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RESEARCH PAPER

Myoendothelial coupling in the mesenteric arterial bed; segmental differences and interplay between nitric oxide and endothelin-1

RHP Hilgers and JGR De Mey

Department of Pharmacology and Toxicology, Cardiovascular Research Institute Maastricht (CARIM), University of Maastricht, The Netherlands

Background and purpose: We tested the hypothesis that activated arterial smooth muscle (ASM) stimulates endothelial vasomotor influences via gap junctions and that the significance of this myoendothelial coupling increases with decreasing arterial diameter.
Experimental approach: From WKY rats, first-, second-, third- and fourth-order branches of the superior mesenteric artery (MA1, MA2, MA3 and MA4 respectively) were isolated and mounted in wire-myographs to record vasomotor responses to 0.16–20 $\mu mol \cdot L^{-1}$ phenylephrine.
Key results: Removal of endothelium increased the sensitivity (pEC_{50}) to phenylephrine in all arteries. The nitric oxide (NO) synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) (100 $\mu mol \cdot L^{-1}$) did not modify pEC_{50} to phenylephrine in all denuded arteries, and increased it in intact MA1, MA2 and MA3 to the same extent as denudation. However, in intact MA4, the effect of L-NAME was significantly larger (ΔpEC_{50} 0.57 $\pm$ 0.02) than the effect of endothelium removal (ΔpEC_{50} 0.20 $\pm$ 0.06). This endothelium-dependent effect of L-NAME in MA4 was inhibited by (i) steroidal and peptidergic uncouplers of gap junctions; (ii) a low concentration of the NO donor sodium nitroprusside; and (iii) by the endothelin-receptor antagonist bosentan. It was also observed during contractions induced by (i) calcium channel activation (BayK 8644, 0.001–1 $\mu mol \cdot L^{-1}$); (ii) depolarization (10–40 $mmol \cdot L^{-1}$ K^+); and (iii) sympathetic nerve stimulation (0.25–32 Hz).
Conclusions and implications: These pharmacological observations indicated feedback control by endothelium of ASM reactivity involving gap junctions and a balance between endothelium-derived NO and endothelin-1. This myoendothelial coupling was most prominent in distal resistance arteries.
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Keywords: mesenteric resistance artery; smooth muscle; endothelium; gap junctions; nitric oxide; endothelin-1; regional heterogeneity

Abbreviations: 18α-GA, 3β-hydroxy-11-oxo-18α,20β-olean-12-en-29-oic acid (18α-glycyrrhetinic acid); 18β-GA, 3β-hydroxy-11-oxo-18β,20β-olean-12-en-29-oic acid (18β-glycyrrhetinic acid); 20-HEDE, 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid; 20-HETE, 20-hydroxyeicosa-6(Z),15(Z)-tetraenoic acid; ASM, arterial smooth muscle; DDMS, *N*-methylsulphonyl-12,12-dibromododec-11-enamide; ET-1, endothelin-1; KRB, Krebs Ringer buffer; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MEGJ, myoendothelial gap junction; SNP, sodium nitroprusside

Introduction

Stimulation of the endothelium, with for instance acetylcholine (ACh), causes an increase in endothelial cell calcium (Busse *et al.*, 1989; Busse and Fleming, 2003). This, in turn, can stimulate endothelium-dependent vasomotor effects. Endothelium-derived vasoactive factors include the vasodilators nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin and the vasoconstrictors prostaglandin endoperoxide and endothelin-1 (ET-1) [see (Furchgott and Vanhoutte, 1989; 2006)]. Conduction of endothelial cell hyperpolarization through heterocellular myoendothelial gap junctions (MEGJ) is an additional mechanism of endothelium-dependent vasodilatation (Dora *et al.*, 2003; Feletou and Vanhoutte, 2006). This EDHF-like reactivity and the density of MEGJ increases with decreasing arterial size in at least the rat mesenteric arterial bed (Shimokawa *et al.*, 1996; Sandow and Hill, 2000; Hilgers *et al.*, 2006). Through

Correspondence: Professor Dr Jo GR De Mey, Department of Pharmacology and Toxicology, Cardiovascular Research Institute Maastricht, University of Maastricht, PO box 616, 6200 MD, Maastricht, The Netherlands. E-mail: j.demey@farmaco.unimaas.nl
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the MEGJ not only current but also small molecules such as calcium ions can pass from endothelial to smooth muscle cells (Dora *et al.*, 1997; 2003; Lamboley *et al.*, 2005; Feletou and Vanhoutte, 2006; Isakson *et al.*, 2007), and vice versa (Dora *et al.*, 1997; 2000).

We hypothesized that in small resistance arteries, activation of arterial smooth muscle (ASM) was accompanied by endothelial feedback vasomotor influences involving gap junctions. We therefore used rat mesenteric resistance arteries that are well known to display endothelium-dependent relaxing responses that are resistant to inhibition of NO synthase (Garland and McPherson, 1992). We used arteries of different branching order in view of reported segmental differences in density of MEGJ (Sandow and Hill, 2000) and pharmacological properties of EDHF-like activity (Shimokawa *et al.*, 1996; Hilgers *et al.*, 2006). The arteries were stimulated with phenylephrine that acts on α_1-adrenergic receptors, which are expressed by ASM cells but do not directly stimulate influx of calcium ions in endothelial cells (Dora *et al.*, 2000). Responses were studied in the presence and absence of endothelium, uncouplers of gap junctions, and of inhibitors of NO synthases and endothelin receptors. In additional experiments in the smallest resistance arteries, we used a dihydropyridine calcium channel activator, depolarization and sympathetic nerve stimulation that directly activate the ASM but not the endothelium (Burnstock and Ralevic, 1994; Nilius and Droogmans, 2001).

