Norepinephrine, via β-Adrenoceptors, Regulates Bumetanide-Sensitive Cotransporter Type 1 Expression in Thick Ascending Limb Cells

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Abstract—The sympathetic nervous system, via norepinephrine, regulates renal sodium transport, and chronic sympathetic activation causes sustained increases in blood pressure by reducing sodium excretion. Our previous studies show that chronic norepinephrine infusion increases the abundance of the bumetanide-sensitive cotransporter type 1, the apical sodium transporter of the thick ascending limb of Henle’s loop. The present study was initiated to elucidate the mechanisms by which norepinephrine regulates the protein levels of this transporter in an immortalized thick ascending limb epithelial cell line. Treatment with norepinephrine, either alone or in the presence of actinomycin D or cycloheximide, had no effect on cotransporter mRNA levels. Treatment with norepinephrine, however, increased bumetanide-sensitive cotransporter type 1 protein levels (70% increase versus control; \( P=0.012 \)), and pretreatment with cycloheximide blocked the effect of norepinephrine on bumetanide-sensitive cotransporter type 1 protein levels. To further elucidate the mechanism, thick ascending limb cells were treated with norepinephrine in the presence of phentolamine (α-adrenoceptor blocker), propranolol (β-adrenceptor blocker), SQ22536 (adenylyl cyclase inhibitor), PD098059 (mitogen-activated protein kinase pathway inhibitor), H-89 (protein kinase A inhibitor), or staurosporine (protein kinase C inhibitor). Treatment with propranolol, SQ22536, and H-89 abolished the effects of norepinephrine on bumetanide-sensitive cotransporter type 1 protein levels, whereas staurosporine had no effect. Treatment with PD098059 partially inhibited the effects of norepinephrine (40% decrease versus norepinephrine; \( P=0.03 \)), and treatment with phentolamine potentiated the effects of norepinephrine (30% increase versus norepinephrine; \( P=0.02 \)) on bumetanide-sensitive cotransporter type 1 protein levels. We conclude that regulation of bumetanide-sensitive cotransporter type 1 by norepinephrine proceeds via the β-adrenceptor receptor–cAMP–protein kinase A pathway that involves in part mitogen-activated protein kinases and that α-adrenceptor activation negatively regulates bumetanide-sensitive cotransporter type 1 protein levels. (Hypertension. 2007;49:1351-1357.)

Key Words: BSC-1/NKCC2 ■ thick ascending limb ■ norepinephrine ■ α-adrenoceptor ■ β-adrenoceptor ■ cAMP ■ protein kinase A ■ cAMP

The bumetanide-sensitive cotransporter type 1 (BSC-1) Na-K-2Cl cotransporter is the principal apical Na⁺ entry pathway in the thick ascending limb (TAL). Long-term dysregulation of BSC-1 may contribute to 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. Moreover, BSC-1 is upregulated in rats with small-to-moderate myocardial infarctions, dehydration, and cardiac failure and in an animal model of liver cirrhosis. Moreover, our results in the spontaneously hypertensive rat (SHR) show that expression of BSC-1 is also elevated in this animal model of essential hypertension and that the natriuretic response to furosemide (which blocks BSC-1) is significantly higher in the SHR compared with its normotensive counterpart, suggesting that BSC-1 could be involved in the development and/or maintenance of hypertension in the SHR.

Although our studies establish a role for BSC-1 in the regulation of blood pressure in the SHR, the underlying factors responsible for this increase in BSC-1 expression are unknown. A possible mechanism leading to increased BSC-1 expression is overactivation of the renal sympathetic nervous system. Extensive evidence points to the renal nerves as a link between the sympathetic nervous system and long-term blood pressure control by the kidneys. Studies in the SHR suggest that the sympathetic nervous system, particularly renal sympathetic nerve activation, play an important role in the development of hypertension in the SHR. For example, SHR have increased sympathetic outflow and renal sympathetic nerve activity compared with normotensive rats, and renal denervation attenuates hypertension in SHR.

