Blood pressure regulates the activity and function of the Na-K-2Cl cotransporter in vascular smooth muscle


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Blood pressure regulates the activity and function of the Na-K-2Cl cotransporter in vascular smooth muscle. Am J Physiol Heart Circ Physiol 286: H1552–H1557, 2004; 10.1152/ajpheart.00695.2003.—The Na-K-2Cl cotransporter (NKCC1) is one of several transporters that have been linked to hypertension, and its inhibition reduces vascular smooth muscle tone and blood pressure. NKCC1 in the rat aorta is stimulated by vasoconstrictors and inhibited by nitrovasodilators, and this is linked to the contractile state of the smooth muscle. To determine whether blood pressure also regulates NKCC1, we examined the acute effect of hypertension on NKCC1 in rats after aortic coarctation. In the hypertensive aorta (28-mmHg rise in mean blood pressure), an increase in NKCC1 activity (measured as bumetanide-sensitive 46Rb efflux) was apparent by 16 h and reached a plateau of 62% greater than control at 48 h. In contrast, there was a slight decrease in NKCC1 activity in the hypotensive aorta (21% decrease in mean blood pressure). Measurement of NKCC1 mRNA by real-time PCR revealed a fivefold increase in the hypertensive aorta compared with the hypotensive aorta or sham aorta. The inhibition by bumetanide of isometric force response to KCl application was significantly greater in the hypertensive aorta than in the control aorta or hypotensive aorta. We conclude that NKCC1 in rat aortic smooth muscle is regulated by blood pressure, most likely through changes in transporter abundance. This upregulation of NKCC1 is associated with a greater contribution to force generation in the hypertensive aorta. This is the first demonstration that NKCC1 in vascular smooth muscle is regulated by blood pressure and indicates that this transporter is important in the acute response of vascular smooth muscle to hypertension.

hypertension; rat; aorta; aortic coarctation

THE FUNCTION OF VASCULAR SMOOTH MUSCLE is dependent on membrane potential and changes in the concentration of intracellular calcium. Because both are controlled in part by the transport of monovalent ions and because of the salt sensitivity of hypertension, changes in ion transport by vascular smooth muscle have long been suspected to play a role in hypertension (35). However, definitive data are generally lacking, primarily because much of the data come from cultured cells or circulating blood cells that are of questionable relevance to vascular smooth muscle in vivo. When abnormalities have been observed in intact smooth muscle, it is not clear whether they are the cause or the result of hypertension. Altered fluxes of Na⁺, K⁺, and Cl⁻ have all been described (7, 11, 15, 16, 20, 24, 32), and specific transporters that have been implicated include the Na-K pump (32), K⁺ channels (20, 24), and the Na-K-2Cl cotransporter NKCC1 (7). The relevance of any of these transporters to essential hypertension remains unknown.

Of these transporters, only NKCC1 could account for altered fluxes of all three ions. This electroneutral transporter mediates the coupled flux of Na⁺, K⁺, and Cl⁻ in virtually all cells, including vascular smooth muscle cells (2, 8), and is characterized by its sensitivity to “loop” diuretics, particularly bumetanide. NKCC1 is a critical component of vectorial salt transport in epithelia, but in other cells its principal function appears to be the regulation of intracellular Cl⁻ concentration ([Cl⁻]) and cell volume. This is true in vascular smooth muscle cells as well (2, 7, 19, 31, 34) and has important implications for the changes seen in hypertension.

In rat arterial smooth muscle, NKCC1 is acutely stimulated by vasoconstrictors and inhibited by nitrovasodilators (2), and inhibition of NKCC1 with bumetanide reduces the contractile response to phenylephrine (PE) (2, 19). A reduction in force generation is also seen in mice that lack NKCC1 (28), and blood pressure is also reduced in these mice (10, 28), indicating that NKCC1 contributes to vascular tone in vivo. It is likely that NKCC1 promotes smooth muscle contraction by maintaining or increasing [Cl⁻], because the effect of bumetanide is mimicked by depletion of intracellular Cl⁻ (19) and because bumetanide has no effect on the contraction in response to KCl (2), which increases [Cl⁻]. Because Cl⁻ currents contribute to the initial agonist-induced depolarization, vascular smooth muscle contraction is dependent on [Cl⁻], [Cl⁻], must therefore be maintained well above its electrochemical equilibrium and a coupled, electroneutral transporter such as NKCC1 is ideally suited for this purpose. Consistent with this is the observation that bumetanide lowers [Cl⁻], in vascular smooth muscle (7).

