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# Neurogenic Mechanisms Contribute to Hypertension in Mice With Disruption of the K-Cl Cotransporter KCC3

Marco B. Rust,\* Jörg Faulhaber,\* Mareike K. Budack, Carsten Pfeffer, Tanja Maritzen, Michael Didié, Franz-Xaver Beck, Thomas Boettger, Rudolf Schubert, Heimo Ehmke, Thomas J. Jentsch, Christian A. Hübner

**Abstract**—The neurodegenerative disorder Andermann syndrome is caused by mutations of the K-Cl cotransporter KCC3. Mice with a targeted disruption of the corresponding gene, *Slc12a6*, reproduce neurodegeneration of the peripheral and central nervous system (CNS) and display arterial hypertension. *Kcc3* is expressed in numerous tissues, including the CNS and vascular smooth muscle cells. As the intracellular chloride concentration may influence myogenic tone and hence blood pressure, we measured the chloride concentration in vascular smooth muscle cells. It was indeed increased in superficial brain arteries and saphenous arteries of *Kcc3*<sup>-/-</sup> mice. Isolated saphenous arteries and their third-order branches, however, reacted indistinguishably to changes in intravascular pressure, stimulation of  $\alpha_1$ -adrenoreceptors, exogenous nitric oxide, or blockade of calcium-activated chloride channels. Likewise, the responses to  $\alpha_1$ -adrenergic stimulation or exogenous nitric oxide in vivo were identical in both genotypes. These results argue against a major vascular-intrinsic component of arterial hypertension in *Kcc3*<sup>-/-</sup> mice. In contrast, either  $\alpha_1$ -adrenergic blockade or inhibition of ganglionic transmission abolished the difference in arterial blood pressure between both genotypes. This demonstrates a neurogenic component in the maintenance of this phenotype, which is further supported by an increase of urinary norepinephrine and epinephrine excretion in *Kcc3*<sup>-/-</sup> mice. Our data indicate that local control of myogenic tone does not require KCC3 and that hypertension in *Kcc3*<sup>-/-</sup> mice depends on an elevated sympathetic tone. (*Circ Res.* 2006;98:549-556.)

**Key Words:** K-Cl cotransport ■ Andermann syndrome ■ blood pressure ■ vascular tone ■ neurogenic hypertension

The combination of peripheral neuropathy and variable agenesis of the corpus callosum with autosomal recessive inheritance was described by Andermann in 1972.<sup>1,2</sup> Andermann syndrome is caused by loss-of-function mutations of the K-Cl cotransporter KCC3.<sup>3</sup> Its disruption in mice caused peripheral polyneuropathy and a locomotor deficit.<sup>3,4</sup> Our *Kcc3*<sup>-/-</sup> mouse model in addition displayed neurodegeneration of the central nervous system, which has already been extensively described.<sup>4</sup> *Kcc3*<sup>-/-</sup> mice had a reduction of the seizure threshold and spike-wave-complexes in electrocorticograms, resembling EEG abnormalities in patients with Andermann syndrome. *Kcc3*<sup>-/-</sup> mice also developed a slowly progressive hearing loss and arterial hypertension,<sup>4</sup> features that have not yet been described in the human syndrome.

KCC3 belongs to the family of cation-chloride cotransporters, which are important for a wide variety of cellular

processes, like cell volume regulation, establishment of cytoplasmic chloride concentration ( $[Cl^-]_i$ ), or transepithelial transport. Their stoichiometric coupling of cation to anion translocation results in an electrically silent, passive transport driven by the transmembrane gradients of these ions. K-Cl cotransporters (KCC1, -2, -3, and -4) usually lower  $[Cl^-]_i$  below the electrochemical equilibrium by extruding  $Cl^-$  from the cell. An exception may be the role of KCC4 in supporting cells of the inner ear, which is thought to take up the  $K^+$  ions that have left the outer hair cells.<sup>5</sup> A striking example for the importance of KCCs in establishing low  $[Cl^-]_i$  is the modulation of GABAergic transmission by the neuronal K-Cl cotransporter KCC2. As GABA<sub>A</sub> receptors are ligand-gated  $Cl^-$  channels,  $[Cl^-]_i$  determines whether GABA depolarizes or hyperpolarizes the cell. By reducing  $[Cl^-]_i$  below its electrochemical equilibrium, the increase of KCC2 expres-

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sion during brain development renders the initially depolarizing GABA response hyperpolarizing at later ages.<sup>6–8</sup>

$[Cl^-]_i$  may also affect the contractility of vascular smooth muscle cells (VSMCs) of small arteries and arterioles. The tone of these “resistance vessels” determines the peripheral resistance to blood flow and thereby influences arterial blood pressure.<sup>9</sup> It depends on a complex interplay of vasodilator and vasoconstrictor stimuli, which are integrated by VSMCs and transformed into the activity of the contractile apparatus. Like all muscle cells, VSMCs use  $[Ca^{2+}]_i$  as a trigger for contraction.  $[Ca^{2+}]_i$  rises because of  $Ca^{2+}$  influx via voltage-dependent  $Ca^{2+}$  channels in the plasma membrane or by  $Ca^{2+}$  release from intracellular stores. In addition to direct stimulation of muscle contraction,  $[Ca^{2+}]_i$  may also activate  $Ca^{2+}$ -activated  $Cl^-$  channels in the plasma membrane. Such channels have been identified functionally in VSMCs and their role in enhancing vasoconstriction was shown in different species.<sup>10</sup> The electrochemical  $Cl^-$  gradient in VSMCs is such that the opening of plasma membrane  $Cl^-$  channels will result in  $Cl^-$  efflux and thereby depolarize the cell. If  $[Cl^-]_i$  is raised further, eg, because of the absence of KCC3, which was shown to be expressed in VSMCs,<sup>11</sup> opening of  $Ca^{2+}$ -activated  $Cl^-$  channels is expected to lead to a more pronounced depolarization and hence enhanced  $Ca^{2+}$  entry via voltage-gated  $Ca^{2+}$  channels. As a consequence, vascular tone should be increased and might cause arterial hypertension.

