Decreased blood pressure and vascular smooth muscle tone in mice lacking basolateral Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter

JAMIE W. MEYER, MICHAEL FLAGELLA, ROY L. SUTLIFF, JOHN N. LORENZ, MICHELLE L. NIEMAN, CRAIG S. WEBER, RICHARD J. PAUL, and GARY E. SHULL

Departments of \(^1\)Molecular Genetics, Biochemistry, and Microbiology and \(^2\)Molecular and Cellular Physiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Received 25 February 2002; accepted in final form 3 July 2002

Meyer, Jamie W., Michael Flagella, Roy L. Sutliff, John N. Lorenz, Michelle L. Nieman, Craig S. Weber, Richard J. Paul, and Gary E. Shull. Decreased blood pressure and vascular smooth muscle tone in mice lacking basolateral Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter. Am J Physiol Heart Circ Physiol 283: H1846–H1855, 2002. First published July 11, 2002; 10.1152/ajpheart.00083.2002.—The basolateral isoform of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter (NKCC1) functions in the main-tenance of cellular electrolyte and volume homeostasis. NKCC1-deficient (Nkcc1\(^{-/-}\)) mice were used to examine its role in cardiac function and in the maintenance of blood pressure and vascular tone. Tail-cuff measurements demonstrated that awake Nkcc1\(^{-/-}\) mice had significantly lower systolic blood pressure than wild-type (Nkcc1\(^{+/+}\)) mice (114.5 ± 2.2 and 131.8 ± 2.5 mmHg, respectively). Serum aldosterone levels were normal, indicating that extracellular fluid-volume homeostasis was not impaired. Studies using pressure transducers in the femoral artery and left ventricle showed that anesthetized Nkcc1\(^{-/-}\) mice have decreased mean arterial pressure and left ventricular pressure, whereas myocardial contraction parameters were not significantly different from those of Nkcc1\(^{+/+}\) mice. When stimulated with phenylephrine, aortic smooth muscle from Nkcc1\(^{-/-}\) and Nkcc1\(^{+/+}\) mice exhibited no significant differences in maximum contractility and only moderate dose-response shifts. In phasic portal vein smooth muscle from Nkcc1\(^{-/-}\) mice, however, a sharp reduction in mechanical force was noted. These results indicate that NKCC1 can be important for the maintenance of normal blood pressure and vascular tone.

vasculature; hypotension; bumetanide

THE BASOLATERAL ISOFORM of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransport-er (NKCC1) mediates the transport of 1 Na\(^+\), 1 K\(^+\), and 2 Cl\(^-\) into the cell under normal conditions. NKCC1 is expressed in most tissues, including heart (10, 42), vascular smooth muscle (39), and endothelial cells (37). Although the roles of NKCC1 in transepithelial ion transport and in the regulation of cell volume and intracellular ion concentrations are well established (9, 14, 15, 17, 19–21, 24, 36, 37), its cardiovascular functions are not well understood. Vasoactive agents alter the activity of NKCC1 in vascular smooth muscle (2, 39, 47) and in endothelial cells (6, 27, 35). There is evidence that NKCC1 is responsible for the maintenance of intracellular Cl\(^-\) concentrations and cell volume during agonist stimulation of endothelial cells (38) and that its activity is regulated in response to changes in vascular smooth muscle contractility (1). The loop diuretic bumetanide, an inhibitor of NKCC1, causes a reduction in the sensitivity of rat aortic rings to phenylephrine-induced contraction (2). Furosemide, another loop diuretic, has been shown to relax canine venous smooth muscle preparations while having little effect on arteries (18). The results of these and other in vitro studies (reviewed in Ref. 13) suggest that NKCC1 could play a direct role in the modulation of vascular tone.

In vivo studies using bumetanide and other loop diuretics provide support for this hypothesis. In patients with congestive heart failure, furosemide caused a reduction in left ventricular filling pressure and an increase in caval venous compliance, which preceded the natriuretic and diuretic effects (11). After acute myocardial infarction, furosemide caused a decrease in left ventricular filling pressure, which was attributed to venodilation, but also led to a rapid increase in blood pressure and systemic vascular resistance (34). These and other studies (reviewed in Ref. 13) indicate that loop diuretics affect systemic hemodynamics and cardiac function not only by their natriuretic and diuretic activities but also by effects on the vasculature. Although some of these hemodynamic effects appeared to be caused by the release of vasoactive compounds from the kidney (5), it has been suggested that direct inhibition of NKCC1 by loop diuretics may cause vasodila-
tion of capacitance veins (13).