Little is known about NKCC1 in hypertensive smooth muscle. NKCC1 activity is increased in smooth muscle from rats made hypertensive by administration of deoxycorticosterone acetate and results in an increase in [Cl⁻] (7). Whether this is a direct effect of mineralocorticoid or the result of hypertension is not known. The former is suggested by our recent demonstration that aldosterone stimulates NKCC1 in rat aortas in vivo in the absence of hypertension and in normal aortas in vitro (13). To what extent upregulation of NKCC1 affects smooth muscle tone is unknown. We sought to determine whether blood pressure regulates NKCC1 by studying the effect of aortic coarctation. This procedure produces both hypertensive...
and hypotensive segments of the aorta in the same animal and, assuming that the proximal and distal aorta have similar sensitivities to vasoactive substances, the effect of blood pressure can be studied independently of any systemic effects. Early time points were examined to avoid effects that hypertrophy and remodeling might have on the cotransporter. We show that acute hypertension increases NKCC1 activity and mRNA and increases its contribution to force generation. This is the first demonstration of the upregulation and functional significance of NKCC1 in hypertensive smooth muscle.

METHODS

Animals. All animal studies were performed under a protocol approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 250–300 g were anesthetized with ketamine and xylazine, and the abdominal aorta was exposed through a left flank incision. The portion of the aorta between the two renal arteries was dissected and then tied off together with a 0.45-mm stainless steel wire. The wire was immediately removed to produce a fixed stenosis equal to the cross-sectional area of the wire (0.64 mm²). In sham-operated rats, the aorta was mobilized but was not tied off. Blood pressure was measured just before death via catheters placed in the femoral and carotid arteries under urethane anesthesia. In sham-operated rats, the aorta was mobilized but was not tied off. Blood pressure was measured just before death via catheters placed in the femoral and carotid arteries under urethane anesthesia.

NKCC1 assay. Activity was measured as bumetanide-sensitive 86Rb efflux as previously described (2). This has several advantages over measurement of influx. First, multiple time points can be generated from a single sample so that efflux can be measured before and after the addition of bumetanide. Thus bumetanide-sensitive flux can be determined in single samples, greatly reducing error and the amount of tissue required. Second, efflux data do not require normalization to weight, which also reduces error. Briefly, the aorta was opened longitudinally, and the endothelium was removed with a cotton swab. Segments were then loaded with 86Rb for 2 h in a HEPES-buffered physiological saline solution (PSS) containing 142 mM NaCl, 121 Cl⁻, 5.4 mM K⁺, 1.8 mM Ca²⁺, 0.8 mM Mg²⁺, and 5 mM glucose. The inability to maintain a constant CO₂ environment during the flux measurements precluded bicarbonate as a buffer. Steady-state loading of Rb requires 3–4 h, but concern that changes occurring in vivo might dissipate over this time dictated a shorter loading period. Previous studies have revealed a single pool of intracellular Rb and no differences between fluxes after different loading times. After segments were extensively washed, the efflux of 86Rb was measured over 10 min at 2-min intervals before and after the addition of 50 μM bumetanide. Although the same inhibition occurred with 10 μM bumetanide (not shown), the higher concentration was used to minimize the time to a new steady state. Results are expressed as the fraction of 86Rb leaving the vessel per minute. A new baseline was achieved 4 min after the addition of bumetanide so that the flux due to NKCC1 was determined by subtracting the mean of the three values after 4 min of bumetanide from the mean of the three values just before the addition of bumetanide (13).

