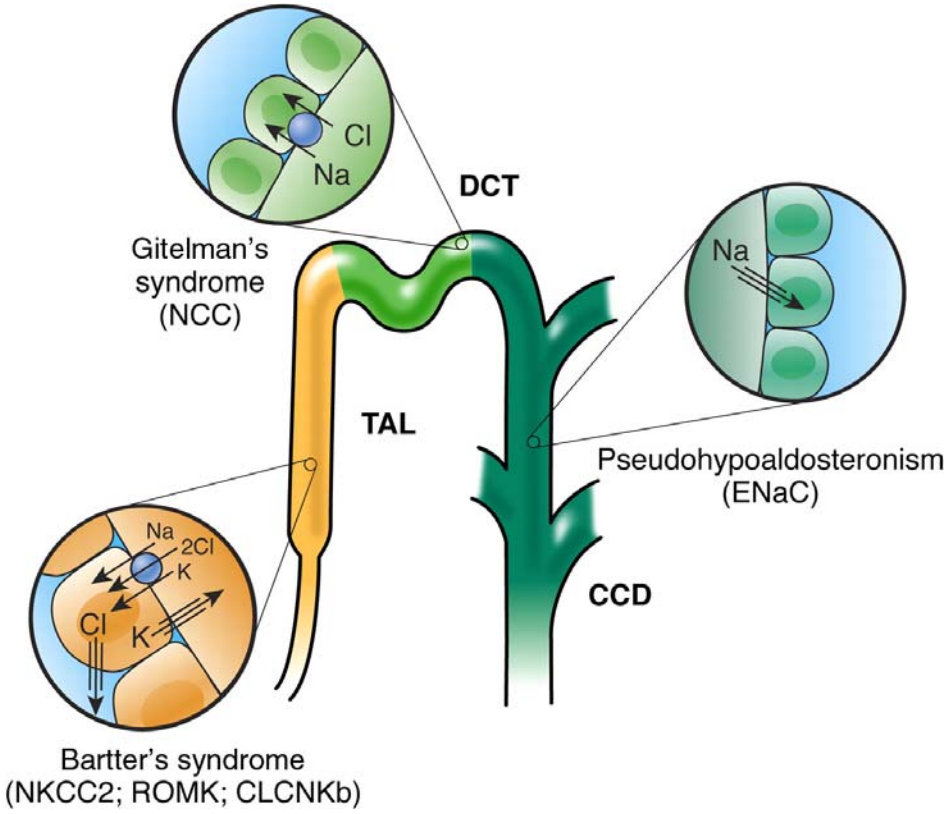

Figure 3. Disorders associated with mutations in renal sodium transport systems

Mutations in genes encoding for TSC result in Gitelman's syndrome; mutations in genes encoding for either NKCC2 or ROMK, or CLCNKb cause Bartter's syndrome and finally, mutations in the gene encoding ENaC result in pseudohypoaldosteronism. Adapted from Mullins LJ *et al*.(58)

1.8 THE RENAL BUMETANIDE-SENSITIVE NA-K-2CL COTRANSPORTER TYPE-1 (BSC-1/NKCC2)

The bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter is the major salt transport pathway in the apical membrane of the mammalian thick ascending limb of Henle's loop (TAL). The function of

this cotransporter in the TAL is critical for salt reabsorption, for the production and maintenance of the countercurrent multiplication mechanism, and is also involved in the regulation of the acid-base and divalent mineral cation metabolism(92). The disruption of the Na^+-K^+-$2Cl^-$ cotransporter gene in humans(93) and mice(65) produces Bartter's syndrome, an autosomal recessive disease characterized by metabolic alkalosis, hypokalemia, hypercalciuria, and severe volume depletion, accompanied by a reduction in arterial blood pressure. In addition, the Na^+-K^+-$2Cl^-$ cotransporter protein in the TAL is the main pharmacological target of loop diuretics, which are used extensively in the treatment of edematous states.

The primary structure of the kidney-specific, bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter (BSC-1 or NKCC2) has been elucidated by cloning cDNA from rat(94), rabbit(95), mouse(96), and human kidney(93). BSC1 belongs to the superfamily of electroneutral cation-coupled chloride cotransporters (SLC12A) for which nine genes have been identified(97). Two of these genes encode Na^+-K^+-$2Cl^-$ cotransporters: BSC-1, a kidney-specific cotransporter expressed only at the apical membrane of the TAL, and BSC-2 (also known as NKCC1), a ubiquitously expressed gene at the basolateral membrane of epithelial cells and in several nonepithelial cells. The degree of identity between these proteins is ~60%, and ~50% between these genes and the thiazide-sensitive Na^+-Cl^- cotransporter (TSC), the other Na^+-coupled to chloride transporter of the SLC12 family. The basic topology of BSC-1, shown in figure 4, has been deduced from hydropathy analysis of the 3,825 bp coding segment of BSC-1 cDNA and predicts a NH_2-terminal hydrophilic region of 174 amino acids, followed by a central hydrophobic domain with 12 putative membrane-spanning helices, and a long hydrophilic COOH terminus of 454 amino acids.

Additionally BSC-1 or NKCC2 has four splice variants: NKCC2F, A and B formed as a result of alternative splicing of three cassettes of exon 4 giving rise to a variable 96 base pair sequence that encodes the second transmembrane domain and 11 amino acids of the following connecting segment, and NKCC2AF, which possesses both A and F exons in tandem(95, 96, 98, 99). Each of the variants is differentially distributed along the thick ascending limb (TAL), with some degree of overlap, and has different kinetic properties(95, 96, 100-102). F displays the lowest affinity for ions under controlled conditions and B the highest (Kms: F>A>B), whereas A displays the highest transport capacity and B the lowest (Vmax: for A>F>B). Regarding AF, intriguingly, influx studies in the *Xenopus laevis* oocyte expression system have shown that this

variant is nonfunctional even if it is able to reach the cell surface(99). B is expressed predominantly in macula densa cells, F in the inner stripe of outer medulla and A in the cortex and outer stripe of outer medulla. It is believed that the differential localization and kinetic properties of the variants contribute to the fine tuning mechanism for the control of sodium transport in the TAL.

BSC-1 and BSC-2 form dimers although each monomer is thought to be fully functional. The predicted core molecular weight of NKCC2 monomer is ~120kDa, although western blot analysis of proteins extracted from rat kidneys show an apparent molecular weight of ~160kDa(103, 104). The rBSCl protein sequence contains six potential N-linked glycosylation sites(94); two are located in the hydrophilic loop between membrane-spanning segments M7 and M8 (Asn-442 and Asn-452); of the four other potential N-linked glycosylation sites, two are positioned within potential membrane-spanning segments (Asn-396 and Asn-79) and two (Asn-864 and Asn-875) are located in the putative cytosolic COOH terminus. Recently, studies have confirmed that BSC-1 is a glycosylated protein and that prevention of glycosylation reduces its functional expression by affecting both the insertion into plasma membrane and the intrinsic activity of the transporter(105). Finally, BSC-l protein contains seven potential protein kinase C phosphorylation sites(94): two in the NH_2, terminus (Ser-57 and Thr-75) and five in the COOH terminus (Thr-639, Thr-927, Ser-983, Ser-999, and Ser-1029). In addition, three potential cAMP-dependent protein kinase phosphorylation sites are present in the putative cytosolic COOH terminus of rBSC1: Ser-874, Ser-1013, and Ser-1060(94). In addition, the phosphoregulatory domain in the NH_2-terminal domain of BSC-2 containing three threonines (T-184, T-189, T-202), is highly conserved in both rat (T-101 corresponding to T-189 of BSC-2)(94) and rabbit BSC-1 (T-99, T-104, T-107 corresponding to T-184, T-189, T-202 in BSC-2)(106). Recently, studies confirmed that BSC-1 is phosphorylated *in vivo* and that phosphorylation increases both trafficking of BSC-1 to the plasma membrane and its activity(106, 107).

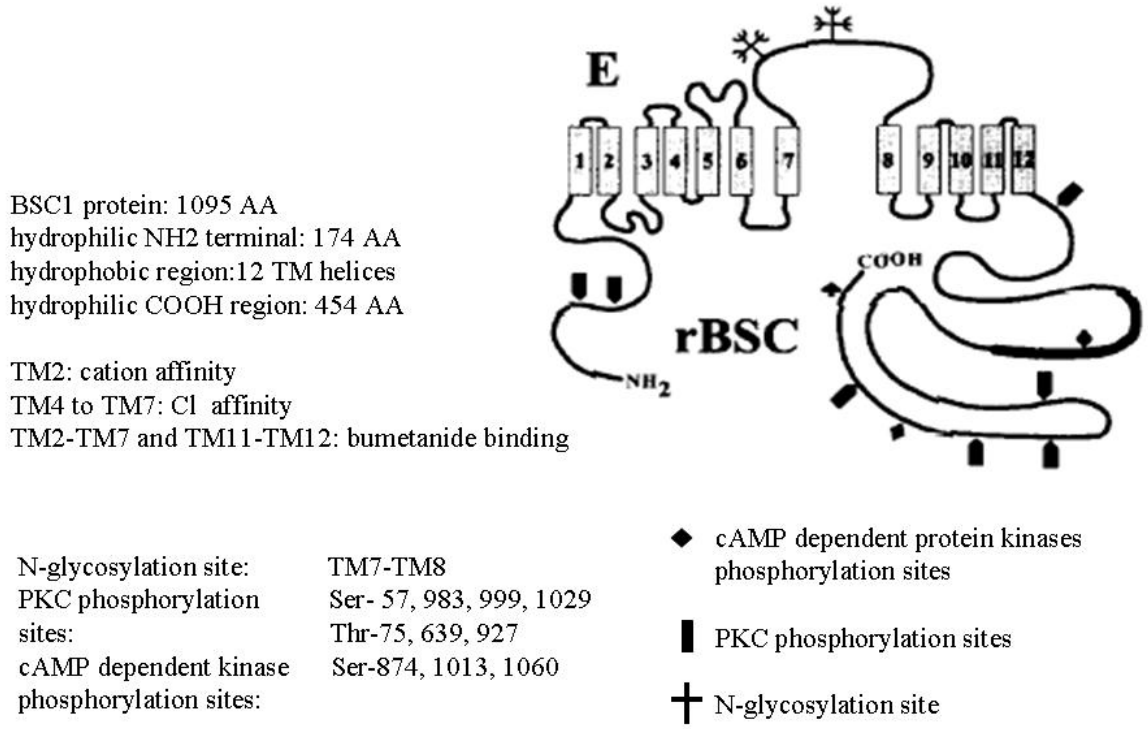

Figure 4. Structure of BSC-1 protein

1.8.1 Regulation of BSC-1

The thick ascending limb plays a central role in the regulation of water excretion by concentrating the renal medulla (the countercurrent multiplier mechanism) and by diluting the tubule fluid delivered to the distal convoluted tubule. Thus, regulation of BSC-1 is implicated in the maintenance of water balance. Increasing net NaCl reabsorption in TAL by hormones generating cAMP *via* their respective Gs-coupled receptors such as vasopressin, glucagon, parathyroid hormone, β-adrenergic, and calcitonin is a fundamental mechanism for regulating salt transport in this nephron segment. Of these hormones, the most widely studied is the antidiuretic hormone vasopressin(108, 109). As demonstrated in isolated perfused tubule studies mediated by cAMP, vasopressin increases NaCl absorption by TAL(108, 110, 111) following a mechanism that appears to involve trafficking of Na^+-K^+-$2Cl^-$ cotransporter BSC-1, from an

intracellular vesicular pool to apical plasma membrane(107, 112, 113). In a recent study using a polyclonal antibody that recognizes BSC-1 when phosphorylated at threonine residues located in the amino-terminal domain, Gimenez and Forbush observed that vasopressin's effect in mouse TAL may be dependent in part on phosphorylation of BSC-1 and that vasopressin action in TAL induces phosphorylation of Na^+-K^+-$2Cl^-$ cotransporter protein that is associated with migration of cotransporter-containing vesicles to apical membrane(106, 107). Other hormones that generate cAMP *via* their respective Gs-coupled receptors stimulate concomitant increases in NaCl absorption rate, such as parathyroid hormone, calcitonin, and glucagon, presumably using similar mechanisms to those demonstrated for vasopressin(114-116). Prostaglandin E2 has been demonstrated to have a short-term inhibitory effect on NaCl absorption in TAL(117), presumably *via* its ability to inhibit cAMP production in TAL cells(118). Another mediator that regulates TAL NaCl transport *via* effects in BSC-1 is nitric oxide, which directly inhibits NaCl absorption in isolated perfused preparations(119).

In addition to the short-term effect of vasopressin on BSC-1 trafficking or activity, long-term increases in vasopressin levels have been demonstrated to upregulate BSC-1 protein expression in TAL cells(120). This action results in long-term potentiation of NaCl transport in TAL, as demonstrated by Besseghir *et al.* in isolated perfused tubule studies, in which the investigators observed that chronic *in-vivo* administration of antidiuretic hormone to Brattleboro rats significantly increased basal voltage and chloride transport in TAL(121). In addition, long-term change in prostaglandin E2 levels appears to modulate BSC-1 expression levels in TAL because the cyclooxygenase inhibitors indomethacin or diclofenac increased BSC-1 abundance, an effect that was reversed by misoprostol, a prostaglandin E2 analog(122). Supporting this observation, Escalante *et al.* previously showed in isolated rabbit mTAL cells that arachidonic acid metabolites produced a concentration-dependent inhibition of Na^+-K^+-$2Cl^-$ cotransporter activity, an effect that was prevented by selective inhibition of cytochrome *P*-450 monooxygenases(123). In addition to actions of hormones that generate cAMP in TAL, regulatory mediators using other signal mechanisms also modulate BSC-1 expression in TAL. Glucocorticoids increase BSC-1 mRNA and protein expression by a mechanism that requires vasopressin, while aldosterone has no effect on BSC-1 expression levels(124). By stimulating cGMP production, nitric oxide increases BSC-1 expression, as observed by Turban *et al.* as a marked decrease in this cotransporter abundance in response to inhibition of nitric oxide

synthases by *N*G-nitro-L-arginine methyl ester (L-NAME)(125). In addition, although angiotensin II infusion was found to increase BSC-1 abundance in TAL(126), absence of AT_{1a} receptors in mice(127) or blockade of angiotensin II AT_1 receptors by candesartan(128) did not produce opposite effects, suggesting that the angiotensin II effect on BSC-1 expression is indirect and related to local changes in nitric oxide or PGE2 levels. Finally, expression of BSC-1 is also regulated by acid-base status. Chronic metabolic acidosis has been shown to enhance expression of BSC-1 mRNA and protein in medullary TAL(129) by glucocorticoid-dependent and -independent mechanisms(124). In this regard, it has been recently reported that metabolic acidosis increases the stability of BSC-1 mRNA, without affecting *SLC12A1* transcription rate(130). Under physiological conditions, most of the ammonium produced in the proximal tubule is reabsorbed in TAL to be later secreted in medullary collecting ducts and excreted into urine(131, 132). Thus, during acidosis, in which production of ammonium by proximal tubule is increased, enhancing of BSC-1 expression arises as a compensatory mechanism to increase ammonium reabsorption. Finally chronic hypercalcemia has been shown to cause a reduction in BSC-1 abundance, likely to play a major role in the urinary concentration defects associated with hypercalcemia(133).

1.8.2 BSC-1 and disease

The physiological importance of BSC-1 in regulation of salt (and water) transport and counter current multiplication has been well established with the use of loop diuretics bumetanide and furosemide that act as functional blockers of BSC-1. Because BSC-1 is the principal apical Na^+ entry pathway in the thick ascending limb of Henle, it is a prime candidate for long-term dysregulation of arterial blood pressure. The absence of functional BSC-1 protein resulting from mutations or deletion of the NKCC2 gene is associated with a serious salt-wasting disorder and low blood pressure(134). Decreased abundance of BSC-1 has also been observed in a rodent model of ischemia-induced acute renal failure, characterized by impaired renal tubular sodium reabsorption(135). On the other hand, excessive BSC-1 activity has been linked with inherited hypertension in humans and rodent models. Recent studies demonstrate that enhanced expression of BSC-1 in the thick ascending limb causes sodium retention in rats with congestive heart failure (CHF)(136). Moreover, BSC-1 is up-regulated in rats with small-to-moderate myocardial

infarctions(137), dehydration, cardiac failure(138) and liver cirrhosis(139), suggesting that high BSC-1 expression underlies edema formation. An increased abundance of BSC-1, however, has also been noted in rats with the syndrome of inappropriate secretion of antidiuretic hormone, hyponatremia without edema. Recently, studies in an animal model of brain infarction, showed increased NKCC2 abundance and enhanced body fluid accumulation, likely *via* the sodium loading-dependent concentration of the urine(140). Aquaporin-2 levels in the animal model of brain infarction remained unaltered, suggesting that the physiological process of edema formation is based on specific BSC-1 expression. Increased levels of BSC-1 mRNA and protein have also been reported in an experimental model of prenatal programming of hypertension in the rat, induced by a maternal low-protein diet during pregnancy(141). Increased expression and activity of BSC-1 have also been reported in isolated thick ascending limb tubules of Dahl-salt sensitive rats, thus explaining, at least in part, their genetic renal inability to excrete sodium(142). Increased BSC-1 activity could be accounted for by changes in the regulatory mechanisms, by changes in protein abundance, or by a combination of both. The molecular mechanisms controlling BSC-1 gene transcription and regulation in response to chronic challenges, however, are not known; nor has it been explored how they interact with the regulatory mechanisms.

1.9 THE DISSERTATION

The present study is based on the concept that the pathogenesis of hypertension involves the kidney. In this regard, transporters in the apical (and basolateral) membranes of epithelial cells in nephrons are responsible for sodium reabsorption and fluid balance, and therefore may be important candidates for involvement in the development of hypertension. Additionally, many lines of evidence indicate that the sympathetic nervous system, *via* the renal nerves, plays an important role in the pathogenesis of essential hypertension. The goals of the dissertation were to: *1)* identify whether renal sodium ion transporter expression is altered in essential hypertension and if so, its physiological significance in an animal model of essential hypertension, the Spontaneously Hypertensive Rat (SHR); *2)* determine the role of the

sympathetic nervous system (and norepinephrine) in regulation of renal transport systems; and *3)* elucidate the underlying molecular mechanism.

Our results show that the abundance of sodium transporters BSC-1, Na-K-ATPase-α1, NHE-3, NBC-1; the potassium channel ROMK-1 and the water channel AQP-2 are elevated in the SHR, suggesting that the pathophysiology of altered renal excretory function in essential hypertension may involve alterations in several transporters located along the nephron. Further, these alterations in protein levels are not accompanied by changes in mRNA, suggesting that a post-transcriptional mechanism(s) is responsible for the over-expression of these transporters.

Among the renal sodium transporters profiled in the SHR, BSC-1 was found to be elevated more than the other transporters, suggesting that the increase in BSC-1 abundance may be the most important contributing factor to altered tubular function in SHR. In support of this conclusion, our results also demonstrate that the natriuretic response to furosemide is greater in SHR versus WKY, and normalizes blood pressure in the SHR. Thus, both our biochemical and pharmacological data are consistent with an important role of BSC-1 in the pathophysiology of hypertension in SHR. Additionally, the progression from pre-hypertensive to hypertensive state in the SHR is accompanied by a proportional increase in both steady-state protein levels of BSC-1 as well as its distribution to the plasma membrane, indicating that BSC-1 expression and distribution are stage dependent and increase as hypertension progresses. The increased presentation of BSC-1 at the plasma membrane could result in increased sodium reabsorption and thereby contribute to the pathogenesis of hypertension in the SHR, and drugs that target/alter BSC-1 expression or alternatively block BSC-1 function may be useful for the treatment of essential hypertension.

The renal sympathetic nervous system promotes sodium and water retention by directly enhancing renal epithelial cell transport; however the mechanisms are unclear. We hypothesized that long-term exposure of the kidney to norepinephrine upregulates the expression of key renal epithelial transport systems. To test this hypothesis, we used immunoblotting of renal cortical and medullary tissue to investigate the abundance of major transport systems expressed along the renal tubule in response to chronic infusions of norepinephrine. Our results indicate that norepinephrine infusion significantly increased protein abundance of BSC-1, along with modest increases in NHE-3, NBC-1 and aquaporin-2. We conclude that norepinephrine-induced increases in the expression of NHE-3, NBC-1, BSC-1 and aquaporin-2 are likely to play an

important role in the regulation of salt and water transport by norepinephrine in the kidney and may partially explain the altered renal sodium and water handling associated with over-activation of the sympathetic system.

Finally, to understand the underlying mechanism of regulation of renal transporters by sympathetic activation, we focused our attention on the regulation of BSC-1 by norepinephrine since we hypothesize that BSC-1 may be one of the critical players in the progression/maintenance of increased sodium handling and hypertension in the SHR along with an overactive sympathetic system. Our studies with an immortalized thick ascending limb cell line show that the effect of norepinephrine on BSC-1 involves post-transcriptional control mechanisms *via* the β-adrenoceptor-cAMP-PKA pathway that involves at least in part the MAP kinases. Finally, we found that the α-adrenoceptor negatively regulates BSC-1. Further elucidation of the mechanisms involved could eventually lead to the identification of new targets and the development of therapies that could help better treat diseases associated with over-activation of the sympathetic nervous system such as essential hypertension. These studies are a necessary first step in this direction.

2.0 INCREASED EXPRESSION OF THE SODIUM TRANSPORTER BSC-1 IN SPONTANEOUSLY HYPERTENSIVE RATS

2.1 INTRODUCTION

The kidneys play an important role in regulating blood pressure by controlling sodium balance(7, 54). Although many systems can influence blood pressure in the short term, the long-term blood pressure setting ultimately depends on renal sodium excretion. In this regard, transporters in the apical and basolateral membranes of epithelial cells in nephrons are responsible for sodium reabsorption and fluid balance and are therefore important candidates for involvement in the development of hypertension(143).

The results of renal transplantation experiments in genetically hypertensive and normotensive rat strains are consistent with the concept that the kidneys regulate long-term levels of arterial blood pressure and that a defect in the kidney is importantly involved in the pathogenesis of genetic hypertension(6, 144, 145). Moreover, studies using isolated perfused kidneys from spontaneously hypertensive rats (SHR) reveal an intrinsic renal abnormality in Na^+ excretion that may contribute to the maintenance of hypertension in SHR(146). Furthermore, examination of the pressure-natriuresis relationship and the effect of furosemide (an inhibitor of the bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter, also called BSC-1 or NKCC2) on this relationship demonstrates a resetting of the pressure-natriuresis process in SHR by a mechanism involving in part BSC-1(147).

BSC-1 in the thick ascending limb of Henle's loop mediates reabsorption of approximately 25% of the filtered Na^+ load and is selectively inhibited by loop diuretics(61, 62). Because BSC-1 is the principal apical Na^+ entry pathway in the thick ascending limb of Henle, it is a prime candidate for long-term dysregulation of arterial blood pressure. In support of this concept, recent studies demonstrate that enhanced expression of BSC-1 in the thick ascending

limb causes sodium retention in rats with congestive heart failure (CHF)(136). Moreover, BSC-1 is up-regulated in rats with small-to-moderate myocardial infarctions(137), dehydration and cardiac failure(138). Finally, mutations in the *NKCC2* gene, which encodes BSC-1, presumably by inducing a loss of function of the transporter, result in Bartter's syndrome, an inherited disease characterized by hypokalemic metabolic alkalosis, hypercalciuria, salt wasting, and volume depletion resulting in hypotension(93, 148). Clearly alterations in BSC-1 activity can influence long-term levels of arterial blood pressure.

Because BSC-1 influences arterial blood pressure, it is conceivable that increases in BSC-1 activity and/or expression contribute to genetic hypertension. The expression of BSC-1 in SHR has not been previously examined, but it can be hypothesized that changes in BSC-1 expression may play a critical role in the development of altered sodium handling in the SHR thereby contributing to the pathogenesis of genetic hypertension. Accordingly, in the present study, we determined the expression of BSC-1 protein and mRNA in the outer cortex, inner strip of outer medulla and inner medulla of kidneys obtained from both SHR and Wistar-Kyoto (WKY) normotensive rats. To determine the specificity of any observed changes in BSC-1 expression, we also compared protein expression of the thiazide sensitive Na^+-Cl^- cotransporter (TSC), the type-3 Na^+-H^+ exchanger (NHE-3), Na^+-K^+-ATPase-α_1, the inwardly rectifying K^+ channel (ROMK-1), the type-1 Na^+-HCO_3^--cotransporter (NBC-1), aquaporin-1 and aquaporin-2. Finally, because we observed a marked increase in the expression of BSC-1 protein in the inner strip of the outer medulla of SHR, we also compared the acute effects of the loop diuretic furosemide on hemodynamics and renal function in SHR versus WKY.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Male WKY rats (11-13 weeks of age) and age-matched SHR were obtained from Taconic Farms (Germantown, NY). Rats were allowed to acclimate to the University of Pittsburgh Animal Facility for at least 1 week before initiation of the experimental protocols. Protocols were approved by the Institutional Animal Care and Use Committee. Animals were divided into two

groups: one to be used for the immunoblotting and RT-PCR experiments and the other group for the furosemide infusion study.

2.2.2 Kidney dissection and tissue preparation for immunoblotting

Blood pressures in WKY and SHR rats were measured as described below; following which kidneys were rapidly excised and washed in ice-cold PBS. The left kidneys were dissected to obtain outer cortex, inner stripe of outer medulla and inner medulla and the dissected tissues were homogenized in lysis buffer containing (Tris HCl, 2% SDS, glycerol, PMSF and protease inhibitors). Protein concentrations were measured using the BCA protein assay. Whole homogenates from the cortex, outer medulla and inner medulla were used to study the specific regional expression of the different proteins.

