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Bradykinin-induced relaxation of coronary microarteries: *S*-nitrosothiols as EDHF?

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1 To investigate whether *S*-nitrosothiols, in addition to NO, mediate bradykinin-induced vasorelaxation, porcine coronary microarteries (PCMAs) were mounted in myographs.

2 Following preconstriction, concentration–response curves (CRCs) were constructed to bradykinin, the NO donors *S*-nitroso-*N*-penicillamine (SNAP) and diethylamine NONOate (DEA-NONOate) and the *S*-nitrosothiols L-*S*-nitrosocysteine (L-SNC) and D-SNC. All agonists relaxed PCMAs. L-SNC was $\approx$5-fold more potent than D-SNC.

3 The guanylyl cyclase inhibitor ODQ and the NO scavenger hydroxocobalamin induced a larger shift of the bradykinin CRC than the NO synthase inhibitor L-NAME, although all three inhibitors equally suppressed bradykinin-induced cGMP responses.

4 Complete blockade of bradykinin-induced relaxation was obtained with L-NAME in the presence of the large- and intermediate-conductance Ca^{2+}-activated K^+-channel (BK_{Ca}, IK_{Ca}) blocker charybdotoxin and the small-conductance Ca^{2+}-activated K^+-channel (SK_{Ca}) channel blocker apamin, but not in the presence of L-NAME, apamin and the BK_{Ca} channel blocker iberiotoxin.

5 Inhibitors of cytochrome *P*450 epoxygenase, cyclooxygenase, voltage-dependent K^+ channels and ATP-sensitive K^+ channels did not affect bradykinin-induced relaxation.

6 SNAP-, DEA-NONOate- and D-SNC-induced relaxations were mediated entirely by the NO-guanylyl cyclase pathway. L-SNC-induced relaxations were partially blocked by charybdotoxin + apamin, but not by iberiotoxin + apamin, and this blockade was abolished following endothelium removal. ODQ, but not hydroxocobalamin, prevented L-SNC-induced increases in cGMP, and both drugs shifted the L-SNC CRC 5–10-fold to the right.

7 L-SNC hyperpolarized intact and endothelium-denuded coronary arteries.

8 Our results support the concept that bradykinin-induced relaxation is mediated *via de novo* synthesized NO and a non-NO, endothelium-derived hyperpolarizing factor (EDHF). *S*-nitrosothiols, *via* stereoselective activation of endothelial IK_{Ca} and SK_{Ca} channels, and through direct effects on smooth muscle cells, may function as an EDHF in porcine coronary microarteries.
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Abbreviations: B_2 receptor, bradykinin type 2 receptor; BK_{Ca}, large conductance Ca^{2+}-activated K^+-channel; CRC, concentration–response curve; DEA-NONOate, diethylamine NONOate; EDHF, endothelium-derived hyperpolarizing factor; Hoe140, D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin; IK_{Ca}, intermediate conductance Ca^{2+}-activated K^+-channel; K_{IR}, inwardly rectifying K^+ channel; L-NAME, N^{ω}-nitro-L-arginine methyl ester HCl; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PCMAs, porcine coronary microarteries; SK_{Ca}, small conductance Ca^{2+}-activated K^+-channel; SNAP, *S*-nitroso-*N*-penicillamine; D-SNC, D-*S*-nitrosocysteine; L-SNC, L-*S*-nitrosocysteine; U46619, 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F_{2\alpha}$

Introduction

Bradykinin relaxes coronary arteries in an endothelium-dependent manner. This effect is mediated *via* bradykinin type 2 (B_2) receptors. B_2 receptor activation results in NO synthesis by endothelial NOS, and NO relaxes vascular smooth muscle cells through guanylyl cyclase activation and subsequent cGMP generation (Danser *et al*., 2000). NOS inhibitors, however, do not completely block bradykinin-induced vasorelaxation, suggesting the existence of either NO-storage sites (Davisson *et al*., 1996a; Danser *et al*., 1998) or a non-NO 'endothelium-derived hyperpolarizing factor' (EDHF) (McGuire *et al*., 2001; Busse *et al*., 2002).

EDHF-mediated responses in different arteries have been linked to the release of K^+, the generation of cytochrome-*P*450 products from arachidonic acid (epoxyeicosatrienoic acids, EETs), and to the production of H_2O_2 (Campbell *et al*., 1996; Randall *et al*., 1996; Mombouli & Vanhoutte, 1997; Edwards *et al*., 1998; 2001; Fisslthaler *et al*., 1999; Matoba &

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Shimokawa, 2003). The identity of EDHF and its contribution to overall relaxation differs between species, between vascular beds and between vessels of different sizes (Hwa *et al*., 1994).

EDHF-mediated relaxation depends on the activation of intermediate- and small-conductance Ca^{2+}-activated K^+-channels (IK_{Ca}, SK_{Ca}) (Busse *et al*., 2002). These channels are located on endothelial cells (Bychkov *et al*., 2002; Sollini *et al*., 2002), and (as a consequence of endothelial hyperpolarization), may be responsible for the subsequent relaxation that is generally attributed to the release of an EDHF (Edwards *et al*., 1998; 2001). This EDHF induces smooth muscle hyperpolarization by activating inwardly rectifying K^+ channel (K_{IR}) channels, Na^+-K^+-ATPase and/or large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels (Busse *et al*., 2002; Archer *et al*., 2003). With regard to the latter, it is important to note that NO itself is capable of inducing hyperpolarization *via* activation of Ca^{2+}-activated K^+ channels in vascular smooth muscle (Bolotina *et al*., 1994).

Bradykinin induces release of NO-containing factors (e.g., *S*-nitrosothiols) from cellular storage sites (Davisson *et al*., 1996a; Danser *et al*., 1998; Tom *et al*., 2001). Depletion of NO storage sites occurs only after repeated exposure to bradykinin or following prolonged inhibition of NOS (Davisson *et al*., 1996a; Danser *et al*., 1998). *S*-nitrosothiols induce relaxation through decomposition to NO (Rafikova *et al*., 2002), or by activating stereoselective recognition sites (Davisson *et al*., 1996b). These recognition sites could either be cysteine residues within Ca^{2+}-activated K^+ channels (Lang *et al*., 2003) or a novel class of receptors which specifically recognize L-*S*-nitrosocysteine (L-*S*NC) and structurally similar *S*-nitrosothiols such as L-*S*-nitroso-β,β-dimethylcysteine (Travis *et al*., 1997).

In the present study, we set out to investigate the possibility that *S*-nitrosothiols act as an EDHF in porcine coronary microarteries (PCMAs). PCMAs rather than large porcine coronary arteries were used, because the contribution of EDHF to vasorelaxation is larger in smaller vessels (Hwa *et al*., 1994; Danser *et al*., 2000). We compared the relaxant effects of L-*S*NC to those of bradykinin and the NO donors *S*-nitroso-*N*-acetylpenicillamine (*N*-acetyl-3-(nitrosothio)-D-valine or SNAP) and diethylamine NONOate (DEA-NONOate), both in the absence and presence of an inhibitor of NOS, an inhibitor of guanylyl cyclase, and inhibitors of a wide range of EDHF candidates. To rule out residual NO (i.e., non-EDHF)-mediated effects as much as possible we also made use of the NO scavenger hydroxocobalamin. Guanylyl cyclase activation by NO or NO-containing factors was quantified by measuring cGMP generation. To verify the stereoselectivity of L-SNC-induced effects, parallel experiments were performed with D-*S*-nitrosocysteine (D-SNC). Finally, electrophysiological measurements were performed in intact and endothelium-denuded porcine coronary arteries to verify direct hyperpolarization by bradykinin and L-SNC.

