Reduced expression of SK_{Ca} and IK_{Ca} channel proteins in rat small mesenteric arteries during angiotensin II-induced hypertension

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Hilgers RH, Webb RC. Reduced expression of SK_{Ca} and IK_{Ca} channel proteins in rat small mesenteric arteries during angiotensin II-induced hypertension. Am J Physiol Heart Circ Physiol 292: H2275–H2284, 2007. First published January 12, 2007; doi:10.1152/ajpheart.00949.2006.—Ca^{2+}-activated K^{+} channels (K_{Ca}), in particular, the small and intermediate K_{Ca} (SK_{Ca} and IK_{Ca}, respectively) channels, are key players in endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation in small arteries. Hypertension is characterized by an endothelial dysfunction, possibly via reduced EDHF release and/or function. We hypothesize that during angiotensin II (14 days)-induced hypertension (ANG II-14d), the contribution of SK_{Ca} and IK_{Ca} channels in ACh-induced relaxations is reduced due to decreased expression of SK_{Ca} and IK_{Ca} channel proteins in rat small mesenteric arteries (MAs). Nitric oxide- and prostacyclin-independent vasorelaxation to ACh was similar in small MAs of sham-operated and ANG II-14d rats. Catalase had no inhibitory effects on these relaxations. The highly selective SK_{Ca} channel blocker UCL-1684 almost completely blocked these responses in MAs of sham-operated rats but partially in MAs of ANG II-14d rats. These changes were pressure dependent since UCL-1684 caused a greater inhibition in MAs of 1-day ANG II-treated normotensive rats compared with ANG II-14d rats. Expression levels of both mRNA and protein SK3 were significantly reduced in MAs of ANG II-14d rats. The IK_{Ca} channel blocker 1-(2-chlorophenyl)diphenylmethylyl-1H-pyrazole (TRAM-34) resulted in comparable reductions in the relaxation responses to ACh in MAs of sham-operated and ANG II-14d rats. Relative mRNA expression levels of IK1 were significantly reduced in MAs of ANG II-14d rats, whereas protein levels of IK1 were not but tended to be lower in MAs of ANG II-14d rats. The findings demonstrate that EDHF-like responses are not compromised in a situation of reduced functional activity and expression of SK3 channels in small MAs of ANG II-induced hypertensive rats. The role of IK1 channels is less clear but might compensate for reduced SK3 activity.

Calcium-activated potassium channels; endothelium

Calcium-activated K^{+} channels (K_{Ca}) are key players in the control of endothelium-dependent vasorelaxation. The endothelial cell intermediate- and small-conductance K_{Ca} channels (IK_{Ca} and SK_{Ca}, respectively) are especially important in endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation and hyperpolarization in resistance-sized arteries (1, 7, 13, 18, 28, 36, 37). This hyperpolarization is communicated to adjacent smooth muscle cells likely through myoendothelial gap junctions (30, 37) or via diffusible factor(s) (11, 17, 34, 41). These K_{Ca} channels regulate membrane potential and Ca^{2+} homeostasis in response to hemodynamic stresses and vasoactive factors. For instance, an increase in intracellular Ca^{2+} leads to an increase in the activity of K_{Ca} channels, causing smooth muscle cell hyperpolarization and prevention of further influx of Ca^{2+} (2, 4, 29).

