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Different modulation by Ca^{2+}-activated K^+ channel blockers and herbimycin of acetylcholine- and flow-evoked vasodilatation in rat mesenteric small arteries

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1 The present study addressed whether endothelium-dependent vasodilatation evoked by acetylcholine and flow are mediated by the same mechanisms in isolated rat mesenteric small arteries, suspended in a pressure myograph for the measurement of internal diameter.

2 In pressurized arterial segments contracted with U46619 in the presence of indomethacin, shear stress generated by the flow evoked relaxation. Thus, in endothelium-intact segments low (5.1 ± 0.6 dyn cm^{-2}) and high (19 ± 2 dyn cm^{-2}) shear stress evoked vasodilatations that were reduced by, respectively, 68 ± 11 and $68 \pm 8\%$ ($P<0.05$, $n=7$) by endothelial cell removal. Acetylcholine ($0.01 - 1\,\mu$M) evoked concentration-dependent vasodilatation that was abolished by endothelial cell removal.

3 Incubation with indomethacin alone did not change acetylcholine and shear stress-evoked vasodilatation, while the combination of indomethacin with the nitric oxide (NO) synthase inhibitor, N^G,N^G-asymmetric dimethyl-L-arginine (ADMA 1 mM), reduced low and high shear stress-evoked vasodilatation with, respectively, 52 ± 15 and $58 \pm 10\%$ ($P<0.05$, $n=9$), but it did not change acetylcholine-evoked vasodilatation.

4 Inhibition of Ca^{2+}-activated K^+ channels with a combination of apamin ($0.5\,\mu$M) and charybdotoxin (ChTX) ($0.1\,\mu$M) did not change shear stress- and acetylcholine-evoked vasodilatation. In the presence of indomethacin and ADMA, the combination of apamin ($0.5\,\mu$M) and ChTx ($0.1\,\mu$M) increased contraction induced by U46619, but these blockers did not change the vasodilatation evoked by shear stress. In contrast, acetylcholine-evoked vasodilatation was abolished by the combination of apamin and charybdotoxin.

5 In the presence of indomethacin, the tyrosine kinase inhibitor, herbimycin A ($1\,\mu$M), inhibited low and high shear stress-evoked vasodilatation with, respectively, 32 ± 12 and $68 \pm 14\%$ ($P<0.05$, $n=8$), but it did not change vasodilatation induced by acetylcholine. In the presence of indomethacin and ADMA, herbimycin A neither changed shear stress nor acetylcholine-evoked vasodilatation.

6 The present study suggests that Ca^{2+}-activated K^+ channels sensitive for the combination of apamin and ChTx are involved in acetylcholine-evoked, mainly non-NO nonprostanoid factor-mediated, vasodilatation, while an Src tyrosine kinase plays a role for flow-evoked NO-mediated vasodilatation in rat mesenteric small arteries.
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Keywords: Flow; acetylcholine; endothelium; resistance arteries; rat; herbimycin A

Abbreviations: ADMA, N^G,N^G-asymmetric dimethyl-L-arginine; AUC, area under curve; $BaCl_2$, barium chloride; ChTx, charybdotoxin; EDHF, endothelium-derived hyperpolarizing factor; EDTA, ethylenediaminetetraacetic acid; NO, nitric oxide; NOS, nitric oxide synthase; PSS, physiological salt solution; U46619, 9,11-di-deoxy-11α, 9α-epoxymethano prostaglandin $F_{2\alpha}$

Introduction

The vascular endothelium plays an important role in the regulation of vascular tone through release of nitric oxide (NO) and prostaglandins. In addition, there are endothelium-dependent vasodilatation resistant to the inhibition of cyclooxygenase and NO synthase (NOS), non-NO, nonprostanoid endothelium-derived hyperpolarizing factor (EDHF)-type relaxation, which is most pronounced in the distal part of the systemic arterial circulation (Parsons *et al.*, 1994; Hwa *et al.*, 1994; Shimokawa *et al.*, 1996; Buus *et al.*, 2000; McGuire *et al.*, 2001; Busse *et al.*, 2002). Several candidates for the EDHF-type relaxation have been suggested to mediate agonist-induced relaxation of small arteries such as potassium ions (Edwards *et al.*, 1998), hydrogen peroxide (H_2O_2) (Matoba *et al.*, 2000), and products of the cytochrome *P*450 pathway (Fulton *et al.*, 1992; Bauersachs *et al.*, 1994; Fisslthaler *et al.*, 1999; Bolz *et al.*, 2000). Other studies have attributed agonist-evoked non-NO nonprostanoid relaxation to myoendothelial gap junction communication (Chaytor *et al.*, 1998; Yamamoto *et al.*, 1999; Sandow & Hill, 2000; Coleman *et al.*, 2001; Taylor *et al.*, 2001). Elevation of shear stress evokes NO-mediated relaxation in large systemic arteries

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(Rubanyi *et al.*, 1986; Cooke *et al.*, 1991), coronary small arteries (Muller *et al.*, 1999) and human subcutaneous arteries (Paniagua *et al.*, 2001), but it was shown to lead to EDHF-type vasodilatation in rat mesenteric arteries (Matrougui *et al.*, 1997; Takamura *et al.*, 1999; Izzard & Heagerty, 1999) and human coronary arterioles (Miura *et al.*, 2001). In human coronary arterioles, shear stress-evoked vasodilatation is attributed to cytochrome *P*450 metabolites (Miura *et al.*, 2001), but the non-NO nonprostanoid EDHF-type vasodilatation is not fully characterized in mesenteric small arteries and it has not been addressed whether acetylcholine and shear stress is causing vasodilatation by the same endothelium-dependent mechanism in small arteries.

The inhibition of small- and intermediate-conductance Ca^{2+}-activated K^+ channels by the combination of apamin and charybdotoxin (ChTx) has been considered as a unique characteristic of the non-NO nonprostanoid-type relaxations (Edwards *et al.*, 1998; Buus *et al.*, 2000), and hyperpolarization evoked by agonists in small arteries (Zygmunt & Högestatt, 1996; Edwards *et al.*, 1998; Yamamoto *et al.*, 1999). A general blocker of Ca^{2+}-activated K^+ channels, tetraethylammonium, and ChTx was also found to inhibit the non-NO nonprostanoid vasodilatation and hyperpolarization induced by flow in coronary arteries (Dube & Canty, 2001; Miura *et al.*, 2001), while the combination of apamin and ChTx only caused partial inhibition of flow-induced vasodilatation in rat mesenteric arteries (Takamura *et al.*, 1999). Therefore, these studies could suggest that flow activates another relaxation mechanism than acetylcholine. Agonist activation of isolated endothelial cells by acetylcholine is dependent on rises in intracellular Ca^{2+} (Nilius & Droogmans, 2001), while shear stress-evoked endothelial cell activation is partly dependent of Ca^{2+} and on the activation of tyrosine kinase or serine/threonine kinase (Corson *et al.*, 1996; Fleming *et al.*, 1998; Dimmeler *et al.*, 1999). However, in small arteries it has not been addressed whether different kinds of endothelial cell activation such as by the endothelium-dependent agonist, acetylcholine, and flow lead to vasodilatation mediated by distinct endothelium-derived factors.