Methods

Drugs

Bradykinin, SNAP, DEA-NONOate, L-cysteine, D-cysteine, $NaNO_2$, 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F_{2\alpha}$ (U46619), N^{ω}-nitro-L-arginine methyl ester HCl (L-NAME), N^{ω}-nitro-L-arginine (L-NA), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), hydroxocobalamin, indomethacin, diclofenac, glibenclamide, 4-aminopyridine, charybdotoxin, apamin, iberiotoxin, ouabain, $BaCl_2$, sulfaphenazole, miconazole and 3-isobutyl-1-methyl-xanthine were from Sigma-Aldrich (Zwijndrecht, The Netherlands). D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe140) was a kind gift of Dr W. Linz, Hoechst, Frankfurt, Germany. Indomethacin, glibenclamide, ouabain and ODQ were dissolved in dimethylsulfoxide. Sulfaphenazole and miconazole were dissolved in ethanol. Hydroxocobalamin was dissolved in methanol. All other chemicals were dissolved in water.

Tissue collection

Pig hearts ($n=123$) were collected at the local slaughterhouse. Epicardial arteries (diameter ≈ 1.5 mm) and tertiary branches of the left anterior descending coronary artery (PCMAs; diameter 337 ± 8.4 μm) were removed and either used directly or stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4.

Organ bath studies

PCMAs were cut into segments of ≈ 2 mm length and mounted in microvascular myographs (J.P. Trading, Aarhus, Denmark) with separated 6-ml organ baths containing Krebs bicarbonate solution aerated with 95% O_2/5% CO_2 and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30-min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mmHg effective transmural pressure (Mulvany & Halpern, 1977).

The normalized vessel segments were exposed to 30 mM KCl twice. In some vessels, the endothelium was removed by gently rubbing a hair through the lumen of the mounted artery. Endothelial integrity or removal was verified by observing relaxation (or lack thereof) to 10 nM substance P after preconstriction with 10 nM of the thromboxane-A_2 analogue U46619. The maximal contractile response to KCl was determined by exposing the tissue to 100 mM KCl. Thereafter, vessels were allowed to equilibrate in fresh organ bath fluid for 30 min in the absence or presence of one or more of the following inhibitors: the NOS inhibitor L-NAME (100 μM), the NO scavenger hydroxocobalamin (200 μM), the guanylyl cyclase inhibitor ODQ (10 μM), the COX inhibitor indomethacin (10 μM), the $IK_{Ca} + BK_{Ca}$ channel inhibitor charybdotoxin (100 nM), the SK_{Ca} channel inhibitor apamin (100 nM), the BK_{Ca} channel inhibitor iberiotoxin (100 nM), the voltage-dependent K^+ channel (K_v) inhibitor 4-aminopyridine (5 mM), the ATP-sensitive K^+ channel (K_{ATP}) inhibitor glibenclamide (1 μM), the K_{IR} inhibitor $BaCl_2$ (30 μM), the Na^+-K^+-ATPase inhibitor ouabain (0.5 mM), the cytochrome *P*450 epoxygenase inhibitors sulfaphenazole or miconazole (10 μM) or the B_2 receptor antagonist Hoe140 (1 μM). Vessels were then preconstricted with U46619, and concentration-response curves (CRCs) were constructed to bradykinin, SNAP, DEA-NONOate, L-SNC or D-SNC. L-SNC and D-SNC were prepared immediately prior to the experiment and stored in the dark below 0°C. In short, 50 μl of a 0.2 M solution of L- and

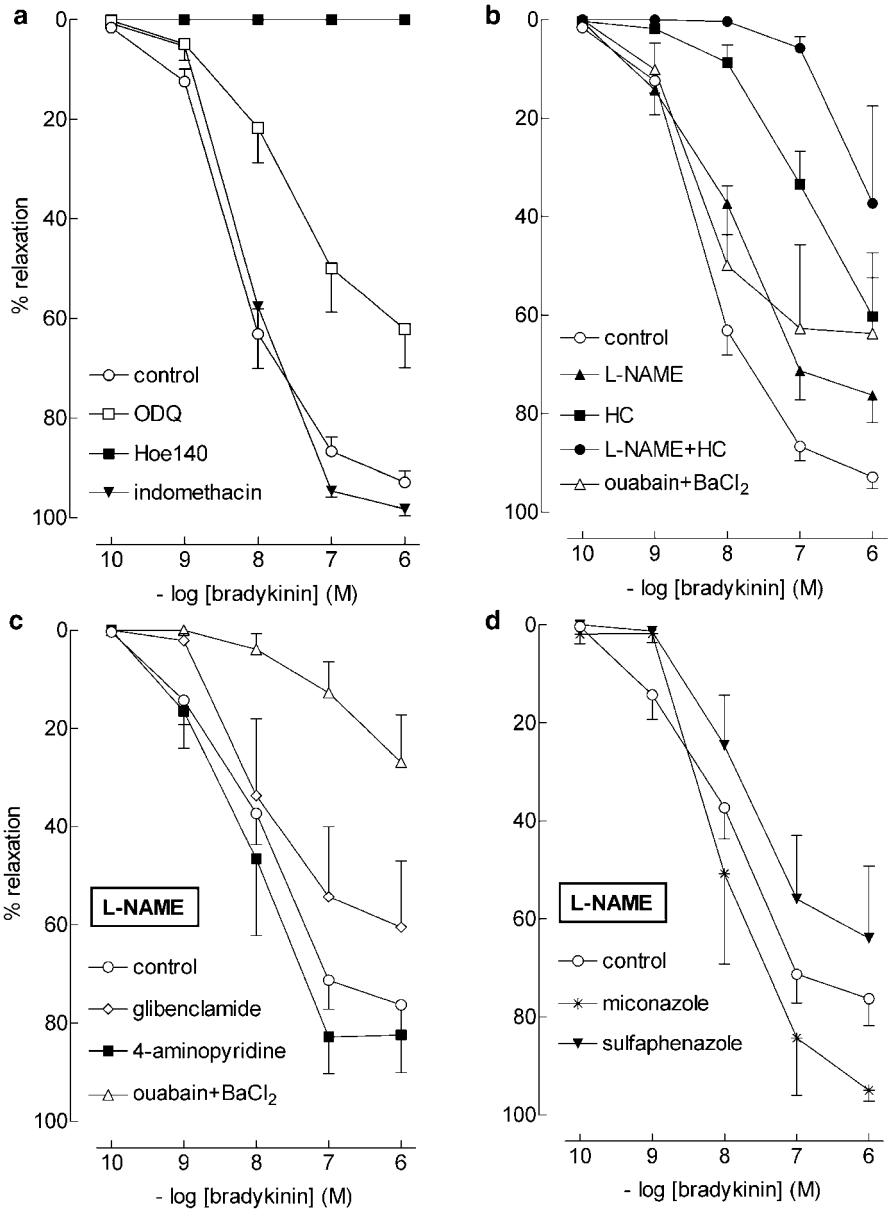


Figure 1 Relaxations of PCMAs, preconstricted with U46619, to bradykinin in absence (control; a, b) or presence of 100 μM L-NAME (c, d) with one or more of the following inhibitors: 1 μM Hoe140, 10 μM ODQ, 10 μM indomethacin, 100 μM L-NAME, 200 μM hydroxocobalamin (HC), 0.5 mM ouabain, 30 μM $BaCl_2$, 1 μM glibenclamide, 5 mM 4-aminopyridine, 10 μM miconazole or 10 μM sulfaphenazole. Data (mean $\pm$ s.e.mean; $n = 5$–45) are expressed as a percentage of the contraction induced by U46619.