2.2.3 Electrophoresis and immunoblotting

Proteins were solubilized at 60°C for 15 min in Laemmli sample buffer. SDS-PAGE was performed on gradient polyacrylamide gels (4-12%) loaded with 20μg protein per lane. For immunoblotting, proteins were transferred electrophoretically to PVDF membranes. Membranes were blocked in 5% milk for 2 hours, probed overnight at 4°C with the respective primary antibodies in PBS containing 1% milk: BSC-1 (1:2000), thiazide sensitive Na^+-Cl^- cotransporter (TSC; 1:600), aquaporin-1 (AQP-1; 1:2000) and aquaporin-2 (AQP-2; 1:2000), type-1 Na^+-$HCO3^-$-cotransporter (NBC-1; 1:1000), type-3 Na^+-H^+ exchanger (NHE-3; 1:1000), Na^+-K^+-ATPase-α_1 (1:5000) and inwardly rectifying K^+ channel (ROMK-1; 1:1000). Membranes were probed with β-actin (1:10,000 Sigma Chemical Co., St. Louis, MO) for 1 hour to determine loading efficiency. BSC-1, TSC, AQP-1 and AQP-2 were the kind gift of Dr. M.A Knepper (NIH). All other primary antibodies were from Chemicon (Temecula, CA). All antibodies were found to be highly specific for the protein of interest and their specificity has been extensively characterized. Subsequently, membranes were exposed to a secondary HRP conjugated donkey anti-rabbit polyclonal antibody (1:5000, Pierce Biotechnology Inc., Rockford, IL) in PBS containing 1% milk for 1 hour at room temperature. Bound antibodies were visualized using a luminol-based enhanced chemiluminescence substrate (SupersignalWest Dura Extended

Duration Substrate, Pierce Biotechnology Inc., Rockford, IL) before exposure to X-ray film (Kodak 165-1579; Eastman Kodak Co., Rochester, NY). Densitometric analysis was performed using ImageQuant TL (Amersham Biosciences, Piscataway, NJ) and band densities were normalized to β-actin.

2.2.4 RNA isolation and RT-PCR

The right kidneys were used for RNA isolation. Kidneys were dissected to obtain outer cortex, inner stripe of outer medulla and inner medulla. RNA was isolated from the dissected tissues using TRIzol reagent (GIBCO Life Technologies, Carlsbad, CA) as per the manufacturer's instructions. By using the primer sequences listed in table 1, RNA (0.5 μg) was reverse transcribed and amplified using Titanium One-step RT-PCR kit (Clontech, Palo Alto, CA). Each PCR cycle (total 30 cycles) consisted of denaturing at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 68°C for 60 seconds. RT-PCR products were separated on a 1.2% agarose gel and visualized by incorporating ethidium bromide in the gel. Densitometric analysis was performed using ImageQuant TL and band densities were normalized to β-actin.

Table 1. Primers used for RT-PCR analysis of BSC-1, ROMK-1 and AQP-2

	Accession number	Primer Sequence (5′-3′)	Nucleotides	Product size
BSC-1	U10096	Forward:GCATTGTCTTAACAGGAGGACC	2254	464
		Reverse:GAACTGGAGAGATGTCAAACCC	2676	
ROMK-1	AF081365	Forward:AGCTCTATAAGGCTGCATACGG	1305	421
		Reverse:ACCTTGGGTTCAGAGAGGTACA	1725	
AQP-2	NM01209	Forward:AAGAGAAAGAGAGAGGGAGGGA	46	753
		Reverse:GGGGAACAGCAGGTAGTTGTAG	798	

2.2.5 Effects of furosemide in SHR and WKY rats

Acute renal and hemodynamic responses to the loop diuretic furosemide (Sigma Chemical Co., St. Louis, MO) at bolus doses of 3 and 50 mg/kg were measured in SHR and WKY. Each rat was anesthetized with pentobarbital (45 mg/kg; Research Biochemicals, Natick, MA) and placed on an isothermal pad. Temperature was monitored with a rectal probe thermometer and kept at 37 degrees C with a heat lamp. A short section of polyethylene tubing (PE-240) was placed in the trachea to facilitate respiration. The left carotid artery was exposed and cannulated with PE-50 tubing for blood sample collections and for mean arterial BP (MABP) and heart rate (HR) measurements *via* a digital BP analyzer (Micro-Med, Inc., Louisville, KY). A PE-50 catheter was placed in the left jugular vein for infusion of [^{14}C] inulin (0.5 μCi bolus and 0.035 μCi/100 μl of 0.9% saline/min; NEN, Boston, MA). A PE-20 catheter also was inserted into the jugular vein to administer bolus doses of furosemide. An incision was made in the rat's abdomen, and a PE-10 catheter was placed in the left and right ureters to facilitate collection of urine. A flow probe (model 1RB; Transonic Systems, Inc., Ithaca, NY) was placed on the left renal artery for determination of renal blood flow (RBF).

Infusions of saline and [^{14}C] inulin were initiated, and following a 2-hr stabilization period, a urine sample and mid-point blood sample were collected during a 30-min baseline clearance period. MABP, HR, and RBF were recorded at 5-min intervals, and averaged. A bolus dose of furosemide (3 mg/kg) was administered, a 10-min stabilization period was allowed, following which, MABP, HR, and RBF were recorded, and a urine sample and mid-point blood sample were collected during an additional 30-min clearance period. Another bolus dose of furosemide (50 mg/kg) was administered, and the above procedure repeated.

Rats were euthanized and the left kidneys were weighed. Urine volume (UV) was determined gravimetrically for each of the collection periods, and samples were analyzed for [^{14}C] inulin radioactivity (model 2500TR liquid scintillation analyzer; Packard Instrument Company, Downers Grove, IL) and sodium/potassium concentrations (Model IL943 flame photometer; Instrumentation Laboratory, Lexington, MA). Renal clearance of [^{14}C] inulin was used as an estimate of glomerular filtration rate (GFR). The RBF, GFR, UV, and excretion rates of sodium ($U_{Na}V$), and potassium (U_KV), were corrected to kidney weight measured in grams (g kid).

2.2.6 Statistical analysis

All data are presented as mean ± SEM. Comparisons between groups were made by unpaired *t*-test. Group comparisons for the furosemide study were performed using analysis of variance and Fisher's least-significant difference (LSD) test. *P* values <0.05 were considered significant.

2.3 RESULTS

2.3.1 Expression of BSC-1 and TSC proteins

BSC-1 and TSC are sodium transporters that are expressed predominantly in the apical membranes of the thick ascending limbs and distal tubules, respectively. Semi-quantitative immunoblotting showed that the expression of BSC-1 protein was found to be significantly higher in the outer medulla of the SHR compared to WKY (6-fold, $P< 0.05$; Figure 5). We could not detect expression of BSC-1 in the outer cortex and inner medulla of either group. In contrast to BSC-1, TSC protein was expressed primarily in the outer cortex, and the expression in SHR versus WKY was similar (Figure 5).

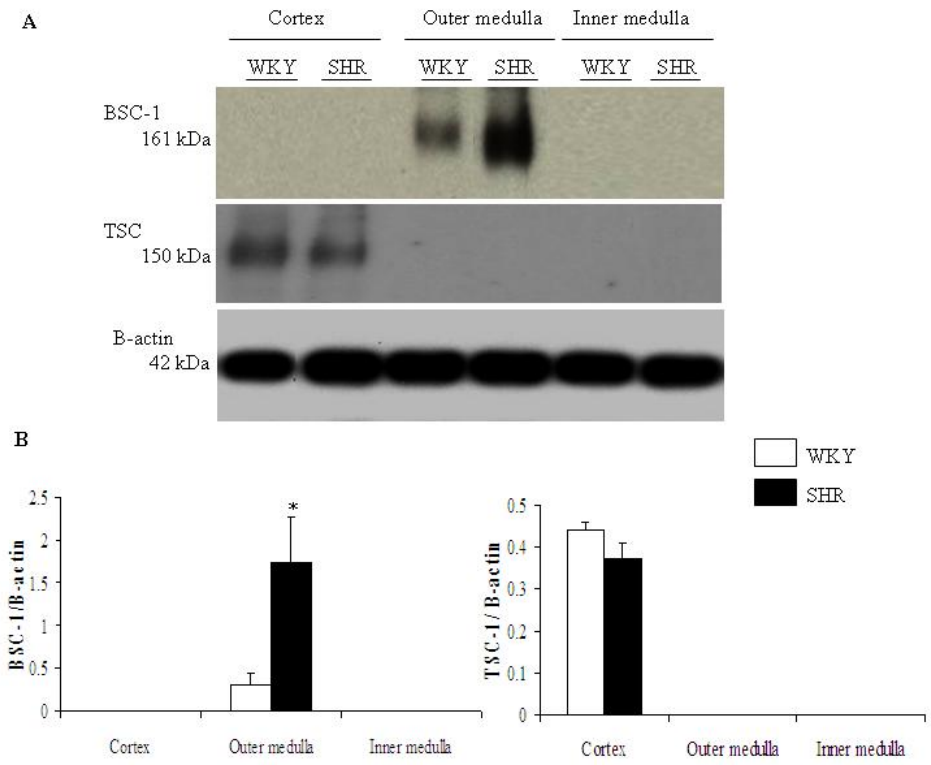

Figure 5. Expression of BSC-1 and TSC in the kidney outer cortex, inner strip of outer medulla and inner medulla of WKY and SHR rats

In Panel A, each lane was loaded with 20 μg of protein from a different rat. Blots were probed with rabbit anti-BSC-1, rabbit-anti-TSC and mouse-anti-β-actin antibodies. Panel B summaries densitometric analysis of BSC-1 and TSC normalized to β-actin. Densitometric analysis revealed that expression of BSC-1 was significantly higher in the outer medulla of SHR, while expression of TSC in the cortex was similar in SHR and WKY. *Indicates $P<$ 0.05 compared with WKY. Values represent means ± SEM for 7 observations.

2.3.2 Expression of NHE-3, Na^+-K^+-ATPase-α_1 and NBC-1 proteins

Previous studies have shown that expression of NHE-3 in the proximal tubule and Na^+-K^+-ATPase in the collecting duct are upregulated in the SHR kidney(149, 150). Semi-quantitative immunoblotting revealed expression of NHE-3 protein in the outer cortex, outer medulla and inner medulla with significantly higher expression in the cortex and inner medulla of SHR kidneys (2-fold, $P<$ 0.05; Figure 6). Na^+-K^+-ATPase-α_1 protein expression was detected in the

outer cortex and outer and inner medulla, and Na^+-K^+-ATPase-α_1 protein expression was significantly, albeit modestly (approximately 25%), higher in the outer and inner medulla of the SHR compared to WKY ($P< 0.05$; Figure 2). We also detected expression of NBC-1 protein, which is localized to basolateral membranes of proximal tubules in the outer cortex, but not outer or inner medulla, and found the expression of NBC-1 protein to be higher in the outer cortex of the SHR compared to WKY (2-fold, $P< 0.05$; Figure 7).

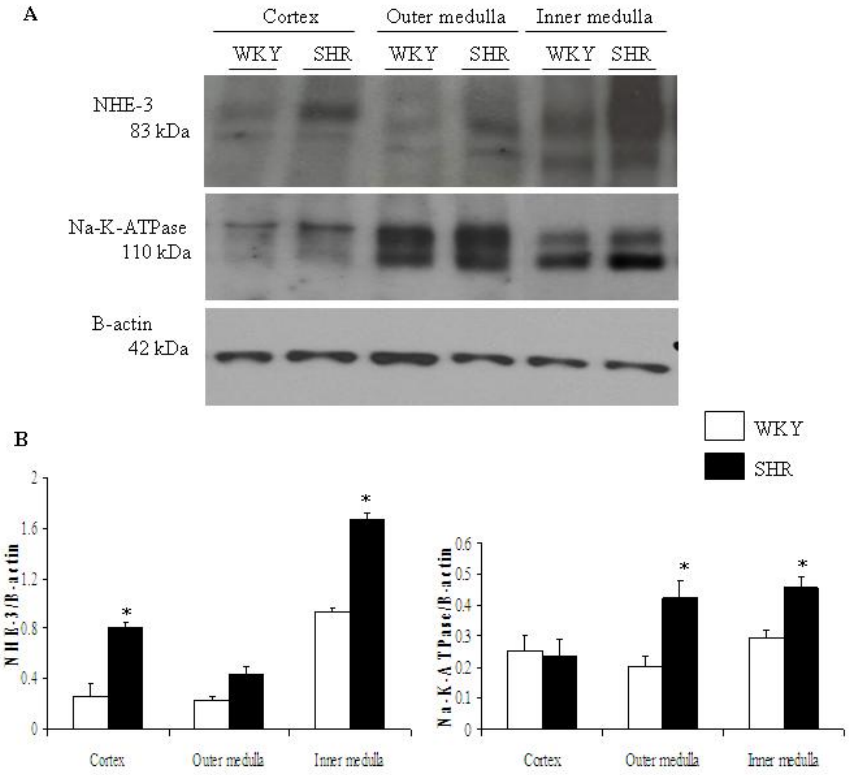

Figure 6. NHE-3 and Na^+-K^+-ATPase (α_1-subunit) expression in the kidney outer cortex, inner strip of outer medulla and inner medulla of WKY and SHR rats

In Panel A, each lane was loaded with 20 μg of protein from a different rat. Blots were probed with rabbit-anti-NHE-3, rabbit-anti-Na^+-K^+-ATPase-α_1 subunit and mouse-anti-β-actin antibodies. Panel B summaries densitometric analysis of NHE-3 and Na^+-K^+-ATPase normalized to β-actin. Densitometric analysis revealed that expression of NHE-3 was higher in the cortex, outer medulla and inner medulla in SHR, and expression of α_1-Na^+-K^+-ATPase was slightly higher in the outer and inner medulla in SHR. *Indicates $P< 0.05$ compared with WKY. Values represent means ± SEM for 7 observations.

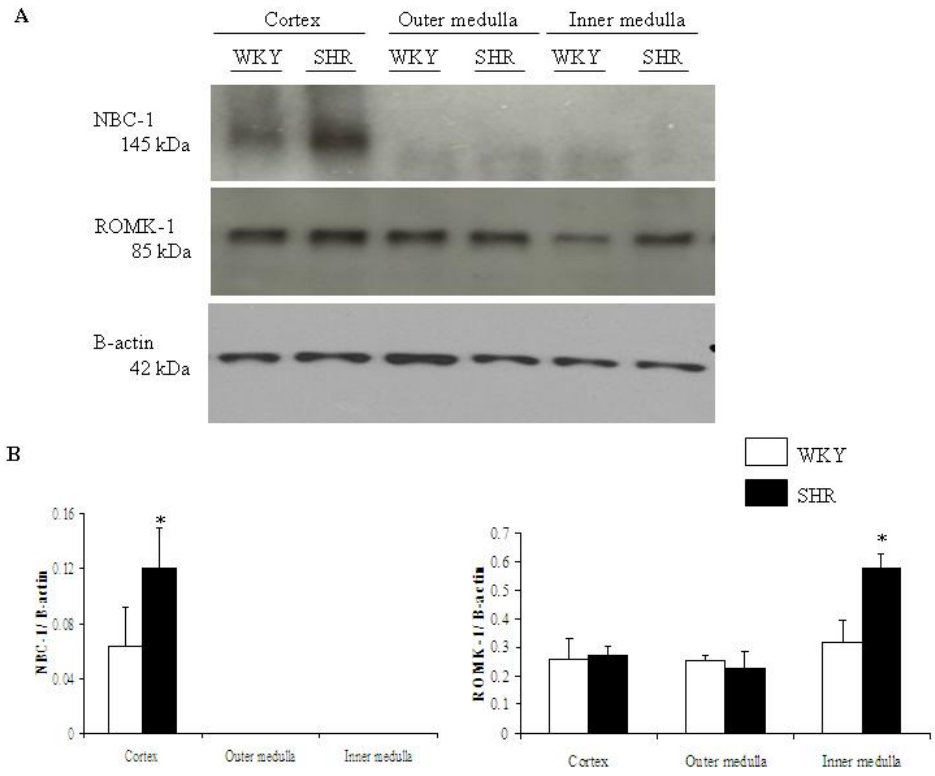

Figure 7. NBC-1 and ROMK-1 expression in the kidney outer cortex, inner strip of outer medulla and inner medulla of WKY and SHR rats

In Panel A, each lane was loaded with 20 μg of protein from a different rat. Blots were probed with rabbit-anti-NBC-1, rabbit-anti-ROMK-1 and mouse-anti-β-actin antibodies. Panel B summaries densitometric analysis of NBC-1 and ROMK-1 normalized to β-actin. Densitometric analysis revealed that expression of NBC-1 was higher in the cortex of the SHR and that expression of ROMK-1 was higher in the inner medulla of SHR. *Indicates $P<$ 0.05 compared with WKY. Values represent means ± SEM for 7 observations.

2.3.3 Expression of ROMK-1 protein

ROMK-1 in the apical membranes of the thick ascending limb allows for apical K^+ recycling for the efficient functioning of BSC-1, and ROMK-1 in the distal convoluted tubule and collecting duct participates in K^+ secretion. Semi-quantitative immunoblotting detected ROMK-1 expression in the outer cortex and outer and inner medulla, which is consistent with its localization. Although ROMK-1 has been originally cited as being a 45 kDa protein (151),

under our experimental conditions, the band of interest was found to be around 85 kDa which is approximately double the size of the ROMK monomer, and is believed to represent homodimeric and/or heterodimeric complexes formed by ROMK isoforms(152). Expression of ROMK-1 protein in the outer cortex and outer medulla was similar between both groups. However, expression of ROMK-1 in the inner medulla was significantly higher in the SHR compared to WKY (2-fold, $P< 0.05$; Figure 7).

2.3.4 Expression of AQP-1 and AQP-2 proteins

Several studies have demonstrated altered expression and apical targeting of aquaporins in water balance disorders(136, 153). AQP-1 and AQP-2 protein expression was detected in the outer cortex and outer and inner medulla. The expression of AQP-2 was significantly higher in the inner medulla, but not outer cortex or outer medulla, of the SHR compared to WKY (2-fold, $P< 0.05$; Figure 8). Expression of AQP-1 between both groups was similar in all three kidney regions (Figure 8).

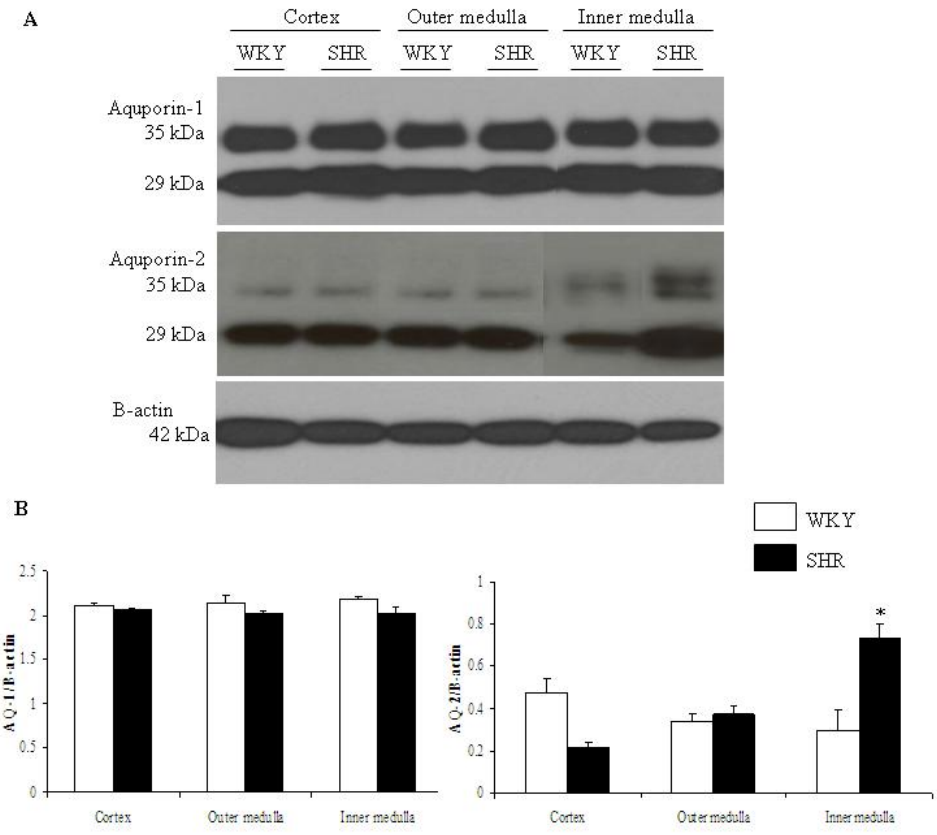

Figure 8. Expression of AQP-1 and AQP-2 in the kidney outer cortex, inner strip of outer medulla and inner medulla of WKY and SHR rats

In Panel A, each lane was loaded with 20 μg of protein from a different rat. Blots were probed with rabbit anti-AQ-1, anti-AQ-2 and mouse-anti-β-actin antibodies. Panel B summaries densitometric analysis of AQP-1 and AQP-2 normalized to β-actin. Densitometric analysis revealed that expression of AQP-1 was not different in SHR compares with WKY, while expression of AQP-2 was higher in the inner medulla in SHR. *Indicates $P < 0.05$ compared with WKY. Values represent means ± SEM for 7 observations.

2.3.5 Expression of BSC-1, AQP-2 and ROMK-1 mRNA

To determine whether the increases in BSC-1, AQP-2 and ROMK-1 protein expression were accompanied by similar increases in mRNA expression, we examined the mRNA expression for these proteins in outer cortex, outer medulla and inner medulla using RT-PCR. RT-PCR demonstrated that levels of BSC-1, ROMK-1 and AQP-2 mRNA in both groups were similar

(Figure 9), suggesting that post-transcriptional events are likely to be responsible for the increase in the expression of these proteins.

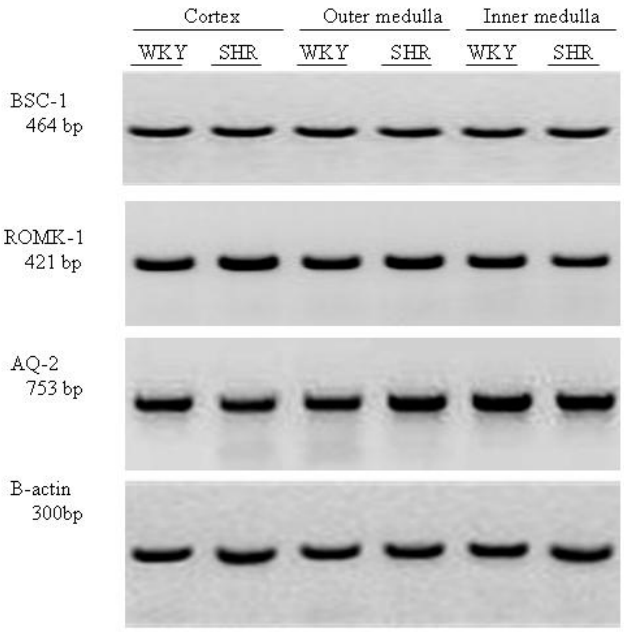

Figure 9. BSC-1, ROMK-1 and AQP-2 mRNA expression in the kidney outer cortex, inner strip of outer medulla and inner medulla of WKY and SHR rats as determined by RT-PCR

2.3.6 Effects of BSC-1 inhibition

As an index of the relative activity of the BSC-1 transporter, we measured the response to low and high doses of furosemide (3 and 50 mg/kg, respectively) in WKY and SHR. Administration of furosemide lowered the mean arterial blood pressure significantly and resulted in normalization of blood pressure in SHR (Figure 10A and 10B). The effects of furosemide on renal blood flow (figure 10C and 10D) and renal vascular resistance (Figure 11A and 11B) in WKY and SHR were similar. However the reduction in glomerular filtration rate (Figure 11D) was greater in SHR compared with WKY with both doses of furosemide (44.4 and 69.6% versus

20.6 and 49.7%, respectively). In contrast, diuretic responses to both low and high doses of furosemide (Figure 12B) were augmented in WKY (1099 and 1043%) compared with SHR (740 and 904%). Furosemide also increased absolute (Figure 12C and 12D) and fractional (Figure 13A and 13B) sodium excretion in the SHR and WKY. However the percentage increase in absolute and fractional sodium excretion with 50 mg/kg furosemide was higher in the SHR compared with WKY (1350% versus 766%, respectively).