The aim of the present study was to clarify whether acetylcholine- and flow-induced vasodilatation is mediated by the same mechanisms in rat mesenteric small arteries. For this purpose, the effect of blockers of Ca^{2+}-activated K^+ channels and Src tyrosine kinase as well as inhibitors of cyclooxygenase and NO synthase were evaluated on flow- and acetylcholine-evoked vasodilatation in pressurized vascular segments.

Methods

The 12- to 16-week-old male Wistar rats were killed by a blow to the head followed by exsanguination. The procedures were in accordance with Danish Animal Law and regulations. The mesenteric vascular bed was removed and throughout the subsequent dissection, the tissue was bathed in cold physiological salt solution (PSS, 4°C) of the following composition (mM): $CaCl_2$ 1.6, NaCl 119, KCl 4.7, glucose 5.5, $MgSO_4 \cdot 7H_2O$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, and ethylenediaminetetraacetic acid (EDTA) 0.026. The solution was gassed with 5% CO_2 in air to maintain pH at 7.4.

Flow-induced vasodilatation

Third-order mesenteric arteries were isolated by microdissection. After dissection arterial segments with a length of 1.5 – 3 mm were transferred to the chamber of a pressure myograph for cannulation (Danish Myotechnology, Aarhus, Denmark (Buus *et al.*, 1994). At this stage, the chamber was filled with PSS equilibrated with room air at ambient temperature, and the perfusion line was filled with PSS. The chamber contained two glass pipettes (tip outer diameter $\sim 120 - 130\,\mu$m): one was fixed and the other was mounted on a manipulator allowing adjustment of the vessel length. The vessel was cannulated with the inflow pipette and tied by a surgical thread. The inflow pressure was elevated to 15 – 20 mmHg to wash out blood, and the other end of the vessel was cannulated with the outflow pipette. After cannulation, the pressure myograph was transferred to the stage of an inverted microscope, the pressure was elevated to 30 mmHg, and the myograph bath was heated to 37°C. Then the pressure was elevated to 80 mmHg, and the axial length was adjusted to eliminate any buckling of the preparation. When pressurized, the vessel segment was longitudinally stretched to 110% of the initial passive length at 80 mmHg. Under an inverted microscope (Telaval 31, Zeiss) the preparation was examined for leaks, which were easily identified by outflow of solution.

The middle of the vessel segment was viewed through the inverted microscope (magnification $\times 100$), and its inner and outer diameter was measured by a videomicroscope technique. The signal from a video camera attached to the inverted microscope was fed to a frame grabber and then to a dimension-analyzing program (VesselView, Danish Myotechnology, Aarhus, Denmark) allowing continuous sampling of diameters and pressures at 3 Hz. Hydrostatic pressures of both inlet and outlet reservoirs were measured by pressure transducers connected to the perfusion line on the inlet and outlet side, respectively.

The arterial segment was exposed to U46619 (0.1 μM) added both intra- and extraluminally, since we observed that intraluminal washout contributed to the vasodilatation obtained by increasing flow. When the contraction was stable, acetylcholine (1 μM) was added extraluminally. The following criteria were applied to accept an arterial segment for the investigation of endothelium-dependent vasodilatation: (1) The contractile response induced by U46619 should reduce vessel diameter with at least 25%. (2) Relaxation evoked by acetylcholine (1 μM) above 50%. (3) If an air bubble passed the arterial segment, it led to exclusion of the experiment.

To investigate the role of the endothelial cell layer, a first response to flow and acetylcholine was obtained in the U46619-contracted artery. Flow was generated by suction with a peristaltic pump and flow was adjusted in the ranges of either $25 - 50\,\mu$l or $100 - 200\,\mu$l min^{-1}, which correspond to shear stress levels of, respectively, 4 and 16 dyn cm^{-2} in the mesenteric small arteries. Shear stress was calculated from the following equation:

$$\tau = 4\eta Q/\pi r^3,$$

where r is the internal vessel diameter, Q the average flow velocity, and η the viscosity. In between the two responses to flow, the pump was stopped allowing the preconstriction to regain. The latter procedure was included, since we observed that very high flow, corresponding to $30 - 50$ dyn cm^{-2},

generated irreversible damage to the vascular wall. The suction from the peristaltic pump introduced a small pressure variation with an amplitude of 3 mmHg and frequency of 0.04 – 0.3 Hz. The segment was bubbled with small air bubbles, allowed to dilate and was constricted with U46619 (0.1 μM) and acetylcholine (1 μM) was added. Lack of vasodilatation to acetylcholine was taken as evidence of successful removal of the endothelial cell layer. A second response to flow and acetylcholine was obtained, and finally the segment was dilated with papaverine (100 μM).

For characterization of the mechanisms involved in the endothelium-dependent vasodilatation by flow and acetylcholine, the arterial segment was constricted with U46619 applied both extra- and intraluminally. When the constriction induced by U46619 was stable a first response was obtained for flow generating a shear stress corresponding to 4 and 16 dyn cm^{-2}, and then acetylcholine (0.01 – 1 μM) was added. The preparation was washed and incubated with either indomethacin (3 μM), ADMA (1 mM), indomethacin and ADMA, or indomethacin and herbimycin (10 μM) for 20 min, and a second response was obtained for flow and acetylcholine. In another series of experiments, indomethacin and ADMA were present throughout the experiments, and a first response for flow and acetylcholine was obtained, after which a second response was obtained either in the absence of drugs or after incubation with apamin and ChTx, barium chloride ($BaCl_2$,100 μM), or herbimycin A.

Drugs

The following drugs were used: acetylcholine HCl, indomethacin, asymmetric dimethyl L-arginine (ADMA), herbimycin A, 9,11-di-deoxy-11α, 9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619), noradrenaline HCl, and phenylarsine oxide were from Sigma, U.S.A. Apamin and ChTx were from Latoxan, France, and $BaCl_2$ from Merck, Germany.

Noradrenaline was prepared in 0.25 N HCl and further diluted in distilled water. U46619 was dissolved in 50% ethanol and further diluted in distilled water. The other drugs were dissolved in distilled water. None of the solvents, in the concentration applied, had any effect on the preparations.

Analysis of data

All responses are expressed as mean $\pm$ s.e.m., where n equals the number of rats. Relaxations are expressed as changes in internal diameter or area under the relaxation response was calculated and expressed as changes in internal diameter (μm) with time (s) (GraphPad Prism, San Diego, CA, U.S.A.). The differences between means were analyzed with two-way ANOVA and complementary Bonferroni t-test. A value of $P<0.05$ was considered to indicate statistical significance.

