Twenty-Four-Hour Exposure to Altered Blood Flow Modifies Endothelial Ca\(^{2+}\)-Activated K\(^+\) Channels in Rat Mesenteric Arteries\(^{[S]}\)

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ABSTRACT

We tested the hypothesis that changes in arterial blood flow modify the function of endothelial Ca\(^{2+}\)-activated K\(^+\) channels (calcium-activated K\(^+\) channel (K\(_{Ca}\)), small-conductance calcium-activated K\(^+\) channel (SK3), and intermediate calcium-activated K\(^+\) channel (IK1)) before arterial structural remodeling. In rats, mesenteric arteries were exposed to increased [HF] or reduced blood flow [LF] and analyzed 24 h later. There were no detectable changes in arterial structure or in expression level of endothelial nitric-oxide synthase, SK3, or IK1. Arterial relaxing responses to acetylcholine and 3-oxime-6,7-or in expression level of endothelial nitric-oxide synthase, SK3, or 24 h later. There were no detectable changes in arterial structure /HF] or reduced blood flow [LF]. Reduced blood flow selectively blunts EDHF relaxation in resistance arteries through inhibition of the function of K\(_{Ca}\) channels. An increase in blood flow leads to a more prominent role of IK1 channels in this relaxation.

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The endothelial small- and intermediate-conductance calcium-activated K\(^+\) channels (K\(_{Ca}\), 2.3 or SK3 and K\(_{Ca}\), 3.1 or IK1, respectively) have been proposed to initiate endothelium-derived hyperpolarizing factor (EDHF) signaling in resistance-sized arteries (Edwards et al., 1998; Burnham et al., 2002; Crane et al., 2003; Eichler et al., 2003; Hilgers et al., 2006). These calcium-activated K\(^+\) channel (K\(_{Ca}\)) channels can be activated by pharmacological stimuli to mediate K\(^+\) efflux and result in endothelial hyperpolarization with subsequent smooth muscle hyperpolarization, leading to closure of voltage-activated Ca\(^{2+}\)-channels and relaxation. K\(_{Ca}\) channels and other mechanosensitive cation channels, such as endothelial transient receptor potential V4 channels, can play a crucial role in acute endothelium-dependent vasodilator responses to elevated shear stress (Olesen et al., 1988; Köhler et al., 2006). Indeed, pharmacological blockade of K\(_{Ca}\) channels blunts shear stress-induced vasodilatations in isolated small arteries (Popp et al., 1998), suggesting release of EDHF and/or spread of an electrical current via activation of K\(_{Ca}\) channels. Whether the expression of K\(_{Ca}\) channels is modulated by in vivo shear stress alterations remains elusive. Given their distinct spatial location in the endothelial layer (SK3 at endothelial cell borders and IK1 at sites of

Abbreviations: K\(_{Ca}\), calcium-activated K\(^+\) channel; SK3, small-conductance calcium-activated K\(^+\) channel; IK1, intermediate calcium-activated K\(^+\) channel; EDHF, endothelium-derived hyperpolarizing factor; ACh, acetylcholine; NS309, 3-oxime-6,7-dichlore-1H-indole-2,3-dione; LF, low flow; HF, high flow; NF, normal flow; MA, mesenteric artery(ies); KRb, Krebs-Ringer buffer; NE, norepinephrine; PHE, phenylephrine; INDO, indomethacin; l-NAME, N\(^{\text{g}}\)-nitro-l-arginine methyl ester; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinazoline-1-one; UCL 1684, 6,12,19,20,25,26-hexahydro-5,27:13,18,21,24-trietheno-11,7-metheno-7H-dibenzo[b,n] [1,5,12,16]tetraazacyclotricosine-5,13-dium dibromide; TRAM-34, 1-[[2-chlorophenyl]diphenylmethyl]-1H-pyrazole; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; pEC\(_{50}\), sensitivity; E\(_{\text{max}}\), maximal effect.
myoendothelial gap junctions; Sandow et al., 2006; Dora et al., 2008), it may be expected that the expression and function of KCa channels is under differential control by the frictional force of blood flow. Hence, we investigated whether subchronic (24-h) alterations in shear stress modulate expression and function of SK3 and IK1 channels in rat mesenteric arteries. This time point of 24 h after ligation reflects a transition from acute flow-mediated responses to structural flow-induced remodeling processes. Previously, relationships were proposed between acute vasomotor responses and chronic arterial structural responses to altered blood flow (De Mey et al., 2005). Understanding the molecular events that occur in response to early changes in arterial blood flow may provide us with more insight into the structural arterial changes in response to chronic alterations in blood flow. The function of each KCa channel was analyzed by recording relaxing responses to acetylcholine (ACh) and the KCa channel activator NS309 during inhibition of NO synthases, cyclooxygenases, and soluble guanylate cyclase. We used a surgical ligation method in which first-order mesenteric arteries were either exposed to low flow (LF; -90%), high flow (HF; +90%), or normal flow (NF) in vivo (Pourageaud and De Mey, 1997; Buus et al., 2001). After several days, this intervention resulted in adaptive changes of the structural arterial diameter and wall mass (Unthank et al., 1996; Buus et al., 2001; Tuttle et al., 2001). After 1 day, LF, HF, and NF first-order mesenteric arteries were isolated to study EDHF responses to ACh and NS309 in the absence and presence of pharmacological inhibitors of KCa channel subtypes and protein expression levels of SK3 and IK1 channels using Western blots and immunohistochemistry.