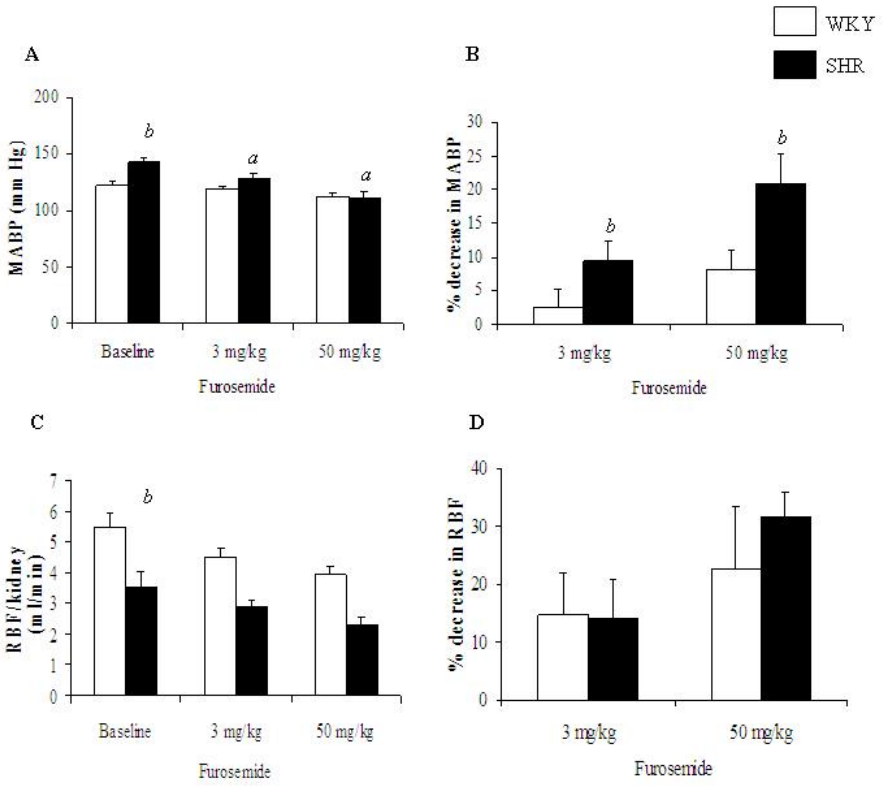

Figure 10. Mean arterial blood pressure (MABP) and renal blood flow (RBF) (A and C) and percent changes in MABP and RBF (B and D) following furosemide administration to WKY and SHR rats

$^{a}P< 0.05$ versus baseline; $^{b}P< 0.05$ versus corresponding value in WKY. Values represent means ± SEM for 6 observations

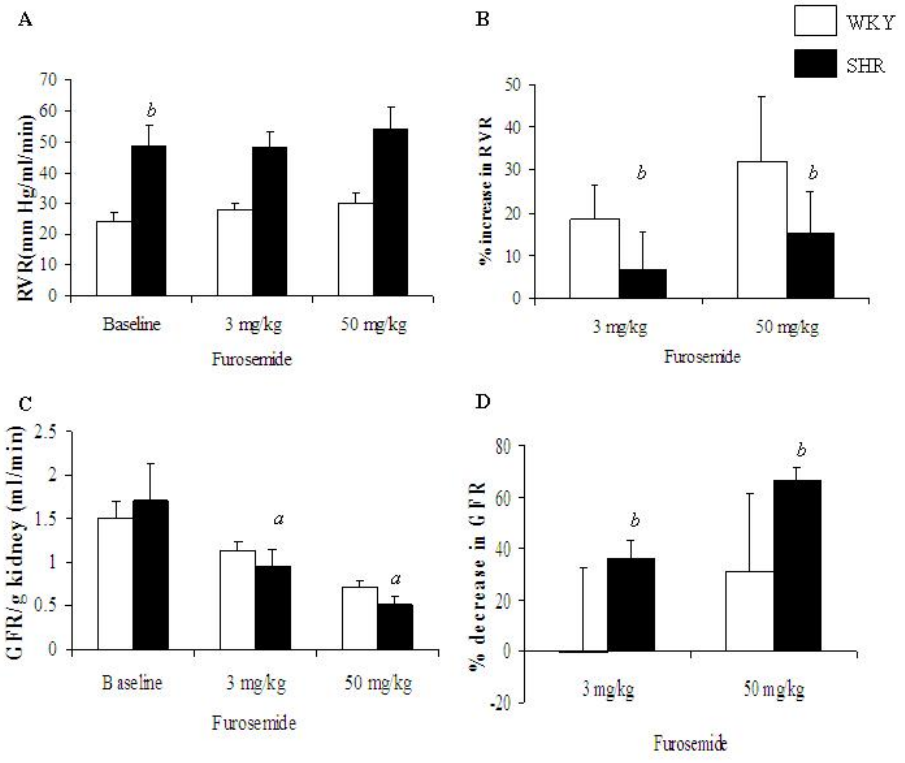

Figure 11. Renal vascular resistance (RVR) and glomerular filtration rate (GFR) (A and C) and percent changes in renal vascular resistance and glomerular filtration rate (B and D) following administration of furosemide to WKY and SHR rats

[a]*P*< 0.05 versus baseline; [b]*P*< 0.05 versus corresponding value in WKY. Values represent means ± SEM for 6 observations

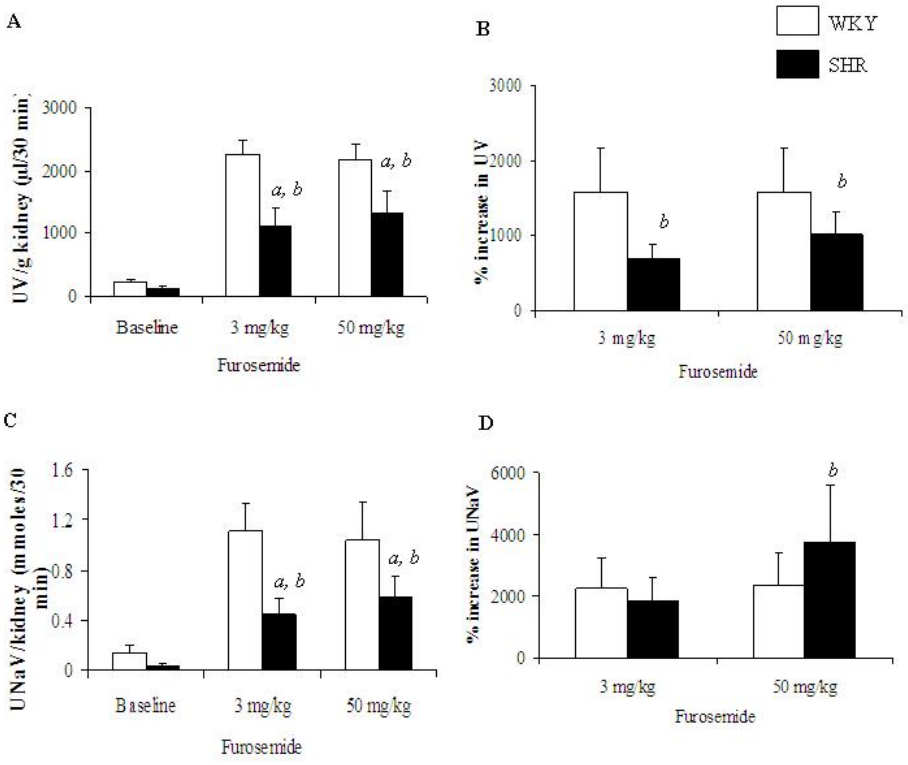

Figure 12. Urine volume (UV) and sodium excretion rate ($U_{Na}V$) (A and C) and percent changes in urine volume and sodium excretion rate (B and D) following administration of furosemide to WKY and SHR rats

$^{a}P< 0.05$ versus baseline; $^{b}P< 0.05$ versus corresponding value in WKY. Values represent means ± SEM for 6 observations

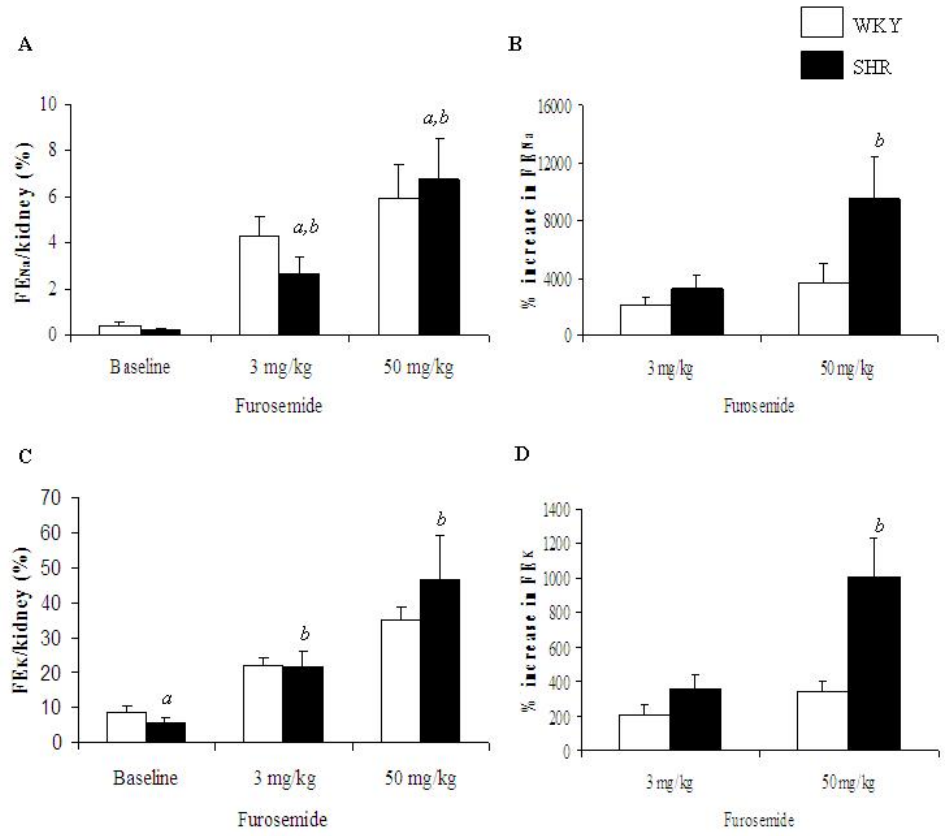

Figure 13. Fractional sodium (FE_{Na}) and potassium (FE_K) excretion rates (A and C) and percent changes in fractional sodium and potassium excretion rates (B and D) following furosemide administration to WKY and SHR rats

$^{a}P< 0.05$ versus baseline; $^{b}P< 0.05$ versus corresponding value in WKY. Values represent means ± SEM for 6 observations

2.4 DISCUSSION

The present study was based on the concept that the pathogenesis of sustained hypertension involves the kidneys. Altered renal sodium handling in the SHR has been previously reported(9, 154); however, the factors contributing to this effect are unknown. We have shown through semi-quantitative immunoblotting that the expression of sodium transporters BSC-1, Na-K-ATPase-α1, NHE-3, NBC-1, the potassium channel ROMK-1 and the water channel AQP-2 are

elevated in the SHR, suggesting that the pathophysiology of altered renal excretory function in genetic hypertension may involve alterations in several transporters located along the nephron. TSC protein expression was not changed, suggesting that the distal convoluted tubule sodium transport mediated by TSC is not affected in this genetic model of hypertension.

In the present study, expression of BSC-1 protein was elevated in the SHR more so than any other protein examined. This finding suggests that the increase in BSC-1 expression may be the most important contributing factor to altered tubular function in SHR. In support of this conclusion, our results also demonstrate that when the response to furosemide (an inhibitor of BSC-1) is expressed as a percentage of the baseline sodium excretion (either absolute or fractional), the natriuretic response to furosemide is greater in SHR versus WKY. Thus, both our biochemical and pharmacological data are consistent with an important role of BSC-1 in the pathophysiology of hypertension in SHR.

In the present study, acute administration of furosemide lowered arterial blood pressure in SHR, but not WKY. Generally, changes in renal excretory function do not alter arterial blood pressure within the time frame of the current study. However, anesthetized rats are more sensitive to volume depletion. Therefore, it is possible that the acute reduction in blood pressure induced by furosemide in SHR was secondary to more severe volume depletion following furosemide-induced natriuresis. At any rate, it is important to note that despite the greater reduction in renal perfusion pressure (which would tend to attenuate sodium excretion), furosemide still had a greater effect on sodium excretion in SHR.

Importantly, the increase in steady-state levels of BSC-1, ROMK-1 and AQP-2 proteins in the SHR were not accompanied by an increase in their respective steady state mRNA levels, suggesting that a post-transcriptional mechanism is responsible for the heightened-expression of these transporters. In this regard, our findings are consistent with previous reports demonstrating that post-transcriptional mechanisms are responsible for the increase in protein expression and activity of the NHE-3 transporter as well as Na-K-ATPase in the SHR(155, 156). Whether higher steady state levels of BSC-1, ROMK-1 and AQP-1 in SHR are due to greater translational efficiency or enhanced stability of the proteins or both cannot be deduced from the present study.

Our study is consistent with the idea that an intrinsic abnormality in sodium handling by the kidney contributes to the pathogenesis of hypertension in SHR. Studies in an experimental model of prenatally-programmed-hypertension suggest that prenatal programming of

hypertension involves transcriptional upregulation of sodium transporters BSC-1 and TSC in the thick ascending limb and distal convoluted tubule, respectively(141). Also, development of gene-targeting techniques in mice has enabled direct assessment *in vivo* of the roles of different apical renal Na^+ transporters in the control of extracellular fluid volume and blood pressure(65, 157). In this regard, gene targeting experiments show that the most detrimental mutation is the inactivation of *NKCC2* gene, which directly affects the countercurrent urine-concentrating mechanism and triggers profound disorganization of renal tissue.

The mechanisms mediating the upregulation of BSC-1 expression in genetic hypertension in SHR are yet to be determined. Several hormones such as vasopressin, angiotensin, prostaglandins, catecholamines and atrial natriuretic factor (ANP) may be involved in BSC-1 regulation. In this regard, our finding that the water channel AQP-2 is also upregulated in the SHR may implicate vasopressin. Both BSC-1 and AQP-2 are vasopressin-regulated proteins, and administration of vasopressin increases sodium and water reabsorption in the thick ascending limb and the collecting ducts(120, 158). The vasopressin-induced expression of BSC-1 in the thick ascending limb may be responsible for the enhanced urinary concentrating ability associated with sustained antidiuresis. Moreover, vasopressin regulates water permeability across the collecting duct by trafficking AQP-2 from intracellular vesicles to the apical plasma membrane(159). Thus, the increased expression of BSC-1 and aquaporin-2 could well be a vasopressin-mediated response. However, our finding that AQP-2 is only elevated in the inner medulla, not outer medulla, weighs against this conclusion because vasopressin increases AQP-2 expression all along the medullary collecting duct.

The renin-angiotensin system also regulates expression of epithelial transporters and could be involved in upregulation of transporter expression in SHR. Studies by several groups demonstrate that administration of angiotensin II increases BSC-1 expression in rat kidneys(126) and that expression of BSC-1 is reduced in mice lacking angiotensin converting enzyme(160). Additionally, studies with angiotensin converting enzyme inhibitors in rats with cardiac failure show that blockade of angiotensin II synthesis normalizes both BSC-1 expression and renal sodium excretion suggesting that angiotensin II influences renal sodium handling in cardiovascular disease *via* BSC-1(161).

Prostaglandins may also regulate renal sodium excretion in SHR. Recent studies with cyclooxygenase inhibitors show that COX-2 inhibitors increase BSC-1 expression in rat kidneys, thus implicating a role for prostaglandins in BSC-1 regulation(122).

Although our study demonstrates a role for BSC-1 in hypertension in the SHR, it does not rule out the involvement of other sodium transporters. Previous studies show that sodium reabsorption in the proximal tubule is increased in the SHR(154, 162). Studies also demonstrate that sodium transporters of the proximal tubule, namely the NHE-3, and Na^+-K^+-ATPase are upregulated in the SHR kidney(150, 155). Our data are in concordance with these findings. In addition, our studies indicate that expression of the potassium-channel ROMK-1 is also higher in the inner medulla of the SHR. Thus, it appears that the pathophysiology of genetic hypertension is complex and that several transporters located along the nephron may be involved.

As noted above, previous studies clearly indicate that sodium reabsorption in the proximal tubule is increased in the SHR. Thus, it is conceivable that the observed increases in BSC-1 expression in the inner strip of the outer medulla (corresponding to the thick ascending limb) in SHR are merely secondary to increased proximal tubular transport in SHR. However, this seems unlikely because numerous studies with loop and thiazide diuretics indicate that in normal animals and humans, changes in sodium transport in one nephron segment are always accompanied by opposite changes in sodium transport in all other nephron segments. For example, chronic inhibition of sodium transport in the thick ascending limb with loop diuretics or in the distal convoluted tubule with thiazide diuretics causes a compensatory increase in sodium reabsorption and/or transporters in other nephron segments(163-165). This phenomenon provides the basis for the synergy that is achieved when diuretics acting at different nephron segments are combined to provide sequential blockade(166). Therefore, it is unlikely that increases in proximal tubule transport would cause increases in BSC-1 expression in the thick ascending limb because the expected response would be a decrease, not an increase, in BSC-1 expression.

In summary, the present study demonstrates a marked increase in the expression of BSC-1 and more modest increases in the expressions of Na-K-ATPase-α1, NHE-3, NBC-1, ROMK-1 and AQP-2 in the SHR kidney. Therefore, dysregulation of the steady state levels of renal epithelial transporters may importantly contribute to the pathogenesis of hypertension in genetic hypertension.

3.0 EXPRESSION OF BSC-1 DURING DEVELOPMENT OF HYPERTENSION IN THE SHR

3.1 INTRODUCTION

The Spontaneously Hypertensive Rat (SHR) is one of the most widely used animal models of essential hypertension. These rats were developed in the 1950s by selective inbreeding of Wistar Kyoto Rats (WKY) with increased blood pressure and exhibit symptoms similar to human essential hypertensive populations such as spontaneous elevation of blood pressure with age, sensitivity to antihypertensive drugs effective in humans, and involvement of the renin-angiotensin and sympathetic nervous systems in the development and maintenance of hypertension. Several lines of evidence suggest that altered renal functions may play a role in the pathogenesis of essential hypertension in the SHR. This has been documented by the development of high blood pressure in normotensive rats after renal cross-transplantation between normotensive and hypertensive strains where the genetic predisposition of hypertension is transferred with the donor kidney from the hypertensive strains(6, 30, 31, 36, 167). Moreover, this was demonstrated not only in adult rats with established hypertension(168) but also in young prehypertensive rats(169). These data therefore support the hypothesis that altered renal functions contribute significantly to the development and maintenance of primary hypertension.

Our previous results indicate that in the adult SHR kidney as compared to WKY rats, protein levels of several sodium ion transporters are altered, namely the type-1 bumetanide sensitive cotransporter (BSC-1), type-1 inwardly rectifying potassium channel (ROMK-1), type-3 sodium-hydrogen exchanger (NHE-3), type-1 sodium-bicarbonate cotransporter (NBC-1), Na-K-ATPase and the water channel aquaporin-2. Since protein levels of BSC-1 were found to be most altered in adult SHR compared to WKY (170), we hypothesized that BSC-1 may play an important role in the pathogenesis of hypertension. In support of this hypothesis, our results also

demonstrate that when the response to furosemide (an inhibitor of BSC-1) is expressed as a percentage of the baseline sodium excretion (either absolute or fractional), the natriuretic response to furosemide is greater in SHR versus WKY(170).

The present study was initiated to further characterize the expression of BSC-1 in SHR prior to and during the development of hypertension. Our results show that BSC-1 protein abundance increases as hypertension progresses in the SHR and remains elevated in the SHR after the development of mild or severe hypertension as compared to age-matched normotensive WKY rats. The increase in BSC-1 steady state protein level in the SHR is additionally accompanied by increased presentation of BSC-1 at the plasma membrane, suggesting a physiological significance.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Male WKY rats (4-13 weeks of age) and age-matched SHR were obtained from Taconic Farms (Germantown, NY). Rats were allowed to acclimate to the University of Pittsburgh Animal Facility for at least 1 week before initiation of the experimental protocols. Protocols were approved by the Institutional Animal Care and Use Committee.

3.2.2 MABP measurement

Each rat was anesthetized with pentobarbital (45 mg/kg; Research Biochemicals, Natick, MA) and placed on an isothermal pad. Temperature was monitored with a rectal probe thermometer and kept at 37 degrees C with a heat lamp. A short section of polyethylene tubing (PE-240) was placed in the trachea to facilitate respiration. The left carotid artery was exposed and cannulated with PE-50 tubing for for mean arterial blood pressure (MABP) and heart rate (HR) measurements *via* a digital BP analyzer (Micro-Med, Inc., Louisville, KY). Following a 30

minute stabilization period, MABP and HR were recorded at 1-minute intervals for 20 minutes and averaged.

3.2.3 Kidney dissection and tissue lysate preparation

Blood pressures in age-matched WKY and SHR rats were measured as described; following which kidneys were rapidly excised and washed in ice-cold PBS. Kidneys were dissected to obtain cortex and inner stripe of outer medulla. Dissected tissues were divided into three groups for: *1)* preparation of tissue lysates, *2)* isolation of plasma membrane and intracellular vesicles, and *3)* RNA isolation. Tissue lysates were prepared by homogenizing the dissected tissues in lysis buffer containing Tris HCl, 2% SDS, glycerol, PMSF and protease inhibitors. Protein concentrations were measured using the BCA protein assay method.

3.2.4 Plasma membrane and intracellular vesicle isolation

Plasma membrane and intracellular vesicles were isolated by differential ultracentrifugation. This protocol has been shown to yield fractions that contain exclusively plasma membrane (at 17,000g) and membranes associated with intracellular vesicles (at 200,000g)(159, 171) (Appendix B). Cortex and inner stripe of outer medulla were dissected from each kidney, minced finely, and homogenized in isolation solution (250 mM sucrose/10 mM triethanolamine, pH 7.6) containing protease inhibitors (1 μg/ml leupeptin, 0.1 mg/ml phenylmethylsulfonyl fluoride). For subcellular fractionation, sequential centrifugations of the homogenates were carried out at 1000 x *g* for 10 min at 4°C, discarding the pellet and centrifuging the supernatant at 4,000 x g for 20 min at 4°C, discarding the pellet and centrifuging the supernatant at 17,000 x *g* at 4°C for 20 min to yield plasma membrane pellet, and 200,000 x *g* for 60 min to obtain fractions enriched in intracellular vesicle. The resulting pellet was resuspended in isolation solution and protein concentrations determined using the BCA Protein Assay method. Following total protein concentration determination, pellets from these centrifugations were solubilized in Laemmli sample buffer.

3.2.5 Electrophoresis and immunoblotting

Proteins were solubilized at 60°C for 15 min in Laemmli sample buffer. SDS-PAGE was performed on gradient polyacrylamide gels (4-12%) loaded with 20μg protein (whole cell lysates) or 2μg protein (fractionation lysates) per lane. For immunoblotting, proteins were transferred electrophoretically to PVDF membranes. Membranes were blocked in 5% milk for 2 hours, probed overnight at 4°C with the BSC-1 primary antibody (1:2000) in PBST containing 1% milk or with β-actin (1:10,000 Sigma Chemical Co., St. Louis, MO) for 1 hour to determine loading efficiency. BSC-1 antibody was the kind gift of Dr. Mark A. Knepper, NIH. Subsequently, membranes were exposed to a secondary HRP conjugated donkey anti-rabbit polyclonal antibody (1:5000, Pierce Biotechnology Inc., Rockford, IL) in PBST containing 1% milk for 1 hour at room temperature. Bound antibodies were visualized using a luminol-based enhanced chemiluminescence substrate (SupersignalWest Dura Extended Duration Substrate, Pierce Biotechnology Inc., Rockford, IL) before exposure to X-ray film (Kodak 165-1579; Eastman Kodak Co., Rochester, NY).

3.2.6 RNA isolation and RT-PCR

RNA was isolated from the dissected tissues using TRIzol reagent (GIBCO Life Technologies, Carlsbad, CA) as per the manufacturer's instructions. By using the primer sequences listed in table 2, RNA (0.5 μg) was reverse transcribed and amplified using Titanium One-step RT-PCR kit (Clontech, Palo Alto, CA). Each PCR cycle (total 30 cycles) consisted of denaturing at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 68°C for 60 seconds. RT-PCR products were separated on a 1.2% agarose gel and visualized by incorporating ethidium bromide in the gel.

Table 2. Primers used for RT-PCR analysis of BSC-1

	Accession number	Primer Sequence (5′-3′)	Nucleotides	Product size
BSC-1	U10096	Forward:GCATTGTCTTAACAGGAGGACC Reverse:GAACTGGAGAGATGTCAAACCC	2254 2676	464

3.2.7 Statistical analysis

All data are presented as mean ± SEM. Comparisons between groups were made by unpaired *t*-test. *P* values <0.05 were considered significant.

3.3 RESULTS

3.3.1 Development of hypertension in the SHR

Previous studies in young SHR showed that SHR and Wistar-Kyoto rats (WKY) had similar BP at or before 4 wk of age, with hypertension developing at or around 6 weeks of age(155, 172). Our results are consistent with these findings. At about 4-6 weeks of age, there was no difference in the mean arterial blood pressures between the SHR and WKY. However, there was a marked difference in mean arterial blood pressures as the animal aged, at about 6-8 weeks, MABP was significantly higher in the SHR compared to WKY and continued to increase from there on. At 14-16 weeks, SHR animals exhibited severe hypertension with MABP in the range of 190mmHg compared to WKY, which remained constant at 100 mmHg (Figure 14).

