

Genetic Deficit of SK3 and IK1 Channels Disrupts the Endothelium-Derived Hyperpolarizing Factor Vasodilator Pathway and Causes Hypertension

Sebastian Brähler*; Anuradha Kaistha, MSc*; Volker J. Schmidt, MD; Stephanie E. Wölfle, PhD; Christoph Busch, MD; Brajesh P. Kaistha, MSc; Michael Kacik, MD; Anna-Lena Hasenau; Ivica Grgic, MD; Han Si, PhD; Chris T. Bond, PhD; John P. Adelman, PhD; Heike Wulff, PhD; Cor de Wit, MD; Joachim Hoyer, MD; Ralf Köhler, PhD

Background—It has been proposed that activation of endothelial SK3 ($K_{Ca}2.3$) and IK1 ($K_{Ca}3.1$) K^+ channels plays a role in the arteriolar dilation attributed to an endothelium-derived hyperpolarizing factor (EDHF). However, our understanding of the precise function of SK3 and IK1 in the EDHF dilator response and in blood pressure control remains incomplete. To clarify the roles of SK3 and IK1 channels in the EDHF dilator response and their contribution to blood pressure control in vivo, we generated mice deficient for both channels.

Methods and Results—Expression and function of endothelial SK3 and IK1 in IK1^{-/-}/SK3^{TT} mice was characterized by patch-clamp, membrane potential measurements, pressure myography, and intravital microscopy. Blood pressure was measured in conscious mice by telemetry. Combined IK1/SK3 deficiency in IK1^{-/-}/SK3^{TT} (+doxycycline) mice abolished endothelial K_{Ca} currents and impaired acetylcholine-induced smooth muscle hyperpolarization and EDHF-mediated dilation in conduit arteries and in resistance arterioles in vivo. IK1 deficiency had a severe impact on acetylcholine-induced EDHF-mediated vasodilation, whereas SK3 deficiency impaired NO-mediated dilation to acetylcholine and to shear stress stimulation. As a consequence, SK3/IK1-deficient mice exhibited an elevated arterial blood pressure, which was most prominent during physical activity. Overexpression of SK3 in IK1^{-/-}/SK3^{TT} mice partially restored EDHF- and nitric oxide-mediated vasodilation and lowered elevated blood pressure. The IK1-opener SKA-31 enhanced EDHF-mediated vasodilation and lowered blood pressure in SK3-deficient IK1^{+/+}/SK3^{TT} (+doxycycline) mice to normotensive levels.

Conclusions—Our study demonstrates that endothelial SK3 and IK1 channels have distinct stimulus-dependent functions, are major players in the EDHF pathway, and significantly contribute to arterial blood pressure regulation. Endothelial K_{Ca} channels may represent novel therapeutic targets for the treatment of hypertension. (*Circulation*. 2009;119:2323-2332.)

Key Words: blood pressure ■ endothelium-derived factors ■ ion channels ■ $K_{Ca}3.1$ protein, mouse

The arterial endothelium modulates the contractile state of the underlying vascular smooth muscle and thus blood pressure by releasing the vasodilating autacoids nitric oxide (NO)¹ and prostacyclin (prostaglandin₁₂ [PGI₂]).² Besides NO and PGI₂, a third factor or signaling pathway termed endothelium-derived hyperpolarizing factor (EDHF)³ adds to endothelium-dependent vasodilation by causing hyperpolarization and relaxation of vascular smooth muscle. Although EDHF has been proposed to be as powerful³⁻⁵ as NO and PGI₂ in controlling vascular tone and blood pressure, direct evidence for this is missing. Moreover, the molecular identi-

ties of EDHF and the EDHF-signaling pathways are still a matter of ongoing debate. Indeed, EDHF dilatory responses have been attributed to a variety of diffusible EDHFs such as cytochrome P450-generated metabolites of arachidonic acid,^{6,7} lipoxygenase products,⁸ NO itself,⁹ hydrogen peroxide,¹⁰ and C-type natriuretic peptide,¹¹ as well as to electrotonic coupling¹² of endothelium and smooth muscle (for extensive review, see References 3 and 5). Whatever EDHF might be, activation of the Ca^{2+} -activated K^+ channels SK3 ($K_{Ca}2.3$)¹³ and IK1 ($K_{Ca}3.1$),¹⁴ located in the endothelium,¹⁵⁻¹⁸ has been proposed to initiate EDHF signaling in a variety of

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From the Department of Internal Medicine/Nephrology, Philipps University, Marburg, Germany (S.B., A.K., C.B., B.P.K., M.K., A.H., I.G., J.H., R.K.); Department of Physiology, University of Lübeck, Lübeck, Germany (V.J.S., S.E.W., C.d.W.); Division of Nephrology, Albert Einstein College of Medicine, Bronx, NY (H.S.); Vollum Institute, Oregon Health and Science University, Portland (C.T.B., J.P.A.); and Department of Pharmacology, University of California, Davis (H.W.).

*The first 2 authors contributed equally to this article.

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Correspondence to Ralf Köhler, PhD, Biomedical Research Center, Hans-Meerweinstrasse, 35037 Marburg, Germany. E-mail rkoehler@med.uni-marburg.de

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vascular beds and species.^{17–20} On intracellular Ca^{2+} mobilization in response to receptor stimulation by pharmacological stimuli, SK3 and IK1 mediate K^+ efflux and produce membrane hyperpolarization.^{16,18} This endothelial hyperpolarization and/or the concomitant K^+ efflux¹⁷ causes subsequent smooth muscle hyperpolarization, leading to the closure of voltage-activated Ca^{2+} channels and finally relaxation. However, the concept that IK1 and SK3 are required to initiate the EDHF dilator response is based mainly on studies of isolated vessels and pharmacological evidence.^{3,5,17,18} Recently, we reported that IK1-deficient mice ($IK1^{-/-}$) exhibit a partial defect in EDHF signaling and a moderately elevated blood pressure.²⁰ Genetic modulation of SK3 expression in mice²² has also been shown to alter blood pressure.²² In these $SK3^{T/T}$ mice, expression levels of SK3 are controlled by site-specific insertion of a tetracycline-based genetic switch.²¹ Dietary doxycycline administration dramatically diminished SK3 expression, whereas the channel is overexpressed ≈ 3 -fold in the absence of doxycycline.²¹ Although SK3 suppression by doxycycline treatment causes a substantial elevation of blood pressure,²² it is unknown whether the higher blood pressure in $SK3^{T/T}$ (+doxycycline) mice is related to defects in EDHF signaling. Thus, the understanding of the specific roles of SK3 and IK1 in the EDHF dilator response and the contribution of EDHF to endothelial control of vascular tone, and ultimately blood pressure in vivo, is incomplete.^{3,5} To elucidate the roles of SK3 and IK1, we generated mice deficient for both channels ($IK1^{-/-}/SK3^{T/T}$ +doxycycline) and studied signaling related to EDHF as well as arterial blood pressure in these mice. The present study identifies endothelial SK3 and IK1 channels as major effectors in EDHF signaling in vivo and reveals their significant impact on systemic blood pressure control.

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Methods

$IK1^{-/-}SK3^{T/T}$ animals were generated by interbreeding $SK3^{T/T}$ mice and $IK1^{-/-}$ mice and genotyped by polymerase chain reaction. For suppression of SK3, mice received doxycycline in the drinking water (2 mg/mL, 2% sucrose) for at least 6 days. SK3/IK1 expression was detected by Western blot analysis of brain (SK3) and liver extracts (IK1). Vascular SK3/IK1 expression was detected by immunohistochemistry on carotid arteries (CA). Whole-cell IK1/SK3 currents were recorded in freshly isolated CA endothelial cells with the use of patch-clamp and smooth muscle membrane potentials in CA by sharp-electrode techniques. Endothelium-dependent and -independent vasodilation was studied by pressure myography in CA and intravital microscopy of resistance arterioles in the cremaster muscle in vivo. Arterial blood pressure, heart rate, and locomotor activity were measured telemetrically in freely moving mice for at least 3 consecutive days in the presence or absence of doxycycline or 24 hours before and after injection of the IK1-opener SKA-31.

Statistical Analysis

Data are given as mean \pm SEM. One-way ANOVA tests followed by Newman-Keuls or Bonferroni post hoc tests or paired Student *t* test were used as indicated to assess differences between groups.

For a detailed description of Methods, see the online-only Data Supplement.

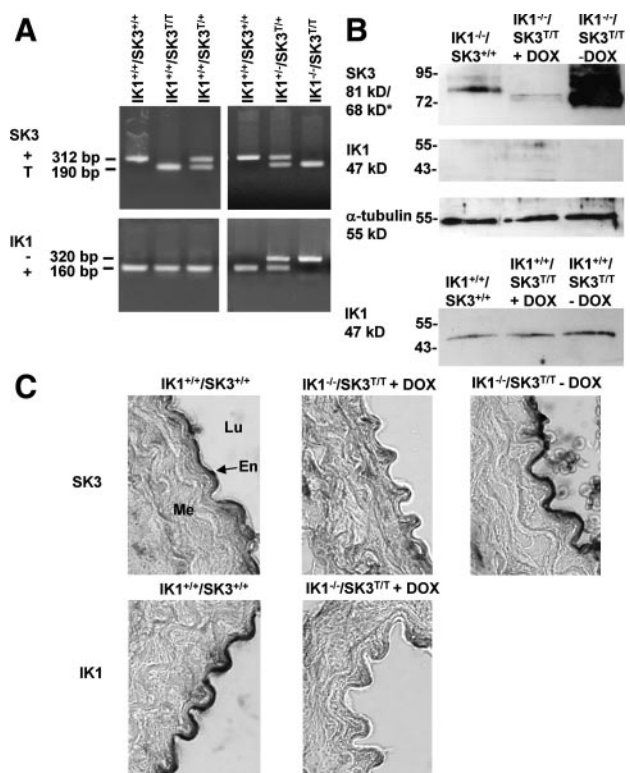


Figure 1. Generation of $IK1^{-/-}/SK3^{T/T}$ mice. A, Polymerase chain reaction amplified SK3 WT (+) and tTA-targeted (T) alleles (top) as well as IK1 WT (+) and targeted alleles (-) (bottom). Note homozygous $IK1^{-/-}/SK3^{T/T}$ mice on right. B, top, Western blot of SK3 protein in brain from $IK1^{-/-}/SK3^{T/T}$ mice +/-doxycycline (DOX) for 2 weeks. *The apparent smaller size of the protein in $IK1^{-/-}/SK3^{T/T}$ -doxycycline is due to the deletion of 40 AA from the n-terminus. Middle, IK1 was not detected in liver from $IK1^{-/-}/SK3^{T/T}$ mice. Visualization of α -tubulin demonstrates equal loading. Bottom, IK1 in liver from $IK1^{+/+}/SK3^{T/T}$ mice. C, Immunohistochemistry of SK3 (top) and IK1 (bottom) expression in endothelium of CA. Note the absence of specific IK1 and SK3 staining in endothelium of $IK1^{-/-}/SK3^{T/T}$ +doxycycline and the intense SK3 staining in $IK1^{-/-}/SK3^{T/T}$ -doxycycline compared with WT. Me indicates media; Lu, lumen; and En, endothelium.