D-cysteine was mixed with 50 μl 0.2 M $NaNO_2$. The subsequent addition of 10 μl 1 M HCl resulted in a stable 0.1 M solution (pH $\approx$ 5) of the respective SNC isomers (Davisson *et al.*, 1996b). Preliminary studies with $NaNO_2$, L- and D-cysteine ($n = 3$ each) revealed that, separately, these drugs did not exert relaxant effects in preconstricted PCMAs (data not shown).

Cyclic GMP measurement

To study bradykinin- and L-SNC-induced cGMP production, vessel segments (5–10 mg) were exposed to bradykinin (1 μM) or L-SNC (10 or 100 μM) in 10 ml oxygenated Krebs bicarbonate solution for 1 min at 37°C in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (100 μM), following a 30-min preincubation in the absence (control) or presence of hydroxocobalamin, ODQ, Hoe140 and/or L-NAME at the above concentrations. Tissues were subsequently frozen in liquid nitrogen, and stored at -80°C. To determine cGMP, frozen tissues were homogenized in 0.5 ml 0.1 M HCl using a stainless-steel ultraturrax (polytron). Homogenates were centrifuged at 3300 g, and cGMP was measured in 300 μl supernatant by ELISA following acetylation (R&D Systems, Minneapolis, U.S.A.). Experiments were performed in quadruplicate, and results are expressed as pmol mg^{-1} protein. The lower limit of detection was 0.1 pmol mg^{-1} protein.

Electrophysiological measurements

Freshly isolated epicardial artery segments ($\approx$ 40 mm length) were excised, slit and mounted in heated (37°C) chambers and

maintained in modified Tyrode's solution (in mM: NaCl 132, KCl 4, $CaCl_2$ 1.6, $MgCl_2$ 1.2, NaH_2PO_4 0.36, $NaHCO_3$ 23.8, Ca^{2+}-EDTA 0.05, glucose 10; gassed with 20% O_2/5% CO_2/75% N_2, pH 7.4) containing the NOS inhibitor L-NA (300 μM), the COX inhibitor diclofenac (10 μM), and U46619 (1 μM) to mimic conditions in the organ chamber experiments as closely as possible. Both endothelium intact and endothelium-denuded segments were used. Smooth muscle membrane potential was recorded by impaling cells through the intima as described (Fisslthaler *et al*., 1999). Bradykinin (100 nM) and L-SNC (50 μM) were applied as bolus injections into the bath.

Data analysis

Data are given as mean ± s.e.m. Contractile responses are expressed as a percentage of the contraction to U46619. CRCs were analyzed as described using the logistic function described by de Lean *et al*. (1978) to obtain pEC_{50} ($-^{10}\log EC_{50}$) values (Table 1). L-NAME, ODQ, hydroxocobalamin and/or ouabain + $BaCl_2$ increased basal tone by 10–40%, whereas 4-aminopyridine increased basal tone by 80%. In such cases the concentration of U46619 (range 10–30 nM) was adjusted to obtain a preconstriction corresponding to ≈95% of the maximal contractile response in all vessels. Statistical analysis was by paired *t*-test, once one-way ANOVA, followed by Dunnett's *post hoc* evaluation, had revealed that differences existed between groups. $P<0.05$ was considered significant.

Results

Mechanism of bradykinin-induced relaxation

Bradykinin relaxed preconstricted vessel segments in a concentration-dependent manner ($pEC_{50}=8.2\pm0.1$, $n=45$; Figure 1). Bradykinin-induced relaxations were unaffected by indomethacin and abrogated by Hoe140. L-NAME shifted the bradykinin CRC ≈5-fold to the right, whereas ODQ and hydroxocobalamin induced a ≈10-fold rightward shift (see Table 1 for pEC_{50} values). Apamin, iberiotoxin and charybdotoxin, separately or in combination, did not significantly affect the bradykinin CRC (Figure 2, Table 1), nor did ouabain + $BaCl_2$ (Figure 1, Table 1).

In the presence of L-NAME, apamin and charybdotoxin, when given separately, did not affect the bradykinin CRC (pEC_{50}'s 7.8 ± 0.3 and 8.2 ± 0.2, respectively, $n=6$ for each; Figure 2), nor did glibenclamide, 4-aminopyridine, sulfaphenazole and miconazole (pEC_{50}'s 7.8 ± 0.5, 8.0 ± 0.3, 7.8 ± 0.4 and 7.9 ± 0.6, respectively, $n=5$ for each; Figure 1). In contrast, when given in addition to L-NAME, charybdotoxin + apamin fully blocked the bradykinin-induced responses (Figure 2, $n=5$), whereas hydroxocobalamin ($n=5$) and ouabain + $BaCl_2$ ($n=5$) shifted the bradykinin CRC >100-fold ($P<0.01$; Figure 1) to the right. Iberiotoxin, without ($n=6$) or with ($n=6$) apamin, reduced the maximum effect of bradykinin in the presence of L-NAME ($P<0.01$; Figure 2), without altering its pEC_{50} (7.8 ± 0.5 and 8.1 ± 0.2, respectively).

Thus, NO and/or NO-containing factors as well as Ca^{2+}-activated K^+-channels (BK_{Ca}, IK_{Ca} and SK_{Ca}), K_{IR} channels, and Na^+-K^+-ATPase are involved in the bradykinin-induced relaxation, and the NO-induced effects are mediated, at least in part, *via* activation of guanylyl cyclase. No evidence for a role of K_v channels, K_{ATP} channels, COX products, or cytochrome *P*450 epoxygenase products was obtained.

Mechanism of NO-induced relaxation

SNAP and DEA-NONOate relaxed preconstricted coronary microvessels in a concentration-dependent manner (Figure 3 and 4; Table 1). Both hydroxocobalamin and ODQ shifted the SNAP and DEA-NONOate-induced CRC to the right and, in combination, completely blocked SNAP-induced relaxation. Charybdotoxin + apamin, either as combination or together

