Impaired Hyperpolarization in Regenerated Endothelium
After Balloon Catheter Injury

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Abstract—Ca\(^{2+}\)-activated K\(^+\) (K\(_{Ca}\)) channels control endothelial Ca\(^{2+}\) homeostasis and the formation of vasodilators. After angioplasty, dysfunction of the regenerated endothelium leads to abnormal vasoregulation. In this study, we tested the expression and function of K\(_{Ca}\) channels in regenerated endothelium at 6 weeks after balloon catheter injury of rat carotid arteries (CAs) by using single-cell reverse transcription–polymerase chain reaction, patch-clamp techniques, and analysis of vasoreactivity. In single regenerated endothelial cells (ECs), the percentage of ECs expressing the K\(_{Ca}\) genes, rSK3 (12±8%) and rIK1 (22±9%), was significantly lower compared with the percentage of native ECs expressing these genes (rSK3 58±8%, rIK1 64±10%). In patch-clamp experiments, K\(_{Ca}\) currents and acetylcholine-induced hyperpolarization were markedly reduced in regenerated ECs (shift of membrane potential −6±3 mV) compared with those in native ECs (shift of membrane potential −21±5 mV). In pressure myograph experiments, acetylcholine-induced dilation was impaired in reendothelialized CAs compared with normal CAs. Intraluminal application of the K\(_{Ca}\) blocker apamin and charybdotoxin inhibited dilation by 30% in normal CAs but was without effect in reendothelialized CAs. Intraluminal application of 1-ethyl-2-benzimidazolinone (100 μmol/L), an opener of K\(_{Ca}\) channels, evoked dilation by 29% in normal CAs but had no effect in reendothelialized CAs. In conclusion, the impaired expression of K\(_{Ca}\) channels in regenerated endothelium results in defective hyperpolarization and impaired dilation. Thus, the impaired K\(_{Ca}\) channel function contributes to functional alterations of regenerated endothelium after angioplasty. (Circ Res. 2001;89:174-179.)

Key Words: angioplasty ■ neointima ■ endothelium ■ Ca\(^{2+}\)-activated K\(^+\) channels ■ endothelium-dependent vasodilation

The endothelium controls vascular tone by releasing vasodilating factors, such as NO, prostacyclin, and the endothelium-derived hyperpolarizing factor (EDHF). In the control of endothelial function itself, Ca\(^{2+}\)-activated K\(^+\) (K\(_{Ca}\)) channels play an important role in regulating endothelial hyperpolarization. Endothelial hyperpolarization increases the electrochemical driving force for Ca\(^{2+}\) influx into the endothelium and thus indirectly augments Ca\(^{2+}\)-dependent formation of vasodilating factors. Moreover, endothelial hyperpolarization might directly induce dilation, given that endothelial hyperpolarization is propagated via gap-junctional coupling to the underlying vascular smooth muscle cells (VSMCs), leading to the closure of voltage-gated Ca\(^{2+}\) channels.

Balloon catheterization, a procedure to relieve arterial stenosis and to increase blood flow, induces endothelial ablation and stimulates the proliferation of intimal VSMCs, which increases the risk of restenosis. After angioplasty, migration and proliferation of adjacent endothelium occur to reline the injured area within weeks. However, the function of this regenerated endothelium has been shown to be abnormal. For instance, in porcine coronary arteries with a regenerated endothelium, decreased relaxations to serotonin, diminished activity of G proteins, alterations of endothelium-dependent hyperpolarization, increased uptake of modified LDL, and reduced NO formation have been described. However, relaxations to other endothelium-dependent vasodilators, such as bradykinin, ADP, and calcium ionophores, were normal or moderately reduced. The function of K\(_{Ca}\) channels has not been characterized in regenerated endothelium after angioplasty, so far. We hypothesized that a disturbed function of K\(_{Ca}\) channels might be present in regenerated endothelium, leading to a decreased endothelium-dependent relaxation. Therefore, we performed a study to compare the expression and function of K\(_{Ca}\) channels in native and regenerated endothelium after balloon catheter injury (BCI) of the rat carotid artery (CA). K\(_{Ca}\) expression and function were measured by single-cell reverse transcription (RT)–polymerase chain reaction (PCR), patch-clamp techniques in situ, and analysis of in vitro vasoreactivity with use of a pressure myograph.
Material and Methods
Three- to 4-month-old male Sprague-Dawley rats (450 to 500 g; Charles River Laboratories, Sulzfeld, Germany) were subjected to BCI of the left CA by use of a 2F embolectomy catheter. For controls, the right CA was exposed but not catheterized. Rats were killed 6 weeks after BCI. Left and right CAs were excised and cleaned of connective tissue. Neointimal thickening and reendothelialization were determined in paraffin-embedded and differential nonserial cross sections by light microscopy (not shown). Animal study protocols were approved by the Animal Care and Use Committee.