The relative contribution of endothelium-derived relaxing factors regulating arterial tone depends largely on the vessel type and the (patho)physiological state. An inverse correlation between the arterial diameter and the relative contribution of EDHF has been proposed (42). We recently observed a regional heterogeneity in the expression level of SK_{Ca} and IK_{Ca} channels and their contribution in ACh-induced relaxations in rat mesenteric arteries, with higher expression levels in fourth-order mesenteric arteries (MAs) compared with first-order vessels, stressing the importance of these channels in smaller vessels where the role of EDHF is more pronounced (27). A number of cardiovascular diseases, including diabetes, atherosclerosis, and hypertension, are characterized by a reduced EDHF response (20, 21). Recently, a reduced expression of SK_{Ca} channel mRNA, but not IK_{Ca}, was observed in MAs of streptozotocin-induced diabetic apolipoprotein E-deficient mice (16). The role of SK_{Ca} and IK_{Ca} channels during hypertension is far from clear. A study using transgenic SK3^{T/T} mice, in which the SK3 expression levels can be manipulated with dietary doxycycline, showed that SK3 channel suppression caused a pronounced hypertension, indicating the significance of this SK3 channel in regulating vasomotor tone and blood pressure (45). Knowledge of the mechanisms underlying EDHF and K_{Ca} channel function would improve our understanding of the regulation of arterial tone in health and disease. Changes in molecular composition of K_{Ca} channels may therefore be a fundamental event contributing to the progression of arterial dysfunction during hypertension. Whether these changes in K_{Ca} channel function and expression during hypertension occur at the level of resistance-sized arteries, where they are needed in regulating vascular tone and hence local blood flow, is not completely understood and requires further studies.

We measured EDHF-mediated vasorelaxing responses in the absence or presence of specific antagonists of IK_{Ca} and SK_{Ca} channels in small (fourth order) MAs of sham-operated normotensive and angiotensin II (ANG II)-induced hypertensive rats. To distinguish between rapid (hours–days) ANG II-induced and slow (days–weeks) hypertension-related changes in K_{Ca} channel function, we included a third group of rats treated with ANG II (60 ng·kg^{-1}·min^{-1}) via osmotic minipumps for 24 h. Since these rats still remained normotensive (22), the effects of ANG II, independently of pressure on SK_{Ca} and IK_{Ca} channel
function, could be studied. Furthermore, RT-PCR and immuno-precipitation followed by Western blot analysis were performed to analyze the expression of both IKCa and SKCa channel mRNAs and protein levels in isolated small MAs of sham-operated and 14-day ANG II-treated rats. We hypothesized that after 14 days of ANG II treatment, but not after 1 day, the contribution of SKCa and IKCa channels in ACh-induced relaxations is reduced, due to decreased expression of SKCa and IKCa channel proteins in small rat MAs.

**MATERIALS AND METHODS**

**Animal preparation and blood pressure measurement.** Sprague-Dawley rats (225–250 g, Harlan, Indianapolis, IN) were implanted subcutaneously with osmotic minipumps (Alzet, Alza) after they were anesthetized with a mixture of ketamine (80 mg/kg) and xilazine (10 mg/kg). The rats were divided into three groups: a control group infused with saline only, and two groups of rats infused with ANG II (60 ng·kg⁻¹·min⁻¹) for a period of 1 and 14 days, respectively. Systolic blood pressure was measured by tail-cuff plethysmography in conscious rats to monitor the progression of hypertension. The basal systolic blood pressure was measured by tail-cuff plethysmography in each rat when they were allowed to incubate for 45 min. Arterial integrity was assessed by relaxation with ACh (1 μM) followed by relaxation with ACh (1 μM/l). After being washed, the arterial rings were contracted with the thromboxane analog 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin F2α (U-46619, 0.1 μmol/l), U-46619 was observed to produce stable contractions for a long period in MAs of both normo- and hypertensive rats, unlike phenylephrine, which showed unstable contractions, in particular, in MAs of hypertensive rats (personal observations). Cumulative concentration-response curves (CRCs) to ACh were performed under control conditions in the combined presence of indomethacin (inhibitor of cyclooxygenase, 10 μmol/l) and Nω-nitro-L-arginine methyl ester ([L-NNAME, inhibitor of nitric oxide (NO) synthase, 100 μmol/l]) to study the contribution of H2O2 derived from superoxide anions resulting in vasorelaxation, CRCs to ACh were performed in the combined presence of L-NNAME and indomethacin and catalase (100 U/ml). To study the role of KCa channels in ACh-induced relaxations, we always determined EDHF-mediated relaxations in the combined presence of L-NNAME and indomethacin to rule out any potential interference of NO and prostaglandins with KCa channels (3). Since U-46619 has been shown to cause an inactivation of SKCa channels in small rat MAs (14), one single cumulative CRC for ACh was performed for each arterial segment incubated with L-NNAME, indomethacin, and KCa channel inhibitor(s).