Gene-targeted mice lacking NKCC1 have been developed by several groups (9, 15, 40). NKCC1-deficient (Nkcc1\(^{-/-}\)) mice exhibit reduced epithelial chloride secretion (15, 17, 19, 20), male sterility (40), and both profound deafness and a balance defect (9, 15). In our own study (15), we also reported a significant reduction in mean arterial pressure (MAP) of anesthetized Nkcc1\(^{-/-}\) mice, measured using a femoral artery cath-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
eter; however, another group of investigators (40) observed no significant difference in systolic blood pressure of awake \(Nkcc1^{-/-}\) and wild-type (\(Nkcc1^{+/+}\)) mice, measured using a tail-cuff apparatus. With the exception of these limited analyses of blood pressure carried out using different procedures, the cardiovascular phenotype of \(Nkcc1^{-/-}\) mice has not been examined. Thus the major objectives of the present study were to perform a comprehensive analysis of blood pressure to resolve the apparent discrepancies between the two studies and to determine whether the loss of NKC1 leads to alterations of cardiac and/or vascular smooth muscle contractility. The results demonstrate that the \(Nkcc1^{-/-}\) mouse has a hypertensive phenotype and suggest that this may be due, at least in part, to a reduction in vascular tone.

**MATERIALS AND METHODS**

**Mice and genotype analysis.** Development of the NKC1 null mutant mouse line by gene targeting was described previously (17). Null mutant and wild-type control mice were generated by breeding of heterozygous mutant mice that were on a mixed background of 129/SvJ and Black Swiss strains. Genotypes were determined by PCR analysis of DNA from tail biopsies as described previously (15). The use of mice in these experiments was approved by the University of Cincinnati Animal Care and Use Committee.

**Tail-cuff blood pressure measurements.** Systolic blood pressure of adult \(Nkcc1^{+/+}\) (n = 17) and \(Nkcc1^{-/-}\) mice (n = 16) was recorded for 21 days using a Visitech Systems (Apex, NC) computerized tail-cuff apparatus (28). Because of the defect in the vestibular system of the inner ear of \(Nkcc1^{-/-}\) mice (9, 15, 40), the mutants were easily agitated and would sometimes attempt to spin in the apparatus. Thus extreme care in handling the mice was needed to obtain accurate recordings of systolic blood pressure. Each day, 10 preliminary blood pressure measurements were performed to acclimate the mice to the apparatus, and these were followed by 10 recorded systolic blood pressure and heart rate measurements. There were no time delays between each set of measurements. The blood pressure waveform was carefully observed during the preliminary measurements to identify mice that were not retaining the proper position in the apparatus. During the second set of measurements, data were accepted if a blood pressure was identified by the computer in at least 5 of the 10 measurements and was >50 or <-200 mmHg. Recordings not meeting these criteria (fewer than 1%) were discarded.

**Analysis of blood pH, gases, and electrolytes.** Conscious mice of both sexes ranging in age from 8 to 16 wk were placed on a 37°C heating pad to enhance peripheral blood circulation. Blood (50 \(\mu\)l) was collected from the tail vein in a heparinized capillary tube (Ciba-Corning; Medfield, MA) and immediately analyzed for acid/base status, blood gases, and plasma electrolytes using a blood gas analyzer (model 348; Chiron Diagnostics, Oberlin, OH).

**Serum aldosterone levels.** Concentrations of aldosterone in serum from mice of both sexes ranging in age from 8 to 16 wk were determined using a \(^{125}\)I radioimmunoassay, performed in duplicate according to the manufacturer’s suggested protocol (Diagnostics Products; Los Angeles, CA).

**Measurement of mean arterial blood pressure and cardiac function in the intact closed-chest mouse.** Adult male and female mice weighing ≥20 g were anesthetized with an intraperitoneal injection of 50 \(\mu\)g ketamine/g body wt and 100 \(\mu\)g thiobutabarbital/g body wt (Inactin; Research Biochemicals International, Natick, MA). As described earlier (30), polyethylene tubing (0.4 mm outer diameter) was inserted into the abdominal aorta from the right femoral artery and connected to a low-compliance pressure transducer (COBE Cardiovascular; Arvada, CO) for measurement of MAP. A high-fidelity, 1.8-French Millar Mikro-Tip transducer (model SPR-612; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the left ventricle to monitor cardiac performance. The right femoral vein was cannulated for infusion of dobutamine. MAP and intraventricular pressure signals from the COBE transducer and the Millar transducer were analyzed using a MacLab 4/s data acquisition system connected to a Macintosh 7100/80 computer. Average values for heart rate, MAP, and systolic left ventricular pressure (LVP) were measured directly from the pressure waveforms and were determined for each animal from at least 50 consecutive beats during the final 30 s of each 3-min dosage period. Maximum \(dP/dt\) (\(+dP/dt\)) and \(dP/dt\) at 40 mmHg (\(dP/dt_{40}\)) were calculated from the first derivative of the pressure waveforms.