Materials and Methods

Animals. In total, 32 male Wistar Kyoto rats of 12 weeks of age were obtained from Charles River (Mastricht, The Netherlands). All animals were caged separately and had free access to standard food (SRMA-1210; Hope Farms, Woerden, The Netherlands) and tap water. Experimental protocols were performed in accordance with institutional guidelines and were approved by the Ethics Committee on Experimental Animal Welfare of the Maastricht University.

Ligation Model. Small mesenteric arteries (MA) were exposed to altered blood flow via a surgical ligation method as described previously (Pourageaud and De Mey, 1997). In brief, in anesthetized (isoflurane; Abbott Laboratories Ltd., Maidenhead, UK) rats, laparotomy was performed and the mesentery was spread out on gauze. Local blood flow was lowered (LF) by distal ligation of three alternate second-order MA branches. The MA running between these had compensatory higher blood flows (HF). We previously observed in Wistar Kyoto rats that the blood flow averages 10 and 200% in LF and HF compared with second-order MA outside of the surgical area (NF) (Pourageaud and De Mey, 1997; Buus et al., 2001). Animals received buprenorphine (0.05 mg/kg s.c. Temgesic; Schering Plough, Utrecht, The Netherlands) as an analgetic before and after surgical intervention.

Pressure Myograph Experiments. Twenty-four hours after surgery, rats were killed by CO2 inhalation, and the mesentery was removed and placed in cold (4°C) Krebs-Ringer buffer (KRB) with the following composition: 118.5 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25.0 mM NaHCO3, and 5.5 mM glucose. From each experimental rat, one segment of first-order MA (4–5 mm in length) that had been exposed to LF, one segment to HF, and one segment to NF were carefully dissected. In an organ chamber, segments were cannulated at both ends on two glass micropipettes (outer diameter, 200 μm), tied with nylon knots, and incubated in 10 ml of calcium-free physiological salt solution of the following composition: 144 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 14.9 mM HEPES, and 5.5 mM glucose, pH 7.4. The organ chamber was placed on the stage of an inverted microscope (Nikon, Tokyo, Japan) equipped with a black-and-white video camera (Stemmer, Puchheim, Germany). An arteriograph system (Living Systems Instruments, Burlington, VT) analyzed the signal obtained from the video image and continuously determined the wall thickness and internal diameter, while intraluminal pressure was controlled (Halpern et al., 1984). Internal diameters and wall thickness were recorded using PowerLab and Chart 5 software (ADInstruments Ltd., Chalgrove, Oxfordshire, UK). Passive pressure-diameter relations were constructed as described previously (Hilgers et al., 2004).

Wire-Myograph Experiments. From each experimental rat, segments of first-order MA (2 mm in length) that had been exposed to LF, HF, or NF in vivo (for 1 day) were carefully dissected and mounted in a wire-myograph (Danish Myotech, Aarhus, Denmark) for the recording of isometric force development (Hilgers et al., 2006). Segments were incubated for 0.5 h in KRB that was continuously aerated with 95% O2, 5% CO2 and maintained at 37°C. Each experiment started by progressively stretching the arterial segment to the diameter at which the largest contractile response to 10 μM norepinephrine (NE) could be obtained (optimal diameter). To exclude any vasodilator influences of sensory motor nerves (De Mey et al., 2008), arteries were exposed to 1 μM capsaicin (during 20 min).