Methods

All animal care and experimental protocols were in accordance with institutional guidelines and were approved by the Ethics Committee on Experimental Animal Welfare of the University of Maastricht.

Wire myography

Twelve to 16 weeks old Wistar Kyoto rats (n = 44; Charles River, Maastricht, The Netherlands) were killed by CO_2 inhalation. First-, second-, third- and fourth-order side branches of the superior mesenteric artery (MA1, MA2, MA3 and MA4 respectively) were isolated and mounted in wire myographs for the recording of isometric force development (Hilgers *et al.*, 2006). Each experiment started by progressively stretching the arterial segment to the diameter at which the largest contractile response to 10 $\mu mol \cdot L^{-1}$ noradrenaline could be obtained (optimal diameter).

Pharmacological strategy

All experiments were performed after exposure of the arterial segments to capsaicin (1 $\mu mol \cdot L^{-1}$, during 20 min) and in the continuous presence of indomethacin (1 $\mu mol \cdot L^{-1}$), to exclude influences of sensory-motor nerves (Wang and Wang, 2004; De Mey *et al.*, 2008;) and of vasodilator and vasoconstrictor prostaglandins. In every type of experiment, we used four arterial segments: two with endothelium and two without endothelium, incubated with or without the inhibitor of NO synthases, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 $\mu mol \cdot L^{-1}$). This was performed for MA1, MA2, MA3 and MA4, such that a total of sixteen arteries were used for each rat. In some experiments only MA1 and/or MA4 were used. The endothelium was removed by sliding a horse hair through the lumen of the vessel before mounting the arterial segment in the myograph (Osol *et al.*, 1989). Endothelium-denuded arteries that had a remaining relaxation of >20% in response to 10 $\mu mol \cdot L^{-1}$ ACh were discarded.

We recorded contractile responses to smooth muscle-selective stimulation with phenylephrine (0.16–20 $\mu mol \cdot L^{-1}$). In a subset of endothelium-denuded and endothelium-intact MA4, contractile responses were assessed using the L-type voltage-operated calcium channel opener BayK8644 (1–1000 $nmol \cdot L^{-1}$), high potassium solution (K^+, 10–40 $mmol \cdot L^{-1}$) and electrical field stimulation (EFS, 0.25–32 Hz). EFS was applied through two platinum electrodes placed along the long axis of the arterial segments and a stimulator (Danish Myotechnology, Aarhus, Denmark; stimulus intensity 85 mA, duration 2 ms) (De Mey *et al.*, 2008).

18α-glycyrrhetinic acid (18α-GA, 20 $\mu mol \cdot L^{-1}$), 18β-glycyrrhetinic acid (18β-GA, 5 $\mu mol \cdot L^{-1}$), carbenoxolone (20 $\mu mol \cdot L^{-1}$) and synthetic peptides with homology to conserved extracellular regions of connexins were used as putative uncouplers of gap junctions (Yamamoto *et al.*, 1998; Tare *et al.*, 2002). We used a cocktail of mimetic peptides (100 $\mu mol \cdot L^{-1}$), which consisted of ^{43}Gap 26 (VCY DKS FPI SHV R), ^{40}Gap 27 (SRP TEK NVF IV) and ^{37,43}Gap27 (SRP TEK TIFII) (Chaytor *et al.*, 2001).

Endothelin receptors were blocked by the non-selective antagonist bosentan (10 $\mu mol \cdot L^{-1}$) (Clozel *et al.*, 1994). Cytochrome P450 arachidonic acid ω-hydroxylase (CYP4A) and receptors for 20-hydroxyeicosa-6(Z),15(Z)-tetraenoic acid (20-HETE), which can participate in the cellular effects of ET-1 were inhibited with *N*-methylsulphonyl-12, 12-dibromododec-11-enamide (DDMS, 3 $\mu mol \cdot L^{-1}$) and 20-hydroxyeicosa-6(Z),15(Z)-dienoic acid (20-HEDE, 3 $\mu mol \cdot L^{-1}$) respectively (Imig *et al.*, 2000; Zhao *et al.*, 2004).

To address the role of basal release of endothelium-derived NO, we recorded responses to phenylephrine in MA4 in the absence and presence of endothelium and L-NAME during exposure to a low but constant concentration of the NO-donor sodium nitroprusside (SNP, 30 $nmol \cdot L^{-1}$).

Data and statistical analysis

Contractile responses were expressed as a percentage of the maximal contractile response to 10 $\mu mol \cdot L^{-1}$ noradrenaline prior to the administration of any pharmacological inhibitor. Individual concentration-response curves were fitted to a non-linear sigmoid regression curve (Graphpad Prism 5.0). Sensitivity (pEC_{50}) and maximal effect (E_{max}) are shown as mean ± SEM. Statistical significance of effects and differences was analysed using either one-way ANOVA (comparison of pEC_{50} and E_{max}) or two-way ANOVA (comparison of concentration-response curves). A Bonferroni post-test was used to compare multiple groups. A P value <0.05 was considered statistically significant.

