Impaired Endothelium-Derived Hyperpolarizing Factor–Mediated Dilations and Increased Blood Pressure in Mice Deficient of the Intermediate-Conductance Ca$^{2+}$-Activated K$^+$ Channel

Han Si,* Willm-Thomas Heyken,* Stephanie E. Wölffle, Marcin Tysiac, Rudolf Schubert, Ivica Grgic, Larisa Vilianovich, Günter Giebing, Tanja Maier, Volkmar Gross, Michael Bader, Cor de Wit, Joachim Hoyer, Ralf Köhler

Abstract—The endothelium plays a key role in the control of vascular tone and alteration in endothelial cell function contributes to several cardiovascular disease states. Endothelium-dependent dilation is mediated by NO, prostacyclin, and an endothelium-derived hyperpolarizing factor (EDHF). EDHF signaling is thought to be initiated by activation of endothelial Ca$^{2+}$-activated K$^+$ channels (K$_{Ca}$), leading to hyperpolarization of the endothelium and subsequently to hyperpolarization and relaxation of vascular smooth muscle. In the present study, we tested the functional role of the endothelial intermediate-conductance K$_{Ca}$ (IK$_{Ca}$/K$_{Ca}^{3.1}$) in endothelial hyperpolarization, in EDHF-mediated dilation, and in the control of arterial pressure by targeted deletion of K$_{Ca}^{3.1}$. K$_{Ca}^{3.1}$-deficient mice (K$_{Ca}^{3.1}$−/−) were generated by conventional gene-targeting strategies. Endothelial K$_{Ca}$ currents and EDHF-mediated dilations were characterized by patch-clamp analysis, myography and intravital microscopy. Disruption of the K$_{Ca}^{3.1}$ gene abolished endothelial K$_{Ca}^{3.1}$ currents and significantly diminished overall current through K$_{Ca}$ channels. As a consequence, endothelial and smooth muscle hyperpolarization in response to acetylcholine was reduced in K$_{Ca}^{3.1}$−/− mice. Acetylcholine-induced dilations were impaired in the carotid artery and in resistance vessels because of a substantial reduction of EDHF-mediated dilation in K$_{Ca}^{3.1}$−/− mice. Moreover, the loss of K$_{Ca}^{3.1}$ led to a significant increase in arterial blood pressure and to mild left ventricular hypertrophy. These results indicate that the endothelial K$_{Ca}^{3.1}$ is a fundamental determinant of endothelial hyperpolarization and EDHF signaling and, thereby, a crucial determinant in the control of vascular tone and overall circulatory regulation. (Circ Res. 2006;99:0-0.)

Key Words: hypertension ■ endothelium ■ EDHF ■ intermediate-conductance Ca$^{2+}$-activated K$^+$ channel ■ K$_{Ca}^{3.1}$−/− mice

The vascular endothelium plays a key role in the control of organ perfusion and contributes to the regulation of arterial blood pressure by releasing vasoactive factors that modulate the contractile state of the underlying smooth muscle. In response to classical agonists, such as acetylcholine (ACh) and bradykinin, as well as to hemodynamic stimuli, the endothelium produces, in principle, 3 vasodilating factors, nitric oxide (NO), prostacyclin (PGI2), and an endothelium-derived hyperpolarizing factor (EDHF). Although the modes of action of NO and PGI2 became clear 20 years ago, the nature of the latest identified factor, EDHF, is still controversial and debated thoroughly since its discovery in the late eighties.5,6 Regarding the relative contribution of NO and EDHF within the vascular tree, it appears that EDHF becomes more important when vessel diameter decreases, whereas NO seems to be predominant in large arteries.7 Importantly, defects in the NO system and also in EDHF signaling, as demonstrated more recently, are pathologically relevant in disease states such as hypertension,8,9 diabetes,10 and renal insufficiency.11 Studies in transgenic mice deficient in either endothelial NO synthase (eNOS)12 or the PGI2-generating cyclooxygenase-1 (COX-1),13,14 or in both enzymes,14 revealed that endothelium-dependent dilation is still preserved, which was attributed to a compensatory effect by the respective other dilator and supposedly by EDHF.
Despite considerable progress in recent years, the identity of EDHF and the cellular mechanisms underlying EDHF signaling upstream of smooth muscle hyperpolarization and relaxation remains elusive. Several chemical factors among them, K⁺ ions and cytochrome P450-generated metabolites of arachidonic acid, have been suggested to serve as EDHF or contribute to EDHF signaling by intraendothelial mechanisms. In addition, a nonchemical mechanism characterized by the direct spread of hyperpolarization from the endothelium to the subjacent smooth muscle via myoendothelial gap junctions was proposed to transfer the hyperpolarization amplified from the targeted and from wild-type allele, respectively. D, Electrophoretic analysis of PCR products derived from genomic DNA. The 320- and the 160-bp bands were completely blocked by inhibitors of this channel, suggesting that the 2 KCa channels have specific functions, at least in certain vascular beds. However, the exact role of KCa3.1 as opposed to KCa2.3 to mediate endothelial hyperpolarization and its contribution to the control of vascular tone, EDHF signaling, and, possibly, the regulation of arterial pressure remain unclear.

To dissect the relative roles of KCa3.1 and KCa2.3, we generated mice with a targeted disruption of the KCa3.1 gene. Here, we show that deletion of the KCa3.1 gene and thus loss of KCa3.1 functions results in an impaired EDHF-mediated dilation and elevated systemic blood pressure.