During contraction with a single concentration of phenylephrine (PHE; 20 μM), relaxing responses to the endothelium-dependent muscarinic vasodilator ACh (0.001–10 μM) were recorded in the absence of any inhibitors (control). After washing and a 30-min rest period, arterial segments were again contracted with 20 μM PHE. When a stable contraction was achieved, relaxing responses to the KCa channel activator NS309 (0.1–12.8 μM; Strøe et al., 2004) were recorded. The same segments were then incubated for 0.5 h with 10 μM indomethacin (INDO; inhibitor of cyclooxygenases), 100 μM Nω-nitro-l-arginine methyl ester (l-NAME; inhibitor of NO synthases), and 10 μM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; inhibitor of the NO-sensitive soluble guanylate cyclase), and relaxing responses to ACh and NS309 were repeated. To study the contribution of each KCa channel subtype, EDHF-mediated relaxations were always studied in the combined presence of l-NAME, INDO, and ODQ to rule out any potential interference of NO and prostaglandins with KCa channels (Bolotina et al., 1994). UCL 1684 at 1 μM (Romey et al., 1984) was used to block small-conductance KCa channels, and 10 μM TRAM-34 (Wulff et al., 2000) was used to block intermediate-conductance KCa channels. This concentration for TRAM-34 was chosen in accordance with our recently published article (Hilgers and Webb, 2007). According to the Guide to Recepiors and Channels (Alexander et al., 2008), the nomenclature for small-conductance KCa channels is KCa2.2 (SKX) and, for intermediate-conductance KCa channels, it is KCa3.1 (SK4, IK1). The endothelial SK3 isoform is expressed in arteries. For simplicity, SK3 and IK1 are used in the remainder of the text. These antagonists were tested individually or in combination.

Because NS309 can activate both human KCa subtypes, but with a slight (2- to 4-fold) selectivity for IK1 over SK3 (Strøe et al., 2004), the individual contribution of a KCa channel subtype upon NS309 activation was always analyzed in the combined presence of l-NAME, INDO, and ODQ and the opposing KCa channel blocker. For example, to address the IK1-mediated responses, segments were incubated with l-NAME, INDO, ODQ, and UCL 1684. In a subset of segments, the endothelium was mechanically removed by gently rubbing the lumen with an equine hair (Oso et al., 1989).

Western Blotting. At 1 day postflow-modifying surgery, three 10-mm-long first-order MA that had been exposed to LF, HF, or NF were freed of adipose and connective tissue and quickly snap-frozen in liquid nitrogen and kept at -80°C until protein expression analysis. Segments were homogenized in cold (4°C) radioimmunoprecipitation assay buffer [50 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 0.25%...
deoxycholic acid, 1% Nonidet P-40, and 1 mM EDTA enriched with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM Na$_2$VO$_4$. The homogenate was centrifuged at 15,000 g for 30 min at 4°C. The supernatant was kept on ice. Protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Twenty micrograms of protein was loaded and separated by SDS-polyacrylamide gel electrophoresis (10%) and subsequently transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked by treatment with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20, anti-K$_{C_3.1}$ (1:200, Alomone Labs, Jerusalem, Israel), anti-K$_{C_2.3}$ N-term (Alomone Labs, Temecula, CA) and kept overnight at 4°C. After incubation with secondary antibodies, signals were revealed with chemiluminescence autoradiography and quantified densitometrically by determining the ratio between protein and GAPDH pixel density.

**Immunostaining.** After the passive pressure-diameter curves, the segments were fixed at a pressure of 80 mm Hg in 4% phosphate-buffered formalin. Fixed vessels were embedded in paraffin, and cross-sections (4 μm) were subjected to immunohistochemistry using a peroxidase second step approach (swine anti-rabbit horseradish peroxidase). Endothelial and smooth muscle cell nuclei were stained with hematoxylin. Primary antibodies were directed against IK1 (anti-K$_{C_3.1}$, 1:1600; Alomone Labs) or SK3 (anti-K$_{C_2.3}$ N-term, 1:3000; Alomone Labs). Negative control stainings were performed where the primary antibody was omitted to check whether the staining was specific. Video images were taken from cross-sections using an axioline (Carl Zeiss, Jena, Germany) and a standard charge-coupled digital camera (model DFC 290; Leica, Wetzlar, Germany).

