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Potassium- and acetylcholine-induced vasorelaxation in mice lacking endothelial nitric oxide synthase

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1 The contribution of an endothelium-derived hyperpolarizing factor (EDHF) was investigated in saphenous and mesenteric arteries from endothelial nitric oxide synthase (eNOS) (−/−) and (+/+) mice.

2 Acetylcholine-induced endothelium-dependent relaxation of saphenous arteries of eNOS(−/−) was resistant to N^{ω}-nitro-L-arginine (L-NNA) and indomethacin, as well as the guanylyl cyclase inhibitor, 1H-(1,2,4)oxadiazolo(4,3-a) quinoxalin-1-one(ODQ).

3 Potassium (K^+) induced a dose-dependent vasorelaxation which was endothelium-independent and unaffected by either L-NNA or indomethacin in both saphenous and mesenteric arteries from eNOS(−/−) or (+/+) mice.

4 Thirty μM barium (Ba^{2+}) and 10 μM ouabain partially blocked potassium-induced, but had no effect on acetylcholine-induced vasorelaxation in saphenous arteries.

5 Acetylcholine-induced relaxation was blocked by a combination of charybdotoxin (ChTX) and apamin which had no effect on K^+-induced relaxation, however, iberiotoxin (IbTX) was ineffective against either acetylcholine- or K^+-induced relaxation.

6 Thirty μM Ba^{2+} partially blocked both K^+- and acetylcholine-induced relaxation of mesenteric arteries, and K^+, but not acetylcholine-induced relaxation was totally blocked by the combination of Ba^{2+} and ouabain.

7 These data indicate that acetylcholine-induced relaxation cannot be mimicked by elevating extracellular K^+ in saphenous arteries from either eNOS(−/−) or (+/+) mice, but K^+ may contribute to EDHF-mediated relaxation of mesenteric arteries.

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Keywords: Endothelium-derived hyperpolarizing factor; inward rectifier potassium channel (K_{IR}) and Na^+/K^+ ATPase; eNOS knockout mice; saphenous and mesenteric arteries

Abbreviations: ChTX, charybdotoxin; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; IbTX, iberiotoxin; K_{Ca}, calcium-activated potassium channel; K_{IR}, inward rectifier potassium channel; L-NNA, N^{ω}-nitro-L-arginine; ODQ, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one; PGI_2, prostacyclin

Introduction

Furchgott & Zawadzki (1980) established the role of the endothelium in mediating endothelium-dependent relaxation of vascular smooth muscle to acetylcholine. Subsequently, nitric oxide (NO) was identified as an important endothelium-derived relaxing factor or EDRF (Palmer *et al*., 1987) and together with prostacyclin (PGI_2) (Moncada *et al*., 1976), have been identified as important endothelium-derived vasorelaxants. However, there is now an increasing body of evidence suggesting that a factor(s) other than NO or PGI_2, plays an important role in mediating endothelium-dependent vascular smooth muscle hyperpolarization (see recent reviews: Mombouli & Vanhoutte, 1997; Triggle *et al*., 1999). In comparison to NO, relatively little is known about the biochemistry, physiology or pharmacology of EDHF(s) which are now thought to contribute to vasodilation, notably in resistance vessels, *via* the hyperpolarization of the vascular smooth muscle through K^+ channel activation (Garland *et al*., 1995; Feletou & Vanhoutte, 1996; Mombouli & Vanhoutte, 1997; Quilley *et al*., 1997; Triggle *et al*., 1999). Considerable tissue and species variability, as well differences reflecting experimental protocol procedure, are noted in the literature with respect to the identification of the K^+ channels that mediate the effects of EDHF on vascular smooth muscle. Thus, Adeagbo & Triggle (1993) in a study with the isolated perfused rat mesenteric bed, concluded that apamin-sensitive Ca^{2+}-activated K^+ channels mediated the effects of EDHF on vascular smooth muscle. Activation of the Na^+/K^+ ATPase pump (Feletou & Vanhoutte, 1988) has also been proposed to explain the EDHF-induced hyperpolarization. Recently, acetylcholine-mediated endothelium-dependent hyperpolarization of rat hepatic and mesenteric arteries was reported to be mediated by endothelial cell-derived K^+ exiting the cell following the opening of calcium-activated ChTX- and apamin-sensitive potassium channels (Edwards *et al*., 1998). However, K^+ may not be the universal EDHF because recent data from guinea-pig carotid and porcine coronary arteries indicate differences between the vasorelaxation and hyperpolarization attributed to EDHF and tissue responsiveness to either 5 or 10 mM K^+ (Quignard *et al*., 1999).

It is very well known that NO plays an important role in the regulation of endothelium-dependent relaxation. Although the assumption is that eNOS is responsible for the production of endothelial-derived NO, the contributions of the other two isoforms of nitric oxide NOS: neuronal (nNOS) and inducible (iNOS), should also be considered. For instance in pial vessels from eNOS(−/−) mice, neuronal NOS derived NO compensates for the lack of endothelial NOS derived NO (Meng *et al*.,

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1996). In contrast, acetylcholine induces relaxation of small peripheral arteries isolated from mice lacking eNOS and this relaxation is insensitive not only to indomethacin and NOS inhibitors but also to the guanylyl cyclase inhibitor ODQ and possesses the characteristics of being mediated by an EDHF (Waldron *et al*., 1999).

In the current study, we have investigated K^+-induced relaxation of mesenteric and saphenous arteries from eNOS(−/−) and eNOS(+/+) mice and compared the relaxation with that produced by acetylcholine.