Here we show that disruption of KCC3 indeed increases  $[Cl^-]_i$  in VSMCs. However, in vitro studies on isolated arterial blood vessels as well as pharmacological interventions in vivo demonstrated no major differences of vascular contractility in *Kcc3*<sup>-/-</sup> mice. In contrast our data support that hypertension is maintained by a stimulation of the sympathetic nervous system. This proposed neurogenic component is further supported by increased urinary norepinephrine and epinephrine excretion rates in *Kcc3*<sup>-/-</sup> mice.

## Materials and Methods

### Animals

The generation of *Kcc3*<sup>-/-</sup> mice used in this study was described previously.<sup>4</sup> Studies were performed in a mixed 129SV/C57Bl6 background using littermates as controls. Mice were housed in an animal facility with a 12-hour light-dark cycle and allowed water and food ad libitum. Animal treatment and care was provided in accordance with institutional guidelines. The Institutional Animal Care and Use Committee of Hamburg approved all protocols and experimental procedures.

### Blood Pressure Measurements and Determination of Plasma Aldosterone

Chronic catheters were implanted into the left femoral artery and vein as described.<sup>12</sup> Arterial blood pressure and heart rate were determined in awake, unrestrained male *Kcc3*<sup>-/-</sup> (n=11) and control (n=10) mice (body weight [BW] 24 to 36 g; age 3 to 4 months) for 1 hour on day 2 or 3 after surgery. After a baseline recording (100 s), sodium nitroprusside (SNP) (30 ng·g BW<sup>-1</sup> per minute, Sigma) and at least 60 minutes later phenylephrine (PE) (30 ng·g BW<sup>-1</sup> per minute; Sigma) were given as ramp infusions at a rate of 1.5  $\mu$ L·g BW<sup>-1</sup> per minute for 100 s. Mean arterial pressure and heart rate responses were averaged over the last 20 s of the infusion period. 60 minutes later the baseline blood pressure was recorded for 100 s and prazosin (Sigma) was infused intravenously as a bolus (0.1 mg·kg BW<sup>-1</sup> in 1.5  $\mu$ L·g BW<sup>-1</sup> per minute saline) over 3 minutes,

followed by a continuous infusion (0.2 mg·kg BW<sup>-1</sup> per hour in 2.5  $\mu$ L·g BW<sup>-1</sup> per minute saline) of 15 minutes duration. Mean arterial pressure and heart rate responses were averaged over the last 900 s of the infusion period. On day 4 after surgery, sympathetic and parasympathetic ganglionic transmission was blocked by hexamethonium (25 mg·kg BW<sup>-1</sup>; Sigma) as a bolus infusion over 60 s after a baseline recording of 100 s. Mean arterial pressure and heart rate responses were averaged over 20 s after the end of the infusion. In a separate group of *Kcc3*<sup>-/-</sup> and wild-type (WT) mice atenolol (Sigma) was given as an intravenous bolus infusion (2 mg·kg BW<sup>-1</sup> in 2.5  $\mu$ L·g BW<sup>-1</sup> per minute saline) over 60 s after a baseline recording for 100 s (day 2 after surgery). Mean arterial pressure and heart rate response were averaged after the infusion over 300 s. On day 5 after surgery, 250  $\mu$ L of arterial blood was collected for determination of plasma aldosterone levels. Plasma aldosterone concentrations were measured as duplicates with a RIA kit (Coat-a-Count; Immunotech).

### Morphology

LacZ stainings of blood vessels, kidney and adrenal glands were performed as described.<sup>4</sup> Sections of paraffin-embedded saphenous arteries were prepared and stained by hematoxylin/eosin (HE). The thickness of the media and the blood vessel diameter were determined using an Axiophot microscope (Zeiss) and the Kappa Imaging Software ImageBase (Kappa) to calculate the wall to lumen ratio.

### Electron Microprobe Analysis

Tissues of adult *Kcc3*<sup>-/-</sup> mice and controls (n=5) were shock frozen in an isopentane/propane mixture (1:4) at -196°C. One micrometer sections were cut at -90°C and freeze dried. Element concentrations were determined using a scanning transmission electron microscope fitted to an x-ray detector system as described.<sup>13</sup>