Semiquantitative RT-PCR amplification of KCa channel genes. Fourth-order (and smaller branches along the gut wall) MAs were carefully freed of adipose and connective tissue and quickly snap frozen in liquid nitrogen. To generate sufficient amounts of total RNA, isolated MA segments were pooled from 10 rats and subse-

quentely homogenized. This pool was considered as n = 1, three individual pools of a total of 30 different rats were used to isolate total RNA, which yielded a total of n = 3. Total RNAs were isolated with TRIzol reagent (Invitrogen). The amount of RNA was determined spectrophotometrically at an absorbance of 260 nm. An amount of 0.5 μg of total RNAs was reverse transcribed [oligo(dT)2–18 primer using Moloney murine leukemia virus reverse transcriptase, Amersham Biosciences], and cDNAs were subsequently amplified in Ready-to-Go RT-PCR beads in a two-step procedure (Amersham Biosciences). Primer sequences for the SK3 gene (Genbank accession number AF292389) were as follows: forward, nt: 1389–1406; 5’-CCTCTACATCAGCCTGGA-3’; reverse, nt: 2090–2073; 5’-ACTT-TGGCCGTGTTCAATC-3’. The PCR product generated a fragment of 702 bp. Primer sequences for the SK4 gene, which is encoded by the Kcnq4 gene, are actually the same as the rat IKCa subtype encoded by the rat (r)IK1 gene [see Genbank accession numbers AF914058 (5MIK) and NM023021 (rIK1)], were as follows: forward, nt: 1193–1212; 5’-GAAACAGTGAATCTCAGGT-3’; reverse, nt: 1390–1372; 5’-CTATGGGCTCCTGGATG-3’. The PCR product generated in a fragment of 198 bp. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Genbank accession number AF106860) were as follows: forward, nt: 84–104; 5’-GGCTGCTTC-TCTCTTTGACA-3’; reverse, nt: 282–263; 5’-CGCTCTGGGAG-GATGGGTAT-3’. The PCR product generated a fragment of 199 bp. The general protocol for PCR amplification was 35 cycles at 94°C for 1 min, at 60°C for 1 min, and at 72°C for 1 min, followed by a final extension at 72°C for 10 min. Amplified DNA fragments were separated in a 2% agarose gel. The gel images were recorded by video camera (Sony Video Camera Module CCD, Tokyo, Japan) connected to an IBM AT computer (New York, NY) with a 512 × 512-pixel array imaging board with 256 gray levels. The PCR products were quantified by densitometric scanning of gel images using UN-SCAN-IT software (Silk Scientific). Results were then expressed as the densitometric ratio of the gene of interest to GAPDH.

**Immunoprecipitation of SK3 and IK1 proteins.** Fourth-order and higher-order MAs running along the gut were carefully freed of adipose and connective tissue and quickly snap frozen in liquid nitrogen and kept at −80°C until protein expression analysis. Proteins were isolated from arteries pooled from 10 rats to ensure sufficient amount of proteins. This pool was considered as n = 1, three individual pools of a total of 30 different rats were used to isolate total protein extract, which yielded a total of n = 3. Arteries were homogenized in cold RIPA buffer [50 mmol/l Tris·HCl (pH 7.4), 0.15 mmol/l NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40, and 1 mmol/l EDTA] enriched with 1 mmol/l PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mmol/l NaVO4. The homogenate was separated by centrifugation at 15,000 g for 30 min at 4°C. The supernatant was kept on ice. Protein concentration was determined via the BSA protein assay kit (Pierce Chemical, Rockford, IL). Aliquot samples of 100 μg of total protein extract were then incubated with 1 μg of antibody (anti-SK3, Sigma-Aldrich, St. Louis, MI, or anti-IK1, Santa Cruz Biotechnology) for 6 h at 4°C under constant rotation, followed by the addition of Sepharose A and B beads (1:1) for 6 h at 4°C (Upstate Biotechnology, Lake Placid, NY). Immunoprecipitates were collected by centrifugation (12,000 rpm; 5 min), washed three times with phosphate-buffered saline solution, and resuspended in Laemmli’s sample buffer with β-mercaptoethanol (Bio-Rad, Hercules, CA). Samples were loaded and separated by SDS-PAGE (10%) and subsequently transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked by treatment with 5% non-dry-fat milk in Tris-buffered saline containing 0.05% Tween 20, probed with anti-SK3 or anti-IK1 (1:1,000), and kept overnight at 4°C. After incubation with secondary antibody, signals were revealed with chemiluminescence autoradiography and quantified densitometrically.