Solutions and drugs

The Krebs Ringer bicarbonate-buffered physiological salt solution [Krebs Ringer buffer (KRB)] that was continuously aerated

with 95% O_2/5% CO_2 and maintained at 37°C contained (in mmol·L^{-1}): 118.5 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO4, 25.0 $NaHCO_3$ and 5.5 glucose. 18α- and 18β-GA and BayK 8644 were purchased from Sigma and dissolved in DMSO. DDMS and 20-HEDE were a gift from J. Falck (Department of Biochemistry, University of Texas Soutwestern Medical Center, Dallas, TX, USA) and were dissolved in DMSO. Bosentan was obtained from Actelion Pharmaceuticals and was dissolved in DMSO. Capsaicin and indomethacin were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands) and dissolved in ethanol. Ach, carbenoxolone, noradrenaline, phenylephrine and SNP were all purchased from Sigma and dissolved in KRB. The connexin mimetic peptides were purchased from Severn Biotech (Kidderminster, Worcestershire, UK) and dissolved in KRB. High K^+-KRB solution was KRB in which all NaCl was replaced by KCl. Solutions containing 10–40 mmol·L^{-1} K^+ were prepared by mixing appropriate volumes of KRB and K^+-KRB. The maximal concentrations of the solvents never exceeded 0.1% and did not alter arterial reactivity.

Results

General observations

Arterial optimal lumen diameter decreased with increasing branching order and did not differ significantly between intact and denuded arteries (Table 1). However, in denuded third-order arteries (MA3), optimal diameters were significantly smaller compared with intact third-order arteries (Table 1). Contractile responses to 10 μmol·L^{-1} noradrenaline were significantly larger in MA1 than in MA4, while MA2 and MA3 displayed intermediate responses. In denuded arteries, these contractile responses were smaller than in intact arteries (Table 1). Mechanical removal of the endothelium and all (combinations of) inhibitors of endothelium-dependent vasomotor responses did not alter basal tension.

In all arteries, phenylephrine (0.16–20 μmol·L^{-1}) induced large contractions (Figure 1). Pretreatment with capsaicin slightly, but significantly, increased sensitivity for phenylephrine compared with non-treated arteries (data not shown). All subsequent data were obtained after pretreatment with capsaicin and in the continuous presence of 1 μmol·L^{-1} indomethacin.

Effect of denudation and L-NAME

In endothelium-intact arteries, sensitivity (pEC_{50}) to phenylephrine was significantly higher in the smaller MA4 compared with the larger MA1 (Figures 1 and 2). Mechanical removal of the endothelium increased pEC_{50} to phenylephrine in all MAs. This leftward shift was not statistically significant in MA4 compared with the other MAs (Figure 2). However, pEC_{50} in denuded arteries did not differ between groups (Figure 2).

In denuded arteries, L-NAME did not significantly modify pEC_{50} in all MAs (Figures 1 and 2). In endothelium-intact arteries, L-NAME significantly increased pEC_{50}, but not E_{max}, to phenylephrine (Figures 1 and 2). This leftward shift in pEC_{50} to phenylephrine caused by L-NAME, increased with decreas-

Table 1 Optimal diameter and maximal tensions to 10 μmol·L^{-1} noradrenaline for endothelium-denuded (–E) and endothelium-intact (+E) first-, second-, third- and fourth-order mesenteric arteries (MA1, MA2, MA3 and MA4 respectively)

Parameter	*MA1*		*MA2*		*MA3*		*MA4*	
	–E	*+E*	*–E*	*+E*	*–E*	*+E*	*–E*	*+E*
Optimal diameter (μm)	376 ± 10	367 ± 7	308 ± 10	322 ± 8[b]	235 ± 7	261 ± 5[a–c]	209 ± 4	217 ± 3[b–d]
Tension (N·m^{-1})	4.04 ± 0.26	4.47 ± 0.23	3.01 ± 0.28	3.92 ± 0.24[a]	1.86 ± 0.27	2.92 ± 0.15[a–c]	2.17 ± 0.18	2.51 ± 0.11[b–d]
n	17	22	12	15	12	15	19	25

Values are shown as mean ± SEM.
[a]$P < 0.05$ versus –E.
[b]$P < 0.05$ versus MA1.
[c]$P < 0.05$ versus MA2.
[d]$P < 0.05$ versus MA3.