Results

Generation of $IK1^{-/-}/SK3^{T/T}$ Mice

$IK1^{-/-}$ and $SK3^{T/T}$ mice were generated as described previously^{20,21} and interbred to generate $IK1^{-/-}/SK3^{T/T}$ (Figure 1A). $IK1^{-/-}/SK3^{T/T}$ offspring were viable, fertile, and had no overt behavioral or neurological defects with or without doxycycline administration. In $IK1^{+/+}/SK3^{T/T}$ mice, dietary doxycycline for at least 6 days virtually abolished SK3 expression in brain (Figure 1B). In $IK1^{-/-}/SK3^{T/T}$, loss of IK1 protein was confirmed in liver (Figure 1B). Immunohistochemistry demonstrated loss of endothelial SK3 and IK1 from $IK1^{-/-}/SK3^{T/T}$ +doxycycline and overexpression of SK3 in $IK1^{-/-}/SK3^{T/T}$ -doxycycline (Figure 1C).

Combined IK1 and SK3 Suppression Abolishes Endothelial K_{Ca} Currents and Reduces Endothelium-Mediated Smooth Muscle Hyperpolarization

Endothelial IK1/SK3 functions were determined by whole-cell patch-clamp experiments²⁰ on freshly isolated CA endo-

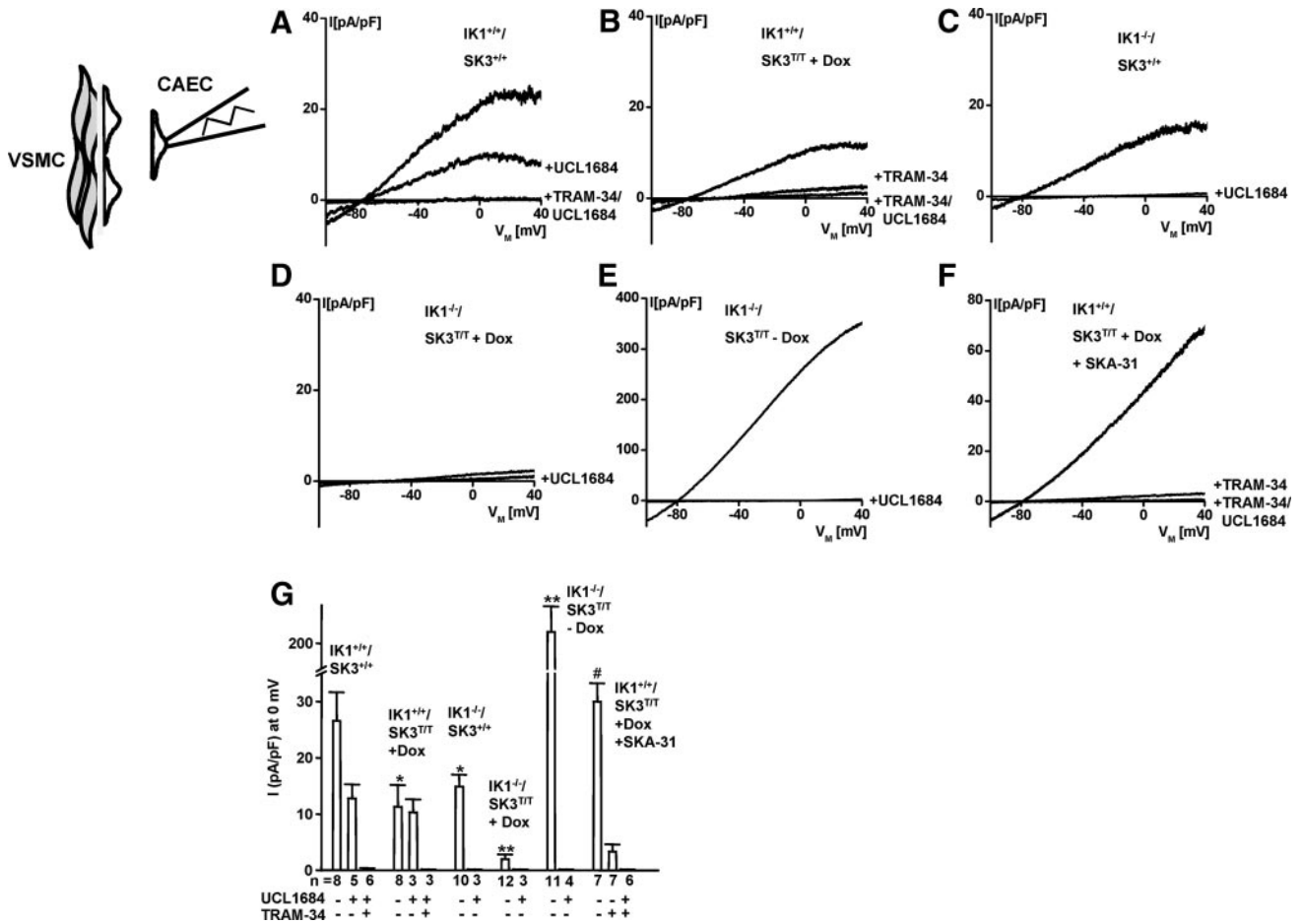


Figure 2. IK1 and SK3 currents in CA endothelial cells. Scheme on left illustrates whole-cell patch-clamp experiments in freshly isolated CA endothelial cells. VSMC indicates vascular smooth muscle cells. A, Current-voltage relationship of composite IK1 and SK3 currents in CA endothelial cells from WT. Currents were reduced by the SK blocker UCL1684 (1 μ mol/L) and abolished by additional application of the IK1 blocker TRAM-34 (1 μ mol/L). B, Reduced currents in CA endothelial cells from IK1^{+/+}/SK3^{T/T}+doxycycline (Dox). The residual, largely IK1-mediated current was inhibited by TRAM-34. A minor TRAM-34-resistant current was blocked by UCL1684. C, Reduced currents in IK1^{-/-}/SK3^{+/+} CA endothelial cells and inhibition of the residual SK3 current by UCL1684. D, Absence of IK1 and almost complete suppression of SK3 currents in IK1^{-/-}/SK3^{T/T}(+doxycycline). E, Overexpression of SK3 in IK1^{-/-}/SK3^{T/T}-doxycycline resulted in large, UCL1684-sensitive currents. F, In IK1^{-/-}/SK3^{T/T}+doxycycline, the IK1-opener SKA-31 (500 nmol/L) strongly potentiated the IK1-mediated current (sensitive to TRAM-34). UCL1684 blocked the small residual current. G, Summary data for K⁺ currents at 0 mV in all groups. Values are given as mean \pm SE; numbers (n) in charts refer to number of CA endothelial cells. **P*<0.05, ***P*<0.01 vs WT; #*P*<0.05 vs IK1^{+/+}/SK3^{T/T}+doxycycline, 1-way ANOVA followed by Newman-Keuls post hoc test.

thelial cells (Figure 2A through 2G). On Ca²⁺ dialysis, wild-type (WT) CA endothelial cells showed a K⁺ current that was abolished by a combination of the IK1 blocker TRAM-34²³ (1 μ mol/L) and the SK blocker UCL1684²⁴ (1 μ mol/L). After suppression of SK3 by dietary doxycycline, the amplitude of this K⁺ current was reduced by \approx 50% in SK3^{T/T} mice, and the remaining K⁺ current was inhibited by TRAM-34. TRAM-34 unmasked a small UCL1684-sensitive current, indicating that the doxycycline treatment reduced SK3 expression by \approx 90%.²² The total K⁺ current was reduced to a similar extent in IK1^{-/-} mice, and the residual current was abolished by UCL1684. In doxycycline-administered IK1^{-/-}/SK3^{T/T} mice, the K⁺ current was virtually abolished, and the very small residual K⁺ current was sensitive to UCL1684. In contrast, the UCL1684-sensitive K⁺ current was 10-fold larger in untreated IK1^{-/-}/SK3^{T/T} mice compared with the composite K⁺ current in WT CA endothelial cells, which reflects the overexpression of SK3. In

doxycycline-administered IK1^{+/+}/SK3^{T/T} mice, the IK1-opener SKA-31(naphtho[1,2-*d*]thiazol-2-ylamine)²⁵ (500 nmol/L) enhanced the TRAM-34-sensitive K⁺ currents by 2- to 3-fold, thus strongly potentiating IK1 currents, without an appreciable effect on the residual UCL1684-sensitive SK3 current.