Results

Flow-evoked vasodilatation

A total of 60 vessels were mounted, 43 segments were accepted, and 17 arteries excluded because of lack of response to flow ($n=9$), destruction by an air bubble passing the artery either accidently ($n=5$) or in connection with attempts to remove the endothelial cell layer ($n=3$). The average diameter of the mounted and accepted arteries was 353 $\pm$ 10 μM, and U46619 constricted the arteries to 198 $\pm$ 10 μM ($n=43$). In pressurized arterial segments contracted with U46619 in the presence of indomethacin, shear stress generated by the flow evoked relaxation. Thus, in endothelium-intact segments low (5.1 $\pm$ 0.6 dyn cm^{-2}) and high (19 $\pm$ 2 dyn cm^{-2}) shear stress evoked vasodilatations that were reduced by, respectively, 68 $\pm$ 11 and 68 $\pm$ 8% ($P<0.05$, $n=7$) by endothelial cell removal (Figures 1 and 2a). Acetylcholine (0.01 – 1 μM) evoked concentration-dependent vasodilatation that was abolished by endothelial cell removal (Figures 1b and 2b). Incubation with indomethacin (3 μM) did not change vasodilatations induced by flow or by acetylcholine (Figure 3a,b). In the presence of indomethacin, incubation with an inhibitor of NO synthase, ADMA (1 mM), reduced low and high shear stress-evoked vasodilatation with, respectively, 52 $\pm$ 15 and 58 $\pm$ 10% ($P<0.05$, $n=9$; Figure 4a). However, acetylcholine-evoked vasodilatation was largely unchanged in the presence of ADMA, although Student's t-test revealed significant differences comparing 10^{-7} M acetylcholine relaxation obtained in the absence and presence of ADMA (Figure 4b).

Effect of blockers of Ca^{2+}-activated K^+ channels and herbimycin on flow-evoked vasodilatation

In the presence of indomethacin, incubation with the combination of inhibitors of small-conductance Ca^{2+}-activated K^+ channels, apamin (0.5 μM), and intermediate- and largeconductance Ca^{2+}-activated K^+ channels, ChTx (0.1 μM) did not change flow- or acetylcholine-evoked vasodilatation (Figure 5a,b).

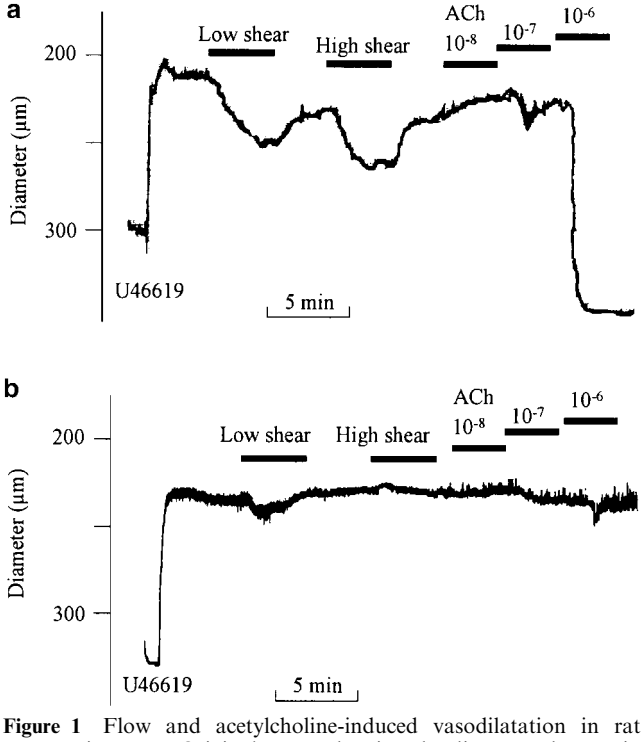

Figure 1 Flow and acetylcholine-induced vasodilatation in rat mesenteric artery. Original traces showing the diameter changes in (**a**) endothelium-intact and (**b**) endothelium-denuded rat mesenteric artery contracted with U46619 (0.1 μM), and relaxed with flow corresponding to 4 and 16 dyn cm^{-2}, and then with increasing concentrations of acetylcholine (ACh). The experiments were performed in the presence of indomethacin (3 μM).

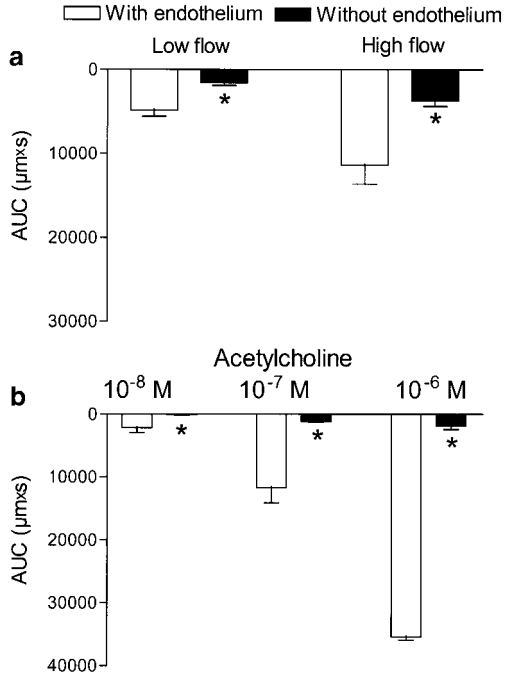

Figure 2 Endothelial cell removal abolished acetylcholine vasodilatation and inhibited flow-induced vasodilatation. Area under the vasodilatation curve (AUC) calculated for each response to (**a**) flow and (**b**) acetylcholine in endothelium-intact and -denuded arterial preparations. Results are mean ± s.e.m. of seven preparations. The experiments were performed in the presence of indomethacin (3 μM). Differences in responses evaluated by two-way analysis of variance: $^*P<0.05$ *versus* endothelium-intact preparations; vasodilatation responses were dependent on the magnitude of shear stress and acetylcholine concentration applied.

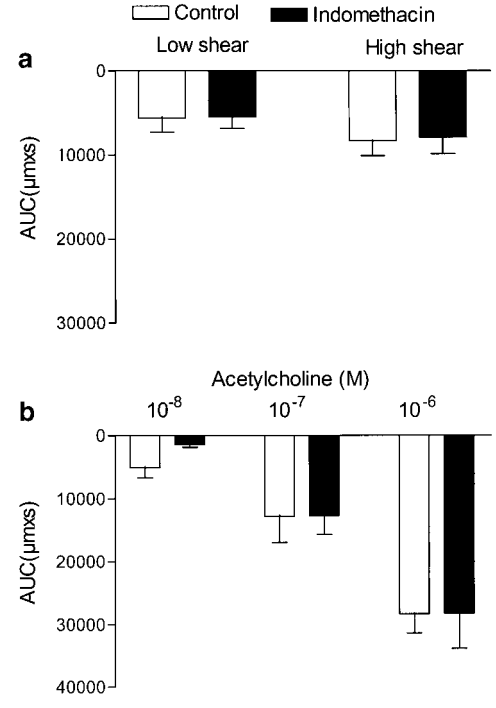

Figure 3 Flow- and acetylcholine-induced vasodilatation resistant to the inhibition of cyclooxygenase. Area under the vasodilatation curve (AUC) calculated for each response to (**a**) flow and (**b**) acetylcholine obtained in the absence and the presence of indomethacin (3 μM). Results are mean ± s.e.m. of five preparations.