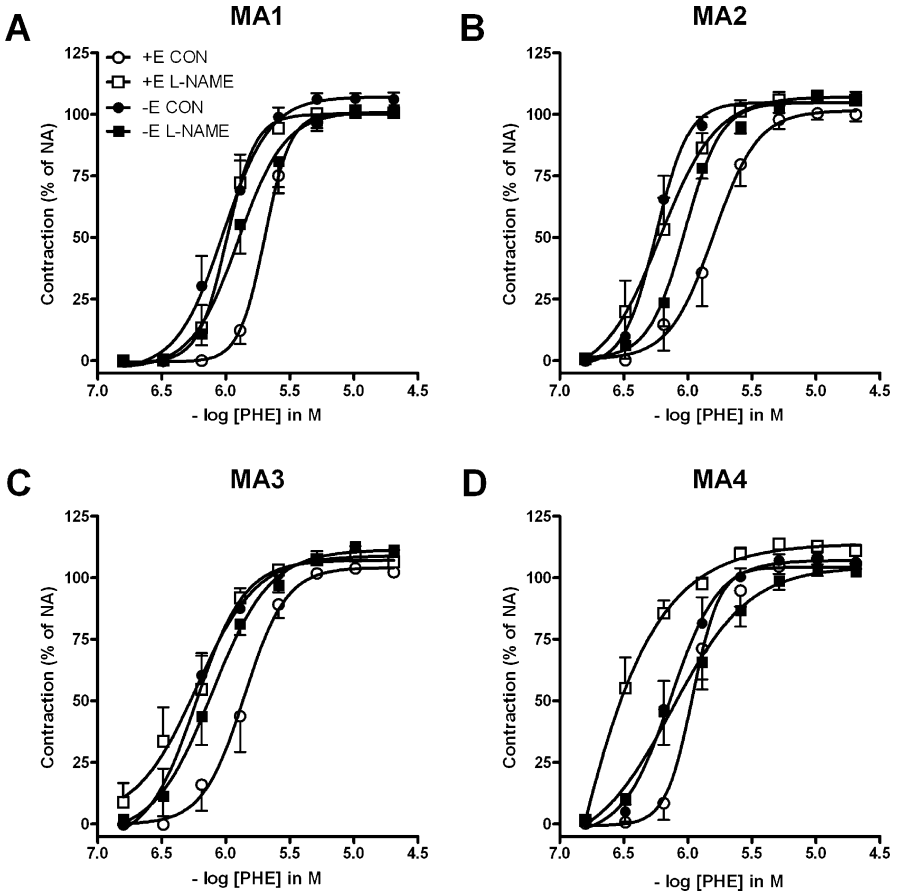

Figure 1 Effect of denudation and L-NAME on responses of α_1-adrenoceptors. Contractile responses of endothelium-intact (+E) and -denuded (–E) first- (MA1; A), second- (MA2; B), third- (MA3; C) and fourth-order (MA4; D) mesenteric arteries (MA) to phenylephrine (PHE). Concentration-response curves tophenylephrine (0.16–20 μmol·L^{-1}) were analysed in the absence (circles) and in the presence of the NO synthase inhibitor L-NAME (100 μmol·L^{-1}; squares). Data are expressed as percentage the response to 10 μmol·L^{-1} noradrenaline (NA) in the absence of inhibitor and are shown as mean ± SEM. L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester; NO, nitric oxide.

ing vessel diameter. For instance, in MA1 the change in pEC_{50} was 0.29 ± 0.04 compared with 0.36 ± 0.03 in MA2, 0.42 ± 0.05 in MA3 and 0.57 ± 0.02 in MA4 (Figure 2). In MA4, but not in the larger arteries, the effect of L-NAME on sensitivity to phenylephrine (ΔpEC_{50} 0.57 ± 0.02) was significantly larger than the effect of endothelium removal (ΔpEC_{50} 0.20 ± 0.06) (Figure 2).

Contributions of ET-1 and 20-HETE

The role of ET-1 in myoendothelial feedback was investigated with the non-selective endothelin receptor antagonist bosentan and with inhibitors of its downstream signalling component, 20-HETE. Bosentan (10 μmol·L^{-1}) did not affect sensitivity to phenylephrine in denuded MAs (Figure 2). In endothelium-intact MA1, MA2 and MA3 bosentan did not affect pEC_{50} (Figure 2A–C). In contrast to MA1, bosentan significantly shifted pEC_{50} to the right in endothelium-intact MA4 (Figure 2D).

Bosentan did not affect the endothelium-dependent enhancement in sensitivity to phenylephrine caused by L-NAME in MA1 (Figure 2A), this finding was in marked contrast to the effects on MA4 ($P < 0.05$; Figure 2D). In MA2 and MA3 intermediate shifts in pEC_{50} were observed ($P < 0.05$).

The CYP4A inhibitor DDMS (3 μmol·L^{-1}) and the 20-HETE receptor antagonist 20-HEDE (3 μmol·L^{-1}) did not affect the contractile response to phenylephrine in denuded MA4 in the absence or presence of L-NAME (data not shown). In endothelium-intact MA4 both DDMS and 20-HEDE did not affect pEC_{50} (data not shown). In the presence of L-NAME, the characteristic leftward shift was decreased in both DDMS and 20-HEDE-treated MA4 (ΔpEC_{50} 0.38 ± 0.10 and 0.22 ± 0.11 respectively).

Effect of inhibitors of gap junctions

In denuded MA1 and MA4, 18α-GA (20 μmol·L^{-1}) did not significantly alter the responses to phenylephrine in either the absence or presence of L-NAME (Figure 2A and D). In endothelium-intact MA1 and MA4, 18α-GA did not affect pEC_{50} to phenylephrine (Figure 2A and D). Most importantly, 18α-GA abolished the endothelium-dependent enhancement of the contractile response by L-NAME, specifically by reducing pEC_{50} to phenylephrine in the smallest MA4 (Figure 2D).