Methods

Experimental procedure

Homozygous eNOS knockout mutant mice, generated by homologous recombination have been previously described (Huang *et al*., 1995). Breeding pairs of homozygous eNOS knockout and control (C57B16/SV129) mice were obtained from Dr Paul Huang (Cardiovascular Research Centre, Massachusetts General Hospital) and bred at the University of Calgary. Mice (20–24 weeks of age) were killed by cervical dislocation. Saphenous and mesenteric arteries were removed and kept in Krebs' solution (composition, mM): NaCl 120, $NaHCO_3$ 25, KCl 4.8, NaH_2PO_4 1.2, $MgSO_4$ 1.2, Dextrose 11.0, $CaCl_2$ 1.8, bubbled with 95%O_2 and 5%CO_2. Arteries were cut into 2 mm rings and mounted on a Mulvany-Halpern myograph as previously described (Mulvany & Halpern, 1977). The passive tension-internal circumference was determined by stretching to achieve an internal circumference equivalent to 90% of that of the vessel under a transmural pressure of 100 mmHg. All experiments were performed at 37°C. In some experiments, the endothelial cell layer was removed by gently rubbing the intimal surface of the rings with a wire. Confirmation that the endothelium was functionally absent was obtained by lack of a relaxation response to acetylcholine (10 μM).

Protocols

After a 45 min equilibration period, endothelium-dependent relaxations to acetylcholine were studied in tissues precontracted with 0.3–1 μM phenylephrine. The first series of experiments compared acetylcholine-induced relaxation with that produced by increasing the extracellular potassium concentration. The contribution of EDHF to the acetylcholine-induced relaxation was determined in tissues pretreated for 30 min with L-NNA (100 μM) and indomethacin (10 μM). The second series of experiments was performed to compare acetylcholine and potassium-induced relaxation in the presence of barium, the K_{IR} inhibitor and ouabain, the Na^+/K^+ ATPase inhibitor. All experiments were performed in the presence of L-NNA (100 μM) and indomethacin (10 μM). The third series of experiments was designed to determine if potassium-induced relaxation is endothelium dependent. The objective of the fourth series of experiments was to determine the effects of the calcium-activated potassium channel (K_{Ca}) inhibitors, charybdotoxin and apamin, on acetylcholine- and K^+-induced vasodilation in eNOS(−/−) and (+/+) mice. Finally, we compared acetylcholine and K^+-induced relaxation in mesenteric versus saphenous arteries. The relaxation-response curve to added KCl was determined in both endothelium intact and denuded tissues which were precontracted with 0.3 μM phenylephrine and based upon this response curve, it was determined that the addition of 10 mM KCl (on top of the 4.8 mM already in the Krebs) gave the maximal relaxation. Thus, a single concentration of potassium was used to evaluate the effects of potassium-induced relaxation.

All drugs were obtained from Sigma. All drugs were dissolved in distilled water except for indomethacin and ODQ which were dissolved in 95% ethanol and DMSO, respectively.

Data analysis

Data are expressed as pD_2 values and pD_2 is defined as the negative logarithm to base ten of the EC_{50} values. In all experiments, n equals the number of animals used in these experiments. Relaxation is expressed as percentage of phenylephrine-induced tone ± s.e.mean. The significance of differences between mean values was calculated by Student's t-test. Statistical significance of differences between the means of data groups was performed using ANOVA for curve analysis. Significance was assumed if $P<0.05$.

Results

Both acetylcholine and potassium can induce vasorelaxation in eNOS(+/+) and (−/−) mice

In endothelium-intact saphenous arteries from eNOS(+/+) mice precontracted with phenylephrine (1 μM), acetylcholine induced a concentration-dependent relaxation response with a pD_2 value of 7.3 ± 0.04 and maximal relaxation of 91.3 ± 1.9% of the phenylephrine-induced contraction. The cyclo-oxygenase inhibitor indomethacin (10 μM) or NO synthase inhibitor, L-NNA (100 μM) attenuated acetylcholine-induced relaxation to 80.0 ± 8.0% or 64.9 ± 6.3%, respectively ($P<0.05$) ($n=6$). In the presence of the indomethacin and L-NNA, the relaxation was attenuated to 51.4 ± 7.1% with a pD_2 value of 6.8 ± 0.05 ($P<0.05$). The relaxation was significantly inhibited to 20.0 ± 3.7% with a pD_2 value of 6.3 ± 0.04 by the combination of ODQ (10 μM), indomethacin and L-NNA ($P<0.05$) (Figure 1a). In contrast to eNOS(+/+) mice, the maximal relaxation induced by acetylcholine in eNOS(−/−) mice was 65.7 ± 5.1% (pD_2 value of 6.6 ± 0.05). Moreover, the combination of L-NNA and indomethacin, or L-NNA, indomethacin plus ODQ, neither affected acetylcholine-induced relaxation (pD_2 value of 6.7 ± 0.04, 6.6 ± 0.07) nor maximal relaxation (55.7 ± 2.3, 57.5 ± 4.2% respectively) ($P>0.05$) (Figure 1b) in vessels from eNOS(−/−) mice. Tetrodotoxin (1 μM) has no effect on either acetylcholine or K^+-induced relaxation (data not shown). Based upon our observation that acetylcholine-mediated relaxation was almost totally abolished by the combination of cyclo-oxygenase, NO synthase and guanylyl cyclase inhibitors, these data indicate that in mouse saphenous arteries, nitric oxide and PGI_2 are important in mediating endothelium-dependent relaxation in eNOS(+/+) mice, whereas a non-NO/PGI_2 factor(s) is entirely responsible for mediating endothelium-dependent vasorelaxation in eNOS(−/−) mice.