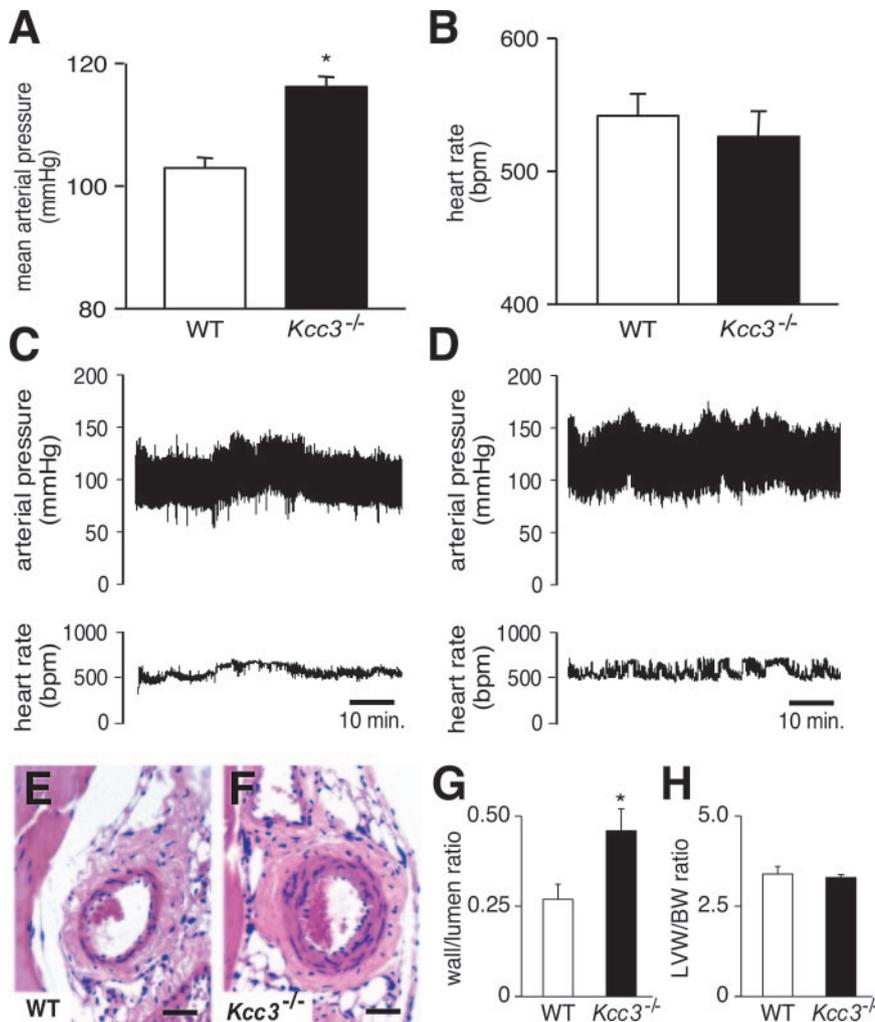
### Echocardiography

A Vevo 660 system (Visual Sonics Inc, Toronto, Canada) with a 40-MHz single-crystal mechanical transducer was used. Animals were kept under temperature- and electrocardiogram-controlled anesthesia (isoflurane, 1.5 volume %) at a heart rate of 500 bpm (496±6 bpm). Two-dimensional echocardiography images were obtained using an electrocardiogram-triggered recording mode which allows a temporal resolution of 1000 frames per second and a spatial resolution of up to 30  $\mu$ m. The thickness of the anterior and posterior wall, the left ventricular diameter and the area of the left ventricular cavity were obtained in a short axis view at the level of the papillary muscles. Pulmonary flow and diameter of the pulmonary artery (PAD) were assessed in a modified parasternal long axis view. Cardiac index (CI) was calculated by:  $CI = VTI \cdot \pi \cdot (1/2 PAD)^2 \cdot HR \cdot BW^{-1}$ . VTI indicates the velocity time integral of the pulmonary flow; HR, the heart rate.

### In Vitro Studies on Isolated Resistance Vessels

The technique has been described previously.<sup>14</sup> Pieces of saphenous arteries (1.8- to 2.0-mm long) from adult *Kcc3*<sup>-/-</sup> and control mice (n=6) were threaded on two 40- $\mu$ m diameter stainless steel wires and mounted on a wire myograph (model 410A, JP Trading) containing physiological salt solution (PSS) consisting of (in mmol/L) 120 NaCl, 4.5 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 1.0 MgSO<sub>4</sub>, 1.6 CaCl<sub>2</sub>, 0.025 EDTA, 5.5 glucose, 26 NaH<sub>2</sub>CO<sub>3</sub>, 5 HEPES (pH 7.4) at 37.0°C, continuously bubbled with carbogen. All drugs were applied directly into the experimental chamber. Isometric tension was recorded with the program Myodaq (JP Trading). The vessels were stretched radially until their optimal lumen diameter corresponding to 90% of the passive diameter of the vessel at 100 mm Hg was reached.

Small third-order branches (n=4 for WT and n=7 for *Kcc3*<sup>-/-</sup>) of the saphenous artery (mean luminal diameter,  $\approx$ 70  $\mu$ m) were fitted onto 2 glass cannulas, which were mounted in the experimental chamber flooded with PSS and placed onto the stage of an inverted microscope. Vessel diameters were measured by video microscopy. The chamber (2 mL) was continuously perfused with PSS at a rate of



**Figure 1.** Loss of KCC3 in mice results in arterial hypertension. Analysis of cardiovascular parameters disclosed a significant increase in mean arterial pressure (mm Hg $\pm$ SEM) of *Kcc3*<sup>-/-</sup> mice (n=11) compared with WT littermates (n=10) (A), whereas the heart rate (bpm $\pm$ SEM) was not changed (B). Representative arterial pressure and heart rate recordings of a single WT (C) and *Kcc3*<sup>-/-</sup> (D) mouse disclosed no difference in the variability of arterial pressure and heart rate. HE staining of paraffin-embedded *Arteria saphena* specimens of a WT (E) and a *Kcc3*<sup>-/-</sup> (F) mouse revealed media hypertrophy in *Kcc3*<sup>-/-</sup> mice resulting in an increase of the wall-to-lumen ratio (G) (10 preparations from 6 animals per genotype). Scale bar corresponds to 50  $\mu$ m in E and F. *Kcc3*<sup>-/-</sup> mice did not display cardiac hypertrophy as indicated by comparable left ventricle weight-to-body weight (LVW/BW) ratios (n=6 per genotype) (H). \*Significance ( $P < 0.05$ ).

2 mL/min. One cannula was connected to a pressure monitor and the second to a reservoir to exert the desired intravascular pressure by gravity. The pressure was increased to 80 mm Hg, and the vessel was lengthened up to its in vivo length. Before experiments vessel viability was tested with 1  $\mu$ mol/L acetylcholine (ACh) and 0.1  $\mu$ mol/L PE. All drugs were applied to the adventitial side of the vessels only. Pressure steps were obtained by quickly changing the height of the reservoir. Experiments were finished by a 20 minutes perfusion with Ca<sup>2+</sup>-free PSS to determine the fully relaxed vessel diameter at 80 mm Hg.

### Determination of Plasma Electrolytes

In a separate group of mice, catheters were implanted into the left femoral artery of *Kcc3*<sup>-/-</sup> and control mice (n=8) and the arterial blood was collected into heparinized tubes. Plasma concentrations of Na<sup>+</sup> and K<sup>+</sup> were measured by flame photometry (Eppendorf).

### Determination of Urinary Catecholamines

Mice were housed in individual metabolic cages for 24 hours (PHYMEP) with free access to water and chow. Urine was collected into tubes containing 15  $\mu$ L of 6N HCl as a preservative. Epinephrine and norepinephrine were determined by ELISA (CatCombi, IBL Hamburg).

