Altered Expression of Small-Conductance Ca\(^{2+}\)-Activated K\(^{+}\) (SK3) Channels Modulates Arterial Tone and Blood Pressure


Abstract—The endothelium is a critical regulator of vascular tone, and dysfunction of the endothelium contributes to numerous cardiovascular pathologies. Recent studies suggest that apamin-sensitive, small-conductance, Ca\(^{2+}\)-activated K\(^{+}\) channels may play an important role in active endothelium-dependent vasodilations, and expression of these channels may be altered in disease states characterized by vascular dysfunction. Here, we used a transgenic mouse (SK3\(^{T/T}\)) in which SK3 expression levels can be manipulated with dietary doxycycline (DOX) to test the hypothesis that the level of expression of the SK subunit, SK3, in endothelial cells alters arterial function and blood pressure. SK3 protein was elevated in small mesenteric arteries from SK3\(^{T/T}\) mice compared with wild-type mice and was greatly suppressed by dietary DOX. SK3 was detected in the endothelium and not in the smooth muscle by immunohistochemistry. In whole-cell patch-clamp experiments, SK currents in endothelial cells from SK3\(^{T/T}\) mice were almost completely suppressed by dietary DOX. SK3 channels contributed to sustained hyperpolarization of the endothelial membrane potential, which was communicated to the arterial smooth muscle. Pressure- and phenylephrine-induced constrictions of SK3\(^{T/T}\) arteries were substantially enhanced by treatment with apamin, suppression of SK3 expression with DOX, or removal of the endothelium. In addition, suppression of SK3 expression caused a pronounced and reversible elevation of blood pressure. These results indicate that endothelial SK3 channels exert a profound, tonic, hyperpolarizing influence in resistance arteries and suggest that the level of SK3 channel expression in endothelial cells is a fundamental determinant of vascular tone and blood pressure. (Circ Res. 2003;93:124-131.)

Key Words: endothelium ■ potassium channels ■ vascular tone ■ blood pressure

Blood pressure and flow are regulated by the constriction and dilation of resistance arteries, generally with internal diameters <300 \(\mu\)m.\(^{1}\) Physiological stimulation through elevations in intravascular pressure or increased sympathetic activity promotes smooth muscle depolarization, intracellular Ca\(^{2+}\) influx, and vasoconstriction. The resulting increase in total peripheral resistance within the vasculature increases blood pressure.\(^{2}\)

The endothelium exerts a dilating influence that opposes arterial constriction. Activation of K\(^{+}\) channels is thought to contribute to this influence through increased release of relaxing factors such as NO and prostacyclin (PGI\(_2\)) and through smooth muscle hyperpolarization.\(^{3,5}\) The small-conductance Ca\(^{2+}\)-activated K\(^{+}\) (SK) channel has received considerable attention as a potential mediator of these responses. SK channels are opened by intracellular Ca\(^{2+}\) via an association with calmodulin\(^6\) and are believed to play a role in the modulation of tissue excitability.\(^7\) Of the three characterized SK channel isoforms (SK1, SK2, and SK3),\(^8\) mRNA for SK2 and SK3 has been identified in endothelial cells.\(^9\) Apamin, a toxin blocker of SK channels, either alone or in combination with charybdotoxin, a blocker of intermediate-conductance (IK) and large-conductance (BK) Ca\(^{2+}\)-activated K\(^{+}\) channels, has been reported to inhibit arterial relaxations to various endothelium-dependent vasodilators, such as acetylcholine, bradykinin, substance P, and ATP in numerous vascular beds.\(^{10-13}\) Acute apamin-sensitive relaxations are most often attributed to hyperpolarization of arterial smooth muscle,\(^{14,15}\) which may involve myoendothelial gap junctional communication,\(^{16}\) or the action of an undefined factor referred to as endothelium-derived hyperpolarizing factor.\(^{17-19}\) Particular interest in endothelial SK3 channels has been heightened by recent observations that the vascular dysfunctions resulting from balloon catheter injury\(^{20}\) and cirrhosis\(^{21}\) are associated with changes in endothelial SK3 expression. However, previous approaches have not allowed...
for specific isolation of these channels for experimental study. Consequently, the role of endothelial SK3 channels in the regulation of cardiovascular function is not known.