**Drugs.** Acetylcholine, phenylephrine, norepinephrine, and L-NNAME were purchased from Sigma (Zwijndrecht, The Netherlands) and dissolved in KRB. TRAM-34 and NS309 (Sigma) were dissolved in DMSO. Indomethacin (Sigma) was dissolved in ethanol. ODQ (Calbiochem, Darmstadt, Germany) was dissolved in DMSO. UCL 1684 (Tocris Bioscience, Bristol, UK) was dissolved in DMSO. The concentrations of DMSO used never exceeded 0.3% (v/v) in the organ bath and were observed previously to not significantly modify contractile responses (Hilgers and De Mey, 2009).

**Data and Statistical Analysis.** Contractile responses were expressed as a percentage of the maximal contractile response to 10 μM NE before the administration of any pharmacological inhibitor. Relaxing responses were expressed as a percentage of the maximal contractile response to 20 μM PHE. Individual concentration-response curves were fitted to a nonlinear sigmoid regression curve (Prism 5.0; GraphPad Software Inc., San Diego, CA). Sensitivity (pEC$_{50}$) and maximal effect (E$_{max}$) are shown as mean ± S.E.M. Statistical significance of differences and analyses were performed using either one-way analysis of variance (comparison of pEC$_{50}$ and E$_{max}$) or two-way analysis of variance (comparison of concentration-response curves). A Bonferroni post hoc test was used to compare multiple groups. A P value <0.05 was considered statistically significant.

**Results**

**Arterial Structure.** Twenty-four hours after flow-modifying surgery, no significant changes in internal diameter were observed. Internal diameter measured at an intraluminal pressure of 120 mm Hg was 408 ± 20 μm (n = 8) for NF, 371 ± 28 μm (n = 6) for LF, and 405 ± 20 μm (n = 8) for HF. Media cross-sectional area was comparable for NF, HF, and LF arteries (11.0 ± 1.8, 10.5 ± 1.8, and 13.2 ± 3.7 × 10$^3$ μm$^2$, respectively).

**Contractile Reactivity.** Optimal diameters of HF arteries measured in the wire-myograph were significantly larger compared with NF vessels (323 ± 5 versus 295 ± 5 μm). Optimal diameters of LF arteries (284 ± 6 μm) were comparable with their NF counterparts. Endothelial denudation did not affect optimal diameters in NF, LF, and HF arteries (296 ± 5, 312 ± 11, and 331 ± 11 μm, respectively). Contractile responses to 10 μM NE correlated well with optimal diameters. The sensitivity (pEC$_{50}$) to phenylephrine did not differ significantly between NF, LF, and HF arteries (5.57 ± 0.04, 5.51 ± 0.04, and 5.56 ± 0.04, respectively). The maximal contraction was significantly larger in HF vessels (4.50 ± 0.21 N/m) compared with LF (3.90 ± 0.16 N/m) but not with NF vessels (4.06 ± 0.15 N/m). Endothelial denudation did not affect maximal contraction to norepinephrine in NF, LF, and HF arteries (3.36 ± 0.49, 4.46 ± 0.49, and 4.20 ± 0.40 N/m, respectively).

Relative maximal contractions to phenylephrine (20 μM), expressed as percentage of maximal contraction to 10 μM NE, in the absence of any inhibitors was significantly larger in LF (99 ± 4%) compared with NF (91 ± 4%) but not with HF (94 ± 2%). Endothelial denudation or coinoculation of endothelium-intact arteries with L-NAME (100 μM), INDO (10 μM), and ODQ (10 μM) did not affect basal tone but significantly increased maximal contraction to comparable levels in NF, HF, and LF arteries (111 ± 5, 113 ± 4, and 112 ± 3%, respectively, for endothelium-denuded and 106 ± 3, 107 ± 3, and 107 ± 3%, respectively, for L-NAME-, INDO-, and ODQ-treated arteries). Additional incubation with UCL 1684 (1 μM) did not significantly alter basal tension and maximal contractile responses to PHE irrespective of the in vivo blood flow. Additional incubation with TRAM-34 (10 μM) did not affect basal tone and maximal contraction to PHE in NF arteries (102 ± 4%) but significantly reduced maximal contraction to PHE in HF (96 ± 3%) and LF (94 ± 3%) arteries. Coincubation with both UCL 1684 and TRAM-34 resulted in comparable maximal contractions to PHE in NF, HF, and LF arteries (96 ± 3, 96 ± 3, and 95 ± 2%, respectively). In some LF arteries (five of eight), but not in NF and HF arteries, combined incubation with L-NAME, INDO, ODQ, UCL 1684, and TRAM-34 resulted in a small increase in basal tone (6 ± 3%).

