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Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase

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1 Acetycholine-mediated relaxations in phenylephrine-contracted aortas, femoral and mesenteric resistance arteries were studied in vessels from endothelial nitric oxide synthase knock-out (eNOS −/−) and the corresponding wild-type strain (eNOS +/+) C57BL6/SV19 mice.

2 Aortas from eNOS (+/+) mice relaxed to acetylcholine in an endothelium-dependent N^G-nitro-L-arginine (L-NOARG) sensitive manner. Aortas from eNOS (−/−) mice did not relax to acetylcholine but demonstrated enhanced sensitivity to both authentic NO and sodium nitroprusside.

3 Relaxation to acetylcholine in femoral arteries was partially inhibited by L-NOARG in vessels from eNOS (+/+) mice, but relaxation in eNOS (−/−) mice was insensitive to a combination of L-NOARG and indomethacin and the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). The L-NOARG/ODQ/indomethacin-insensitive relaxation to acetylcholine in femoral arteries was inhibited in the presence of elevated (30 mM) extracellular KCl.

4 In mesenteric resistance vessels from eNOS (+/+) mice, the acetylcholine-mediated relaxation response was completely inhibited by a combination of indomethacin and L-NOARG or by 30 mM KCl alone. In contrast, in mesenteric arteries from eNOS (−/−) mice, the acetylcholine-relaxation response was insensitive to a combination of L-NOARG and indomethacin, but was inhibited in the presence of 30 mM KCl.

5 These data indicate arteries from eNOS (−/−) mice demonstrate a supersensitivity to exogenous NO, and that acetylcholine-induced vasorelaxation of femoral and mesenteric vessels from eNOS (−/−) mice is mediated by an endothelium-derived factor that has properties of an EDHF but is neither NO nor prostacyclin. Furthermore, in mesenteric vessels, there is an upregulation of the role of EDHF in the absence of NO.

Keywords: eNOS knockout mice; EDHF; NO; smooth muscle; endothelium

Abbreviations: EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; eNOS (−/−) mice, endothelial nitric oxide synthase knock-out mice; eNOS (+/+) mice, wild-type (C57BL/SV19) mice; L-NAME, N^G-nitro-L-arginine methyl ester; L-NOARG, N^G-nitro-L-arginine; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; TEA, tetraethylammonium

Introduction

It is generally believed that endothelium-dependent relaxation to acetylcholine is primarily mediated by the release of nitric oxide (NO). However, other mediators including prostacyclin and a poorly characterized endothelium-dependent hyperpolarizing factor (EDHF) have been proposed. Nonetheless, the existence of EDHF has been questioned and various explanations have been put forward to account for the hyperpolarization and nitric oxide synthase (NOS) inhibitor-resistant relaxations that have been observed in several arteries. These include incomplete pharmacological blockade of NOS (Martin *et al*., 1992; Cohen *et al*., 1997), NO or prostacyclin activation of potassium channels (Tare *et al*., 1990; Parkington *et al*., 1993; Bolotina *et al*., 1994), gap junctional communication between endothelial and smooth muscle cells (Davies *et al*., 1988), and a small increase in extracellular K^+ (Edwards *et al*., 1998). Here we describe acetylcholine-induced relaxation of small peripheral arteries isolated from mice which lack endothelial NOS. This relaxation possesses the characteristics of being mediated by an EDHF.

Methods

Breeding pairs of homozygous endothelial NOS knockout (eNOS−/−) and control (C57BL6/SV19) mice were obtained from Dr Paul Huang (Cardiovascular Research Centre, Massachusetts General Hospital) and bred at the University of Calgary. The generation of eNOS (−/−) mice by gene targeting in embryonic stem cells has been previously described (Huang *et al*., 1995). In accordance with guidelines established by the University of Calgary Animal Care Committee, mice of either sex (25–37 g) were killed by cervical dislocation and the aorta, femoral and first order branches of the mesenteric artery were dissected out into Krebs' solution of composition (in 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_2/5% CO_2. The aorta was cut into 2 mm rings and placed on wires in an organ bath under a resting tension of 1 g. Isometric force was measured by Grass FT-03 transducers and a Grass model 7D polygraph. Femoral arteries were mounted in a