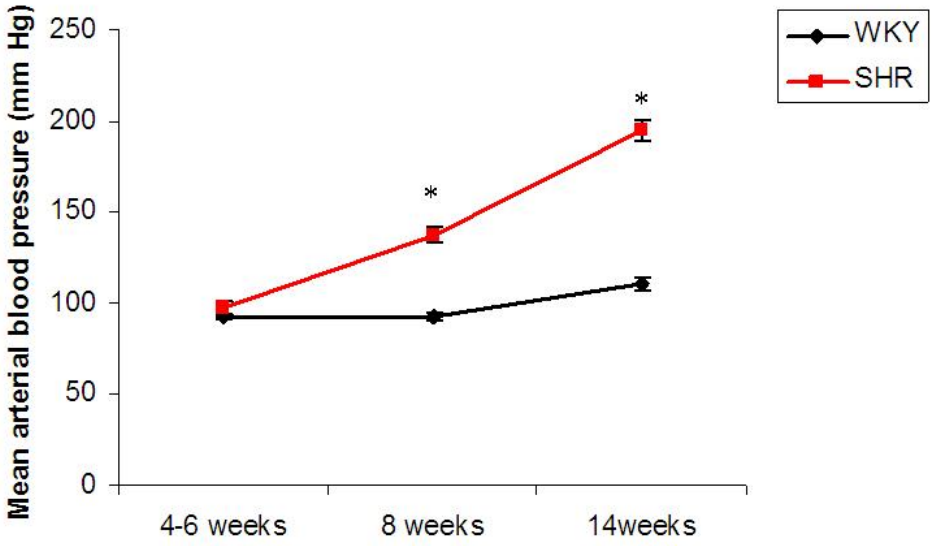

Figure 14. MABP in SHR and WKY over time

3.3.2 Steady-state protein levels of BSC-1 increase as hypertension progresses in the SHR

Our results indicated that in the pre-hypertensive stage, there was no difference in the steady-state abundance of BSC-1 between the two strains. However, as hypertension progressed in the SHR, the levels of BSC-1 protein also increased proportionally, i.e. the abundance of BSC-1 protein mirrored the onset/development of hypertension in the SHR. At 8 weeks of age, BSC-1 protein abundance in the SHR was about 3-fold higher ($P<0.05$) and as the animal became severely hypertensive at about 14-16 weeks of age, steady-state protein levels of BSC-1 became 5-fold higher ($P<0.05$) compared to WKY. These results indicate that the abundance of BSC-1 protein is stage dependent and increases as hypertension progresses in the SHR (Figure 15).

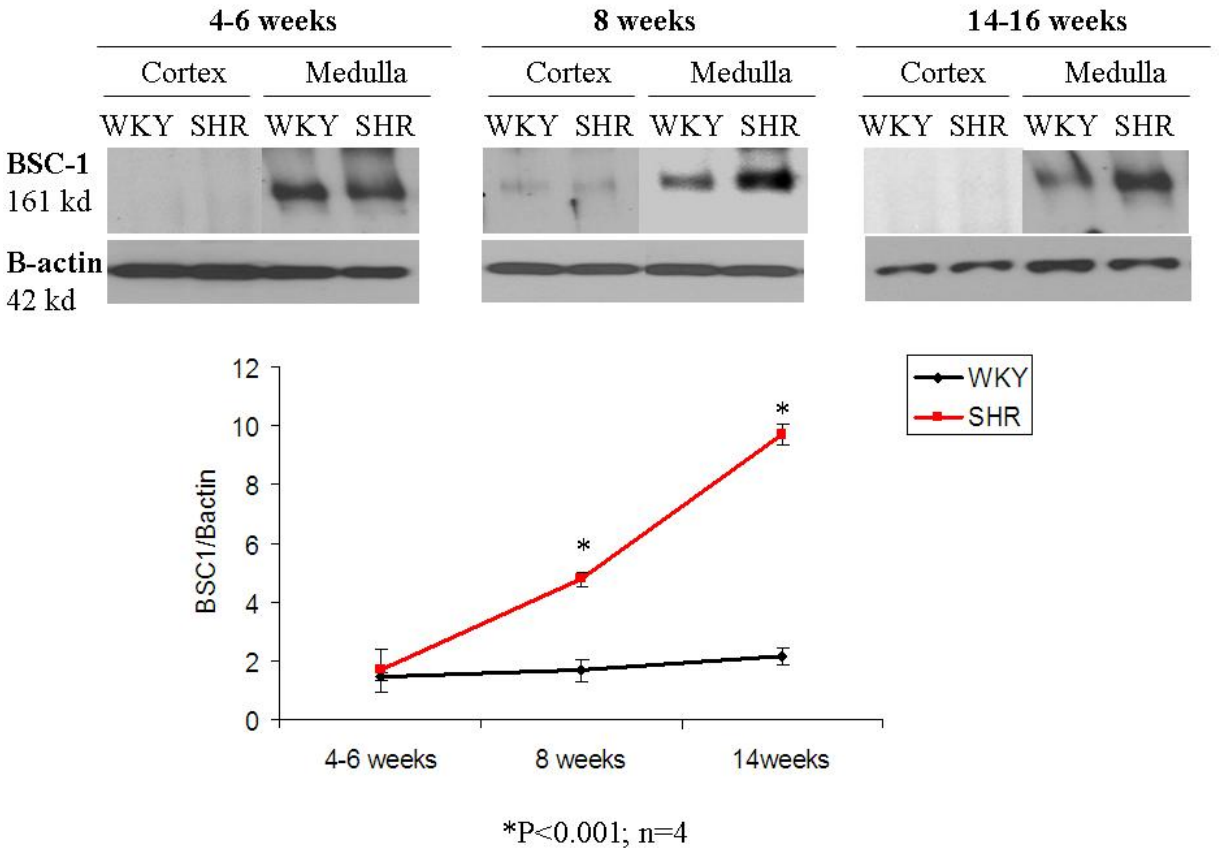

Figure 15. Abundance of BSC-1 protein in the SHR and WKY over time

Top panel: each lane was loaded with 20 μg of protein and blots were probed with rabbit anti-BSC-1 and mouse-anti-β-actin antibodies. Bottom panel: densitometric analysis of BSC-1protein in outer medulla normalized to β-actin. Values represent means ± SEM. *denotes $P < 0.05$ vs. Control (n=4, each group)

3.3.3 Distribution of BSC-1 to the plasma membrane increases as hypertension progresses in the SHR

With respect to the distribution of BSC-1 to the plasma membrane, our results showed that in the pre-hypertensive stage, there was no significant difference in the distribution of BSC-1 between the two strains as determined by the ratio of BSC-1 protein in plasma membrane to intracellular vesicles. However, as hypertension progresses, we observed a marked increase in the localization of BSC-1 to the plasma membrane, resulting in a plasma membrane to intracellular vesicle ratio

(PM: IV) of 4:1 in SHR as compared to 1.4:1 in the WKY. In the severely hypertensive stage, the ratio was further increased, with the SHR exhibiting a PM: IV ratio of 6:1 compared to the PM: IV ratio of WKY at 2:1. This suggests that in addition to an increase in steady-state protein levels, there is an increased presentation of BSC-1 at the plasma membrane as hypertension progresses in the SHR. Thus, both the increase in steady-state protein levels of BSC-1 and the alteration in distribution (increased presentation of BSC-1 to plasma membrane) correlate with the development of hypertension in the SHR (Figure 16).

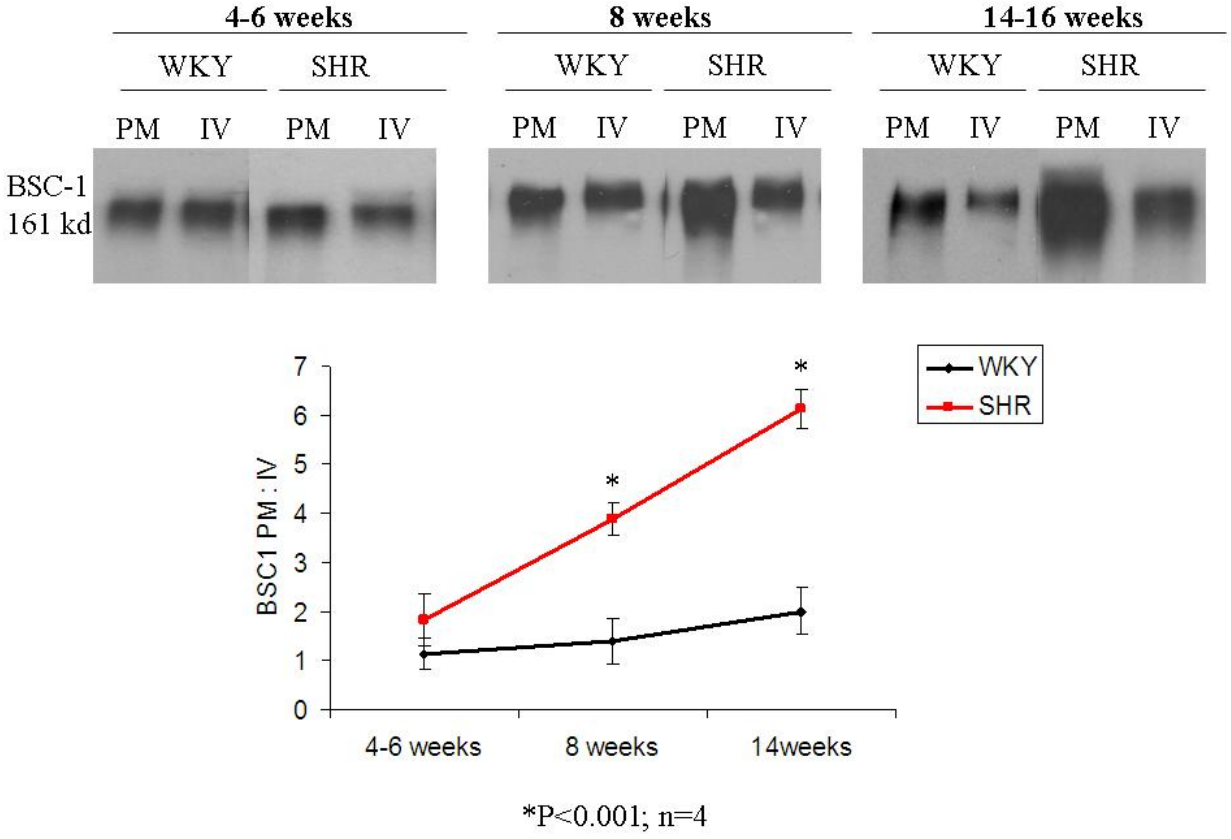

Figure 16. Distribution of BSC-1 protein in plasma membrane and intracellular vesicles in SHR and WKY over time

Top panel: each lane was loaded with 2 µg of protein following isolation of plasma membrane and intracellular vesicles and blots were probed with rabbit anti-BSC-1 antibody. Bottom panel: densitometric analysis of BSC-1 protein abundance in outer medulla expressed as a ratio of plasma membrane (PM) to intracellular vesicle (IV). Values represent means ± SEM. *denotes $P < 0.05$ vs. Control (n=4, each group)

3.3.4 Increase in BSC-1 protein is not accompanied by changes in mRNA as hypertension progresses in the SHR

Finally, these changes in steady state BSC-1 protein levels and increased distribution to the plasma membrane were not accompanied by corresponding changes in BSC-1 mRNA at any age. Thus, levels of BSC-1 mRNA in SHR and WKY were found to be similar in each of the three groups, irrespective of age and degree of hypertension (Figure 17).

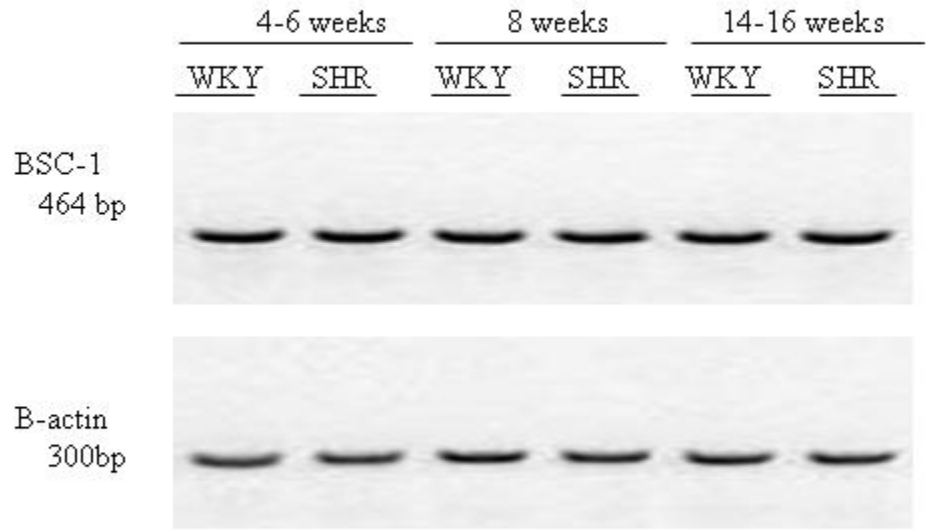

Figure 17. Expression of BSC-1 mRNA in inner stripe of outer medulla in SHR and WKY over time

RT-PCR analysis of BSC-1 mRNA levels in outer medulla of WKY and SHR rats at various stages of hypertension. (n=4, each group)

3.4 DISCUSSION

Essential hypertension results from the culmination of a series of pathological changes in the body that leads to a sustained elevation of blood pressure. The spontaneously hypertensive rat (SHR) is a suitable model to study hypertension development because it is similar to humans

with essential hypertension. These similarities include a genetic predisposition to high BP without specific etiology, increased total peripheral resistance without volume expansion, and similar responses to drug treatment(4). A precise knowledge of the defect(s) in early blood pressure development is essential to understand hypertension. For a causal role to be ascribed to a defect, this defect should occur at the initiation of blood pressure elevation. Defects that occur only after large blood pressure elevations should be considered secondary to the disease process(173).

The present study is based on the hypothesis that hypertension in the SHR tracks the kidney and that a defect(s) in the kidney is responsible for the development of hypertension. We had previously shown that in the adult SHR, expression of several renal sodium ion transporters and water channels are elevated, with the Na-K-2Cl cotransporter BSC-1 expression being the most altered(170). We therefore examined the expression of the renal bumetanide-sensitive Na-K-2Cl cotransporter BSC-1 in the SHR before and after the onset of hypertension. Our data show that the progression from pre-hypertensive to hypertensive state in the SHR is accompanied by a proportional increase in both steady-state protein levels of BSC-1 as well as its distribution to the plasma membrane, indicating that BSC-1 expression and distribution are stage dependent and increase as hypertension progresses. We hypothesize that the increased presentation of BSC-1 at the plasma membrane results in increased sodium reabsorption and thereby contributes to the pathogenesis of hypertension in the SHR.

These results are in agreement with the previous studies using isolated perfused kidneys from spontaneously hypertensive rats (SHR) that reveal an intrinsic renal abnormality in Na^+ excretion may contribute to the maintenance of hypertension in SHR(146). An examination of the pressure-natriuresis relationship and the effect of furosemide (an inhibitor of BSC-1) on this relationship demonstrates a resetting of the pressure-natriuresis process in SHR by a mechanism involving BSC-1(147).

Thus, the strong stimulation of BSC-1 could potentially contribute to the significant rise in blood pressure. Several lines of evidence support this hypothesis. First of all, the thick ascending limb (TAL) is an important site of sodium transport; roughly 30% of the filtered sodium load is normally reabsorbed at this level. Second, apical sodium entry in the TAL is mainly mediated by BSC-1 (92) as demonstrated by the natriuresis associated with the use of loop diuretics, an effect that is enhanced by their inhibiting action on tubuloglomerular feedback

(TGF)(174). Third, patients carrying a mutation with a loss of function of the BSC-1 gene (type I Bartter's syndrome) are characterized by orthostatic hypotension(175). Finally, mice lacking the BSC-1 gene suffer from severe salt wasting resulting in rapid death(65). It is noteworthy to underline that upregulation of BSC-1 has been also reported in the early phase of prenatally programmed hypertension induced by a maternal low-protein diet during pregnancy(141) and in the early phase of hypertension in the Milan strain of rats (MHS) rats(176), a model in which hypertension develops because of a primary alteration in renal tubular sodium reabsorption(168, 177).

The stimulation of BSC-1 may also be involved in the increased glomerular filtration rate (GFR) found in the SHR. In fact, activation of ion transport along the TAL is expected to decrease the delivery of sodium chloride to the macula densa, thus enhancing GFR through the TGF mechanism. In young SHR rats, the alteration of TGF, together with an increase in net interstitial pressure, has already been described(178, 179), and the present studies identify a molecular mechanism involved in this process.

An important unresolved question is the modulatory mechanism of BSC-1 regulation in the SHR. Our results show that the increased protein levels of BSC-1 in the SHR are not accompanied by changes in mRNA, suggesting that post-transcriptional mechanisms might be involved. A post-transcriptional mechanism has been similarly proposed for the over-expression of NHE-3 protein in the SHR(155, 180). However the underlying signaling mechanism(s) responsible for this effect are unknown and several factors could be involved such as vasopressin, angiotensin II, prostaglandins, catecholamines and sympathetic activation, atrial natriuretic factor (ANP), nitric oxide (NO), reactive oxygen species (ROS), cAMP, or could result from morphological changes in the kidney and tubular cells. Regulation of BSC-1 by vasopressin, angiotensin II, cAMP, nitric oxide and prostaglandins has been documented(113, 120, 122, 125, 126, 181, 182). Regulation of BSC-1 by the sympathetic system has been implicated in studies using an animal model of liver cirrhosis induced by common bile duct ligation(CBL), which showed that renal denervation attenuated the sodium retention in CBL rats, and was associated with normalization of the natriuretic effect of furosemide, as well as a significant reduction in the expression of BSC-1, suggesting an important role for renal sympathetic nerve activation in BSC-1 regulation(183). Recently, it has been demonstrated that the TAL is the major source of superoxide production(184); in addition, superoxide stimulates

sodium chloride absorption along the TAL(185, 186), and the kidney of SHR generates more superoxide compared to WKY(187). Together, these studies may indicate a role of superoxide in the modulation of the function of BSC-1. Additionally, increased sodium reabsorption by TAL could be related to morphological changes in the kidney and tubular cells; it has been demonstrated that the size of the kidneys and the volume of the tubular cells in SHR are smaller than WKY rats and SHR rats have decreased nephron number(188, 189). This would necessitate a higher GFR-to-kidney size ratio to filter the blood; it is therefore reasonable to postulate that the increased single-nephron sodium load may trigger the reported upregulation of TAL sodium reabsorption. These findings are intriguing, because in rats with low birth weight induced by a maternal low-protein diet, the development of hypertension has been related to upregulation of BSC-1(141).

In summary, the present study demonstrates an increase in BSC-1 protein levels, in the absence of mRNA level changes, as hypertension progresses in the SHR and may explain at least in part the inappropriate sodium handling of the kidney in this model of essential hypertension.

4.0 REGULATION OF THE RENAL BUMETANIDE-SENSITIVE NA-K-2CL COTRANSPORTER BSC-1/NKCC2 BY NOREPINEPHRINE

4.1 INTRODUCTION

The sympathetic nervous system plays a fundamental role in the homeostatic control of blood pressure. Decreases in arterial pressure or effective arterial blood volume increase sympathetic activity, whereas increases in pressure suppress activity, thus regulating blood pressure in the short term. In addition to regulating short-term blood pressure, the sympathetic system also participates in the long-term control of blood pressure. Both chronic increases and decreases in renal adrenergic activity alter renal excretory function and produce sustained elevations and reductions in arterial pressure, respectively. For example, chronically increased activity of the sympathetic nervous system produces hypertension and chronic intrarenal norepinephrine infusion increases arterial pressure(190, 191). Other studies show that sustained electrical stimulation of sympathetic nerves produces hypertension(192-194). These results suggest that chronic, inappropriate increases in sympathetic activity can produce sustained increases in arterial pressure.

Many lines of evidence indicate that the sympathetic nervous system, *via* the renal nerves, plays an important role in the pathogenesis of essential hypertension in humans and laboratory animals(22, 195). Patients with established essential hypertension have increased sympathetic nervous system activity, as evidenced by increased plasma and urinary norepinephrine levels, elevated excretion of catecholamine metabolites, and an exaggerated depressor response to centrally acting sympatholytic agents(196-199). The observation that converting enzyme inhibitors can cause both blood pressure and plasma norepinephrine levels to return to normal in patients with essential hypertension is consistent with the interpretation that activation of the sympathetic nervous system in these subjects is, at least in part, dependent on

angiotensin(200-202). In the spontaneously hypertensive rat of the Okamoto strain (SHR) and in the DOCA/NaCl hypertensive model, increased renal efferent nerve activity contributes to the development of hypertension by causing increased renal sodium retention(203, 204). In both of these experimental models, renal denervation delays the development and blunts the severity of hypertension and is associated with increased urinary sodium excretion, suggesting a renal efferent mechanism(205-207).

At the level of the kidney, sympathetic nerves innervate the tubules, the vessels, and the juxtaglomerular apparatus and changes in renal sympathetic nerve activity can directly influence the functions of these innervated renal effector units(208). An increase in renal sympathetic nerve activity increases renal tubular water and sodium reabsorption throughout the nephron, decreases renal blood flow and glomerular filtration rate by constricting the renal vasculature, and increases activity of the renin-angiotensin system by stimulating renin release from juxtaglomerular granular cells(22).

The vast majority of *in vivo* and *in vitro* studies directed at elucidating the role of the adrenergic/sympathetic system in the kidney focus on the regulation of renal blood flow and glomerular filtration(209, 210). In contrast, few studies address the direct effects of the adrenergic system on salt and water transport across the nephron. Adrenergic receptors exist on most nephron segments, including proximal tubule, the thick ascending limb, distal convoluted tubule and the inner medullary collecting duct, indicating that epithelial function is under some degree of control by the adrenergic system(22, 211-213). Norepinephrine released from renal sympathetic nerve terminals or reaching the kidney from the circulation may thus importantly modulate salt and fluid transport. The observation that low-level adrenergic stimulation of renal adrenergic nerves or infusion of low doses of the adrenergic agonist norepinephrine produces an increase in renal tubular sodium reabsorption without alterations in renal hemodynamics, and independent of contributions from humoral factors such as angiotensin II or prostaglandins(214, 215), suggests that the adrenergic system is capable of direct regulation of renal tubular sodium transport.

The precise molecular downstream targets that mediate the direct effects of norepinephrine on renal excretory function are unknown. However, integral membrane proteins in renal epithelial cells that mediate transport of ions and water are attractive candidates because they are responsible for salt and water balance and are involved in the urinary concentrating

mechanism. In this regard, several transporters exist in the kidney and are localized to specific segments of the nephron, mediating the entry of sodium across the apical membrane(216). Moreover, water transport across the apical surface of the nephron occurs *via* specialized channels called aquaporins that are selective for water and are located along the proximal tubule, distal convoluted tubule and collecting duct(53).

The present study was initiated to test the hypothesis that norepinephrine, the principal neurotransmitter of the sympathetic/adrenergic system, modulates the expression of renal epithelial transport systems. To test this hypothesis, we utilized semi-quantitative immunoblotting in a rat model of chronically elevated norepinephrine (*via* infusion).

4.2 MATERIALS AND METHODS

4.2.1 Animals and Treatment

Pathogen-free male Sprague-Dawley rats (200-300 g body wt) were used. Rats were allowed to acclimate to the University of Pittsburgh Animal Facility for at least one week before initiation of the experimental protocols. Protocols were approved by the Institutional Animal Care and Use Committee. Initially, all rats were maintained in filter-top microisolator cages with autoclaved feed and bedding, and free access to drinking water. Rats were divided into 2 groups (n=6) and given either norepinephrine (600 ng/min) or saline (control) for a period of 15 days with implanted osmotic mini pumps (Alzet).

4.2.2 MABP and HR measurement

At the end of treatment, each rat was anesthetized with pentobarbital (45 mg/kg; Research Biochemicals, Natick, MA) and placed on an isothermal pad. Temperature was monitored with a rectal probe thermometer and kept at 37° C with a heat lamp. A short section of polyethylene (PE)-240 tubing was placed in the trachea to facilitate respiration. The left carotid artery was exposed and cannulated with PE-50 tubing for blood sample collections and for mean arterial BP

(MABP) and heart rate (HR) measurements *via* a digital BP analyzer (Micro-Med, Inc., Louisville, KY). Infusion of saline was initiated, and following a 30-minute stabilization period, mean arterial blood pressure and heart rate were recorded at one-minute intervals and averaged and reported in the table 2.

4.2.3 Kidney dissection and tissue preparation for immunoblotting

Blood pressures in control and hormone-infused rats were measured as described above; following which kidneys were rapidly excised and washed in ice-cold PBS. The left kidneys were dissected to obtain outer cortex and inner stripe of outer medulla and inner medulla, and the dissected tissues were homogenized in lysis buffer containing (Tris HCl, 2% SDS, glycerol, PMSF and protease inhibitors). Protein concentrations were measured using the BCA protein assay.

4.2.4 Electrophoresis and immunoblotting

Proteins were solubilized at 60° C for 15 min in Laemmli sample buffer. SDS-PAGE was performed on gradient polyacrylamide gels (4-12%) loaded with 20 μg protein per lane. For immunoblotting, proteins were transferred electrophoretically to PVDF membranes. Membranes were blocked in 5% milk for 2 hours, probed overnight at 4° C with the respective primary antibodies in PBS containing 1% milk: BSC-1 (1:2000), thiazide-sensitive Na^+-Cl^- cotransporter (TSC; 1:600), aquaporin-1 (AQP-1; 1:2000) and aquaporin-2 (AQP-2; 1:2000), type-1 Na^+-HCO_3^--cotransporter (NBC-1; 1:1000), type-3 Na^+-H^+ exchanger (NHE-3; 1:1000), Na^+-K^+-ATPase-α_1 (1:5000) and inwardly rectifying K^+ channel (ROMK-1; 1:1000). Membranes were probed with β-actin (1:10,000 Sigma Chemical Co., St. Louis, MO) for 1 hour to determine loading efficiency. BSC-1, TSC, AQP-1 and AQP-2 were the kind gift of Dr. Mark A. Knepper (NIH). All other primary antibodies were from Chemicon (Temecula, CA). Subsequently, membranes were exposed to a secondary HRP conjugated donkey anti-rabbit polyclonal antibody (1:5000, Pierce Biotechnology Inc., Rockford, IL) in PBS containing 1% milk for 1 hour at room temperature. Bound antibodies were visualized using a luminol-based enhanced chemiluminescence substrate (SupersignalWest Dura Extended Duration Substrate, Pierce

Biotechnology Inc., Rockford, IL) before exposure to X-ray film (Kodak 165-1579; Eastman Kodak Co., Rochester, NY). Densitometric analysis was performed using ImageQuant TL (Amersham Biosciences, Piscataway, NJ) and band densities were normalized to β-actin.