Table 1 pEC_{50} values of bradykinin, SNAP, L-SNC, D-SNC or DEA-NONOate in the absence or presence of several inhibitors

			pEC_{50}		
Inhibitor	*Bradykinin*	*SNAP*	*L-SNC*	*D-SNC*	*DEA-NONOate*
None	8.2 ± 0.1 (45)	7.1 ± 0.1 (14)	6.5 ± 0.1 (22)$^{\#}$	6.0 ± 0.1 (18)	6.6 ± 0.1 (4)
Hoe140	<6 (3)*				
Indomethacin	8.0 ± 0.1 (5)				
ODQ	7.2 ± 0.2 (15)*	5.7 ± 0.1 (11)*	5.3 ± 0.1 (9)*	5.3 ± 0.1 (13)*	5.8 ± 0.1 (4)†
Hydroxocobalamin	6.8 ± 0.2 (16)*	6.5 ± 0.2 (11)*	5.4 ± 0.2 (8)†	5.5 ± 0.1 (13)*	5.9 ± 0.1 (4)†
L-NAME	7.7 ± 0.1 (23)*		7.5 ± 0.3 (5)		
Apamin	8.5 ± 0.2 (7)		7.2 ± 0.1 (4)		
Charybdotoxin	8.2 ± 0.2 (8)				
Iberiotoxin	8.4 ± 0.2 (6)		7.1 ± 0.1 (4)		
Charybdotoxin + apamin	7.6 ± 0.3 (7)	7.2 ± 0.1 (6)	6.0 ± 0.1 (10)*	5.9 ± 0.1 (13)	6.5 ± 0.1 (4)
Iberiotoxin + apamin	8.8 ± 0.2 (6)		7.1 ± 0.2 (5)		
Ouabain + $BaCl_2$	8.7 ± 0.1 (6)		6.7 ± 0.1 (4)†		
Glibenclamide			7.2 ± 0.1 (5)		
4-Aminopyridine			7.1 ± 0.5 (4)		
No endothelium			7.3 ± 0.1 (9)†		
No endothelium + charybdotoxin + apamin			7.0 ± 0.1 (4)		
ODQ + hydroxocobalamin		<5 (5)*‡	4.6 ± 0.3 (5)*§	4.3 ± 0.2 (12)*‡	
ODQ + charybdotoxin + apamin		5.6 ± 0.2 (5)*	5.3 ± 0.1 (5)*	5.2 ± 0.1 (5)*	
Hydroxocobalamin + charybdotoxin + apamin		6.3 ± 0.2 (6)†	5.3 ± 0.2 (5)†	5.7 ± 0.1 (5)†	
ODQ + hydroxocobalamin + charybdotoxin + apamin		<5 (3)*‡	4.5 ± 0.2 (5)*‡	4.3 ± 0.2 (8)*‡	

Data are mean ± s.e.m. (*n* value); *$P<0.01$, †$P<0.05$ vs none; ‡$P<0.01$, §$P<0.05$ vs hydroxocobalamin or ODQ; $^{\#}P<0.05$ vs D-SNC.

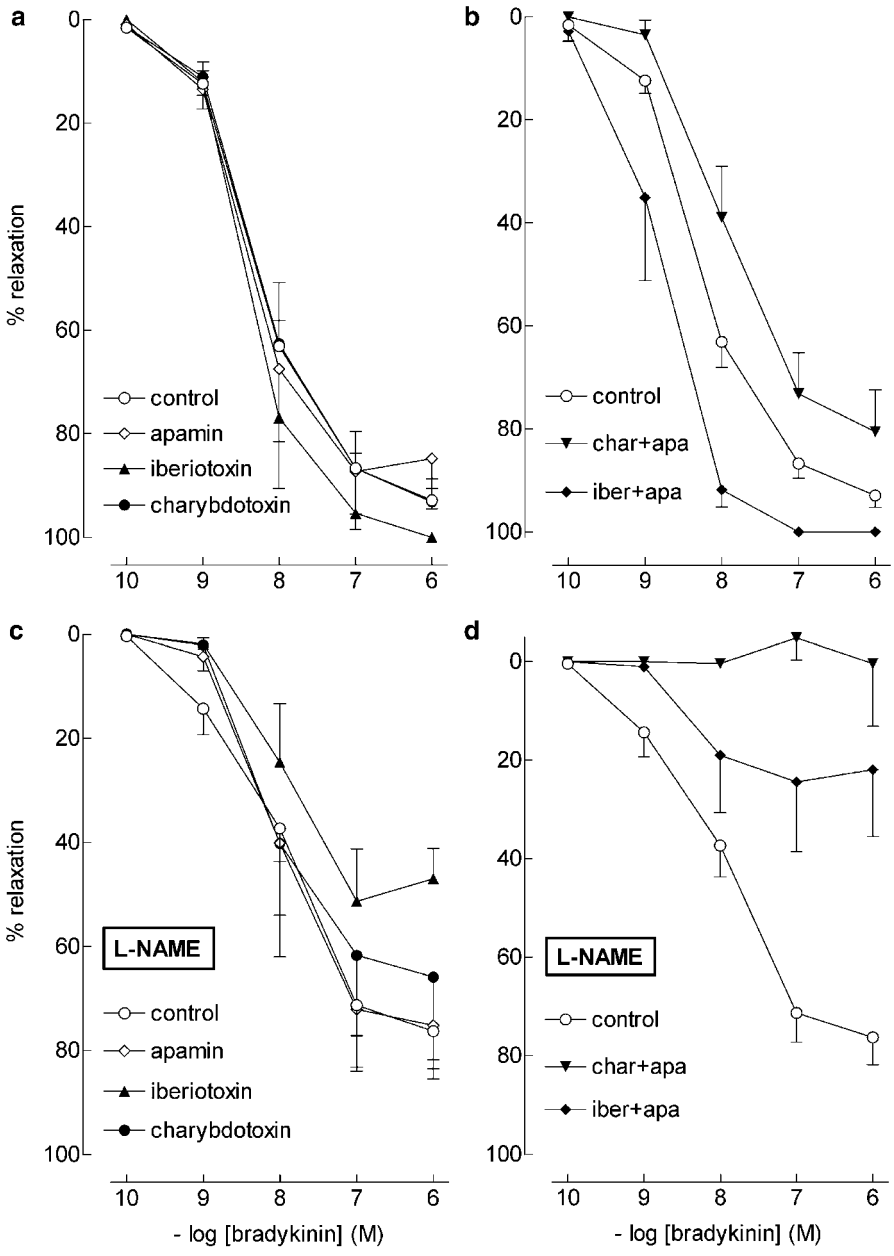


Figure 2 Relaxations of PCMAs, preconstricted with U46619, to bradykinin in absence (control; a, b) or presence of 100 μM L-NAME (c, d) with one or more of the following inhibitors: 100 nM charybdotoxin (char), 100 nM apamin (apa), or 100 nM iberiotoxin (iber). Data (mean ± s.e.mean; $n = 5$–45) are expressed as a percentage of the contraction induced by U46619.

with hydroxocobalamin or ODQ, did not elicit a rightward shift in the SNAP or DEA-NONOate CRC.

Thus, the relaxation induced by exogenous NO depends entirely on activation of guanylyl cyclase, and does not involve Ca^{2+}-activated K^+-channels.

Mechanism of S-*nitrosothiol-induced relaxation*

L-SNC and D-SNC relaxed preconstricted PCMAs in a concentration-dependent manner. L-SNC was 5 times more potent than D-SNC ($P<0.05$; Figure 5, Table 1). ODQ and hydroxocobalamin shifted the CRCs of both L-SNC and D-SNC 5–10-fold to the right (P = NS for the difference in rightward shift between L-SNC and D-SNC) and, when combined, caused a further rightward shift (Table 1).