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Mulvany-Halpern myograph (Model 400A, J.P. Trading, Denmark) under normalised tension as previously described (Mulvany & Halpern, 1977). Mesenteric arteries were mounted in a pressure myograph (Living Systems, Vermont, U.S.A.) under constant pressure of 60 mmHg, perfusion of 50 μl min^{-1} and superfusion of 5 ml min^{-1} and their diameter was measured by videomicroscopy. All experiments were performed at 37°C. For the relaxation/vasodilation experiments, tissues were precontracted with phenylephrine (0.3–10 μM). Care was taken to ensure that a submaximal contraction/constriction was obtained, and that equi-effective concentrations of phenylephrine or KCl were used in all protocols. High [K^+] solutions were made by iso-osmolar replacement of Na^+ by K^+. Data are expressed as pD_2 values which are defined as the negative logarithm to base 10 of the EC_{50} values. Relaxation is expressed as percentage of phenylephrine-induced tone (aorta and femoral arteries) or vasoconstriction (mesenteric artery) ±s.e.mean. In the femoral artery experiments there was a tachyphylaxis between the first and second concentration-response curve to acetylcholine, though subsequent control curves were not different from the second. The significance of differences between mean values was calculated by Student's *t*-test. All drugs were from Sigma except 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Tocris, MO, U.S.A.) and NO gas (Union Carbide, Canada).

Results

pD_2 values for phenylephrine in wild-type (+/+) and eNOS knockout (−/−) mice aortas were 7.14±0.02 and 6.54±0.06, respectively ($n=5$, $P<0.05$). The maximum contractions to phenylephrine were significantly greater in eNOS (−/−) vs eNOS (+/+) (1.51±0.16 Nm^{-1} vs 1.24±0.15 Nm^{-1}, $n=5$, $P<0.05$). Aortas from eNOS (+/+) mice were contracted with phenylephrine and relaxed in response to acetylcholine (10 μM), whereas aortas from endothelial NOS knockout mice did not (67±3.5 vs 6±3.5%, $n=5$ and 6). However, as illustrated in Figure 1, aortas from both groups of animals were maximally relaxed by exogenous NO, aortas from eNOS (−/−) mice being significantly more sensitive than C57BL6/SV19 mice (control pD_2 7.26±0.04 vs 8.07±0.06, $n=5$ and 6, $P<0.001$). Aortic tissue from eNOS (−/−) was also more sensitive to sodium nitroprusside than was tissue from eNOS (+/+) (pD_2 of 8.46±0.04 vs 10.21±0.02, $n=5$–6, $P<0.001$ for eNOS (+/+) and eNOS (−/−) respectively).

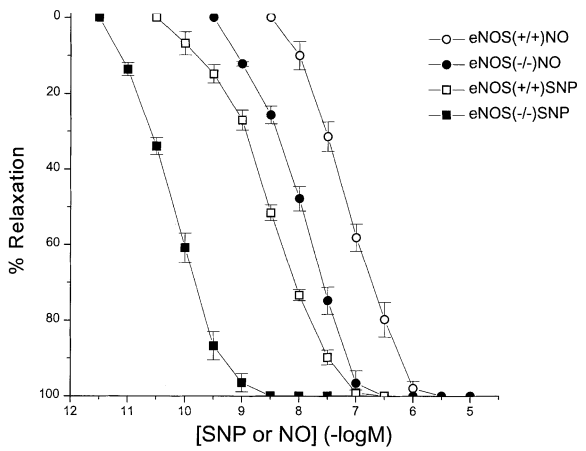

Figure 1 Concentration-relaxation curve to authentic nitric oxide (NO) solution and sodium nitroprusside in phenylephrine-precontracted aorta from eNOS (+/+) and eNOS (−/−) mice.