**Analysis of blood vessel contractile properties.** Mice of 8–12 wk of age were euthanized with \(CO_2\) inhalation followed by cervical dislocation. Thoracic aortas, in segments of 5–7 mm, were dissected and mounted for isometric force recording as described previously (29). Studies were completed in both intact and endothelium-denuded aortas. Phenylephrine concentration-isometric force relationships were generated in the absence and presence of bumetanide (10 \(\mu\)M; 20-min incubation). The portal vein was dissected from each mouse by tying 4-0 sutures at the hepatic bifurcation and the anterior mesenteric vein. The portal vein was cut free, and the end was secured with the suture material to the myograph. From the time of the dissection, the vessel was maintained in physiological salt solutions (PSS). PSS contained the following (in mmol/l): 118 NaCl, 4.73 KCl, 1.2 MgCl\(_2\), 0.026 EDTA, 1.2 KH\(_2\)PO\(_4\), 2.5 CaCl\(_2\), 5.5 glucose and was buffered with 25 mM NaHCO\(_3\); pH was 7.4 at 37°C, when bubbled with 95% \(O_2/5\% CO_2\). Experiments were completed at optimal tension based on adjusting the vessels to a point where maximum peak-to-peak oscillations were observed. Force measurements were obtained using a Harvard Apparatus differential capacitor force transducer (South Natick, MA) connected to a Biopac MP100 data acquisition system that allowed measurements or calculations of contractile parameters including frequency of spontaneous contractions and tension-time integral (T-I integral). The effects of bumetanide on contractile parameters were examined after a 20-min preincubation with 10 \(\mu\)M bumetanide.

**Statistics.** Data are presented as means ± SE. Student’s \(t\)-test was used to compare mutant mice to the corresponding control mice. Mixed two-factor ANOVA with repeated measures on the second factor was used to compare genotype and dosage in dobutamine-treated, closed-chest, anesthetized mice and tail-cuff blood pressure studies.

**RESULTS**

**Tail-cuff measurements of systolic blood pressure.** In a recent study (40), it was reported that systolic blood pressure in \(Nkcc1^{-/-}\) mice, measured using a tail-cuff apparatus, was not significantly different from that of wild-type controls. Because this result differed from our previous results showing that MAP in anesthetized \(Nkcc1^{-/-}\) mice was significantly reduced (15), we measured tail-cuff pressures in \(Nkcc1^{-/-}\) and \(Nkcc1^{+/+}\)
mice to determine whether the apparent discrepancy was due to the different methods that were used.

Mice were analyzed in three separate experiments with at least five mice of each genotype in each experiment, and blood pressures and heart rate were recorded for 21 consecutive days. When the data for all three experiments were pooled, Nkcc1−/− mice (n = 16) had a significantly lower systolic blood pressure than Nkcc1+/+ mice (n = 17) throughout the course of the 21-day period (Fig. 1A), with average blood pressures of 114.5 ± 2.2 mmHg in Nkcc1−/− mice and 131.8 ± 2.5 mmHg in Nkcc1+/+ mice. There were no significant differences in heart rates (data not shown). Because the original study describing use of the tail-cuff apparatus included a 7-day period for acclimation of the mice to the apparatus (28), we performed separate analyses of the data for the first 7 days and the following 14 days. As shown in Fig. 1B, blood pressures for both genotypes were slightly lower during the initial 7-day period; however, the differences between the two genotypes were essentially the same during both periods. When the data from individual experiments were analyzed, systolic blood pressure was significantly reduced (P < 0.01) in the Nkcc1−/− mice in all three experiments (Nkcc1+/+ and Nkcc1−/−, respectively: experiment 1, 134.8 ± 2.6 and 116.8 ± 3.4 mmHg; experiment 2, 135.4 ± 4.3 and 119.3 ± 2.9 mmHg; experiment 3, 122.5 ± 2.5 and 106.3 ± 2.4 mmHg). On the basis of these data, we conclude that the loss of NKCC1 causes a significant reduction in systolic blood pressure.

Blood acid-base, electrolyte status and serum aldosterone levels. Although NKCC1 does not play a direct role in NaCl reabsorption in the kidney, as in the case of NKCC2 (51), it is conceivable that its absence impairs Na+-fluid volume homeostasis by some indirect means, thereby leading to the blood pressure defect. To examine this possibility, we analyzed blood from Nkcc1+/+ and Nkcc1−/− mice (Table 1). There were no significant differences in blood gases, pH, HCO3−, Na+, Cl−, or Ca2+; however, K+ concentrations were significantly elevated in the Nkcc1−/− mice. Serum aldosterone levels were essentially the same in both genotypes, indicating that extracellular volume depletion was unlikely to be a significant factor in the observed hypotension.