Charybdotoxin + apamin shifted the L-SNC CRC, but not the D-SNC CRC, 5–10 fold to the right (Figure 6, Table 1). Endothelium-denudation shifted the L-SNC CRC 5–10 fold to the left, and abolished the rightward shift induced by charybdotoxin + apamin (Figure 6, Table 1). Charybdotoxin + apamin did not have additional effects on top of ODQ, hydroxocobalamin (Table 1) or ODQ + hydroxocobalamin (Figures 5 and 6, Table 1) with either L-SNC or D-SNC.

Glibenclamide, 4-aminopyridine, L-NAME, apamin, iberiotoxin, and iberiotoxin + apamin did not affect the L-SNC CRC (Figure 7, Table 1).

Ouabain + $BaCl_2$ shifted the L-SNC CRC five-fold to the right (Figure 7, Table 1) but did not exert an additional effect on top of hydroxocobalamin (pEC_{50} 5.9 ± 0.2, $n = 4$; Figures 5 and 7).

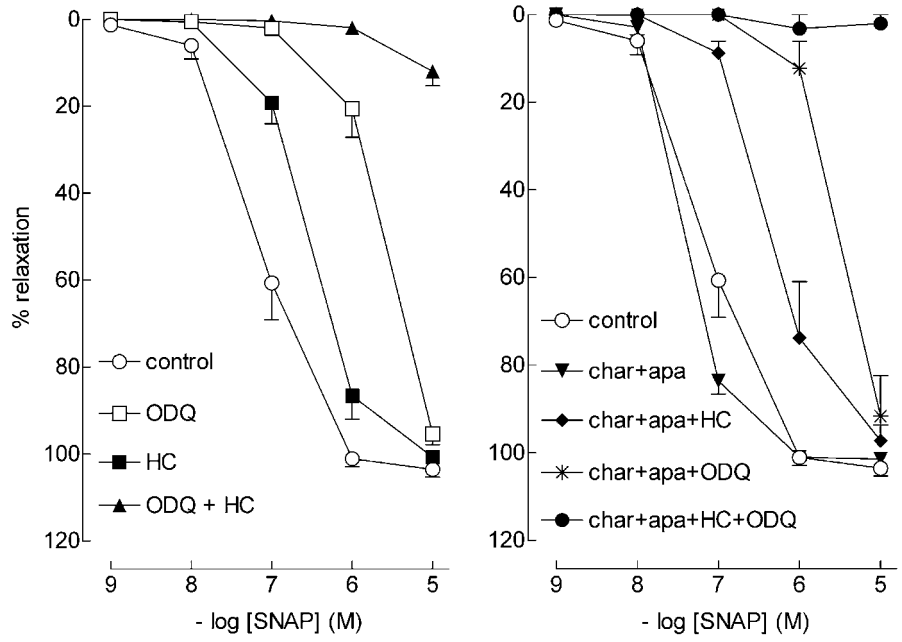
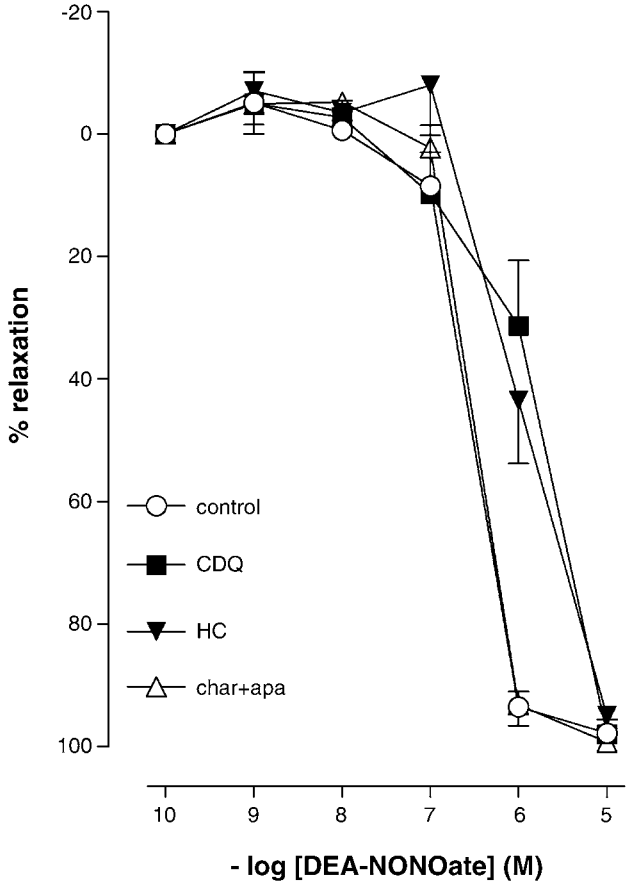


Figure 3 Relaxations of PCMAs, preconstricted with U46619, to SNAP in the absence (control) or presence of one or more of the following inhibitors: 10 μM ODQ, 200 μM hydroxocobalamin (HC), 100 nM charybdotoxin (char) or 100 nM apamin (apa). Data (mean $\pm$ s.e.mean; $n = 5$–14) are expressed as a percentage of the contraction induced by U46619.



Thus, *S*-nitrosothiol-induced relaxation occurs in a stereoselective manner, and is mediated *via* activation of guanylyl cyclase, endothelial IK_{Ca} and SK_{Ca} channels, K_{IR} channels and the Na^+-K^+-ATPase. Neither NOS, K_v channels, K_{ATP} channels, nor BK_{Ca} channels appear to mediate this response.

Cyclic GMP

Baseline cGMP levels were 9.4 ± 2.7 pmol mg^{-1} protein ($n = 16$). Bradykinin increased the microvascular cGMP levels four- to 5-fold (Figure 8; $P < 0.05$ vs control). Hoe140 largely prevented this increase, whereas L-NAME, hydroxocobalamin and ODQ reduced the microvascular cGMP content following bradykinin stimulation to levels below baseline. The combination of hydroxocobalamin and L-NAME did not further decrease cGMP levels. L-SNC increased cGMP >5-fold ($P < 0.05$ vs control). ODQ fully prevented the increase induced by 10 μM L-SNC, and a similar tendency was observed for hydroxocobalamin (P = NS). ODQ did not fully prevent the increase induced by 100 μM L-SNC.

Electrophysiological measurements

Both bradykinin ($n = 6$) and L-SNC ($n = 6$) hyperpolarized vascular smooth muscle cells ($P < 0.01$) in porcine coronary

Figure 4 Relaxations of PCMAs, preconstricted with U46619, to DEA-NONOate in the absence (control) or presence of one or more of the following inhibitors: 10 μM ODQ, 200 μM hydroxocobalamin (HC), 100 nM charybdotoxin (char) or 100 nM apamin (apa). Data (mean $\pm$ s.e.mean; $n = 4$) are expressed as a percentage of the contraction induced by U46619.