Cell Harvesting
Freshly isolated CA segments (1 to 2 mm in length) were cut open longitudinally and fixed on a holding capillary to give direct access to the luminal surface. For cell harvesting, vessel slices were preincubated with 0.05% trypsin and 0.02% EDTA in PBS without Ca2+/Mg2+ for up to 15 minutes. For washout, CAs were superfused with PBS for 5 minutes. Under microscopic control, a single endothelial cell (EC) was selectively fixed with the patch pipette and mechanically detached from the CA. For negative controls, samples of bath solution were aspirated next to the EC.

Reverse Transcription
ECs were transferred to a tube containing 1 μL “first-strand” buffer, 0.5 μL dNTPs (10 mmol/L each), 1 μL “random” hexamer primer (10 pmol/μL), 1 μL dithiothreitol (0.1 mmol/L), and 0.5 μL RNase inhibitor (40 U/μL). After one freeze-thaw cycle to induce breakdown of the cell membrane, 0.5 μL SuperScript RT (200 U/μL, Life Technologies) was added, and the final volume (~10 μL) was incubated for 1 hour at 37°C.

Polymerase Chain Reaction
Single-cell RT-PCR was performed as described previously. First and “nested” primer pairs (TIB MOLBIOL) for rat endothelial NO synthase (eNOS), the small-conductance K Ca channel genes (rSK1–3), and the intermediate-conductance K Ca channel gene (rIK1) were selected to span intronic sequences. The use of these primer pairs yielded no PCR product of expected size from genomic DNA of cell samples processed without RT (n = 5) or of 50 ng/μL rat DNA after two PCR rounds. GenBank accession numbers are as follows: rIK1, AF156554; rSK1, AF000973; rSK2, U69882; rSK3, U69884; and eNOS, AJ011116.

In a single-cell sample, cDNAs of rIK1 and rSK1–3 were coamplified along with eNOS cDNA. A first “multiplex” PCR was performed in a final volume of 50 μL containing 5 μL PCR buffer (10 ×), 2 μL dNTPs (10 mmol/L each), 3 μL MgCl2 (1 mol/L), 1 μL of each sense and antisense primer (10 pmol), ~10 μL RT product, and 0.5 μL Taq DNA polymerase (5 U/μL) (all Life Technologies) by using a Biozym Maxicycler PTC 9600. After initial denaturation for 5 minutes at 94°C, PCR amplification was carried out for 30 seconds at 94°C, 1 minute at 55°C, 1 minute at 72°C, and 10 minutes at 72°C for 50 cycles. In a second multiplex PCR round with nested primers, 5 μL of the first PCR product was used for reamplification (45 cycles, annealing temperature 60°C). PCR products were analyzed on a 2% agarose gel containing ethidium bromide. A 50-bp DNA ladder served as molecular weight markers. The identities of PCR products were verified by sequencing with the use of an ABI 377 automatic sequencer (ABI Prism). Respective forward and reverse primers were as follows: for rIK1, first primers were 5'-ACACCATCAGCTTCAGT-3' and 5'-GAGGAGCTTCTCAGTTGTA-3'; for rSK1, first primers were 5'-GACACACCTAC-GTTGGGAGG-3' and 5'-AGCTCCGACCACCTCCATA-3'; and nested primers were 5'-GCTGGAGAAACACGTGCAACA-3' and 5'-TTGCTGTATCCTCAGT-3'. For eNOS, first primers were 5'-GGAGGAGGCTTCTCAGTTGTA-3' and 5'-GAGGAGCTTCTCAGTTGTA-3'; for rSK1, first primers were 5'-GACACACCTAC-GTTGGGAGG-3' and 5'-AGCTCCGACCACCTCCATA-3'; and nested primers were 5'-GCTGGAGAAACACGTGCAACA-3' and 5'-TTGCTGTATCCTCAGT-3'.