To study endothelial IK1/SK3-mediated hyperpolarization of smooth muscle to acetylcholine (100 nmol/L), we performed sharp-electrode measurements of smooth muscle membrane potentials in pressurized (80 mm Hg) and precontracted (phenylephrine, 1 μ mol/L) CA, as shown in Figure 3. In WT, acetylcholine elicited a robust hyperpolarization from a resting membrane potential of -40 ± 1 to -61 ± 4 mV. Suppression of SK3 (SK3^{T/T}+doxycycline) slightly reduced the hyperpolarization to acetylcholine (from -40 ± 1 to -57 ± 1 mV; *P*<0.05 versus WT). In IK1^{-/-}, the hyperpolarization response was markedly reduced (from -40 ± 1 to -51 ± 1 mV; *P*<0.01 versus WT and SK3^{T/T}+doxycycline).

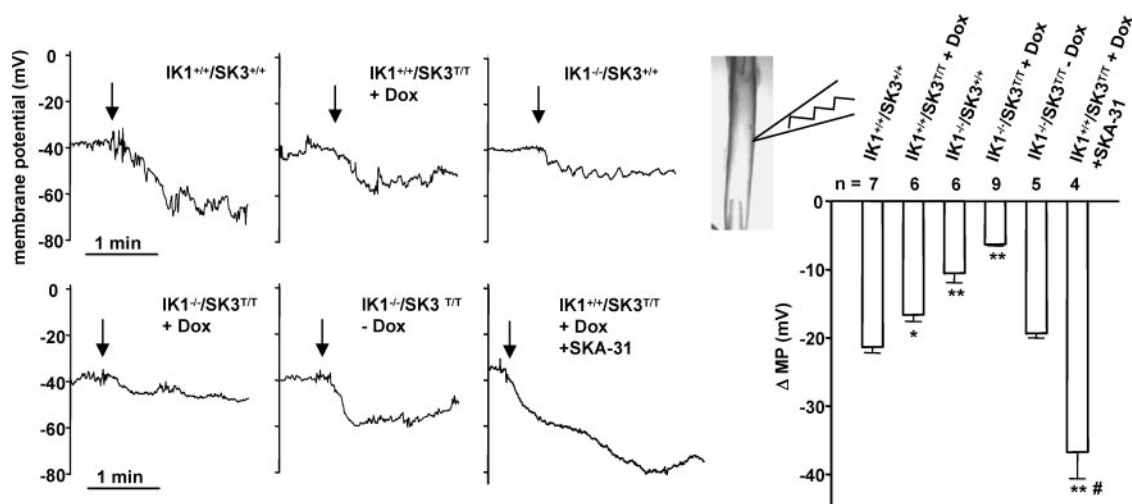


Figure 3. Sharp-electrode measurements of smooth muscle membrane potentials in pressurized, constricted CA (illustrated by picture in top panel). Left, Representative recordings before and during application of acetylcholine (100 nmol/L, arrow) are shown. Luminal concentration of SKA-31 was 500 nmol/L. Right, Change of membrane potential (Δ MP) from different groups. Dox indicates doxycycline. Values are given as mean \pm SE; numbers (n) in charts refer to number of CA. * P <0.05, ** P <0.01 vs WT, 1-way ANOVA followed by Newman-Keuls post hoc test; # P <0.01 vs $IK1^{+/+}/SK3^{T/T}$ +doxycycline.

In CA from $IK1^{-/-}/SK3^{T/T}$ +doxycycline, the hyperpolarization response was attenuated even further (from -39 ± 1 to -45 ± 1 mV; P <0.01 versus other groups). However, in CA that lacked IK1 and overexpressed SK3 ($IK1^{-/-}/SK3^{T/T}$ -doxycycline), we observed a restoration (from -39 ± 1 to -58 ± 1 mV), and the response was not different from WT. Interestingly, potentiation of IK1 functions by SKA-31 in $IK1^{+/+}/SK3^{T/T}$ +doxycycline strongly enhanced acetylcholine-induced hyperpolarization (from -41 ± 2 to -78 ± 1 mV; P <0.01).

Loss of Endothelial IK1 and SK3 Channels Disrupts the EDHF Dilator Response

We next examined EDHF-type vasodilation in pressurized and precontracted CA²⁰ using a "classic" EDHF protocol³ in which we blocked NO synthase using *N* ω -nitro-L-arginine (L-NNA) (300 μ mol/L) and cyclooxygenase using indomethacin (10 μ mol/L) to eliminate NO-mediated and, although unlikely in these murine vessels, prostacyclin-mediated vasodilations.²⁰ Figure 4A shows representative tracings of acetylcholine-induced EDHF-type vasodilations for WT, $IK1^{-/-}$, $IK1^{-/-}/SK3^{T/T}$ +doxycycline, and $IK1^{-/-}/SK3^{T/T}$ -doxycycline. For concentration-response curves from all groups, see Figure 4B. Intraluminally applied acetylcholine (100 nmol/L) produced a robust EDHF-type vasodilation in WT. In contrast, in CA from $IK1^{-/-}/SK3^{+/+}$, vasodilation to 100 nmol/L acetylcholine was reduced by $\approx 75\%$ (P <0.001), and in CA from $IK1^{-/-}/SK3^{T/T}$ +doxycycline, 100 nmol/L acetylcholine induced virtually no dilation ($\approx 99\%$ reduction; P <0.001; Figure 4B, left panel). EDHF-type vasodilation to 100 nmol/L acetylcholine was not significantly altered in $IK1^{+/+}/SK3^{T/T}$ +doxycycline as well as in SK3-overexpressing $IK1^{+/+}/SK3^{T/T}$ -doxycycline (Figure 4B, right panel). However, in CA from mice lacking $IK1^{-/-}$ and overexpressing SK3, the EDHF-type vasodilation was restored. Potentiation of IK1 functions by SKA-31 in $SK3^{T/T}/IK1^{+/+}$ +doxycycline caused a significant increase of amplitude and duration of EDHF-

type vasodilation to 10 and 100 nmol/L acetylcholine (Figure 4C), which reflects a left shift of the concentration-response curve.

We performed a second set of experiments in the absence of L-NNA and indomethacin to evaluate combined NO- and EDHF-mediated vasodilation to acetylcholine (Figure 4D). Under these conditions, acetylcholine (100 nmol/L) produced a ≈ 1.5 -fold larger vasodilation in WT because of the contribution of NO. With intact NO synthesis, the vasodilation to 100 nmol/L acetylcholine was equally reduced by $\approx 30\%$ in CA from $IK1^{-/-}$ (P <0.05) and $IK1^{-/-}/SK3^{T/T}$ +doxycycline (P <0.01). Interestingly, the deficit of SK3 and IK1 impaired the NO-mediated vasodilation at a lower acetylcholine concentration of 10 nmol/L (P <0.05; insert in Figure 4D, left panel). A similar impairment was caused by the lack of SK3 alone in $SK3^{T/T}$ +doxycycline (P <0.05; insert in Figure 4D, right panel), which suggests an additional role for SK3 in NO production elicited by low levels of circulating vasoactive factors. In CA from $IK1^{X}/SK3^{T/T}$ -doxycycline, which overexpressed SK3, vasodilation was similar to WT. Endothelium-independent vasodilations to sodium nitroprusside (10 μ mol/L), vasoconstriction on phenylephrine (1 μ mol/L), and elevated K^{+} (60 mmol/L) were similar in all genotypes (Figure 4A and Figure I in the online-only Data Supplement).

Because EDHF has been reported to play a prominent role in the microcirculation, we next investigated the contribution of IK1 and SK3 in EDHF-type and NO/EDHF-mediated vasodilation in resistance-sized arterioles of the cremaster microcirculation in vivo by intravital microscopy.^{26,27} This in vivo technique allows monitoring of arteriolar diameters during superfusion of vasoactive compounds. In the presence of inhibitors of NO synthase/cyclooxygenase, acetylcholine (1 μ mol/L) produced significant vasodilation in WT, which was abolished in $IK1^{-/-}/SK3^{T/T}$ +doxycycline (P <0.01) (Figure 4E). With intact NO synthase/cyclooxygenase, acetylcholine induced a comparable dilation in WT (Figure 4E, left panel), which