In the present study, we tested the hypothesis that SK3 channels influence tonic endothelium-dependent vasoregulation and that manipulation of SK3 channel gene expression alters vascular tone and blood pressure. We used a transgenic mouse (SK3<sup>+/−</sup>) harboring genetically targeted alleles for the SK3 channel, in which SK3 gene expression can be experimentally controlled by dietary doxycycline (DOX).<sup>22</sup> In the absence of DOX, the SK3 gene is ≈3-fold overexpressed in this mouse compared with the wild-type mouse, as evidenced by expression patterns in brain and uterine tissues, with no change in the normal temporal and cell-type–specific profile of SK3 channel expression. Addition of dietary DOX greatly suppresses or abolishes SK3 gene expression. Through acute and specific control of a single gene product, the functional impact of SK3 channel expression could be specifically assessed. Moreover, reversible upregulation and downregulation of SK3 expression allowed us to assess the effects of a range of SK3 expression levels in vivo.

Materials and Methods

Conditional SK3 expression (SK3<sup>T/T</sup>) was achieved through homologous recombination by insertion of a gene switch targeting the SK3 gene, as previously described.<sup>22</sup> A regulatory cassette was inserted into the 5′ untranslated sequence of the gene, encoding the binary tetacycline-sensitive transactivator (tTA) protein. The native SK3 promoter drives expression of the functional gene via tTA such that promoter function and tissue distribution are conserved, and experimental modulation of SK3 expression is achieved via dietary exposure to the tetracycline derivative DOX for at least 5 days. Homozygous SK3-targeted mice (SK3<sup>+/−</sup>) and wild-type mice used for the present study were of the same background strain (c57BL/6). Genotypes were confirmed by reverse transcription (RT)–polymerase chain reaction (PCR). Mice were euthanized via intraperitoneal injection of sodium pentobarbital and subsequent decapitation, in accordance with the University of Vermont Institutional Animal Care and Use Committee and the National Institutes of Health's Guide on the Humane Treatment of Experimental Animals. Some mice were given 0.5 mg/mL DOX and 2% sucrose in the drinking water for at least 6 days.

Western Blotting and Quantitative PCR

Homogenized mesenteric arteries were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After exposure to rabbit anti-SK3 (primary) antibody (Alomone Laboratories) and to goat anti-rabbit (secondary) antibody, visualization was achieved by chemiluminescence. SK3 protein was quantified relative to α-GAPDH. For PCR, the mesenteric artery (~75 mg) was pulverized under liquid nitrogen. Total RNA was extracted in 200 μL TriReagent and converted to single-stranded cDNA using murine Moloney leukemia virus reverse transcriptase. Real-time PCR, using SYBR Green, was performed on each sample in duplicate for transcriptase. Real-time PCR, using SYBR Green, was performed on stranded cDNA using murine Moloney leukemia virus reverse standard curve for each amplicon was prepared.

Membrane Potential Measurements

For endothelial cell measurements, mesenteric arteries (200 to 300 μm) were cut longitudinally and pinned, luminal side up, to the bottom of a small chamber lined with Sylgard. The chamber was continuously superfused with warm (37°C), gassed (95% O<sub>2</sub>/5% CO<sub>2</sub>) physiological saline solution (PSS) containing (mmol/L) NaCl 119, KCl 4.7, NaHCO<sub>3</sub> 24, KH<sub>2</sub>PO<sub>4</sub> 0.2, EDTA 1.1, MgSO<sub>4</sub> 1.2, CaCl<sub>2</sub> 1.6, and glucose 10.6. Sharp glass electrodes (~100-MΩ resistance) containing 1 mol/L KCl were advanced to the intimal surface using a micromanipulator, and measurements were made using an electrometer (World Precision Instruments). Data were recorded via computer using Axotape and Dataq software. For some experiments, electrode tips were loaded with the fluorescent dye propidium iodide (1% in 1 mol/L KCl), allowing for unambiguous identification of endothelial cells.<sup>23</sup> Multiple measurements (3 to 6) from each artery were averaged before and after apamin and were counted as a single n. For smooth muscle measurements, mesenteric arteries (100 to 200 μm) were cannulated, pressurized, and superfused with PSS. Membrane potentials were measured using electrodes containing 0.5 mol/L KCl.