Patch-Clamp Experiments
Membrane currents were recorded with an EPC-9 (HEKA) patch-clamp amplifier with the use of voltage ramps (duration 1000 ms) from ~100 to 100 mV and were low-pass-filtered (~3 dB, 1000 Hz) at a sample time of 0.5 ms. Membrane potentials (V m) were recorded in the current-clamp mode. Plate pipettes had a tip resistance of 2 to 4 MΩ in symmetrical KCl solution. If not otherwise stated, the pipette solution contained (mmol/L) KCl 135, MgCl2, 4, EGTA 1, CaCl2 0.955 ([(Ca2+]o) = 3 mmol/L], and HEPES 5 (pH 7.2). The NaCl bath solution contained (mmol/L) NaCl 137, Na2HPO4 4.5, KCl 3, KH2PO4 1.5, MgCl2 0.4, and CaCl2 0.7 (pH 7.4). Experiments were performed at 37°C. Data analysis was performed as described previously. If not otherwise stated, leak currents were not subtracted before or during data acquisition.

Pressure Myograph Experiments
Injured (n = 5) and normal (n = 9) CA segments of 3 to 4 mm in length were cannulated with micropipettes in an experimental chamber mounted on the stage of a Zeiss Axiosvert 100. Vessel diameter was continuously monitored with a video camera. The bath and perfusion solution contained (mmol/L) NaCl 145.0, NaH2PO4 1.2, KCl 4.7, MgSO4 1.2, CaCl2 2.0, glucose 5.0, pyruvate 2.0, and MOPS buffer 3.0, along with 1 g/100 mL BSA (pH 7.4 at 37°C). CAs were pressurized to 80 mm Hg with a pressure myograph system (J.P. Trading P100) and continuously perfused at a flow rate of 0.6 mL/min and at constant intraluminal pressure. After an initial equilibration period, CAs were preconstricted with 1 μmol/L phenylephrine in the bath solution. After development of stable tone, vasodilatory responses were determined by perfusion with increasing concentrations of acetylcholine (ACh, 1 × 10−6 to 2 × 10−4 mol/L) alone or in combination with 2 μmol/L apamin (APA) and 0.1 μmol/L charybdotoxin (CTX). 1-Ethyl-2-benzimidazolinone (1-EBIO, 1 to 200 μmol/L) was made as ~1000-fold stock solution in dimethyl sulfoxide and applied intraluminally. In a subset of experiments, the bath and perfusion medium contained the NO synthase inhibitor Nω-nitro-L-arginine (L-NNA, 100 μmol/L) and the cyclooxygenase inhibitor indomethacin (10 μmol/L). Diameter changes were expressed as a percentage of the maximal dilation measured in response to 10 mmol/L sodium nitrite. 1-EBIO was obtained from TOCRIS; all other chemicals and toxins were from Sigma Chemical Co.

Statistical Analysis
Data are given as mean ± SE. The Mann-Whitney U test was used to assess differences between groups. A value of P < 0.05 was considered significant.