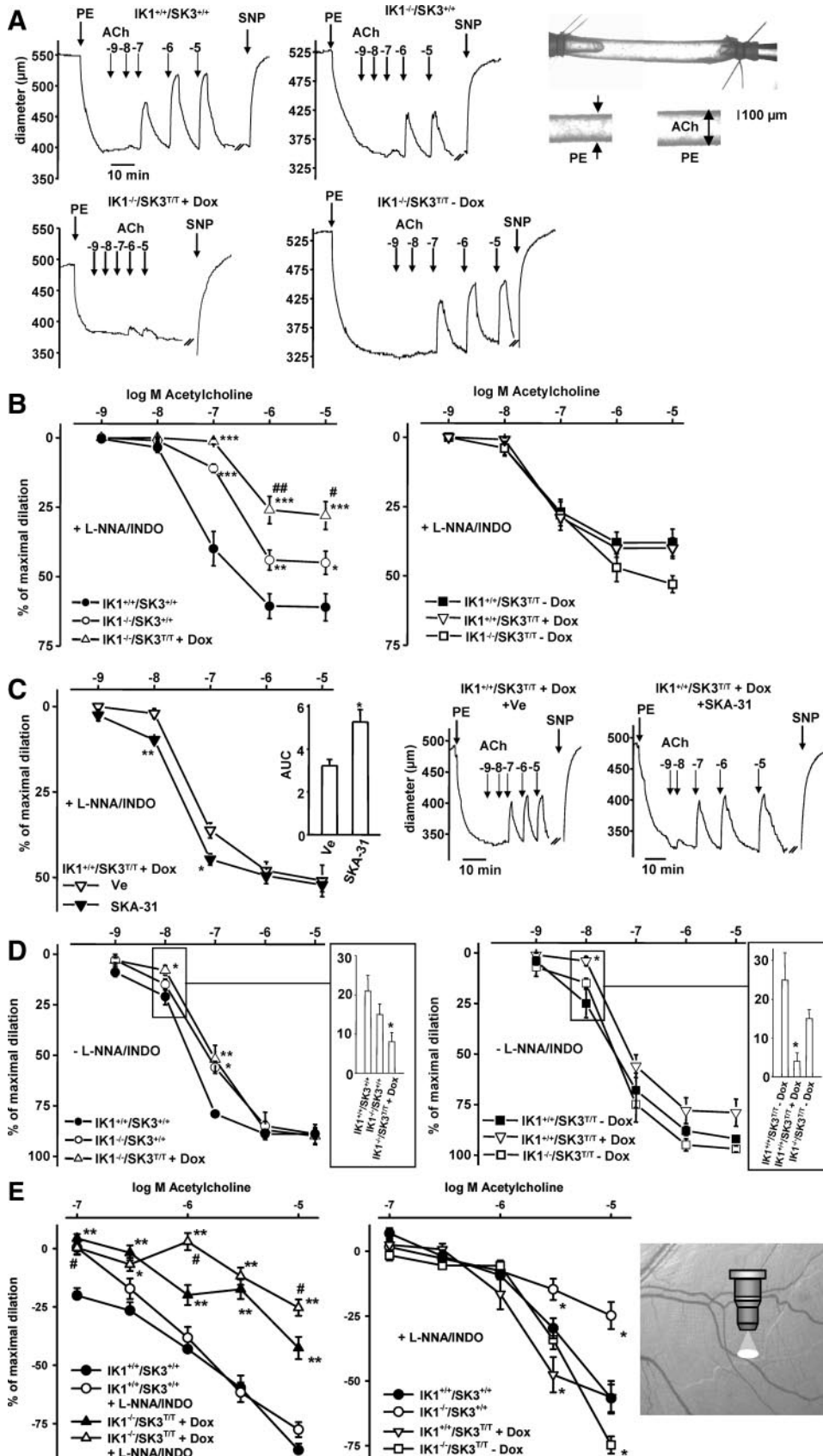


Figure 4. Impact of IK1/SK3 deficiency on acetylcholine-induced vasodilation in CA and in arterioles. A, Original tracings show increases in diameter (EDHF-type vasodilation) in response to increasing concentrations of acetylcholine (ACh) (numbers in graphs, -9 to -5=logM [acetylcholine]) and to 10 µmol/L sodium nitroprusside (SNP). CA were precontracted with phenylephrine (PE) (1 µmol/L) and pressurized to 80 mm Hg (illustrated in top panel on right). B, Concentration-response curves of acetylcholine-induced

was also reduced by $\approx 50\%$ in $IK1^{-/-}/SK3^{T/T} + \text{doxycycline}$. At low concentrations of acetylcholine (100 nmol/L) only, dilations were mainly NO mediated in WT, which was virtually absent in $IK1^{-/-}/SK3^{T/T} + \text{doxycycline}$, indicating a contribution of endothelial K_{Ca} channels to NO formation.

In a second set of experiments, we tested the contribution of the individual K_{Ca} channels to EDHF-mediated vasodilation (Figure 4E, right panel). The deficit of IK1 in $IK1^{-/-}$ strongly reduced EDHF dilator responses to acetylcholine ($P < 0.05$), whereas the deficit of SK3 in $IK1^{+/+}/SK3^{T/T} + \text{doxycycline}$ did not reduce the dilation. The response at 3 $\mu\text{mol/L}$ acetylcholine was apparently larger than in WT. Overexpression of SK3 in IK1-deficient mice ($IK1^{-/-}/SK3^{T/T} - \text{doxycycline}$) increased dilations to WT levels or enhanced them even further (at 10 $\mu\text{mol/L}$, $P < 0.05$). Acetylcholine dilations obtained during intact NO synthase and cyclooxygenase were comparable to pure EDHF-type responses in these genotypes (Figure IV in the online-only Data Supplement), again demonstrating the insignificant role of NO and prostaglandins in the absence of either endothelial K_{Ca} channel. Endothelium-independent vasodilations in response to sodium nitroprusside (10 $\mu\text{mol/L}$) and dilations to adenosine (1 and 10 $\mu\text{mol/L}$) were similar in all groups (data not shown). These findings in a conduit artery and in the cremaster microcirculation suggest that IK1 channels are of major importance for EDHF-mediated dilations to acetylcholine and that SK3 only contributes to the response if IK1 channels are absent.

Loss of Endothelial SK3 but Not of IK1 Channels Impairs Wall Shear Stress–Induced Dilator Responses

Endothelial ion channels have been suggested to contribute to wall shear stress–induced arterial vasodilation and thus to mechanisms of endothelial mechanotransduction of altered hemodynamics.²⁸ However, the specific contribution of either SK3 or IK1 channels is unknown. To elicit wall shear stress–mediated dilation in different groups, we increased the viscosity of the perfusion medium by adding 5% dextran. In CA of WT, the increase in viscosity elicited a small but appreciable EDHF-type dilator response of $\approx 14\%$ (Figure 5A, presence of L-NNA/indomethacin). In $SK3^{T/T} + \text{doxycycline}$, this wall shear stress–induced dilation was reduced to $\approx 2\%$ ($P < 0.01$) and was attenuated to a similar extent in $IK1^{-/-}/SK3^{T/T} + \text{doxycycline}$ ($\approx 5\%$; $P < 0.05$). In contrast, CA of $IK1^{-/-}$ displayed dilations similar to that observed in WT CA. Overexpression of SK3 in

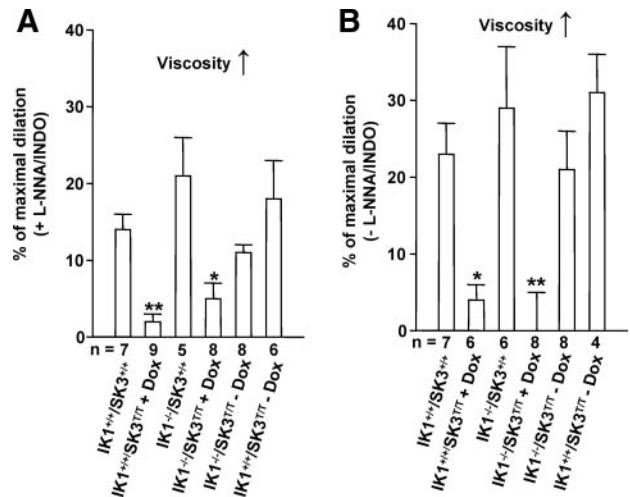


Figure 5. Impact of IK1 and/or SK3 deficiency on wall shear stress–induced vasodilation of CA in the presence (A) and absence (B) of L-NNA/indomethacin (INDO). Numbers (n) in charts refer to number of CAs. Note that inhibition of NO and prostacyclin synthesis strongly reduced dilation. For wall shear stress–induced vasodilation, the viscosity of the perfusion medium was increased from 0.7 to 2.9 mPa by adding 5% dextran, which enhanced wall shear stress in CA from ≈ 3 to 7 dyne/cm². Dox indicates doxycycline. Values are given as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs WT, 1-way ANOVA followed by Newman-Keuls post hoc test.

$IK1^X/SK3^{T/T} - \text{doxycycline}$ did not affect this dilation. Similarly, wall shear stress–induced dilation elicited by increasing flow from 30 to 600 $\mu\text{L}/\text{min}$ was impaired in $IK1^X/SK3^{T/T} + \text{doxycycline}$, whereas it remained intact in $IK1^{-/-}$ and $IK1^X/SK3^{T/T} - \text{doxycycline}$ (Figure IIA in the online-only Data Supplement). In the absence of inhibitors of NO/prostacyclin synthesis, WT CA responded to wall shear stress stimulation by high viscosity (Figure 5B) as well as by an increase of flow (Figure IIB in the online-only Data Supplement) with more pronounced dilations of $\approx 23\%$ and $\approx 25\%$, respectively, due to the contribution of NO.²⁸ Again, this response was severely impaired in $IK1^X/SK3^{T/T} + \text{doxycycline}$, whereas it remained intact in $IK1^{-/-}$ and $IK1^X/SK3^{T/T} - \text{doxycycline}$. In addition, the IK1-opener SKA-31 had no effect on wall shear stress–induced dilation, elicited by either high viscosity or flow, in $SK3^{T/T} + \text{doxycycline}$ (Figure IIC in the online-only Data Supplement). These findings suggest that SK3 channels are particularly crucial