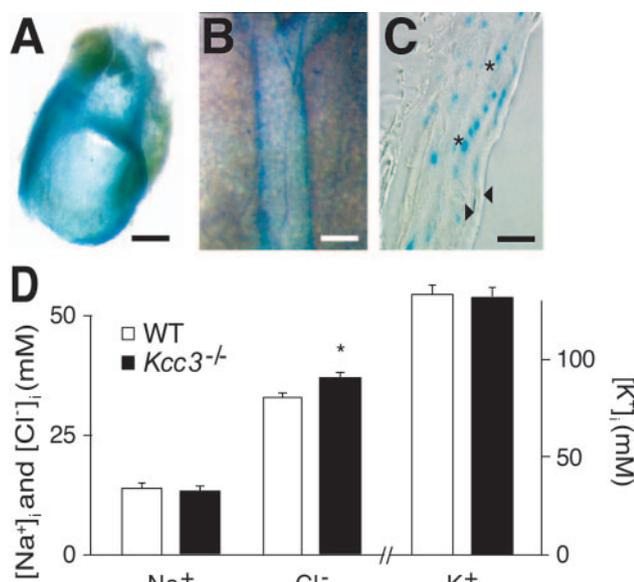
### Quantitative RT-PCR Analysis of Renin Transcript Levels

Total kidney RNA was extracted from 3-month-old *Kcc3*<sup>-/-</sup> and control littermates (n=8) using TRIzol (Gibco) and transcribed into

cDNA using the SuperScript II cDNA kit (Invitrogen). Real-time PCR was performed using the ABI PRISM 7700 and the SYBR green PCR master mix (Applied Biosystems). Renin transcript levels in WT and *Kcc3*<sup>-/-</sup> samples were compared after normalization to GAPDH and HPRT transcript levels. The following primers were used: renin-forward (TGAAAGTTGATCATGCAAGCCC), renin-reverse (GCCTCCCAGGTCAAAGGAAAT), GAPDH-forward (AGCCTCGTCCCGTAGACAAAA), GAPDH-reverse (TGGCAACAATCTCCACTTTGC), HPRT-forward (GTTCTTTGCTGACCTGCTGGA), and HPRT-reverse (TCCCCGTTGACTGATCATT).

### In Vitro Analysis of Chromaffin Cell [Ca<sup>2+</sup>]<sub>i</sub> Transients in Response to GABA and ACh

Chromaffin cells were isolated from P1 mice and grown on glass cover slips for 1 to 2 days as described.<sup>15</sup> Then cells were loaded with the Ca<sup>2+</sup>-sensitive fluorescent dye fura-2 (Molecular Probes) for 45 minutes. Cover slips were rinsed, placed into a chamber, and superfused at room temperature with saline (in mmol/L): 138 NaCl, 5 KCl, 1.3 CaCl<sub>2</sub>, 0.3 KH<sub>2</sub>PO<sub>4</sub>, 0.8 MgSO<sub>4</sub>, 0.3 Na<sub>2</sub>HPO<sub>4</sub>, 5.6 glucose, 20 HEPES (pH 7.4). Fura-2-loaded cells were excited at 340 and 380 nm and the emission recorded at >510 nm with the  $\times 100$  objective (Axiophot microscope, Zeiss) and a charge-coupled device camera (C4742-95, Hamamatsu) at 3-s intervals. Increasing concentrations of ACh (in  $\mu$ mol/L: 1, 2.5, 10; n=29) or GABA (in  $\mu$ mol/L: 1, 10, 50; n=29) were added and changes in [Ca<sup>2+</sup>]<sub>i</sub> were monitored. The fluorescence intensity was determined using the MetaMorph 6.0 software (Universal Imaging Corp). Relative baseline [Ca<sup>2+</sup>]<sub>i</sub> and maximal [Ca<sup>2+</sup>]<sub>i</sub>



**Figure 2.** KCC3 is involved in the regulation of  $[Cl^-]_i$  of vascular smooth muscle cells (VSMCs). *Kcc3* is expressed in the aorta (A) and a small mesenteric artery (B), as indicated by lacZ stainings from adult *Kcc3*<sup>+/-</sup> mice. Higher magnification of a saphenous artery section (C) demonstrated expression of *Kcc3* in VSMCs (stars) but not in endothelial cells (arrowheads). Scale bar corresponds to 300  $\mu$ m (A), 80  $\mu$ m (B), and 10  $\mu$ m (C). X-ray microprobe analysis of element concentrations revealed an increase of  $[Cl^-]_i$  in *Kcc3*<sup>-/-</sup> VSMCs of saphenous arteries (mmol kg<sup>-1</sup> wet weight; WT: 33.1 $\pm$ 1.1 mmol/L; *Kcc3*<sup>-/-</sup>: 36.9 $\pm$ 1.3 mmol/L; n=39 VSMCs, *P*<0.05) (D). No significant differences were found in cytoplasmic K<sup>+</sup> or Na<sup>+</sup> concentrations. \*Significance (*P*<0.05).

within 30 s of chromaffin cell stimulation were calculated from their 340 nm/380 nm fluorescence ratio.

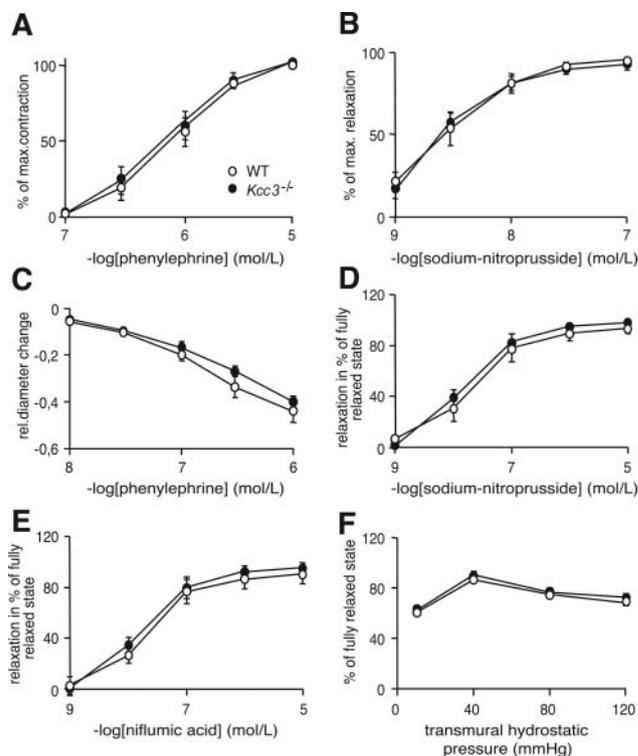
### Statistical Analysis

Data are mean $\pm$ SEM, and n represents the number of animals, specimens, or cells. Statistical analysis was performed by the unpaired Student's *t* test or ANOVA followed by the Newman-Keuls test. An error level of *P*<0.05 was considered significant.