In a subset of experiments using intact and denuded MA4 from additional rats, other potential gap junction uncouplers

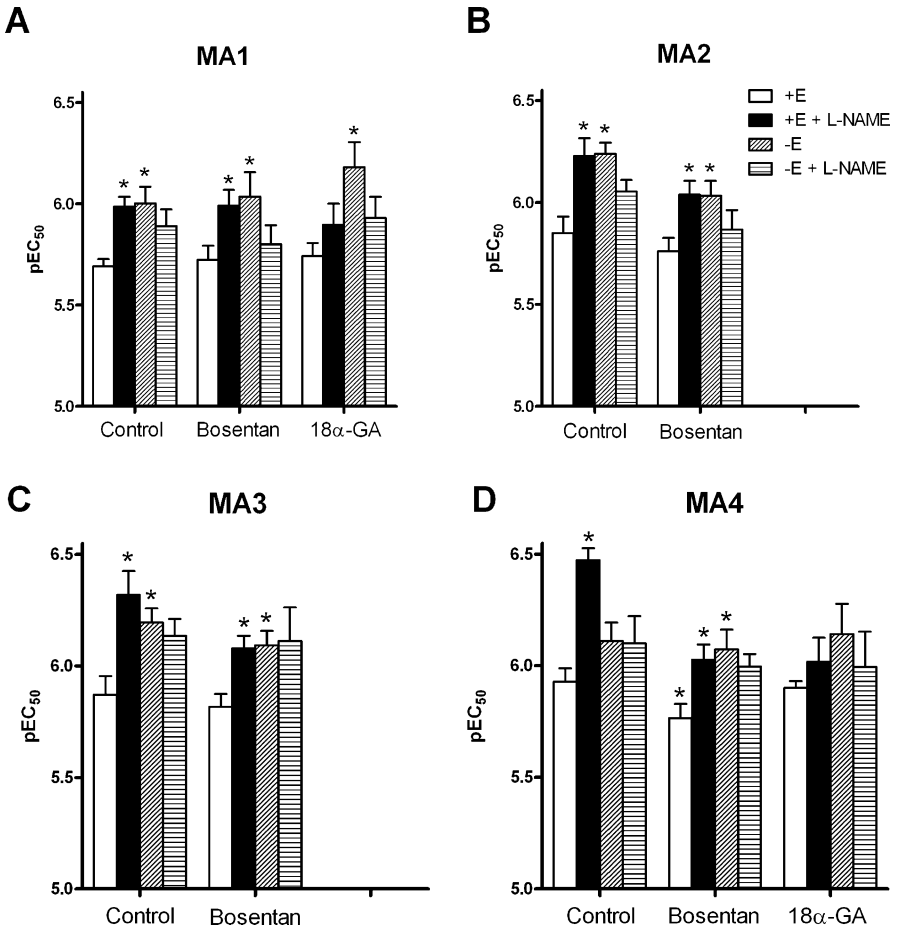

Figure 2 Sensitivity to phenylephrine. Sensitivity (pEC_{50}) to phenylephrine (PHE) for endothelium-intact (+E) and endothelium-denuded (–E) first- (MA1; A), second- (MA2; B), third- (MA3; C) and fourth-order (MA4; D) mesenteric arteries (MA) in the absence and presence of 100 μmol·L^{-1} L-NAME. pEC_{50} values were calculated for control conditions (control), in the presence of the non-selective ET-receptor antagonist bosentan (10 μmol·L^{-1}) and of the gap junction uncoupler 18α-glycyrrhetinic acid (18α-GA, 20 μmol·L^{-1}). Data are shown as mean ± SEM. *$P < 0.05$ versus +E. L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester.

were tested. 18β-GA (5 μmol·L^{-1}), its water-soluble analogue carbenoxolone (20 μmol·L^{-1}) and a cocktail of gap junction blocking peptides (^{43}Gap 26, ^{40}Gap 27 and ^{37,43}Gap 27, 100 μmol·L^{-1} each) all significantly reduced pEC_{50} to phenylephrine in the presence of L-NAME (ΔpEC50 0.10 ± 0.11, 0.25 ± 0.15 and 0.09 ± 0.16 respectively), indicating that gap junctions are involved in the endothelium-dependent effect of L-NAME in MA4.

Basal release of NO

We assessed the role of basal endothelium-derived NO by performing the stimulus-contraction assay during NO synthase blockade with L-NAME in endothelium-intact and denuded MA4 isolated from a different set of WKY rats in the absence and presence of a low, but constant, concentration of the NO donor, SNP (30 nmol·L^{-1}). The NO donor decreased contractile responses to phenylephrine in endothelium-intact MA4 by significantly lowering pEC_{50} (Figure 3). In endothelium-denuded MA4 the NO donor did not affect pEC_{50} (Figure 3). Most importantly, the NO donor abolished the differences between intact and denuded MA4 (Figure 3).

Effects of BayK 8644, depolarization and nerve stimulation

To verify whether the endothelium-dependent effect of L-NAME in MA4 is specific for α_1-adrenoceptor stimulation, we used three other ASM stimuli. The activator of L-type voltage operated calcium channels, BayK 8644 (0.001–1 μmol·L^{-1}), depolarizing K^+ solution (10–40 mmol·L^{-1}) and electrical field stimulation (0.25–32 Hz) all induced comparable contractile responses in endothelium-denuded and endothelium-intact MA4 (Figure 4). L-NAME significantly increased the sensitivity to all three stimuli in the endothelium-intact MA4, but not in the denuded MA4 (Figure 4).