Cardiac performance in the intact closed-chest anesthetized mouse. To determine whether Nkcc1−/− mice might exhibit a cardiac disease phenotype that could be a contributing factor in the blood pressure defect, we analyzed heart rate, MAP, LVP, left ventricular end-diastolic pressure (LVEDP), and maximum and minimum dP/dt, and dP/dt40 under basal conditions and after the administration of dobutamine, a β-adrenergic agonist. There were no significant differences between the heart rates of Nkcc1−/− and Nkcc1+/+ mice under...
either basal conditions or after β-adrenergic stimulation (Fig. 2A). MAP was significantly decreased in Nkcc1<sup>−/−</sup> mice under basal conditions (Nkcc1<sup>+/+</sup>, 70.4 ± 3.0 mmHg; Nkcc1<sup>−/−</sup>, 84.0 ± 3.9 mmHg) and at all but the highest doses of dobutamine (Fig. 2B). Systolic LVP (Fig. 2C) was significantly reduced in Nkcc1<sup>−/−</sup> mice under basal conditions (Nkcc1<sup>+/+</sup>, 95.6 ± 3.0 mmHg; Nkcc1<sup>−/−</sup>, 107.3 ± 4.1 mmHg) and at all dobutamine doses, consistent with a reduction in afterload (indicated by the reduced MAP). There were no significant differences in LVEDP (Fig. 2D), maximum dP/dt (Fig. 2E), minimum dP/dt (data not shown), or dP/dt<sub>40</sub> (Fig. 2F). These data suggest that the reduced blood pressure in anesthetized Nkcc1<sup>−/−</sup> mice is not the result of an impaired myocardium.

Mechanical studies in Nkcc1<sup>−/−</sup> aortas and portal veins. Regulation of vascular smooth muscle contractility plays a major role in the maintenance of normal blood pressure. The observations that NKCC1 activity is altered in response to vasoactive compounds (1) and that inhibitors of NKCC1 alter vascular contractility (reviewed in Ref. 13) suggest that eliminating NKCC1 could affect vascular tone. To test this hypothesis, we examined the mechanical properties of aortas (tonic smooth muscle) and portal veins (phasic smooth muscle) from Nkcc1<sup>−/−</sup> and Nkcc1<sup>+/+</sup> mice.

<table>
<thead>
<tr>
<th></th>
<th>Nkcc1&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>n</th>
<th>Nkcc1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;, mM</td>
<td>146.1 ± 2.1</td>
<td>32</td>
<td>144.8 ± 1.9</td>
<td>29</td>
<td>0.661</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;, mM</td>
<td>5.6 ± 0.2</td>
<td>32</td>
<td>6.5 ± 0.3</td>
<td>29</td>
<td>0.009</td>
</tr>
<tr>
<td>Cl&lt;sup&gt;−&lt;/sup&gt;, mM</td>
<td>118.5 ± 3.7</td>
<td>11</td>
<td>111.3 ± 1.1</td>
<td>11</td>
<td>0.089</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;, mM</td>
<td>1.2 ± 0.03</td>
<td>21</td>
<td>1.2 ± 0.03</td>
<td>18</td>
<td>0.264</td>
</tr>
<tr>
<td>pH</td>
<td>7.30 ± 0.03</td>
<td>32</td>
<td>7.31 ± 0.03</td>
<td>29</td>
<td>0.812</td>
</tr>
<tr>
<td>HCO&lt;sub&gt;3&lt;/sub&gt;−, mM</td>
<td>20.6 ± 1.7</td>
<td>32</td>
<td>21.4 ± 1.6</td>
<td>29</td>
<td>0.707</td>
</tr>
<tr>
<td>PCO&lt;sub&gt;2&lt;/sub&gt;, mmHg</td>
<td>68.6 ± 1.9</td>
<td>32</td>
<td>61.8 ± 3.1</td>
<td>29</td>
<td>0.067</td>
</tr>
<tr>
<td>Aldosterone, pg/ml</td>
<td>398 ± 116</td>
<td>7</td>
<td>430 ± 106</td>
<td>12</td>
<td>0.817</td>
</tr>
</tbody>
</table>

Values are means ± SE for blood or serum samples from 8–16-week-old mice; n, number of mice in each group. P = statistical significance of differences between groups.