Results
KCa Function and Expression in Single Native and Regenerated ECs
To determine KCa function, we performed whole-cell patch-clamp experiments in situ in electrically uncoupled single CAs. For activation of KCa currents, cells were dialyzed with a pipette solution containing 3 μmol/L [Ca2+]o. In 9 of 10 native ECs from normal CAs, dialysis with Ca2+ induced a significant activation of outward current with slight inward rectification at positive Vm s. The reversal potentials (Vrev s) extrapolated from current-voltage relations were ~45 ± 6 mV, thus indicating K+ channel activation (Figure 1A). However, in addition to K+ channel activation, dialysis with Ca2+ also induced a significant activation of Ca2+-dependent nonselect-
tive currents previously identified in ECs, thus explaining the apparent discrepancy between the estimated K+ equilibrium potential of −89 mV and the measured \( V_{rev} \), K+ current density determined at a holding potential of 0 mV to minimize contamination by nonselective currents and standardized to cell capacity was 24±6 pA/pF at 0 mV in 4.5 mmol/L K+ bath solution and 140 mmol/L K+ pipette solution. The Ca\(^{2+}\)-activated outward current was blocked by 53±9% in the presence of 1 µmol/L APA (n=4, \( K_d \) 0.57±0.08 µmol/L; Figure 1B), by 42±11% in the presence of 100 nmol/L CTX (n=5, \( K_d \) 7.0±2 nmol/L), and by 41±9% in the presence of 1 µmol/L clotrimazole (CLT), a more selective blocker of the intermediate-conductance \( K_{Ca} \) (maxi K) had no effect on \( K_{Ca} \) currents in ECs. Mean current densities at 0 mV (7±2 pA/pF at 0 mV and 53±6 pA/pF at 0 mV) and \( V_{rev} \) of those three ECs exhibiting a \( K_{Ca} \) current fraction were 15±1 pA/pF and −17±5 mV, respectively (Figure 2A). To ensure the harvest of ECs, cell samples were analyzed for eNOS expression by use of the multiplex RT-PCR technique. EC-specific eNOS expression was detected in 43% and 54% of cell samples from native and regenerated endothelium, respectively. Medium samples (n=15) yielded no PCR products.

Expression rSK3 and rIK1 was detected in 58±8% and 64±10%, respectively, of native eNOS+ EC samples. However, in regenerated eNOS+ ECs, the expression of rSK3 and rIK1 was detected in only 12±8% (P<0.01) and 22±9% (P<0.05), respectively (Figure 2B). In eNOS+ cell samples from normal CAs, expression of rSK3 and rIK1 was detected in 8±4% and 3±2%, respectively. In eNOS− cell samples from injured CAs, the expression of rSK3 was detected in 1±1%. The expression of rIK1 was not detected in these samples. This indicates that the expression of rSK3 and rIK1 is greatly restricted to eNOS+ samples and thus to ECs from which mRNA was harvested successfully. The expression of other SK subtypes, rSK1 and rSK2, was not detected in either native or regenerated ECs by single-cell RT-PCR (n=10, Figure 2C).

**In Vitro Vasoreactivity in Normal CAs and in CAs After BCI**

In vitro vasoreactivity to endothelium-independent responses to phenylephrine and sodium nitrite was similar in pressurized non–endothelium-denuded CAs (n=5) at 6 weeks after BCI compared with normal CAs (n=12): phenylephrine constricted injured CAs by 82±11 µm and normal CAs by 100±12 µm. Sodium nitrite dilated injured CAs by 86±12 µm and normal CAs by 123±32 µm. For determination of endothelium-dependent dilation, ACh was applied to the lumen of injured CAs and normal CAs. ACh induced a dose-dependent dilation (EC50 14 nmol/L) in normal CAs (Figure 3, left), which was completely abolished after removal of the endothelium (n=3, not shown). At the highest dose tested (200 nmol/L ACh), the outer diameter increased by 72±11 µm. When ACh in combination with 2 µmol/L APA and 100 nmol/L CTX was applied to the endothelium (n=7), ACh-induced dilation was significantly reduced by 30%, and a right shift of the dose-response curve was observed (EC50 50 nmol/L, P<0.05; Figure 3, left). After preincubation with 100 µmol/L L-NNa and 10 µmol/L indomethacin to assess the contribution of EDHF-mediated vasodilation, the remaining NO- and prostacyclin-independent vasodilation induced by 0.2 µmol/L ACh (18±2%, n=7) was abolished when ACh was applied in combination with CTX and APA (1±1%, P<0.05; n=4).