Figure 4 (Continued). EDHF-type vasodilation in the presence of L-NNA/indomethacin (INDO) (300 $\mu\text{mol/L}$, 10 $\mu\text{mol/L}$). Left panel shows WT ($IK1^{+/+}/SK3^{+/+}$), $IK1^{-/-}/SK3^{+/+}$, $IK1^{-/-}/SK3^{T/T} + \text{doxycycline}$ ($n = 7$ to 8), and right panel shows $IK1^{+/+}/SK3^{T/T} + \text{doxycycline}$ ($n = 9$), $IK1^{+/+}/SK3^{T/T} - \text{doxycycline}$ ($n = 6$), and $IK1^{-/-}/SK3^{T/T} - \text{doxycycline}$ ($n = 5$). C, left, Potentiation of acetylcholine-induced EDHF-type vasodilation in $IK1^{+/+}/SK3^{T/T} + \text{doxycycline}$ by the IK1-opener SKA-31 (500 nmol/L; Ve indicates vehicle [dimethyl sulfoxide]; $n = 4$ each). Insert shows prolongation of responses (area under curve [AUC]) to 90-second exposure to acetylcholine in the presence of SKA-31. Right panel displays representative tracings. Note that time span of the experiment is considerably longer because of the enhanced duration of dilations in the presence of SKA-31. D, Concentration–response curves of acetylcholine-induced vasodilation in CA in the presence of NO (absence of blockers) in $IK1^{+/+}/SK3^{+/+}$, $IK1^{-/-}/SK3^{+/+}$, and $IK1^{-/-}/SK3^{T/T} + \text{doxycycline}$ ($n = 5$ per group, left) and in $IK1^{+/+}/SK3^{T/T} - \text{doxycycline}$, $IK1^{+/+}/SK3^{T/T} + \text{doxycycline}$, and $IK1^{-/-}/SK3^{T/T} - \text{doxycycline}$ ($n = 5$ each group, right). Inserts visualize differences at 10 nmol/L acetylcholine. Values are given as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs WT; # $P < 0.05$, ## $P < 0.01$ vs $IK1^{-/-}/SK3^{+/+}$ in B, 1-way ANOVA followed by Newman-Keuls post hoc test. E, Impact of combined IK1/SK3 deficiency on acetylcholine-induced vasodilation in resistance-sized arterioles in the cremaster microcirculation (illustrated in scheme on right). Left, Concentration–response curves in WT ($IK1^{+/+}/SK3^{+/+}$) and $IK1^{-/-}/SK3^{T/T} + \text{doxycycline}$ mice in the presence of L-NNA/indomethacin (30 $\mu\text{mol/L}$, 3 $\mu\text{mol/L}$) (EDHF-type dilation) and in the absence of blockers. Right, Impact of IK1 or SK3 deficiency as well as SK3 overexpression on acetylcholine-induced EDHF-type vasodilation. Concentration–response curves in WT ($IK1^{+/+}/SK3^{+/+}$), $IK1^{+/+}/SK3^{T/T} + \text{doxycycline}$, $IK1^{-/-}/SK3^{+/+}$, and $IK1^{-/-}/SK3^{T/T} - \text{doxycycline}$ ($n = 26$ to 59 arterioles in 4 to 6 animals for each genotype). Values are given as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ vs WT, 1-way ANOVA followed by Bonferroni post hoc test; # $P < 0.05$ vs same genotype without blockers, paired *t* test.

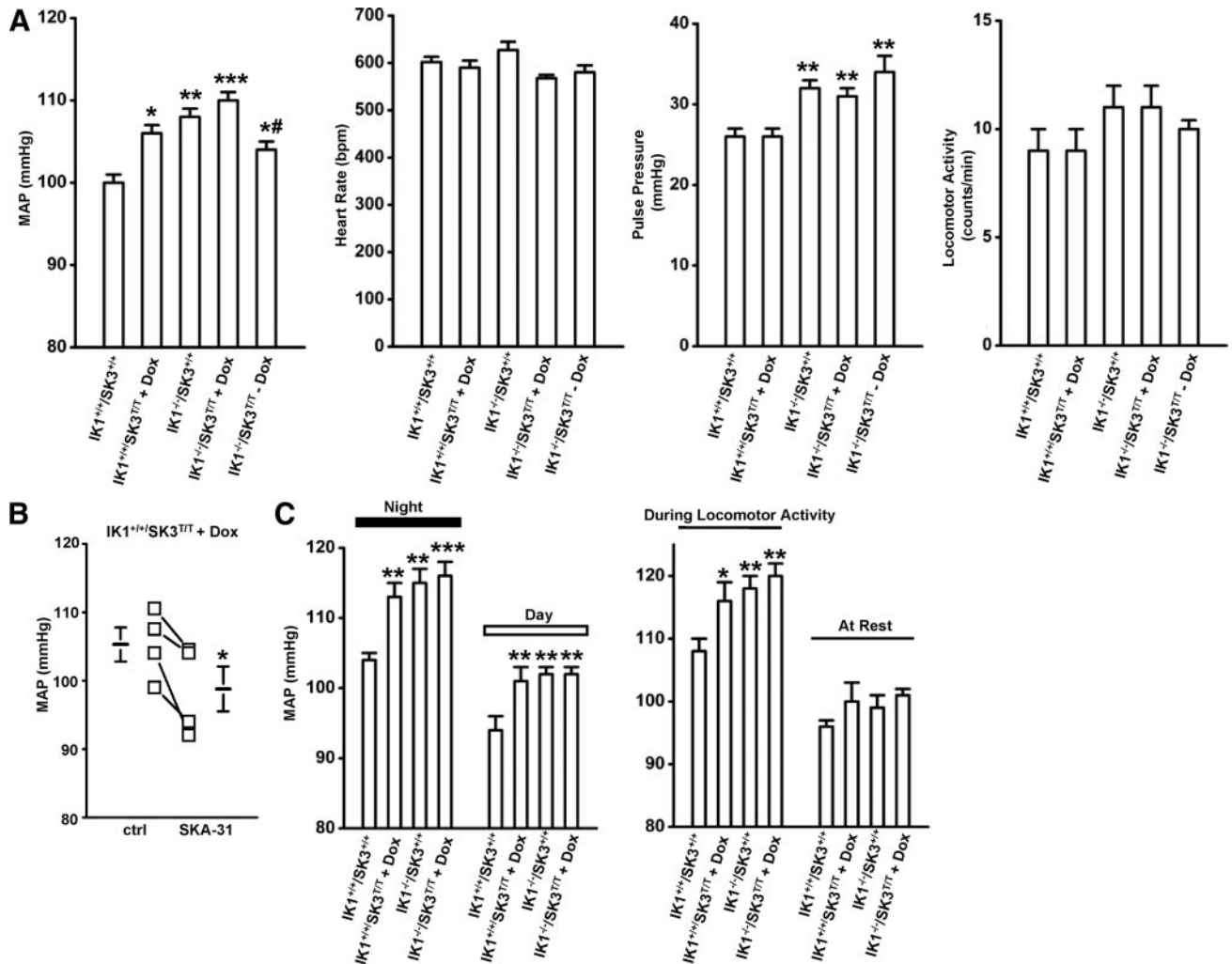


Figure 6. Arterial blood pressure in IK1/SK3-deficient mice. **A**, Telemetric measurements (over 72 hours) of mean arterial blood pressure (MAP), heart rate, pulse pressure, and locomotor activity in WT (IK1^{+/+}/SK3^{+/+}; n=8; 4 males/4 females), IK1^{+/+}/SK3^{T/T}+doxycycline (Dox) (n=6; 4 males/2 females), IK1^{-/-}/SK3^{+/+} (n=6; 2 males/4 females), IK1^{-/-}/SK3^{T/T}+doxycycline (n=9; 5 males/4 females), and IK1^{-/-}/SK3^{T/T}-doxycycline (n=5; 3 males/2 females). **B**, Blood pressure-lowering effect of a single injection of the IK1-opener SKA-31 (30 mg/kg IP in IK1^{+/+}/SK3^{T/T}+doxycycline mice; n=4). Ctrl indicates 24-hour mean arterial pressure before application. **C**, Circadian variation of mean arterial pressure and impact of physical activity on mean arterial pressure in the different genotypes. Mean arterial pressure at night and daytime (on left) and mean arterial pressure during locomotor activity (>1 count per minute) and at rest (<1 count per minute) (on right) are shown. Values are given as mean±SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 vs WT in **A** and **C**; #*P*<0.05 vs IK1^{-/-}/SK3^{T/T}+doxycycline (**A**), 1-way ANOVA followed by Newman-Keuls post hoc test, paired Student *t* test in **B**.

for EDHF/NO-mediated dilations of CA to wall shear stress stimulation.

Combined IK1 and SK3 Suppression Increases Arterial Blood Pressure

We next assessed whether the loss of both endothelial K_{Ca} channels and thus of EDHF signaling mediated by IK1 and SK3 alters systemic blood pressure. For this purpose, we conducted continuous telemetric blood pressure measurements for at least 3 days in mice of both genders after doxycycline treatment (>6 days) (Figure 6A). WT had a mean arterial pressure of 100±1 mm Hg, which was unaffected by doxycycline administration (99±2; n=5). SK3^{T/T}-doxycycline (n=5) had a comparable mean arterial pressure of 102±1 mm Hg, whereas SK3^{T/T}+doxycycline had an elevated mean arterial pressure of 106±1 mm Hg (*P*<0.01 versus WT; *P*<0.05 versus SK3^{T/T}-doxycycline). Likewise,

IK1^{-/-} had a higher mean arterial pressure of 108±1 mm Hg (*P*<0.01 versus WT), similar to a previous report.²⁰ Doxycycline administration did not alter mean arterial pressure in IK1^{-/-} (107±5; n=3). However, IK1^{-/-}/SK3^{T/T}+doxycycline exhibited an even higher mean arterial pressure of 110±1 mm Hg (*P*<0.001 versus WT; *P*<0.05 versus SK3^{T/T}+doxycycline, but not significantly different from IK1^{-/-} [*P*=0.15]). Doxycycline removal (>2 weeks) lowered mean arterial pressure to 105±1 mm Hg in these animals (n=5; *P*<0.05 versus IK1^{-/-}/SK3^{T/T}+doxycycline), but mean arterial pressure was still higher than in the WT (*P*<0.05). In addition, in IK1^{-/-}/SK3^{T/T}, which never received doxycycline, mean arterial pressure was lower (104±1 mm Hg) than in the doxycycline-administered group (*P*<0.05) but also higher than in the WT (*P*<0.05; Figure 6A). This indicated that overexpression of SK3 in IK1^{-/-}/SK3^{T/T}-doxycycline reduced mean arterial pressure but was

not able to return it to normotensive levels. In contrast, pharmacological potentiation of IK1 in IK1^{+/+}/SK3^{T/T}+doxycycline (n=4) by a single injection of the IK1-opener SKA-31 (30 mg/kg IP) lowered mean arterial pressure to normotensive levels over 24 hours (Figure 6B).

Heart rate was similar in all genotypes (Figure 6A, second panel). Pulse pressure (Figure 6A, third panel) was comparable in SK3^{T/T}+doxycycline and WT but elevated in IK1^{-/-}, IK1^{-/-}/SK3^{T/T}+doxycycline, and IK1^{-/-}/SK3^{T/T}-doxycycline. Locomotor activity (Figure 6A, fourth panel) was similar in SK3^{T/T}+doxycycline and WT and tended to be higher in IK1^{-/-} and IK1^{-/-}/SK3^{T/T}+doxycycline ($P=0.08$ and $P=0.09$ versus WT, respectively). SKA-31 injections in IK1^{+/+}/SK3^{T/T}+doxycycline did not significantly alter heart rate, pulse pressure, and locomotor activity (Figure III in the online-only Data Supplement), although a trend toward lower values was observed.

