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Effect of tumour necrosis factor-α and interleukin 1β on endothelium-dependent relaxation in rat mesenteric resistance arteries *in vitro*

[1]R. Wimalasundera, [1]S. Fexby, [2]L. Regan, [1]S.A. McG. Thom & *,[1]A.D. Hughes

[1]Clinical Pharmacology, National Heart & Lung Institute, U.K. and [2]Department of Obstetrics and Gynaecology, Faculty of Medicine, Imperial College of Science, Technology & Medicine, London W2 1NY, U.K.

1 Pre-eclampsia is associated with elevated proinflammatory cytokine levels and endothelial dysfunction. This study examined the effect of two cytokines, tumour necrosis factor-α (TNF) and interleukin-1β (IL-1) on endothelium-dependent relaxation in response to acetylcholine (ACH), bradykinin (BK) and histamine (HIS) in rat mesenteric small arteries *in vitro*.

2 Rat mesenteric arteries were mounted in an isometric myograph. Tone was induced with phenylephrine (PE) or a depolarizing solution containing 80 mM KCl (K_{80}). Relaxation was measured in response to ACH, BK, HIS and sodium nitroprusside (SNP), an endothelium-independent relaxant. Inhibition of NO synthase by a combination of N^{ω}-monomethyl-L-arginine (L-NMMA) and N^{ω}-nitro-L-arginine methyl ester (L-NAME) significantly inhibited relaxation in response to ACH and BK. Addition of an inhibitor of cyclooxygenase, indomethacin, had no additional effect when added to L-NMMA and L-NAME. Inhibition of endothelium-derived hyperpolarizing factor (EDHF) by K_{80} partially reduced responses to ACH and BK. Inhibition of HIS-induced relaxation was more marked with K_{80}. L-NMMA and L-NAME largely abolished the remaining relaxation to ACH, BK and HIS in arteries contracted with K_{80}.

3 Preincubation with TNF for 30 min caused an inhibition of relaxation in response to ACH and BK in arteries contracted with PE. Responses to HIS and SNP were not affected by TNF under these conditions. TNF also inhibited ACH-induced relaxation in arteries contracted with K_{80}. IL-1 had no effect on responses to ACH and the combination of TNF and IL-1 was not more effective than TNF alone.

4 The inhibitory effect of TNF on ACH-induced relaxation was abolished by coincubation with superoxide dismutase (SOD) and was not seen if NO synthase was inhibited by L-NMMA and L-NAME.

5 TNF inhibits the NO-dependent component of endothelium-dependent relaxation in response to ACH and BK, but does not inhibit the EDHF-dependent component. This effect may be attributable to the ability of TNF to increase levels of superoxide anions (O_2^-) and the ability of O_2^- to inactivate NO. This mechanism could contribute to the endothelial dysfunction seen in situations where TNF is elevated, such as pre-eclampsia.

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Introduction

Pre-eclampsia is a common medical disorder of pregnancy affecting 5–7% of pregnancies and is a major cause of maternal and perinatal mortality and morbidity. Pre-eclampsia is characterized by a rise in blood pressure in the second half of pregnancy, associated with proteinuria, thrombocytopenia, abnormal liver function tests and increased peripheral vascular resistance (Higgins & de Swiet, 2001). The cause of the increased vascular resistance in pre-eclampsia remains uncertain, but it has been proposed that endothelial dysfunction could be a contributory factor. Loss of endothelium-dependent relaxation in response to acetylcholine (ACH) (McCarthy *et al*., 1993; Oguogho *et al*., 1996; Pascoal *et al*., 1998), bradykinin (BK) (Knock & Poston, 1996; Pascoal *et al*., 1998; Ashworth *et al*., 1999), histamine (HIS) (Oguogho *et al*., 1996; Suzuki *et al*., 2000) and flow (Cockell & Poston, 1997) has been reported in a range of human arteries from pre-eclamptic women studied *in vitro*, but the cause of this endothelial dysfunction is unknown.