Materials and Methods

Detailed methods for generation of KCa3.1−/− mice, Southern blot analysis, routine genotyping, RT-PCR, Western blot analysis, patch-clamp electrophysiology, membrane-potential measurements, pressure myography, intravital microscopy, and tail-cuff and telemetric blood pressure measurements are included in the online data supplement, available at http://circres.ahajournals.org.

Statistics

Data are given as mean±SE or SEM as indicated. The Student’s t test was used to assess differences between groups. Probability values of P<0.05 were considered significant.

Results

Generation of KCa3.1−/− Mice

Mice deficient in KCa3.1−/− were generated by homologous recombination in embryonic stem cells using conventional techniques (Figure 1A). This strategy deleted exon 4 of the KCa3.1 gene, which encodes the channel pore. Successful gene targeting of KCa3.1 was verified by Southern blot and PCR analysis (Figure 1B and 1C). Loss of KCa3.1 gene expression in KCa3.1−/− was confirmed by RT-PCR in total
mRNA extracts from spleen (Figure 1D). The lack of the protein was proven by Western blot analysis (Figure 1E) of erythrocyte ghost membranes known to contain the KCa3.1 protein (also referred to as “Gardos” channel).

Heterozygous KCa3.1/H11002/H11001 breeding pairs produced offspring in a Mendelian manner: 25% were homozygous wild-type (H11001/H11001), 52% heterozygous (H11002/H11001), and 23% homozygous KCa3.1/H11002/H11002. Homozygous KCa3.1/H11002/H11002 were viable and fertile.

Mating of KCa3.1/H11002/H11002 males with KCa3.1/H11002/H11002 females produced normal offspring and litter sizes, similar to a different KCa3.1/H11002/H11002 strain.34

Deficiency of Endothelial KCa3.1 and Diminished Endothelial Hyperpolarization in KCa3.1/−/− Mice

To evaluate whether the KCa3.1 gene deletion results in diminished functional KCa currents in the endothelium of KCa3.1/−/− mice, we performed whole-cell patch-clamp experiments in freshly isolated aortic endothelial cells (AEC) of KCa3.1/+/+ (n=8) and KCa3.1/−/− mice (n=7) and in carotid artery endothelial cells (CAEC) in situ of these mice (n=3 each). For the activation of KCa channels, cells were dialyzed with a pipette solution containing 3 μmol/L Ca2+. To verify the lack of KCa3.1 currents, whole-cell patch-clamp experiments were conducted first in the presence of the KCa2.3 blocker UCL1684 (100 nmol/L)34 to unmask KCa3.1 currents from KCa2.3 currents in AEC and CAEC in situ. KCa3.1 currents were not detectable in AEC and CAEC of KCa3.1/−/− mice, whereas KCa3.1 currents were present in AEC and in CAEC of KCa3.1/+/+ littermates. Original current traces and mean KCa current standardized to cell capacitance are shown in Figure 2A and 2B. TRAM-34, a selective blocker of KCa3.1 channels,36 completely blocked this current (n=10; Figure 2), demonstrating that the UCL1684-insensitive current is indeed mediated by KCa3.1. Moreover, this indicates that KCa3.1 together with KCa2.3 confers the KCa currents in wild-type AEC,24,37 as in rat EC, as shown previously.21 In a second set of experiments, we determined mixed KCa3.1/KCa2.3-current densities in AEC and in CAEC in situ of KCa3.1/−/− mice and KCa2.3-current densities in AEC and in CAEC in situ of KCa3.1/+/+ mice. Compared with the composite KCa3.1/KCa2.3-current density in EC of wild-type mice, the KCa2.3-current density in KCa3.1/−/− mice was significantly smaller (Figure 2B). KCa2.3-current densities (determined in the presence of TRAM-34) were similar in AEC of both genotypes (KCa3.1/−/−: 14±2 pA/pF, n=5; wild type: 10±3 pA/pF, n=13). Also, cell capacitance was not different between genotypes in AEC (KCa3.1/−/−: 9±1; wild type: 8±1 pF; n=21 and 25, respectively) and in CAEC in situ (KCa3.1/−/−: 8±1;
wild type: 9 ± 1 pF; n=8 and 12, respectively). These results indicate that deletion of KCa3.1 gene reduced the overall endothelial KCa-current density by ~50%.

To test whether the loss of endothelial KCa3.1 currents and the reduced overall KCa currents in KCa3.1−/− mice led to an impaired agonist-induced endothelial hyperpolarization, we conducted membrane-potential measurements in AEC clusters (>20 cells) and in the endothelium of carotid artery in situ (CAE). Capacitance values of >150 pF indicated intact electrical coupling in AEC clusters and in EC of the carotid artery (CA) of KCa3.1−/− and KCa3.1+/+ mice. As shown in Figure 3, the amplitude of the hyperpolarization in response to ACh (100 nmol/L) was significantly smaller in both preparations studied in KCa3.1−/− (at 100 nmol/L ACh: ≈Δ−10 mV versus ≈Δ−25 mV in wild type). Moreover, the ACh-induced hyperpolarization was completely reversed by the KCa2.3 blocker UCL1684 (100 nmol/L) in KCa3.1-deficient tissues, whereas in wild-type tissues, a combination of UCL1684 and TRAM-34 (1 μmol/L) was required to fully reverse the hyperpolarization response. Similarly, membrane hyperpolarization in response to the KCa3.1/KCa2.3 opener DC-EBIO (10 μmol/L) was significantly reduced in AEC clusters obtained from KCa3.1−/− mice (Figure 3). Nevertheless, the endothelial resting potential did not differ between the genotypes (Figure 3B) and was likewise not altered in the presence of 1 or both KCa blockers, indicating that KCa3.1 and KCa2.3 do not contribute considerably to the setting of the resting potential under these conditions in these cells.