Further analysis of circadian variation of blood pressure revealed that the enhancement of mean arterial pressure in the SK3^{T/T}+doxycycline, IK1^{-/-}, and IK1^{-/-}/SK3^{T/T}+doxycycline was mainly caused by a higher mean arterial pressure in the dark period (Figure 6C, left) and during locomotor activity (Figure 6C, right).

Discussion

Endothelial IK1 and SK3 channels have been hypothesized to contribute to EDHF signaling mainly on the basis of pharmacological evidence. However, the contribution of the EDHF dilator system to arterial tone *in vivo* and to systemic blood pressure control has not been elucidated previously. Therefore, we generated IK1^{-/-}/SK3^{T/T} mice in which activation of a tetracycline-based genetic switch²¹ by doxycycline treatment effectively abolished SK3 expression, resulting in deficiency of both endothelial K_{Ca} channels. This approach enabled us to study the effect of deficiency of both channels on endothelium-dependent dilation and blood pressure control *in vivo* as well as the potential rescuing effects of SK3 overexpression and of pharmacological potentiation of IK1 activity in mice lacking the respective other channel. We demonstrate that K_{Ca} currents are mediated by SK3 and IK1 (Figure 2). However, despite their equal ability to generate endothelial K_{Ca} currents, the 2 channels make distinct contributions to acetylcholine-induced EDHF-mediated smooth muscle hyperpolarization. The activation of IK1 seems to be more important because its absence impaired the hyperpolarization of smooth muscle more severely than the loss of SK3 alone (Figure 3). However, SK3 channels also contribute to smooth muscle hyperpolarization as demonstrated by the fact that lack of SK3 alone slightly reduces hyperpolarization and further impairs the reduced hyperpolarization response in IK1^{-/-}. In keeping with this finding, strong overexpression of SK3 in IK1^{-/-}/SK3^{T/T}-doxycycline (enhancing the endothelial K_{Ca} currents >10-fold) restored the acetylcholine-induced smooth muscle hyperpolarization to near-normal values despite the lack of IK1. Conversely, potentiation of IK1 activity by the IK1-opener SKA-31 strongly enhanced hyperpolarization in IK1-expressing and SK3-deficient mice to above the level seen in WT, thus further demonstrating a key role

of IK1 in mediating acetylcholine-induced smooth muscle hyperpolarization.

The different roles of IK1 and SK3 became further apparent in the EDHF dilator responses in the different genotypes (Figure 4A). The loss of IK1 alone resulted in a pronounced effect at intermediate acetylcholine concentrations. However, the additional deletion of SK3 in IK1^{-/-}/SK3^{T/T}+doxycycline further impaired dilations, highlighting the importance of both channels for generating EDHF-type dilations. In contrast to IK1 deficiency, the suppression of SK3 in IK1-expressing mice was without appreciable effects. However, the impaired dilation in IK1 deficiency could be augmented by overexpression of SK3 but hardly reached WT levels. Moreover, overexpression of SK3 in IK1 WT did not produce a larger EDHF response, which suggests that the importance of SK3 is only demonstrated in the absence of IK1. In contrast, potentiation of IK1 by SKA-31 clearly enhanced the EDHF response in SK3-deficient mice (shown here), as well as in WT, although not in IK1^{-/-}, as shown recently by us.²⁵ Importantly, the present experiments in the microcirculation clearly validate the requirement of both IK1 and SK3 for the EDHF dilator response in resistance-sized arterioles *in vivo*. Only at high concentrations of acetylcholine was a remaining small dilation observed, which was comparable to responses in conduit arteries. Moreover, the findings in mice lacking either IK1 or SK3 or overexpressing SK3 during IK1 deficiency are in agreement with the findings in the conduit artery and further underscore a major role of IK1 in the EDHF response to acetylcholine, whereas SK3 contributes only in the absence of IK1.

Thus, these findings support the notion that IK1 in particular is of pivotal importance for the EDHF response elicited by stimulation of G-protein-coupled receptors (by acetylcholine) and subsequent Ca²⁺ release from the endoplasmic reticulum. The distinct roles may be related to the recently suggested spatial separation of the 2 channels within the endothelium: SK3 at endothelial cell junctions²⁹ (and perhaps in caveolae³⁰) and IK1 at endothelial projections through the holes in the elastic lamina (also the sites of myoendothelial gap junctions²⁹) and in close proximity to the endoplasmic reticulum.³¹ Thus, acetylcholine-triggered Ca²⁺ release events (recently termed Ca²⁺ pulsars³¹) could preferentially activate colocalized IK1 channels.

Depending on the type of vessel, the contribution of NO and prostaglandins in endothelium-dependent dilations varies substantially.^{4,18,32} In the conduit vessels used here, NO contributes considerably to acetylcholine-induced dilation.²⁰ At maximal acetylcholine stimulation and with unimpeded NO synthesis, the dilation of the CA remains intact despite the lack of K_{Ca} channels (Figure 4D). However, at lower acetylcholine concentrations, dilations were reduced in SK3- and IK1/SK3-deficient mice despite intact NO synthase, suggesting that K_{Ca} channels, in particular SK3, are required for NO synthesis. In contrast to CA, the lack of K_{Ca} channels substantially reduces dilations in the murine microcirculation with a functioning NO system (Figure 4E), highlighting the special importance of the EDHF-dependent dilation in arterioles, the minor role of NO-dependent mechanisms,³² and the inability of NO to compensate for the lack of K_{Ca} channels. This

suggests that, at least in the macrocirculation, an endothelial hyperpolarization contributes to the activation of NO synthase possibly by providing the driving force for Ca^{2+} influx, as suggested previously.³³ Interestingly, SK3 channels were found to be more important in this phenomenon because both SK3- and IK1/SK3-deficient mice exhibited reduced vasodilations to low concentrations of acetylcholine, whereas IK1-deficient mice did not show impairment.

The differential roles of the 2 endothelial K_{Ca} channels are further highlighted by the finding that SK3 deficiency but not IK1 deficiency impaired dilations of CA in response to shear stress stimulation by increasing either viscosity or flow. The attenuation in animals lacking SK3 was evident in the presence as well as in the absence of NO, suggesting that SK3 is required not only for the EDHF-mediated but also for the larger NO-mediated flow-induced dilation. Overexpression of SK3 in either IK1-lacking or IK1-expressing mice failed to further increase shear stress-induced dilation, suggesting either that basal SK3 expression levels are sufficient to produce hyperpolarization or that the ensuing vasodilation simply abrogates the mechanical stimulus.³⁴ Potentiation of IK1 functions by SKA-31 in SK3-deficient mice was ineffective to restore the response, which further supports the notion that IK1 is not activated by shear stress-induced $[\text{Ca}^{2+}]_i$ increases. Thus, SK3, unlike IK1, seems to be crucial for adequate endothelial mechanotransduction in response to shear stress stimulation and may therefore be important for tonic EDHF activity and endothelial NO formation under continuous blood flow in vivo.

Taken together, our data suggest that activation of IK1 and SK3 in a stimulus-dependent fashion constitutes the major electric trigger for EDHF dilator responses. The importance of the SK3/IK1-initiated EDHF system for blood pressure control is supported by our finding of a significant increase in blood pressure in mice lacking 1 or both channels. Intriguingly, the defect in the SK3/IK1 EDHF dilator system increased blood pressure mainly during locomotor activity, suggesting that the EDHF system is especially required to ensure adequate vasoregulation and thereby blood pressure control during physical activity. Interestingly, pulse pressure was elevated in mice lacking IK1 but not in SK3-lacking animals (for additional discussion, see the online-only Data Supplement). Because the combined loss of SK3 and IK1 had few additive effects on blood pressure, the 2 channels exert distinct but essential roles in blood pressure control. This hypothesis is supported by the inability of SK3 overexpression to completely compensate for the loss of IK1 and to restore normal blood pressure. This may be related to a more significant role of IK1 in EDHF-type dilation, which is underscored by the ability of the IK1-opener SKA-31 to return the elevated blood pressure in SK3-deficient and IK1-expressing mice back to normotensive levels. Additionally, SKA-31 lowered blood pressure in WT mice but not the elevated blood pressure in IK1-deficient mice,²⁵ which emphasizes its 10-fold higher potency for IK1 over SK3. Alternatively, both channels may use distinct downstream EDHF signaling pathways, ie, electrotonic coupling mechanisms (SK3) and/or K^+ release (IK1), as suggested recently.^{3,5,12,17,27,29} Moreover, SK3 may add to blood pressure control by exerting a tonic

influence on vascular tone,²² possibly by its role in NO dilator responses to low levels of vasoactive factors and flow alterations.

In summary, the present study demonstrates that EDHF-mediated dilations are virtually absent in SK3/IK1-deficient mice and that these mice exhibit enhanced blood pressure. The analysis of these animals further provides substantial evidence that SK3 and IK1 play distinct roles in vessel physiology despite their overlapping function as endothelial K_{Ca} channels. We suggest that SK3/IK1-deficient mice represent an “EDHF knockout,” which may provide a useful model to study the physiological actions of putative EDHFs^{4,5,11} in the circulation.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The arterial endothelium modulates vascular tone and blood pressure by releasing nitric oxide and prostacyclin, as well as by a third factor or signalling pathway termed “endothelium-derived hyperpolarizing factor” (EDHF). Herein, we show that EDHF-type signalling and dilations strongly depend on the presence of endothelial K_{Ca} -channels (SK3/IK1) and that their lack has a significant impact on vascular dilation and blood pressure control in vivo. Thus, SK3/IK1-deficient mice may help to shed light on the role of EDHFs in cardiovascular pathologies such as atherosclerosis, hypertension, and stroke. Strikingly, the EDHF-potentiating effects of the IK1-opener SKA-31 and the partial rescue of EDHF deficiency by overexpression of SK3 returned blood pressure to almost normotensive levels, suggesting that selective pharmacological activators of endothelial SK3 and IK1 may be of therapeutic value in the treatment of hypertension. In support of this hypothesis, we recently demonstrated that SKA-31 significantly lowers blood pressure in angiotensin II hypertensive mice. Pharmacological activation of endothelial K_{Ca} channel activity might be particularly desirable in situations where the EDHF response is impaired because of a decreased activity of endothelial K_{Ca} channels, as has been reported after cardiopulmonary bypass, balloon catheter angioplasty, and chronic renal failure.