**Relaxing Responses to ACh during α1-Adrenergic Contraction.** In the absence of any inhibitors, sensitivity (pEC$_{50}$) to ACh did not differ between NF and HF vessels (pEC$_{50}$ of 7.67 ± 0.04 versus 7.71 ± 0.03; Fig. 1, A and B) but was significantly reduced in LF vessels (7.25 ± 0.05; Fig. 1C). Mechanical endothelial denudation resulted in a complete absence of ACh-induced relaxation in NF and LF arteries but a small residual relaxation (E$_{max}$ of 22 ± 10%) was still observed in HF arteries (Fig. 1).

**ACh-Induced EDHF-Mediated Relaxations.** EDHF-mediated relaxations were measured in vessels treated with L-NAME (100 μM), INDO (10 μM), and ODQ (10 μM). In NF vessels, these inhibitors resulted in a statistically significant rightward shift of the concentration-response curves to ACh (∆pEC$_{50}$ of 0.54 ± 0.17) without affecting E$_{max}$ (97 ± 1%; Fig. 1A). In HF vessels, the ∆pEC$_{50}$ (0.44 ± 0.12) and E$_{max}$ (96 ± 1%) values were similar to those of NF vessels (Fig. 1B), but sensitivity to ACh was significantly higher in HF compared with NF (7.26 ± 0.04 versus 7.13 ± 0.04, respectively). In LF arteries, a marked reduction of the EDHF-mediated response...
was observed ($\Delta pEC_{50}$ could not be determined, whereas $E_{\text{max}}$ was only $21 \pm 8\%$; Fig. 1C).

**Contribution of IK1 Channels to EDHF-Mediated Relaxations.** To study the contribution of IK1 channels, EDHF-mediated relaxations were recorded in the presence of $N^\text{-nitro}-l$-arginine methyl ester (100 μM), indomethacin (10 μM), and ODQ (10 μM; $+l-NH_2+O$; closed squares). Segments that were exposed to NF (A), HF (B), or LF (C) were analyzed 24 h after ligation. Values are expressed as mean $\pm$ S.E.M.

![Fig. 1.](image1)

![Fig. 2.](image2)

As observed ($\Delta pEC_{50}$ could not be determined, whereas $E_{\text{max}}$ was only $21 \pm 8\%$; Fig. 1C).

**Contribution of IK1 Channels to EDHF-Mediated Relaxations.** To study the contribution of IK1 channels, EDHF-mediated relaxations were recorded in the presence of $N^\text{-nitro}-l$-arginine methyl ester (100 μM), indomethacin (10 μM), and ODQ (10 μM; $+l-NH_2+O$; circles), the selective IK$_{Ca}$ channel blocker TRAM-34 (10 μM; squares), the selective SK$_{Ca}$ channel blocker UCL 1684 (1 μM; filled circles), and the combined incubation of TRAM-34 and UCL 1684 (diamonds). Segments that were exposed to NF (A), HF (B), or LF (C) were analyzed 24 h after ligation. Values are expressed as mean $\pm$ S.E.M.

![Fig. 1.](image1)

![Fig. 2.](image2)
EDHF-mediated relaxations were recorded in the presence of L-NAME, INDO, ODQ, and the selective SK3 channel antagonist UCL 1684 (1 μM). In NF vessels, UCL 1684 caused a statistically significant rightward shift of the responses to ACh (ΔpEC50 of 0.62 ± 0.18; Fig. 2A), without significantly lowering Emax (73 ± 15%; Fig. 2A). In HF arteries, UCL 1684 caused a far greater inhibition of the EDHF response as evidenced by a greater rightward shift (ΔpEC50 of 1.56 ± 0.15) and a larger reduction of Emax (42 ± 11%; Fig. 2B). Again, in LF arteries no noticeable changes occurred in the remaining small EDHF-mediated relaxation in the presence of UCL 1684 (Fig. 2C).