Femoral arteries isolated from eNOS (−/−) and eNOS (+/+) mice contracted to both KCl and phenylephrine (Figure 2a,b), and there was no significant difference ($P>0.05$) in the pD_2 values obtained for KCl (1.47±0.9 vs 1.56±0.7, $n=8$, $P>0.05$ respectively). For phenylephrine in the absence or presence of L-NOARG (100 μM), the pD_2 values were 5.89±0.03; 5.92±0.02; 5.60±0.06; 5.87±0.03 for eNOS (−/−) in the absence and presence of L-NOARG and eNOS (+/+) in the absence of L-NOARG, respectively ($n=10$). In the absence of L-NOARG, femoral arteries from the (−/−) mice were significantly ($n=10$, $P<0.05$) more sensitive to phenylephrine, however, the difference in sensitivity was lost after pretreatment with L-NOARG. The maximum contraction to phenylephine in the femoral artery were for eNOS (+/+) 2.05±0.2 Nm^{-1} ($n=3$) and for eNOS (−/−) 3.01±0.16 Nm^{-1} ($n=8$, $P<0.05$). In the presence of indomethacin, femoral arteries isolated from eNOS (+/+) mice (D_{100} 357±22 mm, $n=16$ vessels from eight animals) relaxed in response to acetylcholine (Figure 3a). The pD_2 value and maximum response for the concentration-response curve to acetylcholine was 6.82±0.06 and 88.1±3.3% ($n=4$). Exposure of the vessels to 30 mM K^+ containing Krebs' solution elicited a contraction (4.4±0.4 Nm^{-1}) that was not significantly different from that elicited by 3 μM phenylephrine (3.7±0.2 Nm^{-1}, $n=4$, $P>0.05$). In the presence of 30 mM K^+, acetylcholine relaxed the tissues with a pD_2 of 6.42±0.03 ($P=0.001$ vs phenylephrine-contracted tissue) and a maximum relaxation of 58.8±4.0% ($n=4$, $P>0.05$). In the presence of a combination of indomethacin, L-NOARG (100 μM) with nitro-L-arginine methyl ester (L-NAME, 100 μM) the acetylcholine concentration response curve was not shifted to the right (pD_2 value of 6.77±0.12, $P>0.05$) though the maximum response was decreased to 39.4±2.6% ($n=3$, $P<0.001$). In the presence of ODQ alone, the maximum response was reduced to 20.0±3.7% ($n=7$, $P<0.001$) with the pD_2 value of 6.3±0.39. Combination of indomethacin, L-NOARG, L-NAME and 30 mM K^+ abolished all relaxations induced by acetylcholine ($n=3$).

Femoral arteries isolated from eNOS (−/−) mice (D_{100} 290±15 μm, $n=26$ vessels from 13 animals) relaxed in response to acetylcholine, though to a lesser maximum than the control mice (pD_2 6.73±0.14 and 54.2±4.9%, $n=9$; $P<0.05$ vs eNOS (+/+) mice; Figure 3b). These relaxations were insensitive to indomethacin (3 μM), indomethacin plus L-NOARG (100 μM) or indomethacin, L-NOARG and ODQ (3 μM) in combination (pD_2 values of 6.78±0.29, 6.53±0.17 and 6.89±0.20 and maximal relaxations of 49.6±4.3, 51.2±3.3 and 45.9±8.3%, $n=3$ for acetylcholine, $P>0.05$ for all). Precontraction with 30 mM K^+ containing Krebs' solution, in the absence of inhibitors of NOS or guanylyl cyclase, completely abrogated the relaxation to acetylcholine in femoral arteries isolated from mice lacking endothelial NOS.

First order mesenteric arterioles from eNOS (−/−) mice had a resting diameter of 259±18.8 μm ($n=8$) when pressurized to 60 mmHg. Figure 4a shows that after constriction with phenylephrine (1–10 μM, diameter 112±9.8 μm, $n=8$), these vessels dilated to acetylcholine with a pD_2 value of 6.40±0.15 and a maximum relaxation of 80.7±3.2% ($n=11$). This relaxation was slightly reduced after incubation with indomethacin (10 μM) with the pD_2 value not different at 6.59±0.30 ($n=4$) and only the relaxation due to 10 μM acetylcholine significantly reduced. The acetylcholine-induced relaxation was virtually abolished by L-NOARG (100 μM) in combination with indomethacin (maximum