Membrane potential measurements in smooth muscle of CAs as performed by using conventional sharp electrode technique (from the adventitial side) revealed that smooth muscle hyperpolarization to ACh (100 nmol/L) was blunted in CAs (n=6) of KCa3.1−/− (−44 ± 2 mV −Δ−5 mV) versus −58 ± 3 mV (±Δ−15 mV) in wild-type CA; n=6; P<0.01). Resting potentials were similar (−39 ± 2 mV in KCa3.1−/− versus −43 ± 2 mV in wild-type mice; P=NS).

Together, these results suggest that the loss of endothelial KCa3.1 leads to an impaired endothelial and smooth muscle hyperpolarization in response to agonists.

**Impaired EDHF-Mediated Dilation in KCa3.1−/− Mice**

To test whether the lower KCa-current densities and the reduced endothelial hyperpolarization response in KCa3.1−/− led to impaired endothelial function and especially a defective EDHF-mediated dilation, we determined ACh- and DC-EBIO–induced dilator responses in CAs of KCa3.1−/− and wild-type mice. In the presence of the NO synthase blocker N’-nitro-L-arginine (L-NNA) (100 μmol/L) and the COX inhibitor INDO (10 μmol/L), ACh-induced dilations were significantly reduced in KCa3.1−/− mice. Original traces and concentration-response curves are shown in Figure 4A. At 100 nmol/L ACh, the dilation of CAs of KCa3.1−/− mice was approximately half of the dilatory response observed in wild-type mice. The EDHF-type dilation was partially suppressed by UCL1684 (100 nmol/L) and completely abrogated by the combination of UCL1684 and TRAM-34 (1 μmol/L) in wild-type mice. In contrast, UCL1684 was sufficient to...
effectively block EDHF-type dilations in KCa3.1−/− mice, and TRAM-34 had no further inhibitory effects (Figure 4A, lower right). Notably, UCL1684 alone or in combination with TRAM-34 did not reduce vessel diameter in wild-type or KCa3.1−/− mice, suggesting that these channels do not initiate or exert a tonic endothelial dilator effect in this preparation.

In the absence of blockers of NOS and COX, endothelium-dependent dilation was larger overall in wild-type and KCa3.1−/− mice. This is in line with the notion that, particularly, NO synthesis and release contribute significantly to ACh-induced dilation in this artery (Figure 4B). Prostacyclin did not contribute to this enhanced dilator response in wild-type and KCa3.1−/− mice because INDO alone had no inhibitory effect (also see Figure I in the online data supplement).

However, when compared with wild-type mice, the overall larger (“total”) ACh-induced dilation was still reduced in KCa3.1−/− mice (Figure 4B). Because inhibition of NOS reduced ACh-induced dilation to a similar extent in wild-type and KCa3.1−/− mice, it seems that the reduction of the total endothelium-dependent dilation in KCa3.1−/− mice reflects an impaired EDHF-type response in these mice. Moreover, the persisting impairment of the ACh-induced dilation in KCa3.1−/− mice in the presence of intact NO synthesis indicates that the diminished EDHF-type dilation is not compensated by an enhanced NO or prostacyclin release.

Dilation in response to direct pharmacological activation of both endothelial KCa by the potent KCa3.1/KCa2.3 opener DC-EBIO (10 μmol/L) was also significantly diminished in CAs of KCa3.1−/− mice (Figure 4C), which shows that the loss
Figure 5. Impaired EDHF-mediated dilation in resistance-sized vessels in the cremaster microcirculation of Kc3.1−/− mice. Concentration-response curves of ACh-induced EDHF-type dilation (in the presence of L-NNa [30 μmol/L] and INDO [3 μmol/L]) in wild-type and Kc3.1−/− mice (n=80 arterioles in 8 mice, each genotype). Values are given as mean±SEM. **P<0.001 vs wild type (Student’s t test).

of Kc3.1 also blunts pharmacologically induced EDHF-type dilation.

Vasoconstriction of CAs in response to phenylephrine (PE) (1 μmol/L) did not differ between Kc3.1−/− mice and wild-type littermates. PE contracted CAs of Kc3.1+/+ mice by 114±8 μm (from 572±9 to 458±10 μm; n=20) and CAs of Kc3.1−/− mice by 125±9 μm (from 568±8 to 448±10 μm; n=23). Endothelium-independent dilation in response to sodium nitroprusside (SNP) (1 μmol/L) was also unchanged in Kc3.1−/− mice. SNP dilated PE-precontracted CAs of Kc3.1+/+ mice by 106±8 μm and CAs of Kc3.1−/− mice by 119±10 μm (P=NS). Likewise, pressure-induced vasoconstriction (myogenic tone), as determined in myogenically active small arteries (gracilis artery, ~100 μm in diameter), was not altered in Kc3.1−/− mice (data not shown). These results indicate that deficiency of Kc3.1 does not alter the tonic dilatatory effect of the endothelium on myogenic tone or tone initiated by α1-adrenergic receptor stimulation using PE. In this regard, it is noteworthy that Kc3.23 have been proposed to a play role herein24 (for additional discussion, see the online data supplement).