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To test the hypothesis that the sympathetic nervous system may regulate BSC-1 expression, we recently examined the effects of chronic norepinephrine (the principal neurotransmitter of the sympathetic nervous system) infusion on BSC-1 protein levels in vivo. These experiments demonstrate that chronic administration of norepinephrine increases mean arterial blood pressure and profoundly upregulates BSC-1 protein levels.

Regulation of BSC-1 expression by the sympathetic neurotransmitter norepinephrine could explain the upregulation of BSC-1 in SHRs, as well as 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. Therefore, the present study was initiated to determine the underlying molecular mechanism responsible for the regulation of BSC-1 by norepinephrine. In the present study, we used a murine immortalized TAL cell line, a stable cell line derived from microdissected loops of Henle of the Tg(SV40E)Bri7 murine immortalized TAL cell line, a stable cell line derived (courtesy of Dr Glenn T. Nagami, University of California Los Angeles, Los Angeles, CA). Studies were performed using an immortalized TAL cell line.

Methods

Chemicals and Reagents
All of the chemicals and reagents were obtained from Sigma-Aldrich Corporation unless otherwise indicated.

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, University of California Los Angeles, Los Angeles, CA). Cells were grown in T-25 flasks in DMEM/F-12 supplemented with 10% FCS, 1 mmol/L of HEPES, and antibiotics and incubated in a humidified 5% CO2/95% 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 (Dr Glenn T. Nagami, personal communication, University of California Los Angeles, Los Angeles, CA). Cells were grown in T-25 flasks in DMEM/F-12 supplemented with 10% FCS, 1 mmol/L of HEPES, and antibiotics and incubated in a humidified 5% CO2/95% 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 (Dr Glenn T. Nagami, personal communication, University of California Los Angeles, Los Angeles, CA). Studies were performed on cells between passages 12 and 15. Before treatment, cells were washed with PBS, trypsinized, and plated in 6-well plates in regular culture medium for 24 to 48 hours to reach 60% to 70% confluence. Cells were serum starved for an additional 24 to 48 hours and media was changed every day, after which cells were treated with various pharmacological agents for 1 to 24 hours and finally harvested for RNA and protein isolation for RT-PCR and immunoblotting, respectively. For RNA measurements, some cells were pretreated with actinomycin D or cycloheximide for 30 minutes, followed by treatment with norepinephrine for 1 to 24 hours. 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 minutes, followed by overnight incubation with norepinephrine, after which, cell lysates were prepared for immunoblotting. In a separate set of experiments, cells were treated overnight with vasopressin (arginine vasopressin), DDAVP, forskolin or 8-bromo-cAMP, after which cells were lysed for subsequent immunoblotting.

RNA Isolation and RT-PCR
After treatment, cells were washed with PBS, and RNA was isolated using Trizol reagent (GIBCO Life Technologies) as per the manufacturer’s instructions. By using the primer sequences listed in the Table, RNA (1 μg) was reverse transcribed and amplified using Titanium One-step RT-PCR kit (Clontech). 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.

Protein Isolation and Immunoblotting
After treatment, cells were washed twice with cold PBS and lysed in lysis buffer (10 mmol/L of Tris HCl [pH 7.4], 1% Triton X-100, 2 mmol/L of EDTA, 10 μg/mL of aprotinin, 1 μg/mL of leupeptin, 1 mmol/L of PMSF, 50 mmol/L of Na3PO4, 50 mmol/L of NaF, and 1 mmol/L of NaVO3). 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 minutes in Laemmli sample buffer. SDS-PAGE was performed on gradient polyacrylamide gels (4% to 12%) loaded with 10 μg of protein per lane. For immunoblotting, proteins were transferred electrophoretically to PVDF membranes. Membranes were blocked in 5% milk for 2 hours and probed for 2 hours 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, Conn) or mouse anti-β-actin monoclonal antibody (1:3000, A5441 Sigma Chemical Co). Subsequently, membranes were exposed to a secondary horseshadish peroxidase–conjugated donkey anti-rabbit or sheep–anti-mouse polyclonal antibody (1:5000, Pierce Biotechnology Inc) 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) before exposure to x-ray film (Kodak 165-1579; Eastman Kodak Co). Densitometric analysis was performed using ImageQuant TL (Amersham Biosciences), and band densities were normalized to β-actin.