An increase in extracellular K^+ by adding an additional 2–15 mM KCl (final bath concentration of 6.8–19.8 mM K^+) induced a dose-dependent relaxation in saphenous arteries in both eNOS(+/+) and (−/−) mice with maximum relaxation of 68.5 ± 4.0, 74.5 ± 6.3% and pD_2 value of 4.9 ± 1.4, 6.1 ± 0.6 respectively ($P>0.05$) (Figure 2). Maximal responses were induced by the addition of 10–12 mM KCl for a final bath concentration of 14.8–16.8 K^+. In subsequent experimental protocols, the

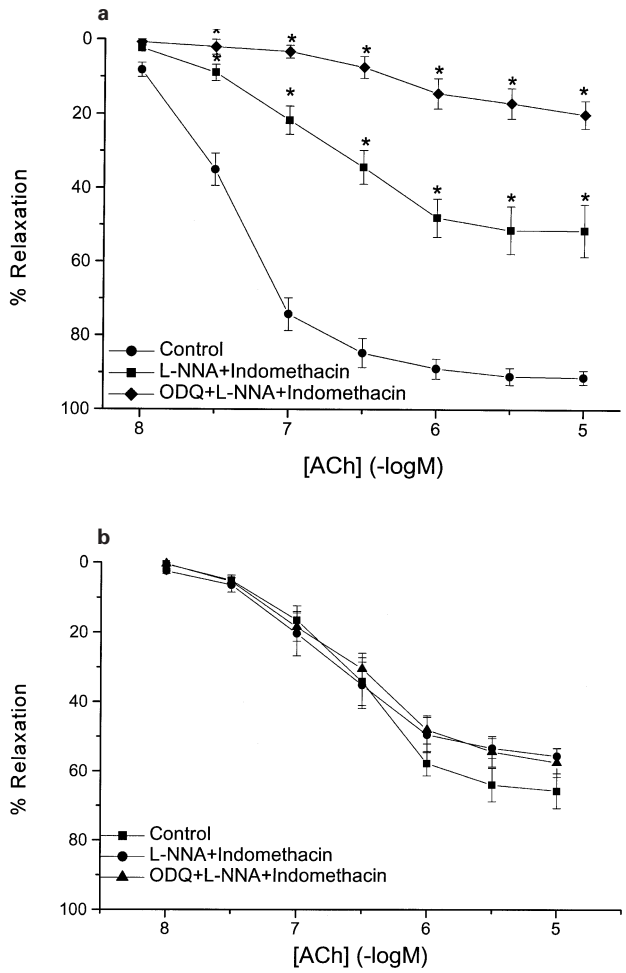

Figure 1 Concentration-response curves showing the effects of NOS (L-NNA, 100 μM) in combination with COX (indomethacin, 10 μM) inhibition, and NOS, COX and GC inhibition (ODQ, 10 μM) versus control (no inhibitors) on acetylcholine-induced vasorelaxation of phenylephrine (1 μM) precontracted mouse saphenous arteries. ($n=10$). (a) In vessels from eNOS +/+ mice both NOS and COX and the combination of NOS, COX, and GC inhibition significantly reduced acetylcholine-induced vasorelaxation (*$P<0.05$). (b) In vessels from eNOS −/− mice neither NOS plus COX nor NOS, COX and GC inhibition had a significant effect on acetylcholine-mediated relaxation ($P>0.05$).

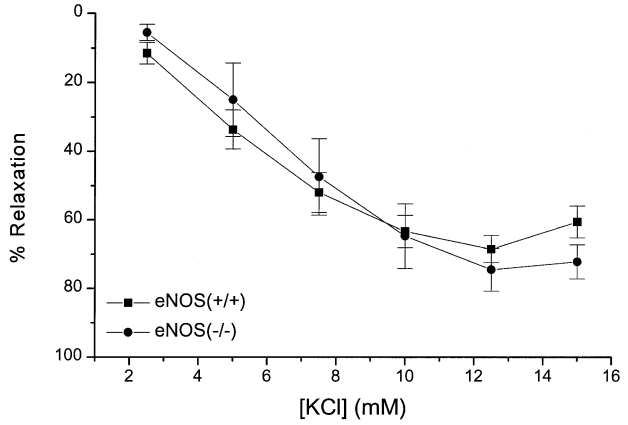

Figure 2 Concentration-response curves showing that increasing extracellular K^+ by the addition of 2–15 mM KCl (for a final bath concentration of 6.8–19.8 mM K^+) produces equivalent vasorelaxant responses in saphenous arteries from both eNOS +/+ and −/− mice ($P>0.05$) ($n=7$). Data are means±s.e.mean.

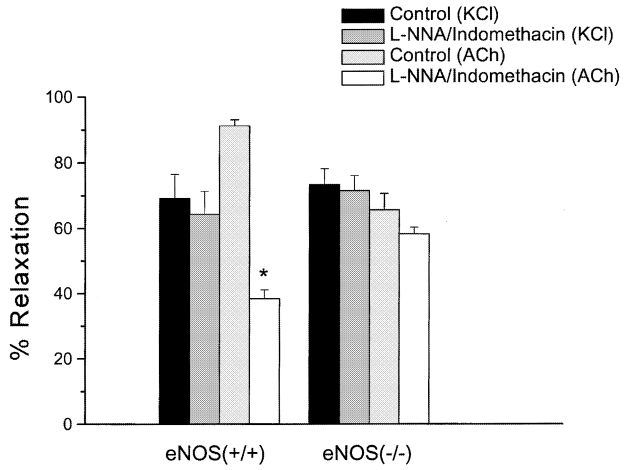

Figure 3 Relaxation responses to either 100 μM acetylcholine or the addition of 10 mM KCl in saphenous arteries in the absence or presence of a combination of NOS and COX inhibition. Acetylcholine, but not K^+, induced relaxation in vessels from eNOS +/+ was significantly inhibited (*$P<0.05$) in the presence of NOS and COX inhibition, whereas in vessels from eNOS −/− mice the relaxation responses to both acetylcholine and K^+ were insensitive to the combination of NOS and COX inhibition ($P>0.05$, $n=6$).

addition of 10 mM KCl to the tissue bath was used to study the pharmacological properties of K^+ induced vasorelaxation.