**Contribution of Both IK1 and SK3 Channels to EDHF-Mediated Relaxations.** To study the combined role of IK1 and SK3 channels in EDHF-mediated ACh-induced relaxations, arteries were incubated with L-NAME, INDO, ODQ, TRAM-34, and UCL 1684. No residual relaxation was observed in response to ACh in all types of artery (Fig. 2).

**IKCa Channel Activation by NS309.** Relaxing responses of PHE (20 μM)-contracted endothelium-intact mesenteric arteries to increasing concentrations of the opener of both SK3 and IK1 channels (NS309; 0.1–12.8 μM) are shown in Fig. 3. In LF vessels, the sensitivity to this agent was significantly lower compared with NF and HF vessels (pEC50 5.80 ± 0.07 versus 6.24 ± 0.06 and 6.25 ± 0.06, respectively). In endothelium-denuded vessels, the sensitivity to NS309 was significantly reduced in all vessels. Strikingly, the pEC50 value was comparable for NF, HF, and LF vessels (5.48 ± 0.26, 5.50 ± 0.17, and 5.35 ± 0.06, respectively; Fig. 3). In all vessels, inhibition of NO and prostanoid action did not result in a statistically significant shift in pEC50 values (ΔpEC50 of −0.12 ± 0.12, 0.09 ± 0.10, and −0.11 ± 0.17 in NF, HF, and LF, respectively; Fig. 3).

**Role of IKCa Channel Subtypes in NS309-Evoked Responses.** To study the contribution of IK1 and SK3 to NS309-evoked relaxations, arteries were analyzed in the presence of L-NAME, INDO, and ODQ. Coincubation with TRAM-34 caused a marked rightward shift in the concentration-response curves to NS309 in all vessels (Fig. 4). This rightward shift was similar for NF, HF, and LF vessels (ΔpEC50 of 0.45 ± 0.10, 0.53 ± 0.11, and 0.39 ± 0.05, respectively; Fig. 4). SK3 channel blockade with UCL 1684 caused a significant rightward shift in NF (ΔpEC50 of 0.26 ± 0.05; Fig. 4A). However, in HF and LF vessels no apparent shift was observed (ΔpEC50 of 0.02 ± 0.13 and 0.18 ± 0.08, respectively; Fig. 4, B and C).

Pharmacological blockade of both IK1 and SK3 channels with TRAM-34 and UCL 1684 resulted in comparable residual relaxing responses induced by NS309 in NF, HF, and LF vessels (pEC50 of 5.51 ± 0.09, 5.57 ± 0.09, and 5.34 ± 0.21, respectively; Fig. 4). These pEC50 values did not differ from those in endothelium-denuded arteries (Fig. 3).

**Expression of Endothelial IKCa and NO Synthase in Conditions of Altered Blood Flow.** Expression of SK3, IK1, and eNOS protein was comparable for NF, LF, and HF arteries (Fig. 5). Immunohistochemical binding of paraffin-embedded cross-sections with polyclonal rabbit antibodies against IK1 showed strong staining at the endothelial layer (see Supplemental Fig.). Low staining of IK1 was observed in the medial and adventitial layers. In the cross-section of the LF artery, an IK1-positive rolling lymphocyte was visible. SK3 staining was evident at the endothelial layer, but also

**Discussion**

In the vasculature, IKCa (KCa3.1 or IK1) and SKCa channels, especially the SK3 subunit (KCa2.3), are constitutively

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**Figure 3.** Relaxing responses to the SKCa and IKCa channel opener NS309 (0.1–12.8 μM) in phenylephrine (20 μM)-contracted first-order mesenteric arteries with endothelium (+E; open symbols), without endothelium (−E; closed circles), and in intact arteries incubated in Nω-nitro-γ-arginine methyl ester (100 μM), indomethacin (10 μM), and ODQ (10 μM) (+L-N+1+O; closed squares). Segments that were exposed to NF (A), HF (B), or LF (C) were analyzed 24 h after ligation. Values are expressed as mean ± S.E.M.
expressed in endothelial cells (Köhler et al., 2000; Burnham et al., 2002). In this study, the names IK1 and SK3 are used. The importance of endothelial IK1 and SK3 channels in EDHF-mediated hyperpolarization and subsequent smooth cell relaxation in response to pharmacological stimuli is well accepted (Bychkov et al., 2002; Crane et al., 2003; Eichler et al., 2003). ACh has been the preferred choice of pharmacological stimulus to initiate an increase in endothelial cell [Ca^{2+}], which activates both endothelial IK1 and SK3 channels leading to an EDHF-mediated relaxation in contractile conditions (Crane et al., 2003). This is in contrast to quiescent mesenteric arteries where ACh activates only SK3 channels, leading to a hyperpolarization of the resting membrane potential (Crane et al., 2003). Whether chronic alterations in blood flow can modulate the expression and function of IK1 and SK3 channels has not been elucidated previously and hence was the objective of this study.