Epidemiological evidence points to an immune aetiology of pre-eclampsia. There is increasing evidence that normal pregnancy is a proinflammatory state and that pre-eclampsia may be an exaggeration of this response (Redman *et al*., 1999). In pre-eclampsia, there is increased release of some pro-inflammatory cytokines such as tumour necrosis factor-α (TNF) (Kupferminc *et al*., 1994; Visser *et al*., 1994; Vince *et al*., 1995; Hamai *et al*., 1997; Conrad *et al*., 1998), interleukin-6 (Conrad *et al*., 1998; Benyo *et al*., 2001),

*Author for correspondence; Clinical Pharmacology, Imperial College of Science, Technology & Medicine, St Mary's Hospital QEQM Wing, South Wharf Road, London, W2 1NY, U.K.; E-mail: a.hughes@ic.ac.uk

interleukin-2 (Hamai *et al*., 1997) and interleukin-12 (Daniel *et al*., 1998). Plasma levels of other cytokines, such as interleukin-1β (IL-1) have not been found to be elevated (Kupferminc *et al*., 1994; Conrad *et al*., 1998), although placental expression of IL-1 has been reported to be increased (Rinehart *et al*., 1999), possibly as a result of placental hypoxia (Benyo *et al*., 2001).

Cytokines such as TNF and IL-1 are known to have a variety of procoagulant and proinflammatory effects on the vascular endothelium (reviewed in Mantovani *et al*., 1998). Moreover, elevated levels of cytokines may also contribute to the increased oxidative stress that has been reported to exist in pre-eclampsia (reviewed in Poston & Chappell, 2001). The aim of this study was to determine the effect of TNF and IL-1 on endothelium-dependent relaxation in response to vasodilators and to explore the possible role of superoxide (O_2^-) in mediating any effects of these cytokines.

Methods

Male Wistar rats ($\sim$250 g) were killed by cervical dislocation following CO_2 narcosis and the mesenteries removed. Small arteries (third order, internal diameter = 346 $\pm$ 65 μm (mean $\pm$ s.d.), n = 168) were mounted in parallel as ring segments in separate chambers of an isometric dual-channel myograph (Mulvany & Halpern, 1977), allowing one segment to act as time control for the other. Only one pair of arteries from an individual animal was used for each individual relaxant. All studies (unless otherwise specified) were conducted in physiological saline solution (PSS) comprising (mM): NaCl, 118; KCl, 4.7; $CaCl_2 \cdot 6H_2O$, 2.5; $MgSO_4 \cdot 7H_2O$, 1.17; $NaHCO_3$, 25.0; $NaH_2PO_4 \cdot 2H_2O$, 1.0; Na_2EDTA, 0.03; and glucose 5.5, maintained at 37°C and bubbled with 95% O_2 and 5% CO_2.

After mounting, the vessels were allowed to equilibrate for 1 h and then set to a 'normalized' internal circumference estimated to be 90% of the circumference they would maintain if relaxed and exposed to 100 mmHg transmural pressure ($0.9L_{100}$). This was calculated for each individual vessel on the basis of the passive length–tension characteristics of the artery and the Laplace relation (Mulvany & Halpern, 1977). This procedure optimized active force generation by these vessels.

Before beginning the studies, vessel viability was assessed by exposing arteries for $\sim$2 min to high potassium solution (PSS with equimolar substitution of 118 mM KCl for NaCl). This was repeated until stable responses were achieved (usually three exposures). The artery was then contracted with high potassium solution containing noradrenaline (10 μM) to assess maximum contraction. After washout, the functional integrity of the endothelium was established by adding ACH (10 μM) to a vessel contracted by noradrenaline (10 μM) in PSS. Vessels that failed to reproducibly produce tension equivalent to more than 100 mmHg effective pressure (calculated by Laplace's law) in response to high potassium solution containing noradrenaline, or relax completely to ACH were discarded.

Role of NO, cyclooxygenase and EDHF in responses to relaxants

Initial studies were designed to investigate the contribution of NO, cyclooxygenase products and endothelium-derived hyperpolarizing factor (EDHF) to dilatation induced by ACH (1 nM–10 μM), HIS (10 nM–10 μM) and BK (1 nM–10 μM) in rat mesenteric small arteries. Sodium nitroprusside (SNP; 1 nM–10 μM) was used as an endothelium-independent relaxant in these studies. Preliminary experiments (not shown) confirmed that relaxation in response to ACH, HIS and BK were completely abolished by physical removal of the endothelium as described previously (Prieto *et al*., 1997). Responses to substance P were also examined in this preliminary study, but maximal relaxation rarely exceeded 10%; so further studies were not conducted using this peptide.