Fig. 2. Cardiovascular performance of the intact closed-chest mouse. Dobutamine dose-response relationships for heart rate (A), mean arterial pressure (B), maximum systolic left ventricular pressure (LVP) (C), left ventricular end-diastolic pressure (LVEDP; D), maximum dP/dt (E), and dP/dt at 40 mmHg (F) were determined in Nkcc1<sup>−/−</sup> (n = 11) and Nkcc1<sup>+/+</sup> (n = 13) mice. Values are means ± SE. *Significant difference in MAP (P < 0.001) at these dobutamine dosages. **Significant group effect (P < 0.02) between Nkcc1<sup>−/−</sup> with Nkcc1<sup>+/+</sup> systolic LVP. HR, heart rate; MAP, mean arterial pressure.
Figure 3 shows the concentration-force relationships for Nkcc1−/− and Nkcc1+/+ aortic rings after stimulation with phenylephrine in either the presence or absence of 10 μM bumetanide, an inhibitor of NKCC1. When aortic rings with intact endothelium (Fig. 3A) were examined, the Nkcc1−/− aorta appeared to be more sensitive to phenylephrine than the Nkcc1+/+ aorta, regardless of whether the experiment was performed in the absence or presence of bumetanide; however, the concentration yielding 50% of maximum contraction (EC50) was significantly different (P < 0.01) only in the presence of bumetanide. After treatment with bumetanide, there was a slight leftward shift in the phenylephrine concentration-force curve for Nkcc1−/− aortas and a slight rightward shift in the curve for Nkcc1+/+ aortas; however, neither of these shifts were statistically significant.

Because NKCC1 is expressed in both vascular smooth muscle and endothelium, experiments were also performed to determine whether removal of the endothelium would lead to differences in contractility between the two genotypes. Figure 3B shows the concentration-force relationships for Nkcc1−/− and Nkcc1+/+ aortic rings devoid of endothelium, using the same pharmacological conditions as in Fig. 3A. Relative to intact aortas, both genotypes exhibited greater sensitivity to phenylephrine. Endothelium-denuded Nkcc1−/− and Nkcc1+/+ aortas treated with bumetanide exhibited a rightward shift in the phenylephrine concentration-force curve (toward reduced sensitivity) when compared with aortas of the same genotype that were not treated with bumetanide. The Nkcc1+/+ aorta exhibited a greater sensitivity to bumetanide (approximately fourfold shift in EC50) than the Nkcc1−/− aorta (approximately twofold shift in EC50); the differences were significant in the Nkcc1+/+ (P < 0.001) but not in the Nkcc1−/− mice.

Figure 4 shows the maximum force/area (in mN/mm2) generated by Nkcc1−/− and Nkcc1+/+ aortic rings, with or without endothelium, after stimulation with 10 μM phenylephrine and in the presence or absence of 10 μM bumetanide. Although Nkcc1−/− aortas exhibited a trend toward a decreased maximum force of contraction relative to Nkcc1+/+ controls, the differences were slight and not significant. Similarly, bumetanide had no significant effect on maximal force generated by aortas of either genotype.

The contractile performance of isometrically mounted Nkcc1+/+ and Nkcc1−/− portal veins was examined before and after the administration of 10 μM bumetanide. Tracings of the spontaneous contraction profiles are shown in Fig. 5. Within 10 min of exposure to bumetanide, a marked decrease in mechanical force was apparent for Nkcc1+/+ portal veins, whereas little change was observed in Nkcc1−/− portal veins. In the absence of bumetanide, mechanical force (Fig. 6A), as estimated by the T-t integral, was significantly lower in Nkcc1−/− portal veins (94.0 ± 18.4 mN·s/mm2) than in Nkcc1+/+ controls (237.7 ± 37.7 mN·s/mm2). In the presence of 10 μM bumetanide, there was an 85% reduction in force in the Nkcc1+/+ portal vein when compared with basal force, whereas only a 30% reduction (from a much lower basal level) was observed in Nkcc1−/− portal veins (Fig. 6A). Measurements of on-time (Fig. 6B) and off-time (Fig. 6C) demonstrated that the two genotypes had different contraction cycles. On-time (contraction phase) for Nkcc1−/− portal veins (10.5 ± 2.0 s) was significantly less than that of the Nkcc1+/+ (16.8 ± 4.2 s), and off-time (relaxation phase) for Nkcc1−/− portal veins (23.4 ± 7.7 s) was significantly greater than that of the Nkcc1+/+ (10.4 ± 3.3 s). In the presence of 10 μM bumetanide, there was a significant change in both Nkcc1−/− on time (9.41 ± 0.43 s) and off time (41.8 ± 6.0 s) when compared with the basal state. Nkcc1−/− on time (9.03 ± 1.2 s) and off time (31.4 ± 3.6 s) after bumetanide administration was not significantly different from the baseline Nkcc1−/− values.