**Data analyses and statistics.** Experimental values were calculated relative to the maximal changes from the contraction produced by
U-46619 in each segment, which was taken as 100%. The phospho-
EC50 (pEC50) values for ACh were expressed as -log of the molar
concentration to produce 50% of the maximal response. Data are
expressed as means ± SE of n arterial segments. Statistical analysis
was performed by using a two-way analysis of ANOVA to compare
the CRCs between the groups. The analyses were performed by using
GraphPad Prism software. Values of P < 0.05 were considered a
statistically significant difference.

Drugs. l-Phenylephrine hydrochloride, ACh, sodium nitroprus-
side, L-NAME, indomethacin, sodium nitroprusside, catalase, and 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) were
all purchased from Sigma-Aldrich. 9,11-Dideoxy-9α,11α-methano-
epoxyprostaglandin F2α, (U-46619) was purchased from Calbiochem
(San Diego, CA). 6,12,19,20,25,26-Hexahydro-5,27:13,18:21,24-
trietheno-11,7-metheno-7H-dibenzo [bn][1,5,12,16]tetraazacycloti-
tricosine-5,13-dium ditrifluoroacetate (UCL-1684) was purchased from
Tocris (Ellisville, MI). Indomethacin was dissolved in ethanol, and
TRAM-34 and UCL-1684 were dissolved in DMSO. All other stock
solutions were prepared by using PSS.

RESULTS

Increased blood pressure in ANG II-treated rats. Systolic
blood pressure was elevated in ANG II (14 days)-treated rats
compared with sham-operated rats. The systolic blood pressure
after 14 days of treatment was 191 ± 6 mmHg in hypertensive
rats compared with 120 ± 2 mmHg in normotensive rats (n = 8,
P < 0.01). There were no significant changes of the systolic
blood pressure in sham-operated rats during the 14-day treat-
ment and in rats after 1-day infusion with ANG II (data not
shown).

ACh-induced vasorelaxations. U-46619 (0.1 μmol/l) re-
resulted in stable contractions in fourth-order MAs. The active
force generated with this concentration of U-46619 was similar
in isolated MAs of sham-operated and ANG II (14 days) -
treated rats (15.6 ± 0.7 vs. 14.6 ± 1.3 mN, respectively).
Under control conditions, ACh (0.001–10 μmol/l) caused con-
centration-dependent relaxations and resulted in almost com-
plete relaxations in U-46619 (0.1 μmol/l)-contracted MAs of
both groups of rats (Fig. 1). Sensitivity for ACh was compa-
rable for MAs of sham-operated and ANG II (14 days)-treated
rats (pEC50 value 7.02 ± 0.04 vs. 7.21 ± 0.06, respectively).
The CRCs to ACh in the presence of L-NAME and indo-
methacin showed a similar rightward shift in MAs of both
groups of rats (Fig. 1). Maximal relaxation to ACh (10 μmol/l) was comparable between MAs of both
groups of rats (Fig. 1).

Involvement of H2O2 in non-NO, non-PGI2-mediated ACh-
induced relaxations. The involvement of H2O2 in non-NO,
non-PGI2-mediated ACh-induced relaxations was assessed by
incubating MAs of sham-operated and ANG II (14 days) -
treated rats with catalase (100 U/ml) in the combined presence
of L-NAME and indomethacin. In both groups of rats, catalase
did not result in any significant changes in the sensitivity and
maximal relaxation to ACh, but they tended to be reduced in
the MAs of ANG II (14 days)-treated rats compared with
sham-operated rats [maximum effect (E_{max}), 79 ± 5% vs. 89 ±
2%, respectively; P = 0.08; Fig. 2].