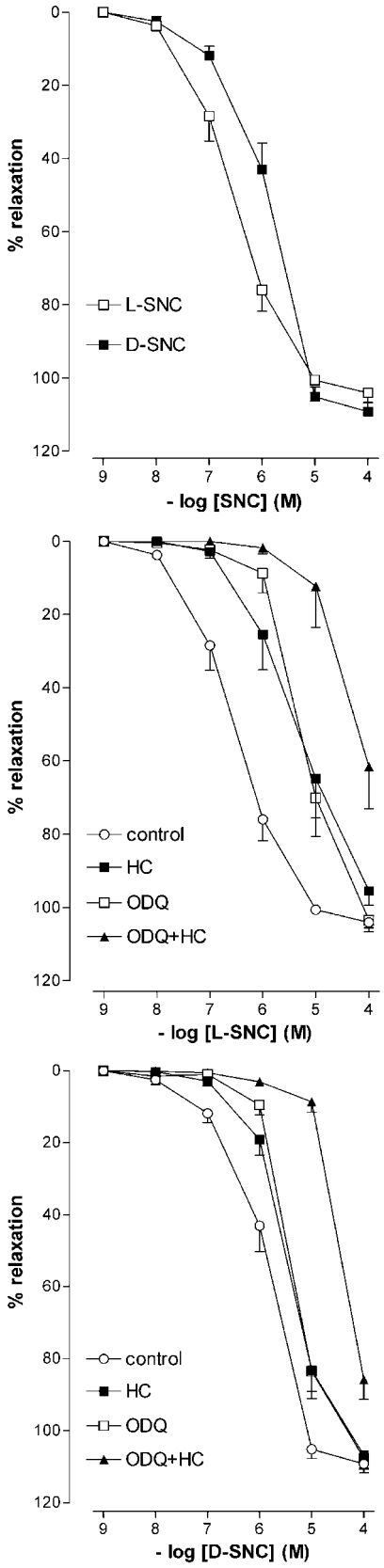


Figure 5 Relaxations of PCMAs, preconstricted with U46619, to L-SNC or D-SNC in the absence (control) or presence of 10 μM ODQ and/or 200 μM hydroxocobalamin (HC). Data (mean ± s.e.mean; $n = 4$–18) are expressed as a percentage of the contraction induced by U46619.

arteries (Figure 9). The effect of L-SNC was not affected by the removal of the endothelium ($n = 5$).

Discussion

The present study shows that B_2 receptor-mediated vasodilation in porcine coronary microarteries involves the NOS/NO/guanylyl cyclase/cGMP pathway and Ca^{2+}-activated K^+-channels, but not COX products or K_{ATP} channels. This is in full agreement with the concept that both NO and an EDHF that is not *de novo* synthesized NO determine bradykinin-induced relaxation. The two pathways appear to be interchangeable, since blocking each pathway separately (with L-NAME and charybdotoxin + apamin, respectively) only marginally affected bradykinin-mediated relaxation, whereas blocking both pathways together abrogated the effects of bradykinin. The modest effect of blocking NOS in the present study opposes earlier data in large porcine coronary arteries, where L-NAME alone induced a $\approx$10-fold rightward shift of the bradykinin CRC (Danser *et al*., 2000). Apparently, as has been suggested before, *de novo* synthesized NO is of greater importance in large arteries, and the contribution of EDHF is larger in microarteries (Hwa *et al*., 1994).

The BK_{Ca} channel inhibitor iberiotoxin, with or without the SK_{Ca} channel inhibitor apamin, reduced the maximum effect but not the potency of bradykinin in the presence of L-NAME. This finding, combined with the lack of effect of the K_v channel inhibitor 4-aminopyridine, suggests that the complete inhibition of bradykinin-induced relaxation obtained with charybdotoxin (a nonselective inhibitor of BK_{Ca}, IK_{Ca} and K_v channels) in the presence of apamin and L-NAME can be attributed to the blockade of all three types of K_{Ca} channels.

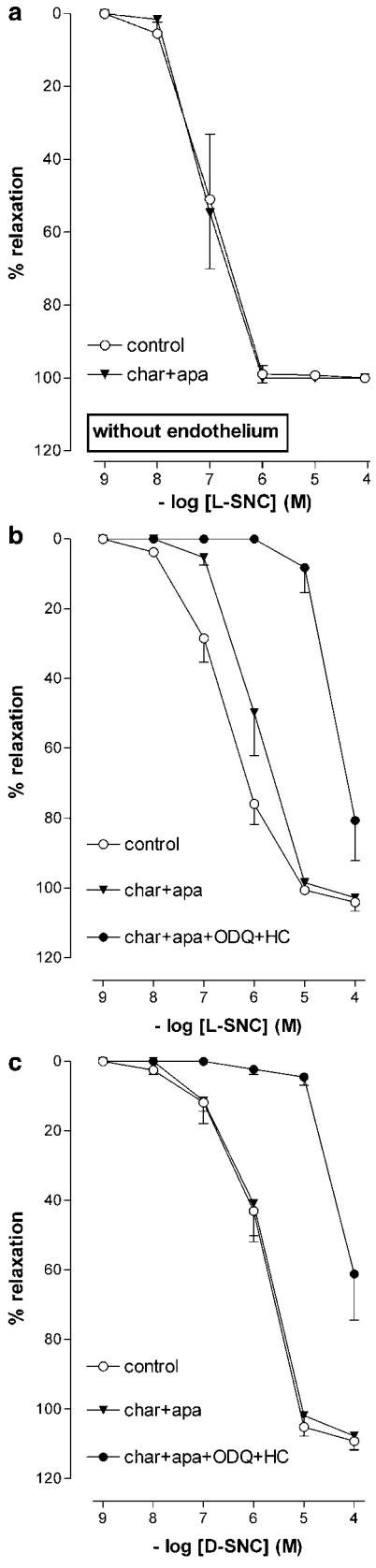
BK_{Ca} channels are located on vascular smooth muscle cells (Archer *et al*., 2003). Although endothelial EETs are believed to activate these channels (Edwards *et al*., 1998; Miura & Gutterman, 1998; Fisslthaler *et al*., 1999; Imig *et al*., 2001; Busse *et al*., 2002; Gauthier *et al*., 2002; Archer *et al*., 2003), the lack of effect of the cytochrome *P*450 epoxygenase inhibitors miconazole and sulfaphenazole excludes this possibility in our experimental setup.

IK_{Ca} and SK_{Ca} channels are expressed in endothelial cells (Bychkov *et al*., 2002; Sollini *et al*., 2002), and their activation results in endothelial hyperpolarization and the accumulation of K^+ in the myo-endothelial space. This K^+ is believed to subsequently hyperpolarize vascular smooth muscle cells by activating K_{IR} channels and/or the Na^+-K^+-ATPase (Edwards *et al*., 1998; Edwards *et al*., 2001; Busse *et al*., 2002). The inhibitory effect of $BaCl_2$ and ouabain towards bradykinin in the presence of L-NAME confirms this concept in PCMAs.

S-*nitrosothiols as EDHF?*

We propose that NO-containing/releasing factors, *S*-nitrosothiols in particular, act as an EDHF in PCMAs. The contribution of such factors is supported by our observations that the guanylyl cyclase inhibitor ODQ and the NO scavenger hydroxocobalamin inhibited the bradykinin-induced effects to a much greater degree than L-NAME, and that, in combination, L-NAME + hydroxocobalamin almost completely prevented bradykinin-induced relaxations. Since *S*-nitrosothiol-induced relaxations occur through activation of stereoselective

recognition sites and/or *via* their decomposition to NO (Davisson *et al*., 1996b; 1997), we used both L-SNC and D-SNC to verify this proposal.



