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Heterogeneity in relaxation mechanisms in the carotid and the femoral artery of the mouse

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Abstract

The participation of prostanoids, nitric oxide and non-prostanoid non-nitric oxide factors in endothelium-dependent relaxations was investigated in phenylephrine (PE)-constricted carotid and femoral arteries of C57BL6 mice. The carotid artery was more sensitive to acetylcholine as compared to the femoral artery, and cyclooxygenase inhibition did not influence the relaxation in either vessel. In the carotid artery, high doses of acetylcholine caused transient constrictions, which were abolished by indomethacin or piroxicam. In the carotid but not the femoral artery, N^{ω}-nitro-L-arginine or 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) enhanced PE-induced contractions enormously, suggesting that endogenous nitric oxide production is much higher in the carotid artery. While in the carotid artery all relaxation was abolished by N^{ω}-nitro-L-arginine or ODQ, a residual response ($34 \pm 5\%$ and $74 \pm 4\%$, respectively) but with a different shape, was maintained in the femoral artery. This N^{ω}-nitro-L-arginine-resistant relaxation was abolished by the combination of apamin and charybdotoxin. In both arteries, ODQ abolished relaxation to *S*-nitroso-*N*-acetyl-D-penicillamine, while N^{ω}-nitro-L-arginine enhanced the sensitivity to this donor of exogenous nitric oxide. In 30 mM KCl, the relaxation to acetylcholine was abolished by N^{ω}-nitro-L-arginine or ODQ in either artery. In conclusion, in the carotid artery endothelium-dependent relaxation is mediated predominantly by nitric oxide acting via cyclic GMP-dependent pathways, while in the femoral artery part of the relaxation can be attributed to a non-prostanoid non-nitric oxide factor operating via apamin/charybdotoxin-sensitive potassium channels.

Keywords: Hyperpolarization; Nitric oxide (NO); Prostanoid; N^{ω}-nitro-L-arginine; ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one); Carotid artery; Femoral artery

1. Introduction

Since many years, the endothelium has been recognized as an important modulator of vascular tone (Furchgott and Zawadzki, 1980). It releases several vasodilator substances, such as prostacyclin, nitric oxide, and endothelium-derived hyperpolarizing factor (EDHF). The relative importance of these vasorelaxing factors in the total endothelium-dependent relaxation differs among species and even vessel types within a certain species. As far as the identity of EDHF is concerned, many suggestions have been made (Cohen et al., 1997; Edwards et al., 1998; Vanhoutte, 1998; Fisslthaler et al., 1999). Presumably, as for its relative importance, the identity of EDHF also differs among species and vessel types.

In the present study, vascular responses were studied in murine blood vessels, as a basic tool for future studies in models for atherosclerosis in which vascular dysfunction is a central feature. Both endothelium-dependent and -independent relaxations were investigated, and the relative roles of prostanoids, nitric oxide and non-prostanoid non-nitric oxide factors were elucidated step by step. Most of the previous studies in mice focused on shifts in the relaxation response as a whole (Bonthu et al., 1997), or did not investigate the different relaxation pathways simultaneously (Faraci et al., 1998; Chataigneau et al., 1999).

As relaxation mechanisms often differ among vessel types, responses were compared between the carotid and the femoral artery. Both vessels are susceptible to the development of atherosclerosis, and leave the possibility for the experimental induction of intimal thickening (Lindner et al., 1993; Carmeliet et al., 1997; Moroi et al., 1998; Kondo et al., 1999). There are, however, marked differences between both arteries. While the carotid artery can

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be defined as an elastic artery, the femoral artery is more of the muscular type. Hence, it can be anticipated that vascular responses in both vessel types might differ both in magnitude and underlying mechanisms.

2. Materials and methods

2.1. Chemicals

The following pharmacological agents were used: sodium pentobarbital (Nembutal®, Sanofi, Brussels, Belgium), indomethacin (Merck Sharp and Dohme, Brussels, Belgium), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and piroxicam (both purchased from Tocris Cooson, Bristol, UK). Prostaglandin $F_{2\alpha}$ (Dinolytic®) was a kind gift from Shering AG. Acetylcholine chloride was obtained from Sterop (Brussels, Belgium), serotonin creatinine sulphate monohydrate from Acros Organics (Geel, Belgium), and L-phenylephrine (PE), N^{ω}-nitro-L-arginine, papaverine and *N*-acetyl-D-penicillamine were purchased from Sigma (Bornem, Belgium). The equimolar reaction of *N*-acetyl-D-penicillamine with $NaNO_2$ in an acidified environment results in the formation of *S*-nitroso-*N*-acetyl-D-penicillamine. Charybdotoxin was obtained from Alomon (Jerusalem, Israel) and apamin from Sanvertech (Boechout, Belgium).

2.2. Isolation and mounting of blood vessels

Female C57BL6 mice (Charles River), aged 19–23 weeks (weight: 22.0 ± 0.14 g) were anaesthetised with sodium pentobarbital (Nembutal®, 50 mg/kg, i.p.). Carotid and femoral arteries were carefully removed and cleaned of adherent adipose and connective tissue. Vessels were mounted as 2 mm segments in a small wire (40 μm) myograph (Mulvany and Halpern, 1977) for isometric tension recording. All blood vessels were immersed in a Krebs–Ringer solution (37°C and continuously aerated with a 95% O_2/5% CO_2 gas mixture, pH 7.4) with the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NAHCO_3$ 25, CaEDTA 0.025, and glucose 11.1. Indomethacin 10 μM was added to the solution, except in those experiments where the influence of prostanoids on vascular responses was investigated. Carotid and femoral arteries were set to their normalised diameter according to the method of Mulvany and Halpern (1977). In short, vessels were stretched gradually until the calculated internal diameter reached a value equal to the internal circumference the vessel would have in vivo, when fully relaxed and under a transmural pressure of 100 mm Hg. Under these conditions, mean diameters were 414 ± 15 μm for the carotid arteries ($n = 19$) and 253 ± 5 μm for the femoral arteries ($n = 19$), achieved by force of 2.00 ± 0.11 and 0.90 ± 0.06 mN mm^{-1} respectively. All data were normalised for vessel length and expressed in units of mN mm^{-1}, which is defined as absolute force divided by twice the vessel length. After a 25 min equilibration period, vessels were exposed to a mixture of KCl 50 mM, PE 3 μM, serotonin 10 μM and prostaglandin $F_{2\alpha}$ 100 μM, to fully activate smooth muscle cells and sensitise vessel segments to the different agonists used. This procedure was repeated twice, as this was shown to be the most effective in preliminary experiments (data not shown). In some cases, the endothelium was removed by rubbing the inner surface of the vessel carefully with a human hair. Efficient endothelium removal was checked by the absence of relaxation in response to acetylcholine (10 μM).