NS309, the opener of both IK1 and SK3 channels, has a 1000-fold higher potency than 1-ethyl-2-benzimidazolinone and a 30-fold higher potency than the dichloronated ethyl-2-benzimidazolinone analog (Strøeek et al., 2004). It has a slight (4-fold) preference for human IK1 channels over human SK3 channels in cultured human embryonic kidney 293 cells (Strøeek et al., 2004). In the guinea pig carotid artery, NS309 (10 \mu M) induced endothelium-dependent hyperpolarizations that could be completely inhibited by endothelial denudation and a cocktail of charybdotoxin and apamin (Leuranguer et al., 2008).

To create chronic alterations in arterial blood flow, we used a surgical rat mesenteric artery ligation procedure (Unthank et al., 1996; Pourageaud and De Mey, 1997). This model enabled us to study, in the same rats, arteries that were exposed to a normal, reduced (90%) or increased (90%) blood flow (Pourageaud and De Mey, 1997). In this setting, reduced and increased blood flow ultimately (14 days) lead to inward hypotrophic and outward hypertrophic remodeling, respectively (Unthank et al., 1996; Buus et al., 2001; Tuttle et al., 2001). Here, we focused on the early changes (after...
24 h) induced by shear stress alterations in the arterial wall, which are characterized by differential expression of genes involved in endothelial cell activation (Malek et al., 1993) and vascular smooth muscle cell dedifferentiation and extracellular matrix remodeling (Buus et al., 2001; Wesselman et al., 2004), before structural changes in lumen diameter and wall mass (Buus et al., 2001). Because adaptive remodeling was incomplete after 24 h, it is expected that wall shear stress levels have not yet been normalized. Hence, flow-reduced arteries experience lower shear stress levels and flow-loaded arteries experience higher shear stress levels compared with normal flow arteries.

Differences in relaxing activity were not due to differences in the intensity of the precontraction, because flow-loaded and flow-reduced arteries had similar sensitivity and maximal contractile responses to phenylephrine compared with normal flow arteries. In flow-loaded and normal flow arteries comparable NO- and EDHF-mediated relaxing responses to ACh were observed. However, in flow-reduced arteries the ACh-induced relaxing response was mainly dependent on NO and prostanooids, because the EDHF response was drastically reduced. This differential impairment in the EDHF response could be the result of a difference in the coupling between muscarinic-receptor activation and to the opening of IK1 and SK3 channels. Therefore, we used NS309 representing muscarinic receptor-independent activation (Strøaek et al., 2004). In this study, comparable relaxing responses to NS309 were observed for normal flow and flow-loaded arteries in the absence of any inhibitors, but the sensitivity (but not maximal relaxation) for NS309 was significantly reduced in flow-reduced arteries. Endothelial denudation significantly blunted the NS309-evoked relaxing responses in all arteries to comparable levels, confirming earlier results in human coronary arterioles (Feng et al., 2008) and guinea pig carotid arteries (Leuranguer et al., 2008). In contrast to ACh-induced EDHF responses in flow-reduced arteries, the sensitivity to NS309 was reduced, whereas a maximal relaxation persisted. These observations demonstrate different signaling mechanisms for ACh- and NS309-mediated activation of endothelial KCa channels. Because we only studied relaxing responses in the wire-myo- manograph, we are unable to conclude whether the almost absent ACh-induced EDHF response in flow-reduced arteries is due to impaired EDHF release and/or impaired conduction of hyperpolarization to the smooth muscle cells via myoendothelial gap junctions. Future electrophysiological studies are needed to address this issue. In contrast, proinflammatory influences that might occur during situations of drastic blood flow reductions (Bakker et al., 2008) could lead to the impaired endothelial-dependent relaxation. However, it is beyond the scope of this article to study markers of inflammation and their role in endothelial KCa channel activity.