Various agents were used to inhibit the putative mediators responsible for endothelium-dependent relaxation. NO synthase (NOS) was inhibited by using N^{ω}-monomethyl-L-arginine (L-NMMA; 100 μM) and N^{ω}-nitro-L-arginine methyl ester (L-NAME; 10 μM). Since arginine analogues may not fully inhibit NO synthesis in some preparations (Vanheel & Van de Voorde, 2000), the effectiveness of these concentrations of inhibitor was examined by comparing the inhibition seen to ACH in the presence of L-NMMA and L-NAME in the presence and absence of oxyhaemoglobin (10 μM), an NO scavenger (Simonsen *et al*., 1999). Preincubation with oxyhaemoglobin, L-NMMA and L-NAME did not significantly inhibit further relaxation in response to ACH compared with that when the NO synthase inhibitors were present alone ($-\log EC_{50}$ and maximum relaxation to ACH in the presence of L-NMMA & L-NAME = -7.06 ± 0.11 and 96.9 ± 2.1% compared with -7.21 ± 0.18 and 93.1 ± 4.5% (mean $\pm$ s.e.m., n = 4), respectively, in oxyhaemoglobin, and L-NMMA & L-NAME). It was concluded therefore that the concentrations of L-NMMA and L-NAME used were adequate to inhibit NO synthesis in this preparation. Indomethacin (INDO; 5 μM) was used as an inhibitor of cyclooxygenase. The relaxant effects of EDHF were inhibited by contracting vessels with a depolarizing potassium solution (K_{80}) comprising PSS with equimolar substitution of 80 mmol KCl for NaCl.

Concentration–response curves to relaxants were constructed following establishment of stable contraction with phenylephrine (PE; 10 μM), or K_{80}. These contractile stimulants induced maximal or near-maximal tone. The sequence of addition of relaxants was conducted using a Latin square design to overcome possible time- or order-dependent effects. For all studies, one of the pair of arteries (test vessel) was exposed to inhibitors, while the other acted as control. NO synthase inhibitors were added 30 min prior to addition of PE or K_{80} and remained in the bath throughout the experiment.

Effects of TNF and IL-1 on endothelium-dependent and endothelium-independent relaxation

In order to assess the effects of TNF and IL-1 on endothelium-dependent relaxation, the test vessel was incubated in TNF (1 nM), IL-1 (100 pM) or vehicle (distilled H_2O) for 30 min prior to contraction with PE (10 μM), or K_{80} in some experiments. When arteries were exposed to TNF and NO synthase inhibitors, all inhibitors were present in the bath for 30 min prior to induction of tone. The concentrations of TNF and IL-1 used had no effect on resting tone and did not affect contraction in response to PE or K_{80} (data not shown). Cumulative concentration–response curves to relaxants were then constructed as described above with the arteries remaining exposed to the cytokine/vehicle. Separate sets of vessels

were used for each relaxant to avoid confounding effects resulting from prolonged exposure to TNF or IL-1.

The possibility that the effect of TNF involved release of O_2^- was investigated by assessing whether superoxide dismutase (SOD; 150 IU ml^{-1}) could influence the effects of TNF on ACH-induced relaxation. The test vessel was incubated in SOD for 10 min prior to incubation with TNF or vehicle for 30 min. The effect of TNF on relaxation in response to ACH in the continued presence or absence of SOD was then compared.

Data analysis and statistics

All data are presented as means ± s.e.m.'s of (n) observations. Relaxation was calculated as per cent reduction in PE- or K_{80}-induced contraction (there was negligible basal tone in these preparations). Cumulative concentration–response data were fitted to a logistic function by nonlinear regression using GraphPad Prism 3.02 (GraphPad Software Inc., Institute for Scientific Information, San Diego, U.S.A.) to estimate $\log EC_{50}$ values. $\log EC_{50}$ values were not calculated if maximum responses (E_{max}) were less than 20%. Data were compared statistically using a paired or unpaired two-tailed Student's t-test as appropriate, and $P<0.05$ was considered significant.