Acetylcholine-induced relaxation in eNOS(+/+) mice was sensitive to L-NNA and indomethacin with a maximum relaxation of 38.4±2.7% compared to almost 100% relaxation under control conditions, but K^+-induced relaxation was insensitive to a combination of L-NNA and indomethacin in both eNOS(+/+) and (−/−) mice with maximal relaxations of 64.3±7.0% compared to 68.8±6.7% in (+/+) mice, and 66.7±6.9% compared to 73.4±4.8% in (−/−) mice in the absence of L-NNA and indomethacin ($P>0.05$) (Figure 3). Endothelium-denuded preparations were also used to determine if the relaxation-induced by acetylcholine and potassium was endothelium-dependent. After removal of the endothelium, acetylcholine-induced relaxation was abolished, but K^+-induced relaxation remained: 50.5±9.2% in eNOS(+/+) mouse saphenous arteries and 69.5±6.5% in eNOS(−/−) mice and compared to endothelium intact preparations, the difference was not significant ($P>0.05$) (Figure 4).

K^+-induced relaxation in saphenous arteries is sensitive to K_{IR} inhibitor Ba^{2+} and the Na^+/K^+ ATPase inhibitor ouabain

Ten mM potassium-induced relaxation of mouse saphenous arteries was partially inhibited in the presence of 30 μM Ba^{2+} or 10 μM ouabain in eNOS(+/+) mice with maximal relaxations of 34.5±5.5 and 42.6±6.5%, respectively (Figure 5). In eNOS(−/−) mice, Ba^{2+} or ouabain inhibited K^+-induced relaxation to comparable levels as exhibited in tissues from eNOS(+/+) mice (36.5±5.1 and 37.9±7.2%, respectively). The combination of Ba^{2+} and ouabain almost totally abolished 10 mM K^+-induced relaxation in both eNOS(+/+) and (−/−) mice (maximal relaxations were 7.6±3.0 and 4.3±1.4%, respectively) ($P<0.05$). In contrast, barium, ouabain, and the combination of barium and ouabain produced only a comparably smaller inhibition of acetylcholine-induced relaxation, in the presence of L-NNA and

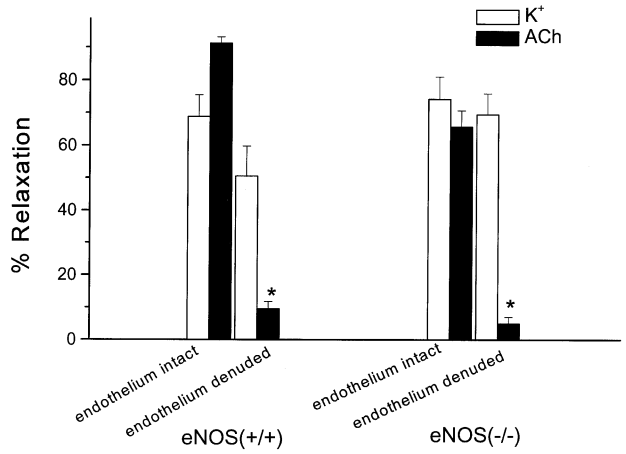

Figure 4 Acetylcholine- and K^+-induced vasorelaxation responses were compared in endothelium-intact and endothelium-denuded preparations of mouse saphenous arteries. The removal of the endothelium eliminated the acetylcholine-induced relaxation in saphenous arteries from both eNOS +/+ and −/− mice whereas the K^+-induced relaxation was shown to be endothelium-independent ($P>0.05$, $n=8$). Data are means±s.e.mean.

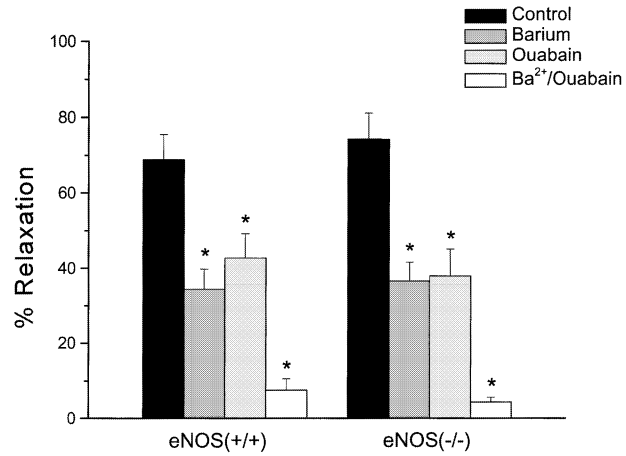

Figure 5 The relaxation response induced by the addition of 10 mM K^+ to saphenous arteries from eNOS +/+ and eNOS −/− mice was partially inhibited in the presence of 30 micromolar Ba^{2+} or 10 μM ouabain (*$P<0.05$, $n=7$). The combination of Ba^{2+} and ouabain, however, almost totally abolished the vasorelaxant response to K^+ in saphenous arteries from both eNOS +/+ and eNOS −/− mice. (*$P<0.05$, $n=7$). Data are means±s.e.mean.

indomethacin, in eNOS(+/+) mice with maximum relaxations of 36.0±8.5, 37.6±5.5 and 38.0±8.8% from 51.4±7.1% and pD_2 value of 6.3±0.09, 6.6±0.05, 6.50±0.03 (Figure 6). However, in mouse saphenous arteries from eNOS(−/−), acetylcholine-induced relaxation was insensitive to either barium, ouabain alone or the combination of barium and ouabain with maximum relaxation 50.7±8.4, 55.0±6.6 and 50.0±7.7% and pD_2 value of 6.7±0.03, 6.6±0.03, 6.4±0.05 ($P>0.05$).