Real-time PCR. Total RNA was prepared using a modified phenolchloroform extraction from rat aortas previously frozen in liquid N₂ and stored at −80°C. RNA (2 μg) was converted into cDNA using ThermoScript RT reverse transcriptase (Invitrogen; Carlsbad, CA), and 200 ng were then amplified in a PE Biosystems (Warrington, UK) real-time PCR unit using SYBR green dye. Forward and reverse primers for NKCC1 were 5'-CCACCCACACCATCTACTA-3' and 5'-TGGCACAACAGCATCTCT-3', respectively, corresponding to nucleotides 743–761 and 956–973 of rat NKCC1 cDNA (GenBank Accession No. U13174). Results were normalized to real-time PCR of rat β-actin (GenBank Accession No. NM_031144) using the forward and reverse primers 5'-TGGTTCCTCCTAGGCTCTGC-3' and 5'-ATGTCAGCAGATCCTCCTCA-3', corresponding to nucleotides 416–439 and 635–658.

Isometric force generation. These measurements were performed immediately after the measurement of blood pressure. Aortas were rapidly excised and rinsed in cold bicarbonate-buffered PSS, loose fat and connective tissue were removed, and the endothelium was denuded. Aortas were maintained in PSS for the remainder of the experiment. PSS contained (in mmol/l) 118 NaCl, 4.73 KCl, 1.2 MgSO₄, 0.025 EDTA, 1.2 KH₂PO₄, 2.5 CaCl₂, 11 glucose, and 25 NaH₂CO₃-NaHCO₃. The PSS pH was 7.4 when bubbled with 95%O₂-5% CO₂ at 37°C. Aortic rings (5 mm) were isometrically mounted, and tension was adjusted to 50 mM. After a series of stimulations with 50 mM KCl, concentration-isometric force curves were generated in response to PE (0.1 nM–10 μM) in the absence and presence of bumetanide (10 μM). Developed forces were expressed as a percentage of the maximal force generated in response to PE. Individual EC₅₀ values were determined and compared using ANOVA. Significance was defined as P < 0.05.

RESULTS

Blood pressure. Blood pressures measured 72 h after coarctation are shown in Table 1. Systolic, diastolic, and mean carotid artery pressures were all significantly elevated after coarctation. Coarctation produced a greater increase in diastolic than systolic pressure, and mean blood pressure increased 31%. Responses were more variable in the femoral artery with only systolic pressure showing a significant decrease (25%). Blood pressure was not measured at earlier time points.

NKCC1 activity. Measurements of bumetanide-sensitive efflux in the aorta proximal to the coarctation (hypertensive aorta) are shown in Fig. 1. NKCC1 activity was increased at all time points, but this was significant only at 48 and 72 h. Activity increased slightly in proximal aortas from sham-operated animals at 30 h. This increase was also observed in the distal aorta, suggesting a systemic response to surgery. In contrast, NKCC1 activity was not increased distal to the coarctation and actually was slightly, but not significantly lower, than in distal aortas from sham-operated rats (Fig. 2). Figure 3 compares proximal and distal aortas from coarcted and sham animals. NKCC1 activity proximal to the coarctation was more than twice that distal to the coarctation, whereas in sham animals the activities did not differ. Efflux in the presence of bumetanide (bumetanide-insensitive efflux) was not altered in the hypertensive aorta proximal to the coarctation (Fig. 4), indicating that the increase in NKCC1 activity in the hypertensive aorta was not part of a generalized increase in K⁺ flux.

NKCC1 mRNA. Attempts to quantitate cotransporters by immunoblotting were unsuccessful, probably due to low abun-

Table 1. Blood pressures measured 72 h after aortic coarctation

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<tr>
<th></th>
<th>Sham</th>
<th>Coarcted</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Carotid artery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>112±6</td>
<td>133±5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Diastolic</td>
<td>74±5</td>
<td>106±6</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Mean</td>
<td>91±3</td>
<td>119±2</td>
<td>&lt;0.01</td>
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<tr>
<td>Femoral artery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>103±2</td>
<td>77±10</td>
<td>0.05</td>
</tr>
<tr>
<td>Diastolic</td>
<td>88±1</td>
<td>72±8</td>
<td>0.1</td>
</tr>
<tr>
<td>Mean</td>
<td>96±0</td>
<td>75±10</td>
<td>&lt;0.01</td>
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Values are means ± SE (in mmHg); n, no. of arteries.
dance and difficulties in working with whole tissue. As a surrogate indicator of an increased number of transporters, NKCC1 mRNA was quantitated by real-time PCR. As shown in Table 2, the proximal aorta contained over fivefold more NKCC1 mRNA than the corresponding portion of the aorta from sham-operated rats 72 h after coarctation. Because the content of mRNA for β-actin was not altered, a similar fivefold increase occurred in the ratio of NKCC1 mRNA to β-actin mRNA.