Statistical Analysis
All of the data are presented as mean±SEM. Where appropriate, comparisons between groups were made by unpaired t test or 1-way ANOVA followed by Bonferroni’s multiple comparison posttest to determine statistical significance. P<0.05 was considered significant.

Results

Vasopressin, Forskolin, and 8-Bromo-cAMP Regulate BSC-1 Levels in the TAL Cell Line
Previous studies show that vasopressin regulates BSC-1 protein levels via the V2 receptor. In addition, studies demonstrate that BSC-1 protein levels, trafficking, and function are regulated by cAMP. 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 and the selective V2 receptor vasopressin analog DDAVP. In addition, cells were also treated with the adenyl cyclase activator forskolin and the cell-permeable cAMP analog 8-bromo-cAMP. Our results show that in the TAL cell
line, both arginine vasopressin and DDAVP increased BSC-1 protein levels over control (Figure 1). Treatment with the adenylyl cyclase activator forskolin and 8-bromo-cAMP also increased BSC-1 protein levels over control (Figure 1). Thus, in the immortalized TAL cell culture model, we were able to reproduce the effects of vasopressin and cAMP on BSC-1 protein, as has been reported previously 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.

**Norepinephrine Does Not Alter BSC-1 mRNA**

To determine whether norepinephrine alters BSC-1 mRNA expression, TAL cells were treated with norepinephrine in the presence or absence of actinomycin D or cycloheximide. Treatment with norepinephrine, either alone or in the presence of actinomycin D or cycloheximide, failed to alter BSC-1 mRNA levels (Figures 2 and 3). This suggests that any regulation of BSC-1 protein levels by norepinephrine proceeds via a posttranscriptional mechanism and does not involve regulation at the mRNA level.

**Norepinephrine Increases BSC-1 Protein Levels**

Treatment with 1 μmol/L of norepinephrine significantly increased BSC-1 protein levels compared with control (70% increase versus control; \( P = 0.012; n = 4 \); Figure 4). Pretreatment of TAL cells with the protein synthesis inhibitor cycloheximide completely blocked the effects of norepinephrine on BSC-1 protein, while having no effect on control cells (Figure 4), suggesting that norepinephrine regulates BSC-1 protein levels via a translational mechanism and/or involves additional protein factors.
BSC-1 protein (Figure 5), whereas pretreatment with phentolamine did not inhibit the effect of norepinephrine on BSC-1 protein (Figure 5) and resulted in a significant increase in BSC-1 protein levels compared with norepinephrine alone (30% increase; \( P \leq 0.02; n=4 \)). These results indicate that, in TAL cells, BSC-1 protein levels are positively regulated by the \( \beta \)-adrenoceptor and negatively regulated by the \( \alpha \)-adrenoceptor.

**Regulation of BSC-1 by Norepinephrine Proceeds via a cAMP-Dependent Pathway and Involves, in Part, Mitogen-Activated Protein Kinases**

To elucidate the mechanism of regulation of BSC-1 by norepinephrine, TAL cells were treated with 1 \( \mu \)mol/L of norepinephrine in the presence of an adenylyl cyclase inhibitor SQ22536 (50 \( \mu \)mol/L) and a mitogen-activated protein (MAP) kinase/extracellular regulated kinase pathway inhibitor PD090859 (25 \( \mu \)mol/L) to determine the role of cAMP and MAP kinases, respectively. Treatment with the adenylyl cyclase inhibitor completely inhibited the effect of norepinephrine on BSC-1 protein (Figure 6), suggesting that activation of cAMP by norepinephrine is the mechanism involved. Treatment with the MAP kinase/extracellular regulated kinase pathway inhibitor, however, only partially inhibited the effect of norepinephrine (40% decrease; \( P=0.03; n=4; \) Figure 6), suggesting that, in addition to MAP kinases, other factors are involved in mediating the effects of norepinephrine.