In injured CAs, ACh-induced dilation was severely impaired (Figure 3, right). At the highest dose tested (200 nmol/L), outer diameter increased by 14±5 µm (P<0.01). CTX and APA had no significant effect on the remaining ACh-induced dilation (n=4).

Intraluminal application of 1-EBIO induced dose-dependent dilation of normal CAs (EC50 110 µmol/L; Figure 3, right). The outer diameter increased by 61±12 µm at the highest dose (200 µmol/L) tested. This dilation was greatly reduced when 1-EBIO was applied together with APA and...
CTX. Perfusion of CA with vehicle substance (dimethyl sulfoxide #0.1%) alone was without effect on vessel diameter (n = 4). In injured CAs (Figure 3), 1-EBIO–induced dilation was absent in four of five vessels, and only a small dilation was observed in one vessel at the highest dose tested (200 μmol/L, increase of outer diameter by 5 μm).

Discussion

After BCI, an abnormal function of the regenerated endothelium could be shown by us in this rat carotid model of BCI and has also been reported in different animal models by others.11–15 We tested the hypothesis that this disturbed function of the regenerated endothelium might be due to an impaired function of endothelial KCa channels. Our findings of a decreased expression and function of endothelial rSK3 and rIK1 and the lack of dilatory responses to a KCa-selective channel opener in injured CAs support this hypothesis.

Endothelial KCa in Rat CAs

In situ whole-cell patch-clamp experiments in native ECs from normal CAs, we observed hyperpolarizing Ca2+-dependent K+ currents, which exhibited the characteristics of SK and IK1 with respect to Ca2+ sensitivity, slight inward

mean±SE (**P<0.01, Mann-Whitney U test). Inhibition of ACh-induced hyperpolarization in native ECs by 1 μmol/L APA and 1 μmol/L CLT is shown. H, Augmentation of ACh-induced hyperpolarization in native EC by 100 μmol/L 1-EBIO.
Figure 3. A, Original tracings illustrating vasodilator responses to intraluminal application of 0.2 μmol/L ACh and 100 μmol/L 1-EBIO in normal CA (left) and injured CA (right) under control conditions and in combination with 2 μmol/L APA and 100 nmol/L CTX. Vessels were preconstricted with 1 μmol/L phenylephrine (PE). Endothelium-independent vasodilation and maximal vasodilatation were induced by extraluminal application of 10 mmol/L sodium nitrite (SN) and 60 mmol/L KCl, respectively. Boxes indicate exposure intervals followed by washout. B and C, Dose-dependent vasodilator responses to intraluminal application of ACh (B) or 1-EBIO (C) in normal and injured CAs. Values (percentage of maximal dilation) are given as mean±SE (*P<0.05 and **P<0.01, Mann-Whitney U test).

Current-clamp experiments in endothelial layers supported this interpretation because the combination of APA and CLT, with respect to IK1, reversed ACh-induced hyperpolarization. This indicates that in contrast to the aorta, endothelial hyperpolarization in the CA is mediated by activation of both small and intermediate KCa (SK and IK1, respectively) channels. In single-cell RT-PCR experiments, we demonstrated that single ECs in situ expressed rSK3 and rIK1 genes, whereas expression of the other SK subtypes, rSK1 and rSK2, was not detected. In previous studies, rSK3 expression was detected in rat neuronal cells and in skeletal muscle,24 in which SK activity mediates afterhyperpolarization. IK1 expression was observed in human lymphocytes20,21 and human fibroblasts,23 in which IK1 expression was related to mitogenic cell growth and proliferation. In a recent study on human ECs of the mesenteric artery, we could show that IK1 mediates endothelial hyperpolarization in response to bradykinin.17 The results of the present study indicate that expression of rSK3 and rIK1 genes confers endothelial hyperpolarization in rat CAs.