## Results

### *Kcc3*<sup>-/-</sup> Mice Are Hypertensive

In our first report on *Kcc3*<sup>-/-</sup> mice, we mentioned that these mice display arterial hypertension.<sup>4</sup> In a larger group of 3-month-old male *Kcc3*<sup>-/-</sup> mice, blood pressure again was significantly (*P*<0.05) higher (116 $\pm$ 2 mm Hg, n=10) compared with WT littermates (103 $\pm$ 2 mm Hg, n=10) (Figure 1A). There was no significant difference in heart rates (Figure 1B). Blood pressure or heart rate variability, defined as the SD of individual recordings around the respective mean,<sup>16</sup> were not changed either (Figure 1C and 1D). Averaged within a group, the variability was 12 $\pm$ 1 mm Hg for arterial pressure and 44 $\pm$ 4 bpm for heart rates in *Kcc3*<sup>-/-</sup> mice, similar to that of WT mice (13 $\pm$ 2 mm Hg and 50 $\pm$ 7 bpm). Chronic arterial hypertension in *Kcc3*<sup>-/-</sup> mice was supported by the presence of media hypertrophy of the saphenous artery (Figure 1E through 1G). Left ventricular hypertrophy was not observed in 3-month-old *Kcc3*<sup>-/-</sup> mice (Figure 1H).



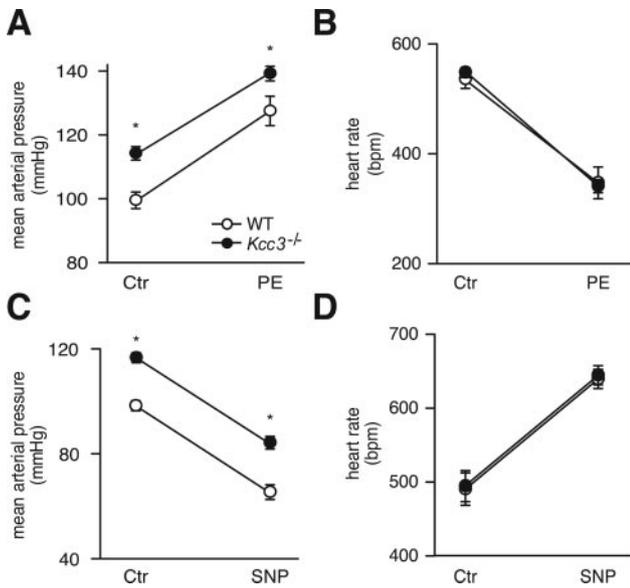
**Figure 3.** Elevated  $[Cl^-]_i$  of VSMCs does not affect vascular contractility. Application of increasing concentrations of the vasoconstrictor PE or the vasodilator SNP induced comparable changes in vessel tension of *Kcc3*<sup>-/-</sup> and WT saphenous arteries under isometric conditions (A and B) (n=6 per genotype). PE, SNP, and NFA induced diameter changes on third-order branches of the saphenous artery under isobaric conditions were unchanged (C through E) (n=4 for WT and 7 for *Kcc3*<sup>-/-</sup>). Additionally, the myogenic response of *Kcc3*<sup>-/-</sup> vessels (n=6) to an increase of the transmural pressure was comparable to that of WT vessels (n=5) (F). Open circles indicate WT; filled circles, *Kcc3*<sup>-/-</sup>.

### Disruption of *Kcc3* Increases $[Cl^-]_i$ in Vascular Smooth Muscle Cells

Our gene targeting allowed the analysis of *Kcc3* expression by lacZ staining of tissues from *Kcc3*<sup>+/-</sup> mice. Expression of *Kcc3* was found in VSMCs of various blood vessels (Figure 2A through 2C) and was absent in endothelial cells (Figure 2C). To test the significance of KCC3 for determining  $[Cl^-]_i$  in VSMCs, Cl<sup>-</sup> concentrations of shock frozen blood vessels were measured by electron microprobe analysis. In *Kcc3*<sup>-/-</sup> mice,  $[Cl^-]_i$  was significantly elevated in VSMCs of superficial brain arteries (WT: 28.1 $\pm$ 1.1 mmol/L, n=41 VSMCs; *Kcc3*<sup>-/-</sup>: 32.0 $\pm$ 0.9 mmol/L, n=59 VSMCs, *P*<0.05) and saphenous arteries (Figure 2D). In contrast, K<sup>+</sup> and Na<sup>+</sup> concentrations were not altered (Figure 2D).

### Vascular Contractility in *Kcc3*<sup>-/-</sup> Mice Is Not Altered

Isolated saphenous arteries were used for in vitro analysis of vascular contractility in *Kcc3*<sup>-/-</sup> and WT mice. Changes in vessel tension in response to PE, an  $\alpha_1$ -adrenergic agonist, or SNP were recorded under isometric conditions. Increasing concentrations of PE or SNP did not reveal any difference in contractility between the genotypes (Figure 3A and 3B).



**Figure 4.** *Kcc3*<sup>-/-</sup> mice do not exhibit dysfunction of the baroreflex. In vivo application of PE (A) or SNP (C) did not diminish the difference in MAP (mm Hg±SEM) and resulted in comparable acute changes of the heart rate (bpm±SEM) (*Kcc3*<sup>-/-</sup>; n=11 [B]; WT; n=10 [D]), indicating that the baroreflex was not affected in *Kcc3*<sup>-/-</sup> mice. Open circles indicate WT; filled circles, *Kcc3*<sup>-/-</sup>. \*Significance ( $P<0.05$ ).