**Regulation of BSC-1 Proceeds via a Protein Kinase A–Dependent Pathway**

BSC-1 protein contains potential cAMP-dependent kinase and protein kinase C (PKC) phosphorylation sites in the C- and N-terminal domains, thus supporting the hypothesis that protein kinase A (PKA) and/or PKC could be involved in regulation of BSC-1 by norepinephrine. 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 staurosporine before norepinephrine treatment. Treatment with the PKA inhibitor H-89 abolished the effect of norepinephrine on BSC-1 (Figure 7), whereas treatment with the PKC inhibitor staurosporine had no effect on BSC-1 protein levels (Figure 7), suggesting that, in TAL cells, PKA, but not PKC, is involved in the regulation of BSC-1 by norepinephrine.

Discussion

The results of the present study establish that norepinephrine upregulates the steady-state levels of BSC-1 protein in TAL cells. The mechanism does not rely on increases in BSC-1 mRNA levels and is mediated by the β-adrenoceptor receptor–cAMP–PKA pathway. Moreover, our results indicate that MAP kinases, but not PKCs, also participate and suggest that α-adrenoceptor activation negatively regulates BSC-1 protein levels.

As an alternative approach to study the regulation of BSC-1 by norepinephrine, we used an immortalized TAL cell line derived from the kidney of a mouse transgenic for SV40 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), and express both Tamm–Horsfall protein and BSC-1 on the apical membrane.

As a preliminary approach to validate the use of the TAL cell line, we examined whether known regulators, such as vasopressin and cAMP (in the form of 8-bromo-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 has been reported previously in the chronic vasopressin infusion model and in oocytes transfected with BSC-1 mRNA. Thus, we were able to establish that the TAL cell line was a reliable model system to study the effects of the native sympathetic neurotransmitter norepinephrine on BSC-1 mRNA and protein levels and accordingly used specific agonists, antagonists, and inhibitors to determine the underlying molecular mechanisms involved in the regulation of BSC-1 by norepinephrine.

The BSC-1/Na-K-2Cl cotransporter gene promoter contains consensus binding sites for transcription factors, such as cAMP-response element binding protein (CREB), nuclear factor 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. Even so, norepinephrine failed to regulate BSC-1 mRNA levels, either in the absence or presence of actinomycin D or cycloheximide. Thus, we were unable to establish a transcriptional mechanism of regulation of BSC-1 by norepinephrine. This result seems inconsistent with the fact that the BSC-1 gene promoter contains the cAMP response element (CRE). However, CREB phosphorylation does not necessarily activate transcription of genes with CRE in the promoter region. For example, methylation of CRE sites inhibits CREB binding, and a recent analysis indicates that phosphorylation of CREB alone is insufficient to activate target genes with CRE and suggests that the selective recruitment of CREB binding protein and perhaps other cofactors may determine whether phosphorylated CREB causes gene activation. It is also conceivable that despite the presence of CRE in the BSC-1 gene promoter, cAMP has little or no effect on BSC-1 mRNA expression because of insufficient expression of CREB or compartmentalization of PKA.

Despite the lack of effect of norepinephrine on BSC-1 mRNA, treatment of TAL cells with norepinephrine resulted in a marked increase in BSC-1 protein levels. This indicates that the effect of norepinephrine proceeds via a posttranscriptional mechanism. In support of this conclusion, pretreating cells with cycloheximide completely inhibited the effect of norepinephrine on BSC-1 protein, suggesting that regulation of BSC-1 by norepinephrine proceeds via enhancement of translation and/or that additional proteins may be involved in the regulation of BSC-1 degradation/recycling. 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 and the epithelial sodium channel of the collecting duct. Altered degradation/recycling of BSC-1 has been proposed to be the mechanism involved in regulation of BSC-1 by vasopressin and could be the mechanism involved in the regulation of BSC-1 by norepinephrine.

