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 $^{86}$Rb$^+$. 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 $^{86}$Rb$^+$ 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;41:1131-1135.)

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 contraction 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 [Ca] 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, indicating 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.

Received July 1, 2002; first decision July 30, 2002; revision accepted February 28, 2003.
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© 2003 American Heart Association, Inc.
Hypertension is available at http://www.hypertensionaha.org DOI: 10.1161/01.HYP.0000066128.04083.CA
By guest on June 7, 2017

Results are expressed as the fraction of \(^{86}\text{Rb}\) with and without addition of 50 µmol/L bumetanide. Basal NKCC1 activity was similar to that in freshly isolated aortas from adrenalectomized rats. Bumetanide-insensitive \(^{86}\text{Rb}\) efflux was not significantly changed by aldosterone in vitro but less so than in vivo (29% increase \(P_{<0.01}\)). 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 \(^{86}\text{Rb}\) 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

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.\(^{13,14}\) 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 the adventitia and media with no proliferation or loss of smooth muscle cells (not shown).

NKCC1 Assay

Activity was measured as bumetanide-sensitive \(^{86}\text{Rb}\) efflux as previously described.\(^{14}\) Briefly, vessel segments were opened longitudinally and the endothelium removed with a cotton swab. They were then loaded with \(^{86}\text{Rb}\) for 2 hours in a HEPES-buffered physiological saline solution containing 5.4 mmol/L K\(^+\), 1.8 mmol/L Ca\(^2+\), and 0.8 mmol/L Mg\(^2+\). Steady-state loading of \(^{86}\text{Rb}\) 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 \(^{86}\text{Rb}\) and no differences between fluxes after different loading times. After extensive washing, efflux of \(^{86}\text{Rb}\) 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 \(^{86}\text{Rb}\) leaving the vessel per minute and the flux caused by NKCC1 is determined by subtracting the mean of the 3 values after 4 minutes of bumetanide from the mean of the 3 values just before addition of bumetanide.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA was prepared using a modified phenol-chloroform extraction from rat aorta previously frozen in liquid N\(_2\) and stored at \(-80^\circ\text{C}\). 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 CCAACACACACCTACTAC and TGCGACCACAGCATCTCT, 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 CTGTATGCTCAGTCTGT and ATGGTACGCAGATTTTCCCTCTCA, corresponding to (nucleotides 416 to 439 and 635 to 656).

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 \(^{86}\text{Rb}\) 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 \(^{86}\text{Rb}\) 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).
been proposed in vascular smooth muscle cells, but aldosterone had no immediate effect on $^{86}$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 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 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...

**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...
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.\(^5\),\(^7\),\(^1\)\(^1\) \(^1\) Although we measured cotransporter activity as unidirectional efflux of \(\text{Rb}^+\) (as a tracer for \(\text{K}^+\)), the transporter is bidirectional and the net flux under physiological conditions is inward because of the inward gradients for both \(\text{Na}^+\) and \(\text{Cl}^-\). This is demonstrated by the reduction in \([\text{Cl}^-]\) after treatment of vascular smooth muscle with bumetanide.\(^2\),\(^2\) Thus stimulation of NKCC1 by mineralocorticoid would be expected to increase \([\text{Cl}^-]\), and at least partly explains the increased \([\text{Cl}^-]\), in vascular smooth muscle from deoxycorticosterone acetate (DOCA)-hypertensive rats.\(^1\)\(^6\) Intracellular \([\text{Cl}^-]\) in vascular smooth muscle is substantially lower than extracellular \([\text{Cl}^-]\), but above electrochemical equilibrium, enabling agonist-sensitive \(\text{Cl}^-\) channels to initiate a depolarization that leads to subsequent \(\text{Ca}\) influx via voltage-sensitive channels.\(^2\),\(^2\) A reduction in \([\text{Cl}^-]\) probably explains the reduced sensitivity of isometric force generation to phenylephrine in mice lacking NKCC1\(^2\),\(^4\) and in normal aortas treated with bumetanide.\(^1\)\(^4\),\(^17\) Likewise, an increase in \([\text{Cl}^-]\), resulting from stimulation of NKCC1 could account for the increased sensitivity to vasoconstrictors produced by mineralocorticoids.\(^2\),\(^4\)

Stimulation of NKCC1 activity could explain the increase in intracellular \([\text{Na}^+]\) noted in mineralocorticoid-treated vascular smooth muscle.\(^5\),\(^1\)\(^1\) This has been demonstrated in cardiac myocytes, in which bumetanide blocked the increase in \(\text{Na}^+\) influx produced by aldosterone.\(^2\)\(^5\) An increase in intracellular \(\text{Na}^+\) could contribute to the contractile effect of NKCC1 by secondarily increasing \(\text{Ca}\) influx through Na-Ca exchange. However, \(\text{Cl}^-\) is the more likely mediator because \(\text{Ca}\) stores do not appear to be increased by mineralocorticoids.\(^1\)\(^2\) and because bumetanide does not inhibit the contractile response to KCl.\(^1\)\(^4\) The contraction produced by KCl is \(\text{Ca}\)-dependent but KCl directly depolarizes the membrane and therefore bypasses any effect of intracellular \(\text{Cl}^-\). An acute increase in \([\text{Ca}^{2+}]\), produced by aldosterone in cultured smooth muscle cells could also contribute to increased smooth muscle tone\(^3\) but cannot be ascribed to NKCC1 because there was no acute stimulation of this transporter by aldosterone.

The coupled influx of \(\text{Na}^+\), \(\text{K}^+\), and \(\text{Cl}^-\) 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.\(^2\)\(^6\) and stimulation of NKCC1 by growth factors\(^2\)\(^7\) produces cell enlargement that may be required for cell growth.\(^2\)\(^8\),\(^2\)\(^9\) 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 \(\text{Na}^+\) channels.\(^3\)\(^0\) NKCC1 is acutely regulated through direct phosphorylation by a kinase that is activated by cell shrinkage and inhibited by intracellular \(\text{Cl}^-\).\(^3\)\(^1\),\(^3\)\(^2\) Because neither smooth muscle shrinkage nor a decrease in \([\text{Cl}^-]\) seems likely, aldosterone could be inducing phosphorylation of NKCC1 through a different mechanism that is under genomic control. Vasoconstrictors also phosphorylate NKCC1 in rat clearance.

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**Figure 5.** Effect of receptor antagonists on the stimulation of NKCC1 by aldosterone. Efflux of \(\text{Rb}^+\) in the absence and presence of 50 \(\mu\)mol/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 control by paired analysis.

**Figure 6.** Effect of aldosterone on the activation of NKCC1 by phenylephrine. Efflux of \(\text{Rb}^+\) was measured in the absence and presence of 50 \(\mu\)mol/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 \(\mu\)mol/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.
aorta, 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⁺, K⁺, and Cl⁻ fluxes previously demonstrated in mineralocorticoid-treated rats, as well as upregulation of the Na-K pump secondary to increased [Na⁺]. The fact that this cotransporter 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⁻], indicating that aldosterone should be considered to be a Cl-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. The degree to which bumetanide blocks the vasoconstrictive and hypertensive effects of mineralocorticoids is an important issue that is currently being investigated.

**References**