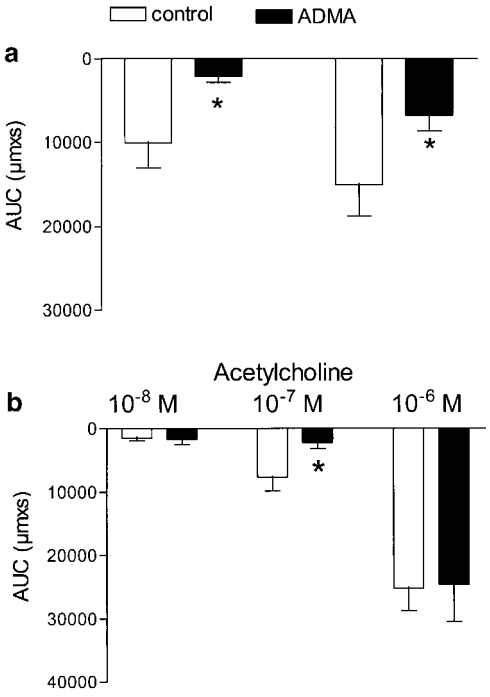

Figure 4 N^G, N^G-asymmetric dimethyl-L-arginine (ADMA) inhibits flow-evoked vasodilatation in rat mesenteric arteries. Area under the vasodilatation curve (AUC) calculated for each response to (**a**) flow and (**b**) acetylcholine in the absence or presence of ADMA (1 mM). Results are mean ± s.e.m. of eight to nine preparations. Differences in responses evaluated by a two-way analysis of variance: $^*P<0.05$ *versus* control response.

In the presence of indomethacin and ADMA, incubation with the combination of apamin (0.5 μM) and ChTx (0.1 μM) increased the vasoconstriction induced by U46619 (0.1 μM) from 198 ± 13 to 182 ± 13 μM ($n=5$). However, both low and high shear stress levels caused vasodilatation to similar levels in the absence and the presence of apamin and ChTx (Figure 5c). In contrast, in the presence of indomethacin and ADMA, the combination of apamin and ChTx abolished acetylcholine-evoked vasodilatation (Figure 5d). Incubation with a blocker of inward rectifier K^+ channels, $BaCl_2$ (100 μM), did not change vasodilatations evoked by flow or acetylcholine ($n=3$, results not shown).

In the presence of indomethacin, an inhibitor of the tyrosine kinase c-Src, herbimycin A, had a pronounced effect on flow-induced vasodilatation (Figure 6a). Thus, herbimycin A reduced low and high flow-evoked relaxation by, respectively, 32 ± 12 and 68 ± 14% ($P<0.05$, $n=7$). In contrast, acetylcholine-evoked vasodilatation in the same vascular preparations was not affected in the presence of herbimycin A (Figure 6b). In the presence of indomethacin and ADMA, incubation with herbimycin A did not cause additional inhibition of flow-evoked vasodilatation, and acetylcholine vasodilatation remained unchanged (Figure 6c, d). In segments treated with indomethacin, incubation with another tyrosine kinase inhibitor, genistein (5 μM), lowered U46619-induced constriction with 50%, but genistein did not affect flow-evoked vasodilatation in rat mesenteric arteries ($n=2$, results not shown). In rat mesenteric small arteries constricted with U46619, a putative activator of tyrosine kinase, phenylarsine oxide (30 μM), abolished tone irreversibly and therefore, this pathway could not be explored further ($n=3$).

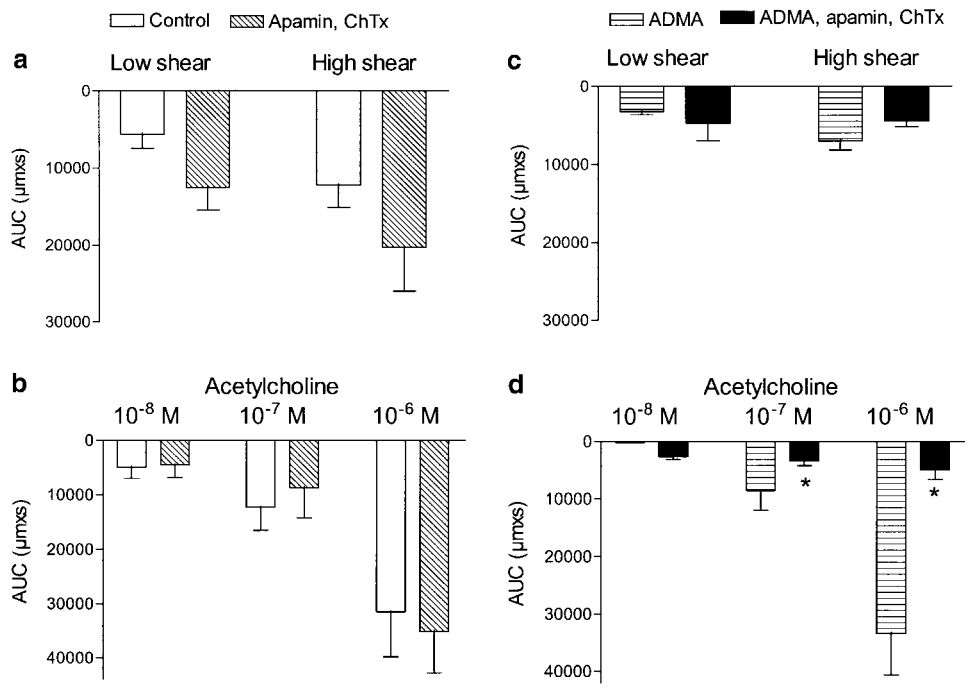

Figure 5 The combination of the blockers of Ca^{2+}-activated K^+ channels, apamin and ChTx, abolished acetylcholine, but not flow-evoked vasodilatation persisting in the presence of indomethacin and ADMA. Area under the vasodilatation curve (AUC) for (**a, c**) flow and (**b, d**) acetylcholine in the absence or presence of a combination of a blocker of small-conductance, apamin (0.5 μM), and intermediate- and large-conductance, ChTx (0.1 μM) Ca^{2+}-activated K^+ channels. The experiments were performed in (**a, b**) the presence of indomethacin (3 μM) and (**c, d**) the presence of indomethacin and ADMA (1 mM). Results are mean$\pm$s.e.m. of four to five preparations. Differences in responses evaluated by two-way analysis of variance: $*P<0.05$ *versus* responses obtained in the presence of indomethacin and ADMA.