Collectively, these data from pressure myograph experiments show that the lack of Kc3.1 impairs ACh-induced dilation predominantly by affecting the EDHF-type dilation.

In addition, the contribution of Kc3.1 to endothelium-dependent dilations was investigated in resistance-sized vessels in the cremaster microcirculation in the presence of inhibitors of NOS and COX. The maximal diameter of the arterioles investigated ranged from 16 to 75 μm and was similar in wild-type and Kc3.1−/− mice (36±1 versus 38±1 μm, respectively; n=80 arterioles in 8 mice, each genotype). The arteriolar dilation in response to ACh was significantly reduced in Kc3.1−/− mice, which was most pronounced at intermediate concentrations (Figure 5). In contrast, the dilation in response to SNP (10 μmol/L) was similar in both genotypes (wild-type: 63±3%; Kc3.1−/−: 66±3% of maximum dilation; P=NS). Thus, the Kc3.1 channel is also required in arterioles for an intact EDHF-type dilation.

Cardiovascular Parameters in Wild-Type and Kc3.1−/− Mice

<table>
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<tr>
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<th>Kc3.1+/+</th>
<th>Kc3.1−/−</th>
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<tr>
<td>n</td>
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<td>28</td>
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<tr>
<td>SBP (mm Hg)</td>
<td>129±3†</td>
<td>142±3†</td>
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<tr>
<td>DBP (mm Hg)</td>
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<td>MAP (mm Hg)</td>
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<tr>
<td>n</td>
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<tr>
<td>HW (mg/g BW)</td>
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<td>4.9±0.1</td>
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<tr>
<td>BW (g)</td>
<td>29±1</td>
<td>29±1</td>
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<tr>
<td>n</td>
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<td>8</td>
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<tr>
<td>LV area (mm²)</td>
<td>18.0±0.9</td>
<td>22.4±1.8*</td>
</tr>
<tr>
<td>RV area (mm²)</td>
<td>3.9±0.2</td>
<td>3.8±0.9</td>
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MAP indicates mean arterial pressure; HW, heart weight; BW, body weight; LV, left ventricle; RV, right ventricle. *P<0.05, †P<0.01 vs wild-type (Student’s t test).

Elevated Blood Pressure in Kc3.1−/− Mice

Because deficiency in Kc3.1−/− resulted in reduced Kc3.1 current density, decreased hyperpolarization responses, and an impaired EDHF-type dilation in conducting and resistance vessels, we questioned whether this has functional consequences on the overall circulation. We tested this hypothesis by measuring systolic blood pressure (SBP) and diastolic blood pressure (DBP) using tail-cuff plethysmography. Compared with wild-type littermates, SBP and DBP were significantly increased by ~13 mm Hg and ~12 mm Hg in Kc3.1−/− mice, respectively. The calculated mean arterial pressure was increased by ~14 mm Hg (Table). In smaller sets of animals (n=4, per group), we conducted 24-hour pressure measurements by telemetry which revealed a comparable increase of the mean pressure in Kc3.1−/− mice (115±2 versus 108±1 mm Hg; P<0.05) and confirmed the tail-cuff measurements. However, heart rate was unchanged (Kc3.1−/−: 637±22; wild-type: 633±17 bpm). Interestingly, we also found a slight increase in heart weight and a larger cross-sectional area of the left ventricle on histological examination in Kc3.1−/− (Table). This might be indicative of mild left-ventricular hypertrophy, presumably resulting from increased blood pressure because the cross-sectional area of the right ventricle was not altered in Kc3.1−/− mice.

Discussion

In the present study, we show that the targeted disruption of the Kc3.1 gene abolished Kc3.1 functions and greatly reduced endothelial Kc3 currents. This reduction of Kc3 currents was associated with an impaired endothelial hyperpolarization and EDHF-type dilation in conducting and resistance vessels in response to the classical endothelial agonist ACh. In addition, systemic blood pressure was elevated in Kc3.1−/− mice. Thus, by using a conventional gene-targeting approach, we demonstrate that the Kc3.1, on its own, is an important determinant of the capability of the endothelium to hyperpolarize on stimulation and, thereby, apparently supports EDHF-type dilation. Moreover, we show for the first time that targeted disruption of a crucial endothelial component of the EDHF-signaling pathway, ie, Kc3.1, has an impact on systemic blood pressure regulation.
In response to agonist stimulation, endothelial hyperpolarization is considered to be a prerequisite for the initiation of the EDHF-signaling pathway. The endothelial KCa channels mediating this initial endothelial hyperpolarization were identified by electrophysiologic, pharmacological, and molecular approaches as KCa3.1 and KCa2.3 channels in arteries from mice, rats, pigs, and humans. These 2 KCa channels are not expressed in mature contractile vascular smooth muscle cells of CAs of mice (also see supplemental Figure II) and of rats. In mice as in rats, endothelial KCa3.1 and KCa2.3 seem to contribute almost equally to the composite endothelial KCa currents, whereas in human EC in intact mesenteric artery preparations, endothelial KCa currents and hyperpolarization are largely mediated by KCa3.1. The present study, found that deficiency of KCa3.1 caused a reduction of KCa currents in AEC and CAEC in response to ACh and a pharmacological activator of KCa. The amplitude of the residual KCa current in KCa3.1−/− mice, which was mediated by KCa2.3, was approximately half of the amplitude of the composite KCa3.1/KCa2.3 current observed in wild-type mice, which suggests that KCa3.1 and KCa2.3 channels contribute equally to endothelial hyperpolarization. Moreover, the current in KCa3.1−/− mice had roughly the same amplitude as the hyperpolarizing current in wild-type EC after pharmacological inhibition of KCa3.1. This important finding suggests that the lack of KCa3.1 is not compensated for by a higher activity and/or expression level of the KCa2.3.