Diameter Measurements

Mesenteric arteries were dissected in cold PSS. Artery segments (100 to 200 μm) were cannulated on glass pipettes, pressurized (with no flow), and superfused with warmed, gassed PSS as described above. Arterial diameter was measured using a video dimension analyzer (Living Systems Instrumentation) and displayed and recorded by computer using Axotape software. Demudation of the endothelium was accomplished by placing an air bubble in the vessel lumen for 3 minutes and was confirmed by the lack of acetylcholine-mediated dilation. All concentration-effect curves to phenylephrine (PE) were performed at an intravascular pressure of 20 mm Hg.

Blood Pressure Measurements

Pressures were measured using a noninvasive tail-cuff monitor (NIBP-2, Columbus Instruments). Measurements were performed in 8 SK3<sup>T/T</sup> mice (4 males and 4 females, aged ≥4 months) over a 5- to 7-day period. Pressures were measured again after exposure to DOX (>2 weeks) and again after removal of DOX (>2 weeks) from the drinking water. The average of 10 measurements was taken as a representative pressure for each animal per day. Similar measurements were made in 8 wild-type mice before and after DOX exposure.
Figure 1. SK3 overexpression and suppression in endothelial cells. A, top left, Western blots from a wild-type (WT) control mouse, possessing only native alleles for the SK3 channel, an untreated SK3T/T mouse (−DOX), possessing two tTA-targeted alleles, and an SK3T/T mouse treated with 0.5 mg/mL DOX (+DOX) for 6 days. The apparent size difference between the SK3 proteins from SK3T/T and WT mice is due to a 40-amino acid deletion as previously described.19 A, right, Immunofluorescence labeling of mesenteric artery cross sections from untreated (SK3T/T) and DOX-treated (SK3T/T + DOX) mice. SK3-labeled fluorescence is shown in green. Autofluorescence of the mesenteric artery wall (red) clearly defines the internal elastic lamina. Bar=20 μm. A, bottom left, En face confocal images of whole-mount mesenteric arteries from WT and SK3T/T mice. Endothelial (left) and smooth muscle (right) cell nuclei are shown in red. Substantial SK3-positive staining (green) was detected in endothelial cells, aligned with the axis of the vessel (arrows), but not in smooth muscle cells. B, Endothelial cell currents. B, left, Apamin-sensitive current-voltage relationships in endothelial cells from untreated (blue) and DOX-treated (red) SK3T/T mice. Currents were recorded in response to a 200-ms voltage ramp from −100 to 50 mV before and after exposure to apamin. Apamin-sensitive currents reversed close to the calculated electrochemical equilibrium potential for K⁺, −83 mV (pipette [Ca²⁺]=3 μmol/L). B, right, Mean apamin-sensitive current densities in endothelial cells from untreated (blue, n=7) and DOX-treated (red, n=7) SK3T/T mice. ***P<0.001. C, Endothelial cell membrane potentials. Continuous recordings (left) and summary data (right) show resting membrane potentials and effects of apamin on arteries from untreated and DOX-treated SK3T/T mice (SK3T/T, n=6; SK3T/T + DOX, n=9).
Materials
SYBR Green, Cy5, and YOYO-1 were purchased from Molecular Probes. Hyaluronidase and elastase were obtained from Worthington, and anti-SK antibody was supplied by Alomone Laboratories. Unless otherwise noted, all other substances and reagents were purchased from Sigma.

Data Analysis
Comparisons between groups were made with unpaired or paired t tests. Multiple-group data were subjected to 2-way ANOVA with repeated measures, and individual comparisons were made by Bonferroni post hoc test analysis where appropriate. Values of P<0.05 were considered statistically significant. Data are given as mean±SEM.

Results
Overexpression and Suppression of SK3 Channels in Resistance Arteries
SK3 protein levels were detected in small (~200-μm) mesenteric arteries from wild-type mice and were elevated in arteries from SK3T/T mice (Figure 1A). Dietary DOX reduced SK3 protein to well below the levels found in wild-type mice. Quantitative RT-PCR showed that DOX exposure decreased SK3 mRNA levels by 97% in arteries from SK3T/T mice. In arteries from wild-type and SK3T/T mice, SK3 protein was largely localized to the endothelial cell layer that lines the arterial lumen, with little or no expression detected in the smooth muscle. SK3 immunofluorescence was essentially undetectable in DOX-treated mice. Thus, SK3 was highly expressed in the endothelium of mesenteric resistance arteries from SK3T/T mice and suppressed by dietary DOX.