Acetylcholine-induced relaxation in saphenous arteries is abolished by a combination of apamin and ChTX, but not IBTX and apamin

Acetylcholine- (in the presence of L-NNA and indomethacin) and K^+-induced relaxations were also compared in the presence of the following potassium channel inhibitors: ChTX, apamin and IbTX. Increasing extracellular K^+ to 30 mM

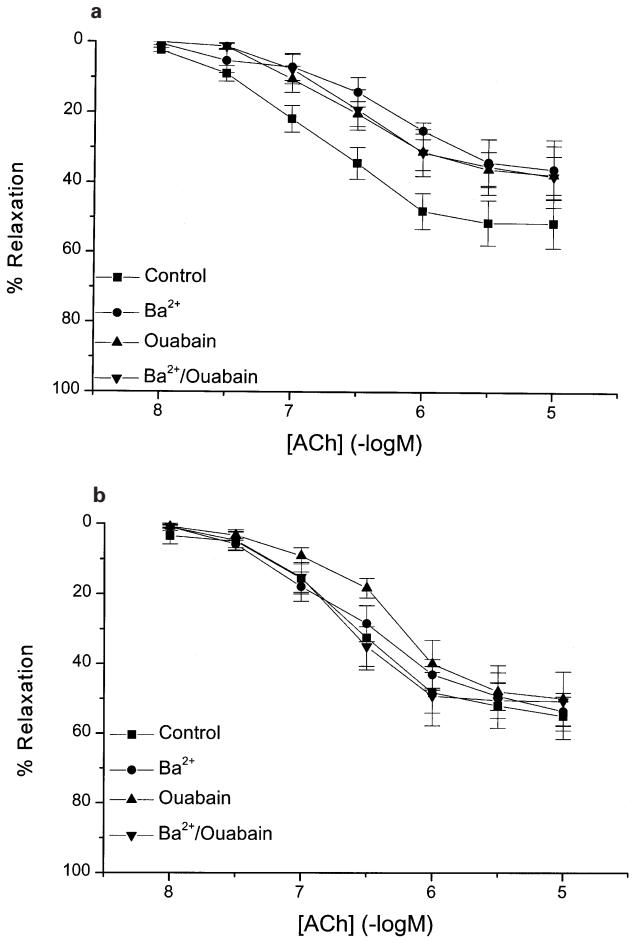

Figure 6 Concentration-response curves showing the lack of effect of barium, or ouabain alone or the effect of a combination of barium and ouabain, on acetylcholine-induced vasorelaxation in saphenous arteries isolated from (a) eNOS +/+ and (b) eNOS −/− mice ($n=7$) obtained in the presence of a combination of NOS (L-NNA, 100 μM) and COX (indomethacin, 10 μM).

completely abolished acetylcholine-induced relaxation in eNOS(−/−) mice (data not shown). Exposure of the vessels to 100 nM ChTX elicited a contraction that was equal to that produced by 10 μm phenylephrine, however, neither apamin nor IbTX produced a comparable response. Furthermore, the addition of ChTX to a tissue contracted with a sub-maximal concentration of phenylephrine resulted in an additional contraction. Neither ChTX (100 nM) nor apamin (1 μM) alone significantly affected the vasorelaxation induced by acetylcholine: maximal relaxations of 61.5±6.5 and 50.5±7.2% and pD_2 values of 6.4±0.08 and 6.3±0.05 in eNOS(+/+) mice (Figure 7a) and maximal relaxations of 67.3±6.7 and 42.7±4.5% and pD_2 value of 6.4±0.07 and 6.2±0.02 in eNOS(−/−) mice (Figure 7b) ($P>0.05$). However, a combination of apamin and ChTX completely abolished the acetylcholine-induced relaxation in both eNOS(+/+) (Figure 7a) and (−/−) mice (Figure 7b) with maximal relaxation responses of 10.1±5.0% and 3.0±1.7% respectively ($P<0.05$). Substitution of ChTX by IbTX, or the combination of IbTX with apamin produced no additional inhibition to that produced by ChTX or apamin alone (37.3±8.5 and 27.5±4.5% respectively versus 39.5±9.6% for control in eNOS(+/+) mice). In the presence of ChTX and apamin, the relaxation produced by 10 mM K^+ was unaffected with maximal relaxation responses of 70.4% and 74% in eNOS(+/+) and (−/−) mice.