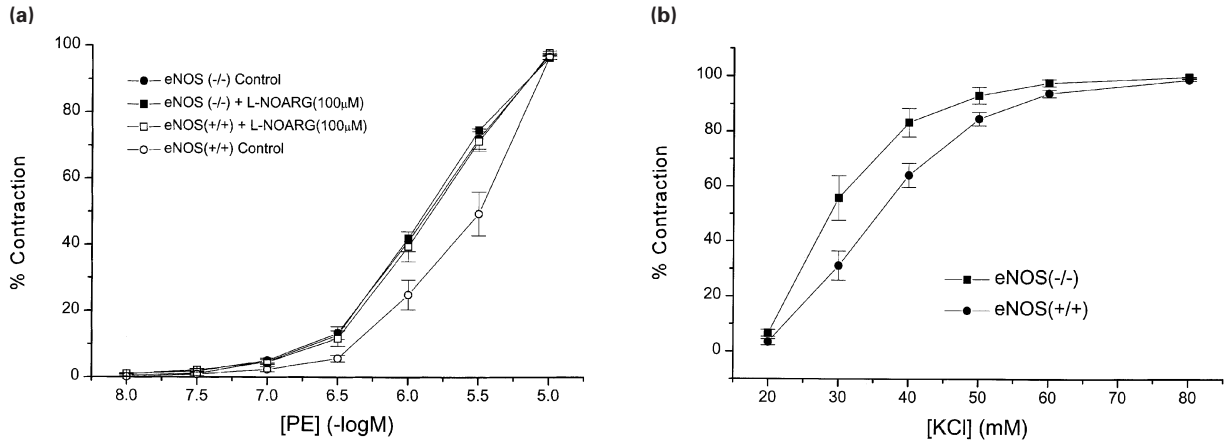

Figure 2 Concentration-response curves to (a) KCl and (b) phenylephine in the absence or presence of L-NOARG (100 μM) in femoral arteries from eNOS (+/+) and eNOS (−/−) mice.

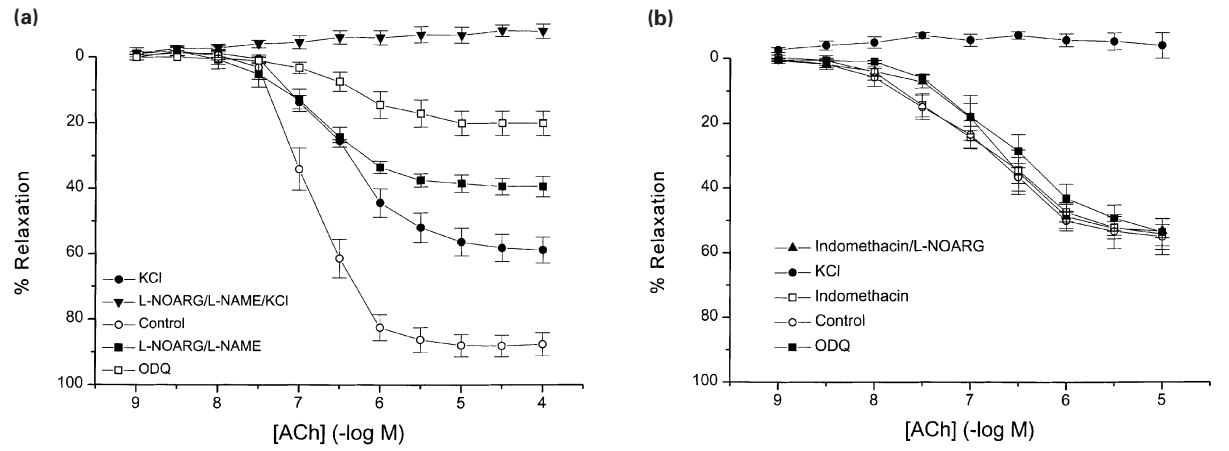

Figure 3 Effect of inhibition of cyclo-oxygenase, NOS, guanylyl cyclase or potassium efflux and combination of these treatments on acetylcholine-induced relaxation of phenylephrine precontracted femoral arteries isolated from (a) eNOS (+/+) and (b) eNOS (−/−) mice.