Discussion

Removal of endothelium and inhibition of NO synthase increased arterial sensitivity to phenylephrine -induced contraction. The effect of L-NAME increased with increasing arterial branching order (Figure 5). In the smallest arteries, the effect of L-NAME was larger than that caused by removal of endothelium. This endothelium-dependent effect of NO syn-

thase blockade was inhibited by uncouplers of gap junctions, antagonism of endothelin receptors and by an NO donor. It was also observed during contractions induced by sympathetic nerve stimulation, depolarization or a dihydropyridine calcium channel agonist. These pharmacological findings indicate that activation of ASM can be accompanied by endothelial vasomotor influences involving gap junctions and a balance between endothelium-derived NO and ET-1 and that this myoendothelial coupling is more prominent in distal, compared with proximal resistance arteries.

Direct chemical and mechanical stimulation of the endothelium influences vasomotor activity of the underlying smooth muscle through transferable endothelium-derived factors and conducted hyperpolarization (Furchgott and Vanhoutte, 1989; Busse and Fleming, 2003). Myo-endothelial gap junctions (MEGJ), as potential sites for electrical communication between the endothelium and smooth muscle, are more common in small resistance arteries than in large conduit arteries (Sandow and Hill, 2000). Here we tested the hypothesis that activation of the ASM stimulates endothelial feedback vasomotor effects in resistance arteries. A pharmacological approach was used in view of the variety of mediators and processes that might be involved. Confounding

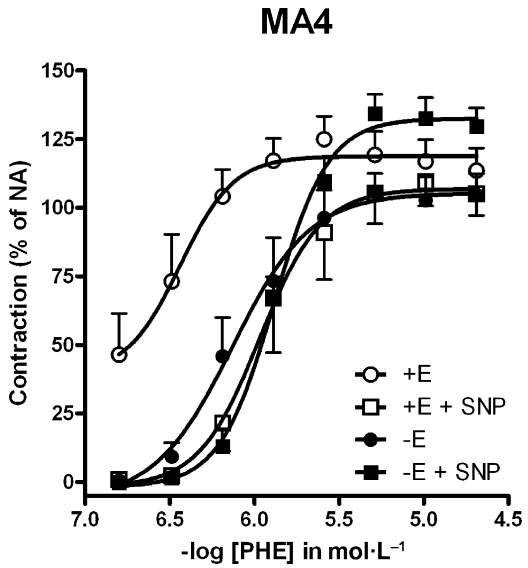

Figure 3 Effect of sodium nitroprusside on responses to α_1-adrenoceptor stimulation in the presence of L-NAME. Concentration-response curves for phenylephrine (PHE) in endothelium-denuded (–E) and endothelium-intact (+E) fourth-order mesenteric arteries (MA4) treated with the NO synthase inhibitor L-NAME (100 μmol·L^{-1}). Vessels were studied in the absence and presence of the NO donor sodium nitroprusside (SNP, 30 nmol·L^{-1}, squares). Values are shown as mean ± SEM. L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester; NO, nitric oxide.

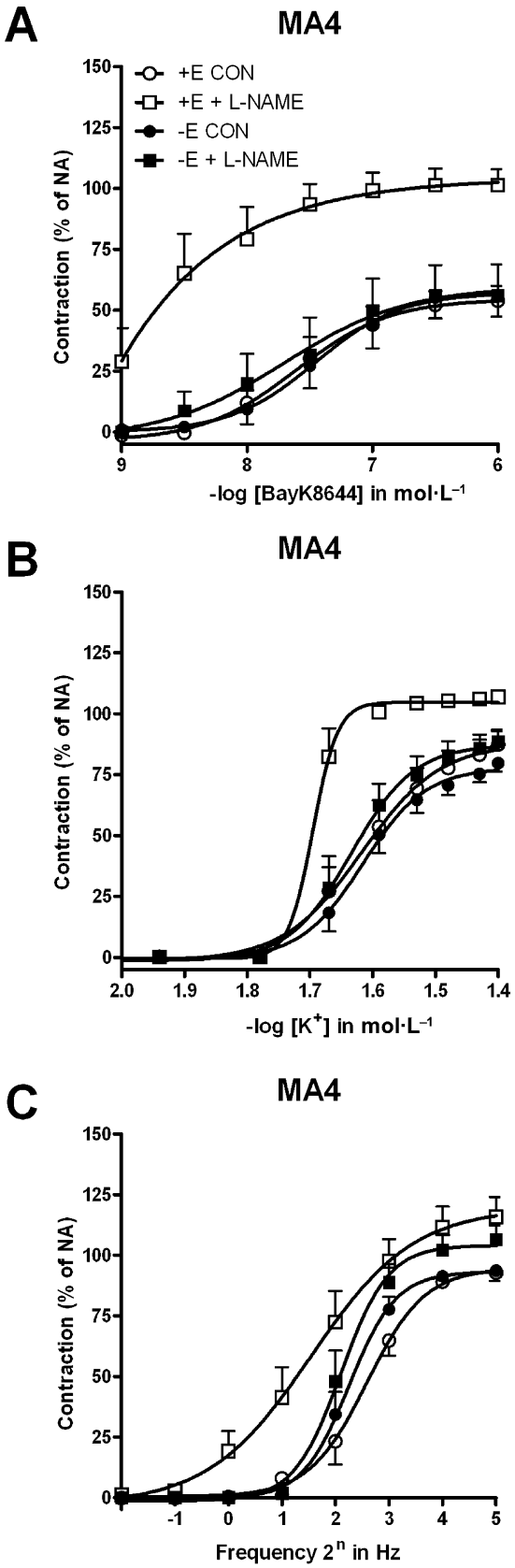

Figure 4 Effects of BayK 8644, depolarization and nerve stimulation. Contractile responses to the L-type voltage-operated calcium channel activator BayK 8644 (A), depolarization with K^+ (B) and electrical field stimulation (C) in endothelium-intact (+E) and endothelium-denuded (–E) fourth-order mesenteric arteries (MA4) in the absence and presence of the NO synthase antagonist L-NAME (100 μmol·L^{-1}). Values are shown as mean ± SEM. L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester; NO, nitric oxide.