L-SNC was ≈5 times more potent than D-SNC. This difference disappeared in the presence of charybdotoxin + apamin (but not in the presence of iberiotoxin with or without apamin), suggesting that L-SNC, but not D-SNC, hyperpolarizes endothelial cells *via* IK_{Ca} and SK_{Ca} channel activation. The comparable rightward shift of the L-SNC CRC in the presence of ouabain + $BaCl_2$ and charybdotoxin + apamin is in agreement with the concept that such hyperpolarization results in endothelial K^+ release and subsequent smooth muscle cell hyperpolarization. In further support of this hypothesis, endothelium denudation abolished the effect of charybdotoxin + apamin towards L-SNC, and L-SNC reduced the membrane potential of smooth muscle cells in intact porcine coronary arteries.

Unexpectedly, however, the removal of the endothelium potentiated L-SNC five to 10- fold. This suggests that L-SNC, like other endothelium-dependent vasodilators, not only hyperpolarizes endothelial cells, but also induces the release of an endothelium-derived contractile factor (Sunano *et al*., 2001). Alternatively, endothelial denudation might uncover direct L-SNC-induced effects on smooth muscle cells, as evidenced by the fact that L-SNC also hyperpolarized endothelium-denuded coronary arteries. One such direct effect is BK_{Ca} channel activation *via* *S*-nitrosylation of cysteine residues (Lang *et al*., 2003). However, the lack of effect of charybdotoxin + apamin towards L-SNC in endothelium-denuded vessels does not support this concept in PCMAs.

Taken together, the relaxant effects of L-SNC, like those of bradykinin, involve K_{Ca} channels, and they occur, at least in part, in a stereoselective manner.

The greater potency of L-SNC *vs* D-SNC is in agreement with previous *in vivo* studies (Davisson *et al*., 1996b; 1997), and may indicate the existence of binding sites that specifically recognize L-SNC and structurally related *S*-nitrosothiols. These binding sites may either be novel receptors or 'nitrosation motifs' in functional proteins such as receptors and ion channels (Stamler *et al*., 1992b; Travis *et al*., 1997; Lang *et al*., 2000; 2003).

NO release from S-*nitrosothiols?*

The effects of L-SNC, at the concentrations used in the present study, are unlikely to be entirely due to its decomposition to NO, nor do they involve *de novo* NO generation by NOS. First, NO did not activate Ca^{2+}-activated K^+ channels in PCMAs, because the dilatory effects of the NO donors SNAP and DEA-NONOate were unaffected by charybdotoxin and apamin. Second, L-NAME did not affect L-SNC-mediated responses. Third, detectable NO production has been reported to occur at *S*-nitrosothiol concentrations above 100 μM only (Ceron *et al*., 2001), that is, at concentrations that are >100 times above the EC_{50} value of L-SNC in the present study. Fourth, ODQ, but not hydroxocobalamin, fully prevented the L-SNC-induced increases in cGMP. This suggests direct,

Figure 6 Relaxations of PCMAs without (a) or with (b, c) endothelium, preconstricted with U46619, to L-SNC or D-SNC in the absence (control) or presence of one or more of the following inhibitors: 10 μM ODQ, 200 μM hydroxocobalamin (HC), 100 nM charybdotoxin (char) or 100 nM apamin (apa). Data (mean ± s.e.mean; n = 4-18) are expressed as a percentage of the contraction induced by U46619.

NO-independent, activation of guanylyl cyclase by L-SNC, in agreement with a previous study in cultured vascular smooth muscle cells (Travis *et al*., 1996). Alternatively, the concentration of hydroxocobalamin used in the present study may have been too low to scavenge all NO generated following L-SNC application (Li & Rand, 1999).

Taken together, the following mechanisms may underlie L-SNC-induced vasorelaxation: direct activation of endothelial IK_{Ca} and SK_{Ca} channels, direct activation of guanylyl cyclase in smooth muscle cells, and decomposition to NO. Simultaneous inhibition of all mechanisms (with charybdotoxin + apamin, ODQ and hydroxocobalamin, respectively) did not fully prevent the relaxations induced by the highest concentration of L-SNC (100 μM). This could relate to the inability of hydroxocobalamin to scavenge all NO (Li & Rand, 1999) and/or the competitive inhibition of guanylyl cyclase by ODQ (Garthwaite *et al*., 1995), allowing full blockade of the cGMP increases and relaxations induced by 10 μM L-SNC, but not of those induced by a 10-fold higher L-SNC concentration (Figures 6 and 8).

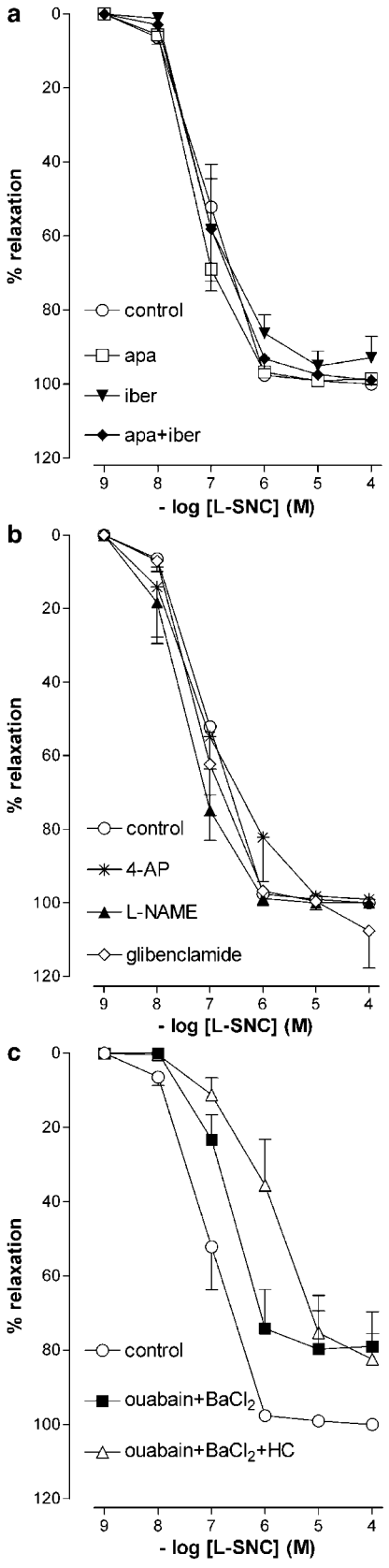


Figure 7 Relaxations of PCMAs, preconstricted with U46619, to L-SNC in the absence (control) or presence of one or more of the following inhibitors: 100 nM iberiotoxin (iber), 100 nM apamin (apa), 5 mM 4-aminopyridine (4-AP), 100 μM L-NAME, 1 μM glibenclamide, 0.5 mM ouabain or 30 μM $BaCl_2$. Data (mean ± s.e.mean; $n = 4$–9) are expressed as a percentage of the contraction induced by U46619.

Release of S-*nitrosothiols?*

Finally, despite the fact that L-SNC is capable of exerting EDHF-like effects, direct evidence demonstrating that L-SNC (or a related compound) mediates bradykinin-induced, EDHF-dependent relaxation is currently lacking. Previous studies support the existence of preformed pools of NO-containing factors (such as *S*-nitrosothiols) in endothelial and vascular smooth muscle cells (Rubanyi *et al*., 1991; Davisson *et al*., 1996a; Danser *et al*., 2000; Andrews *et al*., 2003). These pools become depleted after repeated exposure to endothelium-dependent agonists such as acetylcholine and bradykinin, following prolonged NOS inhibition, or after exposure to UV light (Davisson *et al*., 1996a; Danser *et al*., 2000; Andrews *et al*., 2003).