4.2.5 Statistical analysis

All data are presented as mean ± SEM. Comparisons between groups were made by unpaired *t*-test. *P* values <0.05 were considered significant.

4.3 RESULTS

4.3.1 Chronic norepinephrine infusion increases MABP and HR

Chronic infusion of norepinephrine significantly increased mean arterial blood pressure (MABP) and heart rate (HR) over control (Table 3), consistent with the physiological actions of norepinephrine.

Table 3. Effect of chronic norepinephrine infusion on MABP, Systolic BP, Diastolic BP and Heart Rate (HR)

Values represent means ± SEM. * denotes $P < 0.001$ vs. Control

Treatment	MABP (mm Hg)	Systolic (mm Hg)	Diastolic (mm Hg)	Heart Rate (beats/min)
Control	88.8± 3.0	107.8± 8.3	74.4± 0.9	375.5± 13.7
Norepinephrine	128.3± 10.4 *	165.0± 15.8 *	105.2± 11.3 *	492.5± 15.5 *

4.3.2 Norepinephrine regulates expression of the apical sodium hydrogen exchanger NHE-3 of the proximal tubule

Sodium transport in the proximal tubule has been shown to be a target for the adrenergic system in several physiological studies. In the proximal tubule, the sodium hydrogen exchanger NHE-3

is the principal regulator of sodium uptake and studies have shown that the adrenergic system can modulate its activity in the short-term(217, 218). However, the long-term/chronic effects of norepinephrine on the expression of the transporter have not been examined. Our results show that chronic 15-day norepinephrine infusion resulted in a significant increase in NHE-3 protein levels (2.5-fold; *P*=0.0142, n=5) (Figure 18).

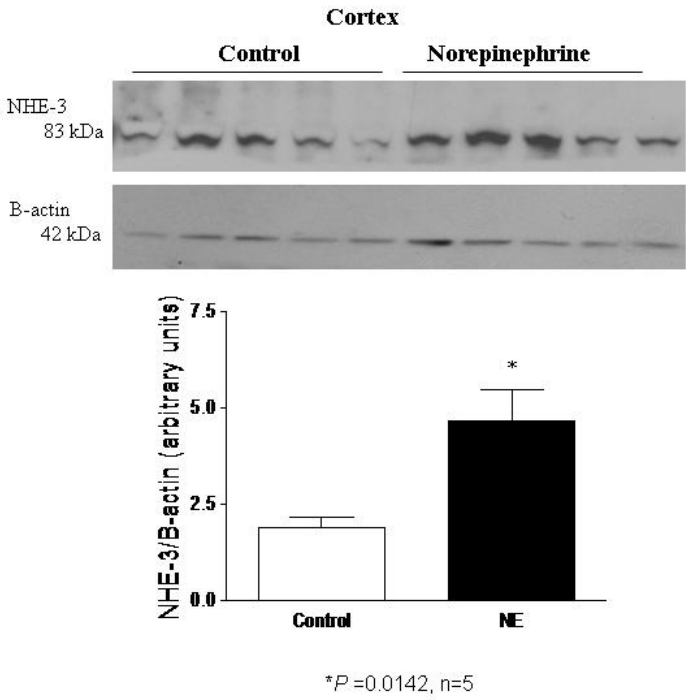

Figure 18. Expression of NHE-3 in the renal outer cortex in control and norepinephrine infused rats

Top panel: each lane was loaded with 20 µg of protein and blots were probed with rabbit anti-NHE-3 and mouse-anti-β-actin antibodies. Bottom panel: densitometric analysis of NHE-3 expression in cortex normalized to β-actin. Values represent means ± SEM. *denotes $P < 0.05$ vs. Control (n=5, each group)

4.3.3 Norepinephrine increases expression of the basolateral sodium bicarbonate transporter NBC-1 of the proximal tubule

The Na^+–HCO_3^- cotransporter NBC-1 mediates bicarbonate absorption from renal proximal tubules. About 85% of the filtered load of HCO_3^- is reabsorbed in the renal proximal tubules. This transepithelial flux is accomplished by the apical membrane Na^+/H^+ exchanger (NHE-3) and the basolateral Na^+-HCO_3^- co-transporter NBC-1. Adrenergic stimulation has been shown to regulate HCO_3− reabsorption in the proximal tubule(219), however the long-term effects of adrenergic/sympathetic activation are unknown. Our results show that chronic 15-day norepinephrine infusion, results in a significant increase in NBC-1 protein levels (2-fold, *P*= 0.0067, n=5) over control (Figure 19).

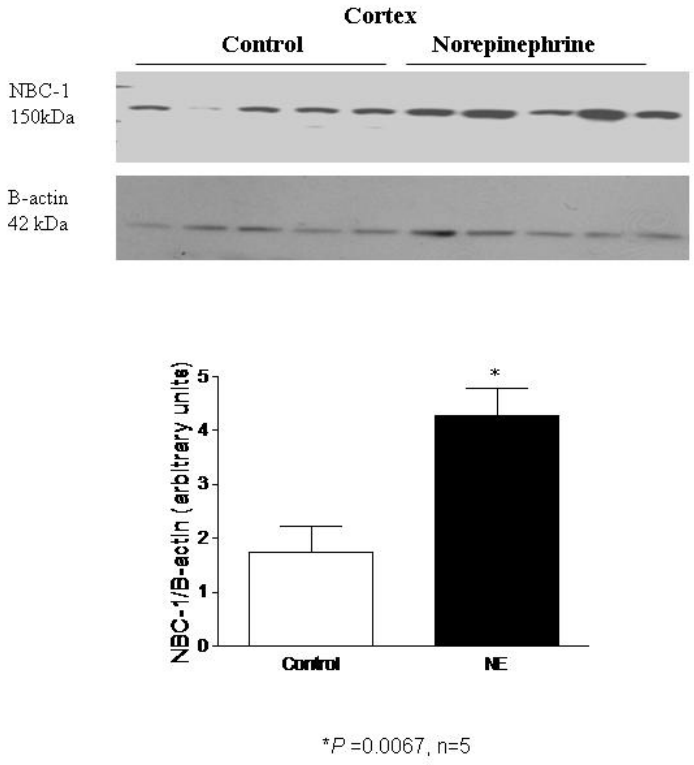

Figure 19. Expression of NBC-1 in the renal outer cortex in control and norepinephrine infused rats

Top panel: each lane was loaded with 20 μg of protein and blots were probed with rabbit anti-NBC-1 and mouse-anti-β-actin antibodies. Bottom panel: densitometric analysis of NBC-1 expression in cortex normalized to β-actin. Values represent means ± SEM. *denotes $P < 0.05$ vs. Control (n=5, each group)

4.3.4 Norepinephrine increases expression of the Na-K-2Cl cotransporter BSC-1 of the thick ascending limb

The bumetanide-sensitive-Na-K-2Cl cotransporter BSC-1 is the principal apical sodium transporter of the thick ascending limb and accounts for 25% of sodium reabsorption in the kidney. Studies in isolated perfused tubules, have shown that adrenergic stimulation (by application of catecholamines) promotes sodium and chloride reabsorption in the thick ascending limb, thus supporting a physiological role for adrenergic innervation of the thick ascending limb(220). Our results show that chronic 15-day norepinephrine infusion significantly increased protein abundances of BSC-1 of the thick ascending limb (3-fold; P=0.0020, n=5) (Figure 20) in the renal medulla.

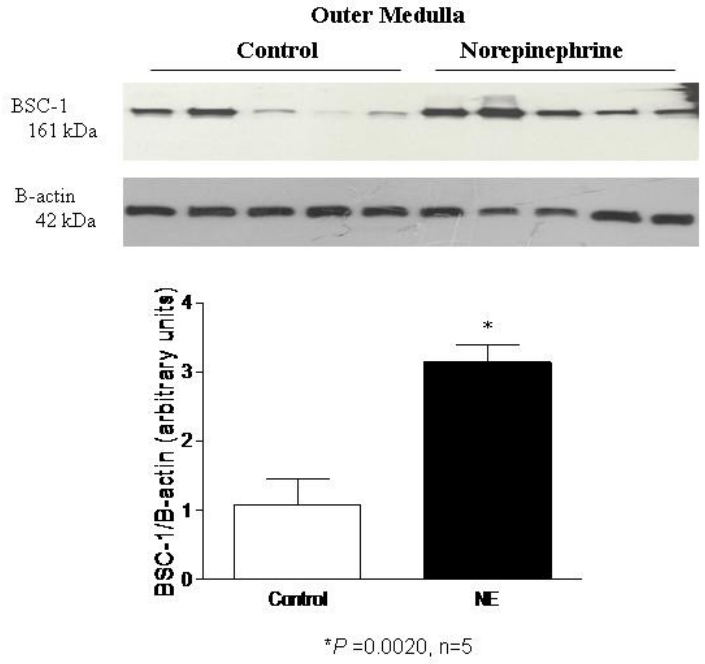

Figure 20. Expression of BSC-1 in the renal inner stripe of outer medulla in control and norepinephrine infused rats

Top panel: each lane was loaded with 20 μg of protein and blots were probed with rabbit anti-BSC-1 and mouse-anti-β-actin antibodies. Bottom panel: densitometric analysis of BSC-1 expression in inner stripe of outer medulla normalized to β-actin. Values represent means ± SEM. *denotes $P < 0.05$ vs. Control (n=5, each group)

4.3.5 Norepinephrine does not alter expression of the inwardly rectifying K channel (ROMK-1) of the thick ascending limb

The inwardly rectifying potassium channel (ROMK) of the thick ascending limb is responsible for the recycling of potassium ions that enter the cell *via* BSC-1/NKCC2. Since we observed changes in protein levels of BSC-1 following norepinephrine infusion, we wished to determine whether changes in BSC-1 were accompanied by similar changes in ROMK-1 expression or not.

Our results of immunoblotting show that, at least in our model, changes in BSC-1 expression are not accompanied by any changes in the expression of ROMK-1 (Figure 21B).

4.3.6 Norepinephrine does not alter the abundance of the thiazide-sensitive Na-Cl cotransporter of the distal tubule or the basolateral Na-K-ATPase

The thiazide-sensitive Na-Cl cotransporter (TSC) accounts for about 3-5% of sodium reabsorption in the distal tubule and has been shown to be an aldosterone-induced protein(138). While studies in isolated distal tubules have shown that salt transport in this segment may be regulated by adrenergic stimulation, the precise targets remain unknown. Studies have implicated a role for the basolateral Na-K-ATPase pump as a driving force for increased sodium reabsorption following adrenergic stimulation that may involve the Na-Cl cotransporter(221). Long-term infusion of norepinephrine did not alter protein abundance of TSC of the distal tubule (Figure 21A). Additionally, chronic norepinephrine infusion did not alter protein levels of the Na-K-ATPase pump of the basolateral membrane in any renal regions (Figure 21A, B and C).

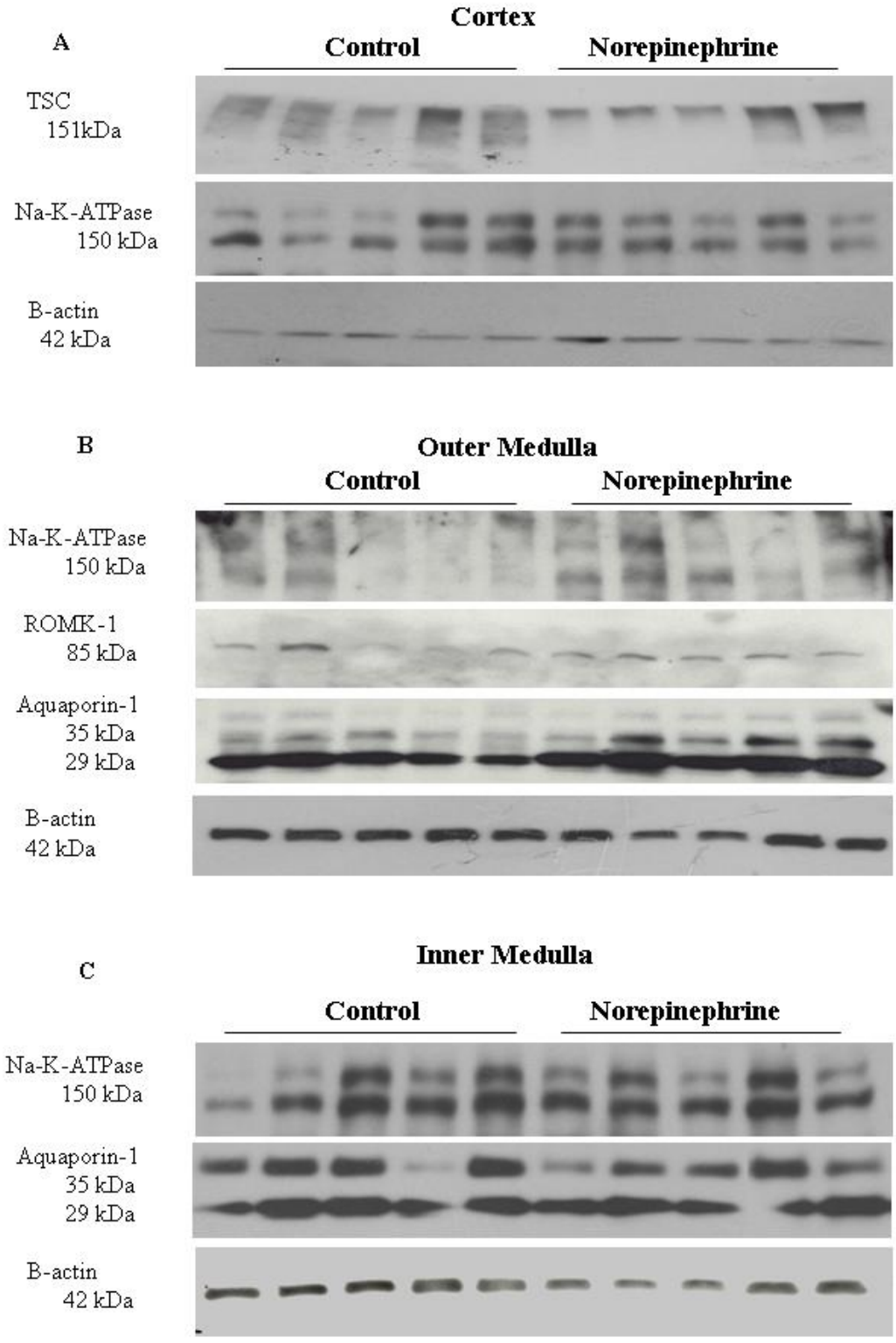
A
Cortex
Control
Norepinephrine
TSC
151kDa
Na-K-ATPase
150 kDa
B-actin
42 kDa
B
Outer Medulla
Control
Norepinephrine
Na-K-ATPase
150 kDa
ROMK-1
85 kDa
Aquaporin-1
35 kDa
29 kDa
B-actin
42 kDa
C
Inner Medulla
Control
Norepinephrine
Na-K-ATPase
150 kDa
Aquaporin-1
35 kDa
29 kDa
B-actin
42 kDa

Figure 21. Expression of TSC, Na-K-ATPase, ROMK-1 and AQ-1 in the renal outer cortex, inner stripe of outer medulla and inner medulla in control and norepinephrine infused rats

Each lane was loaded with 20 μg of protein and blots were probed with rabbit anti-TSC, anti-Na-K-ATPase-α1, anti-ROMK-1, anti-AQ-1 and mouse-anti-β-actin antibodies (n=5).

4.3.7 Norepinephrine increases abundance of the water channel aquaporin-2 of the inner medullary collecting duct

Aquaporins are a family of small membrane proteins that facilitate the reabsorption of water across the nephron. Of the different isoforms, aquaporin-2 is the principal water channel of the inner medullary collecting duct that is involved in both short-term and long-term regulation of water transport by the antidiuretic hormone vasopressin. Altered levels of renal aquaporins have been found to be associated with several diseases involving abnormal water handling by the kidney including essential hypertension, diabetes insipidus, dehydration and chronic heart failure(170, 222-224). Chronic norepinephrine infusion increased abundance of the water channel aquaporin-2 of the inner medullary collecting duct (2-fold; P=0.0039, n=5) (Figure 22). The effect was specific for aquaporin-2 since norepinephrine did not alter the protein levels of aquaporin-1 (Figure 21B, C).

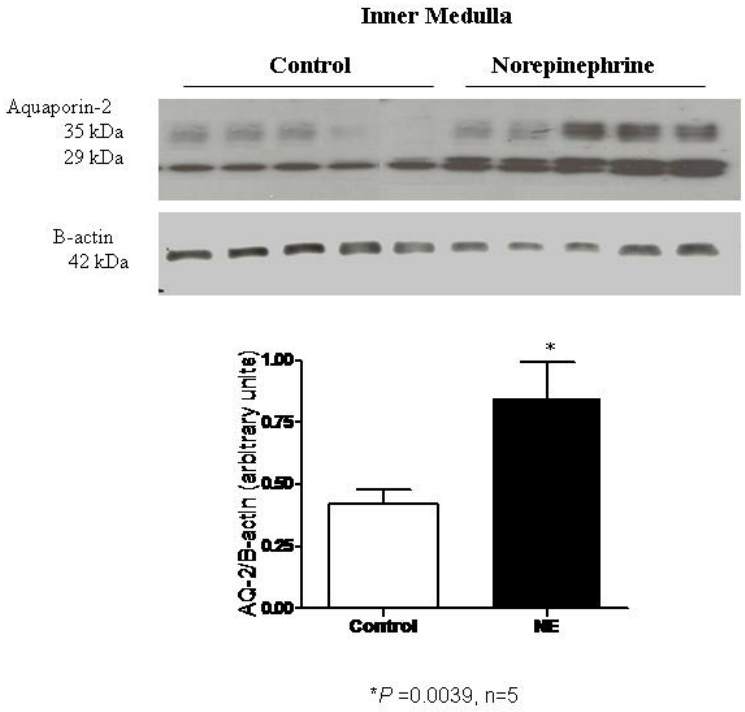

Figure 22. Expression of AQP-2 in the renal inner medulla in control and norepinephrine infused rats

Top panel: each lane was loaded with 20 μg of protein and blots were probed with rabbit anti-AQP-2 and mouse-anti-β-actin antibodies. Bottom panel: densitometric analysis of AQP-2 expression in cortex normalized to β-actin. Values represent means ± SEM. *denotes $P < 0.05$ vs. Control (n=5, each group)

4.4 DISCUSSION

The major conclusion from this study is that efferent renal sympathetic nerve activity may play an important role in the regulation of renal function by directly influencing renal tubular sodium and water reabsorption *via* changes in key transporter systems. The support for this conclusion is our finding that mimicking long-term over-activation of the sympathetic system *via* chronic norepinephrine infusion strongly upregulates expression/abundance of the sodium hydrogen

exchanger NHE-3 and the sodium-bicarbonate co-transporter NBC-1 of the proximal tubule, the bumetanide-sensitive Na-K-2Cl cotransporter BSC-1 of the thick ascending limb, and the water channel aquaporin-2 of the inner medullary collecting duct. In contrast to the aforementioned transport systems, our data show that norepinephrine has no effect on the expression of the thiazide-sensitive NaCl cotransporter TSC, the inwardly rectifying potassium channel ROMK-1, the water channel aquaporin-1 or Na^+-K^+-ATPase-α_1. This suggests that the effects of norepinephrine are specific and that the mechanisms by which norepinephrine regulates renal sodium transporters and water channels may be different than those involved in regulation by vasopressin, aldosterone or angiotensin II.

The molecular mechanism for upregulation of NHE-3, NBC-1, BSC-1 and aquaporin-2 by norepinephrine is unknown. Previous studies show that in epithelial cells, regulation of NHE-3 activity by norepinephrine proceeds *via* activation of the MAPK cascade(225) and regulation of NBC-1 by cholinergic agonists (angitotensin II and CO_2) may be mediated by Src family kinases (SFKs) and MAPKs(226). Whether any of these signaling molecules (SFK and/or MAPK) are involved in the long-term regulation of NHE-3 and NBC-1 by norepinephrine is unknown. Studies aimed at elucidating the regulation of BSC-1 and aquaporin-2 by vasopressin show that the regulation involves activation of adenylyl cyclase and cAMP *via* V_2 receptors(120, 227). Norepinephrine is also known to increase intracellular cAMP levels *via* activation of the β-adrenergic receptor in the proximal straight tubule, thick ascending limb and the collecting duct (212, 228-230), and we hypothesize that regulation of BSC-1 and aquaporin-2 abundance by norepinephrine may involve at least in part cAMP. However, it is possible that long-term regulation of BSC-1 and aquaporin-2 involves additional components such as those of the renin-angiotensin system(231) or the protein synthesis and/or degradation machinery. Additional experiments are required to address these mechanisms.

Recently studies in an animal model of liver cirrhosis induced by common bile duct ligation (CBL), which is associated with increased sodium retention and edema, showed that there was an increase in BSC-1 expression in CBL rats accompanied by an increased natriuretic response to furosemide and that renal denervation attenuated the sodium retention in CBL rats, and resulted in normalization of the natriuretic effect of furosemide, as well as a significant reduction in the expression of BSC-1, suggesting an important role for renal sympathetic nerve activation in BSC-1 regulation(183, 232). It has been suggested that approximately 40% of the

renal sodium retention in edema-forming conditions such as liver cirrhosis(233), congestive heart failure(233), nephrotic syndrome(234) and essential hypertension, characterized by an over-active sympathetic nervous system and increased renal sympathetic nerve activity, is dependent on intact renal sympathetic innervation.

Even though investigators have not yet elucidated the mechanisms leading to the over-activation of the sympathetic nervous system in human and animal models of disease such as essential hypertension, our study is the first to show that norepinephrine regulates the expression/abundance of several key renal sodium transporters of the proximal tubule and thick ascending limb of Henle and the water channel aquaporin-2 of the medullary collecting duct, an effect that can explain the mechanism by which norepinephrine contributes to the enhancement of urinary concentrating ability and sustained antidiuresis in the long-term.

Thus, to our knowledge, this is the first study that highlights a direct relationship between long-term exposure to norepinephrine and increased expression of renal sodium transporters NHE-3, NBC-1 and BSC-1 and water channel aquaporin-2. Our results suggest that the renal sympathetic nervous system may regulate renal excretory function in large part by affecting the expression of transport systems in the nephron. Regulation of sodium transporter and water channel abundances by norepinephrine could additionally explain the abnormal salt and water balance associated with certain pathological disease states involving increased norepinephrine levels such as essential hypertension. A better understanding of how norepinephrine upregulates renal transporters should suggest alternative strategies for the treatment of essential hypertension.

5.0 MECHANISM OF REGULATION OF BSC-1 BY NOREPINEPHRINE

5.1 INTRODUCTION

The thick ascending limb (TAL) of the kidney plays an important role in maintenance of NaCl homeostasis. This segment reabsorbs 25–30% of the filtered NaCl load and generates the corticomedullary osmotic gradient necessary for urine concentration. The bumetanide-sensitive Na-K-2Cl co-transporter (BSC-1/NKCC2) is the major apical Na^+ carrier of the thick ascending limb of the loop of Henle (TAL) and is abundantly expressed in the apical membrane of cortical and medullary TAL and macula densa(103, 104). Because BSC-1 is the principal apical Na^+ entry pathway in the TAL, it is a prime candidate for long-term dysregulation of arterial blood pressure. In support of this concept, recent studies demonstrate that enhanced expression of BSC-1 in the TAL causes sodium retention in rats with congestive heart failure (CHF)(136). Moreover, BSC-1 is up-regulated in rats with small to moderate myocardial infarctions(137), dehydration and cardiac failure(138) and in an animal model of liver cirrhosis(183). Our results in the spontaneously hypertensive rat (SHR), showed that expression of BSC-1 was also elevated in this animal model of essential hypertension and that the natriuretic response to furosemide (which blocks BSC-1) was significantly higher in the SHR compared to its normotensive counterpart, suggesting that BSC-1 could be involved in the development and/or maintenance of hypertension in the SHR(170). Although we were able to establish a role for BSC-1 in regulation of blood pressure in the SHR, the underlying factors responsible for this increase are unknown.

Extensive evidence points to the renal nerves as a link between the sympathetic nervous system and long-term blood pressure control by the kidneys(235). Activation of the renal nerves stimulates renin release(236). Studies in the SHR suggest that the sympathetic nervous system, particularly renal sympathetic nerves, may play an important role in the development of

hypertension in the SHR. Studies have shown that the male SHR has increased sympathetic outflow(237, 238) and renal sympathetic nerve activity compared with normotensive rats(239) and that renal denervation attenuates hypertension in male SHR(205).

In this regard, our studies in the chronic norepinephrine infusion model showed that norepinephrine, the principal neurotransmitter or the sympathetic nervous system regulates BSC-1 protein levels *in vivo* and that administration of norepinephrine resulted in a significant increase in both mean arterial blood pressure and BSC-1 protein levels. Regulation of BSC-1 expression by the sympathetic neurotransmitter norepinephrine could explain the renal effects of the sympathetic nervous system on salt and water excretion and could additionally explain the role of the sympathetic nervous system in a number of disease states associated with altered renal function such as essential hypertension. The present study was thus initiated to determine the underlying molecular mechanism responsible for the regulation of BSC-1 by norepinephrine in an immortalized TAL cell line.