The studies were approved by the Ethical Committee of the University, and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.3. Protocol

In all experiments, results were compared to the appropriate time control, for which the contralateral artery of the same animal was used.

A cumulative concentration–response curve (3 nM–30 μM) was made for PE in the absence or presence of indomethacin 10 μM, piroxicam 100 μM or N^{ω}-nitro-L-arginine 300 μM, and the negative logarithm of the molar dose (pD_2) resulting in 50% of the maximal contraction (E_{max}) was assessed for each vessel segment. For relaxation studies, vessels were precontracted with KCl 30 mM or PE in a concentration corresponding to the pD_2 for that individual vessel segment. Endothelium-dependent and -independent relaxation was assessed by cumulative concentration–response curves for acetylcholine (3 nM–10 μM) or *S*-nitroso-*N*-acetyl-D-penicillamine (3 nM–30 μM), respectively. N^{ω}-nitro-L-arginine 300 μM or ODQ 10 μM was added to the solution after vessels had been precontracted. After an incubation period of at least 15 min or when the precontraction level reached a plateau, a cumulative concentration–response curve for acetylcholine or *S*-nitroso-*N*-acetyl-D-penicillamine was performed in the presence of the respective inhibitors. The specificity of ODQ was checked by performing a cumulative concentration–response curve for papaverine (0.1–100 μM) in the absence and the presence of ODQ 10 μM. Between different concentration–response curves, preparations were washed at least four times until they returned to their basal level of contraction.

2.4. Statistical analysis

All results are expressed as mean ± S.E.M.; *n* represents the number of arteries. For the statistical analysis, the SPSS for Windows package was used. A 5% level of significance was selected. In all cases, results were compared to the corresponding time control and evaluation was

done using analysis of variance (ANOVA) followed by the Student–Newman–Keuls procedure.

3. Results

3.1. Effect of cyclooxygenase inhibition on vasomotor responses

Acetylcholine induced complete and dose-dependent relaxation in both the carotid and the femoral artery. This relaxation was absent in de-endothelialised vessels ($n = 3$). To assess the contribution of prostanoids to the endothelium-dependent relaxation, concentration–response curves for acetylcholine were performed in the absence and presence of indomethacin (10 μM). Vessels were contracted with PE concentrations to reach half-maximal contraction. To achieve this contraction level in the carotid artery in the presence of indomethacin, more PE was needed than was assessed from the concentration–response curve to PE. The PE concentration was adjusted until the contraction level was comparable between the group with and without indomethacin (0.55 ± 0.13 vs. 0.56 ± 0.16 mN mm^{-1} for the carotid artery, and 1.54 ± 0.18 vs. 1.58 ± 0.24 mN mm^{-1} for the femoral artery). Acetylcholine caused complete and dose-dependent relaxation in both arteries. Indomethacin did not change the amplitude or the sensitivity (one-way ANOVA, $P > 0.05$, $n \geq 4$) to acetylcholine (pD_2 values were 7.61 ± 0.08 with vs. 7.58 ± 0.05 without indomethacin for the carotid artery and 6.90 ± 0.11 vs. 7.15 ± 0.08 for the femoral artery). Both in the absence and the presence of indomethacin, the carotid artery was more sensitive to acetylcholine as compared to the femoral artery (one-way ANOVA, $P < 0.01$).

In the carotid but not the femoral artery, higher doses of acetylcholine (0.3 μM and higher) caused immediate transient contraction of the vessel to a level that sometimes exceeded the precontracted level (Fig. 1A). Thereafter, vessels spontaneously returned to the relaxed state. The same phenomenon was observed with higher doses of carbachol (data not shown). In the presence of indo-

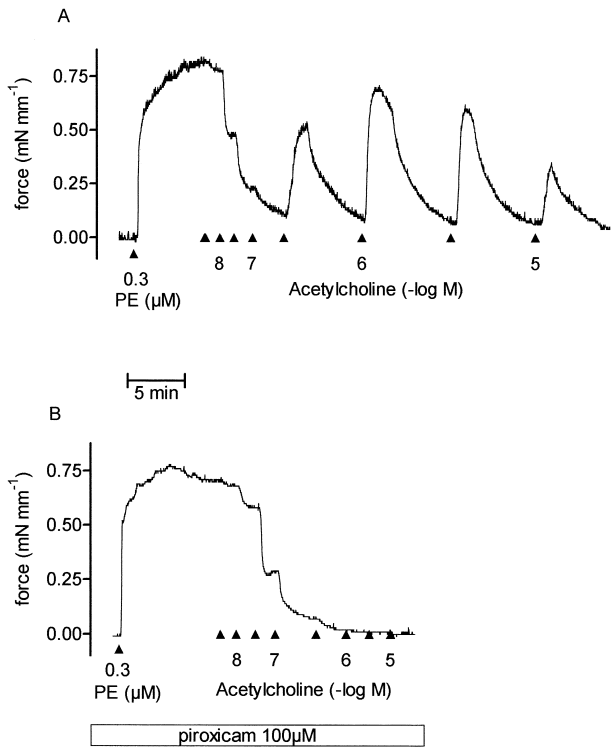

Fig. 1. Representative tracings showing a concentration–response curve to acetylcholine in the carotid artery. In control vessels (A), contracted with PE to half of the maximum, acetylcholine induced relaxations and transient, fully reversible contractions at higher doses. These contractions were suppressed by piroxicam 100 μM (B). Similar results were obtained with indomethacin 10 μM.