Drugs and reagents

All drugs and reagents were obtained from Sigma (Poole, Dorset, U.K.). Aliquots of human TNF (1 μM) and human IL-1 (100 nM) were prepared in sterile buffered saline containing 0.1% bovine serum albumin, stored at −20°C and defrosted immediately prior to use. Other drugs were made up in distilled water.

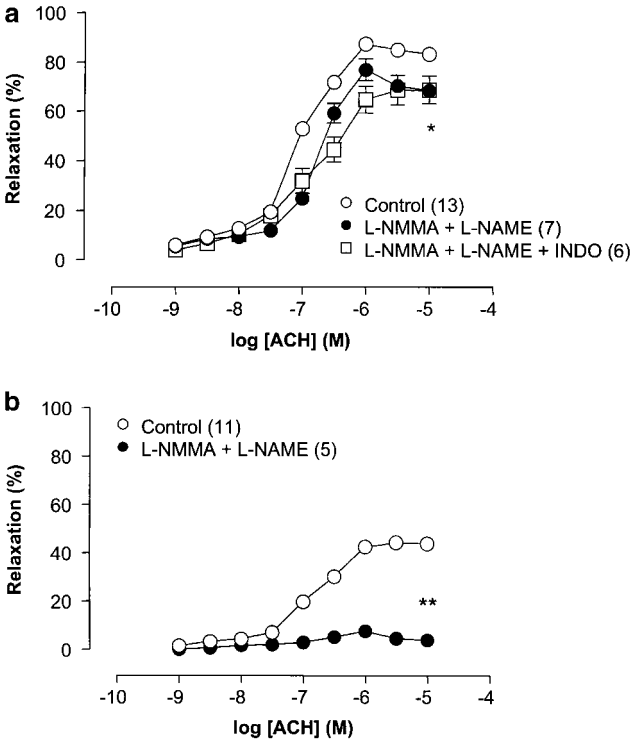

Figure 1 Effect of NO synthase inhibition by L-NMMA and L-NAME in the presence or absence of INDO on relaxation in response to ACH in rat mesenteric small arteries contracted with (a) PE, (b) depolarizing potassium solution containing 80 mM KCl. Data are means ± s.e.m. of (n) observations. $^*P<0.05$ and $^{**}P<0.01$ comparing maximum response between control and L-NMMA + L-NAME.

Results

ACH-induced relaxation

ACH (1 nM–10 μM) induced a concentration-dependent relaxation of rat mesenteric arteries contracted by PE (Figure 1a). L-NAME (10 μM) and L-NMMA (100 μM) inhibited E_{max} to ACH ($P=0.03$) and caused a small rightward shift in the concentration–response relation (Figure 1a, Table 1), although this was not statistically significant ($P=0.07$). Addition of INDO to L-NMMA and L-NAME had no additional effect on the effect of L-NMMA and L-NAME on relaxation in response to ACH (Figure 1a, Table 1).

ACH also induced a concentration-dependent relaxation in arteries contracted by K_{80} (Figure 1b). The potency of ACH was significantly reduced in arteries contracted by K_{80} compared with PE-contracted arteries ($P=0.01$) and E_{max} was also reduced ($P<0.001$; Figure 1b, Table 1). L-NAME and L-NMMA almost completely abolished relaxation in response to ACH following K_{80} contraction ($P=0.004$; Figure 1b, Table 1).

BK-induced relaxation

BK (1 nM–10 μM) induced a concentration-dependent relaxation of rat mesenteric arteries contracted by PE. L-NAME and L-NMMA significantly inhibited E_{max} in response to BK ($P=0.04$) (Figure 2a, Table 1). Addition of INDO to L-NMMA and L-NAME had no additional effect on the impairment of relaxation to BK compared to vessels incubated with L-NMMA and L-NAME alone (Figure 2a, Table 1).

The potency of BK was significantly reduced in arteries contracted by K_{80} compared with PE-contracted arteries ($P<0.001$; Figure 2b, Table 1), and although E_{max} was reduced, this was not statistically significant. L-NAME and L-NMMA almost completely abolished relaxation in response to BK when vessels were contracted with K_{80} (Figure 2b, Table 1).