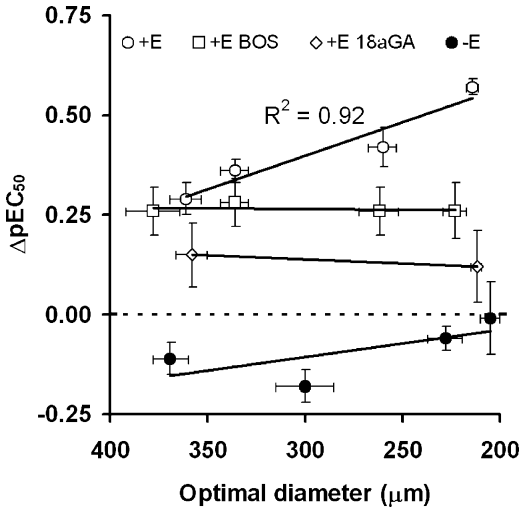

Figure 5 The effect of L-NAME on contractions mediated by α_1-adrenoceptors depends on arterial diameter. The difference of the sensitivity to phenylephrine (ΔpEC50) in the absence and presence of L-NAME (100 μmol·L^{-1}) in endothelium-intact (+E), +E treated with 18α-glycyrrhetinic acid (18α-GA, 20 μmol·L^{-1}), +E treated with bosentan (BOS, 10 μmol·L^{-1}) and endothelium-denuded (–E) mesenteric arteries (MAs) are shown as a function of arterial lumen diameter at which the largest contractile response to 10 μmol·L^{-1} noradrenaline could be obtained (optimal diameter). Values are shown as mean ± SEM. A linear regression line connects the data points. For the +E untreated MAs a R^2 value of 0.92 is shown. L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester.

influences of peri-arterial sensory motor nerves and prostaglandins were excluded by persistent desensitization (Szallasi and Blumberg, 1999; Wang and Wang, 2004; De Mey *et al.*, 2008) and inhibition of cyclooxygenases respectively. Arteries of increasing branching order were made to contract with phenylephrine. This agonist activates α_1-adrenoceptors that are expressed by ASM cells (Stassen *et al.*, 1997) but do not directly stimulate calcium influx in endothelial cells (Dora *et al.*, 2000). Because it can be debated whether the endothelium of small arteries and arterioles expresses α_1-adrenoceptors (Tuttle and Falcone, 2001), additional smooth muscle stimuli were also used.

We first compared responses to phenylephrine in the absence and presence of endothelium and of an inhibitor of NO synthases. In denuded vessels, sensitivity and relative maximal responses to the agonist did not differ between types of artery and L-NAME did not modify the contractile responses. In the largest arteries (MA1), L-NAME increased sensitivity to the agonist to the same extent as endothelium removal. In the smallest vessels (MA4), the effect of L-NAME in the presence of endothelium was considerably larger than the sensitizing effect of endothelium-removal. In MA2 and MA3, intermediate effects were observed. Furthermore, while the effect of denudation did not correlate with arterial size, the effect of L-NAME on sensitivity of intact arteries to phenylephrine displayed a statistically significant inverse relationship with arterial lumen diameter (Figure 5).

Because an endothelium-dependent pro-contractile effect was observed in the presence of indomethacin, the role of endogenous ET-1 was evaluated. The non-selective ET-1 receptor antagonist, bosentan (Clozel *et al.*, 1994) did not significantly modify α_1-adrenoceptor responses in MA1, MA2 and MA3 in absence or presence of endothelium or L-NAME. In the smallest arteries, MA4, however, bosentan reduced the sensitivity to phenylephrine in the presence of endothelium and reduced the endothelium-dependent effect of L-NAME. In these intact vessels, the ET_A receptor antagonist BQ-123 and the ET_B receptor antagonist BQ-788 each reduced but did not inhibit the effect of L-NAME (data not shown). Furthermore, this endothelium-dependent effect was inhibited by the selective inhibitor of CYP4A DDMS (Imig *et al.*, 2000) and by the 20-HETE-receptor antagonist 20-HEDE (Zhao *et al.*, 2004) which had no effects in denuded MA4. These observations are in line with earlier observations that endogenous ET-1 can contribute to vasoconstriction mediated by α_1- adrenoceptors (DeFily *et al.*, 1999), that mesenteric arterial responses to ET-1 involve interactions between ET_A- and ET_B-receptors (Adner *et al.*, 2001) and that the CYP4A–20-HETE pathway is involved in cellular effects of ET-1 (Oyekan *et al.*, 1997; Imig *et al.*, 2000). Our findings suggest that inhibition of NO synthase during stimulation of α_1-adrenoceptors enhances small artery vasoconstriction by increased effects of endothelium-derived ET-1. Most likely NO can inhibit the release of ET-1 from the endothelium via a cyclic GMP-mediated mechanism (Boulanger and Luscher, 1990).