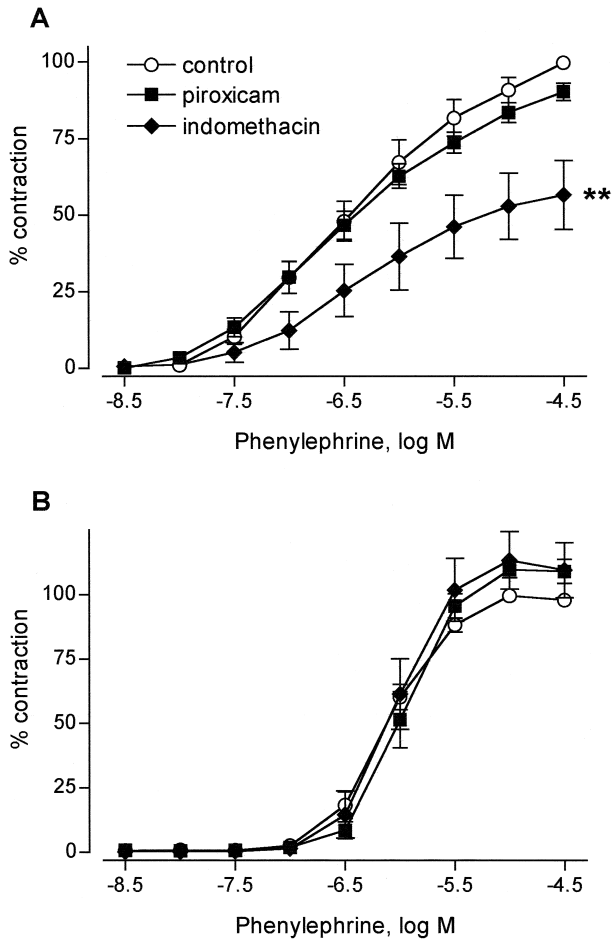

Fig. 2. Contractile responses to PE were attenuated by indomethacin 10 μM, but not by piroxicam 100 μM in the carotid artery (A). Neither drug affected force development in the femoral artery (B). The pD_2 values were unchanged by indomethacin or piroxicam in both vessel types (one-way ANOVA, $P > 0.05$). Data represent the mean ± S.E.M., $n \geq 4$, contractions expressed as percentage of the maximal contraction in the absence of either cyclooxygenase inhibitor. ** Significantly different from control, one-way ANOVA, $P < 0.01$.

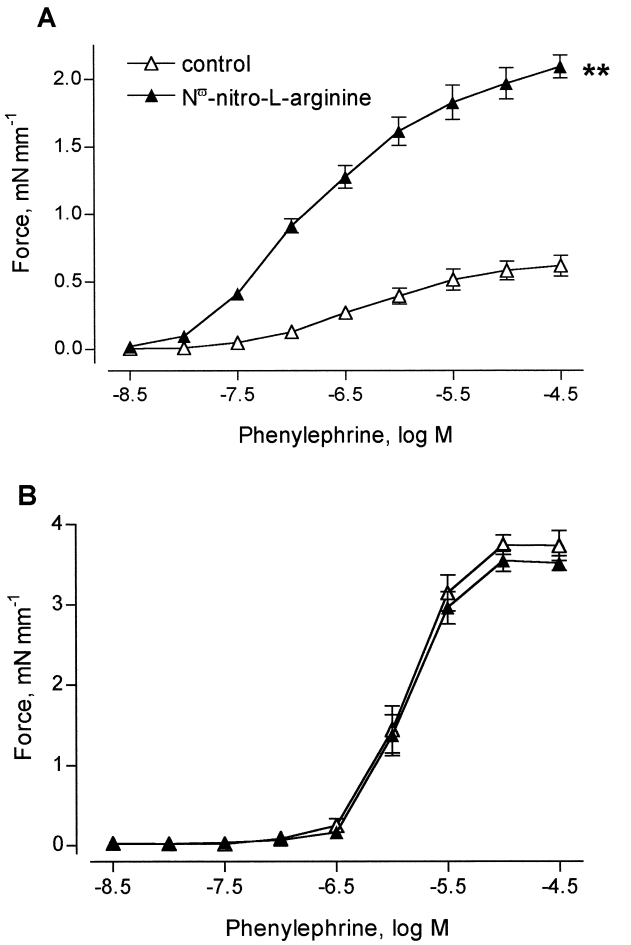

Fig. 3. Contractile responses to PE were raised four fold by N^{ω}-nitro-L-arginine 300 μM in the carotid artery (A), but not changed in the femoral artery (B). N^{ω}-nitro-L-arginine also increased the sensitivity to PE in the carotid (one-way ANOVA, $P < 0.01$), but not the femoral artery. Data represent mean ± S.E.M., $n \geq 5$. * * Significantly different from control, one-way ANOVA, $P < 0.01$.

methacin 10 μM or piroxicam 100 μM, the contractions in response to acetylcholine were not observed (Fig. 1B). Similar contractions to higher concentrations of acetylcholine also occurred in vessels precontracted with KCl, but were not observed at baseline. To avoid the interference of these contractions with the calculation of the pD_2 or the percentage of relaxation, cumulative doses of acetylcholine were only given after vessels had returned to their relaxed state.

As we observed that indomethacin apparently enhanced the PE concentration necessary for contraction of the carotid artery, the contribution of endogenous prostanoids to PE-induced contraction was evaluated using different cyclooxygenase inhibitors. When concentration–response curves to PE were performed in the carotid artery, contraction was inhibited to half of the maximum by 10 μM indomethacin, while it was unaltered by 100 μM piroxicam (Fig. 2A) or lower concentrations of indomethacin (data not shown). In the femoral artery, neither indomethacin nor piroxicam changed the maximal contractile force to PE (Fig. 2B). The sensitivity to the agonist was not influenced (one-way ANOVA, $P > 0.05$; $n \geq 4$) by indomethacin 10 μM or piroxicam 100 μM in either vessel type (pD_2 values are 6.32 ± 0.25 for the control vs. 6.27 ± 0.19 with indomethacin and 6.57 ± 0.16 with piroxicam for the carotid artery, and 6.11 ± 0.07 vs. 6.06 ± 0.11 and 5.95 ± 0.09 for the femoral artery).