HIS-induced relaxation

HIS (1 nM–10 μM) induced a concentration-dependent relaxation of rat mesenteric arteries contracted by PE (Figure 3a, Table 1). L-NAME and L-NMMA caused a small and statistically insignificant inhibition of relaxation in response to HIS in vessels contracted by PE (Figure 3a, Table 1). Addition of INDO had no additional effect compared with L-NAME and L-NMMA (Figure 3a). Responses to HIS were markedly reduced following contraction with K_{80} with both E_{max} ($P<0.001$) and $\log EC_{50}$ ($P=0.008$) being affected (Figure 3b, Table 1). L-NAME and L-NMMA almost completely abolished the remaining small response to HIS (Figure 3b, Table 1).

Table 1 Effect of NO synthase inhibition by N^{ω}-monomethyl-L-arginine (L-NMMA) and N^{ω}-nitro-L-arginine methyl ester (L-NAME) in the presence or absence of indomethacin (INDO) on log EC_{50} and maximum response (E_{max}) to acetylcholine (ACH), bradykinin (BK), histamine (HIS) and sodium nitroprusside (SNP) in rat mesenteric small arteries contracted with phenylephrine (PE), or depolarizing potassium solution containing 80 mM KCl (K_{80})

		Control	*l-NAME+ l-NMMA*	*l-NAME+l-NMMA+INDO*
ACH (PE)	log EC_{50}	−7.3±0.1 (13)	−6.9±0.1 (7)	−6.8±0.2 (6)
	E_{max} (%)	90±3 (13)	77±6 (7)*	69±13 (6)
ACH (K_{80})	log EC_{50}	−6.7±0.2 (11)†	NC	ND
	E_{max} (%)	48±8 (11)††	8±2.2 (5)**	ND
BK (PE)	log EC_{50}	−9.0±0.5 (9)	NC	NC
	E_{max} (%)	56±9 (9)	15±6 (5)*	17±11 (3)
BK (K_{80})	log EC_{50}	−6.0±0.2 (11)††	NC	ND
	E_{max} (%)	39±5 (11)	5±1 (5)**	ND
HIS (PE)	log EC_{50}	−6.5±0.1 (13)	−6.4±0.1 (6)	−6.6±0.5 (6)
	E_{max} (%)	71±8 (13)	58±9 (6)	46±17 (6)
HIS (K_{80})	log EC_{50}	−5.7±0.3 (11)††	NC	ND
	E_{max} (%)	26±5 (11)††	9±2 (5)*	ND
SNP (PE)	log EC_{50}	−7.7±0.4 (13)	−8.0±0.7 (7)	−8.0±0.1 (6)
	E_{max} (%)	92±3 (13)	88±5 (7)	94±4* (6)
SNP (K_{80})	log EC_{50}	−6.8±0.1 (11)†	−6.7±0.1 (5)	ND
	E_{max} (%)	77±3 (11)	82±2 (5)	ND

Data are means±s.e.m. of (n) observations. *$P<0.05$ and **$P<0.01$ comparing responses with control. †$P<0.05$ and ††$P<0.01$ comparing PE- and K_{80}-contracted arteries. NC = not calculated (maximum relaxation < 20%), ND = not done.

SNP-induced relaxation

SNP (1 nM–10 μM) induced a concentration-dependent relaxation of rat mesenteric arteries contracted by PE (Figure 4a, Table 1). L-NAME and L-NMMA had no significant effect on responses to SNP, although addition of INDO to the NO synthase inhibitors caused a small enhancement of E_{max} to SNP ($P=0.03$) (Figure 4a, Table 1). SNP was less potent following K_{80} contraction than PE contraction ($P=0.04$; Figure 4a and b, Table 1). L-NMMA and L-NAME had no significant effect on SNP-induced relaxation in arteries contracted with K_{80} (Figure 4b, Table 1).

Effect of TNF and IL-1 on relaxation

In arteries contracted with PE, TNF induced impairment of relaxation to ACH, causing a significant shift in the ACH concentration–response curve to the right ($P=0.03$), but E_{max} was not significantly inhibited (Figure 5a, Table 2). TNF also inhibited BK-induced relaxation (Figure 5b, Table 2) causing a significant rightward shift in the concentration–response curve ($P=0.02$). In contrast, TNF did not significantly inhibit responses to HIS in arteries contracted by PE (Figure 6a, Table 2) and relaxation in response to SNP was also unaffected by TNF under these conditions (Figure 6b, Table 2). In arteries contracted with K_{80}, preincubation with TNF markedly inhibited relaxation to ACH (Figure 7a, Table 2), but had no effect on responses to SNP (Figure 7b, Table 2).

