Effect of the Na-K-2Cl cotransporter NKCC1 on systemic blood pressure and smooth muscle tone

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Garg P, Martin CF, Elms SC, Gordon FJ, Wall SM, Garland CJ, Sutliff RL, O’Neill WC. Effect of the Na-K-2Cl cotransporter NKCC1 on systemic blood pressure and smooth muscle tone. Am J Physiol Heart Circ Physiol 292: H2100–H2105, 2007. First published January 26, 2007; doi:10.1152/ajpheart.01402.2006.—Studies in rat aorta have shown that the Na-K-2Cl cotransporter NKCC1 is activated by vasoconstrictors and inhibited by nitravasodilators, contributes to smooth muscle tone in vitro, and is upregulated in hypertension. To determine the role of NKCC1 in systemic vascular resistance and hypertension, blood pressure was measured in rats before and after inhibition of NKCC1 with bumetanide. Intravenous infusion of bumetanide sufficient to yield a free plasma concentration above the IC50 for NKCC1 produced an immediate drop in blood pressure of 5.2% (P < 0.001). The reduction was not prevented when the renal arteries were clamped, indicating that it was not due to a renal effect of bumetanide. Bumetanide did not alter blood pressure in NKCC1-null mice, demonstrating that it was acting specifically through NKCC1. In third-order mesenteric arteries, bumetanide-inhibitable efflux of 86Rb was acutely stimulated 133% by phenylephrine, and bumetanide reduced the contractile response to phenylephrine, indicating that NKCC1 influences tone in resistance vessels. The hypertensive effect of bumetanide was proportionately greater in rats made hypertensive by a 7-day infusion of norepinephrine (12.7%, P < 0.001 vs. normotensive rats) but much less so when hypertension was produced by a fixed aortic coarctation (8.0%), again consistent with an effect of bumetanide on resistance vessels rather than other determinants of blood pressure. We conclude that NKCC1 influences blood pressure through effects on smooth muscle tone in resistance vessels and that this effect is augmented in hypertension.

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Mean pressure was measured over a 1-min period after a stable baseline was achieved (10 min) and again after 5 min. Mean pressure was then measured before and 5 min and 10 min after the vehicle bolus and then again after the bumetanide bolus. Bumetanide and vehicle were given as 100-μl boluses followed by an equal volume of heparinized saline to clear the catheter. NKCC1-null (NKCC1−/−) mice (originally provided by Dr. Gary Shull) and wild-type (129Sv1l/Black Swiss) mice were anesthetized with isoflurane, and a pressure transducer (SPR-671, Millar Instruments) was inserted into the right carotid artery for measurement of blood pressure. A catheter was inserted into the right internal jugular vein for infusions. A baseline measurement of blood pressure was taken for 10 min before infusions began. A volume of vessel equal to the volume of bumetanide to be injected was administered over 20–30 s into the jugular vein, and hemodynamic parameters were monitored for 15 min. Thirty minutes after infusion of vehicle, bumetanide (1.2 mg/kg) was infused and hemodynamics were monitored. Data were acquired and analyzed using a PowerLab system and Chart software (ADInstruments, Colorado Springs, CO).

**Bumetanide infusion.** A 10 mM solution of bumetanide was prepared by adding 10 vol of 100 mM bumetanide in DMSO to 89 vol of a HEPES-buffered physiological saline solution and 1 vol of 8 M NaOH. The vehicle control was DMSO, prepared similarly. Because bumetanide is highly protein bound, it was necessary to determine the dose required to achieve an adequate free concentration of bumetanide in plasma. Heparinized blood was obtained from rats before and after a bolus infusion of bumetanide and was centrifuged to obtain plasma. The plasma was then centrifuged through a 10,000-Da filter (Centricon), and the concentration of bumetanide in the filtrate was measured by fluorescence (338-nm excitation and 433-nm emission) after the fluorescence of filtrate was subtracted from normal rat plasma. A dose of 1.2 mg/kg (100 μl of 10 mM bumetanide in a 300-g rat) resulted in a free plasma concentration of 5 μM, which is 25-fold higher than the IC50 of 2.8 μM, determined as described above. As shown in Fig. 2, bumetanide decreased blood pressure an additional 6.8 mmHg beyond that produced by vehicle alone in wild-type mice, similar to that observed in rats. In the NKCC1−/− mice, there was no effect of bumetanide beyond that seen with vehicle alone.