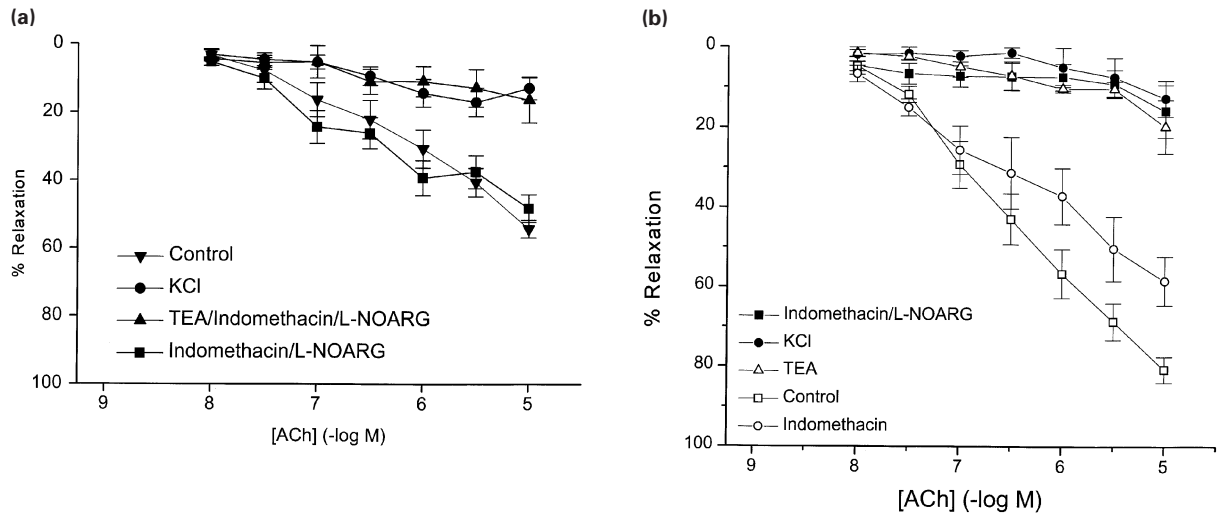

Figure 4 Effect of inhibition of cyclo-oxygenase, NOS or potassium efflux and combination of these treatments of acetylcholine-induced vasodilation of phenylephrine preconstricted mesenteric arteries isolated from (a) eNOS (+/+) and (b) eNOS (−/−) mice.

relaxation of 15.8±6.6%, $n=3$, $P<0.001$). Raising external K^+ to 30 mM decreased the diameter of these vessels to 188±14.6 μm and subsequent addition of phenylephrine (1 μM) constricted the vessels to 84.1±16.4 μm ($n=7$). Following this pretreatment, acetylcholine-induced relaxation was also inhibited with a maximum relaxation of 12.6±4.5% ($n=3$, $P<0.001$). The potassium channel blocker, tetraethylammonium (TEA, 1 mM), in the absence of other blockers, also inhibited the relaxation, reducing the maximal effect of acetylcholine to 19.6±6.7% ($n=4$, $P<0.001$).

Mesenteric arteries isolated from mice lacking endothelial NOS possessed a resting diameter of 238±7.4 μm, $n=13$, $P>0.05$ compared to wild type). The pD_2 values for phenylephrine-induced vasoconstriction in the absence and presence of L-NOARG in mesenteric vessels from eNOS (−/−) mice were 6.36±0.05 and 6.36±0.03 respectively, and thus were not significantly different ($P>0.05$). In contrast to the aortas from eNOS (−/−) mice, mesenteric arteries, when contracted to 84.9±9.6 μm with phenylephrine, relaxed in response to acetylcholine (pD_2 value of 6.40±0.21 and maximum relaxation of 54.2±2.6%; $n=9$; Figure 4b). However, the combination of L-NOARG and indomethacin did not affect the response (pD_2 value of 6.85±0.17 and maximum relaxation of 48.2±4.1%, $n=13$, $P>0.05$ for both). Raising extracellular K^+ to 30 mM constricted the vessels to 197±19.3 μm and further addition of 1 μM phenylephrine constricted the vessels to 79.3±8.8 μm ($n=8$). Under these conditions the response to acetylcholine was greatly reduced (16.9±4.2% maximum relaxation, $n=4$, $P<0.01$). TEA (1 mM), in the presence of indomethacin and L-NOARG, also blocked the relaxation with a maximum effect of 16.3±6.7%, ($n=3$, $P<0.001$).

Discussion

These data indicate that (a) arteries isolated from mice lacking endothelial NOS show a supersensitivity to exogenous NO, and (b) acetylcholine-induced vasorelaxation of arteries can be mediated by a factor that is not NO nor prostacyclin. The relaxation in arteries lacking endothelial NOS is completely sensitive to raised potassium or TEA indicating that a potassium flux is required to elicit this relaxation and hence is presumably mediated by an EDHF.