Genetic Deficit of SK3 and IK1 Channels Disrupts the Endothelium-Derived Hyperpolarizing Factor Vasodilator Pathway and Causes Hypertension

Sebastian Brähler, Anuradha Kaistha, Volker J. Schmidt, Stephanie E. Wölfle, Christoph Busch, Brajesh P. Kaistha, Michael Kacik, Anna-Lena Hasenau, Ivica Grgic, Han Si, Chris T. Bond, John P. Adelman, Heike Wulff, Cor de Wit, Joachim Hoyer and Ralf Köhler

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Supplemental Material

Material and Methods

Transgenic animals: SK3^{T/T} and IK1^{-/-} mice^{1,2} were interbred in a specific-pathogen-free environment of our local animal core facility. Mice received standard mice chow and water ad libitum. Heterozygous IK1^{+/-}/SK3^{T/+} breeding pairs produced offspring at the predicted Mendelian frequencies. All types of double heterozygous transgenic and double transgenic mice were of normal appearance, viable, fertile and showed no obvious neurological or behavioral defects. Genotyping was performed with genomic DNA using standard PCR and primers: Primers were: FP: 5'-ATGGACACTTCTGGGCACTT-3'; RP 5'-AGAGTGCAACAGACCAGGAT-3' for SK3^{+/+} and SK3^{T/T} alleles. Forward and reverse primers for IK1^{+/+} and IK1^{-/-} alleles are stated elsewhere². For suppression of SK3 expression, mice received Dox in the drinking water (2 mg/ml in 2% sucrose) for at least 6 days before experiment. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and animal protocols were approved by the local authorities (Ref.-No.V54-19c20-15MR20/11).

Western Blotting: Mouse brains (for SK3) and livers (for IK1) from each genotype were homogenized in buffer (50 mM Tris, 1 mM EDTA, 2 mM DTT, 0.2 μM benzamidine, 50 mM leupeptin, 0.5 mM PMSF, 0.1 mg/ml trypsin inhibitor pH 8.0). Debris and nuclear material were removed by centrifugation at 1000×g for 2 min. Membrane proteins were collected by centrifugation at 20,000×g for 45 min, resuspended in homogenization buffer and quantified using the Bio-Rad protein assay. 30 μg of membrane proteins were electrophoresed on 8-12 % sodium dodecylsulfate–polyacrylamide gels, and electrophoretically transferred to nitrocellulose membranes (Trans-Blot, all from Bio-Rad, Munich, Germany). Membranes were then blocked in 5% non fat dried milk in TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for one hour at room temperature. An anti-SK3 antibody was purchased from Alomone Labs (Jerusalem, Israel). For detection

of IK1, we used a rabbit antibody directed against the 28 amino acids of the n-terminus (MGGDLVLGLGALRRRKRLLEQEKSLAGW, position 1-28) of the channel (Pineda antibody services, Berlin). Affinity purification of the monospecific IgG fraction was made with the peptide covalent coupled to Sepharose 6B via the cysteine residue of the peptide. The elution of the specific antibodies was carried out with 0.2 M glycine buffer pH 3.0. Primary antibodies diluted in TBST (anti-IK1, 1:200; anti-SK3, 1:200) were applied overnight at 4°C. After rinsing with TBST, the membranes were incubated with a peroxidase-conjugated goat anti-rabbit antiserum (1:10,000 GE Healthcare Europe, Freiburg, Germany) for one hour at room temperature. Immunolabelled proteins were visualized by ECL advance Western blot detection system (GE Healthcare). Equal loading of proteins was validated by stripping and re-probing the Western blots with an anti- α -tubulin antibody (H-300, 1:200; Santa Cruz).

Immunohistochemistry: For detection of IK1 and SK3 proteins, CA were fixed by transcardiac perfusion with 10% neutral-buffered formalin and embedded in paraffin. After pre-incubation with 5% BSA, the cross-sections (7 μ m) were blocked with 30% avidin and biotin (Vector Laboratories; Wertheim-Bettingen, Germany) in 1% BSA for 30 min. Sections were then immunostained using polyclonal antibodies against IK1 (H-120, 1:50 dilution) and SK3 (H-45, dilution 1:50; both from Santa Cruz Biotechnology, Heidelberg, Germany). Detection was performed by using the Vectastain Elite ABC Kit (Vector Laboratories) and the Vector SG Substrate Kit for immunoperoxidase reaction according to the manufacturer's instruction. Labelled sections of CA were photographed at 400x using an AxioCam MRm camera mounted on an AxioObserver.Z1 microscope in ApoTome mode (Zeiss, Jena, Germany).

Electrophysiology: Membrane currents in freshly isolated carotid artery endothelial cells² (CAEC) were determined with an EPC-9 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany) using voltage ramps (1000 ms; -100 to +40 mV). For activation of IK1/SK3-currents, CAEC were dialyzed with a KCl-pipette solution containing 3 μ M $[\text{Ca}^{2+}]_{\text{free}}$ (in mM): 140 KCl, 1 Na₂ATP, 1 MgCl₂, 2 EGTA, 1.91 CaCl₂,

and 5 HEPES, pH 7.2. The NaCl bath solution contained (mM): 137 NaCl, 4.5 Na₂HPO₄, 3 KCl, 1.5 KH₂PO₄, 0.4 MgCl₂, 0.7 CaCl₂, and 10 glucose (pH 7.4). Smooth muscle membrane potentials in CA (precontracted with 1 μM phenylephrine (PE) and pressurized to 80 mmHg) were recorded in the presence of Nω-nitro-L-arginine (L-NNA, 300 μM), indomethacin (INDO, 10 μM) by standard sharp electrode technique using a BA-1S amplifier (npi electronic, Tamm, Germany). Sharp electrodes filled with 0.5 M 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. In experiments using the IK1-opener SKA-31 (naphtho[1,2-d]thiazol-2-ylamine)³, SKA-31 (500 nM) was added to the bath solution (patch-clamp experiments) and to the perfusion buffer (membrane potential measurements and myography) and was present throughout experiments. For control experiments, solutions contained the vehicle (DMSO, 1%).

Vessel studies: Pressure myography in CA was performed as described previously². Bath and perfusion solution contained (in mM): 145 NaCl, 1.2 NaH₂PO₄, 4.7 KCl, 1.2 MgSO₄, 2 CaCl₂, 5 glucose, 2 pyruvate, 3 MOPS buffer. (pH 7.4 at 37°C). CA were pressurized to 80 mmHg and continuously perfused at a flow rate of 0.6 ml/min. CA were pre-constricted with 1 μM PE and perfused with acetylcholine (ACh; 1 nM to 10 μM) in the presence and absence of L-NNA (300 μM) and INDO (10 μM). Diameter changes of CA were expressed as a percentage of the maximal dilation to 10 μM sodium nitroprusside (SNP). To measure wall shear stress(WSS)-mediated vasodilation, the viscosity of the perfusion medium was increased from 0.7 to 2.9 mPa*s by adding 5% dextran which enhanced wall shear stress in CA from ~3 to 7 dyne/cm² (without increasing flow rate). As alternative methods to enhance WSS, flow was enhanced from almost static conditions (~30 μl/min) to physiologically relevant levels (600 μl/min) by increasing the pressure gradient between inflow and outflow capillaries to 20 mmHg within ~5 secs. The shear stress increased from ~0.1 to 3 dyne/cm². The mean intraluminal pressure remains constant at 80 mmHg under these conditions. Shear stress was mathematically estimated according to the Hagen-Poiseuille law: $\tau=4\eta Q/\pi r^3$; τ =shear stress; η =viscosity; Q =flow, and r =radius.

Intravital microscopy: Arteriolar diameters were measured using intravital microscopy in the cremaster microcirculation before and after superfusion of ACh, adenosine, and SNP applied at increasing concentrations as described previously⁴. In brief, animals were anesthetized by intraperitoneal injection of medetomidin (0.5 mg/kg), fentanyl (0.05 mg/kg) and midazolam (5 mg/kg), followed by continuous infusion via a catheter placed in the jugular vein. Mice were intubated by tracheotomy to ensure airway patency and their esophageal temperature was maintained at 37°C by conductive heat. Cremaster muscle was prepared as described⁴ and superfused with bicarbonate-buffered (pH 7.4, 34° C) saline solution (in mM: Na⁺ 138, K⁺ 5, Ca²⁺ 2.5, Mg²⁺ 1.2, Cl⁻ 127, HCO₃⁻ 20, SO₄²⁻ 1.2, H₂PO₄²⁻ gassed with 5% CO₂ and 95% N₂ (pCO₂ ≈ 40 mm Hg, pO₂ ≈ 30 mm Hg). Animals were transferred to a microscope stage (Axioskop FS, Zeiss, Göttingen, Germany) equipped with a charge-coupled video camera. Microscopic images were displayed on a monitor at 700-fold magnification and recorded on videotape for later measurement of luminal diameter after digitization. The resolution after digitization was about 1 μm. Responses were restudied in the presence of L-NNA (30 μM) and INDO (3 μM). Dilator responses are given as the percentage of maximal dilation⁴.