We did not measure *S*-nitrosothiol release following bradykinin stimulation in the present study. Such release may occur in a specific compartment (e.g., the myo-endothelial space, gap junctions, intraendothelial) that does not allow easy detection. Moreover, since it depends on preformed pools, it cannot be monitored by measuring the vascular *S*-nitrosothiol content following bradykinin stimulation. Similar difficulties exist with regard to EETs (Imig *et al*., 2001; Archer *et al*., 2003), and it has therefore been proposed that these cytochrome-*P*450 products contribute to the activation of endothelial K^+ channels as second messengers (Busse *et al*., 2002), rather than being released from endothelial cells in large amounts.

Clinical perspective

S-nitrosylated proteins, the most abundant of which is albumin, are present in micromolar concentrations in normal subjects (Stamler *et al*., 1992a). They are thought to serve both as a source and a sink of NO, thereby buffering the

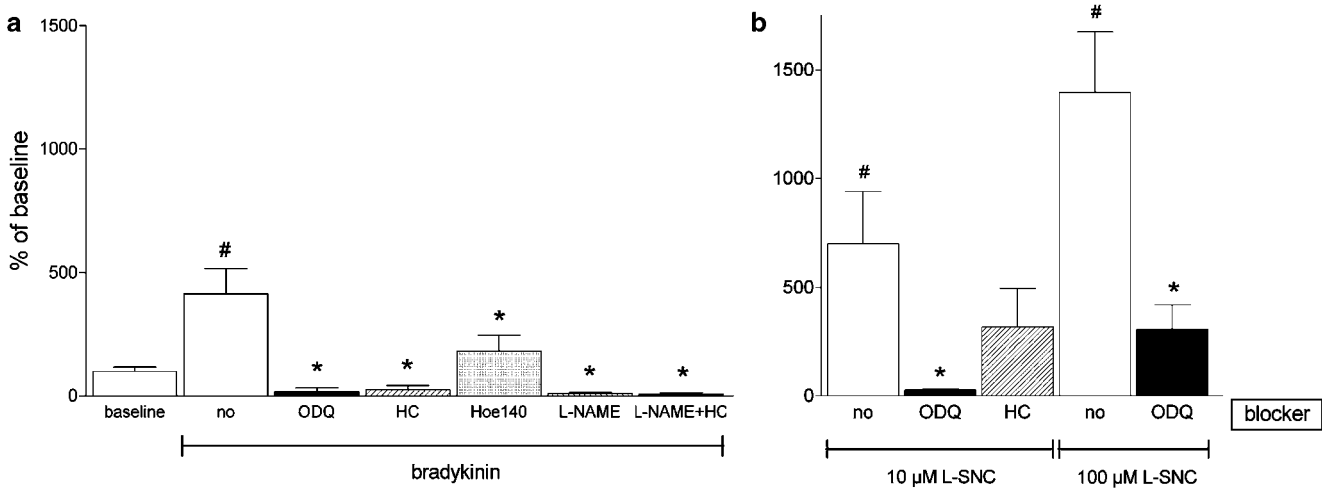
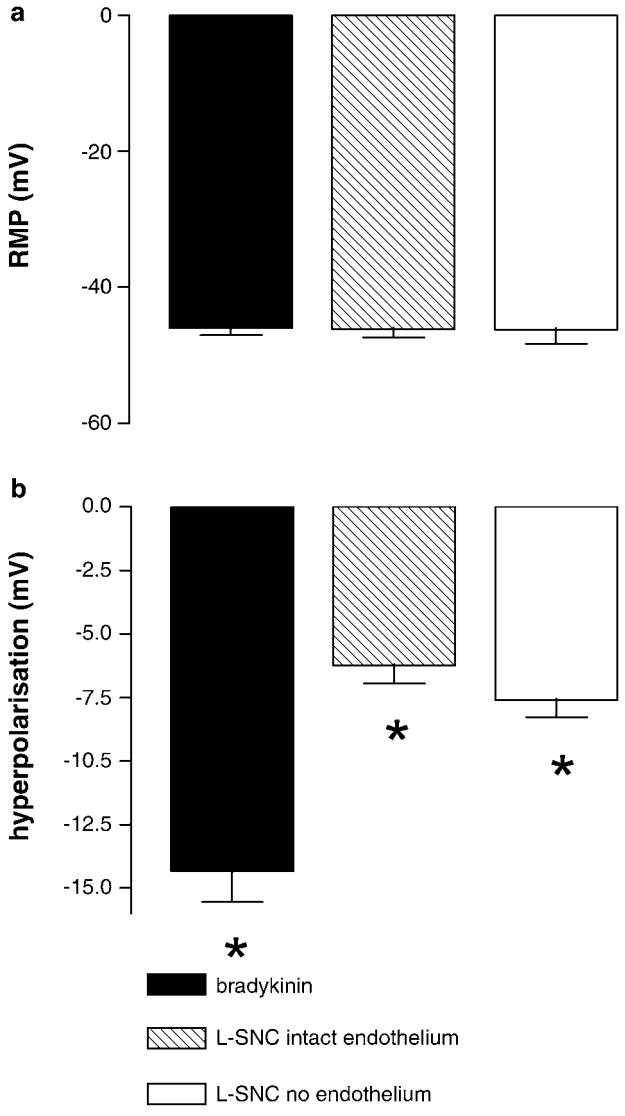


Figure 8 Cyclic GMP levels (expressed as % of baseline) in PCMAs after 1 min exposure to (a) bradykinin (1 μM) or (b) L-SNC (10 or 100 μM) under control conditions (no blocker) and in the presence of 10 μM ODQ, 200 μM hydroxocobalamin (HC), 1 μM Hoe140 and/or 100 μM L-NAME. Data are mean $\pm$ s.e.mean ($n = 3$–10). #$P<0.05$ vs control, *$P<0.05$ vs no blocker.



concentration of free NO. A recent *in vivo* study showed that *S*-nitrosothiols induce dilator responses in human conduit and resistance arteries that are comparable with those of bradykinin and acetylcholine (Rassaf *et al.*, 2002), and it has therefore been suggested (Schechter *et al.*, 2002) that *S*-nitrosothiols provide a new pharmacological route for delivering NO regionally. Our data extend these findings, by implying not only that *S*-nitrosothiols may act by inducing hyperpolarization in micro-arteries (i.e., exert NO-independent effects), but also by showing that their effects occur in a stereoselective manner.

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Figure 9 Hyperpolarization of smooth muscle cells by 100 nM bradykinin and 50 μM L-SNC in porcine coronary arteries with or without endothelium. (a) resting membrane potential (RMP). (b) change in membrane potential. Experiments were performed in the presence of 300 μM L-NA, 10 μM diclofenac and 1 μM U46619. Data are mean $\pm$ s.e.mean of five to six separate experiments; *$P<0.01$ vs control. U46619 did not significantly affect RMP (–44.5 $\pm$ 1.2 mV vs –42.7 $\pm$ 1.7 mV, $n = 6$), and in parallel experiments, using arterial rings from the same pig and following preconstriction with the same U46619 concentration (1 μM), 100 nM bradykinin relaxed the arteries by 89 $\pm$ 9% ($n = 6$).

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