3.2. Impact of N^{ω}-nitro-L-arginine on vasomotor responses

When the contraction to PE (0.3–1 μM; 0.40 ± 0.05 mN mm^{-1} for the carotid artery, and 1.54 ± 0.18 mN mm^{-1} for the femoral artery) reached a plateau, N^{ω}-nitro-

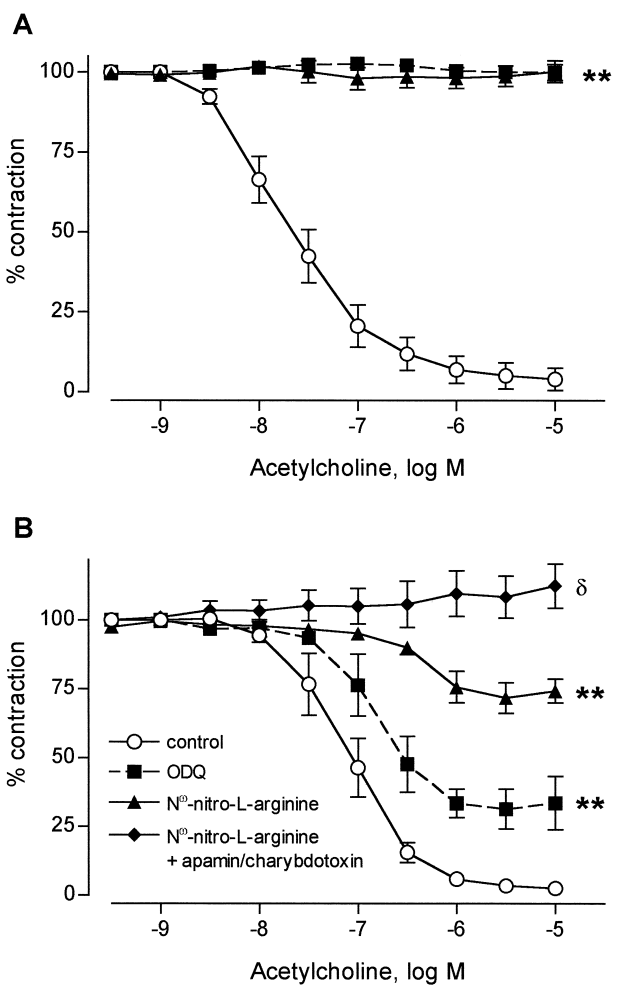

Fig. 4. Relaxation to acetylcholine was completely suppressed by N^{ω}-nitro-L-arginine 300 μM or ODQ 10 μM in the carotid artery (A), while in the femoral (B) artery 34% or 74% of the relaxation was preserved. The N^{ω}-nitro-L-arginine-resistant relaxation in the femoral artery was abolished by the combination of apamin 1 μM and charybdotoxin 60 nM. Data represent mean ± S.E.M.; $n \geq 5$; one-way ANOVA, $P < 0.01$; * * Significantly different from control, δ significantly different from N^{ω}-nitro-L-arginine.

L-arginine 300 μM was added, which resulted in a four-fold increase of the tension to 1.59 ± 0.34 mN mm^{-1} in the carotid artery. This effect was not observed in de-endothelialised rings (data not shown). In the femoral artery, addition of N^{ω}-nitro-L-arginine had little or no effect. These findings were confirmed by the concentration–response curves to PE in the absence or the presence of N^{ω}-nitro-L-arginine (Fig. 3). In the femoral artery, the response to PE was not influenced by N^{ω}-nitro-L-arginine, while in the carotid artery the maximal contractile force increased from 0.62 ± 0.08 to 2.18 ± 0.09 mN mm^{-1}. Also, the sensitivity to PE was significantly increased in the presence of N^{ω}-nitro-L-arginine in the carotid artery, while it was unaltered in the femoral artery (pD_2 values: 6.31 ± 0.08 in controls vs. 6.88 ± 0.01 in the presence of N^{ω}-nitro-L-arginine for the carotid artery, and 5.87 ± 0.07 vs. 5.87 ± 0.06 for the femoral artery). Addition of N^{ω}-nitro-L-arginine did not induce contraction in either vessel type when given at resting tension.

The contribution of nitric oxide to the relaxation was measured by performing concentration–response for acetylcholine in the absence or the presence of N^{ω}-nitro-L-arginine (Fig. 4). For the femoral artery, precontraction levels in the control arteries (i.e. incubated in the absence of N^{ω}-nitro-L-arginine) were comparable (1.58 ± 0.24 mN mm^{-1}) to those in vessels treated with N^{ω}-nitro-L-arginine (1.54 ± 0.18 mN mm^{-1}); however, for the carotid artery, comparable contraction levels could not be achieved (0.70 ± 0.17 mN mm^{-1} in the absence vs. 1.59 ± 0.34 mN mm^{-1} in the presence of N^{ω}-nitro-L-arginine). In the carotid artery, no relaxation ($< 5\%$) could be observed in the presence of N^{ω}-nitro-L-arginine. In the femoral artery, on the other hand, a N^{ω}-nitro-L-arginine-resistant relaxation persisted, but the maximum amplitude ($34 \pm 5\%$) was reduced as compared to relaxation in the absence of N^{ω}-nitro-L-arginine ($97 \pm 1\%$, Fig. 4). Moreover, the shape of this relaxation response was different. While in control conditions, the response to acetylcholine was long-lasting (Fig. 5A) in the presence of N^{ω}-nitro-L-arginine, a large transient relaxation could be observed, followed by a smaller sustained relaxation at higher concentrations of acetylcholine (Fig. 5B). For the construction of the relaxation curve, the maxima of the transient response were selected. The sensitivity (pD_2) to the agonist had also decreased from 7.12 ± 0.14 to 6.26 ± 0.18 in the presence of N^{ω}-nitro-L-arginine (one-way ANOVA, $P < 0.05$, $n = 6$). The N^{ω}-nitro-L-arginine-resistant relaxation in the femoral artery was completely abolished by the combination of apamin 1 μM and charybdotoxin 60 nM (Fig. 4B).