The functional expression of both channels in the same cell implies that they have different functions or that their combined activation is needed to generate a complete hyperpolarization after endothelial stimulation. This latter could be attributable to a necessity to counteract concomitant depolarizing currents through second-messenger-gated cation channels of the transient receptor potential family after stimulation of G protein–coupled receptors. Assuming a tight coupling of the endothelium and smooth muscle via myoendothelial gap junctions, the endothelium may also be subjected to a depolarizing effect of the smooth muscle. In any case, the present study revealed that the loss of KCa3.1 dampened the endothelial hyperpolarization response to ACh as well as to the potent KCa3.1/KCa2.3 opener DC-EBIO. This highlights the requirement of this channel, which adds to endothelial hyperpolarization on stimulation to achieve its full amplitude. Whether the KCa3.1 is simply another source of hyperpolarizing current or acts as an amplifier cannot be clearly decided by the present study. However, it should be noted that 50% of the amplitude of the hyperpolarization response, which is mediated by KCa2.3 was preserved.

Because the endothelial hyperpolarization is critical to induce EDHF-type dilation and may be important for NO-mediated dilation as well, we assessed endothelium-dependent responses in arteries of KCa3.1−/− mice. In the carotid artery of wild-type mice, NO-mediated nearly half of the dilation at intermediate concentrations of ACh (100 nmol/L). Because prostacyclin does not contribute in these vessels, which was verified by the lack of effect of the COX inhibitor INDO, the other half of the response was NO- and prostacyclin-independent and thus apparently attributable to an EDHF-type dilation. This large contribution of EDHF to endothelium-dependent dilation in this artery differs from earlier findings of a lower, or even no, contribution of EDHF in carotid arteries of rats (≈20% EDHF) and mice, respectively. Likely explanations for this discrepancy are the use of different experimental approaches (pressure [this study] versus wire myography) or different compounds and concentrations used for precontraction such as phenylephrine (this study), prostaglandins, or thromboxane A2 mimetics, ie, U46619. Especially the latter was shown to interfere with EDHF signaling in a negative fashion. However, the loss of KCa3.1 reduced the overall endothelium-dependent dilation in the carotid artery. This reduction was not caused by an attenuation of the NO-mediated part of the response because inhibition of NO synthase was equally effective in both genotypes. This suggests an alteration of the EDHF-type dilation, as verified after blockade of NO and prostaglandin synthesis. Importantly, EDHF cannot be compensated for by NO and/or prostaglandins because the overall endothelium-dependent dilation is blunted. Although it is widely accepted that endothelial hyperpolarization mediated by KCa3.1 or KCa2.3 is required for generating an EDHF-mediated dilation, the specific contribution of either KCa3.1 or KCa2.3 is still unclear. Depending on vessel type, species, and experimental approach, a single blocker or only the combination of both blockers is effective in suppressing endothelial hyperpolarization, smooth muscle hyperpolarization, and EDHF-mediated dilation.

In the present study on carotid artery of wild-type mice, blockade of both KCa3.1 and KCa2.3 was required to suppress EDHF-type dilation, which is reflected also by the complete inhibition of endothelial hyperpolarization, and thus both endothelial KCa channels are important in initializing a full EDHF dilation. This hypothesis was further verified by the gene-targeting approach in this present study, as KCa3.1−/− mice exhibit an impaired EDHF-mediated dilation in the CA, which is most likely attributable to the reduced capacity of the endothelium to hyperpolarize. The important role of KCa3.1 is not restricted to conducting arteries. Also, in resistance vessels of the microcirculation, KCa3.1 is critical to initiate a full EDHF-type dilation and its role appears to be prominent at lower concentrations of ACh. Because NO and prostaglandins do not contribute substantially to dilator responses in this preparation, we have not assessed the role of NO in this study. However, a considerable portion of the EDHF-mediated dilation was preserved in both vessel types. This remaining dilation relied on the activation of KCa2.3 because the inhibitor of KCa2.3 almost completely blocked the EDHF-type dilation in KCa3.1−/− mice.

The physiological importance of the impaired endothelial hyperpolarizing capability and diminished EDHF-mediated dilation in KCa3.1−/− mice is reflected by the considerable increase in arterial pressure. This may reflect the importance of endothelial hyperpolarizations to coordinate vascular behavior along the vessel wall through gap junctions. It is noteworthy, that a similar association of impaired endothelial KCa3.1 (and KCa2.3) expression and diminished EDHF-mediated dilation is present in uremic rats and in restenosis disease, which supports the notion that a loss of endothelial KCa3.1 is relevant in cardiovascular diseases. With respect to
the KCa2.3 channel, a recent study using transgenic KCa2.3 mice showed that suppression of KCa2.3 expression increased myogenic and phenylephrine-induced tone and systemic blood pressure, thus highlighting the functional role of this channel in endothelial control of vascular tone (for additional discussion, please see the online data supplement).