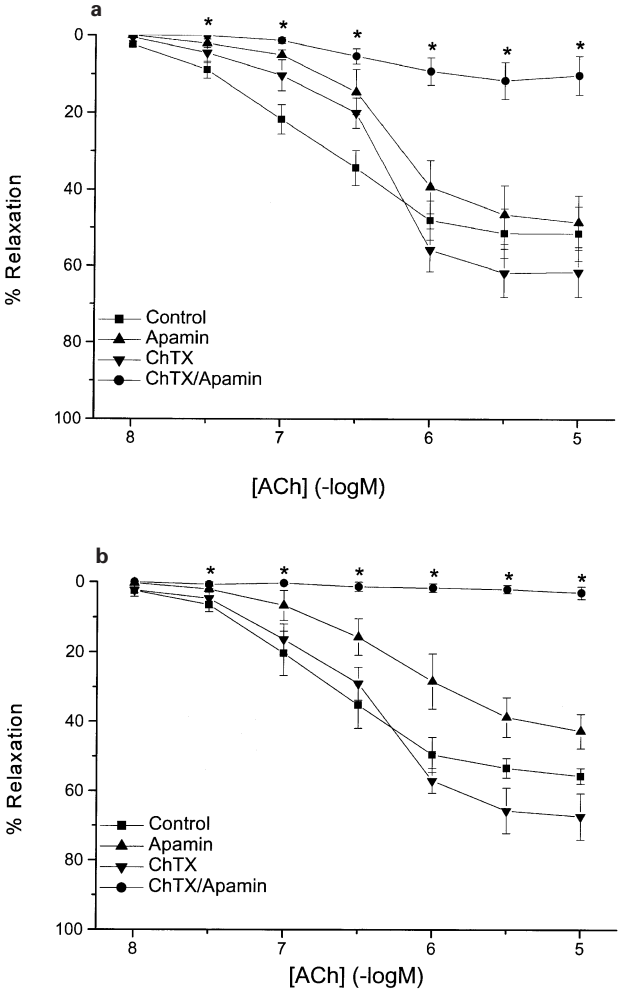

Figure 7 Concentration-response curves showing the effects of potassium channel blockade with ChTX (100 nM), apamin (1 μM), or the combination of ChTX and apamin, in the presence of NOS and COX inhibition (100 μM L-NNA and 10 μM indomethacin), on acetylcholine-induced relaxation in saphenous arteries from (a) eNOS +/+ and (b) eNOS −/− mice ($n=5$). In vessels from both eNOS +/+ and −/− animals a combination of apamin and ChTX almost completely abolished the acetylcholine-induced relaxation (*$P<0.05$) whereas neither apamin nor ChTX alone had a significant inhibitory effect ($P>0.05$).

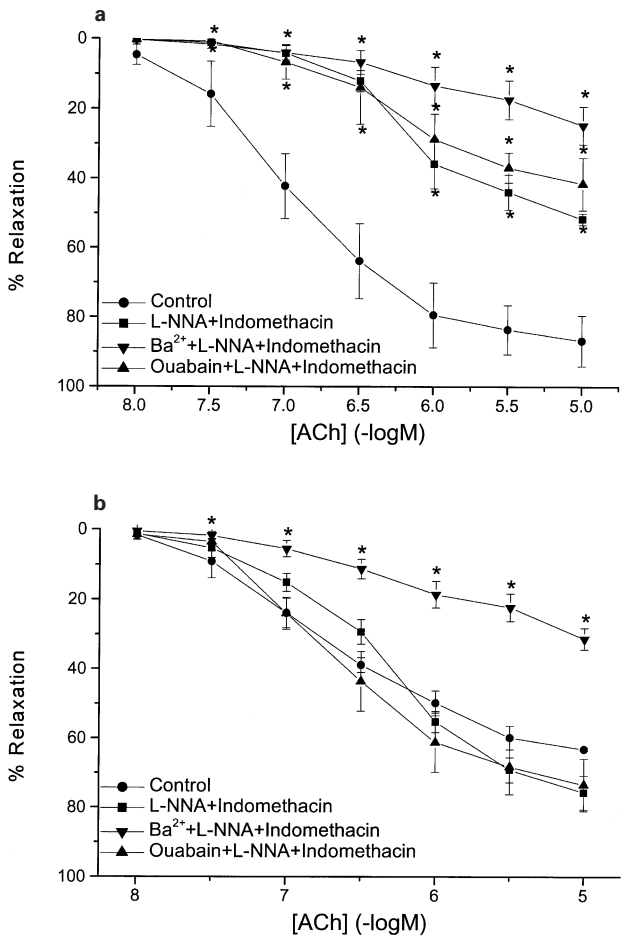

Figure 8 Concentration-response curves showing the effects of barium (30 μM) or ouabain (10 μM), in the presence of NOS and COX (100 μM L-NNA and 10 μM indomethacin), on acetylcholine-induced relaxation in mesenteric arteries isolated from (a) eNOS +/+ mice ($n=7$) and (b) eNOS −/− mice ($n=7$). Barium, but not ouabain, significantly inhibited the maximal relaxation response to acetylcholine in vessels from eNOS +/+ (*$P<0.05$), but neither barium nor ouabain produced a significant inhibitory action on the relaxation response to acetylcholine in vessels from eNOS −/− (*$P<0.05$). Data are mean ± s.e.mean.

Contribution of K^+ to EDHF response in mesenteric arteries

In mesenteric resistance arteries, both acetylcholine and K^+ induced a concentration-dependent relaxation in eNOS(+/+) and (−/−) mice. In the presence of L-NNA and indomethacin, the maximum acetylcholine-induced relaxation was reduced from 86.8 ± 7.3 to 51.7 ± 1.7% in eNOS(+/+) mice ($P<0.05$) (Figure 8a), but was not significantly altered in eNOS(−/−) mice ($P>0.05$): 75.7 ± 4.9 versus 63.2 ± 0.4% (Figure 8b). The acetylcholine-induced relaxation was not attenuated in the presence of ouabain (with maximum relaxations of 41.5 ± 7.6 and 73.5 ± 7.6% and pD_2 values of 6.3 ± 0.06 in and 6.70 ± 0.07 in eNOS(+/+) and (−/−) mice respectively), however, unlike in saphenous arteries, the relaxation was significantly inhibited by 30 μM Ba^{2+} with maximum relaxations of 24.7 ± 5.4 and 31.3 ± 3.1% and pD_2 values of 6.1 ± 0.08 and 6.2 ± 0.08 in eNOS(+/+) and (−/−) mice respectively ($P<0.05$).