There is a growing body of evidence that, in addition to NO and prostacyclin, a third class of endothelium-dependent vasodilators, hyperpolarizing factors, can contribute to the regulation of vascular tone (Garland *et al*., 1995; Mombouli & Vanhoutte, 1997). These factors induce vascular smooth muscle relaxation by activation of a potassium conductance, hyperpolarization of the smooth muscle cells and hence inhibition of calcium influx through voltage-gated calcium channels. Difficulties in bioassaying EDHF and an inconsistent pharmacology between preparations have hampered efforts to identify its molecular nature and led to speculation that a factor separate from NO may not exist. Furthermore, both prostacyclin (Parkington *et al*., 1993) and NO (Bolotina *et al*., 1994) can hyperpolarize smooth muscle cells. Recently, the bradykinin evoked EDHF of the porcine coronary circulation has been bioassayed and tentatively identified as an epoxyeicosatrienoic acid (Hecker *et al*., 1994; Pöpp *et al*., 1996). In addition, a small increase in extracellular K^+ has been tentatively identified as mediating endothelium-dependent hyperpolarization in rat mesenteric and hepatic arteries (Edwards *et al*., 1998). However, the EDHF of other vascular beds has not been convincingly bioassayed and the pharmacology in other arteries is dissimilar leading to speculation about the nature of the hyperpolarizing factor (Martin *et al*., 1992; Cohen *et al*., 1997).

We have utilized the eNOS (−/−) mouse as a way of studying non NO-induced vasodilation without recourse to NOS inhibitors. Some of the cardiovascular consequences of disruption of the endothelial NOS gene have been studied previously (Huang *et al*., 1995; Meng *et al*., 1996; Shesely *et al*, 1996; Faraci *et al*., 1998). Chataigneau *et al*. (1999) reported no difference in the maximal contraction of a variety of arteries to 60 mM KCl between eNOS (+/+) and (−/−) animals; however, there was an enhanced vasoconstrictor effect to noradrenaline and U46619 in the mesenteric and coronary arteries of eNOS (−/−) animals. Our results are similar and we also report an enhanced maximum response and sensitivity to phenylephrine in the eNOS (−/−) aortic and femoral artery tissues when compared to tissues from the eNOS (+/+) animals. Faraci (1998) reported a supersensitivity to sodium nitroprusside in the carotid artery of eNOS (−/−) mice and the present results confirm and extend this observation by demonstrating an increase in sensitivity to not only sodium nitroprusside but also to exogenous NO. Supersensitivity to NO (by a factor of 6.5 in the present study) exists in the eNOS (−/−) mice as one might expect if the basal level of endogenous guanylyl cyclase stimulating activity is decreased as was concluded by Moncada *et al*. (1990) using chronic treatments with NOS inhibitors. However in our study, we report an even greater increase in sensitivity to sodium nitroprusside (48) than to NO (6.5). One explanation we can provide for this result is that tissue sensitivity to NO may be reduced when the source is provided globally to the cell (as in the case of an NO solution) vs the local release from NO that might be expected from an NO donor such as nitroprusside. Another explanation may relate to the different redox states of NO that may predominate in a solution of NO gas vs that locally released by SNP (Goyal & Hee, 1998). Surprisingly, Chataigneau *et al*. (1999) did not find any significant difference in the hyperpolarization produced by SIN-1 in coronary artery cells from eNOS (−/−) vs (+/+) mice.

Acetylcholine induced relaxation of both femoral and mesenteric arteries isolated from wild type eNOS (+/+) mice. However, the responsible mechanisms varied between these vessels and in the mesenteric artery NO predominantly accounts for the vasodilation whereas in the femoral artery a non-NO, non-prostanoid hyperpolarizing factor also contributes. The mesenteric arteries from other species including rat, guinea-pig and rabbit release EDHF and we have demonstrated acetylcholine-induced vasorelaxation in mesenteric arteries from other strains of mice (C57BL6 and C57BL/KsJ, data not shown) under identical conditions that suggests that this lack of EDHF activity is strain-dependent. Although NO appears to be the predominant endothelium-derived vasorelaxant factor released from mesenteric vessels from eNOS (+/+) mice, it is of interest that vasorelaxation response to acetylcholine is inhibited in the presence of 30 mM KCl or TEA. These data suggest that NO mediates vasorelaxation in the mesenteric resistance vessels *via* the activation of a K^+ channel(s). Further studies are required to determine the nature of the K^+ channel activated by NO and whether this process is dependent on the activation of guanylyl cyclase. However, mesenteric vessels from eNOS (−/−) mice did demonstrate a hyperpolarizing factor with different properties from that produced in vessels from eNOS (+/+) mice, as these vessels were insensitive to combined NOS and cyclooxygenase inhibition but were sensitive to raising the concentration of K^+ to 30 mM.