Although studies in whole animals, isolated kidneys or isolated tubules could be used to address the mechanism of regulation of BSC-1 by norepinephrine, each of these techniques has several limitations. Studies involving expression of BSC-1 in oocytes have largely contributed to the understanding of BSC-1 functional kinetics, however it becomes difficult to use such systems to study BSC-1 regulation in the long-term since the endogenous regulators are absent or unknown (102, 112). As a complementary approach to elucidate the underlying mechanism, we used a murine immortalized thick ascending limb cell line, a stable cell line derived from microdissected loops of Henle of the Tg(SV40E)Bri7 mouse, which exhibits furosemide-sensitive Na-K-2Cl activity and endogenous BSC-1 transcript (240). Our results indicate that in the immortalized TAL cell line, norepinephrine regulates BSC-1 levels at the post-transcriptional level *via* the β-adrenoceptor-cAMP-PKA pathway that involves at least in part the MAP kinases and that the α-adrenoceptor negatively regulates BSC-1 protein levels.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and reagents

All chemicals and reagents were obtained from Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise indicated.

5.2.2 Cell culture and treatments

Studies were performed using an immortalized TAL cell line obtained from a transgenic mouse carrying the SV40 large T antigen (courtesy of Dr. Glenn T. Nagami, UCLA)(240, 241). Cells were grown in T-25 flasks in DMEM/F-12 supplemented with 10% FCS, 1mM HEPES, antibiotics and incubated in a humidified 5% CO_2/95% air atmosphere at 37°C. Under these conditions, TAL cells are able to maintain their differentiated state and spontaneously express BSC-1 mRNA and protein (personal communication Dr. Glenn T. Nagami, UCLA). Studies were performed on cells between passages 12-15. Prior to treatment, cells were washed with PBS, trypsinized and plated in 6-well plates in regular cell culture media for 24-48 hrs to reach 60-70% confluence. Cells were serum starved for an additional 24-48 hrs, media was changed everyday, following which cells were treated with various pharmacological agents for 1hr-24 hrs and finally harvested for RNA and protein isolation for RT-PCR and immunoblotting, respectively.

For RNA measurements, cells were pretreated with actinomycin D or cycloheximide for 30 min-1hr, followed by treatment with norepinephrine for 1hr-24 hrs. At various time points during the norepinephrine treatment, RNA was isolated for RT-PCR.

For protein measurements, cells were pretreated with various inhibitors for 30 min, followed by overnight incubation with norepinephrine, following which, cell lysates were prepared for immunoblotting. In a separate set of experiments, cells were treated overnight with vasopressin analogs (AVP, DDAVP) and various cAMP activators (forskolin, 8-Br-cAMP) following which cells were lysed for subsequent immunoblotting.

5.2.3 RNA isolation and RT-PCR

Following treatment, cells were washed with PBS and RNA was isolated using TRIzol reagent (GIBCO Life Technologies, Carlsbad, CA) as per the manufacturer's instructions. By using the primer sequences listed in table 3, RNA (1 μg) was reverse transcribed and amplified using Titanium One-step RT-PCR kit (Clontech, Palo Alto, CA). Each PCR cycle (40 cycles) consisted of denaturing at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 68°C for 60 seconds. RT-PCR products were separated on a 1.2% agarose gel and visualized by incorporating ethidium bromide in the gel.

Table 4. Primers used for RT-PCR analysis of BSC-1

	Accession number	Primer Sequence (5′-3′)	Nucleotides	Product size
BSC-1	U10096	Forward:GCATTGTCTTAACAGGAGGACC Reverse:GAACTGGAGAGATGTCAAACCC	2254 2676	464

5.2.4 Protein isolation and immunoblotting

Following treatment, cells were washed twice with cold PBS, lysed in lysis buffer (10mM Tris HCl, pH 7.4, 1% TritonX-100, 2mM EDTA, 10μg/mL aprotonin, 1μg/mL leupeptin, 1mM PMSF, 50mM $Na_4P_2O_7$, 50mM NaF and 1mM NaV_3O_4). The lysate was then centrifuged at 14,000 rpm for 20 minutes and the supernatant collected for protein concentration determination and sample preparation. Protein concentration was measured using the BCA protein assay method. Proteins were solubilized at 60° C for 15 min in Laemmli sample buffer. SDS-PAGE was performed on gradient polyacrylamide gels (4-12%) loaded with 10μg protein per lane. For immunoblotting, proteins were transferred electrophoretically to PVDF membranes. Membranes were blocked in 5% milk for 2 hours, probed for 2 hrs at 37° C with the respective primary antibodies in PBS containing 1% milk: rabbit anti-BSC-1 monoclonal antibody (1:5000, gift of Dr. Biff Forbush, Yale University, New Haven, CT) or mouse anti-β-actin monoclonal antibody (1:3000, A5441 Sigma Chemical Co., St. Louis, MO). Subsequently, membranes were exposed to a secondary HRP conjugated donkey anti-rabbit or sheep-anti-mouse polyclonal antibody

(1:5000, Pierce Biotechnology Inc., Rockford, IL) in PBS containing 1% milk for 1 hour at room temperature. Bound antibodies were visualized using a luminol-based enhanced chemiluminescence substrate (SupersignalWest Dura Extended Duration Substrate, Pierce Biotechnology Inc., Rockford, IL) before exposure to X-ray film (Kodak 165-1579; Eastman Kodak Co., Rochester, NY). Densitometric analysis was performed using ImageQuant TL (Amersham Biosciences, Piscataway, NJ) and band densities were normalized to β-actin.

5.2.5 Statistical analysis

All data are presented as mean ± SEM. Where appropriate, comparisons between groups were made by unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post-test to determine statistical significance. *P* values <0.05 were considered significant.

5.3 RESULTS

5.3.1 Vasopressin, forskolin and 8-Br cAMP regulate BSC-1 protein levels in TAL cell line

Previous studies show that vasopressin regulates BSC-1 protein levels *via* the V_2 receptor(120). Additionally, studies have also shown that BSC-1 protein levels, trafficking and function are regulated by cAMP(112, 113, 182). To determine whether the immortalized TAL cell line is an effective model system to study regulation of BSC-1, cells were treated with arginine vasopressin (AVP) and the selective V_2 receptor vasopressin analog DDAVP. Additionally, cells were also treated with the adenylyl cyclase activator forskolin and the cell permeable cAMP analog 8-Br cAMP. Our results show that in the TAL cell line, both AVP and DDAVP increased BSC-1 protein levels over control (Figure 23). Treatment with cAMP activator forskolin and 8-Br cAMP also increased BSC-1 protein levels over control (Figure 23). Thus in the immortalized TAL cell culture model we were able to reproduce the effects of vasopressin and cAMP on BSC-

1 protein, as had been previously reported in the chronic vasopressin infusion model and in oocytes transfected with BSC-1 mRNA, validating the use of the TAL cell line to study the effects of norepinephrine on BSC-1 regulation.

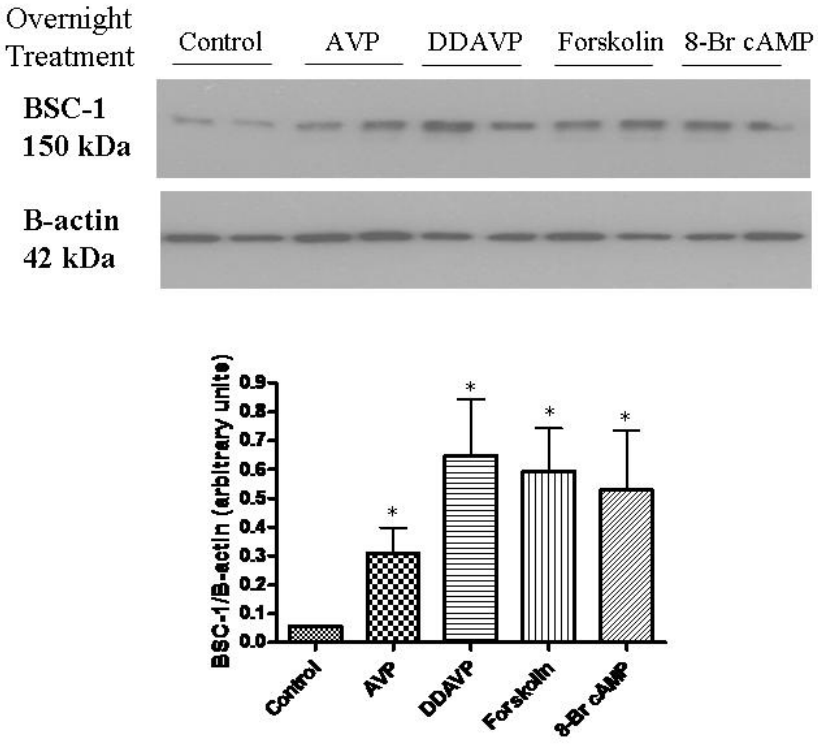

Figure 23. Effect of vasopressin and cAMP activators on BSC-1 protein in TAL cells

Immunoblot analysis of BSC-1 protein levels in serum-starved TAL cells treated overnight with control (C) or 1μM arginine vasopressin (AVP), 1μM vasopressin receptor type-2 selective analog DDAVP, 10μM adenylyl cyclase activator forskolin or 0.5mM cell-permeable analog 8-Br cAMP. Following treatment, cells were washed in cold PBS and protein was isolated for immunoblotting. Top panel: Immunoblot for BSC-1 protein in TAL cells following treatment. Bottom panel: densitometric analysis of BSC-1 protein in TAL cells following treatment. Band densities were normalized to β-actin. Values represent means ± SEM, *P* values <0.05 were considered significant, n=4.

5.3.2 Norepinephrine does not alter BSC-1 mRNA levels or half-life

To determine the cellular mechanism responsible for the regulation of BSC-1 by norepinephrine, TAL cells were treated with norepinephrine in the presence or absence of actinomycin D or cycloheximide. Treatment with norepinephrine alone had no effect on BSC-1 mRNA compared to control (Figure 24A). Additionally inhibition of transcription with actinomycin D or translation with cycloheximide failed to alter BSC-1 mRNA levels (Figure 24 B and 25B) suggesting that regulation of BSC-1 by norepinephrine proceeds *via* a post-transcriptional mechanism and does not involve regulation at the mRNA level.

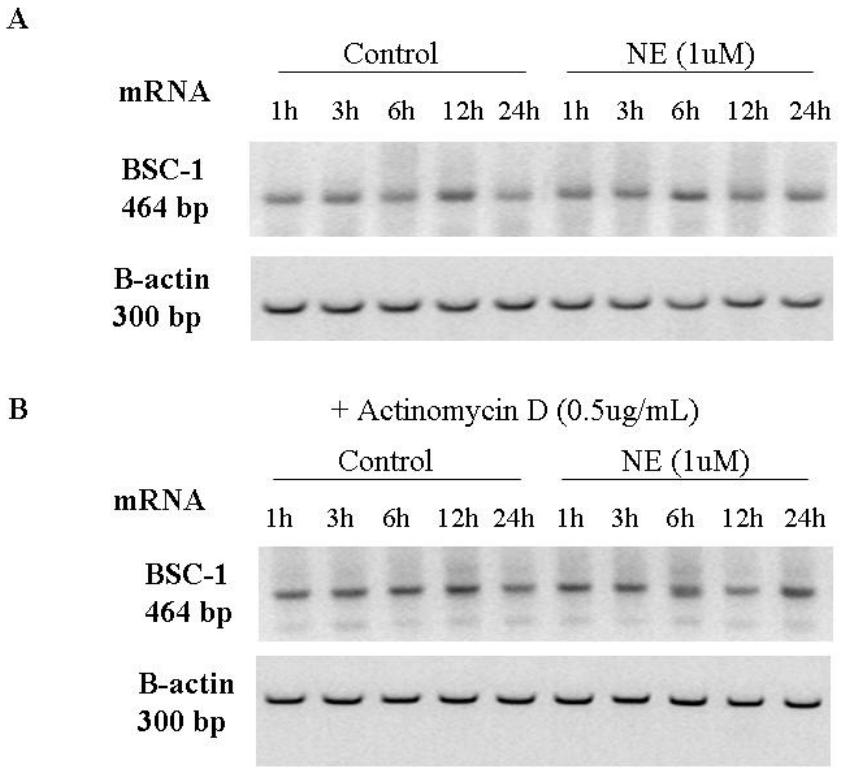

Figure 24. Effect of norepinephrine and actinomycin D on BSC-1 mRNA in TAL cells

RT-PCR analysis of BSC-1 mRNA in serum-starved TAL cells treated with control (vehicle) or 1μM norepinephrine (NE) for 1hr-24hrs in the presence or absence of actinomycin D (0.5μg/mL). At each time point following treatment, cells were washed in cold PBS and RNA was isolated for RT-PCR. Top panel: TAL cells treated with control or norepinephrine alone. Bottom panel: TAL cells treated with control (vehicle) or norepinephrine (1μM) in the presence of actinomycin D (0.5μg/mL, added 1hr prior to norepinephrine treatment).

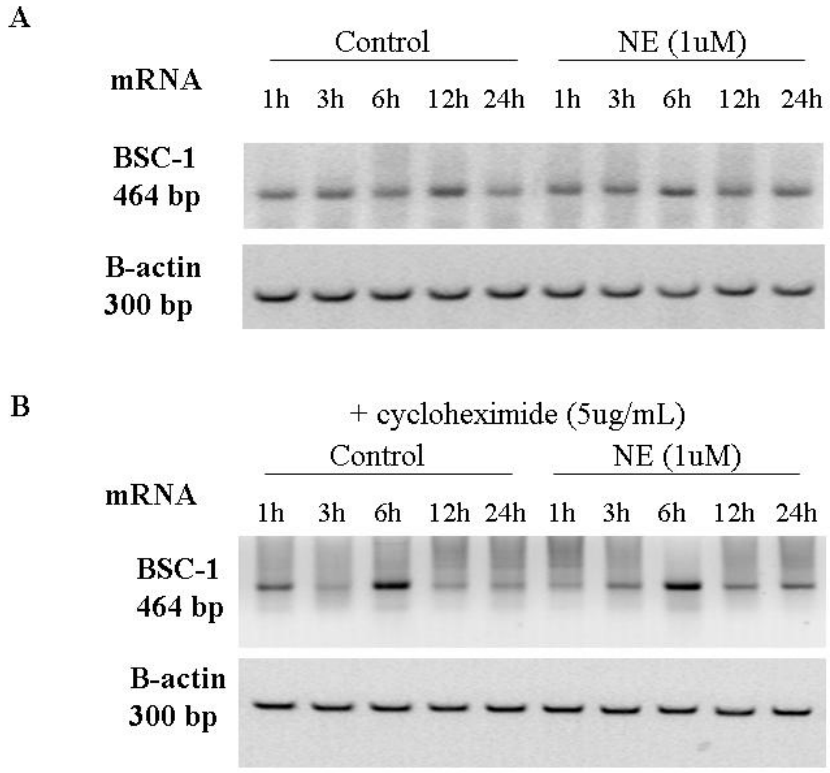

Figure 25. Effect of norepinephrine and cycloheximide on BSC-1 mRNA in TAL cells

RT-PCR analysis of BSC-1 mRNA in serum-starved TAL cells treated with control (vehicle) or 1μM norepinephrine (NE) for 1hr-24hrs in the presence or absence of cycloheximide (5μg/mL). At each time point following treatment, cells were washed in cold PBS and RNA was isolated for RT-PCR. Top panel: TAL cells treated with control or norepinephrine alone. Bottom panel: TAL cells treated with control (vehicle) or norepinephrine (1μM) in the presence of cycloheximide (5μg/mL, added 30min prior to norepinephrine treatment).

5.3.3 Norepinephrine increases BSC-1 protein levels following treatment

Since norepinephrine did not alter BSC-1 mRNA levels, we wished to determine whether norepinephrine could regulate BSC-1 protein levels in the immortalized TAL cell line. Treatment with 1μM norepinephrine significantly increased BSC-1 protein levels compared to control (70%, *P*= 0.012 n=4) (Figure 26). Pretreatment of TAL cells with the protein synthesis inhibitor cycloheximide blocked the effects of norepinephrine on BSC-1 protein, while having no effect

on control-treated cells (Figure 26) suggesting that norepinephrine regulates BSC-1 protein levels *via* a translational mechanism and/or involves additional proteins or factors.

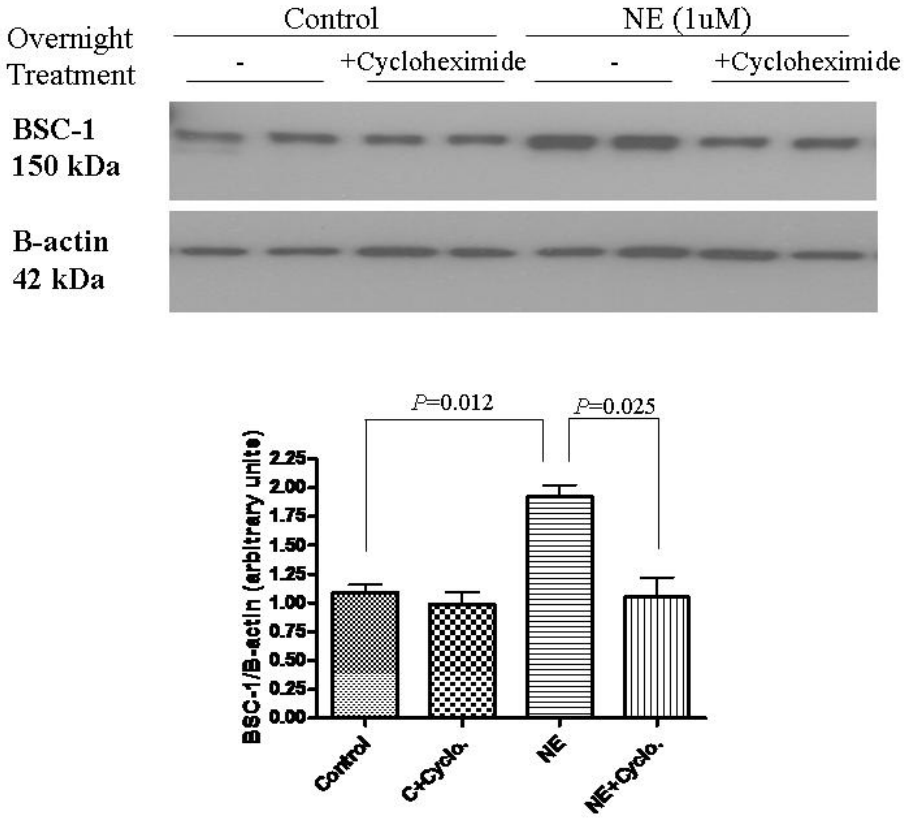

Figure 26. Effect of norepinephrine and cycloheximide on BSC-1 protein in TAL cells

Immunoblot analysis of BSC-1 protein levels in serum-starved TAL cells treated overnight with control (C) or 1μM norepinephrine (NE) in the presence or absence of cycloheximide (5μg/mL, added 30min prior to norepinephrine treatment). Following treatment, cells were washed in cold PBS and protein was isolated for immunoblotting. Top panel: Immunoblot for BSC-1 protein in TAL cells treated with control or norepinephrine in the presence or absence of cycloheximide. Bottom panel: densitometric analysis of BSC-1 protein in TAL cells following treatment. Band densities were normalized to β-actin. Values represent means ± SEM, *P* values <0.05 were considered significant, n=4.

5.3.4 Regulation of BSC-1 by norepinephrine involves both α- and β-adrenoceptors

The TAL is important for salt and water homeostasis and possesses both α and β adrenoceptors. The endogenous neurotransmitter norepinephrine activates both types of adrenergic receptors. To

determine the role of each receptor subtype in regulation of BSC-1, TAL cells were pretreated with 5μM of the α-blocker phentolamine or 5 μM of the β-blocker propranalol for 30 minutes, followed by treatment with 1 μM norepinephrine. Pretreatment with propranolol completely blocked the effect of norepinephrine on BSC-1 protein (Figure 27), while pretreatment with phentolamine resulted in a significant increase in BSC-1 protein levels compared with norepinephrine alone (30%, *P*= 0.02, n=4) (Figure 27). These results indicate that in TAL cells, BSC-1 protein levels are positively regulated by the β-adrenoceptor and negatively regulated by the α-adrenoceptor.

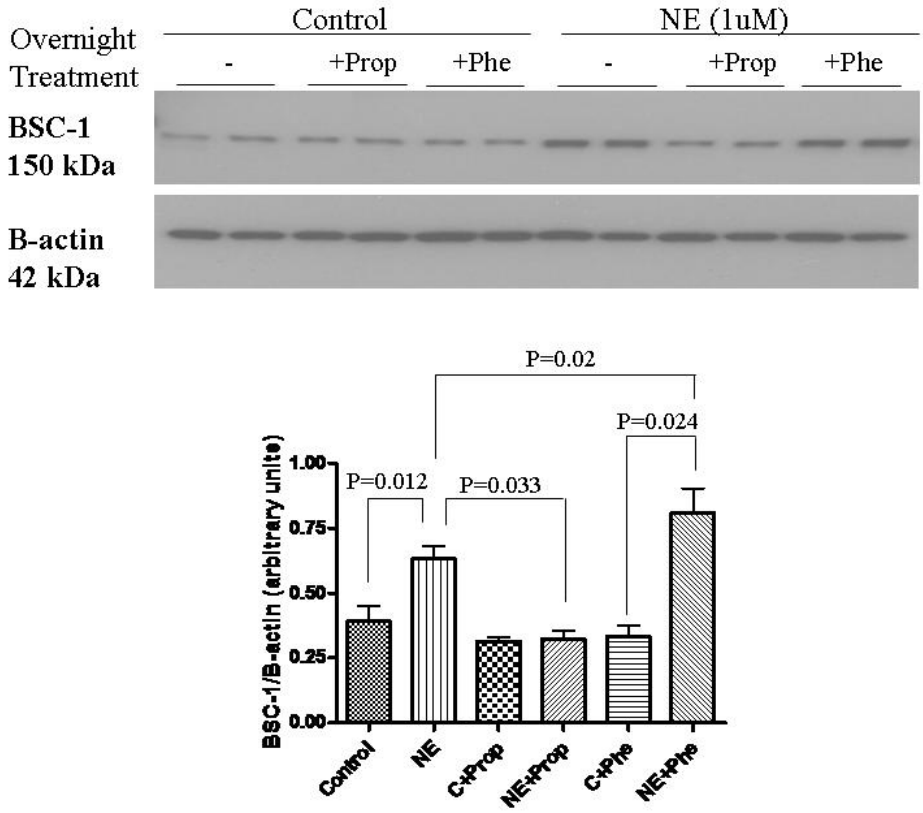

Figure 27. Effect of α and β-blockers on BSC-1 protein levels following norepinephrine treatment

Immunoblot analysis of BSC-1 protein levels in serum-starved TAL cells treated overnight with control (C) or 1μM norepinephrine (NE) in the presence or absence of α-blocker phentolamine (Phe) or β-blocker propranolol (Prop) (5μM each, added 30min prior to norepinephrine treatment). Following treatment, cells were washed in cold PBS and protein was isolated for immunoblotting. Top panel: Immunoblot for BSC-1 protein in TAL cells treated with control or norepinephrine in the presence or absence of phentolamine and propranolol. Bottom panel:

densitometric analysis of BSC-1 protein in TAL cells following treatment. Band densities were normalized to β-actin. Values represent means ± SEM, *P* values <0.05 were considered significant, n=4.

5.3.5 Regulation of BSC-1 by norepinephrine proceeds *via* cAMP dependent pathway and involves in part MAP kinases

To elucidate the mechanism of regulation of BSC-1 by norepinephrine, TAL cells were treated with 1μM norepinephrine in the presence of an adenylyl cyclase inhibitor SQ22536 (50μM) and a MEK inhibitor PD090859 (25μM) to determine the role of cAMP and MAP kinases respectively. Treatment with the adenylyl cyclase inhibitor inhibited the effect of norepinephrine on BSC-1 protein (Figure 28), suggesting that activation of cAMP by norepinephrine is the mechanism involved. Treatment with the MEK inhibitor, however only partially inhibited the effect of norepinephrine (40% decrease, *P*=0.03, n=4) (Figure 28), suggesting that in addition to MAP kinases, additional factors are involved in mediating the effects of norepinephrine.