As in saphenous arteries, 10 mM K^+-induced relaxation of mesenteric arteries was inhibited in the presence of Ba^{2+} (from 73.1 ± 4.2 to 43.0 ± 15.5% in eNOS(+/+) mice and 73.8 ± 4.4

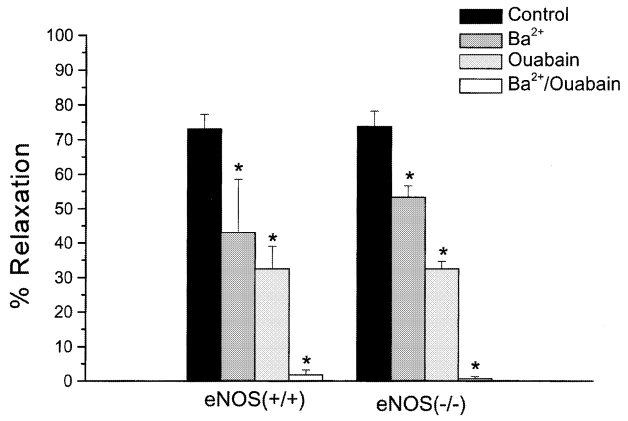

Figure 9 The relaxation responses mediated by the addition of 10 mM K^+, in the presence of a combination of NOS (L-NNA, 100 μM) and COX (indomethacin, 10 μM) inhibition, to mesenteric arteries from eNOS +/+ and −/− mice were partially (*$P<0.05$) inhibited by either barium (30 μM) or ouabain (10 μM), and completely inhibited by the combination of barium and ouabain (*$P<0.05$, $n=6$).

to 53.3±3.3% in eNOS(−/−) mice). Ouabain also inhibited K^+-induced relaxations to 32.5±6.5% in eNOS(+/+) mice and 32.5±2.2% in eNOS(−/−) mice) and the combination of barium and ouabain totally abolished relaxation with maximum relaxation of 1.9±1.4% in eNOS(+/+) and 0.7±0.6% in eNOS(−/−) mice respectively, ($P<0.05$) (Figure 9).

Discussion

In the present study we have compared the vasorelaxant effects of a small increase in extracellular K^+ (10 mM) with that induced by acetylcholine in saphenous and first order mesenteric arteries from eNOS(+/+) and (−/−) mice. The results indicate that under conditions where NOS activity is absent and guanylyl cyclase and cyclo-oxygenase are inhibited, an endothelium-dependent relaxation can still be produced by acetylcholine and this relaxation has the properties of being mediated by an EDHF. In both mesenteric and saphenous vessels, a small increase in extracellular K^+ also relaxed precontracted vessels but in an endothelium-independent manner. K^+-mediated relaxations were sensitive to inhibition by a combination of Ba^{2+} and ouabain thus inferring contributions from the activation of a K_{IR} and an electrogenic Na^+/K^+ ATPase. The NOS, guanylyl cyclase and cyclo-oxygenase inhibitor-insensitive and endothelium-dependent acetylcholine-mediated relaxation of mesenteric arteries was reduced by Ba^{2+} inferring a role for K_{IR} in contributing to EDHF in the mesenteric vascular bed. In contrast, in saphenous arteries from eNOS(+/+) mice, the relaxation pharmacologically attributed to EDHF was only partially inhibited by either Ba^{2+} alone or in combination with ouabain and in the vessels from eNOS(−/−) mice, EDHF-mediated relaxations were completely insensitive to the Ba^{2+} and ouabain combination.

The characteristics of disruption of the eNOS gene have been previously reported and include a loss of endothelium-dependent relaxation in the thoracic aorta (Huang *et al*., 1995; Meng *et al*., 1996). Our results indicate that acetylcholine can induce relaxation of precontracted mesenteric and saphenous arteries from both eNOS(+/+) and (−/−) mice, however, the pharmacological properties of the endothelium-dependent relaxation differ not only in a vessel-dependent fashion but also are influenced by the presence or absence of eNOS expression. Thus, in both saphenous and mesenteric vessels from eNOS(−/−) mice, the relaxation was predominantly mediated by NO, but in eNOS(−/−) mice, the relaxation could be attributed to a non-NO, non-prostanoid hyperpolarizing factor. The endothelium-dependent relaxation in arteries lacking endothelial NOS was completely inhibited by increasing extracellular K^+ to 30 mM or by the combination of apamin and ChTX. These data indicate that a potassium flux was required to elicit endothelium-dependent relaxation, that calcium-activated potassium channels were involved in mediating the response, and that relaxation was presumably mediated by an EDHF.