Likewise, the application of niflumic acid (NFA), a blocker of  $Ca^{2+}$ -activated  $Cl^-$  channels, had a similar vasodilatory effect in both genotypes (data not shown). Smaller third-order branches of the saphenous artery, which are more relevant for the autoregulation of blood flow and capillary hydrostatic pressure, were isolated for isobaric testing. The diameter changes at 80 mm Hg that were inflicted either by PE (Figure 3C), SNP (Figure 3D), or NFA (Figure 3E) were independent of the genotype. Changes of the transmural hydrostatic pressure induced an initial passive vasodilation and a subsequent myogenic contractile response (Bayliss effect), which were of similar magnitudes in WT and *Kcc3*<sup>-/-</sup> preparations (Figure 3F).

### Neurogenic Mechanisms Contribute to Hypertension in *Kcc3*<sup>-/-</sup> Mice

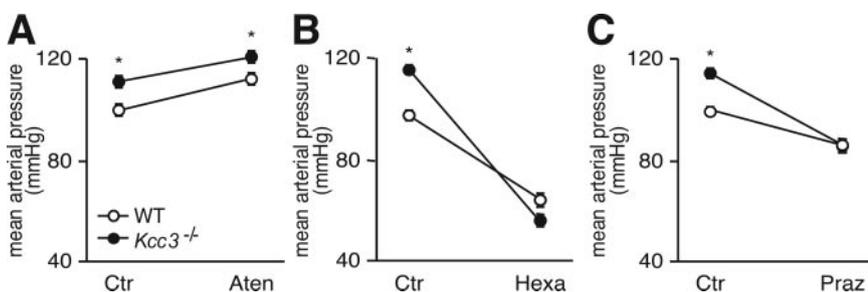
To characterize the arterial hypertension of *Kcc3*<sup>-/-</sup> mice in vivo, we investigated the effects of vasoactive substances on mean arterial blood pressure (MAP). Direct  $\alpha_1$ -adrenergic stimulation by PE resulted in a comparable rise of MAP (Figure 4A) and in a compensatory decrease of heart rate in both genotypes (Figure 4B). Likewise, administration of SNP

decreased the MAP (Figure 4C) and induced a compensatory increase of the heart rate (Figure 4D) independent of the genotype. Both observations strongly argued against a significant difference of the vascular contractility or a compromised baroreceptor reflex.

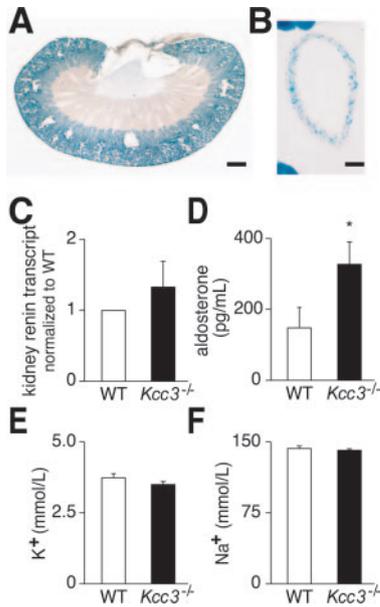
A major alteration of cardiac function could be ruled out by echocardiography, the cardiac index of *Kcc3*<sup>-/-</sup> mice ( $634.4\pm 44.1$  mL·min<sup>-1</sup> per kilogram BW<sup>-1</sup>; n=12) being unaltered compared with WT ( $666.0\pm 36.6$  mL·min<sup>-1</sup> per kilogram BW<sup>-1</sup>; n=12). Furthermore, acute infusion of the  $\beta_1$ -adrenoceptor blocker atenolol, which mainly acts on the heart but not on blood vessels, caused a comparable reduction of the heart rate and a concomitant small increase of the MAP in WT and *Kcc3*<sup>-/-</sup> mice (Figure 5A), again supporting that cardiac function is not altered in *Kcc3*<sup>-/-</sup> mice.

The difference in the MAP could not be attributed to intrinsic differences of vascular contractility. Hence a renal or neurogenic origin appeared likely. As KCC3 is expressed in the renal cortex (Figure 6A), where the protein was localized to the basolateral membrane of proximal tubules<sup>4</sup> and in vascular smooth muscle cells of intrarenal arteries (Figure 6B), we examined the renin-angiotensin-aldosterone system. Plasma aldosterone levels were increased in *Kcc3*<sup>-/-</sup> mice (Figure 6D), whereas renal renin transcript levels, plasma  $K^+$ , and plasma  $Na^+$  concentrations were not altered (Figure 6C, 6E, and 6F).

A contribution of neurogenic mechanisms to the maintenance of hypertension in *Kcc3*<sup>-/-</sup> mice was tested by inhibiting ganglionic transmission with the nicotinic ACh receptor blocker hexamethonium<sup>17</sup> and by  $\alpha_1$ -adrenergic blockade by prazosin.<sup>18</sup> In agreement with an increased neurogenic tone, either substance induced a greater reduction of blood pressure in *Kcc3*<sup>-/-</sup> than in WT mice and abolished the difference in blood pressure between both genotypes (Figure 5B and 5C). The new blood pressure levels were attained within minutes. This excluded chronic volume retention as a main pathogenic factor in the maintenance of arterial hypertension, although not necessarily in its genesis. As elevated sympathetic nerve activity often results in increased systemic and hence increased urinary catecholamine levels,<sup>19,20</sup> urinary epinephrine and norepinephrine excretion were determined and found to be significantly elevated in *Kcc3*<sup>-/-</sup> mice (Figure 7A). Chromaffin cells of the adrenal medulla are the main physiological source of systemic epinephrine and express KCC3 (Figure 7B). KCC3 is not expressed in the adrenal cortex. HE stainings of adrenal gland sections did not reveal any obvious morphological alterations in *Kcc3*<sup>-/-</sup> mice (Figure 7C). To



**Figure 5.** Elevated sympathetic activity is responsible for hypertension in *Kcc3*<sup>-/-</sup> mice. Response to acute  $\beta_1$ -blockade by atenolol (Aten) was not altered in *Kcc3*<sup>-/-</sup> mice (n=7) compared with WT (n=7) (A). Inhibition of ganglionic transmission by hexamethonium (Hexa) (B) or  $\alpha_1$ -adrenergic blockade by infusion of prazosin (Praz) (C) abolished the difference in mean arterial pressure (mm Hg±SEM) between *Kcc3*<sup>-/-</sup> (n=11) and WT (n=10) mice. Open circles indicate WT; filled circles, *Kcc3*<sup>-/-</sup>. \*Significance ( $P<0.05$ ).