Fig. 3. Contraction of aortic rings with (A) and without (B) intact endothelium in response to phenylephrine. Concentration-isometric force relationships for aortic segments from Nkcc1−/− and Nkcc1+/+ mice treated with phenylephrine, a receptor-mediated agonist, were performed in the absence (n = 7 for each genotype) or presence (n = 6 Nkcc1−/− and 7 Nkcc1+/+ mice) of 10 μM bumetanide.
There is controversy about whether the loss of Nkcc1 in mice causes a reduction in blood pressure. In our initial analysis of the Nkcc1−/− mice (15), in which mice of a heterogeneous 129SvJ and Black Swiss background were used, we found that MAP of a small group of anesthetized Nkcc1−/− mice was substantially lower than that of Nkcc1+/+ mice; however, another group reported that systolic blood pressure was not altered in awake Nkcc1−/− mice (of a heterogeneous 129SvJ, C57BL/6, and DBA/2J background). In the latter study (40), tail-cuff measurements yielded values of 100 ± 7 mmHg for Nkcc1−/− mice and 108 ± 8 mmHg for Nkcc1+/+ mice. Although the mean value for the Nkcc1−/− mice was lower than that of Nkcc1+/+ mice, the difference was not statistically significant and it was concluded that blood pressure was not affected by the loss of Nkcc1. However, given the observed variability, the small number of mice analyzed (n = 5 for each genotype), and the direction and magnitude of the measured differences in blood pressure, those data were not inconsistent with a hypotensive phenotype in the awake mouse. The results shown in Fig. 1, using a larger number of mice and a 3-wk time period, demonstrate that systolic blood pressure is, in fact, significantly reduced in unanesthetized Nkcc1−/− mice. Furthermore, the magnitude of the reduction would be expected to be biologically important. It is possible, as noted earlier (40), that differences in mouse strains might affect the blood pressure phenotype. However, our tail-cuff data negate the possibility that the reduction in arterial pressure in the heterogeneous background used in our studies occurs only when the mice are anesthetized, as might have occurred if stimulation of the sympathetic nervous system was providing compensation in the awake mouse.

Loss of ion transporters involved in Na+ reabsorption across the apical membranes of renal epithelial cells leads to extracellular volume depletion and consequent hypotension (7, 45, 46). Nkcc1 is a basolateral transporter and does not contribute directly to Na+ reabsorption; however, because it is expressed in the kidney, we considered the possibility that it could have an indirect effect on the maintenance of Na+-fluid volume homeostasis. Also, Nkcc1 has been implicated

**DISCUSSION**

Our major objectives were to determine whether the loss of Nkcc1 causes a hypotensive phenotype and whether Nkcc1 null mice exhibit impairments of cardiac performance and/or vascular contractility. This is an important issue because loop diuretics, such as bumetanide and furosemide, which inhibit Nkcc1, are used in the treatment of a number of cardiovascular diseases and lead to a reduction in blood pressure. It is often assumed that the therapeutic effect of loop diuretics results entirely from their well-established natriuretic and diuretic activities, which are due to inhibition of Nkcc2, the apical Na+-K+-2Cl− cotransporter of the renal thick ascending limb. However, studies over the past three decades suggest that these drugs may affect the vasculature (13), either by stimulating the release of vasoactive compounds from the kidney or by direct action on vascular endothelium or smooth muscle, and there are indications that loop diuretics might also affect cardiac function under certain conditions (3, 43).
as part of the hepaortal system for sensing plasma Na\(^+\) and K\(^+\) concentrations in the portal vein, which is involved in the regulation of renal electrolyte excretion (32, 33). Although plasma K\(^+\) concentrations were slightly elevated, for reasons that remain unclear, analysis of blood electrolytes and serum aldosterone gave no indication of a major impairment of Na\(^+\)-fluid volume homeostasis. Serum aldosterone levels provide a sensitive indication of major alterations in Na\(^+\)-fluid volume homeostasis. For example, mice lacking the Na\(^+\)/H\(^+\) exchanger (NHE3), the primary mechanism for NaCl absorption in the proximal tubule, have reduced blood pressure and sixfold elevation in aldosterone levels as a result of impaired Na\(^+\)-fluid volume homeostasis (45). No significant elevation of serum aldosterone was observed in \(\text{Nkcc1}^{-/-}\) mice. On the basis of these data, it seems unlikely that the blood pressure defect of NKCC1-deficient mice, which is more severe than that of the NHE3 knockout mice, is due to impaired Na\(^+\)-fluid volume homeostasis.