Isometric force. Measurement of isometric force in the proximal aorta is shown in Fig. 5. Treatment of proximal aortas from sham-operated rats with bumetanide reduced force generation in response to PE, as previously reported (2). Although this was not significant when expressed as the half-maximal concentration (EC50) of PE, there was a significant inhibition of force at the lower concentrations of PE (P < 0.05 by ANOVA). Bumetanide produced a much greater shift in the dose response for PE in aortas from coarcted rats, manifested as a significant, threefold increase in EC50. In a separate set of experiments, proximal and distal segments of the coarcted aorta were compared (Fig. 6). Again, bumetanide produced a significant increase in the EC50 for PE proximal to the coarctation (Fig. 6), but there was no significant increase distal to the coarctation. Neither bumetanide nor coarctation altered maximal force generation.

DISCUSSION

Coarctation of the aorta increased bumetanide-sensitive efflux of 86Rb in vascular smooth muscle proximal to the coarctation. Although the net flux through NKCC1 under physiological conditions is inward owing to the concentration gradient for Cl−, the transporter is bidirectional and there is substantial K+/K+ exchange (23, 30). It is this exchange that is measured in the assay. At this concentration, bumetanide is a selective inhibitor of NKCC1. Bumetanide can also inhibit...
K-Cl cotransporters (KCCs) but at substantially higher concentrations. Smooth muscle cells cultured from the rat aorta express KCC1 and KCC3, with very little KCC2 and no KCC4 (9). Half-maximal inhibition of KCC1 by bumetanide occurs between 170 and 180 μM (17, 27). This parameter has not been determined for KCC3, but there is no inhibition by 30 μM bumetanide (36). The results therefore indicate an increase in the activity of NKCC1 in the hypertensive aorta.

There was no increase in bumetanide-insensitive efflux, indicating that the stimulation of NKCC1 was not part of a generalized increase in K⁺ transport. However, we cannot rule out a change in the Na-K pump because this would not affect K⁺ efflux. The increase in NKCC1 activity did not occur distal to the coarctation, indicating that systemic factors were not involved and that upregulation of NKCC1 is due specifically to increased blood pressure. However, we cannot rule out the possibility that proximal and distal aortas have different sensitivities to circulating factors or that local neurohumoral effects were responsible. Although not significant, there was a decrease in NKCC1 activity at all time points in the distal hypotensive aorta, suggesting regulation of NKCC1 by physiological blood pressure. Circulating levels of renin increase in this model (38), and we (2) have previously shown that angiotensin increases NKCC1 activity in rat aortic smooth muscle in vitro. Although angiotensin II could have contributed to elevated NKCC1 activity in the hypertensive segment, it should have also increased NKCC1 activity distal to the coarctation. The fact that activity was not elevated distally could be due to a concurrent effect of decreased blood pressure to reduce NKCC1 activity. In fact, high angiotensin II levels may explain why there was only a minimal reduction in NKCC1 distal to the coarctation.

Most studies of the relationship between vascular smooth muscle NKCC1 and blood pressure have been limited to cells cultured from hypertensive rats. Its activity is reduced in cells cultured from aortas of spontaneously hypertensive rats (35, 39) but increased in cells cultured from Milan hypertensive rats (4). Because of the phenotypic changes these cells undergo in culture (40), such results probably have little relevance to vascular smooth muscle in vivo. It is of interest that erythrocyte NKCC1 activity correlates with blood pressure in at least two forms of genetic hypertension in rats (3, 18). This suggests that NKCC1 may have a causative role in chronic hypertension, a possibility that is not addressed by our data. However, the fact that blood pressure can influence smooth muscle NKCC1 activity must be considered in future studies of NKCC1 in hypertensive smooth muscle.