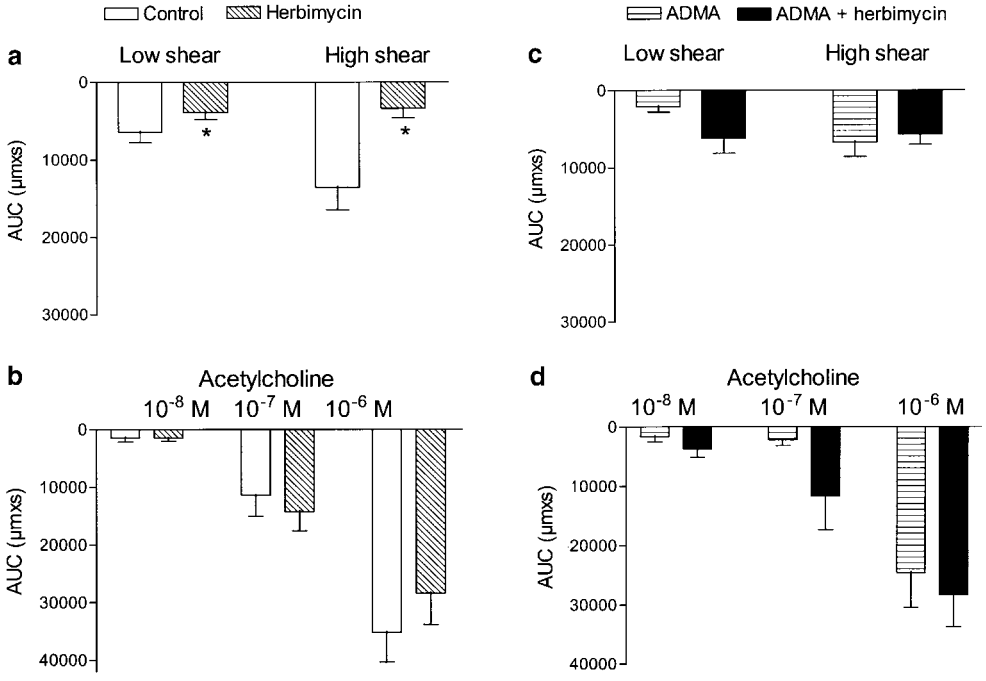

Figure 6 The tyrosine kinase inhibitor, herbimycin A, inhibits the sustained vasodilatation by flow, but it does not change acetylcholine vasodilatation. Area under the vasodilatation curve (AUC) for (**a, c**) flow and (**b, d**) acetylcholine in the absence or the presence of a tyrosine kinase (c-SRC) inhibitor, herbimycin A (1 μM). The experiments were performed in (**a, b**) the presence of indomethacin (3 μM) and (**c, d**) the presence of indomethacin and ADMA (1 mM). Results are mean$\pm$s.e.m. of seven to nine preparations. Differences in responses evaluated by two-way analysis of variance: $*P<0.05$ *versus* responses obtained in the presence of indomethacin.

Discussion

The main findings of the present study are that flow-evoked vasodilatation was markedly reduced in the presence of an inhibitor of NO synthase and of Src kinase, while these treatments did not cause major changes in acetylcholine-evoked vasodilatation. Moreover, the combination of the K^+ channel blockers, apamin and ChTx, inhibited acetylcholine vasodilatation persisting in the presence of inhibitors of cyclooxygenase and NOS. Therefore, these results suggest the endothelial cell signal transduction as well as the contribution of endothelium-derived NO and EDHF-type vasodilatation is different for flow- and acetylcholine-evoked vasodilatation in rat mesenteric small arteries.

Role of endothelium and NO in flow- and acetylcholine-evoked vasodilatation

The range of shear stress applied in the present study corresponds to that *in vivo*, and maximal vasodilatation should be obtained at these shear stress levels (Griffith, 2000). However, the magnitude of vasodilatation in the present study was less than observed in other studies of mesenteric small arteries, where flow-evoked vasodilatation was elicited on spontaneous tone (Matrougui *et al.*, 1997; Izzard & Heagerty, 1999) or constrictions induced by α_1-adrenoceptor antagonists (Takamura *et al.*, 1999). Similar to other studies from this laboratory (Chlopicki *et al.*, 2001), arterial segments that are second to third-order branches of the superior mesenteric artery do not develop spontaneous myogenic tone, and in the case of constrictions elicited by α_1-adrenoceptor agonists, washout contributed to the vasodilatation evoked by the flow levels applied in the present study. Therefore, the thromboxane analog, U46619, was applied in the present study and this could be a limitation, since the processes leading to vasoconstriction evoked by the contractile drugs seem to play a role for relaxations mediated by endothelium-derived relaxing factors (Plane & Garland, 1996; Tomioka *et al.*, 1999). Thus, U46619 was suggested to cause a marked inhibition of acetylcholine relaxation in rat mesenteric small arteries in the presence of NOS blockade (Plane & Garland, 1996). However, in a previous study (Prieto *et al.*, 1998) and also in the present study, we applied lower concentrations of U46619 and obtained maximal vasodilatation to acetylcholine. Moreover, the constriction of the arteries by U46619 to 45% of the passive diameter of the arteries allowed large changes in the resistance of the vascular segments when vasodilatation was evoked by flow.

Vasodilatation by elevation in shear stress following increased flow is considered to be endothelium dependent. There are only few *in vivo* studies which have addressed this issue, but *in vitro* studies have repeatedly shown that flow-induced vasodilatation is either partially inhibited (Pourageaud & Freslon, 1995; Vequaud *et al.*, 1999) or abolished by endothelial cell removal in small arteries (Izzard & Heagerty, 1999; Takamura *et al.*, 1999). In the present study, a small vasodilatation to flow persisted after removal of the endothelial cell layer and can probably be ascribed to the small pressure variation generated by the pump suction in our setup, which on the other hand allowed us to control precisely the generated flow. However, mechanical removal of the endothelium abolished acetylcholine-evoked vasodilatation and markedly reduced flow-induced vasodilatation indicating that both responses are dependent on an intact endothelial cell layer.

NO plays a main role for both agonist and flow-evoked endothelium-dependent vasorelaxation in large arteries (Cooke *et al.*, 1991; Simonsen *et al.*, 1999; Danser *et al.*, 2000). In human small arteries and rat skeletal arterioles, prostanoids and NO were found only to have a minor role in acetylcholine-evoked vasodilatation (Buus *et al.*, 2000; Ungvari *et al.*, 2001), while flow-evoked vasodilatation was abolished in the presence of inhibitors of cyclooxygenase and NO (Paniagua *et al.*, 2001). The same preconstrictor noradrenaline was applied and therefore, these studies suggest that different activation of the endothelial cell layer is associated with the release of different types of endothelium-derived relaxant factor. In rat mesenteric small arteries with spontaneous tone, inhibition of NO synthase and cyclooxygenase was demonstrated either to cause no or partial inhibition of acetylcholine and flow-evoked vasodilatation (Iglarz *et al.*, 1998; Izzard & Heagerty, 1999; Takamura *et al.*, 1999). In the present study, the arteries were constricted with the thromboxane analog, U46619, and an inhibitor of NO synthase, ADMA, caused pronounced inhibition of flow-evoked vasodilatation. The concentration of ADMA, applied in the present study, causes effective inhibition of NO synthase, since it reduces acetylcholine-evoked NO release in the rat superior mesenteric artery to levels similar to that of N^G-nitro-L-arginine (Simonsen *et al.*, 1999; Stankevicius *et al.*, 2002). Therefore, the main part of shear stress-induced vasodilatation of mesenteric small arteries can be attributed to NO, while NO only seems to play a minor role for acetylcholine-evoked vasodilatation.