N^{ω}-nitro-L-arginine did not influence the maximal relaxation to *S*-nitroso-*N*-acetyl-D-penicillamine, but significantly increased the sensitivity to the agonist (Fig. 6) in

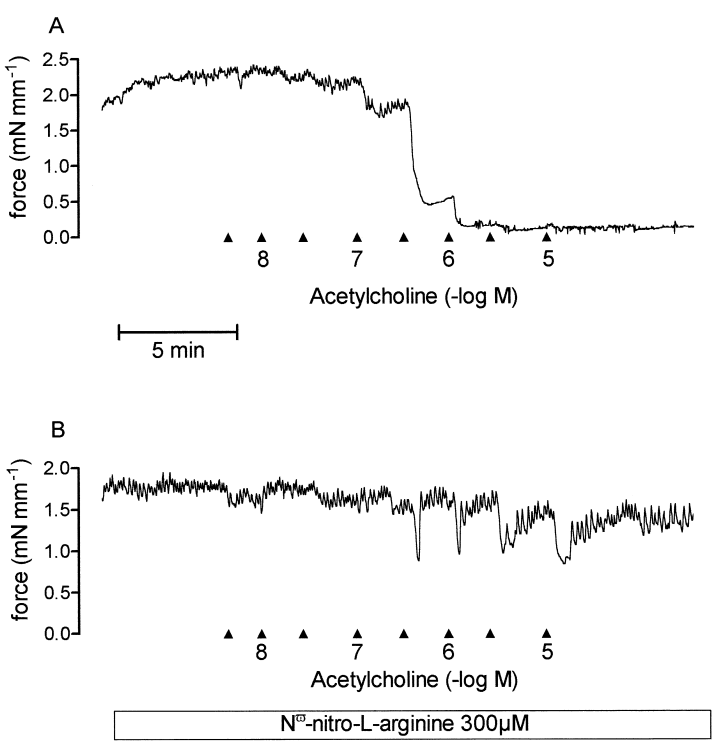

Fig. 5. Representative tracing (1 of 6) showing the effect of N^{ω}-nitro-L-arginine on acetylcholine-induced relaxations in the femoral artery. In the absence of N^{ω}-nitro-L-arginine (A), acetylcholine induced dose-dependent, persistent relaxations, while in the presence of N^{ω}-nitro-L-arginine 300 μM (B), the response became biphasic with a fast transient response followed by a smaller sustained component.

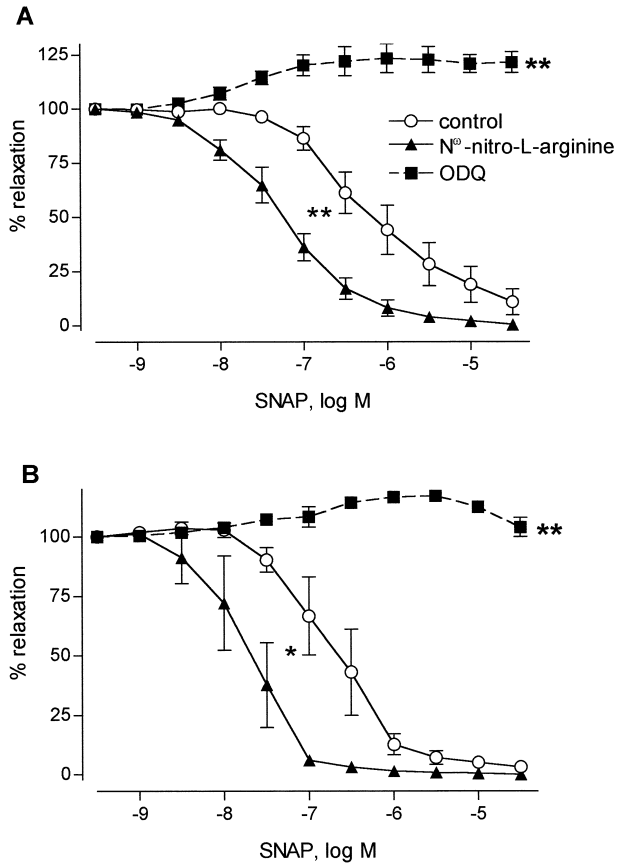

Fig. 6. Concentration–response curves to *S*-nitroso-*N*-acetyl-D-penicillamine in vessels previously contracted with PE 0.3–1 μM were displaced to the left by 300 μM N^{ω}-nitro-L-arginine and abolished by 10 μM ODQ in both the carotid (A) and the femoral artery (B). Data represent mean ± S.E.M.; $n = 4$; one-way ANOVA, significantly different from control: $^{*}P < 0.05$, $^{**}P < 0.01$.

both vessel types (pD_2 values were 6.21 ± 0.23 without vs. 7.28 ± 0.13 with N^{ω}-nitro-L-arginine for the carotid artery and 6.73 ± 0.23 vs. 7.77 ± 0.24 for the femoral artery).

3.3. Effect of guanylyl cyclase inhibition on acetylcholine-induced relaxation

Vessels were precontracted with PE (0.3–1 μM), resulting in precontraction levels of 0.36 ± 0.07mN mm^{-1} for the carotid artery and 1.44 ± 0.03 mN mm^{-1} for the femoral artery. When a plateau contraction was reached, ODQ 10 μM was added, and this resulted in a four fold increase of the contraction level in the carotid artery to 1.50 ± 0.16 mN mm^{-1}. In the femoral artery, addition of ODQ caused little or no rise in the precontraction level. After an incubation period of at least 15 min, or when the contraction levels were stable, cumulative concentration–response curves were made for acetylcholine. In control carotid arteries, the precontraction (0.40 ± 0.04 mN mm^{-1}) could not be raised to the same level as in ODQ-treated vessels. In the carotid artery, relaxations to acetylcholine were abolished (< 5%) in the presence of ODQ, while for femoral arteries 74 ± 4% relaxation could be observed (Fig. 4), which was, however, significantly decreased as opposed to relaxation in the absence of ODQ (97 ± 1%; one-way ANOVA, $P < 0.01$, $n = 5$). Consistent with the observations in the presence of N^{ω}-nitro-L-arginine, the shape of the relaxation response had changed, with a large transient relaxation followed by a smaller sustained relaxation. For the calculations, the maximum of the transient relaxation was chosen for each concentration of acetylcholine. The sensitivity to acetylcholine was statistically not influenced (one-way ANOVA, $P > 0.05$) by ODQ (pD_2 values of 7.12 ± 0.14 in the absence vs. 6.76 ± 0.18 in the presence of ODQ).