**Figure 6.** Activation of the renin-angiotensin-aldosterone system in *Kcc3*<sup>-/-</sup> mice. A, LacZ staining of *Kcc3*<sup>+/-</sup> kidney sections revealed that expression of *Kcc3* in the kidney is restricted to the cortex (A) and VSMCs of renal arteries (B). Scale bar corresponds to 1000  $\mu$ m in A and 150  $\mu$ m B. Although there was a slight tendency toward elevated renin transcript levels in *Kcc3*<sup>-/-</sup> mice (n=8 per genotype), this increase was not significant (C). Plasma aldosterone levels were elevated in *Kcc3*<sup>-/-</sup> mice (D) (n=5 for WT and 6 for *Kcc3*<sup>-/-</sup> mice). Na<sup>+</sup> (F) or K<sup>+</sup> (E) plasma concentrations were unchanged (n=8 per genotype). \*Significance ( $P < 0.05$ ).

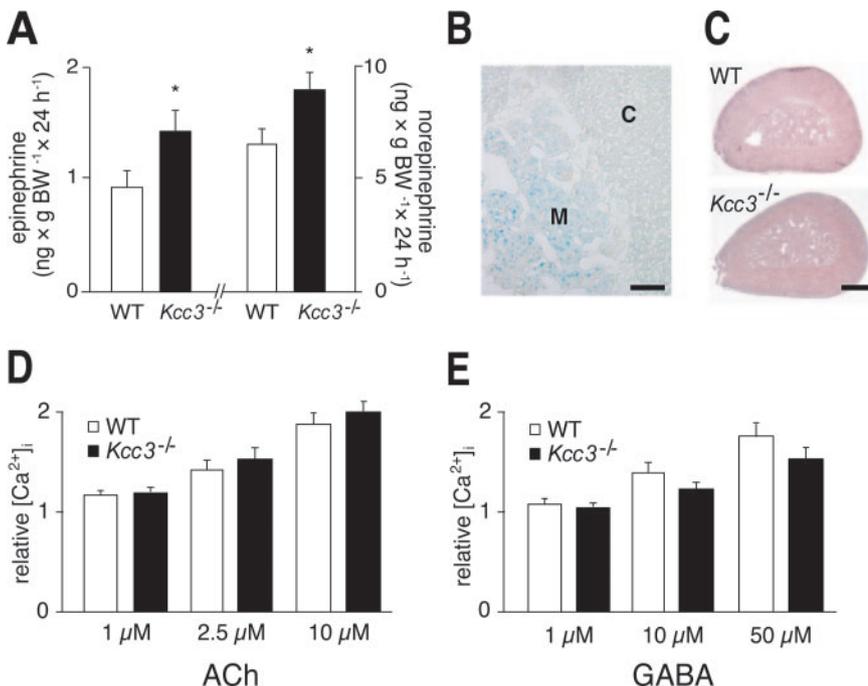
investigate whether the observed elevation of the systemic epinephrine concentration is a cell-intrinsic effect on catecholamine secretion, chromaffin cell [Ca<sup>2+</sup>]<sub>i</sub>, as a trigger for catecholamine secretion, was measured in response to submaximal concentrations of ACh and GABA. As no differences between both genotypes were detected (Figure 7D and

7E), increased systemic catecholamine levels in *Kcc3*<sup>-/-</sup> mice most likely result from an increased activity of the sympathetic system.

**Discussion**

*Kcc3*<sup>-/-</sup> mice displayed a progressive neurodegeneration of the peripheral and the central nervous system,<sup>3,4</sup> just like patients with Andermann syndrome. *Kcc3*<sup>-/-</sup> mice also developed arterial hypertension,<sup>4</sup> a feature that has not yet been described in Andermann syndrome. The aim of our current study was to address the origin of arterial hypertension in *Kcc3*<sup>-/-</sup> mice.

Though arterial hypertension in 3-month-old *Kcc3*<sup>-/-</sup> mice was moderate, it had already caused a significant media hypertrophy in resistance vessels. Left ventricular hypertrophy was not yet detectable. This observation is consistent with clinical findings suggesting that small artery structural remodeling precedes most relevant manifestations of target organ damage, including cardiac hypertrophy.<sup>21</sup> A major contribution of an impaired baroreceptor reflex could be excluded, because an increase in the blood pressure variability under resting conditions is a hallmark of baroreceptor dysfunction<sup>16</sup> but was absent in *Kcc3*<sup>-/-</sup> mice. This conclusion was bolstered by a normal heart rate response to acute infusion of vasoactive drugs in *Kcc3*<sup>-/-</sup> mice. Hence the role of the vascular system in the pathogenesis of the hypertension phenotype was addressed. Both KCC1 and KCC3, but not KCC2 or KCC4, have been shown to be expressed in cultured primary VSMCs.<sup>11,22</sup> Using a reporter gene strategy, we detected *Kcc3* expression not only in VSMCs of large arteries, including the aorta, but also in resistance vessels like the saphenous artery. Endothelial cells were not stained. To test whether KCC3 is constitutively active, intracellular ion concentrations of VSMCs were determined by electron microprobe analysis. In WT preparations, [Cl<sup>-</sup>]<sub>i</sub> was