An alternative hypothesis is that the loss of NKCC1 might cause a direct impairment of the cardiovascular system. NKCC1 is expressed in the heart (10), and there are data showing that inhibition of Na\(^+\)/K\(^+\)/2Cl\(^-\)cotransport activity with bumetanide can affect contractility of cultured cardiac myocytes. The data are contradictory, however, with reversal of the positive inotropic effect of low concentrations of ouabain in one study (41) and a positive inotropic effect reported in another study (26). Analysis of cardiac performance using the closed-chest anesthetized mouse revealed similar values for maximum dP/dt, but \(\text{Nkcc1}^{-/-}\) mice exhibited a significant reduction in systolic LVP. Given the reduction in MAP, however, this change is likely to be due to a reduction in afterload. dP/dt\(_{40}\) was calculated to correct for the reduction in afterload (30) and was found to be identical in \(\text{Nkcc1}^{-/-}\) and \(\text{Nkcc1}^{+/+}\) mice. LVEDP (a measurement of ventricular pressure just before contraction occurs, which is dependent on the filling rate and compliance of the myocardium) was also similar in the two groups of mice, with values well within the normal range (30). Our in vivo data indicate that the genetic loss of NKCC1 causes no major impairment of systolic or diastolic left ventricular function. These results are consistent with an earlier study (31) showing that high concentrations of furosemide have no effect on cardiac performance in dogs.

To determine whether the loss of NKCC1 might affect vascular smooth muscle tone, which could have a major impact on blood pressure, we examined in vitro preparations of aorta and portal vein. In previous studies of rat aorta, vasoactive compounds or alterations in aortic smooth muscle contractility led to changes in the activity of NKCC1, and bumetanide caused an approximately twofold reduction in sensitivity to phenylephrine (1, 2), consistent with a role for NKCC1 in the regulation of vascular tone. In the intact mouse aorta, there were only minor differences in phenylephrine dose-response curves between the two genotypes, and bumetanide had little effect. Furthermore, the sensitivity of \(\text{Nkcc1}^{-/-}\) mice aortas to phenylephrine ap-

---

**Fig. 6. Contractility of isolated portal veins.** A: tension-time integrals for \(\text{Nkcc1}^{+/+}\) and \(\text{Nkcc1}^{+/+}\) portal veins (n = 5 for each genotype) in the absence and presence of 10 \(\mu\)M bumetanide. *P < 0.001 when compared with \(\text{Nkcc1}^{+/+}\) basal value. On time (B) and off time (C) for \(\text{Nkcc1}^{+/+}\) and \(\text{Nkcc1}^{-/-}\) portal veins in the absence or presence of 10 \(\mu\)M bumetanide. *P < 0.01 for both on time and off time when compared with corresponding \(\text{Nkcc1}^{+/+}\) basal value; **P < 0.05 compared with corresponding \(\text{Nkcc1}^{+/+}\) basal value.
peared to be slightly greater than that of the Nkcc1+/+ mouse aortas, rather than slightly less as in the rat aorta treated with bumetanide (2). In the endothelium-denuded Nkcc1+/+ mouse aorta, bumetanide reduced the sensitivity to phenylephrine approximately fourfold, suggesting that the activity of NKCC1 might be required for normal sensitivity of smooth muscle to vascular constrictors; however, a reduction in sensitivity, albeit of lesser magnitude (approximately twofold), was also observed in the Nkcc1−/− mouse aorta. In response to phenylephrine, we observed no significant differences in maximum force of contraction of either intact or endothelium-denuded Nkcc1−/− and Nkcc1+/+ aortic rings, and bumetanide also had no significant effect (Fig. 4). Overall, these data provide little support for a major role for NKCC1 in regulating the contractility of mouse aorta.

In contrast, treatment of Nkcc1+/+ mouse portal veins with bumetanide caused a substantial reduction in mechanical force and, relative to Nkcc1+/+ controls, mechanical activity in untreated Nkcc1−/− portal veins was significantly impaired (Figs. 5 and 6). Furthermore, bumetanide had little effect on contractility of Nkcc1−/− portal veins. These results are consistent with previous studies using loop diuretics, which indicated that NKCC1 plays a major role in the contractility of a subset of vascular tissues. For example, furosemide inhibited the contractile response to vasoconstrictors and reduced the contractility of isolated rat portal vein (4). Furosemide caused the selective relaxation of a number of isolated canine venous preparations (pulmonary, splenic, mesenteric, saphenous) while having little effect on the corresponding arteries (18), and it also inhibited the contraction of human internal mammary artery and saphenous veins in response to angiotensin II (48). Similarly, bumetanide caused relaxation of canine carotid arteries (8). The results of the present study, in which NKCC1 null mutant portal veins were relatively insensitive to bumetanide, indicate that the sharp reduction in contractility of wild-type portal veins after treatment with bumetanide in vitro results from the inhibition of NKCC1.