SK3 Channels Promote Tonic Hyperpolarization of the Endothelium
To determine whether SK3 protein levels in SK3T/T mice are correlated with the expression of functional channels in the vascular endothelium, we measured SK currents in freshly dissociated endothelial cells. SK currents, identified as apamin-sensitive (300-nM) K⁺ currents, were ~25-fold larger in endothelial cells from untreated SK3T/T mice compared with endothelial cells from DOX-treated mice (Figure 1B).

The measured currents suggested that SK3 channels should contribute substantially to the endothelial cell membrane potential. To test this hypothesis, we measured membrane potential in intact endothelium using microelectrodes. Blocking SK channels with apamin depolarized the intact endothelium by ~15 mV in SK3T/T mesenteric arteries (~54.1±1.8 versus ~39.2±3.0 mV, n=6), indicating that the SK conductance contributed substantially to the endothelial membrane potential (Figure 1C). Compared with SK3T/T mice, the membrane potential of endothelial cells from DOX-treated SK3T/T mice (n=5) was depolarized by ~14 mV (~54.1±1.8 versus ~40.2±2.5 mV, P<0.001). Apamin had no effect on membrane potential in these cells (~40.2±2.5 versus ~39.6±2.8 mV), indicating that they were already considerably depolarized as a result of DOX-induced suppression of SK3 expression. These results indicate that SK3 channels promote tonic hyperpolarization of the endothelium depending on their level of expression.

Tonic SK3-Mediated Endothelial Hyperpolarization Is Communicated to Arterial Smooth Muscle
We hypothesized that the SK3 channel effects on endothelial membrane potential may be translated to adjacent smooth muscle, either through direct communication via myoendothelial gap junctions or through the release of a diffusible factor. Apamin substantially depolarized the membrane potential of smooth muscle cells in intact SK3T/T arteries (Figure 2) by 11.9±1.2 mV at an intravascular pressure of 60 mm Hg. Removal of the endothelium led to similar smooth muscle depolarization (~15 mV), which was not further affected by the addition of apamin, indicating that endothelial SK3 channels elicit tonic hyperpolarization of adjacent smooth muscle.

Endothelial SK3 Channels Attenuate Arterial Tone
Tonic hyperpolarization of the smooth muscle via endothelial SK3 channels should decrease arterial tone by reducing Ca²⁺ influx through voltage-dependent Ca²⁺ channels in the smooth muscle cells. To test the functional influence of endothelial SK3 expression in resistance arteries, we measured arterial tone at different levels of intravascular pressure. Pressure-induced increases in arterial tone (“myogenic tone”) contribute substantially to the regulation of peripheral resistance and blood pressure. Mesenteric arteries from SK3T/T mice exhibited little arterial tone (determined by the difference between active and passive diameters at a given pressure) to graded increases in intravascular pressure (Figure 3A), suggesting a tonic dilating influence of SK3 channels. Apamin caused a sustained constriction of SK3T/T arteries at low pressure and elevated arterial tone at all pressure levels tested. Suppression of SK3 expression with DOX
increased pressure-induced tone to a level similar to that observed in arteries exposed to apamin. Apamin had no effect on pressure-induced tone in the DOX-treated group. These results indicate that tonic SK3 channel activity attenuates myogenic tone.

Sympathetic nerve activity, through stimulation of α-adrenergic receptors on smooth muscle cells, is an important regulator of mesenteric artery function and vascular resistance. The α-adrenergic receptor agonist PE increased arterial tone of arteries from SK3 T/T mice, and this PE-induced elevation of tone was markedly enhanced by apamin (Figure 3B), consistent with a decrease in the tonic dilating effect of endothelial SK3 channels. Suppression of SK3 expression by dietary DOX was equivalent to application of apamin, increasing the apparent sensitivity and maximal constriction in response to PE. Removal of the endothelium enhanced PE-induced tone, similar to the effect of blocking SK channels with apamin or suppressing SK3 expression with DOX. Apamin had no significant effect on PE-induced increases in arterial tone in the absence of endothelium, specifically implicating endothelial SK3 channels as the source of the dilating effect.