The endothelium-independent relaxation to *S*-nitroso-*N*-acetyl-D-penicillamine was completely abolished by ODQ in both vessel types (Fig. 6), while the relaxation by papaverine was not influenced at all (Fig. 7).

3.4. Relaxation in high potassium

By depolarizing the vessels with KCl 30 mM, resulting in contraction levels of 1.38 ± 0.28 mN mm^{-1} for the carotid and 2.19 ± 0.36 mN mm^{-1} for the femoral artery, the hyperpolarization-related component in the subsequent agonist-induced relaxation is inhibited. Concentration–response curves for acetylcholine in vessels contracted with KCl 30 mM resulted in a significant decrease (one-way ANOVA, $P < 0.05$, $n = 5$) of the maximal relaxation as compared to precontraction with PE (from 105 ± 2% to 71 ± 4% for the carotid artery, and from 100 ± 1% to 83 ± 5% for the femoral artery; Fig. 8). The sensitivity to the agonist was not changed in either vessel type (pD_2

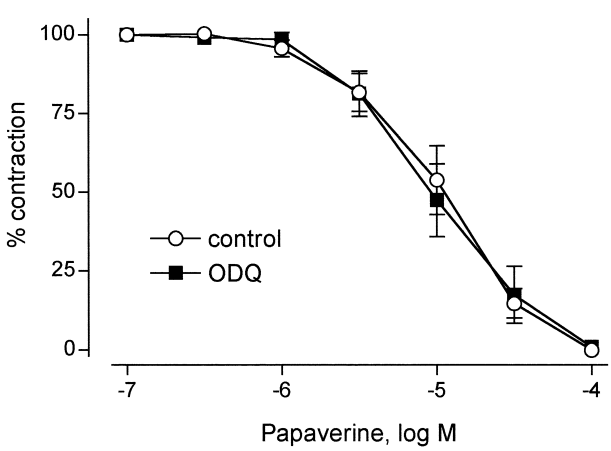

Fig. 7. Concentration–response curve for papaverine in the carotid artery previously contracted with PE 0.3 μM. The cyclic AMP-mediated relaxation by papaverine, was not influenced by ODQ 10 μM (one-way ANOVA, $P > 0.05$). Data represent mean ± S.E.M.; $n = 4$.

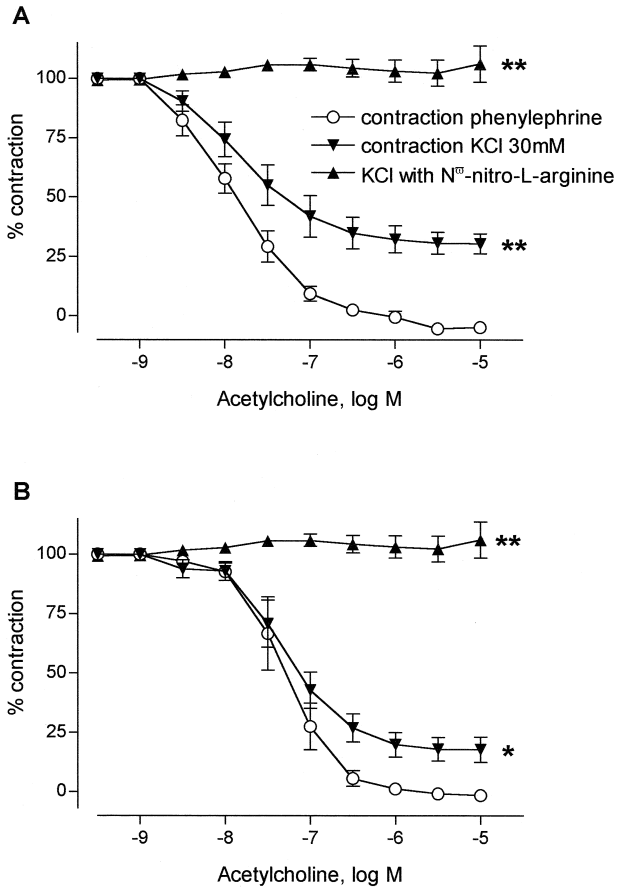

Fig. 8. Concentration–response curves for acetylcholine in vessels contracted with PE 0.3–1 μM or KCl 30 mM. High potassium significantly reduced the relaxation in the carotid (A) and the femoral artery (B), while the sensitivity to acetylcholine was not influenced (one-way ANOVA, $P > 0.05$). Data represent mean ± S.E.M.; $n = 5$; one-way ANOVA, significantly different from control: $^{*}P < 0.05$, $^{**}P < 0.01$.

values are 7.71 ± 0.17 in high potassium vs. 7.91 ± 0.08 in PE for the carotid artery, and 7.28 ± 0.11 vs. 7.27 ± 0.13 for the femoral artery). When concentration–response curves for acetylcholine in high potassium were repeated in the presence of either N^{ω}-nitro-L-arginine 300 μM or ODQ 10 μM, all relaxation was lost in both arteries (Fig. 8).

4. Discussion

In the present study, vascular responses, in particular endothelium-dependent relaxations, were investigated in murine blood vessels and compared between the carotid and the femoral artery. In general, in atherosclerosis research, C57BL6 mice are used because of their higher susceptibility for atherosclerosis as compared to other strains (Breslow, 1996). Therefore, the results can be used as a background for future studies of vascular function and atherogenesis in murine models derived from this strain.

4.1. Contractile responses

In comparing both vessel types, we observed a big difference in contractile force. Though much smaller in diameter, the femoral artery developed more force in response to PE as compared to the carotid artery. The same phenomenon was observed with potassium, indicating that it is indeed a difference in contractile machinery, and not merely a different efficacy of the agonist. The smaller response in the carotid artery was partly explained by a greater impact of spontaneously released nitric oxide (vide infra). Although the contraction of the carotid artery was increased when endogenous nitric oxide production was eliminated, the absolute contractile force was still less than in the femoral artery. While the carotid artery can be defined as an elastic artery, the femoral artery is a muscular type artery. It is possible that the ability of muscular arteries to contract is facilitated by the organisation of the structural components of the arterial wall and the lower elastic tissue volume (Levicky and Dolezel, 1980). Also, it has been described that elastic arteries contain mostly vimentin positive-desmin negative smooth muscle cells, while muscular arteries also contain vimentin positive–desmin-positive smooth muscle cells (Osborn et al., 1981; Johansson et al., 1997), and this phenotypic diversity could have consequences for the contractile capacity of the vessel.