### RESULTS

The effect of NKCC1 on blood pressure was examined by infusing bumetanide into anesthetized rats. In normotensive rats, a bolus injection of vehicle alone did not significantly alter blood pressure (Table 1). This was followed by the bolus injection of bumetanide, which decreased blood pressure in each rat and resulted in a mean decline of 5.2 ± 0.6% compared with vehicle alone after 10 min (P < 0.01). The results are shown graphically in Fig. 1. There was no further decline after 10 min, so this time was used for all subsequent measurements. A similar decrease in blood pressure (7.8%) occurred in rats in which both renal arteries were occluded before the infusions (Table 1 and Fig. 1). Baseline pressure was significantly elevated after renal artery occlusion. To confirm that the decrease in blood pressure produced by bumetanide was the result of inhibition of NKCC1, blood pressure was also measured in the mice lacking NKCC1, before and after intravenous injection of 1.2 mg/kg bumetanide. This resulted in a free plasma bumetanide concentration of 2.8 μM, determined as described above. As shown in Fig. 2, bumetanide decreased blood pressure an additional 6.8 mmHg beyond that produced by vehicle alone in wild-type mice, similar to that observed in rats. In the NKCC1−/− mice, there was no effect of bumetanide beyond that seen with vehicle alone.

### Table 1. Blood pressure measurements in control rats

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Baseline</th>
<th>Vehicle</th>
<th>Bumetanide</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>92</td>
<td>92</td>
<td>93</td>
</tr>
<tr>
<td>5</td>
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<tr>
<td>10</td>
<td>92</td>
<td>84</td>
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</tbody>
</table>

**Intact rats**

**MAP, mmHg**

- Rat 1: 126, 124, 126, 124, 126, 120, 116
- Rat 3: 120, 118, 118, 116, 116, 116, 110, 106

Each row represents an individual rat and indicates mean arterial pressure (MAP) averaged over 1-min periods.
To determine whether NKCC1 influences blood pressure through effects on resistance arteries, NKCC activity and contraction were measured in third-order mesenteric arteries. Efflux of Rb was stable over time, and ~40% was inhibited by bumetanide (Fig. 3A). Immediately after addition of phenylephrine, there was a large increase in both the total flux and the flux in the presence of bumetanide (bumetanide-insensitive efflux). The difference between these two fluxes (bumetanide-sensitive efflux) also increased (Fig. 3B), with an immediate 133% mean increase over the basal rate. The bumetanide-insensitive flux subsequently returned to a level close to baseline, whereas the bumetanide-sensitive flux declined more slowly and remained elevated (54% above basal) 10 min after phenylephrine. Bumetanide-sensitive efflux was increased at each point after phenylephrine; however, because of the error inherent in determining bumetanide-sensitive efflux on top of the rise in bumetanide-insensitive efflux, the difference from baseline was only significant at the last time point, after the bumetanide-insensitive efflux had subsided.

To determine whether NKCC1 participates in the contractile response of resistance vessels, force generation in response to graded increases in phenylephrine concentration was measured in mesenteric arteries before and after incubation with 10 μM bumetanide. As shown in Fig. 4, bumetanide shifted the dose response to the right, resulting in an increase in the EC₅₀ for phenylephrine from 0.59 ± 0.08 to 1.07 ± 0.01 μM (P < 0.01). Maximal force was also reduced by bumetanide (97.1 ± 1.2% vs. 93.4 ± 0.9%, P < 0.02). Neither the basal tension nor the slope was altered by bumetanide.
Last, we examined the effect of bumetanide on blood pressure in hypertensive rats. As shown in Table 2, infusion of norepinephrine at a continuous subcutaneous dose of 2 μg·kg⁻¹·min⁻¹ for 7 days resulted in a substantial increase in mean arterial blood pressure (163 ± 2 mmHg compared with 101 ± 3 mmHg in control rats). Again, there was no effect of vehicle infusion; however, the fractional decrease after bumetanide (12.7 ± 0.7%) was twice that in control, normotensive rats (Fig. 5). Hypertension was also produced by fixed coarctation of the aorta. The mean arterial pressure in these animals (152 ± 8 mmHg) was only slightly less than in the norepinephrine-infused rats, but the decrease after bumetanide was substantially less (8.0 ± 0.3 mmHg, *P < 0.001).

**DISCUSSION**

Inhibition of NKCC1 with bumetanide acutely lowered blood pressure in normal rats. This reduction occurred too quickly to be explained by diuresis and cannot be ascribed to other renal actions, such as inhibition of tubuloglomerular feedback, since it also occurred in the absence of renal blood flow. The fact that bumetanide had no effect on blood pressure in mice lacking NKCC1 indicates that the hypotensive action of bumetanide is due specifically to inhibition of NKCC1. These studies were performed on anesthetized animals and therefore may not reflect the contribution of NKCC1 to blood pressure under normal conditions. However, urethane and isoflurane preserve hemodynamics better than other anesthetics and suppress cardiovascular function only slightly (15).

The reduction in blood pressure suggested an effect of NKCC1 on smooth muscle tone in resistance arteries. The presence of bumetanide-inhibitable fluxes in mesenteric arteries confirmed the presence of NKCC in these vessels and is consistent with our previous findings in aorta (1). Inhibition of NKCC reduced the contractile response of the mesenteric arteries to phenylephrine in vitro, again consistent with previous demonstrations of bumetanide-sensitive contraction to α-agonists or endothelin in conduit arteries (1, 2, 14, 23, 32). This action of bumetanide in resistance arteries is the likely mechanism for its hypotensive effect.