Involvement of SKCa channels in vascular relaxation. UCL-
1684 (0.1 μmol/l), the selective inhibitor of SKCa channels, did
not alter the vasorelaxing response to the NO donor sodium
nitroprusside (data not shown). Maximal tension to U-46619
(0.1 μmol/l) in the presence of UCL-1684 (0.1 μmol/l) did not
differ between values from L-NAME and indomethacin-incu-
bated MAs of both groups of rats (data not shown). CRCs to
ACh in the presence of L-NAME, indomethacin, and UCL-
1684 (0.1 μmol/l) were virtually abolished in MAs of sham-
operated rats (Fig. 3A). However, a residual vasorelaxation was
evident in MAs of ANG II (14 days)-treated rats (Fig. 3B).
Maximal vasorelaxation to ACh (10 μmol/l) was significantly
higher in MAs of ANG II (14 days)-treated rats compared with
sham-operated rats (53 ± 11% vs. 0 ± 2%, respectively).
CRCs to ACh were also assessed in MAs of rats that were
treated with ANG II for 1 day to dissociate between ANG
II-induced and hypertension-related effects on SKCa channel
function. UCL-1684 (0.1 μmol/l) resulted in a greater blunted
ACh-induced relaxation in ANG II (1 day)-treated rats com-
pared with ANG II (14 days)-treated rats (E_{max}, 18 ± 16% vs.
53 ± 11%, respectively; Fig. 3, B and C).

Fig. 1. Concentration-response curves to ACh in U-46619-contracted (0.1
μmol/l) 4th-order mesenteric arteries of sham-operated normotensive (A)
and ANG II 14-day (14-d)-treated rats (B) under control conditions (vehicle)
and in the presence of N\^\textsuperscript{-}nitro-l-arginine methyl ester (l-NAME)
and indomethacin (l-NAME + Indo).
to ACh were significantly blunted in L-NAME, indomethacin, and TRAM-34-incubated (E_{max}, 72 ± 10%, Fig. 4C) and L-NAME and indomethacin-treated MAs (E_{max}, 82 ± 5%, Fig. 4C).

ACh-induced relaxations were completely abolished in the combined presence of L-NAME, indomethacin, UCL-1684, and TRAM-34 in MAs of sham-operated and ANG II (14 days)-treated rats (Fig. 5).

**Fig. 2. Concentration-response curves to ACh in U-46619-contracted (0.1 μmol/l) 4th-order mesenteric arteries of sham-operated normotensive (A) and ANG II (14 days)-treated (B) rats in the presence of L-NAME and indomethacin (L-NAME + Indo) and in the presence of catalase (100 U/ml) and L-NAME + Indo.**

**Involvement of IK_{Ca} channels in vascular relaxation.**

TRAM-34 (10 μmol/l), the selective inhibitor of IK_{Ca} channels, did not result in any changes in the vasorelaxing response to sodium nitroprusside (data not shown). Maximal tension to U-46619 (0.1 μmol/l) in the combined presence of L-NAME, indomethacin, and TRAM-34 (10 μmol/l) were lower but did not differ significantly from values in L-NAME and indomethacin-treated MAs of sham-operated and ANG II (14 days)-treated rats (data not shown). CRCs to ACh were partially inhibited in the presence of TRAM-34 (10 μmol/l) compared with vehicle-treated segments (Fig. 4). Maximal responses to ACh were significantly blunted in L-NAME, indomethacin, and TRAM-34-incubated MAs of sham-operated and ANG II (14-days)-treated rats (E_{max}, 51 ± 16% vs. 44 ± 11%, respectively; Fig. 4, A and B) compared with L-NAME and indomethacin-treated vessels (E_{max}, 79 ± 6% vs. 87 ± 4%, respectively; Fig. 4, A and B). This blunted relaxation tended to be greater in MAs of ANG II (14 days)-treated rats (Δ43% vs. Δ28%). In 1-day ANG II-infused rats, maximal responses to ACh were similar for L-NAME, indomethacin, and TRAM-34-incubated (E_{max}, 72 ± 10%, Fig. 4C) and L-NAME and indomethacin-treated MAs (E_{max}, 82 ± 5%, Fig. 4C).