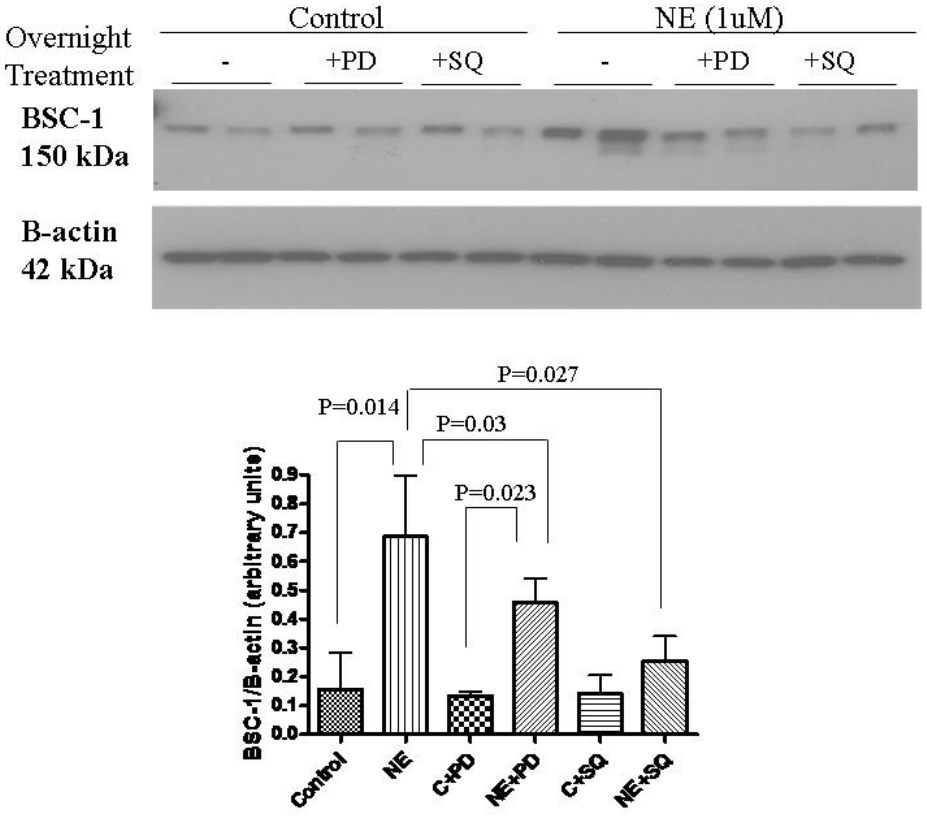

Figure 28. Effect of MEK and adenylyl cyclase inhibition on BSC-1 protein levels following treatment with norepinephrine

Immunoblot analysis of BSC-1 protein levels in serum-starved TAL cells treated overnight with control (C) or 1μM norepinephrine (NE) in the presence or absence of MEK inhibitor PD090859 (PD) or adenylyl cyclase inhibitor SQ22536 (SQ) (25μM and 50μM respectively, added 30min prior to norepinephrine treatment). Following treatment, cells were washed in cold PBS and protein was isolated for immunoblotting. Top panel: Immunoblot for BSC-1 protein in TAL cells treated with control or norepinephrine in the presence or absence of PD090859 and Q22536. Bottom panel: densitometric analysis of BSC-1 protein in TAL cells following treatment. Band densities were normalized to β-actin. Values represent means ± SEM, *P* values <0.05 were considered significant, n=4.

5.3.6 Regulation of BSC-1 proceeds *via* a PKA dependent pathway

BSC-1 protein contains potential cAMP dependent kinase and PKC phosphorylation sites in the C and N-terminal domains, thus supporting the hypothesis that PKA and/or PKC could be involved in regulation of BSC-1 by norepinephrine (94). To determine the role of PKA and PKC in regulation of BSC-1 protein by norepinephrine, TAL cells were treated with a PKA inhibitor H-89 and a PKC inhibitor stuarosporine prior to norepinephrine treatment. Treatment with the PKA inhibitor H-89 abolished the effect of norepinephrine on BSC-1 (Figure 29), while treatment with the PKC inhibitor staurosporine had no effect on BSC-1 protein levels (Figure 29), suggesting that at least in TAL cells, PKC may not be involved in regulation of BSC-1 by norepinephrine. PKC inhibition resulted in a slight increase in BSC-1 protein levels in the presence of norepinephrine, although this failed to reach statistical significance.

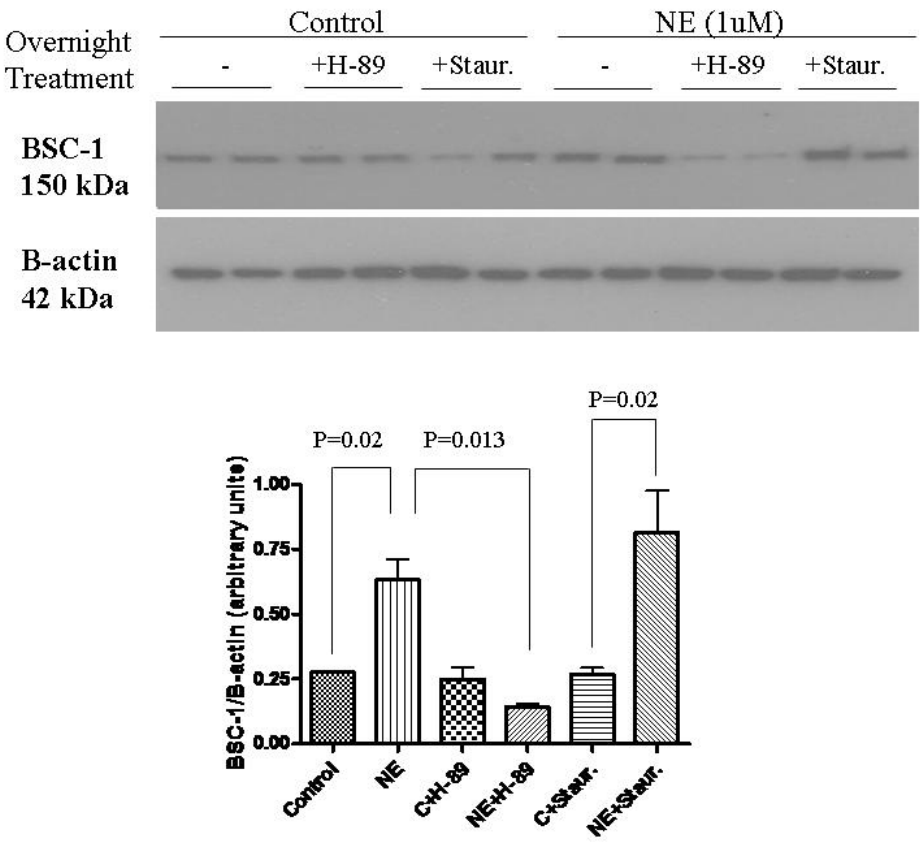

Figure 29. Effect of PKA and PKC inhibition on BSC-1 protein levels following treatment with norepinephrine

Immunoblot analysis of BSC-1 protein levels in serum-starved TAL cells treated overnight with control (C) or 1μM norepinephrine (NE) in the presence or absence of PKA inhibitor H-89 or PKC inhibitor Staurosporine (Staur.) (15μM and 10nM respectively, added 30min prior to norepinephrine treatment). Following treatment, cells were washed in cold PBS and protein was isolated for immunoblotting. Top panel: Immunoblot for BSC-1 protein in TAL cells treated with control or norepinephrine in the presence or absence of H-89 and Staurosporine. Bottom panel: densitometric analysis of BSC-1 protein in TAL cells following treatment. Band densities were normalized to β-actin. Values represent means ± SEM, *P* values <0.05 were considered significant, n=4.

5.4 DISCUSSION

As an alternative approach to study the regulation of BSC-1 by norepinephrine, we employed an immortalized TAL cell line derived from the kidney of a mouse transgenic for SV_{40} T antigen.

These cells have been extensively characterized and display characteristics of differentiated TAL cells; they form polarized monolayers with distinct apical and basolateral domains, have few microvilli at their apical surface, form tight junctions, exhibit numerous mitochondria, a pattern related to an active metabolism, express Tamm-Horsfall protein on the apical membrane and endogenous BSC-1 transcript(240-242). As a preliminary approach to validate the use of the TAL cell line, we examined whether known regulators such as vasopressin and cAMP affect BSC-1 protein levels. Our results indicate that we were able to reproduce the effects of vasopressin and cAMP on BSC-1 protein as had been previously reported in the chronic vasopressin infusion model and in oocytes transfected with BSC-1 mRNA(113, 120). Thus, we were able to establish that the TAL cell line was a good model system to study the effects of the native sympathetic neurotransmitter norepinephrine on BSC-1 mRNA and protein levels and accordingly used specific agonists and antagonists to determine the underlying molecular mechanisms involved in the regulation of BSC-1 by norepinephrine.

Treatment of TAL cells with norepinephrine, resulted in an increase in BSC-1 protein, the effect on BSC-1 mRNA was not significant, suggesting that the effect of norepinephrine proceeds *via* a post-transcriptional mechanism. Although, the BSC-1/NKCC2 promoter contains consensus binding sites for transcription factors such as cAMP-response element binding protein (CRE), NF-κB, interferon-γ activation factor, interferon-α-stimulated gene factor-3, activator protein-1 and activator protein-2, that could function as effector molecules in signal transduction pathways (240), norepinephrine failed to regulate BSC-1 mRNA levels. Thus, in TAL cells, we were unable to establish a transcriptional mechanism of regulation of BSC-1 by norepinephrine since treatment with actinomycin D did not alter levels of BSC-1 mRNA over control. Pretreating cells with cycloheximide completely inhibited the effect of norepinephrine on BSC-1 protein, suggesting that regulation of BSC-1 by norepinephrine proceeds *via* post-transcriptional mechanisms and that additional proteins may be involved in regulation of BSC-1. Altered degradation/recycling has been proposed to be the mechanism responsible for increased expression and/or function of several renal transporters and proteins such as the water channel aquaporin-2 (243) and the epithelial sodium channel (ENaC) of the collecting duct (244). Altered degradation/recycling of BSC-1 has been proposed to be the mechanism involved in regulation of BSC-1 by vasopressin (120) and could be the mechanism involved in regulation of BSC-1 by norepinephrine.

Studies in isolated thick ascending tubules have shown that norepinephrine stimulates cAMP generation *via* the β-adrenergic receptor (212, 245, 246) with β1 being the predominant subtype(247). Treatment with a β-blocker propranolol, adenylyl cylase inhibitor SQ22536, and PKA inhibitor H-89 completely blocked the effects of norepinephrine on BSC-1, suggesting that the β-adrenoceptor-cAMP-PKA pathway is involved in regulation of BSC-1. Treatment with the MAPK inhibitor PD098059 partially blocked the effect of norepinephrine on BSC-1, suggesting that MAPK are partially involved, along with additional signaling molecules in regulation of BSC-1. Recently studies identified WNK kinases as the integrative upstream regulators of renal sodium transport systems(68, 248, 249). WNK, with no lysine (K) kinases, are serine-threonine protein kinases that have been linked to regulation of a number of renal transporters, particularly WNK3 kinase was identified as a positive regulator of NKCC2(250). It remains to be determined whether WNK kinases act downstream of norepinephrine and cAMP activation in the regulation of BSC-1 abundance.

Treatment with the PKC inhibitor staurosporine had no effect on BSC-1 protein levels, suggesting that at least in immortalized TAL cells, PKC may not be involved in the regulation of BSC-1. A similar observation was made in oocytes transfected with BSC-1 cRNA, where PKC activation was found to inhibit BSC-1 function, an effect that could not be inhibited by either the specific PKC inhibitor Gö6976 or the non-specific PKC inhibitors staurosporine and H-7, suggesting that a novel PKC isoform may be involved in regulation of BSC-1(251). Novel PKCs and atypical PKCs have been reported to be expressed in TAL(252), however it is yet to be determined whether such novel or atypical PKCs could be involved in regulation of BSC-1 by norepinephrine.

Pretreatment with the α-adrenoceptor blocker phentolamine resulted in a small but significant increase in BSC-1 protein levels, suggesting that the α-adrenoceptor may negatively regulate BSC-1. Negative regulation of BSC-1 function by the α-adrenoceptor has been previously reported in TAL, where selective α-2 adrenoceptor activation inhibits chloride flux (J_{Cl}) and selective β-adrenoceptor activation stimulates J_{Cl} (245). However, additional studies would be required to characterize the role of α-adrenoceptors in the regulation of BSC-1 abundance.

Thus, in summary, norepinephrine was found to regulate BSC-1 protein levels in an immortalized thick ascending limb cell line *via* a β-adrenoceptor-cAMP-PKA dependent

pathway that involves MAP kinases. The importance of a role for β-adrenoceptors in regulation BSC-1 abundance is especially relevant in essential hypertension. β-adrenergic blockers are frequently used in antihypertensive therapy because of their effect on myocardial contractility and cardiac output. The results of the present study indicate that there may be additional benefits to β-adrenergic blocker therapy *via* alterations in renal tubular transporter systems and tubular function, particularly with respect to inhibition of BSC-1 abundance and/or function in the thick ascending limb, that would promote natriuresis and diuresis, thus aiding in the management of inappropriate salt and water retention associated with essential hypertension.

6.0 SUMMARY AND DISCUSSION

This dissertation focuses on the role of the renal bumetanide-sensitive Na-K-2Cl cotransporter BSC-1/NKCC2 in the pathogenesis of essential hypertension and its regulation by the sympathetic nervous system, the over-activation of which is believed to be an initiating factor in essential hypertension. It has been proposed that the kidneys are normal in the prehypertensive state, but renal alterations are induced by a variety of factors, such as a hyperactive sympathetic nervous system or alterations in the renin-angiotensin system, induced by stress, environmental or genetic factors. The net effect of these factors would be increased sodium retention as a consequence of tubular (increased sodium reabsorption) and glomerular (decreased glomerular filtration rate) mechanisms, resulting in a volume-dependent rise in blood pressure as postulated by Guyton. A better understanding of the underlying molecular mechanisms may lead to improved treatment approaches.

6.1 BSC-1 AS A CRITICAL LINK IN ESSENTIAL HYPERTENSION

The spontaneously hypertensive rat (SHR) is one of the most extensively used genetic models of human essential hypertension. The factors responsible for increased blood pressure in the SHR are not fully understood, although studies indicate both renal and non-renal mechanisms to be involved (6-8). In the SHR, blunting of the pressure-natriuresis curve is observed, such that greater perfusion pressures are required to achieve the same level of diuresis compared to its normotensive counterpart, the Wistar-Kyoto rat (WKY)(9).The pressure-natriuresis curve is altered even in very young SHR, indicating that the resetting of kidney function occurs very early and may be necessary for the development of hypertension in the SHR(10). Additionally, sympathetic nerve activity is elevated in this strain and neurohumoral reactivity to environmental

stress is enhanced compared with normotensive rats (12). Also, brief angiotensin-converting enzyme inhibition in juvenile SHR, as well as neonatal interruption of peripheral sympathetic innervation, chronically reduces arterial pressure associated with a reduction in peripheral vascular resistance (11). Finally, the results of renal transplantation experiments in SHR and normotensive rat strains (WKY) are consistent with the concept that the kidneys regulate long-term levels of arterial blood pressure and that a defect in the kidney is importantly involved in the pathogenesis of genetic hypertension (6, 144, 145). Moreover, studies using isolated perfused kidneys from spontaneously hypertensive rats (SHR) reveal an intrinsic renal abnormality in Na^+ excretion that may contribute to the maintenance of hypertension in SHR (146).

Our studies show that BSC-1 protein expression is higher (6-fold, $P<0.001$) in the adult SHR compared to its normotensive counterpart the WKY (170), and the progression from pre-hypertensive to hypertensive state in the SHR is accompanied by a proportional increase in both steady state protein levels of BSC-1 as well as its distribution to the plasma membrane (moderately hypertensive 4-fold; severely hypertensive 6-fold, each $P<0.001$), indicating that BSC-1 expression and distribution are stage dependent and increase as hypertension progresses. The increased presentation of BSC-1 at the plasma membrane could result in increased sodium reabsorption and thereby contribute to the pathogenesis of hypertension in the SHR.

Finally, our studies show that adult SHR rats are more sensitive to the effects of furosemide, resulting in a 3-fold increase ($P<0.05$) in sodium excretion along with normalization of blood pressure and that the effect of furosemide is specific for SHR, with no effect on mean arterial blood pressure in WKY (170), thus supporting our hypothesis that BSC-1 is involved in the pathogenesis of hypertension in the SHR. Additional data supporting this hypothesis come from a study by Kiprov et al., where long-term administration of furosemide to pre-hypertensive SHR resulted in a delay in the development of hypertension compared to untreated SHR(253).

Studies with other diuretic agents such as thiazide diuretics (which block the thiazide-sensitive Na-Cl cotransporter of the distal tubule) show that thiazide diuretics alone have no effect on mean arterial blood pressures in SHR, while treatment with thiazide diuretics along with other antihypertensive drugs has little or no effect on mean arterial blood pressures in SHR depending on the antihypertensive drug used (254, 255). Thus both the biochemical as well as pharmacological data supports the hypothesis that BSC-1 is involved in the pathogenesis of

essential hypertension and drugs that target/alter BSC-1 expression or alternatively block BSC-1 activity may be useful for the treatment of essential hypertension.

6.2 LINK BETWEEN OVERACTIVE-SYMPATHETIC NERVOUS SYSTEM AND BSC-1 EXPRESSION

Over-activation of the sympathetic nervous system has been associated with several disorders such as essential hypertension (195, 199, 256) and chronic heart failure (198), all of which are characterized by increased salt and water retention by the kidney. However, the precise molecular mechanism of this is poorly understood. Many lines of evidence indicate that the sympathetic nervous system, *via* the renal nerves, plays an important role in the pathogenesis of essential hypertension in humans and laboratory animals(22, 195). Patients with established essential hypertension have increased sympathetic nervous system activity, as evidenced by increased plasma and urinary norepinephrine levels, elevated excretion of catecholamine metabolites, and an exaggerated depressor response to centrally acting sympatholytic agents(196-199). In the SHR and in the DOCA/NaCl hypertensive model, increased renal efferent nerve activity contributes to the development of hypertension by causing increased renal sodium retention(203, 204). In both of these experimental models, renal denervation delays the development and blunts the severity of hypertension and is associated with increased urinary sodium excretion, suggesting a renal efferent mechanism(205-207).

Our results show that chronic administration of norepinephrine, the principal neurotransmitter of the sympathetic nervous system, increases mean arterial blood pressures *in-vivo* (145 mmHg vs. control 115 mmHg, $P<0.001$) and the increase in blood pressure is accompanied by an increase in protein expression of BSC-1 (4-fold, $P<0.001$ over control). The regulation of BSC-1 expression by the sympathetic neurotransmitter norepinephrine is a novel finding that may help explain the renal effects of the sympathetic nervous system on salt and water excretion (22) and highlights a crucial role for the sympathetic nervous system in a number of disease states associated with altered renal function such as essential hypertension.

Further elucidation of the mechanism of BSC-1 regulation by norepinephrine in an immortalized thick ascending limb cell line showed that regulation of BSC-1 proceeds *via* post-

transcriptional mechanisms *via* activation of the β-adrenergic receptor-adenylyl cyclase-cAMP-PKA pathway that utilizes in part MAP kinases (Figure 30). Studies in isolated tubules have previously shown that norepinephrine stimulates cAMP generation in thick ascending limb of Henle *via* the β-adrenergic receptor (212, 245) with β1 being the predominant subtype (246, 247). An important finding of the study is the possible role of the α-adrenergic receptor in regulation of BSC-1. Our results indicate that the α-adrenergic receptor negatively regulates BSC-1 protein levels, although additional studies would be necessary to further elucidate the role of the adrenergic receptor and the underlying molecular mechanisms. This result is also consistent with *in-vivo* data that showed intrarenal administration of selective α-2 agonist clonidine increased osmotic and free water clearance in rats(257). The importance of a role for selective α-2 adrenoceptor stimulation of the TAL can be expressed in the condition of human arterial hypertension. α-2 adrenoceptor agonists are frequently used in antihypertensive therapy, specifically to inhibit central sympathetic outflow. The results of the current study and previous reports(245, 258) indicate that there may be additional benefits to β-blocker and α-2 agonist therapy *via* alterations in renal tubular function. These include the inhibition of BSC-1 expression and sodium chloride absorption from the TAL that would promote natriuresis and, because of the primary role of the TAL in the generation of the corticomedullary solute gradient and urinary concentrating mechanism, water excretion as well. Both alterations in renal function would aid in the management of inappropriate salt and water retention in essential hypertension.

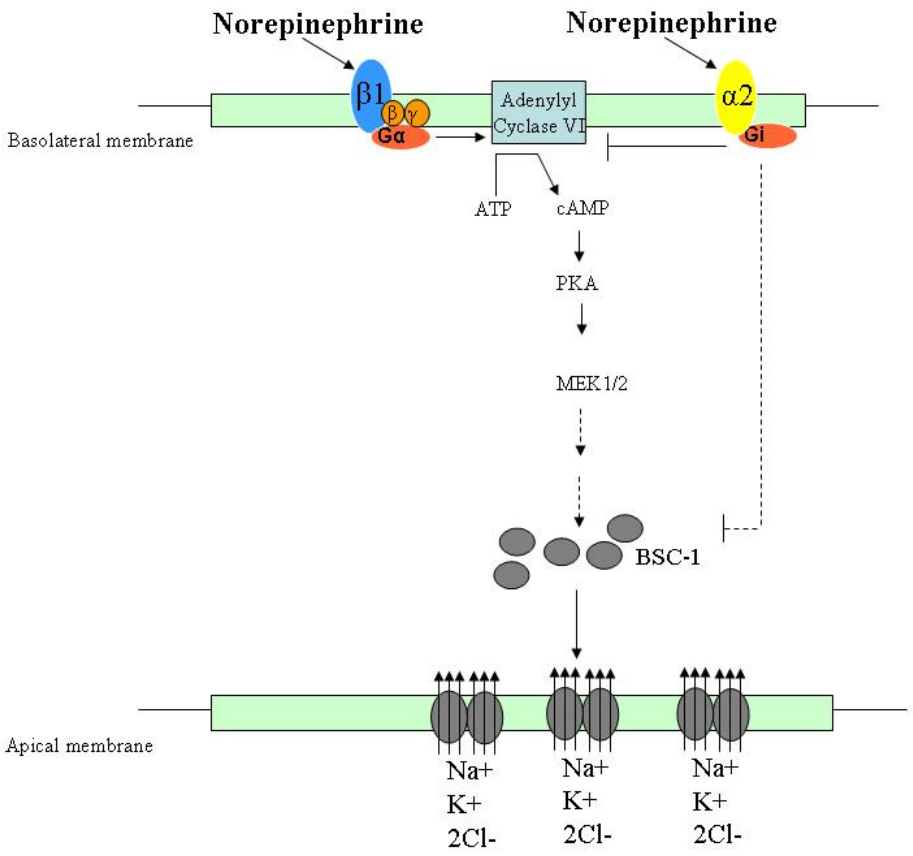

Figure 30. Proposed mechanism of BSC-1 protein regulation by norepineprhine

6.3 ADDITIONAL FACTORS

Although our studies demonstrate a role for BSC-1 (under the influence of an over-active sympathetic nervous system) in hypertension in the SHR, it does not rule out the role of other factors such as the renin-angiotensin system, circulating vasopressin, reactive-oxygen species and inflammatory responses, all of which have also been implicated to play a part in the progression or maintenance of hypertension, or the genetic component to essential hypertension. Recent studies have shown that BSC-1 activity and expression are regulated by vasopressin(120), glucocorticoids(124), angiotensin II(259-261), superoxides(186, 262), nitric oxide(119, 263), prostaglandins(122), metabolic acidosis(129) and chronic saline loading(103) and is down-regulated by potassium depletion(264). Our study also does not rule out the

involvement of other sodium transporters. Previous studies have show that sodium reabsorption in the proximal tubule is also increased in the SHR(154, 162). Studies also show that sodium transporters of the proximal tubule, namely the NHE-3, and Na^+-K^+-ATPase are upregulated in the SHR kidney(150, 155). Our data are in concordance with these findings. In addition, our studies indicate that expression of the potassium-channel ROMK-1 is also higher in the inner medulla of the SHR. Thus, it appears that the pathophysiology of essential hypertension is complex and that several transporters and factors may be involved. Nevertheless, our studies also highlight a crucial role for BSC-1 in the development and/or maintenance of hypertension.

6.3.1 Renin-angiotensin system and hypertension

There is extensive adrenergic innervation of the renal vasculature and renal tubules, and it is well established that renal nerves play an important role in the control of renin release and renal function. Additionally, both norepinephrine released from adrenergic nerve terminals and angiotensin II generated in response to renal nerve stimulation have potent direct effects on real hemodynamics and sodium excretion. Additionally, high plasma levels of angiotensin II are almost always present in patients and animal models of essential hypertension and since blockade of the renin-angiotensin system may temporarily attenuate some of the symptoms of hypertension, it would appear that the renin-angiotensin system is critically involved in the pathogenesis of the vicious cycle of essential hypertension. The decisive action of angiotensin II in this vicious cycle may include any one or a combination of the following pathological (structural) of physiological (functional) events: progressive pathological narrowing of renal blood vessels, progressive afferent arteriole constriction, progressive efferent arteriole constriction, or increased tubular reabsorption of sodium. In this regard, studies in dogs pretreated with the angiotensin converting enzyme inhibitor captopril indicate that the hypertensive crisis associated with intrarenal norepinephrine infusion is critically dependent on the renin-angiotensin system(265). Thus, there is an important interaction between the renin-angiotensin system and the sympathetic nervous system in the control of renal function and arterial blood pressure that may be especially relevant to the pathogenesis of essential hypertension. Recently, studies showed that angiotensin II infusion increases BSC-1 protein

abundance *in vivo*(126) and that angiotensin II controls BSC-1 function in TAL tubules *via* 20-HETE and PKC(261). Thus, the specific contribution of the renin-angiotensin system to the regulation of renal transport systems (BSC-1) under the influence of an over-active sympathetic system, as seen in essential hypertension remains to be determined.