The only study to date of NKCC1 in hypertensive smooth muscle has been in the femoral artery of rats made hyperten-

Table 2. NKCC1 mRNA abundance in the rat aorta

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<th>ShamCoarctedCoarcted/</th>
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<tr>
<td></td>
<td>mRNA, fg/mg total RNA</td>
<td>mRNA,</td>
</tr>
<tr>
<td>NKCC1</td>
<td>0.097±0.023 (n=8)</td>
<td>0.51±0.13 (n=8)</td>
</tr>
<tr>
<td>β-Actin</td>
<td>43.1±7.9 (n=7)</td>
<td>42.1±4.3 (n=7)</td>
</tr>
<tr>
<td>Ka/β-Actin, %</td>
<td>0.037±0.004 (n=7)</td>
<td>0.20±0.05 (n=7)</td>
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</table>

Values are means ± SE; n, no. of aortas. NS, not significant.
sive by nephrectomy, high-salt diet, and administration of deoxycorticosterone acetate (DOCA hypertension model). Measurement of [Cl\textsuperscript{−}], by an ion-selective electrode revealed an increase in hypertensive muscle with an augmented drop after bumetanide (7), indicating increased net influx of Cl\textsuperscript{−} via NKCC1. However, this effect could have been due to the mineralocorticoid rather than hypertension because we (13) have found that aldosterone stimulates NKCC1 in the rat aorta in vitro. Overbeck et al. (32) measured \textsuperscript{86}Rb uptake in the rat aorta 4–5 wk after coarctation and found that ouabain-sensitive uptake (Na-K pump activity) was increased on both sides of the coarctation. However, ouabain-insensitive uptake increased only in the hypertensive aorta, consistent with the results obtained here.

We (1) have previously shown that acute stimulation of NKCC1 by smooth muscle agonists is dependent on contraction, providing a possible link between blood pressure and NKCC1. However, this acute effect would not be detectable in the present study because the vessels were removed from the hypertensive environment for several hours before the assay.

The mechanism by which blood pressure regulates transcription of the NKCC1 gene is unknown because little is known about transcriptional regulation of this gene. However, it is of interest that the murine NKCC1 gene contains several SP1 sites (37) and that this transcription factor is regulated by blood pressure in spontaneous hypertensive rats (29).

Inhibition of NKCC1 reduces contraction of normal vascular smooth muscle (2, 6, 19), indicating that NKCC1 is required for normal contraction. This is probably related to maintenance of [Cl\textsuperscript{−}], above its electrochemical equilibrium (5) because the effect of bumetanide is mimicked by depleting intracellular Cl\textsuperscript{−} (6, 19). The data presented here demonstrate that inhibition of force generation by bumetanide is much greater in acutely hypertensive aortas from coarcted rats compared with either aortas from sham-operated animals or nonhypertensive aortas distal to the coarctation. This latter comparison is important because it again implicates blood pressure itself rather than a systemic factor. It also demonstrates that the contribution of NKCC1 to contraction correlates with its activity.

One interpretation of the data is that increased NKCC1 activity induced by raising blood pressure results in an increase in smooth muscle [Cl\textsuperscript{−}], as previously shown in DOCA hypertensive rats (7), that in turn leads to increased tone. However, neither maximum force nor the sensitivity to PE was increased by coarctation in this study and in a previous study (33). This suggests that rather than increasing force generation, the increase in NKCC1 activity is instead required to maintain normal force generation. Thus the upregulation of NKCC1 may be a secondary, homeostatic response to other alterations related to blood pressure. One possibility is an increase in Cl\textsuperscript{−} efflux, which has been described in hypertensive aortas from mineralocorticoid-treated rats (25). Alternatively, stimulation of NKCC1 may be related to smooth muscle hypertrophy. This transporter is activated by growth factors and mediates a hypertrophic volume increase in both endothelial cells (14) and vascular smooth muscle cells (34) and therefore could contribute to the vascular smooth muscle cell enlargement observed after coarctation (33). Whatever the mechanism for stimulation of NKCC1, it is clear that the tone of acutely hypertensive smooth muscle is more dependent on NKCC1 and therefore more sensitive to bumetanide. This may be of clinical relevance because the mild vasorelaxant property of loop diuretics (8, 12) may be enhanced when blood pressure is elevated.

**REFERENCES**