It is unclear whether therapeutic concentrations of bumetanide observed in vivo are sufficient to have a major effect on vascular contractility, although this does appear to be the case for furosemide. NKCC1 has been reported to have a $K_i$ for bumetanide of 0.044 μM (22). Peak plasma concentrations after a therapeutic dose of bumetanide to human subjects (12) ranged from 39 to 63 ng/ml (0.10–0.17 μM), which is ~2–4 times the levels required for 50% inhibition of the cotransporter. NKCC1 is completely inhibited at 10 μM bumetanide, which is commonly used for in vitro studies, whereas it would be only partially inhibited at therapeutic levels. Therapeutic concentrations of furosemide, which is more commonly used for treatment of cardiovascular diseases, can vary between 1 and 10 μM (52). Several studies have shown that the effects of furosemide on vascular contractility in vitro can be observed at doses within the upper range of therapeutic concentrations (10 μM), consistent with the possibility that part of their therapeutic activity might be due to direct effects on the vasculature (18, 48).

Results of numerous clinical studies support the hypothesis that the therapeutic activity of loop diuretics is due in part, to alterations in vascular tissues, although the mechanisms underlying the effects on the vasculature in vivo are unclear. Treatment of congestive heart failure patients with furosemide led to a rapid reduction in left ventricular filling pressure, which correlated with an increase in venous compliance (11). Other investigators have noted a decrease in left ventricular filling pressure after the administration of loop diuretics in humans (34, 52) or dogs (5), which they also attributed, in part, to an increase in venous compliance. In addition to venodilation, several investigators have reported increases in blood pressure and vascular resistance (34, 52) after loop diuretic treatment. The reduction in contractility of isolated Nkcc1−/− portal veins is consistent with the hypothesis that the increased venous compliance in response to loop diuretics is due to inhibition of NKCC1 in the vasculature; however, the results of several studies (5, 16, 23) suggest that these effects might also be due to the release of vasoactive compounds from the kidney. If the kidney is involved, then inhibition of NKCC1, which is expressed in the extraglomerular mesangium and the glomerularafferent arteriole (25), could contribute to this effect.

Experiments presented here extend our understanding of the phenotypic consequences of the loss of NKCC1. These were previously shown to include impaired chloride secretion in the lung and intestine (15, 17, 19, 20, 53) with reduced fluid secretion in the lung (17) and a low incidence of intestinal blockage (15), severely impaired K+ secretion in the inner ear with accompanying deafness and imbalance (9, 15), male sterility resulting from defective spermatogenesis (40), a sharp reduction in the secretion of saliva (14), and impaired regulatory volume increase and release of excitatory amino acids from astrocytes (49). In the present study, we have shown that the loss of NKCC1 in the mouse causes both hypotension and a reduction in contractility of isolated portal veins but does not appear to impair cardiac performance. The simplest explanation for the hypotensive phenotype is that it is due to a reduction in vascular tone resulting from the loss of NKCC1 in vascular tissue, although the mechanism is likely to be more complex and could involve other organs, such as the kidney. If a vascular defect is the primary mechanism, then it is possible that impaired tone of capacitance veins contributes to the blood pressure deficit by reducing venous return (44) and/or that the tone of resistance vessels is impaired. In this regard, it has been reported that portal veins and resistance vessels share certain functional properties (50).

It should be noted that the observed reductions in blood pressure and the contractility defect are only correlative; a cause and effect relationship has not been established. Additional studies will be needed to
determine the extent of the deficit in vascular contractility, the ionic basis for this defect, and whether it is the major mechanism of the observed hypotension. Given the expression of NKCC1 in renin-secreting cells of the glomerular afferent arteriole, it seems possible that alterations in the secretion of vasoactive compounds from the kidney might, as suggested by others (5, 16, 23), account for some of observed vascular effects of loop diuretics in vivo. The reduction in excitatory amino acid release observed in Nkcc1−/− astrocytes (49) suggests that neuronal mechanisms could also be involved. An understanding of the mechanisms by which the loss of NKCC1 activity affects blood pressure and vascular tone, which may be complex and involve changes in multiple tissues, should be of clinical importance in the treatment of chronic cardiovascular diseases.

We thank Maureen Luehrmann and Angel Whetaker for expert animal husbandry. This research was supported by National Institutes of Health Grants HL-61974, DK-50584, and DK-57532. Present address of R. L. Sutfill: Department of Pathology, Emory University, Atlanta, GA 30322.

REFERENCES