Studies in isolated thick ascending tubules have shown that norepinephrine stimulates cAMP generation via the β-adre-
neceptor²⁵–²⁷ with the β₁ subtype being predominant.²⁸ In the present study, treatment with a β-adrenergic receptor blocker propranolol, adenylyl cyclase inhibitor SQ22536, or PKA inhibitor H-89 completely blocked the effects of norepinephrine on BSC-1 expression, suggesting that the β-adrenergic receptor–cAMP–PKA pathway is involved in the regulation of BSC-1 protein levels. Treatment with the MAP kinase pathway inhibitor PD098059 partially blocked the effect of norepinephrine on BSC-1, suggesting that MAP kinases are partially involved, along with additional signaling molecules, in the regulation of BSC-1. Recently, studies identified with no lysine (WNK) kinases as the integrative upstream regulators of renal sodium transport systems.²⁹–³¹ WNK kinases are serine–threonine protein kinases that have been linked to regulation of a number of renal transporters. In particular, WNK3 kinase was identified as a positive regulator of BSC-1/Na-K-2Cl cotransporter.³² 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 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 attenuate BSC-1 function, an effect that could not be inhibited by either the specific PKC inhibitor Gö6976 or the nonspecific PKC inhibitors staurosporine and H-7, suggesting that a novel PKC isoform may be involved in the regulation of BSC-1.³³ Novel PKCs and atypical PKCs have been reported to be expressed in TAL;³⁴ however, it is yet to be determined whether such novel or atypical PKCs could be involved in the regulation of BSC-1 by norepinephrine.

Pretreatment with the α-adrenergic receptor blocker phentolamine resulted in a small but significant increase in BSC-1 protein levels, suggesting that the α-adrenergic receptor may negatively regulate BSC-1. Negative regulation of BSC-1 function by the α-adrenergic receptor has been reported previously in TAL, where selective α₂-adrenergic receptor activation inhibits chloride flux, and selective β-adrenergic receptor activation stimulates chloride flux.²⁶ However, additional studies would be required to characterize the role of α-adrenergic receptors in the regulation of BSC-1 abundance.

In summary, our results indicate that norepinephrine upregulates and downregulates the expression of BSC-1 protein via β-adrenergic receptors and α-adrenergic receptors, respectively, with the β-adrenergic receptor–mediated stimulation overriding the α-adrenergic-receptor-induced inhibition. The upregulation via β-adrenergic receptors is mediated by the adenylyl cyclase–cAMP–PKA pathway with some involvement of MAP kinases but not PKCs. Although speculative, we propose the following more detailed general scheme based both on the data in this report, as well as results published by others. β₁-Adrenergic receptors and α₂-adrenergic receptors in TALs stimulate and inhibit, respectively, adenylyl cyclase. The increase in cAMP activates PKA and phosphorylates key proteins, for example, BSC-1₀ and vesicle-associated membrane protein,¹⁶ resulting in the altered degradation/recycling of BSC-1. Altered degradation/recycling of BSC-1 in the long term increases steady-state levels of BSC-1. Most likely, MAP kinases and WNK kinases³² are also involved downstream of PKA; however, the details in this regard remain to be elucidated.

As mentioned, our previous studies demonstrate increased BSC-1 expression in SHR,³⁷ and it is conceivable that the augmented levels of BSC-1 in SHR are secondary to increased renal sympathetic tone resulting in norepinephrine-induced upregulation of BSC-1 in the TAL. However, sodium transport in SHRs may be more defective in the proximal as compared with the TAL, whereas the reverse may be true with the Dahl salt-sensitive hypertensive rat.³⁵ We have not yet examined the expression of BSC-1 in other genetic models of hypertension. However, it would be informative to compare the levels of BSC-1 expression in other models of hypertension with known defects in sodium transport in the TAL, such as the Dahl salt-sensitive rat.

**Perspectives**

Norepinephrine was found to regulate BSC-1 protein levels in an immortalized TAL cell line via a β-adrenergic receptor–cAMP–PKA–dependent pathway that involves in part MAP kinases. The importance of a role for β-adrenergic receptors in the regulation of BSC-1 abundance is especially relevant in essential hypertension. β-Adrenergic receptor blockers are frequently used in antihypertensive therapy because of their effect on myocardial contractility, cardiac output, and renin release. The results of the present study indicate that there may be additional benefits to β-adrenergic receptor 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, which would promote natriuresis and diuresis, thus aiding in the management of inappropriate salt and water retention associated with essential hypertension.

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**Disclosures**

None.

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