Role of nonprostanoid non-NO in flow- and acetylcholine-evoked vasodilatation

Endothelial cell calcium plays a pivotal role for agonist-induced release of endothelium-derived relaxing factors including prostacyclin, NO, and EDHF-type vasorelaxation (Nilius & Droogmans, 2001). Simultaneous recordings of endothelial membrane potential and cytosolic Ca^{2+} in the intact rat aorta also showed that acetylcholine-evoked hyperpolarization coincides with rises in Ca^{2+}, suggesting these two events are coupled (Carter & Ogden, 1994; Usachev *et al.*, 1995; Nilius & Droogmans, 2001). Patch-clamp experiments and reverse transcriptase–polymerase chain reaction (RT–PCR) have also provided evidence for the presence of small, intermediate, and large-conductance Ca^{2+}-activated K^+ channels in endothelial cells of intact arteries (Marchenko & Sage, 1996; Kohler *et al.*, 2000), while small- and intermediate-conductance Ca^{2+}-activated K^+ channels are not expressed in mature smooth muscle cells (Burnham *et al.*, 2002; Bychkov *et al.*, 2002). Moreover, measurement of endothelial cell membrane potential in intact mesenteric small arteries suggested endothelial small- and intermediate-conductance Ca^{2+}-activated K^+ channels to be involved in acetylcholine-induced EDHF-type relaxation (Edwards *et al.*, 1998). Therefore, the present study confirms previous studies (Edwards *et al.*, 1998; Buus *et al.*, 2000; Busse *et al.*, 2002), where the blockade of Ca^{2+}-activated K^+ channels with the combination of apamin and ChTx was also found to inhibit acetylcholine vasodilatation resistant to cyclooxygenase and NOS inhibition.

Shear stress-induced vasodilatation is only in part dependent on rises in endothelial cell calcium (Muller *et al.*, 1999; Busse & Fleming, 2003), but Ca^{2+}-activated K^+ channels have been suggested to be involved in flow-evoked vasodilatation. Vasodilatation of large arteries, evoked by superfusate either from cultured endothelial cells exposed to flow or from a perfused endothelium-intact donor artery, was inhibited in the presence of a blocker of Ca^{2+}-activated K^+ channels, tetraethylammonium (Cooke *et al.*, 1991; Hutcheson & Griffith, 1994). Moreover, tetraethylammonium and ChTx was found to inhibit the non-NO nonprostanoid vasodilatation and hyperpolarization evoked by flow in coronary arterioles (Dube & Canty, 2001; Miura *et al.*, 2001). An inhibitor of large-conductance Ca^{2+}-activated K^+ channels, iberiotoxin, was by intraluminal application also found to inhibit flow-dependent dilation in rat mesenteric arterioles, while the effect of the combination of apamin and ChTx on flow was small in medium-sized mesenteric arteries (Takamura *et al.*, 1999). Therefore, arterial size, the vascular bed studied, species differences, and probably also experimental conditions seem to play a role for the implication of Ca^{2+}-activated K^+ channels in flow-evoked vasodilatation. In the present study, we compared in the same preparations the effect of the combination of apamin and ChTx on acetylcholine- and flow-evoked vasodilatation and found that in contrast to acetylcholine-evoked vasodilatation, flow-evoked vasodilatation was not sensitive to the inhibition of Ca^{2+}-activated K^+ channels. These results suggest that opening of Ca^{2+}-activated K^+ channels does not appear to contribute to shear stress-evoked vasodilatation of U46619-constricted rat mesenteric small arteries.

Shear stress enhances the open probability of inward rectifying K^+ channels in cellattached patches (Olesen *et al.*, 1988; Nilius & Droogmans, 2001), and a blocker of these channels, $BaCl_2$, was reported to inhibit flow-evoked vasodilatation in the rabbit iliac arteries (Cooke *et al.*, 1991). However, in the present study a lower concentration (100 μM) of $BaCl_2$, which only inhibits the inward rectifying K^+ channels, did not reduce flow-evoked vasodilatation. Therefore, opening of $BaCl_2$-sensitive inward rectifier and Ca^{2+}-activated K^+ channels does not appear to contribute to shear stress-evoked vasodilatation of U46619-constricted rat mesenteric small arteries, although it cannot be excluded that application of even higher levels of shear stress than applied in the present study will lead to opening of endothelial cell K^+ channels.

Calcium-independent mechanisms are involved in the formation of endothelium-derived relaxing factors. Thus, tyrosine kinase and serine/threonine kinases were found to be involved in phosphorylation and activation of endothelial NOS (Corson *et al.*, 1996; Fleming *et al.*, 1998; Dimmeler *et al.*, 1999). Tyrosine kinases sensitive to genistein were found to be involved in endothelial NOS activation by flow in rat coronary (Muller *et al.*, 1996) and gracialis muscle arterioles (Ungvari *et al.*, 2001). However, in isolated human umbilical vein endothelial cells and porcine aortic endothelial cells shear stress-evoked phosphorylation of extracellular signal-regulated kinase (ERK1/2) and endothelial NOS was found to be dependent on genistein-insensitive tyrosine kinase, which was inhibited in the presence of herbimycin (Takahashi & Berk, 1996; Fleming *et al.*, 1998). Herbimycin is a tyrosine kinase with Src selectivity, and in the present study herbimycin inhibited shear stress-induced vasodilatation without alteration in acetylcholine-evoked vasodilatation. These observations suggest that the flow-evoked, mainly NO-mediated, vasodilatation, in contrast to acetylcholine vasodilatation, depends on the activation of Src tyrosine kinase in rat mesenteric arteries.

The present study suggests that Ca^{2+}-activated K^+ channels sensitive for the combination of apamin and ChTx are involved in acetylcholine-evoked, mainly EDHF-type, vasodilatation, while a Src tyrosine kinase plays a role for flow-evoked NO-mediated vasodilatation in rat mesenteric small arteries.

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References

BAUERSACHS, J., HECKER, M. & BUSSE, R. (1994). Display of the characteristics of endothelium-derived hyperpolarizing factor by a cytochrome *P*450-derived arachidonic acid metabolite in the coronary microcirculation. *Br. J. Pharmacol.,* **113,** 1548 – 1553.

BOLZ, S.S., FISSLTHALER, B., PIEPERHOFF, S., DE WIT, C., FLEMING, I., BUSSE, R. & POHL, U. (2000). Antisense oligonucleotides against cytochrome *P*450 2C8 attenuate EDHF-mediated Ca(2+) changes and dilation in isolated resistance arteries. *FASEB J.,* **14,** 255 – 260.