Next, we addressed whether blood flow alterations resulted in differential contributions of IK1 and SK3 channels in ACh-induced and NS309-evoked relaxing responses. The contribution of individual IK1 and SK3 channels in these EDHF responses was always analyzed in conditions where NO synthases, cyclooxygenases, and soluble guanylate cyclase were blocked with L-NAME, indomethacin, and ODQ, respectively. This was done to rule out any potential interference of NO and vasodilator prostaglandins with KCa channels (Bolotina et al., 1994). We considered an inhibition of the ACh-mediated EDHF response caused by an individual KCa blocker, TRAM-34 at 10 μM (Wulff et al., 2000), for IK1 antagononism or UCL 1684 at 1 μM (Campos Rosa et al., 2000) for SK3 blockade, as a contribution of this KCa channel subtype to the EDHF response. To study the contribution of either IK1 or SK3 channels to NS309-evoked responses a “residual approach” was followed. For example, SK3 channel activation by NS309 was analyzed in the presence of TRAM-34.

In arteries exposed to elevated blood flow, blockade of either IK1 or SK3 resulted in a greater inhibition of the ACh-mediated EDHF response compared with normal flow arteries, suggesting a greater contribution of these channels in muscarinic receptor-mediated EDHF responses in these arteries. These results should be considered with caution because nonspecific antagonistic effects of endothelial KCa channel blockers, such as membrane potential-insensitive actions, might play a role. In flow-loaded arteries a greater residual NS309-evoked response was observed in the presence of UCL 1684, again pointing toward a greater involvement of IK1 channels in the EDHF response.

In all arteries, TRAM-34 caused a greater inhibition of the NS309-evoked relaxations than UCL 1684, supporting the observation that NS309 has a preference for IK1 channels over SK3 channels (Strøaek et al., 2004). Because we observed a greater inhibitory effect of UCL 1684 in the ACh-mediated EDHF response in flow-loaded arteries compared with normal flow arteries, we were unable to observe an anticipated greater residual NS309-evoked EDHF response in the presence of TRAM-34 in flow-loaded arteries. We currently have no explanation for this discrepancy. NS309-induced relaxing responses were comparable in endothelium-denuded arteries and in endothelium-intact arteries exposed to both TRAM-34 and UCL 1684, indicating that all endothelial KCa channels could be blocked with TRAM-34 and UCL 1684. The remaining endothelium-independent relaxation to NS309 (>1 μM) may involve blockade of L-type voltage-operated calcium channels (Morimura et al., 2006). The differences in ACh- and NS309-induced relaxing responses in arteries that experienced alterations in shear stress could not be attributed to differential KCa channel or eNOS expression. The immunohistochemistry in general confirms endothelial expression of SK3 and IK1 channels and the lack of alterations by 24 h of altered blood flow. We would need much better spatial resolution to proof altered channel distribution within the endothelial cells. This is currently beyond our local capacities. Human umbilical vein endothelial cells exposed to laminar shear stress (15 dyn/cm²) display a 8-fold up-regulation of IKCa and enhanced IK1 whole-cell currents after 24 h (Brakemeier et al., 2003). Apparently, in vitro alterations of endothelial IK1 channel expression in response to increased shear stress do not mirror expression profiles in intact arteries. In addition, it can not be excluded that species and/or artery differences may explain the differential expression profiles of endothelial KCa channels in response to altered shear stress. It might well be the case that longer exposures to altered blood flow would lead to differential expression of KCa channels, which can be anticipated due to the location of KCa channels in the endothelial layer.

In conclusion, our data indicate that 24-h alterations in arterial blood flow differentially modulate the activity of endothelial IK1 and SK3 channels in the rat mesenteric arterial bed. It remains to be established whether the ob-
served changes in channel function are causally related to the more slowly developing arterial structural changes. This will require analysis of the in vivo effects of activators and inhibitors of the channels on arterial remodeling in young healthy animals and experimental models displaying impaired flow-induced remodeling, such as occurring during aging (Tuttle et al., 2002), hypertension (Gao et al., 2008), and diabetes (Crijns et al., 1999).

References

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