In pressure myograph experiments, we could demonstrate that the combination of APA and CTX, when selectively applied to the endothelium, significantly blunted the dilatory response to ACh. CLT, the more selective blocker of IK1, was not tested in this experimental setup because it also inhibits cytochrome P-45021 and therefore could diminish the production of EDHF-related compounds.3 Our findings of blunted dilatory response to ACh in the presence of APA/CTX indicated that activation of endothelial SK and IK1 increases the formation of vasodilators in response to stimulation by humoral factors. Moreover, the opening of SK and IK1 by application of 1-EBIO to the endothelium induced dilation, thus indicating that endothelial hyperpolarization induces dilation by itself. Such a role of KCa channels in the endothelium has been proposed to be important in other species and other vascular beds, although the type of endothelial KCa channel being involved may differ substantially between species and the vascular beds investigated.17,25,26

KCa Function in Regenerated Endothelium

In regenerated endothelium, we observed a greatly blunted hyperpolarization in response to ACh, decreased hyperpolarizing KCa whole-cell currents in response to intracellular dialysis with Ca2+, and diminished expression of rIK1 and rSK3 at the single-cell level. Moreover, in pressure myograph experiments, intraluminal application of 1-EBIO failed to induce considerable dilation, and the combination of APA and CTX had no detectable effect on the remaining ACh-induced dilation. Taking together the results obtained from patch-clamp experiments, single-cell RT analysis, and measurements of in vitro vasoactivity, we conclude that the regenerated endothelium has a reduced capacity to hyperpolarize and dilate because of the decreased expression of KCa channels. In native endothelium, KCa activation leads to endothelial hyperpolarization and thereby increases the electrochemical driving force for Ca2+ influx, which was interpreted as a positive-feedback mechanism for the production of vasodilating factors.4–7 However, because blocking of SK and IK1 only partially blunted ACh-induced vasodilation, endothelial hyperpolarization is not an overall prerequisite in mediating endothelium-dependent vasodilation. Thus, this presumably implies that there is still a substantial synthesis of...
NO and prostacyclin even when the electrochemical driving force for Ca\(^{2+}\) entry is not increased by ACh-induced hyperpolarization. Moreover, blocking of endothelial hyperpolarization might affect only EDHF-mediated vasodilation. There are several lines of evidence indicating that K\(_{Ca}\)-mediated endothelial hyperpolarization is directly propagated via gap junctions to VSMCs, leading to the closure of voltage-dependent Ca\(^{2+}\) channels and thus inducing relaxation. Also, K\(^{+}\) efflux through endothelial K\(_{Ca}\) channels has been shown to induce relaxation of VSMCs by stimulating inwardly rectifying K\(^{+}\) channels in VSMCs and was therefore proposed to serve as an EDHF. In the present study, blocking of endothelial hyperpolarization prevented such an EDHF-mediated vasodilation, thus indicating that activation of endothelial K\(_{Ca}\) channels is essential for non-NO and non-prostacyclin-mediated vasodilation.

Therefore, in regenerated endothelium, the decreased expression of K\(_{Ca}\) channels finally results in defective dilation. However, the small extent of remaining and K\(_{Ca}\)-independent ACh-induced vasodilation also suggests that presumably other mechanisms, such as reduced function of G proteins and diminished NO formation, are also impaired.

In conclusion, the impaired K\(_{Ca}\) function could contribute, at least in part, to the severely impaired function of the regenerated endothelium after angioplasty. Moreover, endothelial K\(_{Ca}\) channels can now be considered a new target for pharmacological treatment or gene therapy to improve vessel function after angioplasty.

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