BURNHAM, M.P., BYCHKOV, R., FELETOU, M., RICHARDS, G.R., VANHOUTTE, P.M., WESTON, A.H. & EDWARDS, G. (2002). Characterization of an apamin-sensitive small-conductance Ca(2+)- activated K(+) channel in porcine coronary artery endothelium: relevance to EDHF. *Br. J. Pharmacol.,* **135,** 1133–1143.

BUSSE, R., EDWARDS, G., FELETOU, M., FLEMING, I., VANHOUTTE, P.M. & WESTON, A.H. (2002). EDHF: bringing the concepts together. *Trends Pharmacol. Sci.,* **23,** 374 – 380.

BUSSE, R. & FLEMING, I. (2003). Regulation of endothelium-derived vasoactive autocoid production by hemodynamic forces. *Trends Pharmacol. Sci.,* **24,** 24 – 29.

BYCHKOV, R., BURNHAM, M.P., RICHARDS, G.R., EDWARDS, G., WESTON, A.H., FELETOU, M. & VANHOUTTE, P.M. (2002). Characterization of a charybdotoxin sensitive intermediate conductance Ca(2+)-activated K(+) channel in porcine coronary endothelium: relevance to EDHF. *Br. J. Pharmacol.,* **137,** 1346 – 1354.

BUUS, N.H., SIMONSEN, U., PILEGAARD, H.K. & MULVANY, M.J. (2000). Nitric oxide, prostanoid and non-NO, non-prostanoid involvement in acetylcholine relaxation of isolated human small arteries. *Br. J. Pharmacol.,* **129,** 184 – 192.

BUUS, N.H., VANBAVEL, E. & MULVANY, M.J. (1994). Differences in sensitivity of rat mesenteric small arteries to agonists when studied as ring preparations or as cannulated preparations. *Br. J. Pharmacol.,* **112,** 579 – 587.

CARTER, T.D. & OGDEN, D. (1994). Acetylcholine-stimulated changes of membrane potential and intracellular Ca^{2+} concentration recorded in endothelial cells *in situ* in the isolated rat aorta. *Pflugers Arch.,* **428,** 476 – 484.

CHAYTOR, A.T., EVANS, W.H. & GRIFFITH, T.M. (1998). Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit arteries. *J. Physiol.,* **508,** 561 – 573.

CHLOPICKI, S., NILSSON, H. & MULVANY, M.J. (2001). Initial and sustained phases of myogenic response of rat mesenteric small arteries. *Am. J. Physiol.,* **281,** H2176 – H2183.

COLEMAN, H.A., TARE, M. & PARKINGTON, H.C. (2001). EDHF is not K^+ but may be due to spread of current from the endothelium in guinea pig arterioles. *Am. J. Physiol.*, **280,** H2478 – H2783.

COOKE, J.P., ROSSITCH, JR, E., ANDON, N.A., LOSCALZO, J. & DZAU, V.J. (1991). Flow activates an endothelial potassium channel to release an endogenous nitrovasodilator. *J. Clin. Invest.,* **88,** 1663 – 1671.

CORSON, M.A., JAMES, N.L., LATTA, S.E., NEREM, R.M., BERK, B.C. & HARRISON, D.G. (1996). Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ. Res.,* **79,** 984 – 991.

DANSER, A.H., TOM, B., DE VRIES, R. & SAXENA, P.R. (2000). L-NAME-resistant bradykinin-induced relaxation in porcine coronary arteries is NO-dependent: effect of ACE inhibition. *Br. J. Pharmacol.,* **131,** 195 – 202.

DIMMELER, S., FLEMING, I., FISSLTHALER, B., HERMANN, C., BUSSE, R. & ZEIHER, A.M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature,* **399,** 601 – 605.

DUBE, S. & CANTY, JR, J.M., (2001). Shear stress-induced vasodilatation in porcine coronary conduit arteries is independent of nitric oxide release. *Am. J. Physiol.*, **280,** H2581 – H2590.

EDWARDS, G., DORA, K.A., GARDENER, M.J., GARLAND, C.J. & WESTON, A.H. (1998). K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature,* **396,** 269 – 272.

FISSLTHALER, B., POPP, R., KISS, L., POTENTE, M., HARDER, D.R., FLEMING, I. & BUSSE, R. (1999). Cytochrome *P*450 2C is an EDHF synthase in coronary arteries. *Nature,* **401,** 493 – 497.

FLEMING, I., BAUERSACHS, J., FISSLTHALER, B. & BUSSE, R. (1998). Ca^{2+}-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. *Circ. Res.,* **82,** 686 – 695.

FULTON, D., MCGIFF, J.C. & QUILLEY, J. (1992). Contribution of NO and cytochrome *P*450 to the vasodilator effect of bradykinin in the rat kidney. *Br. J. Pharmacol.,* **107,** 722–725.

GRIFFITH, T.M. (2000). In: *A nitric oxide. Biology and Pathophysiology*. ed. Ignarro, L.J. pp. 570 – 595. San Diego: Academic Press.

HUTCHESON, I.R. & GRIFFITH, T.M. (1994). Heterogeneous populations of K^+ channels mediate EDRF release to flow but not agonists in rabbit aorta. *Am. J. Physiol.,* **266,** H590 – H596.

HWA, J.J., GHIBAUDI, L., WILLIAMS, P. & CHATTERJEE, M. (1994). Comparison of acetylcholine-dependent relaxation in large and small arteries of rat mesenteric vascular bed. *Am. J. Physiol.,* **266,** H952 – H958.

IGLARZ, M., MATROUGUI, K., LEVY, B.I. & HENRION, D. (1998). Chronic blockade of endothelin ETA receptors improves flow dependent dilation in resistance arteries of hypertensive rats. *Cardiovasc. Res.,* **39,** 657 – 664.

IZZARD, A.S. & HEAGERTY, A.M. (1999). Impaired flow-dependent dilatation in distal mesenteric arteries from the spontaneously hypertensive rat. *J. Physiol.,* **518,** 239 – 245.

KOHLER, R., DEGENHARDT, C., KUHN, M., RUNKEL, N., PAUL, M. & HOYER, J. (2000). Expression and function of endothelial Ca(2+)-activated K(+) channels in human mesenteric artery: a single-cell reverse transcriptase – polymerase chain reaction and electrophysiological study *in situ*. *Circ. Res.,* **87,** 496 – 503.

MARCHENKO, S.M. & SAGE, S.O. (1996). Calcium-activated potassium channels in the endothelium of intact rat aorta. *J. Physiol.,* **492,** 53 – 60.

MATOBA, T., SHIMOKAWA, H., NAKASHIMA, M., HIRAKAWA, Y., MUKAI, Y., HIRANO, K., KANAIDE, H & TAKESHITA, A. (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J. Clin. Invest.,* **106,** 1521 – 1531.