An elevation of extracellular K^+ has been reported to dilate rat cerebral arteries by two independent mechanisms, the first *via* K^+ stimulation of the electrogenic Na^+/K^+ ATPase, and the second *via* the activation of the Ba^{2+} sensitive K_{IR} channels (McCarron & Halpern, 1990). In our study an increase of 2–12 mM K^+ (bath concentration of 6.8–16.8 mM) induced an endothelium-independent relaxation in both saphenous and mesenteric arteries in both eNOS(+/+) and (−/−) mice. Furthermore, these K^+-induced relaxations were sensitive to a combination of barium and ouabain, suggesting that K^+ efflux through K_{IR} and Na^+/K^+ ATPase are both involved in mediating the assumed hyperpolarization and relaxation of the smooth muscle cells. Since the K^+-induced relaxation of the vessels was independent of the endothelium, these data also provide indirect evidence for the presence of both K_{IR} channels and an electrogenic Na^+ pump on the vascular smooth muscle cells. The relaxation to both acetylcholine and K^+ is insensitive to tetrodotoxin, thus indicating that the release of a neuronal mediator is not involved in mediating the vasorelaxation responses. Activation of K_{IR} by acetylcholine, or by changing the extracellular K^+ ion concentration, causes hyperpolarization and relaxation. The ouabain-sensitive component of the relaxation may result from the inhibition of the sodium pump, or indirectly due to the closure of K_{IR} through a change in membrane potential. K_{IR} are very steeply voltage dependent, closing on depolarization (Edwards & Hirst, 1988), and ouabain is known to depolarize vascular smooth muscle (Hirst & Van Helden, 1982). Thus an enhanced pump activity or an increase in potassium efflux will produce a hyperpolarization and relaxation of vascular smooth muscle. Our results, which describe the vasorelaxant effects of K^+ in mouse mesenteric and saphenous arteries, are comparable to those described recently by Edwards *et al*. (1998) for rat hepatic and mesenteric vessels.

In spite of an extensive literature concerning the nature of EDHF, the chemical nature and cellular mode of action of this still putative mediator remain unclear (Garland *et al*., 1995; Feletou & Vanhoutte, 1996; Waldron *et al*., 1996; Mombouli & Vanhoutte, 1997; Quilley *et al*., 1997; Triggle *et al*., 1999). EDHF relaxes smooth muscle by directly or indirectly activating K^+ channels and thus hyperpolarizing smooth muscle cells and decreasing the probability of opening of voltage-gated calcium channels. It has recently been suggested, based on the similarity between the vasorelaxant action of a small increase in extracellular K^+ and the cellular actions of the putative EDHF, that endothelium-dependent hyperpolarization results from a small increase in extracellular potassium that has been derived from endothelial cells following the activation of endothelial ChTX-and apamin-sensitive K^+ channels (Edwards *et al*., 1998). Edwards *et al*. (1998) propose that a small increase in extracellular K^+ in the myoendothelial milieu results in the hyperpolarization and relaxation of the adjacent smooth muscle cells *via* the activation of Ba^{2+}-sensitive K^+ channels and ouabain-sensitive Na^+/K^+ ATPase.

Our data indicate that acetylcholine-induced vasorelaxation of arteries from eNOS-(−/−) mice was mediated by a factor (EDHF) that is neither NO nor PGI_2, however, the pharmacological properties of EDHF in saphenous versus mesenteric arteries appears quite different. In saphenous arteries, acetylcholine-induced relaxation was completely insensitive to Ba^{2+} and ouabain, suggesting that K_{IR} and Na^+/K^+ ATPase are not involved in acetylcholine-induced relaxation in this vessel, whereas Ba^{2+} significantly reduced the response to acetylcholine in the mesenteric arteries. Of interest was that the relaxation induced by acetylcholine in both saphenous and mesenteric arteries was totally abolished by the combination of apamin and ChTX but not by apamin or ChTX alone or by the combination of apamin and IbTX. Similar data have been published for rat mesenteric arteries (Edwards *et al*., 1998; Chen & Cheung, 1997) leading to the suggestion that there might be a novel K^+ channel(s) that mediates acetylcholine-induced relaxation (Zygmunt & Hogestatt, 1996). Alternatively, since ChTX is not specific for BK_{Ca} channels and also blocks voltage-dependent K^+ channels

(Kv1.2) (Nelson & Quayle, 1995; Cook, 1990), whereas IbTX is a highly selective and potent blocker of large conductance K_{Ca} channels, the data can be interpreted as evidence for a role of apamin-sensitive SK_{Ca} and ChTX-sensitive voltage-gated K^+ channels. Regardless, in the presence of both apamin and ChTX and in both mouse saphenous and mesenteric arteries, we report that potassium-induced relaxation was unaffected thus clearly indicating the differences between K^+ and EDHF-mediated vasorelaxation. In mesenteric arteries, the combination of apamin and ChTX did block acetylcholine-induced relaxation, and potasssium-induced relaxation was partially blocked by Ba^{2+}, however, apamin and ChTX did not inhibit K^+-mediated relaxations. Since the potassium-induced relaxation was endothelium-independent, the targets for ChTX and apamin in mouse mesenteric arteries were likely K^+ channels in endothelial cells. These data are also consistent with a recent publication with concluded that the combination of apamin and ChTX blocked EDHF-mediated relaxation by an action on the endothelial rather than on the smooth muscle cells (Doughty *et al*., 1999). In contrast to our data with mouse saphenous arteries, we report that precontracted mouse mesenteric vessels relax to acetychloine *via* a mechanism that involves a barium-sensitive component that thus matches that observed for K^+-mediated relaxation of this vessel.

In conclusion, the EDHF-mediated vasorelaxation in mouse mesenteric arteries appears to be at least partially dependent on the activation of a Ba^{2+}-sensitive K_{IR} that is involved in both acetylcholine and K^+-mediated vasorelaxation. In contrast, whatever cellular mechanisms are responsible for mediating EDHF in mouse saphenous arteries do not involve K_{IR} nor the activation of the Na^+/K^+ ATPase pump. Collectively, these data also indicate that EDHF is unlikely to be one single molecular species and, furthermore, that the K^+ channels involved in mediating the effects of EHDF show significant tissue differences that may reflect tissue-dependent specialization of function.

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