In conclusion, the present study demonstrated that the endothelial KCa2.3.1 contributes considerably to endothelial hyperpolarization and EDHF-mediated dilations. Importantly, the loss of KCa2.3.1 cannot be compensated for by enhanced activity of KCa2.3 and/or NO and, therefore, has an impact on overall circulatory function as reflected by the elevation of systemic blood pressure. Thus, this study presents first evidence that genetic deletion of one major component of the EDHF-signaling pathway significantly impairs cardiovascular functions. Conversely, selective openers of endothelial KCa2.3.1 may provide a novel therapeutic approach for the treatment of vascular diseases characterized by endothelial dysfunction and hypertension.

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Disclosures

None.

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Methods

**Generation of \(K_{Ca3.1}^-/-\) mice:** Parts of the \(K_{Ca3.1}\) gene were amplified by PCR from genomic DNA of embryonic stem (ES) cells (kindly provided by Dr. K.P. Knobeloch, Institute of Molecular Pharmacology, Charite, Berlin, Germany). A targeting vector was constructed with a 4.6 kb PCR fragment generated by using the Expand long template PCR system (Roche, Grenzach, Germany) containing exon 5-7 of the \(K_{Ca3.1}\) gene and a 0.9 kb fragment of the intron sequence upstream of exon 4. These fragments flanked the neomycin (\(neo\)) cassette of pTV0. We verified correct insertion of all fragments by sequencing. The NotI-linearized vector was electroporated into 129Sv/ES cells, which were subsequently grown under double selection with G418 and gancyclovir. ES cell clones containing the desired mutation were identified by PCR (FP: 5'–CTTTGGATCCAGATGTTTCTTGGTGTTAAG–3'; RP2 (\(neo\)): 5'–CGTGCAATCCATCTTGTTCA–3') and one correctly targeted clone was injected into C57BL/6 blastocysts. Chimeras were mated to wild-type C57BL/6 (Charles River, Sulzfeld, Germany) and \(K_{Ca3.1}^-/-\) mice were generated by interbreeding heterozygous offspring. Animals were kept in our local specific-pathogen-free (SPF)-animal facility and had free access to chow and water. Routine genotyping was performed by multiplex-PCR using the primers: FP, RP1(exon 4): 5’-GCCACAGTGTGTCTGGAGG–3’, and the RP2(\(neo\)) (Fig. 1). Southern blot analysis with a 1063 bp probe gave a 3.3 kb \(Bgl\)II-fragment in wild-type and a 4.1 kb \(Bgl\)II-fragment in \(K_{Ca3.1}^-/-\) mice (Fig. 1). Primer pairs used for RT-PCR were FP(exon3): 5’-AGATCCTGCTGGAGC TGTTG-3'; FP(exon4): 5’-ACGGGGCACCTCAGCTGGAGCTGGTGTG-3'. Western-blot: Aliquots (150 µl) of erythrocyte ghost membranes from peripheral blood were diluted in 4.5 ml 5 mM HEPES buffer (pH 8.0) and centrifuged at 20,000×g at 4°C for 15 min and the pellet was washed twice. Thereafter the pellet was washed three times in 1.25 mM
HEPES buffer (pH 8.0) to remove the haemoglobin. Membrane fractions were collected and pelleted at 100,000×g for 30 min. The ghost membranes were resuspended in the PBS buffer with 1 mM PMSF and a protease inhibitor cocktail (both Sigma, Seelze, Germany), frozen in liquid nitrogen and stored at -80°C until use. Protein was quantified using the Bio-Rad Protein Assay. 50 µg of total protein was separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to nitrocellulose membranes (Trans-Blot, all from Bio-Rad, Munich, Germany). Immunoblotting was performed applying an anti-KCa3.1 primary antibody (1:200, Sigma, Deisenhofen, Germany). Blots were exposed to a peroxidase-conjugated anti-rabbit antiserum (Roche, Mannheim, Germany) for 1.5 h and the protein was detected using the enhanced chemiluminescence detection system (ECL, Roche). For complete Western-blot results and specificity of this anti-KCa3.1 primary antibody please see supplemental figure III.