Semiquantitative RT-PCR of SK_{Ca} and IK_{Ca} mRNA expression. Expression of SK3 and IK1, the pore-forming subunits of SK_{Ca} and IK_{Ca} channels, respectively, was quantified in total mRNA obtained from sham-operated and ANG II (14 days)-treated rats. Relative expression of mRNA levels of rat SK3 was significantly reduced in isolated small MAs of ANG II (14 days)-treated rats compared with sham-operated rats (Fig. 6A). As seen in Fig. 6B, relative mRNA expression levels of the rat SK4 or IK1 subunit were also significantly reduced in isolated small MAs of ANG II (14 days)-treated rats.

**DISCUSSION**

We demonstrate reduced expression of SK_{Ca} and IK_{Ca} channel proteins, SK3 and IK1, respectively, despite normal ACh-induced endothelium-dependent vasorelaxations in small MAs of ANG II-induced hypertensive rats compared with normotensive rats.

The resting membrane potential of vascular smooth muscle cells are more depolarized, associated with enhanced contractile sensitivity, in arteries from hypertensive animals (6, 26). K_{Ca} channel blockade has been shown to prevent sustained pressure-induced depolarization in cannulated rat small MAs (44). The effect of high blood pressure on membrane potential may be more pronounced in smaller resistance-sized arteries that play a significant role in the regulation of blood pressure. We previously observed a greater mRNA expression of genes encoding for SK_{Ca} and IK_{Ca} channel subunits in smaller MAs compared with larger MAs in normotensive rats (44). The effect of high blood pressure on membrane potential may be more pronounced in smaller resistance-sized arteries. In small MAs, the EDHF response, classically regarded as the non-NO and non-prostacyclin-dependent vasorelaxation, can be completely blocked by a combination of specific SK_{Ca} and IK_{Ca} channel blockers (13, 18, 28), which is in line with the earlier conclusion that large-conductance KCa (BK_{Ca}) channels do not contribute to the EDHF response in rat MAs. A study using transgenic mice (SK3T/T), in which the SK3 channel expression caused a pronounced and reversible hyperpolarizing influence in resistance arteries and that suppression of SK3 channel expression caused a pronounced and reversible hypertension (45). Given the importance of SK_{Ca} and IK_{Ca}...
channels in EDHF-mediated responses and hence blood pressure regulation, we hypothesized that the functional and molecular expression of these KCa channels would be reduced in small MAs of ANG II-induced hypertensive rats compared with normotensive rats. We have chosen the experimental model of chronic ANG II infusion using an osmotic minipump implanted in the neck of rats, which leads to a considerable rise in systolic blood pressure measured after 14 days compared with sham-operated rats. This model is an experimental model of acquired hypertension and differs from the genetic models of hypertension, such as the spontaneously hypertensive rat (SHR). This model of hypertension enables us to study the effect of a sudden drastic rise in blood pressure at adult age on changes in the expression of SKCa and IKCa channels in small resistance-sized arteries. Because of the limited amount of small MA tissue, we pooled isolated small MA segments collected from 10 rats. With this method, we were able to generate sufficient amounts of total RNA and protein extract to perform RT-PCR and Western blot analysis.

We observed no apparent impairment in ACh-induced relaxations in small MAs of ANG II-induced hypertensive rats. Furthermore, the non-NO, nonprostacyclin (PGI₂)-mediated endothelium-dependent relaxation induced by ACh, which can be attributed to the EDHF pathway, was similar in MAs of both normotensive and hypertensive rats. ANG II is a potent activator of NADPH oxidase in vascular smooth muscle cells. The formation of reactive oxygen species (ROS) generated by NADPH oxidase plays an essential role in endothelial dysfunction (23, 24). Superoxide anions can be reduced to the uncharged H₂O₂, where it can act as an EDHF depending on the species and artery (34, 41). To assess whether small MAs from ANG II (14 days)-treated rats had a greater contribution of non-NO, non-PGI₂-mediated ACh-induced relaxation caused by H₂O₂, we incubated small MAs of sham-operated and ANG II-treated rats with catalase to dismutate H₂O₂. We did not observe any significant changes in the CRCs to ACh in the presence of catalase compared with L-NAME and indomethacin-incubated segments, suggesting no significant contribution of H₂O₂ and/or other ROS in the ACh-induced relaxation of resistance-sized arteries of ANG II-treated hypertensive rats.