We next confirmed the regionality of the endothelium-dependent effect of L-NAME and evaluated whether gap junctions are involved. In the proximal MA1, the effect of L-NAME on sensitivity to phenylephrine was again comparable to the effect of endothelium-removal and the putative uncoupler of gap junctions 18α-GA did not significantly modify α_1-adrenoceptor responses in presence or absence of endothelium and L-NAME. In the distal small MA4, the effect of L-NAME was again more pronounced than the effect of endothelium-removal and 18α-GA selectively abolished the endothelium-dependent effect of L-NAME, without significantly affecting sensitivity to stimulation of α_1-adrenoceptors in the absence of the NO synthase inhibitor. Because arterial effects of putative uncouplers of gap junctions are controversial (Chaytor *et al.*, 2001), we used additional chemically unrelated compounds with comparable putative effects on the integrity of gap junctions (Yamamoto *et al.*, 1998; Dora *et al.*, 1999; Chaytor *et al.*, 2000; Tare *et al.*, 2002; Matchkov *et al.*, 2004; 2006; Mather *et al.*, 2005). In intact MA4, also 18β-GA, carbenoxolone and a cocktail of gap junction blocking peptides significantly reduced sensitivity to the α_1-adrenoceptor agonist in the presence of L-NAME but not in its absence. These findings indicate involvement of gap junctions but do not discriminate between homocellular and heterocellular MEGJ.

We used an NO donor to strengthen the suggestions generated with L-NAME that endothelial NO interferes with myoendothelial coupling in small arteries. In MA4, a low and constant concentration of SNP was found not to modify the sensitivity to the α_1-adrenoceptor agonist in intact vessels and in denuded vessels with and without L-NAME, but to selectively reduce the sensitivity in intact arteries exposed to L-NAME. These findings, while not discriminating between candidate mechanisms like inhibition of the synthesis and release of ET-1 (Boulanger and Luscher, 1990) or inhibition of

gap junctional communication (Kameritsch *et al.*, 2005), point to modulation of myoendothelial coupling by basal endothelial production of NO.

We used sympathetic nerve stimulation, depolarizing solution and a dihydropyridine calcium channel agonist to (i) strengthen the possibility that ASM activation was accompanied by endothelial vasomotor influences; and (ii) to start unravelling the mechanisms involved. α_1-adrenoceptors on the ASM can be acted upon by noradrenaline released from nerves, and cause depolarization and calcium-influx through L- type voltage operated calcium channels (see. Mulvany and Aalkjaer, 1990). The endothelium, on the other hand, is not directly innervated (Burnstock and Ralevic, 1994), is inhibited by depolarization, does not express L- type voltage operated calcium channels (Nilius and Droogmans, 2001) and does not display a calcium-response upon direct stimulation of α_1-adrenoceptors (Dora *et al.*, 2000). In isolated MA4, L-NAME caused an endothelium-dependent increase of the contractile responses to sympathetic nerve stimulation, depolarizing solution and BayK 8644. These findings indicate that not only an exogenous α_1- adrenoceptor agonist but also endogenously released sympathetic neurotransmitter can stimulate ASM to induce endothelium-dependent vasomotor effects. They furthermore indicate that neither α_1- adrenoceptor stimulation nor depolarization of the arterial smooth are essential and suggest that second messenger-influx in ASM cells, possibly through MEGJ, can promote endothelial release of ET-1 when synthesis of NO is inhibited. Additional effects of depolarization, inositol triphosphate and Rho proteins that participate in the ASM effects of stimulating α_1-adrenoceptors (Somlyo and Somlyo, 2003), cannot be excluded.

Transfer of calcium ions from smooth muscle to endothelium has previously been documented (Little *et al.*, 1995; Dora *et al.*, 1997; Lamboley *et al.*, 2005; Isakson *et al.*, 2007). It has also previously been proposed that ASM activation can promote endothelial NO production (Fleming *et al.*, 1999; Stankevicius *et al.*, 2006) and that NO inhibits endothelial release of ET-1 (Boulanger and Luscher, 1990). Our suggestion, based on pharmacological observations, that the ASM can promote endothelium-dependent contraction through gap junctions and ET-1 when endothelial NO synthesis is impaired, is novel and merits direct confirmation by electrophysiological and biochemical approaches. Indeed, the nature of the smooth muscle-derived signal and the release of bioactive concentrations of endothelium-derived ET-1 remain to be proven. It raises the possibility that endothelial dysfunction, mostly attributed to haemodynamic and blood borne factors, could also arise from hyperreactivity of the ASM and sympathetic nerves, as seen in resistance arteries in, for instance, essential hypertension (see Mancia *et al.*, 1999).

In conclusion, inhibition of NO synthase in rat mesenteric small resistance arteries causes an endothelium-dependent increase in contractile responses to ASM stimuli that can be prevented by antagonism of ET receptors, uncouplers of gap junctions and an NO donor. These findings suggest an excitatory myoendothelial coupling that might participate in endothelial dysfunction in cardiovascular disease.involving small arteries

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Conflict of interest

None.

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