Unlike mesenteric arteries, femoral arteries demonstrated a non-NO, non-prostanoid relaxing factor in both eNOS (+/+) and eNOS (−/−) mice. In our studies of endothelium-dependent vasorelaxation in femoral vessels, we utilized a combination of NOS inhibitors in eNOS (+/+) mice to ensure complete block of NOS as others have found that a single NOS inhibitor is insufficient (Cohen *et al*., 1997). In addition, we determined the effect of the guanylyl cyclase inhibitor ODQ on acetylcholine-mediated vasorelaxation in eNOS (+/+) mice. ODQ produced a greater inhibition of vasorelaxation than that observed with the L-NOARG/L-NAME combination, thus indicating that NOS inhibitors alone may not be sufficient to completely inhibit the production of NO. These data are therefore in agreement with those of Cohen *et al*. (1997) which indicated that NO production could still be detected in rabbit carotid vessels after pre-treatment of the tissues with L-NAME and L-NOARG. Further, in femoral arteries of mice lacking endothelial NOS, a combination of L-NOARG and the inhibitor of soluble guanylyl cyclase, ODQ, had no effect on the acetylcholine-induced relaxation. The lack of effect of NOS inhibition on the relaxation in mesenteric and femoral arteries shows that other isoforms of NOS do not compensate for the lack of endothelial NOS derived NO in the mutant mice as is the situation in the pial arterioles of endothelial NOS (−/−) mice where neuronal (type I) NOS compensates for the lack of endothelial NOS activity (Meng *et al*., 1996).

Our results are in apparent disagreement with those of Chataigneau *et al*. (1999) who have recently described the complete loss of the endothelium-dependent vasorelaxation response to acetylcholine in aorta, coronary, carotid as well as mesenteric artery from eNOS (−/−) mice. We note that Chataigneau *et al*. (1999) did not study femoral vessels and, furthermore, the protocol pursued for the study of mesenteric vessels was different from that which we used. Thus, we investigated endothelium-dependent vasorelaxation in mesenteric arteries that were pressurized to 60 mmHg with drug effects determined by videomicroscopy following superfusion. It is known that the level of stretch applied to the vessel can affect the nature of the response to acetylcholine. For instance, Parkington *et al*. (1993) have reported that in stretched guinea-pig artery preparations, both a transient and a slow hyperpolarization response to acetylcholine was recorded, whereas in unstretched vessels only the transient hyperpolarization was recorded. Furthermore, Parkington *et al*. (1993) reported that in stretched, but not in unstretched vessels, a hyperpolarization response was recorded for both NO and prostacyclin. We also report that in the eNOS (+/+) mice, the acetylcholine-mediated vasorelaxation appears to be almost entirely mediated by an L-NOARG/indomethacin sensitive process but is also inhibited in the presence of 30 mM KCl. Thus, in the pressurized mesenteric artery from eNOS (+/+) mice, the acetylcholine-mediated vasorelaxation appears to be entirely mediated by NO/PGI_2 and results presumably from K^+ channel activation and hyperpolarization of the vascular smooth muscle cell. Of interest is that our data from femoral arteries obtained from eNOS (+/+) mice indicate a less important role for NO-mediated hyperpolarization and the contribution of a non-NO/PGI_2 mediator which has the expected properties of a distinct EDHF. Further studies are clearly warranted in order to better understand the cellular processes that determine and regulate the release and the cellular effects of NO vs PGI_2 vs EDHF.

The interpretation of gene knockout experiments has been criticized when used to infer that the product of the gene in question is unimportant when the mutant mouse has a similar phenotype as the control as compensatory changes may occur in the mutant animal (Becker *et al*., 1996). However, just such a compensatory role has been speculated for EDHF. It has been proposed that EDHF acts as a backup to NO in pathophysiological states (e.g. pulmonary hypertension; Kemp *et al*., 1995). In the present study, it is clear that an EDHF is upregulated in the mesenteric artery of eNOS knockout mice.

In conclusion, these results show that EDHF is not NO, and that EDHF acts in a compensatory manner in some vessels when endothelial NOS is absent.

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