Studies using the same vessels obtained from SHRs and stroke-prone SHRs have observed a reduced EDHF component (31, 44). The decrease in the EDHF-mediated response has been associated to a change in the expression of gap junctional proteins, such as connexins 37 and 40 (19, 38). In SHRs, the release of vasoconstrictor prostanooids at higher doses of ACh,
and which seemed to progress with advancing age, resulted in endothelial dysfunction (46). The difference in results between our observations and results obtained in SHRs may be due to differences in the duration of the hypertension and/or compensatory mechanisms.

We next addressed the role of SKCa and IKCa in ACh-induced EDHF responses in these artery segments. UCL-1684 is one of the most potent nonpeptidic blockers of SKCa channels, with an IC50 value of 3 nmol/l (12), with little inhibitory action on IKCa channels (32). In small MAs of sham-operated rats, EDHF-mediated responses were completely blocked by UCL-1684, whereas a residual relaxation was evident in similar vessels of ANG II-induced hypertensive rats. The concentration of UCL-1684 (100 nmol/l) was well above the IC50 value of 3 nmol/l, suggesting a full blockade of SKCa channels. The smaller inhibition by UCL-1684 in SKCa-mediated vasorelaxation in small MAs of ANG II (14 days)-treated rats coincided with a downregulation of the SK3 subunit at the level of mRNA and protein.

Blockade of IKCa channels by TRAM-34 resulted in a lower reduction of the ACh-induced relaxation in MAs of ANG II (14 days)-treated rats compared with sham-operated rats. Expression of mRNA encoding for the IK1 subunit was significantly reduced in small MAs of ANG II (14 days)-treated rats, whereas protein levels of IK1 tended to be lower in these segments compared with sham-operated rats.

![Fig. 4. Effect of intermediate-conductance KCa (IKCa) inhibition on EDHF-mediated relaxations.](image-url)
ANG II has numerous actions on the vascular wall, including DNA and protein synthesis in vascular smooth muscle cells independently from pressure changes in both large and small arteries (25, 43), as well as modulation of vasomotor tone via increased ROS by activating NADPH oxidase (22). The latter has been associated with the pathology of hypertension. ROS have been proposed to be regulators of KCa channel function in various tissues (5, 9, 10). NO directly stimulated endothelial BKCa in renal arteries, whereas ROS, especially H2O2, inhibited these KCa channels (5). This ROS-induced inhibition of BKCa channels is unlikely in rat small MAs since EDHF responses are resistant to blockade with the BK Ca channel antagonist iberiotoxin in the MA (27, 49). However, the inhibitory effect of ROS on SKCa and IKCa channels is not well known. To distinguish between ANG II-induced and pressure-related changes in KCa channel function, we included a third group of rats, which were treated with ANG II (60 ng·kg⁻¹·min⁻¹) via osmotic minipumps and were euthanized 24 h later. Since these ANG II-infused rats still remained normotensive (22) and since nonpressor doses of ANG II have been shown to increase protein synthesis after 24 h (15), we could examine the pressure-independent effects of ANG II on KCa channel function. We observed a greater inhibitory effect of SKCa channel inhibition on ACh-induced EDHF-mediated responses in MAs of 1-day ANG II-treated rats compared with 14-day ANG II-treated rats. This blunted relaxation was almost comparable with the sham-operated rats, suggesting that it is

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**Figure 5.** Effect of combined SKCa and IKCa inhibition on EDHF-mediated relaxations. Concentration-response curves to ACh in 4th-order mesenteric arteries from sham-operated normotensive (A) and ANG II (14 day)-treated (B) rats incubated with l-NAME (100 μmol/l) and Indo (10 μmol/l) and contracted with U-46619 (0.1 μmol/l) in the absence (vehicle) and in the presence of UCL-1684 and TRAM-34 (n = 7 artery segments).