Together, our results indicate that SK3 channel activity increases the tonic vasodilating influence of the endothelium through hyperpolarization. This effect could involve enhanced release of the endothelial relaxing factors NO and PGI₂. However, blockade of NO and PGI₂ production with Nω-nitro-l-arginine (L-NNA, 200 μmol/L) and indomethacin (10 μmol/L), respectively, did not prevent the effects of apamin. Apamin constricted arteries from SK3 T/T mice (8.5 ± 2.2% constriction at 20 mm Hg, n = 5) in the presence of L-NNA and indomethacin and increased PE-induced contractions (from 24 ± 6% to 64 ± 2% at 1 μmol/L PE, P < 0.001, n = 5) to approximately the same extent as in the absence of L-NNA and indomethacin. Thus, the SK3-mediated dilating influence does not involve NO or PGI₂ release. Notably, in arteries from wild-type mice, apamin caused a significant, albeit less pronounced, enhancement of PE-induced contractions (from 64 ± 3% to 72 ± 3% at 1 μmol/L PE, P < 0.05, n = 4) in the presence of L-NNA and indomethacin. These data suggest that SK3 channels contribute to the tonic NO- and PGI₂-independent dilating influence of the endothelium in wild-type arteries as well as SK3 T/T arteries and that the magnitude of this dilating influence is related to the level of SK3 expression.
Suppression of SK3 Expression Increases Blood Pressure

Because blocking SK3 channels or suppressing SK3 expression greatly increased the tone of resistance arteries in response to pressure and adrenergic receptor stimulation, we hypothesized that modulation of SK3 channel expression might influence blood pressure. Indeed, we found that suppression of SK3 expression with dietary DOX led to a marked increase in the systolic and diastolic pressures of SK3T/T mice, which was reversed by removal of DOX (Figure 4). DOX had no effect on the blood pressures of wild-type mice. Thus, decreasing and increasing SK3 channel expression caused commensurate changes in blood pressure, consistent with changes in vascular tone and peripheral resistance.

SK3 Channel Overexpression Increases Arterial Diameters

In addition to the direct functional effects of endothelial SK3 expression, we also observed SK3-dependent changes in vessel structure. Arteries from SK3T/T mice were dramatically different in appearance from their wild-type counterparts, exhibiting larger diameters and apparent differences in the degree of branching (Figure 5). The passive internal diameters of mesenteric arteries, for example, were 25% larger in SK3T/T mice than in wild-type mice (179 ± 4 versus 142 ± 3 μm, pressure 100 mm Hg, P < 0.05, n = 6) of the same age and sex. Coronary (septal) arteries, measured in situ from freshly excised hearts, were similarly larger in SK3T/T mice (internal diameters 101 ± 4 versus 70 ± 3 μm, P < 0.05, n = 5). Enlargement of other hollow organs from SK3T/T mice, including the...
vasodilations of carotid arteries after balloon catheter injury appear to be linked to decreased SK3 and IK expression in regenerated endothelial cells. 

It should be noted that IK channels share many properties with SK channels, and they have been implicated in acute endothelium-dependent dilations. Although the IK channel was not the focus of the present study, it may allow for additional fine-tuning of the tonic endothelial hyperpolarizing influence and warrants future study. Endothelium-dependent hyperpolarization has been implicated in flow-induced vasodilation in coronary arteries, suggesting that endothelial channels such as SK3 and IK may play a particularly important vasoregulatory role in vivo. Under conditions of dynamic shear and blood flow, even moderate expression of SK3 and/or IK channels may have profound influences on vascular tone.

In the present study, we provide the first evidence that the expression level of an ion channel (SK3) in the vascular endothelium can profoundly influence cardiovascular function through a sustained hyperpolarization. It is likely that SK3 expression is tightly regulated by hormonal status and is altered with disease, having dramatic and dynamic effects on vascular function and structure. Consequently, altering SK3 expression within a normal physiological range may allow for flexible control of blood pressure and flow, whereas pathological upregulation or downregulation of SK3 expression may lead to severe hemodynamic dysregulation. Future studies will address the mechanisms and influence of dynamic SK3 channel regulation. Finally, these channels could be exploited clinically. Manipulation of endothelial SK3 channel expression may provide a novel therapeutic approach for the treatment of various vascular disorders, including hypertension.

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References


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