Aldosterone Regulates the Na-K-2Cl Cotransporter in Vascular Smooth Muscle

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Abstract—Aldosterone increases cation transport and contractility of vascular smooth muscle, but the specific transporter involved and how it is linked to smooth muscle tone is unknown. Because the Na-K-2Cl cotransporter (NKCC1) contributes to vascular smooth muscle contraction and is regulated by vasoactive compounds, we sought to determine whether this transporter is a target of aldosterone in rat aorta. Treatment of adrenalectomized rats with aldosterone for 7 days resulted in a 63% increase in NKCC1 activity as measured by bumetanide-sensitive efflux of 86Rb⁺. Treatment of normal aortas in culture with aldosterone for 3 and 7 days resulted in 29% and 47% increases in NKCC1 activity, respectively. Aldosterone had no acute effect on 86Rb⁺ efflux. Stimulation of NKCC1 was blocked by spironolactone, a mineralocorticoid receptor antagonist, but not by RU38486, a glucocorticoid receptor antagonist. Aldosterone did not augment the stimulation of NKCC1 by phenylephrine and did not increase NKCC1 mRNA as determined by real-time polymerase chain reaction. We conclude that aldosterone regulates the Na-K-2Cl cotransporter in vascular smooth muscle through classic mineralocorticoid receptors but not through changes in the abundance of NKCC1 mRNA. This could account for the increase in Na⁺, K⁺, and Cl⁻ fluxes previously observed in vascular smooth muscle from mineralocorticoid-treated animals and may contribute to increased vascular tone. (Hypertension. 2003;41:●●●●.●●●●.)

Key Words: aldosterone □ muscle, smooth, vascular □ rats □ hypertension, mineralocorticoid □ vasoconstriction

Mineralocorticoids such as aldosterone are required for the maintenance of sodium balance and vascular tone. Although the major action of aldosterone is to enhance sodium reabsorption in the collecting duct of the kidney, this cannot account for all of its hypertensive effects, and it is clear that vasculature is an important target of mineralocorticoids. Arteries removed from mineralocorticoid-treated animals exhibit increased sensitivity to vasoconstrictors, and a similar response is seen in vessels treated with aldosterone in vitro, indicating a direct action on vascular smooth muscle. Accordingly, mineralocorticoid receptors have been demonstrated in the arterial wall and in isolated smooth muscle cells.

The mechanism responsible for increased contracture is unknown but may involve changes in Na⁺ transport akin to those in other mineralocorticoid-sensitive cells. Both the Na⁺ content and passive flux of Na⁺ are increased in vascular smooth muscle from mineralocorticoid-treated animals, and both the mRNA and activity of the Na⁺ pump are increased. This upregulation of the Na⁺ pump is probably secondary to increased Na⁺ influx because intracellular [Na⁺] is increased rather than decreased. Although increased intracellular [Na⁺] could stimulate Ca influx through the Na-Ca exchange, thereby increasing contractility, Ca stores do not appear to be increased in vascular smooth muscle from mineralocorticoid-treated rats. However, acute increases in intracellular [Ca²⁺] have been observed after treatment of cultured vascular smooth muscle cells with aldosterone. The source of the increased passive Na⁺ flux is unknown, but a likely candidate is the Na-K-2Cl cotransporter NKCC1. We have recently demonstrated that this transporter is acutely activated by vasoconstrictors and inhibited by nitrovasodilators in isolated rat aorta. In addition to the increase in Na⁺ flux, stimulation of NKCC1 could also account for the increased K⁺ and Cl⁻ fluxes also observed in vascular smooth muscle from mineralocorticoid-treated animals. In fact, the increase in intracellular [Cl⁻] in femoral artery of mineralocorticoid-treated, hypertensive rats is abolished by bumetanide, a specific inhibitor of NKCC1. Although this indicates an increased Cl⁻ influx via NKCC1, it is unclear whether this is due to the mineralocorticoid, the high salt diet, or the hypertension. Bumetanide also reduces isometric force generation in normal vascular smooth muscle, indicating a role for NKCC1 in smooth muscle contraction. To determine whether the Na-K-2Cl cotransporter in vascular smooth muscle is regulated by aldosterone, we measured bumetanide-sensitive fluxes in aortas from rats treated with aldosterone. Studies were also performed in normal aortas in culture to show that the regulation of NKCC1 was through a direct action of aldosterone on smooth muscle.
Measurement of Na-K-2Cl cotransport in rat aorta. Efflux of $^{86}$Rb$^+$ is constant and then drops to a lower, constant level after addition of 50 μmol/L bumetanide. Results are the means from a single assay performed in triplicate.