Indomethacin is the most widely used cyclooxygenase inhibitor in in vitro studies, in concentrations varying between 1 and 30 μM. However, we unexpectedly observed that indomethacin 10 μM reduced the contractions of the carotid artery to PE by 50%, while leaving the response in the femoral artery unaffected. This observation, which has not been reported before, could, however, not be reproduced with lower concentrations of indomethacin, or with piroxicam, another cyclooxygenase inhibitor, even in a concentration that was 10 times higher. Therefore, it is concluded that the inhibition of the contraction in the carotid artery is most likely due to an effect of indomethacin not related to cyclooxygenase inhibition. A possible explanation is that indomethacin may have calcium-antagonistic properties (Northover, 1977), but in concentrations that are usually higher than the concentration used to block the prostaglandin biosynthesis (Posner and Peterson, 1982; Sawdy et al., 1998). Because the contractile force of the carotid artery is much smaller as compared to the femoral artery, it is however possible that the carotid artery is more sensitive to the calcium-antagonistic activities of indomethacin.

Another peculiar but consistent finding, were the concentration-dependent, transient contractions to higher doses of acetylcholine in the carotid artery, but not in the femoral artery. This phenomenon was briefly mentioned in the carotid artery of heterozygous C57BL6 mice deficient in one copy of the *eNOS*-gene, but not in their age-matched controls (Faraci et al., 1998). Those authors attributed this

modified response to acetylcholine to an altered endothelial function as a result of deletion of the *eNOS*-gene. We could, however, also observe these contractions in wild type mice of the same strain (C57BL6), indicating that this phenomenon can also occur when endothelial nitric oxide function is intact. Furthermore, we could extend the observation by showing that indomethacin and piroxicam abolish the contractions, and that the temporary nature of those contractions cannot be explained by breakdown of acetylcholine, since the same transient events occurred with carbachol, which is resistant to degradation by cholinesterases. Similar contractions to higher concentrations of acetylcholine that were abolished by flurbiprofen have been described in the saphenous vein of the rabbit (McGrath et al., 1990). Also, indomethacin-sensitive endothelium-dependent — although not transient — contractions to acetylcholine have been described in the aorta of spontaneously hypertensive rats (Lüscher and Vanhoutte, 1986). This was attributed to the production of an endothelium-dependent contracting factor, possibly a prostaglandin endoperoxide that stimulates the thromboxane A_2/prostaglandin H_2 receptors on the vascular smooth muscle cells (Auch-Schwelk et al., 1992). Given the cyclooxygenase-dependent nature of our observation, production of such a factor could possibly explain the acetylcholine-induced contractions in the carotid artery of the mouse. In the femoral artery, the phenomenon was not observed, but whether the causative factor is not formed in the femoral artery, or whether it is the sensitivity of the vessel type to this factor which is determinant, is as yet unclear.

4.2. Impact of spontaneous nitric oxide release

The spectacular, four fold increase in contractile force in the carotid artery in response to nitric oxide synthase inhibition by N^{ω}-nitro-L-arginine or guanylate cyclase inhibition by ODQ points to a large "spontaneous" endothelial production of nitric oxide, which counteracts the contraction induced by PE. A similar though less pronounced increase of the contractions to PE or noradrenaline has been reported in the aorta and the femoral artery (Waldron et al., 1999) and the mesenteric artery (Thorin et al., 1998) of endothelial nitric oxide synthase-deficient C57B16 mice. The smaller amplitude of contractile effect in those mice could relate to adaptive suppression of contractile responses to α-adrenoreceptor stimulation. Neither N^{ω}-nitro-L-arginine nor ODQ caused constrictions in quiescent carotid rings. This could mean that a contractile agonist is required to reveal a continuous basal release of nitric oxide. Alternatively, it could point to a contraction-induced production of nitric oxide. However, in our experiments, the contractile capacity to KCl was unaltered in the presence of N^{ω}-nitro-L-arginine. The absence of basal nitric oxide release in KCl exposed endothelial cells could result from a decreased driving force for Ca^{2+}-entry in depolarized cells. However, this seems less likely since KCl constricted vessels were still capable of substantial nitric oxide production, as shown by their response to acetylcholine (see below). Furthermore, the activation of the endothelial nitric oxide synthase by isometric contraction has been reported to be Ca^{2+}-independent in the rabbit aorta (Fleming et al., 1999). Taken together, the enormous difference in the results obtained with KCl and PE, thus points to a PE-induced rather than a constriction-induced production of nitric oxide. Both α- (Kaneko and Sunano, 1993) and β-adrenergic stimulation (Graves and Poston, 1993) of the endothelial cells has been shown to induce nitric oxide release. Therefore, interaction of PE with α-adrenoreceptors on the endothelial cells might induce nitric oxide production. Also, PE has been shown to facilitate β-adrenoreceptor relaxation, possibly by a direct interaction with β-receptors (Priest et al., 1999). Regional differences in the localisation of functional adrenoreceptors might explain the contrasting results in the two vessel types. In the femoral artery, neither N^{ω}-nitro-L-arginine nor ODQ raised the contraction, suggesting that the "spontaneous nitric" oxide-production in this vessel type is functionally not important. However, measurements of nitric oxide or cyclic GMP and the use of α- and β-receptor antagonists will be necessary to further document these observations.

4.3. Mediators of acetylcholine-induced relaxation

Indomethacin did not alter the relaxation curves for acetylcholine, indicating that prostaglandins are irrelevant in endothelium-dependent relaxation in both carotid and femoral artery, confirming an observation in the carotid artery (Chataigneau et al., 1999). Since neither the maximal response nor the pD_2 was influenced, we concluded that the non-cyclooxygenase-dependent activity (vide supra) of indomethacin did not interfere with the relaxation. In all subsequent experiments, cyclooxygenase was inhibited.