**Figure 7.** Increased urinary epinephrine and norepinephrine excretion rates in *Kcc3*<sup>-/-</sup> mice. Twenty-four-hour urinary excretion rates (ng · g BW<sup>-1</sup> per 24 hours ± SEM) of epinephrine and norepinephrine were significantly elevated in *Kcc3*<sup>-/-</sup> mice (A). LacZ staining of adrenal gland sections (B) demonstrated expression of *Kcc3* in the medulla but not in the cortex of the adrenal gland (M, medulla; C, cortex). HE-stained adrenal glands from *Kcc3*<sup>-/-</sup> mice did not show morphological alterations compared with WT (C). Scale bar corresponds to 50  $\mu$ m in B and 500  $\mu$ m in C. Neither application of ACh (± SEM, n=29) (D) nor of GABA (± SEM, n=21) (E) induced a significant increase in [Ca<sup>2+</sup>]<sub>i</sub> transients in *Kcc3*<sup>-/-</sup> chromaffin cells relative to WT chromaffin cells. This suggested that the increase of urinary epinephrine excretion rates in *Kcc3*<sup>-/-</sup> mice did not result from a chromaffin cell intrinsic effect. \*Significance ( $P < 0.05$ ).

28.1±1.1 mmol/L for superficial brain arteries and 33.1±1.1 mmol/L for saphenous arteries.  $[Cl^-]_i$  of cultured rat aorta VSMCs was determined to be 31.6±3.2 mmol/L using a  $Cl^-$  sensitive dye,<sup>23</sup> in excellent agreement with the present data. Consistent with KCC3 transport activity in VSMCs, we found a 10% to 15% increase in  $[Cl^-]_i$  both in superficial brain arteries and in saphenous arteries of *Kcc3*<sup>-/-</sup> mice. This suggested that the opening of  $Ca^{2+}$ -activated  $Cl^-$ -channels might result in a larger depolarization and hence an enhanced contraction of *Kcc3*<sup>-/-</sup> VSMCs. Evidence for a role of cation-chloride cotransport in vascular blood pressure regulation came from mice that lack the  $Na^+$ -dependent K-Cl cotransporter NKCC1, which is also expressed in VSMCs. The coupling of  $Cl^-$  to  $Na^+$  movement predicts an accumulation of  $Cl^-$  in WT VSMCs.<sup>24</sup> Indeed, 1 study reported hypotension and a reduction of umbilical vein contractility in *Nkcc1*<sup>-/-</sup> mice,<sup>25,26</sup> but these findings could not be reproduced in another *Nkcc1*<sup>-/-</sup> line.<sup>27</sup> If KCC3 activity had a direct, VSMC-intrinsic effect on vascular contractility, an increased myogenic response of *Kcc3*<sup>-/-</sup> arteries to alterations of transmural hydrostatic pressure would be expected. However, myogenic responses and effects of blocking  $Ca^{2+}$  activated  $Cl^-$  channels by NFA were independent of the genotype. Furthermore, neither in vitro dose-response curves of isolated resistance vessels to vasoactive substances such as PE or SNP nor in vivo effects of PE and SNP on MAP were altered in *Kcc3*<sup>-/-</sup> mice. Taken together, these observations argued against an alteration of vascular contractility as a dominating mechanism for arterial hypertension in *Kcc3*<sup>-/-</sup> mice.

As the difference in MAP could not be attributed to differences in vascular contractility or baroreceptor dysfunction, a neurogenic component with increased sympathetic activity, including the outflow to blood vessels and the kidneys, seemed likely. Several recent studies have shown that increased sympathetic nerve activity almost invariably includes renal sympathetic nerves, which stimulate renin secretion and, as a consequence of an increased angiotensin II generation, aldosterone release from the adrenal cortex.<sup>28,29</sup> Indeed, in spite of the increased blood pressure, there was a tendency toward elevated renin transcripts in *Kcc3*<sup>-/-</sup> mice and plasma aldosterone concentrations were moderately increased. The rapid decrease of blood pressure within 80 s after pharmacological blockade of the sympathetic nervous system either by  $\alpha_1$ -adrenergic blockade with prazosin or by blockade of ganglionic transmission with hexamethonium, however, argue against a major contribution to hypertension of chronic volume retention caused by increased aldosterone levels. Primary hyperaldosteronism in *Kcc3*<sup>-/-</sup> mice is very unlikely, because it should result in a compensatory reduction of renin synthesis that was not observed. Furthermore, KCC3 was not detected in the adrenal cortex.

Analogous to other mouse models with neurogenic hypertension,<sup>19,20</sup> urinary epinephrine and norepinephrine excretion rates were increased in *Kcc3*<sup>-/-</sup> mice, thus reflecting elevated levels of circulating catecholamines. GABA or ACh are agonists of catecholamine secretion by chromaffin cells. As  $[Cl^-]_i$  has been shown to be above its equilibrium in chromaffin cells, stimulation of anion-permeable GABA<sub>A</sub> recep-

tors causes depolarization,  $[Ca^{2+}]_i$  transients, and catecholamine release.<sup>30</sup> Disrupting KCC3, which is expressed in WT chromaffin cells, may elevate  $[Cl^-]_i$  and hence increase the depolarizing response to GABA or ACh. However,  $[Ca^{2+}]_i$  responses of *Kcc3*<sup>-/-</sup> chromaffin cells to physiological concentrations of ACh or GABA were not altered. Taken together, these data indicate that the increased catecholamine levels are a consequence of an elevated sympathetic tone and do not result from an altered efficiency of release from chromaffin cells of the adrenal medulla.

Our mouse model for Andermann syndrome indicates that the hypertension caused by *Kcc3*<sup>-/-</sup> ablation is dependent on an elevated sympathetic activation. This may be highly relevant not only for Andermann syndrome but also for other neurodegenerative diseases that are associated with arterial hypertension. Additionally, our work addressed the role of intracellular chloride on vascular reactivity. Although several studies suggested an important influence of  $Ca^{2+}$ -activated  $Cl^-$  channels on vascular smooth muscle reactivity,<sup>9,10</sup> *Kcc3*<sup>-/-</sup> mice revealed that a moderate increase in vascular smooth muscle chloride concentration had no significant impact on vascular tone.

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