MATROUGUI, K., MACLOUF, J., LEVY, B.I. & HENRION, D. (1997). Impaired nitric oxide- and prostaglandin-mediated responses to flow in resistance arteries of hypertensive rats. *Hypertension,* **30,** 942 – 947.

MCGUIRE, J.J., DING, H. & TRIGGLE, C.R. (2001). Endothelium-derived relaxing factors: a focus on endothelium-derived hyperpolarizing factor(s). *Can. J. Physiol. Pharmacol.,* **79,** 443 – 470.

MIURA, H., WACHTEL, R.E., LIU, Y., LOBERIZA, JR, F.R., SAITO, T., MIURA, M. & GUTTERMAN, D.D. (2001). Flow-induced dilation of human coronary arterioles: important role of Ca(2+)-activated K(+) channels. *Circulation,* **103,** 1992 – 1998.

MULLER, J.M., DAVIS, M.J. & CHILIAN, W.M. (1996). Coronary arteriolar flow-induced vasodilatation signals through tyrosine kinase. *Am. J. Physiol.,* **270,** H1878 – H1884.

MULLER, J.M., DAVIS, M.J., KUO, L. & CHILIAN, W.M. (1999). Changes in coronary endothelial cell Ca^{2+} concentration during shear stress- and agonist-induced vasodilatation. *Am. J. Physiol.,* **276,** H1706 – H1714.

NILIUS, B. & DROOGMANS, G. (2001). Ion channels and their functional role in vascular endothelium. *Physiol. Rev.,* **81,** 1415 – 1459.

OLESEN, S.P., CLAPHAM, D.E. & DAVIES, P.F. (1988). Haemodynamic shear stress activates a K^+ current in vascular endothelial cells. *Nature,* **331,** 168 – 170.

PANIAGUA, O.A., BRYANT, M.B. & PANZA, J.A. (2001). Role of endothelial nitric oxide in shear stress-induced vasodilatation of human microvasculature: diminished activity in hypertensive and hypercholesterolemic patients. *Circulation,* **103,** 1752 – 1758.

PARSONS, S.J., HILL, A., WALDRON, G.J., PLANE, F. & GARLAND, C.J. (1994). The relative importance of nitric oxide and nitric oxide-independent mechanisms in acetylcholine-evoked dilatation of the rat mesenteric bed. *Br. J. Pharmacol.,* **113,** 1275–1280.

PLANE, F. & GARLAND, C.J. (1996). Influence of contractile agonists on the mechanism of endothelium-dependent relaxation in rat isolated mesenteric artery. *Br. J. Pharmacol.,* **119,** 191 – 193.

POURAGEAUD, F. & FRESLON, J.L. (1995). Impaired endothelial relaxations induced by agonists and flow in spontaneously hypertensive rat compared to Wistar – Kyoto rat perfused coronary arteries. *J. Vasc. Res.,* **32,** 190 – 199.

PRIETO, D., SIMONSEN, U., HERNANDEZ, M. & GARCIA-SACRISTAN, A. (1998). Contribution of K^+ channels and ouabain-sensitive mechanisms to the endothelium-dependent relaxations of horse penile small arteries. *Br. J. Pharmacol.,* **123,** 1609 – 1620.

RUBANYI, G.M., ROMERO, J.C. & VANHOUTTE, P.M. (1986). Flow-induced release of endothelium-derived relaxing factor. *Am. J. Physiol.,* **252,** H1145 – H1149.

SANDOW, S.L. & HILL, C.E. (2000). Incidence of myoendothelial gap junctions in the proximal and distal mesenteric arteries of the rat is suggestive of a role in endothelium-derived hyperpolarizing factor-mediated responses. *Circ. Res.,* **86,** 341 – 346.

SHIMOKAWA, H., YASUTAKE, H., FUJII, K., OWADA, M.K., NAKAIKE, R., FUKUMOTO, Y., TAKAYANAGI, T., NAGAO, T., EGASHIRA, K., FUJISHIMA, M. & TAKESHITA, A. (1996). The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J. Cardiovasc. Pharmacol.,* **28,** 703 – 711.

SIMONSEN, U., WADSWORTH, R.M., BUUS, N.H. & MULVANY, M.J. (1999). *In vitro* simultaneous measurements of relaxation and nitric oxide concentration in rat superior mesenteric artery. *J. Physiol.,* **516,** 271 – 282.

STANKEVICIUS, E., MARTINEZ, A.C., MULVANY, M.J. & SIMONSEN, U. (2002). Blunted acetylcholine relaxation and nitric oxide release in arteries from renal hypertensive rats. *J. Hyperten.,* **20,** 1571 – 1579.

TAKAHASHI, M. & BERK, B.C. (1996). Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for a herbimycin-sensitive kinase. *J. Clin. Invest.,* **98,** 2623 – 2631.

TAKAMURA, Y., SHIMOKAWA, H., ZHAO, H., IGARASHI, H., EGASHIRA, K. & TAKESHITA, A. (1999). Important role of endothelium-derived hyperpolarizing factor in shear stress-induced endothelium-dependent relaxations in the rat mesenteric artery. *J. Cardiovasc. Pharmacol.,* **34,** 381 – 387.

TAYLOR, H.J., CHAYTOR, A.T., EDWARDS, D.H. & GRIFFITH, T.M. (2001). Gap junction-dependent increases in smooth muscle cAMP underpin the EDHF phenomenon in rabbit arteries. *Biochem. Biophys. Res. Commun.,* **283,** 583 – 589.

TOMIOKA, H., HATTORI, Y., FUKAO, M., SATO, A., LIU, M., SAKUMA, I., KITABATAKE, A. & KANNO, M. (1999). Relaxation in different-sized rat blood vessels mediated by endothelium-derived hyperpolarizing factor: importance of processes mediating precontractions. *J. Vasc. Res.,* **36,** 311 – 320.

UNGVARI, Z., SUN, D., HUANG, A., KALEY, G. & KOLLER, A. (2001). Role of endothelial $[Ca^{2+}]_i$ in activation of eNOS in pressurized arterioles by agonists and wall shear stress. *Am. J. Physiol. Heart Circ. Physiol.,* **281,** H606 – H612.

USACHEV, Y.M., MARCHENKO, S.M. & SAGE, S.O. (1995). Cytosolic calcium concentration in resting and stimulated endothelium of excised intact rat aorta. *J. Physiol.,* **489,** 309 – 317.

VEQUAUD, P., POURAGEAUD, F. & FRESLON, J.L. (1999). Role of nitric oxide and endothelium in the flow-induced dilation of rat coronary arteries under two preconstriction conditions. *Clin. Exp. Pharmacol. Physiol.,* **26,** 470 – 476.

YAMAMOTO, Y., IMAEDA, K. & SUZUKI, H. (1999). Endothelium-dependent hyperpolarization and intercellular electrical coupling in guinea-pig mesenteric arterioles. *J. Physiol.,* **514,** 505 – 513.

ZYGMUNT, P.M. & HÖGESTATT, E.D. (1996). Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br. J. Pharmacol.,* **117,** 1600 – 1606.

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