**Patch-clamp Electrophysiology:** Membrane currents in freshly isolated aortic endothelial cells (AEC) and in carotid artery endothelial cells (CAEC) *in situ* from KCa3.1-/- and KCa3.1+/+ mice were recorded with an EPC-9 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany) using voltage ramps (1000 ms; -100 to +100 mV)2-4. Membrane potentials were recorded in the endothelium of carotid artery (CA) *in situ* and in AEC cluster by using the current-clamp mode. For activation of KCa-currents (composite KCa3.1 and KCa2.3 current), AEC and CAEC were dialyzed with a KCl-pipette solution containing 3 µmol/L [Ca²⁺]Free (in mmol/L): 140 KCl, 1 Na₂ATP, 1 MgCl₂, 2 EGTA, 1.91 CaCl₂, and 5 HEPES, pH 7.2. For current-clamp experiments, the pipette solution contained 0.86 mmol/L CaCl₂ ([Ca²⁺]Free=100 nmol/L). The NaCl bath solution contained (mmol/L): 137 NaCl, 4.5 Na₂HPO₄, 3 KCl, 1.5 KH₂PO₄, 0.4 MgCl₂, 0.7 CaCl₂, and 10 glucose (pH 7.4). It should be noted that neither KCa3.1 nor KCa2.3 but the KCa1.1 (large-conductance KCa-channel, a.k.a. BKCa, Maxi K) is expressed in mature vascular smooth muscle of CA (Fig. II).
**Pressure Myography:** Pressure myography in CA from KCa3.1−/− and KCa3.1+/+ mice was performed as described previously. Bath and perfusion solution contained (in mmol/L): 145 NaCl, 1.2 NaH2PO4, 4.7 KCl, 1.2 MgSO4, 2 CaCl2, 5 glucose, 2 pyruvate, and 3 MOPS buffer (pH 7.4 at 37°C). CA were pressurized to 80 mmHg and continuously perfused at a flow rate of 0.2 ml/min. CA were pre-constricted with 1 µmol/L phenylephrine (PE) and perfused with acetylcholine (ACh; 1 nmol/L to 10 µmol/L) or 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one (DC-EBIO, 10 µmol/L; TOCRIS, Cologne, Germany) in the presence and absence of Nω-nitro-L-arginine (L-NNA, 100 µmol/L) and indomethacin (INDO, 10 µmol/L). In some experiments, ACh was applied together with a combination of the selective IKCa-blocker TRAM-345 (1 µmol/L) and the selective SKCa-blocker UCL1684 (100 nmol/L) or with UCL1684 alone. Pressure myography in A. gracilis was performed as described previously. Diameter changes of CA and A. gracilis were expressed as a percentage of the maximal dilation to 1 µmol/L sodium nitroprusside (SNP).

**Membrane potential measurements:** Membrane potentials in smooth muscle of CA were recorded in the presence of L-NNA (100 µmol/L) and INDO (10 µmol/L) by conventional sharp microelectrode technique using a BA-1S amplifier (npi electronic, Tamm, Germany). Sharp electrodes filled with 0.5 mol/L KCl (tip resistance 80-100 MΩ) were inserted from the adventitial side and a sharp deflection of the zero potential towards negative values indicated successful cell penetration. 3-15 stable potential recordings (>20 sec) per vessel (n=6, each genotype) were made before and after addition of 100 nmol/l ACh and potential values averaged.

**Intravital microscopy:** Intravital microscopy in the cremaster muscle was performed as described. Ten arterioles were studied in each animal and their diameters determined shortly
before and during superfusion of ACh (0.1–10 µmol/L) and SNP (10 µmol/L) in the presence of L-NNA (30 µmol/L) and INDO (3 µmol/L). Diameter changes were normalized to the maximal diameters which was determined by simultaneous superfusion of SNP, ACh, and adenosine (each 30 µmol/L).

**Tail-cuff plethysmography and telemetry:** Systolic (SBP) and diastolic (DBP) blood pressure was recorded in trained conscious and quiescent mice (sex-matched, 3-4 month old, K\textsubscript{Ca}3.1-/-, n=28 (16f/12m); K\textsubscript{Ca}3.1+/+, (11f/11m), n=22) by using a non-invasive tail-cuff monitor (NIBP-device) and analyzed with the PowerLab\textsuperscript{®} data acquisition system (both from ADInstruments, Spechbach, Germany). Evaluation of data was done in a blinded fashion and the average of 10-20 measurements was taken as the representative pressure for a single animal. In addition, telemetric pressure measurements\textsuperscript{9} were performed in smaller sets of animals (n=4, per group). In brief, mice were allowed to recover for 9 days after implantation of a TA11PA-C20 pressure transducer (Data Sciences International, St Paul, Minnesota, USA). Thereafter, pressure values were continuously recorded for 7 days, and the values (24-hrs means) of the last 3 days were averaged and used for statistical analysis.

**Patch-clamp Electrophysiology:** Membrane currents in freshly isolated aortic endothelial cells (AEC) and in carotid artery endothelial cells (CAEC) in situ from K\textsubscript{Ca}3.1-/- and K\textsubscript{Ca}3.1+/+ mice were recorded with an EPC-9 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany) using voltage ramps (1000 ms; -100 to +100 mV)\textsuperscript{2-4}. Membrane potentials were recorded in the endothelium of carotid artery (CA) in situ and in AEC cluster by using the current-clamp mode. For activation of K\textsubscript{Ca}-currents (composite K\textsubscript{Ca}3.1 and K\textsubscript{Ca}2.3 current), AEC and CAEC were dialyzed with a KCl-pipette solution containing 3 µmol/L [Ca\textsuperscript{2+}]\textsubscript{free} (in mmol/L): 140 KCl, 1 Na\textsubscript{2}ATP, 1 MgCl\textsubscript{2}, 2 EGTA, 1.91 CaCl\textsubscript{2}, and 5 HEPES, pH 7.2. For current-clamp experiments, the pipette solution contained 0.86 mmol/L
CaCl₂ ([Ca²⁺]₉₉=100 nmol/L). The NaCl bath solution contained (mmol/L): 137 NaCl, 4.5 Na₂HPO₄, 3 KCl, 1.5 KH₂PO₄, 0.4 MgCl₂, 0.7 CaCl₂, and 10 glucose (pH 7.4). It should be noted that neither K₉Ca3.1 nor K₉Ca2.3 but the K₉Ca1.1 (large-conductance K₉Ca-channel, a.k.a. BK₉Ca, Maxi K) is expressed in mature vascular smooth muscle of CA (supplementary online Fig. S1).