**Methods**

**In Vivo Studies**

Male Sprague-Dawley rats (125 to 150 g; Charles River Laboratories, Wilmington, Mass) were adrenalectomized, then given 0.9% saline to drink and fed 23% protein chow ad libitum as described previously. After 14 days, osmotic minipumps (Model 2001, Alzet) containing 8.3 mg/mL aldosterone (Research Plus Inc) in 10% dimethyl sulfoxide (DMSO) in saline or vehicle alone were implanted subcutaneously in the midscapular region. The infusion rate was 8.3 μg/d (approximately 1.3 mg/kg per day). Rats were sacrificed after 7 days, and blood and aortas were collected. Serum aldosterone levels were determined by radioimmunoassay (Diagnostics Products Corp).

**In Vitro Studies**

Aortas proximal to the celiac axis were removed from normal rats and the adventitia was carefully dissected away, using sterile technique. Rings (0.5 cm in length) were placed in DMEM (low glucose) medium with penicillin and streptomycin, but no serum, and implanted subcutaneously in the midscapular region. The vessels were maintained in a 5% CO2 atmosphere at 37°C with medium changes every 3 days.

**NKCC1 Assay**

Activity was measured as bumetanide-sensitive $^{86}$Rb$^+$ efflux as previously described. Briefly, vessel segments were opened longitudinally and the endothelium removed with a cotton swab. They were then loaded with $^{86}$Rb$^+$ for 2 hours in a HEPES-buffered physiological saline solution containing 5.4 mM/L K, 1.8 mM/L Ca, and 0.8 mM/L Mg. Steady-state loading of Rb$^+$ requires 3 to 4 hours, 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 extensive washing, efflux of $^{86}$Rb$^+$ was measured over 10 minutes at 2 minutes intervals before and after addition of 50 μmol/L bumetanide (Figure 1). Results are expressed as the fraction of $^{86}$Rb$^+$ remaining after bumetanide.

**Real-Time Polymerase Chain Reaction (PCR)**

Total RNA was prepared using a modified phenol-chloroform extraction from rat aorta previously frozen in liquid N2 and stored at −80°C. RNA (2 μg) was converted into cDNA using ThermoScript RT reverse transcriptase (Invitrogen) and 200 ng was then amplified in an PE Biosystems real-time PCR unit using SYBR green dye. Forward and reverse primers for NKCC1 were CCACACACACAC-CTACTAC and TGCGACCACACATCTCT, respectively, corresponding to nucleotides 743 to 761 and 956 to 973 of the 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 TGGTTGCT-CCTGTATGCCTCTTGC and ATGTCAAGCAGATTTCTC-CCTCTCA, corresponding to nucleotides 416 to 439 and 635 to 658.

**Data Analysis**

Results are expressed as the mean of the number of samples indicated. Errors are standard errors. Significance was determined by Student t test (2-tailed).

**Results**

Results from aortas of adrenalectomized rats treated with aldosterone are shown in Figure 2. Aldosterone increased total efflux of $^{86}$Rb$^+$ and decreased bumetanide-insensitive efflux slightly, resulting in a 63% increase in bumetanide-sensitive efflux ($P<0.01$). Plasma levels of aldosterone were 0.46±0.21 nmol/L in control rats; 0.06±0.01 nmol/L in adrenalectomized rats, and 0.6±0.03 nmol/L in adrenalectomized rats receiving aldosterone. To determine whether this was a direct effect of aldosterone on vascular smooth muscle, aortas were removed from normal rats and maintained in culture in the presence or absence of 50 nmol/L aldosterone. As shown in the Table, NKCC1 was also responsive to aldosterone in vitro but less so than in vivo (29% increase after 3 days, $P<0.01; 48%$ after 7 days, $P<0.001$). Basal NKCC1 activity was similar to that in freshly isolated aortas from adrenalectomized rats. Bumetanide-insensitive $^{86}$Rb$^+$ efflux was not significantly changed by aldosterone in culture, but was substantially higher after 7 days in culture. Histology of aortas maintained in culture revealed an intact endothelium and media with no proliferation or loss of smooth muscle cells (not shown).

Most effects of aldosterone are mediated through classic mineralocorticoid receptors that affect DNA transcription. Immediate nongenomic effects on Na$^+$ transport have also
been proposed in vascular smooth muscle cells, but aldosterone had no immediate effect on Rb efflux in rat aorta (Figure 3). The response to different concentrations of aldosterone in culture is shown in Figure 4. A 3-parameter exponential regression yielded a half-maximal concentration of aldosterone for stimulation of NKCC1 of 0.052 ± 0.017 nmol/L, which is in the physiological range and consistent with the affinity of the mineralocorticoid receptor for aldosterone. Stimulation of NKCC1 was blocked by spironolactone (a mineralocorticoid receptor antagonist), but not by RU38486, a glucocorticoid receptor antagonist (Figure 5), indicating that it is mediated by classic mineralocorticoid receptors. The increase in NKCC1 activity with spironolactone is consistent with a partial agonist effect that may occur with spironolactones.