The relative contribution of nitric oxide and EDHF to endothelium-dependent relaxation shows a marked heterogeneity among species and vessels types (Triggle et al., 1999). To evaluate the contribution of hyperpolarization-related pathways in mice, responses to acetylcholine were measured in 30 mM KCl, which excludes smooth muscle cell hyperpolarization (Nelson and Quayle, 1995). Indeed, in potassium-constricted rings, N^{ω}-nitro-L-arginine fully abolished relaxation to acetylcholine in either vessel type. In both arteries, the endothelium-dependent relaxation was slightly diminished in high potassium, suggesting that part of the response is mediated through hyperpolarization of the smooth muscle cells. However, we have to take into account that the diminished relaxation might also be due to the larger preconstriction in KCl-constricted rings or alternatively to a reduced nitric oxide output in high potassium

since the endothelial nitric oxide synthase is Ca^{2+}-dependent (Furchgott, 1983).

To investigate the role of nitric oxide in the relaxation, the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine (Moore et al., 1990) was used. In the PE- and KCl-constricted carotid arteries, all relaxation was abolished by N^{ω}-nitro-L-arginine, confirming observations in the aorta (Barton et al., 1998), the carotid artery (Faraci et al., 1998; Chataigneau et al., 1999) and the mesenteric artery (Banda et al., 1997) of C57BL6 mice. It is unlikely that the loss can only be explained by the higher level of contraction of the carotid artery in the presence of N^{ω}-nitro-L-arginine, since vessels contracted to a similar level with KCl retained their ability to relax to acetylcholine in the absence of N^{ω}-nitro-L-arginine. We therefore conclude that a non-prostanoid non-nitric oxide factor does not contribute to acetylcholine-induced relaxation in the carotid artery and that relaxation through hyperpolarization, if any, is mediated by nitric oxide and/or cyclic GMP.

In contrast, in the femoral artery, N^{ω}-nitro-L-arginine inhibited only 66% of the acetylcholine-induced relaxation, pointing to relaxation by a factor other than nitric oxide or prostacyclin in this vessel type. The existence of a non-nitric oxide non-prostanoid relaxing factor has recently been reported in the mouse femoral artery (Waldron et al., 1999) and we could extend this observation by showing that the N^{ω}-nitro-L-arginine-resistant relaxation was abolished by a combination of apamin and charybdotoxin. This points to the involvement of apamin/charybdotoxin-sensitive potassium channels, with the large and small conductance Ca^{2+}-sensitive potassium channels being the most likely candidates. From our experiments so far, we can, however, not decide whether those potassium channels are located on the endothelial or the smooth muscle cells. The contribution of a separate non-prostanoid non-nitric oxide factor to the relaxation of the femoral, but not the carotid artery, is in agreement with the general hypothesis that the relative role of EDHF decreases as vessel size increases (Shimokawa et al., 1996).

The sensitivity of the carotid artery for exogenous nitric oxide increased in the presence of N^{ω}-nitro-L-arginine, confirming observations in the aorta (Waldron et al., 1999) and the carotid artery (Faraci et al., 1998) of endothelial nitric oxide synthase-deficient mice. It is conceivable that inhibition of the large "spontaneous" release of nitric oxide in the carotid artery (vide supra) sensitises the vessel to lower concentrations of exogenous nitric oxide. Given the apparent absence of "spontaneous" nitric oxide release in the femoral artery, we hypothesised that N^{ω}-nitro-L-arginine would not influence the response to exogenous nitric oxide in this vessel type. This was, however, not confirmed by the experimental data.

To discriminate between the cyclic GMP-dependent and -independent responses to nitric oxide, ODQ, an inhibitor of the soluble guanylate cyclase and the cyclic GMP-dependent relaxation (Garthwaite et al., 1995) was used. In the carotid artery, all relaxation induced by acetylcholine was abolished by ODQ, confirming the results of Faraci et al. (1998). This indicates that nitric oxide itself cannot directly hyperpolarize the smooth muscle cells in this vessel type, which was confirmed by the lack of relaxation to an exogenous nitric oxide donor in the presence of ODQ. In the femoral artery, only 26% of the response to acetylcholine was eliminated by ODQ. The lack of effect of ODQ on papaverine-induced relaxation supports the assumption that ODQ is specific for guanylyl cyclase. However, recently, Feelisch et al. (1999) suggested that higher concentrations of ODQ (30–300 μM) — depending on the experimental conditions — may also inhibit nitric oxide synthase in the rat and rabbit aorta. Given the large inter-species variability, we cannot be sure of the nitric oxide synthase-inhibitory capacity of ODQ in mice, even though we used a concentration that was at least 10 times lower. Nevertheless, the inhibition of the endothelium-dependent relaxation by ODQ was still substantially smaller than the inhibition by the specific NOS-inhibitor N^{ω}-nitro-L-arginine. This observation could suggest that a part of the hyperpolarization-related relaxation in the femoral artery is caused by nitric oxide itself. However, the absence of relaxation in the femoral artery to the exogenous nitric oxide donor *S*-nitroso-*N*-acetyl-D-penicillamine in the presence of ODQ does not support this hypothesis. Reduced formation of contractile prostanoids cannot explain the differences between ODQ and N^{ω}-nitro-L-arginine, since all experiments were performed in the presence of 10 μM indomethacin.

Another important observation is that the shape of the relaxation response had changed in the presence of N^{ω}-nitro-L-arginine and ODQ. In control conditions, the relaxation response to acetylcholine is long lasting. In N^{ω}-nitro-L-arginine- and ODQ-treated vessels on the other hand, acetylcholine evokes relaxations that are clearly biphasic, consisting of a fast, large transient response, followed by a smaller, prolonged response at higher acetylcholine concentrations. From our observations, we assume that in the absence of inhibitors, the fast initial relaxation is due to an EDHF-related mechanism, while the nitric oxide pathway is responsible for the sustained relaxation.

In conclusion, the different mechanisms that contribute to the endothelium-dependent relaxation induced by acetylcholine were elucidated step by step in the carotid and the femoral artery of the mouse. From the present observations, it can be concluded that vasorelaxing prostanoids are not important in the relaxation in either vessel type. In the carotid artery, the endothelium-dependent relaxation is mediated predominantly by nitric oxide acting via cyclic GMP-dependent pathways. In the femoral artery, part of the relaxation was N^{ω}-nitro-L-arginine- and ODQ-resistant, pointing to the existence of a non-prostanoid non-nitric oxide factor operating via apamin/charybdotoxin-sensitive potassium channels, which is responsible for the initial transient part of the relaxation response.

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