1.1.1 Role of WNK kinases

The role of BSC-1 and other renal transport systems in blood pressure regulation has been well established: loss of function mutations in genes encoding NKCC2, ROMK or the basolateral chloride channel ClC-Kb cause Bartter's syndrome, and loss of function mutations in genes encoding thiazide sensitive Na-Cl cotransporter NCC are responsible for Gitelman's syndrome, both inherited disorders featuring low blood pressure due to salt wasting. Although our studies, as well as previous reports, show that hormones such as norepinephrine vasopressin, aldosterone, angiotensin II and glucocorticoids regulate these transporter proteins, thereby maintaining sodium chloride, water and blood pressure homeostasis, until recently the transducers that link hormonal signaling to the downstream targets (transporters) were unknown. Recent studies identified WNK kinases as the integrative upstream regulators of renal sodium transport systems(68, 248, 249). WNK, with no lysine (K) kinases, are serine-threonine protein kinases that have been linked to regulation of a number of renal transporters, particularly WNK3 kinase was recently identified as a positive regulator of both NKCC2 and NCC(250). Mutations in WNK1 and WNK4 have been found to cause pseudohypoaldosteronism type II (PHA II), a disease characterized by hypertension and hyperkalemia, due to a coupled increase in NaCl reabsorption and deficiency in renal K+ secretion(69). It remains to be determined whether WNK kinases act downstream of norepinephrine and cAMP activation in the regulation of BSC-1 abundance. It is also unknown at present whether the activity and/or expression of WNK kinases are altered in the SHR. The role of WNK kinases in essential hypertension has not been previously studied, but we hypothesize that WNK kinases play an important role in the pathogenesis of hypertension in the SHR and further studies are needed to explore this possibility.

6.4 UNIFYING PATHWAY FOR ESSENTIAL HYPERTENSION

According to the Guytonian paradigm, long-term control of arterial pressure takes place *via* renal mechanisms (pressure-natriuresis mechanisms) and that all factors that are believed to play a role in the long-term regulation (or dysregulation) of arterial blood pressure would do so *via* the kidney. We propose a unifying pathway for essential hypertension that unites many of the previous hypotheses, including our hypothesis. In the prehypertensive state, the kidneys are believed to be normal, but that renal alterations and/or injury are initiated in most circumstances by repeated and intermittent renal vasoconstriction induced by a variety of factors, with an overactive sympathetic nervous system being at the forefront. Our hypothesis is that renal-catecholamine interactions are the triggering factor for the pathogenesis of hypertension produced by an overactive sympathetic nervous system that is, in turn, induced by stress, environmental or genetic factors. Activation of the SNS would additionally result in activation of the renin-angiotensin system resulting in elevated circulating angiotensin II levels, enhanced vasoconstriction, vascular damage and inflammation, renal ischemia and renal dysfunction due to direct effects of circulating hormones (including norepinephrine) on renal vasculature and on renal tubular transport systems (BSC-1), resulting in the development and maintenance of a persistent hypertensive state, as is the case in essential hypertension.

6.5 CONCLUSION

The present studies were based on the concept that the pathogenesis of sustained hypertension involves the kidneys. Altered renal sodium handling in the SHR has been previously reported(9, 154); however, the factors contributing to this effect are unknown. Our studies show that in the SHR, there is increased expression of the renal sodium transporter BSC-1, the inhibition of which results in normalization of blood pressure, suggesting that BSC-1 could be a key player in the development and maintenance of hypertension in the SHR. Studies in an experimental model of prenatally-programmed-hypertension suggest that hypertension involves transcriptional upregulation of sodium transporters BSC-1 and TSC in the thick ascending limb and distal convoluted tubule, respectively(141). Also, gene targeting experiments show that inactivation of

NKCC2 gene directly affects the countercurrent urine-concentrating mechanism and triggers profound disorganization of renal tissue(65, 157).

Many lines of evidence indicate that the sympathetic nervous system, *via* the renal nerves, plays an important role in the pathogenesis of essential hypertension in humans and laboratory animals(22, 195). Patients with established essential hypertension have increased sympathetic nervous system activity, as evidenced by increased plasma and urinary norepinephrine levels, elevated excretion of catecholamine metabolites, and an exaggerated depressor response to centrally acting sympatholytic agents(196-199). In the SHR and in the DOCA/NaCl hypertensive model, increased renal efferent nerve activity has been shown to contribute to the development of hypertension by causing increased renal sodium retention(203, 204). In both of these experimental models, renal denervation delays the development and blunts the severity of hypertension and is associated with increased urinary sodium excretion, suggesting a renal efferent mechanism(205-207). Recently, studies showed that increased renal sympathetic activity known to be present in an animal model of liver cirrhosis plays a significant role in sodium retention by stimulating sodium reabsorption in the TAL *via* increased renal abundance of BSC-1(183).

Even though investigators have not yet elucidated the mechanisms leading to the over-activation of the sympathetic nervous system in human and animal models of essential hypertension, our study is the first to show a direct link between the sympathetic nervous system and renal sodium ion transporters. Our studies show that norepinephrine regulates the expression/abundance of one of the key renal sodium ion transporters identified to be involved in the pathogenesis of hypertension in the SHR, namely BSC-1, *via* a β-adrenoceptor-cAMP-PKA dependent pathway, an effect that can explain the mechanism by which norepinephrine and/or the sympathetic nervous system contributes to the enhancement of urinary concentrating ability and sustained antidiuresis in the long-term. Regulation of the sodium transporter abundance by norepinephrine could additionally explain the abnormal salt and water balance associated with certain pathological disease states involving increased norepinephrine levels such as essential hypertension and further elucidation of the mechanisms involved, could lead to the development of newer therapies that could better help treat the progression of diseases associated with over-activation of the sympathetic nervous system such as essential hypertension. These studies are an essential first step in this direction.

APPENDIX A

REGULATION OF RENAL TRANSPORT SYSTEMS

A.1 INTRODUCTION

The regulation of ion and water transport in the kidney is important for maintenance of extracellular fluid volume and arterial blood-pressure regulation. The major ion transporters and water channels in individual renal tubule segments have been identified *via* physiological techniques, and complementary DNAs for all of the key sodium transporters and channels expressed along the renal tubule have been cloned and antibodies are now being used to investigate the molecular basis of renal tubule sodium-transport regulation. These include the apical transporters: sodium-hydrogen exchanger (NHE-3) of the proximal tubule, the bumetanide-sensitive Na-K-2Cl cotransporter (BSC-1) and the inwardly-rectifying K channel (ROMK-1) of the thick ascending limb, the thiazide-sensitive Na-Cl cotransporter (TSC) of the distal tubule, epithelial Na channel (ENaC) and the water channels aquaporin1-4 of the collecting duct; and the basolateral transporters such as Na-K-ATPase (located along entire nephron) and sodium-bicarbonate transporter (NBC-1) of the proximal tubule.

The present study was initiated to validate a chronic hormone infusion model to study the regulation of renal sodium ion transporters and water channels by norepinephrine, the principal neurotransmitter of the sympathetic nervous system. We utilized semi-quantitative immunoblotting in a rat model of chronically elevated norepinephrine (*via* infusion) and as positive controls, we included rat models of chronically elevated vasopressin, angiotensin II and aldosterone, all of which are well known to modulate the expression of sodium ion transporters and water channels along the nephron.

A.2 METHODS

Rats were randomly divided into 5 groups (n=3): Angiotensin II (200 ng/min), aldosterone (150 ng/min), arginine vasopressin (50 ng/min), norepinephrine (600 ng/min) or vehicle-treatment groups. All animals received either the hormones or saline by means of osmotic minipumps (Alzet) for a period of 15 days. Following treatment, blood pressures were measured and kidneys excised for tissue isolation and immunoblotting as previously described in detail in Chapter 4.

A.3 RESULTS AND DISCUSSION

Chronic infusions of norepinephrine, angiotensin II, arginine vasopressin and aldosterone significantly increased arterial blood pressure (Table 5). In this regard, norepineprhine, arginine vasopressin and aldosterone caused similar increases in arterial blood pressure, whereas angiotensin II caused the greatest increase. Chronic norepinephrine infusion resulted in a significant increase in the abundance of NHE-3 in the cortex, but not medulla (Figure 31). In contrast, chronic infusions of arginine vasopressin, angiotensin II and aldosterone did not significantly increase NHE-3 protein expression (Figure 31). Chronic norepinephrine infusion resulted in a 3-fold increase in the protein abundance of the Na-K-2Cl cotransporter BSC-1/NKCC2 ($P<0.05$) in the renal medulla. Similarly, chronic vasopressin and angiotensin II infusions also resulted in an increase in BSC-1 expression by 3-fold and 2-fold respectively, ($P<0.05$) (Figure 32). Aldosterone, on the other hand, did not have any effect on BSC-1 expression. Changes in BSC-1 expression were not accompanied by any changes in the expression of ROMK-1 (Figure 35A). Norepinephrine did not increase the expression of TSC in the cortex (Figure 33). TSC expression, however, was induced by aldosterone and angiotensin II infusions (Figure 33). Although none of the treatments significantly altered the expression of aquaporin-1 (Figure 35B), chronic norepinephrine and vasopressin infusions resulted in a 2-fold increase in aquaporin-2 protein levels in the medulla ($P<0.05$) (Figure 34)

An important aspect of the current study was the inclusion of other hormones (aldosterone, angiotensin and arginine vasopressin) known to regulate renal transport systems in addition to their effects on mean arterial blood pressures. Results from these positive controls

are consistent with previously published results by other laboratories(120, 259, 266, 267). In our experimental set-up, chronic vasopressin infusion upregulated BSC-1 and aquaporin-2 protein abundance in the medulla, aldosterone infusion induced TSC protein abundance and angiotensin II infusion increased BSC-1 and TSC levels as has been previously reported. Vasopressin and angiotensin II have been previously shown to regulate expression of the bumetanide-sensitive Na-K-2Cl cotransporter of the thick ascending limb(120, 259). Vasopressin additionally regulates expression of the water channel aquaporin-2 of the collecting duct(267, 268) and the antidiuretic mineralocorticoid hormone aldosterone regulates expression of the thiazide-sensitive NaCl cotransporter of the distal tubule(266), thereby validating our experimental set-up and protocol.

Table 5. MABP in control and hormone infused rats

Effects of chronic hormone infusions on mean arterial blood pressure (MABP), systolic blood pressure, diastolic blood pressure and heart rate (HR) in rats. Values represent means ± SEM recorded at one-minute intervals. * denotes $P < 0.001$ vs. Control

Treatment	MABP (mm Hg)	Systolic (mm Hg)	Diastolic (mm Hg)	Heart Rate (beats/min)
Protocol 1				
Control	119.6± 6.5	129.4± 1.0	109.8± 3.1	376.7± 7.6
Norepinephrine	142.8± 2.7 *	168.1± 9.6 *	117.5± 2.5 *	487± 37.3 *
Angiotensin II	182.2± 4.0 *	203.2± 8.4 *	161.0± 2.6 *	474.1± 22.9 *
Arginine vasopressin	143.6± 5.0 *	153.8± 3.6 *	133.3± 6.8 *	440± 9.0 *
Aldosterone	143.8± 8.0 *	153.2± 7.2 *	134.4± 8.8 *	423.7± 61.1 *

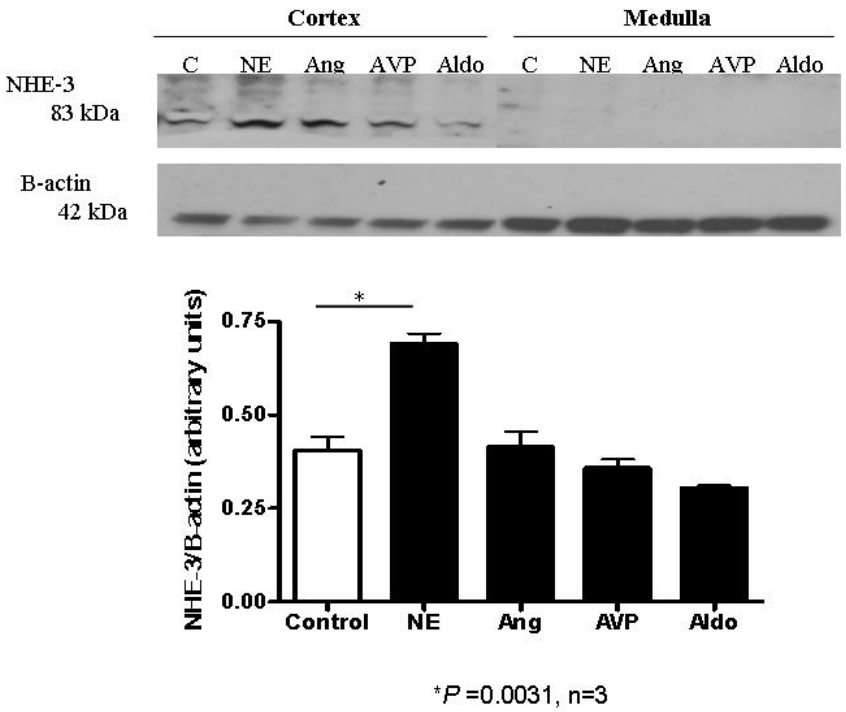

Figure 31. Expression of NHE-3 in the renal outer cortex and medulla in control and hormone-infused rats

Top panel: each lane was loaded with 20 μg of protein and blots were probed with rabbit anti-NHE-3 and mouse-anti-β-actin antibodies. Bottom panel: densitometric analysis of NHE-3 expression in cortex normalized to β-actin. Values represent means ± SEM. *denotes $P < 0.05$ vs. Control (n=3, each group).

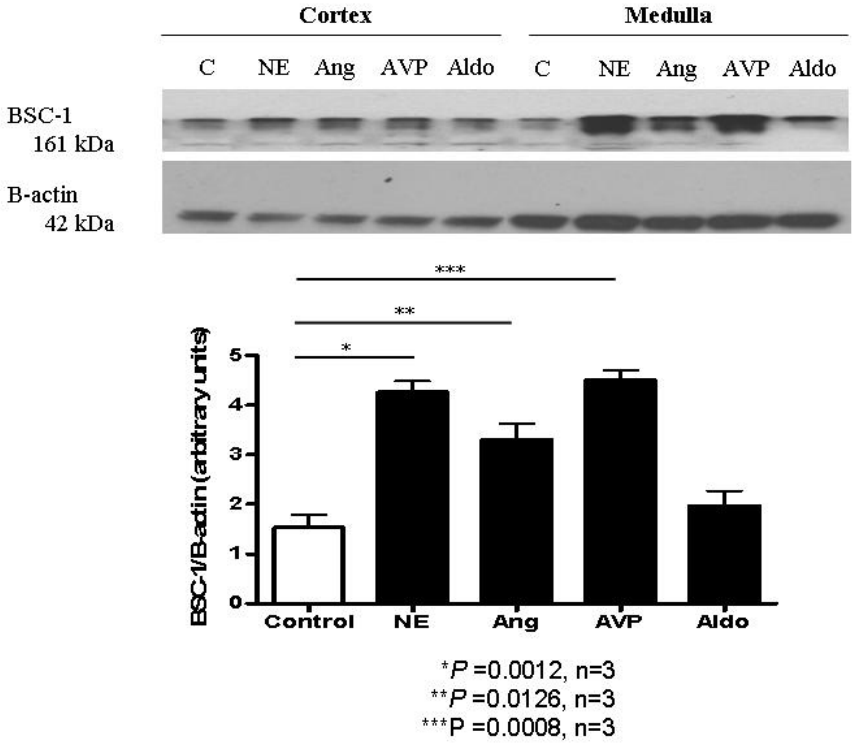

Figure 32. Expression of BSC-1 in the renal outer cortex in control and hormone-infused rats

Top panel: each lane was loaded with 20 μg of protein and blots were probed with rabbit anti-BSC-1 and mouse-anti-β-actin antibodies. Bottom panel: densitometric analysis of BSC-1 expression in medulla normalized to β-actin. Values represent means ± SEM. *denotes $P < 0.05$ vs. Control (n=3, each group).

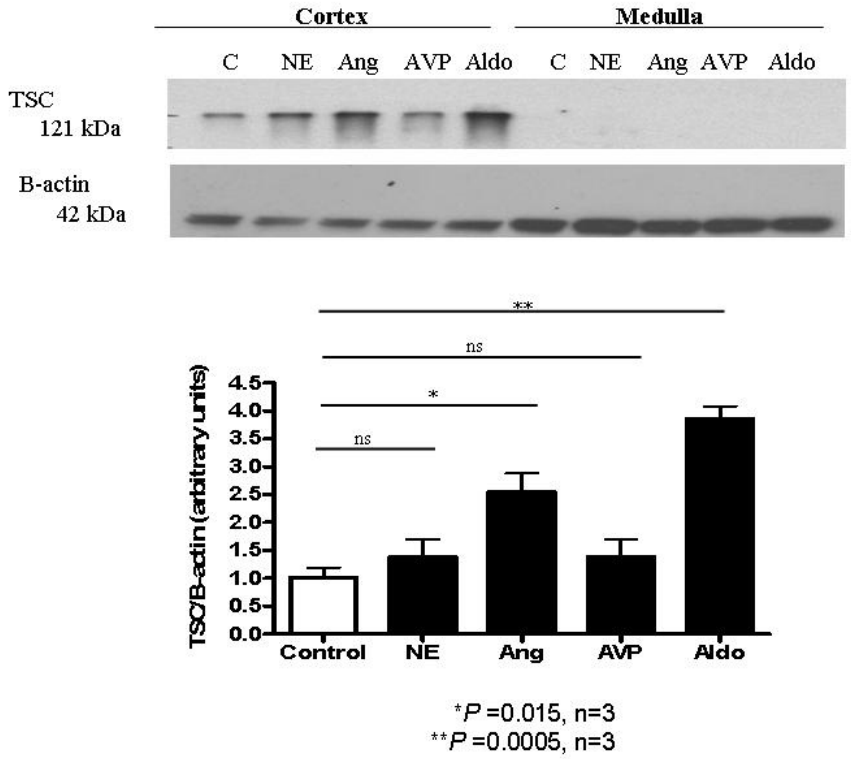

Figure 33. Expression of TSC in the renal outer cortex and medulla in control and hormone-infused rats

Top panel: each lane was loaded with 20 μg of protein and blots were probed with rabbit anti-TSC and mouse-anti-β-actin antibodies. Bottom panel: densitometric analysis of TSC expression in cortex normalized to β-actin. Values represent means ± SEM. *denotes $P < 0.05$ vs. Control (n=3, each group).

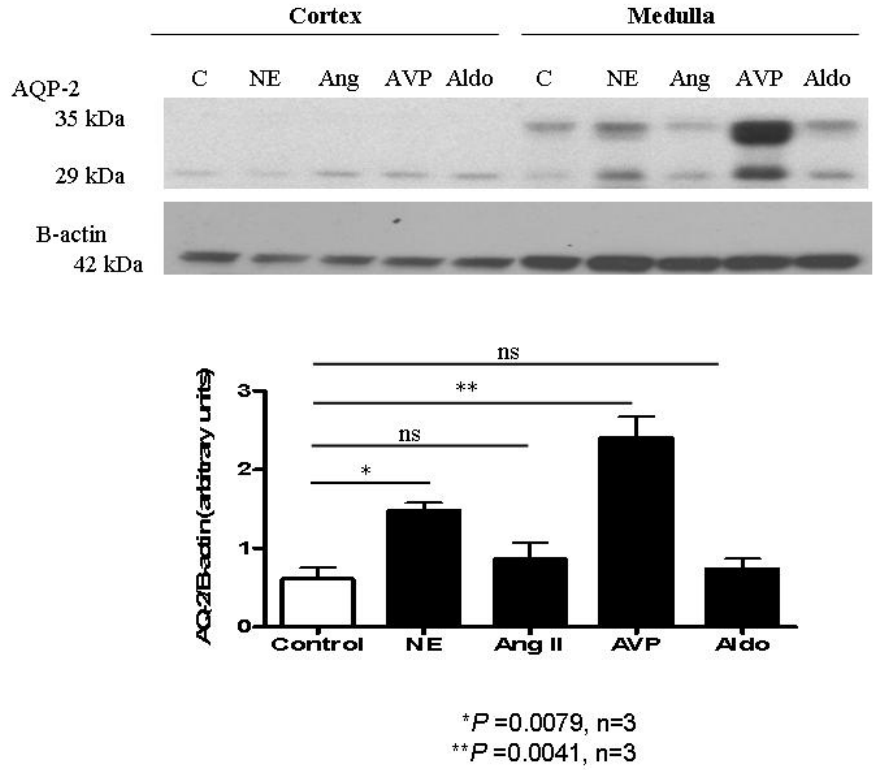

Figure 34. Expression of AQP-2 in the renal outer cortex and medulla in control and hormone-infused rats

Top panel: each lane was loaded with 20 μg of protein and blots were probed with rabbit anti-AQP-2 and mouse-anti-β-actin antibodies. Bottom panel: densitometric analysis of AQP-2 expression in medulla (average of both bands) normalized to β-actin. Values represent means ± SEM. *denotes $P < 0.05$ vs. Control (n=3, each group).

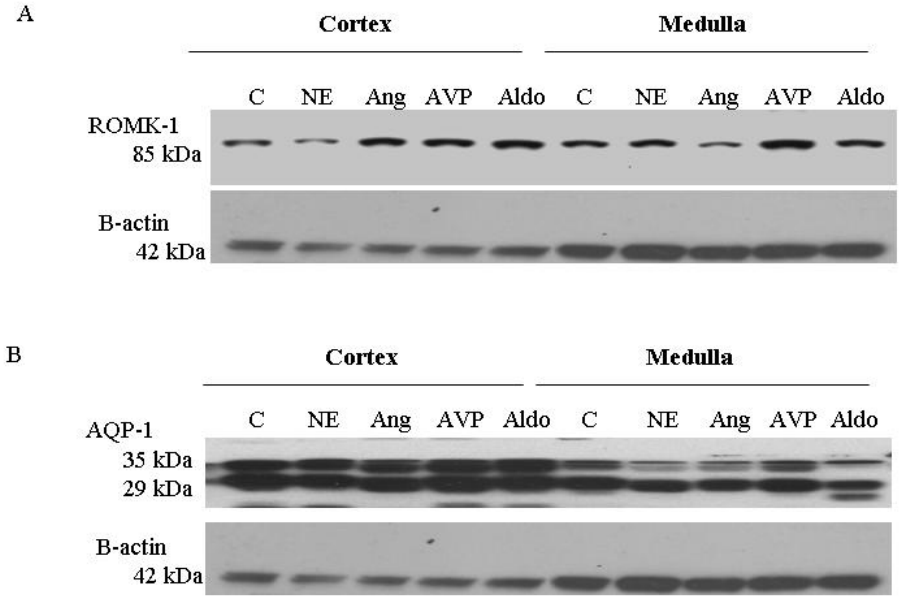

Figure 35. Expression of ROMK-1 and AQP-1 in the renal outer cortex and medulla in control and hormone-infused rats

Each lane was loaded with 20 µg of protein and blots were probed with rabbit anti-ROMK-1, rabbit anti-AQP-1 and mouse-anti-β-actin antibodies.

APPENDIX B

PLASMA MEMBRANE AND INTRACELLULAR VESICLE ISOLATION BY DIFFERENTIAL ULTRACENTRIFUGATION

Isolation of plasma membrane and intracellular vesicle fractions by successive differential centrifugation at 17,000g followed by centrifugation of the resulting supernatant at 200,000g yields fractions that contain exclusively plasma membrane and membranes intracellular vesicles respectively(159, 171). Cortex and inner stripe of outer medulla were dissected fro each kidney, minced finely and homogenized using a saw-toothed homogenizer in isolation buffer (250 mM sucrose/10 mM triethanolamine, pH 7.6) containing protease inhibitors (1 µg/ml leupeptin, 0.1 mg/ml phenylmethylsulfonyl fluoride). This homogenate was centrifuged at 4,000g for 15 min and the supernatant was collected for subsequent centrifugation. To increase the yield of membrane vesicles, the resultant pellet was rehomogenized in fresh isolation buffer, and the centrifugation repeated as described above. The supernatents were pooled, and plasma membrane and intracellular vesicle fractions were prepared consecutively by centrifugation of the supernatant at 17,000g (17,000 rpm Beckman JA-17 rotor) for 30 min and 200,000g (56,800 rpm Beckman Type 90 Ti rotor) for 1 hr, respectively. The resulting pellets were resuspended in 100 µl of isolation buffer and assayed for protein concentration by BCA method. Samples were solubilized in Laemmli sample buffer containing 2.5% SDS. Isolation of protein fractions was verified by immunoblotting with the following markers: water channel AQP-1 (gift of MA Knepper, NHLBI) and vesicle-associated membrane protein VAMP-2 (Chemicon, Temecula, CA) for plasma membrane and intracellular vesicles respectively (Figure 36).

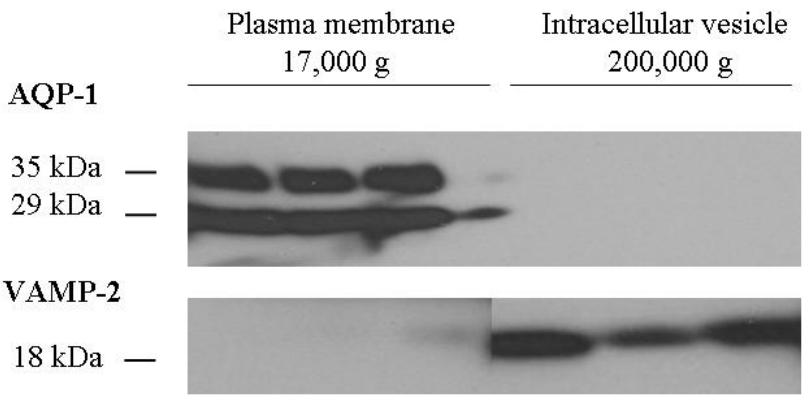

Figure 36. AQP-1 and VAMP-2 immunoblotting in plasma membrane and intracellular vesicle enriched fractions

Each lane was loaded with 1 µg of protein and blots were probed with rabbit anti-AQP-1 and, rabbit anti-VAMP-2 antibodies for plasma membrane and intracellular vesicle respectively.

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