To determine whether aldosterone increases the quantity of cotransporters, we stimulated aldosterone-treated aortas with phenylephrine. This α-adrenergic agent acutely stimulates NKCC1, and we reasoned that an increase in cotransporter quantity would augment the stimulation by phenylephrine. As seen in Figure 6, NKCC1 activity after phenylephrine was not greater in aortas cultured with aldosterone for 3 days. Because basal NKCC1 activity was greater in aldosterone-treated aortas, the stimulation by phenylephrine was actually reduced. Similar results were obtained in an additional experiment and in one experiment in aortas from adrenalectomized rats treated with aldosterone in vivo. To address this issue further, we measured NKCC1 mRNA by real-time PCR. This assay has been used to show increased NKCC1 mRNA expression in hypertensive rats (G. Jiang, F. Akar, S. Cobbs, K. Lomeshriili, R. Lakkis, F. Gordon, R. Sutliff, W. O’Neill, submitted for publication, 2003). In 3 separate experiments, each performed in triplicate, there was no increase in NKCC1 mRNA in aortas treated with aldosterone in culture for 3 days (2.82 ± 0.29 vs 2.86 ± 0.25 pg NKCC1 mRNA/ng β-actin mRNA, aldosterone vs control).

Discussion
Our results demonstrate that activity of the Na-K-2Cl cotransporter in vascular smooth muscle is increased by the administration of aldosterone to adrenalectomized rats. This was due to a direct vascular action of aldosterone because stimulation of NKCC1 was also observed in culture. The concentrations of aldosterone capable of stimulating the cotransporter were physiological and consistent with other actions mediated by the classic mineralocorticoid receptor. The inhibition by spironolactone, but not by RU38486, confirmed involvement of this receptor. Activity of NKCC1 in aortas from adrenalectomized rats was similar to that previously reported in normal rat aorta indicating that mineralocorticoids are not required for NKCC1 activity. Although this is consistent with serum levels of aldosterone in normal rats that

![Figure 3](image-url)  
**Figure 3.** Lack of an immediate effect of aldosterone on rat aortic NKCC1. Efflux of Rb in the absence (solid symbols) and presence (open symbols) of 50 μmol/L bumetanide in normal rat aortas. Aldosterone (50 nmol/L) was added as indicated. Results are from a single aorta assayed in triplicate. Similar results were obtained in an additional experiment. Error bars indicate standard error.

![Figure 4](image-url)  
**Figure 4.** Dependence of NKCC1 activity on aldosterone concentration. Segments of aortas from 4 rats were placed in culture for 7 days with the concentrations of aldosterone indicated. Bumetanide-sensitive efflux of Rb was measured as described in Methods. Results are the means of quadruplicate determinations for each concentration. Error bars indicate standard error.
Figure 5. Effect of receptor antagonists on the stimulation of NKCC1 by aldosterone. Efflux of $^{86}$Rb$^+$ in the absence and presence of 50 μmol/L bumetanide in normal rat aortas cultured for 3 days with vehicle (Control) or aldosterone (+Aldo) and 5 mol/L spironolactone or 100 nmol/L RU38486. Results are presented as bumetanide-sensitive efflux and represent the means of triplicate determinations from a single aorta. Similar results were obtained in an additional experiment. Error bars indicate standard error. *P<0.05 versus basal.

Figure 6. Effect of aldosterone on the activation of NKCC1 by phenylephrine. Efflux of $^{86}$Rb$^+$ was measured in the absence and presence of 50 μmol/L bumetanide in normal rat aortas cultured for 3 days with vehicle (Control) or aldosterone (+Aldo). Efflux was measured for 10 minutes before (+basal) and 10 minutes after (+ PE) addition of 10 μmol/L phenylephrine. Results are the means of a single aorta assayed in triplicate. Similar results were obtained in 2 other aortas after treatment with aldosterone in vitro or in vivo. Error bars indicate standard error. *P<0.05 versus basal.

were indistinguishable from those in adrenalectomized rats, other adrenal steroids could contribute to basal mineralocorticoid action. It appears then that NKCC1 activity in vascular smooth muscle responds to increased aldosterone levels and participates in the adaptation to volume depletion or reduced cardiac output.