**Pressure Myography:** Pressure myography in CA from K₉Ca3.1⁻⁻ and K₉Ca3.1⁺⁺ mice was performed as described previously.³ Bath and perfusion solution contained (in mmol/L): 145 NaCl, 1.2 NaH₂PO₄, 4.7 KCl, 1.2 MgSO₄, 2 CaCl₂, 5 glucose, 2 pyruvate, and 3 MOPS buffer (pH 7.4 at 37°C). CA were pressurized to 80 mmHg and continuously perfused at a flow rate of 0.2 ml/min. CA were pre-constricted with 1 µmol/L phenylephrine (PE) and perfused with acetylcholine (ACh; 1 nmol/L to 10 µmol/L) or 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one (DC-EBIO, 10 µmol/L; TOCRIS, Cologne, Germany) in the presence and absence of Nω-nitro-L-arginine (L-NNA, 100 µmol/L) and indomethacin (INDO, 10 µmol/L). In some experiments, ACh was applied together with a combination of the selective IK₉Ca-blocker TRAM-34⁵ (1 µmol/L) and the selective SK₉Ca-blocker UCL1684⁶ (100 nmol/L) or with UCL1684 alone. Pressure myography in A. gracilis was performed as described previously.⁷ Diameter changes of CA and A. gracilis were expressed as a percentage of the maximal dilation to 1 µmol/L sodium nitroprusside (SNP).

**Intravital microscopy:** Intravital microscopy in the cremaster muscle was performed as described.⁸ Ten arterioles were studied in each animal and their diameters determined shortly before and during superfusion of ACh (0.1–10 µmol/L) and SNP (10 µmol/L) in the presence of L-NNA (30 µmol/L) and INDO (3 µmol/L). Diameter changes were normalized to the maximal diameter which was determined by simultaneous superfusion of SNP, ACh, and adenosine (each 30 µmol/L). TRAM-34 was a kind gift from Heike Wulff (University of
California Davies, CA). All other standard chemicals and toxins were obtained from Sigma (Deisenhofen, Germany).

**Tail-cuff plethysmography and telemetry:** Systolic (SBP) and diastolic (DBP) blood pressure was recorded in trained conscious and quiescent mice (sex-matched, 3-4 month old, K\textsubscript{Ca}3.1\textasciitilde/\textasciitilde, n=28 (16f/12m); K\textsubscript{Ca}3.1+/+, (11f/11m), n=22) by using a non-invasive tail-cuff monitor (NIBP-device) and analyzed with the PowerLab\textsuperscript{®} data acquisition system (both from ADInstruments, Spechbach, Germany). Evaluation of data was done in a blinded fashion and the average of 10-20 measurements was taken as the representative pressure for a single animal. In addition, telemetric pressure measurements\textsuperscript{9} were performed in smaller sets of animals (n=4, per group). In brief, mice were allowed to recover for 9 days after implantation of a TA11PA-C20 pressure transducer (Data Sciences International, St Paul, Minnesota, USA). Thereafter, pressure values were continuously recorded for 7 days, and the values (24-hrs means) of the last 3 days were averaged and used for statistical analysis.

**Drugs:** TRAM-34 was a kind gift from Heike Wulff (University of California Davies, CA). All other standard chemicals and toxins were obtained from Sigma (Deisenhofen, Germany).

**References:**


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Supplementary discussion:

Regarding endothelial KCa2.3, a recent study using transgenic KCa2.3-mice in which KCa2.3-expression can be manipulated with dietary doxycycline, highlighted the functional role of this channel in endothelial control of vascular tone. Suppression of KCa2.3 expression increased myogenic and phenylephrine-induced tone, and systemic blood pressure which suggests that this channel has an impact on the tonic dilating influence (in the absence of an agonist) of the endothelium on vascular tone. However, whether EDHF-signalling upon agonist stimulation is disturbed in these transgenic KCa2.3-mice, has not been investigated so far. Regarding a possible involvement of KCa3.1 in the tonic dilating influence of the endothelium on vascular tone, our present study shows that the myogenic responsiveness to pressure in myogenically active small arteries (ex vivo) as well as to α1-receptor stimulation in small arteries and carotid arteries (ex vivo) was not altered in KCa3.1-/- mice. This suggests that, unlike KCa2.3, the KCa3.1 may not be important for the impact of the unstimulated endothelium on myogenic or vasoconstrictor-induced tone in these preparations.

Inhibition of cyclooxygenase by indomethacin (INDO, 10 µmol/L) did not affect endothelium-dependent dilations of CA from wild-type mice. W/O = without inhibitors.
Freshly isolated VSMC (n=6) of CA from wild-type mice expressed the large-conductance Ca$^{2+}$-activated K$^+$ channel ($K_{Ca}$1.1; a.k.a. BK, BK$_{Ca}$, maxi K$^+$), which was sensitive to iberiotoxin (IbTx, 100 nmol/L; n=6). $K_{Ca}$3.1 and $K_{Ca}$2.3 functions were not detected in these VSMC.
Supplementary online Figure III

Complete Western-blot results using an anti-KCa3.1 primary antibody (1:200, Sigma, Deisenhofen, Germany). Note that the use of this antibody yields also several strong non-specific bands in wild-type and KCa3.1-/- mice in addition to the KCa3.1-specific band (at about 50 kDa) in wild-type mice. Such non-specific binding of this antibody is also indicated by other vendors, e.g., at http://www.alomone.com.