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**Figure 6.** Semiquantitative RT-PCR analysis of SK3 (A) and SK4 (IK1; B) KCa channel subtypes from 4th-order mesenteric arteries from sham-operated (sham) and ANG II (14 days)-treated rats. Total mRNAs were extracted and pooled from 10 rats. Representative agarose gel image of amplicons derived from RT-PCR reactions with cDNA amplified with primers specific for SK3, SK4 (IK1), and GAPDH (see MATERIALS AND METHODS) is shown. Expected fragment sizes are 701 bp (SK3), 195 bp (SK4), and 198 bp (GAPDH). Quantified densitometric analysis of SK3 (A) and SK4 (IK1; B) fragments derived from sham-operated and ANG II (14 days)-treated rats are shown. Relative mRNA expression levels are normalized to GAPDH. rSK3, rat SK3; rIK1, rat IK1. Results are expressed as means ± SE from 3 separate experiments. *P < 0.05 vs. sham.
unlikely that elevated circulating ANG II concentrations cause inactivation of SKCa channels and/or downregulation of SK3 channel expression. Inhibition of IKCa channels by TRAM-34 resulted in comparable ACh-induced and EDHF-mediated responses in sham-operated and 1-day ANG II-treated rats. Further studies are needed to clarify whether elevated and prolonged exposures to ROS inactivate SKCa and IKCa channels and whether the downregulation of SK3 and IK1 channels is caused by a pressure-dependent mechanism.

The observations presented in this study in depolarized MAs suggest that each KCa type may have separate physiological roles, which become particularly clear when they are activated individually. Indeed, Crane and colleagues (13) suggested that true endothelium-dependent hyperpolarization of smooth muscle cells in response to ACh is attributable to SKCa channels, whereas IKCa channels play an important role during ACh-induced repolarization following depolarization. Furthermore, they suggested that this differential activation likely reflects in distinct subcellular localization within endothelial cells (13).

Recently, high-resolution immunohistochemistry demonstrated colocalization of SKCa channels and connexins 37, 40, and 43 at adjacent endothelial cell gap junctions, whereas IKCa and connexin 37 were found at myoendothelial gap junctions (39). This spatial separation of KCa channels suggests a relationship between connexin function and the differential functional activity of SKCa and IKCa. The IKCa channel may allow for additional fine-tuning of the endothelial hyperpolarizing response in situations of reduced SK3 expression.

It is of interest to study the consequences of reduced expression of SK3 and IK1 in more physiological situations such as shear stress-induced vasodilatation. Hemodynamic stresses, such as circumferential wall stress and shear stress, which are altered during hypertension, may be a mechanism to alter KCa channel expression. Indeed, flow-induced arterial remodeling has been shown to activate signaling events leading to smooth muscle cell dedifferentiation in rat small MAs (47).

In summary, we have demonstrated maintained EDHF-like responses despite reduced expression of both SK3 and IK1

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**Fig. 7.** Protein expression levels of SK3 (A) and IK1 (B) subunits from 4th-order mesenteric arteries from sham-operated and ANG II (14 days)-treated rats. Total protein was extracted and pooled from 10 rats, and this pool was considered n = 1. Quantified densitometric analysis of SK3 (A) and IK1 (B) protein expression derived from sham-operated and ANG II (14 days)-treated rats. Relative protein expression levels are normalized to β-actin. Results are expressed as means ± SE from 3 separate experiments. #P < 0.001 vs. sham.
mRNA and protein in small MAs of ANG II-induced hypertensive rats. Endothelial KCa channels, in particular the SK3 channel expression, may be a novel target for therapeutic treatment or molecular manipulation for the treatment of many cardiovascular diseases such as diabetes, preeclampsia, and hypertension.

REFERENCES