Stimulation of NKCC1 could explain the increased Na$^+$, K$^+$, and Cl$^-$ fluxes previously noted in vascular smooth muscle from mineralocorticoid-treated rats. Although we measured cotransporter activity as unidirectional efflux of Rb$^+$ (as a tracer for K$^+$), the transporter is bidirectional and the net flux under physiological conditions is inward because of the inward gradients for both Na$^+$ and Cl$^-$: This is demonstrated by the reduction in [Cl$^-$]$_i$ after treatment of vascular smooth muscle with bumetanide. Thus stimulation of NKCC1 by aldosterone would be expected to increase [Cl$^-$], and at least partly explains the increased [Cl$^-$], in vascular smooth muscle from deoxycorticosterone acetate (DOCA)-hypertensive rats. Intracellular [Cl$^-$] in vascular smooth muscle is substantially lower than extracellular [Cl$^-$], but above electrochemical equilibrium, enabling agonist-sensitive Cl$^-$ channels to initiate a depolarization that leads to subsequent Ca influx via voltage-sensitive channels. A reduction in [Cl$^-$], probably explains the reduced sensitivity of isometric force generation to phenylephrine in mice lacking NKCC1 and in normal aortas treated with bumetanide. Likewise, an increase in [Cl$^-$], resulting from stimulation of NKCC1 could account for the increased sensitivity to vasoconstrictors produced by mineralocorticoids.

Stimulation of NKCC1 activity could explain the increase in intracellular [Na$^+$] noted in mineralocorticoid-treated vascular smooth muscle. This has been demonstrated in cardiac myocytes, in which bumetanide blocked the increase in Na$^+$ influx produced by aldosterone. An increase in intracellular Na$^+$ could contribute to the contractile effect of NKCC1 by secondarily increasing Ca influx through Na-Ca exchange. However, Cl$^-$ is the more likely mediator because Ca stores do not appear to be increased by mineralocorticoids and because bumetanide does not inhibit the contractile response to KCl. The contraction produced by KCl is Ca-dependent but KCl directly depolarizes the membrane and therefore bypasses any effect of intracellular Cl$^-$.

The coupled influx of Na$^+$, K$^+$, and Cl$^-$ ions produced by NKCC1, together with an obligate influx of water, results in an increase in cell volume. Consequently, NKCC1 is an important volume-regulatory transporter and stimulation of NKCC1 by growth factors produces cell enlargement that may be required for cell growth. Thus, stimulation of NKCC1 may contribute to smooth muscle hypertrophy and remodeling in addition to increased tone.

The mechanism by which aldosterone stimulates NKCC1 in vascular smooth muscle is unclear. The absence of an increase in NKCC1 mRNA or augmentation of the phenylephrine response suggests that there is not an increase in the number of transporters. In contrast, hypertension produced by aortic coarctation, which results in a similar stimulation of NKCC1, produces a 5-fold increase in NKCC1 mRNA (Jiang et al, submitted). Although a small increase in NKCC1 mRNA by aldosterone cannot be ruled out, the stimulation of NKCC1 by aldosterone clearly has a different mechanism. It is likely then that aldosterone is producing an indirect genomic stimulation of NKCC1 similar to its regulation of Na$^+$ channels. NKCC1 is acutely regulated through direct phosphorylation by a kinase that is activated by cell shrinkage and inhibited by intracellular Cl$^-$: Because neither smooth muscle shrinkage nor a decrease in [Cl$^-$] seems likely, aldosterone could be inducing phosphorylation of NKCC1 through a different mechanism that is under genomic control. Vasoconstrictors also phosphorylate NKCC1 in rat
aorta.\textsuperscript{14} and the fact that the stimulation of NKCC1 by aldosterone and phenylephrine were not additive suggests a common pathway.

**Perspectives**

The vasculature is an important target of mineralocorticoids, but the mechanism by which mineralocorticoids promote vascular tone is unknown. Previous studies have shown increased ion fluxes consistent with aldosterone action in the kidney, but the identity of the transporter(s) and how these fluxes contribute to increased tone were unclear. The finding that aldosterone increases the activity of the Na-K-2Cl cotransporter in rat aorta provides some important answers. Stimulation of this single transporter can explain the increased passive fluxes of Na\textsuperscript{+}, K\textsuperscript{+}, and Cl\textsuperscript{−} fluxes previously demonstrated in mineralocorticoid-treated rats, as well as upregulation of the Na-K pump secondary to increased [Na\textsuperscript{+}]. The fact that this transporter contributes to force generation provides a plausible link between the increased ion fluxes and the increased vascular tone produced by mineralocorticoids. This inotropic effect of the cotransporter appears to be mediated by an increased [Cl\textsuperscript{−}], indicating that aldosterone should be considered to be a Cl\textsuperscript{−}-retentive hormone as well as a Na-retentive hormone. The contribution of NKCC1 to smooth muscle tone may explain the weak vasodilatory response to loop diuretics, and our new results suggest that they might be particularly useful in treating mineralocorticoid-dependent hypertension. However, these drugs are highly protein-bound, and direct vasodilatory effects may occur only at very high clinical doses.\textsuperscript{14} The degree to which bumetanide blocks the vasoconstrictive and hypertensive effects of mineralocorticoids is an important issue that is currently being investigated.

**References**

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