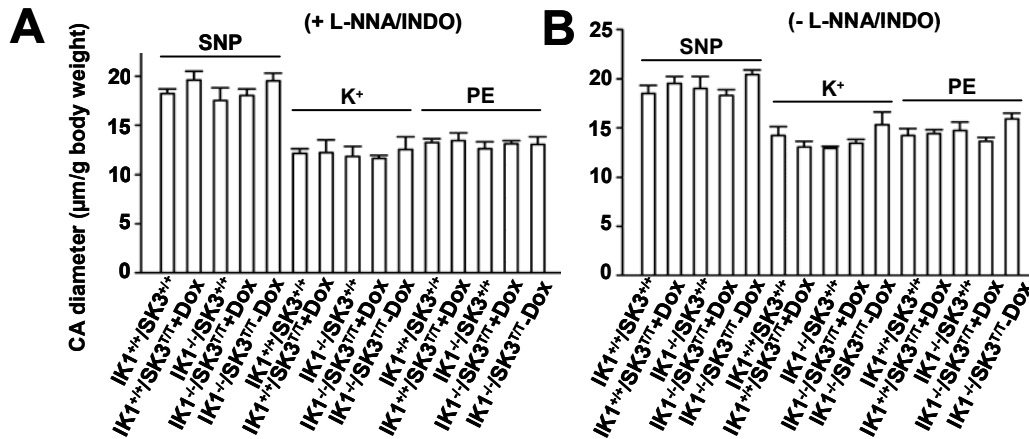
Telemetry: Mice were allowed to recover for 9 days after implantation of a TA11PA-C10 pressure transducer into the left carotid artery (Data Sciences International (DSI), St Paul, Minnesota, USA). Thereafter, pressure values, heart rate, and locomotor activity were continuously recorded for at least 3 consecutive days in Dox-treated or untreated mice. The values of the 3-day recording were averaged and used for statistical analysis. Further analysis of the data (day/night distribution, activity/MAP relationship) was done using the DSI software and Origin®6.1. In experiments using SKA-31, SKA-31 was dissolved in peanut oil and 30mg/kg was injected i.p. (injection volume 100μl) at the end of light period. Telemetry data were recorded 24h before and 24h after injection. The respective 24h-recordings were averaged and used for statistical analysis.

Statistics: Data are given as mean ± SEM. Statistical calculations were done with Origin®6.1 and SigmaStat©2.03. If not stated otherwise, one-way ANOVA followed by

the Newman-Keuls-*post hoc* test was used to assess differences between groups. The paired Student's t-Test was used if observations were obtained in the same animal or vessel before and after a treatment (e.g. assessment of blood pressure changes after SKA-31 injections). *P*-values <0.05 were considered significant.

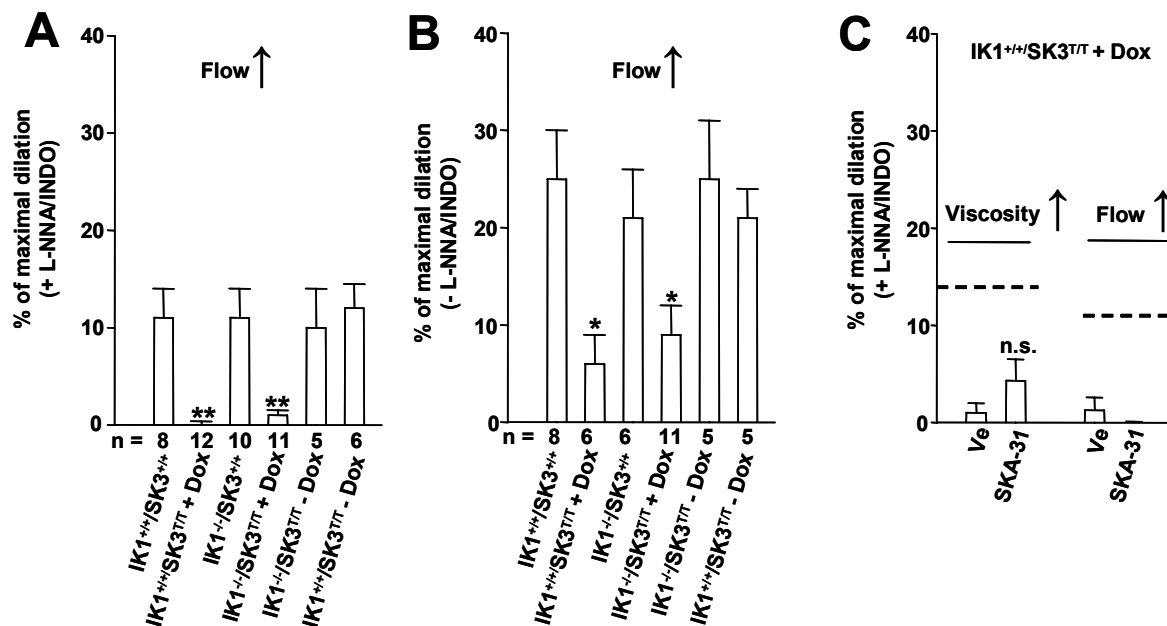
Supplemental Figures

Suppl. Fig. 1:



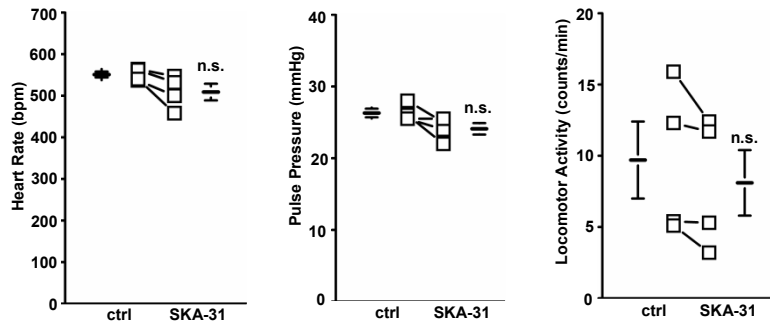
Suppl. Fig. 1: CA diameters (normalized to body weight) in response to SNP (10 µM), 60 mM K⁺ and to PE (1 µM) in the different genotypes and in the presence (A) or absence of blockers of NO-synthase and cyclooxygenase (B). Data are given as mean ± SEM.

Suppl. Fig. 2:



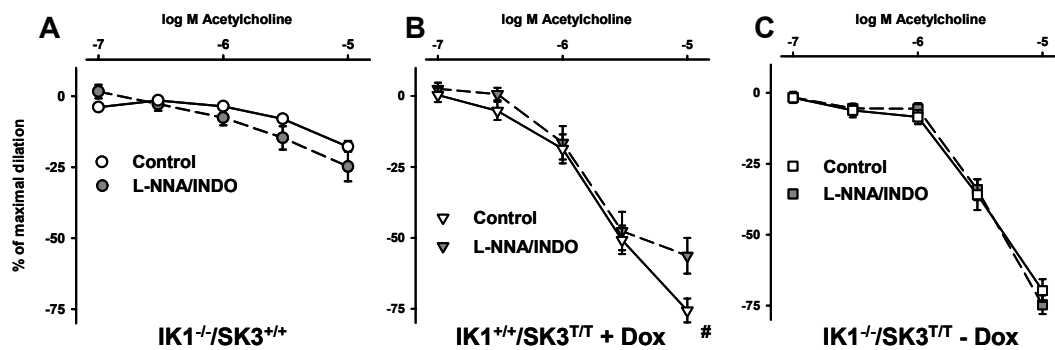
Suppl. Fig. 2: Vasodilation of CA in response to flow stimulation depends on SK3-expression. Flow-induced vasodilation in the presence (A) and absence (B) of L-NNA and INDO. Data are given as mean ± SEM; *P<0.05, **P<0.01, One-Way-ANOVA & Newman-Keuls-*post hoc* test. C) SKA-31 (500 nM) did not significantly alter shear stress-induced vasodilation in SK3-deficient mice (IK1^{+/+}/SK3^{+/+} + Dox, n=4). Dashed line indicates control responses in wt animals. For control values (high viscosity) see also Fig. 5A. Ve = vehicle (DMSO). Data are given as mean ± SEM; SKA-31 (carotid arteries, n=4) vs. Ve (carotid arteries, n=4): n.s., Student's t-test.

Suppl. Fig. 3:



Suppl. Fig. 3: Effect of a single injection of SKA-31 (30 mg/kg/ i.p.) on heart rate, pulse pressure, and locomotor activity over 24 hrs after injection. Ctrl = 24 hrs means ahead of treatment; n.s. = not significant, paired Student's t-test.

Suppl. Fig. 4:



Suppl. Fig. 4: ACh-induced dilation in the absence (white symbols) or presence of L-NNA/INDO (30 and 3 μ mol/L, grey symbols) in the cremaster microcirculation of different genotypes. Inhibition of NO-synthase or cyclooxygenase did not affect ACh-induced dilation. Only in doxycyclin-treated IK1^{+/+}/SK3^{T/T} animals a small attenuation at the highest ACh-concentration was observed (B). Data in the presence of L-NNA/INDO (dashed lines) are replotted herein from Fig. 4E (right panel) for comparison. # P < 0.05 vs. control.

Supplemental Discussion

Interestingly, pulse pressure was elevated in mice lacking IK1, but not in SK3-lacking animals. This could simply be due to enhanced mean arterial pressure as the compliance of the aorta decreases with increasing pressure. However, blood pressure was likewise elevated in animals lacking SK3 without a concomitant rise in pulse pressure, which argues against pressure dependency. In porcine coronary arteries pulsatile stretch releases EDHF and thereby modulates arterial compliance⁵. The current observation indicates a specific role of IK1 in modulating aortic compliance possibly by a related EDHF-type mechanism. Such a modulating effect may alter pulse-wave velocity within the arterial tree and wave reflection. Of note, such factors have recently been suggested to be independent risk factors for cardiovascular disease⁶. Yet, we cannot exclude the possibility that the enhanced arterial compliance is a consequence of the chronic pressure increase, which may have led also to structural changes of the arterial wall in these animals with life-long IK1-deficiency.

Supplemental References

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