The effect of bumetanide is consistent with the known vasodilatory effect of furosemide, a closely related diuretic, in vitro and in vivo (3, 11, 14). However, furosemide is not specific for NKCC1 and can inhibit other Cl⁻ transport pathways. Therefore, the effect of bumetanide and the absence of an effect in NKCC1⁻/⁻ mice provides definitive proof that inhibition of NKCC1 can produce vasodilation and indicates that this is the likely mechanism for the vasodilatory effect of furosemide. Extensive protein binding limits systemic inhibition of NKCC1 by bumetanide at clinical doses, necessitating the use of much larger doses to achieve adequate plasma levels of free bumetanide. With the assumption that that 90% of

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**Table 2. Blood pressure measurements in hypertensive rats**

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Baseline</th>
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<th>Bumetanide</th>
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<tbody>
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<td>160</td>
<td>158 160</td>
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<tr>
<td>0-5</td>
<td>168</td>
<td>164</td>
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</table>

Each row represents an individual rat and indicates MAP averaged over 1-min periods.
bumetanide is protein bound (5) and that the volume of distribution is 0.068 l/kg (30), a standard dose of 0.015 mg/kg in humans would produce a free plasma concentration of ~60 nM. This is well below the half-inhibitory concentration of ~200 nM (20), but a maximal dose could approach this concentration.

The hypotensive action of bumetanide was significantly greater in rats made hypertensive by continuous infusion of norepinephrine. This augmented effect of bumetanide in norepinephrine-treated rats is consistent with the β-adrenergic stimulation of NKCC in mesenteric arteries, and, together, these observations indicate that stimulation of NKCC1 in vascular smooth muscle contributes to the hypertensive action of β-agonists. Whether an increase in NKCC activity is sufficient to produce hypertension is not known. The hypotensive action of bumetanide was substantially less in rats made hypertensive by aortic coarctation. This latter form of hypertension, in the acute stage studied here, results from a fixed increase in resistance in the aorta rather than increased tone in resistance vessels. The greater effect of bumetanide in the norepinephrine model than in the coarctation model is thus consistent with an action of bumetanide in resistance vessels. An equally hypotensive action of bumetanide in the coarctation model would have indicated an effect on cardiac output rather than systemic vascular resistance.

The effect of NKCC1 on smooth muscle contraction is most likely the result of its regulation of intracellular [Cl\(^-\)] since substitution of Cl\(^-\) with other anions or addition of Cl\(^-\)-channel blockers mimics the effect of bumetanide (4, 22, 23), and norepinephrine produces an increase in intracellular [Cl\(^-\)] that is partly blocked by bumetanide (8). Although intracellular [Cl\(^-\)] in smooth muscle is well below extracellular [Cl\(^-\)], its electrochemical potential is still outward because of the negative membrane potential. Being electroneutral, NKCC1 is not hindered by membrane potential and will move Cl\(^-\) inward solely as dictated by ion gradients and thus is ideally suited for maintaining intracellular [Cl\(^-\)] against an electrical potential, with the energy ultimately provided by the Na-K pump (26). Consequently, bumetanide or furosemide produces substantial decreases in intracellular [Cl\(^-\)] (6, 7, 13, 21) in vascular smooth muscle, and this is augmented in hypertension (7). Inhibition of contraction by Cl\(^-\)-channel blockers demonstrates that the high intracellular [Cl\(^-\)] is necessary for agonist-sensitive Cl\(^-\)-channels to initiate the depolarization that leads to subsequent Ca\(^{2+}\) influx via voltage-sensitive channels (4, 22). Consistent with this, furosemide reduces phenylephrine-mediated Ca\(^{2+}\) fluxes in rabbit aorta (11) and bumetanide inhibits influx through L-type Ca\(^{2+}\)-channels in depolarized rat aorta (2). This is also supported by the observation that bumetanide does not inhibit contraction induced by KCl (1), which depolarizes smooth muscle directly without involvement of Cl\(^-\)-channels.

Although these results suggest smooth muscle NKCC1 as a pharmacological target in hypertension, concurrent inhibition of NKCC2 in the ascending limb of Henle is a major obstacle. This would preclude currently available NKCC1 inhibitors because the large doses required to overcome the protein binding and achieve inhibitory free levels in plasma would result in very high free levels in the urinary space and a massive diuresis. Thus selective inhibition of NKCC1 would require a compound that does not inhibit the closely related NKCC2 or is not excreted in the urine. In addition, systemic inhibition of NKCC1 may produce unacceptable toxicity in the form of cochlear dysfunction and infertility (9).

**REFERENCES**


**GRANTS**

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