Preincubation with IL-1 (100 pM) for 30 min had no significant effect on relaxation in response to ACH in PE-contracted arteries (Figure 8a, Table 3). IL-1 co-incubated with TNF shifted the concentration – response curve to ACH to the right (Figure 8b, Table 3). This effect was similar to that seen with TNF alone. IL-1 had no significant effect on relaxation in response to SNP in arteries contracted with PE (Table 3). Similarly, the combination of IL-1 and TNF did not affect responses to SNP (Table 3).

Effect of SOD and NO synthase inhibitors on the effect of TNF

Addition of SOD did not significantly alter potency or efficacy of the concentration–response relation to ACH in arteries contracted with PE or K_{80}. When arteries were exposed to TNF in the presence of SOD, TNF failed to inhibit responses to ACH in arteries contracted with PE or K_{80} (Figure 9a and b, Table 4). Furthermore, when arteries were preincubated with L-NAME and L-NMMA to inhibit NO synthase, prior to contraction with PE, responses to ACH were unaffected by TNF (Figure 9c, Table 4).

Discussion

The main findings of this study were that exposure of rat mesenteric arteries to TNF for a brief period (30 min) significantly impaired endothelium-dependent relaxation to ACH and BK without inhibiting responses to HIS or SNP. The inhibitory effect of TNF on ACH-induced relaxation was more marked when arteries were contracted by a high potassium solution to block the contribution from EDHF. The effect of TNF could be inhibited by preincubation with SOD and was not seen when NO synthase was inhibited. Exposure to another proinflammatory cytokine, which is not elevated in pre-eclampsia, IL-1, had no effect on endothelium-dependent relaxation to ACH and combination of IL-1 with

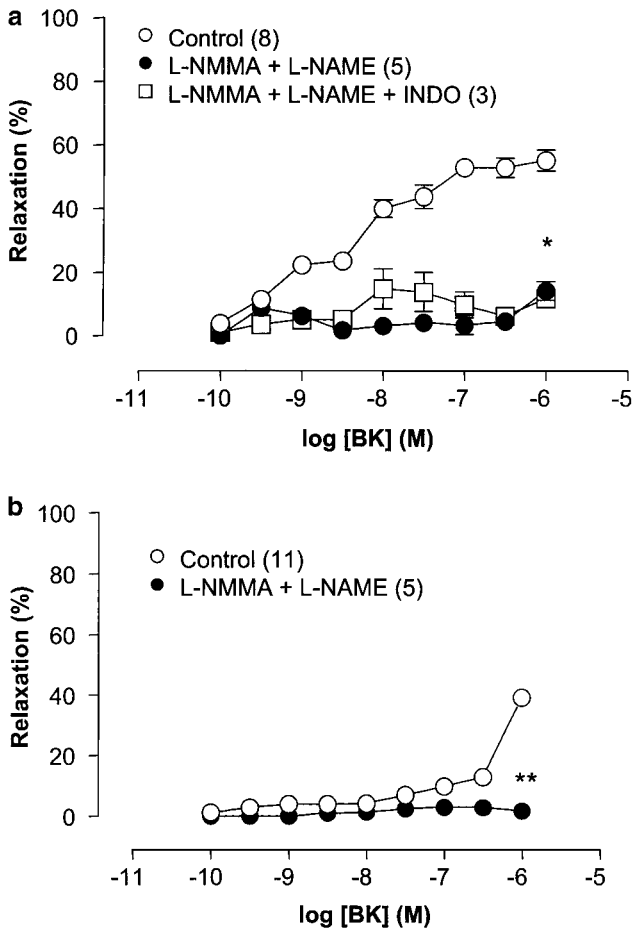

Figure 2 Effect of NO synthase inhibition by L-NMMA and L-NAME in the presence or absence of INDO on relaxation in response to BK in rat mesenteric small arteries contracted with (a) PE, (b) depolarizing potassium solution containing 80 mM KCl. Data are means ± s.e.m. of (*n*) observations. $^{*}P<0.05$ and $^{**}P<0.01$ comparing maximum response between control and L-NMMA + L-NAME.

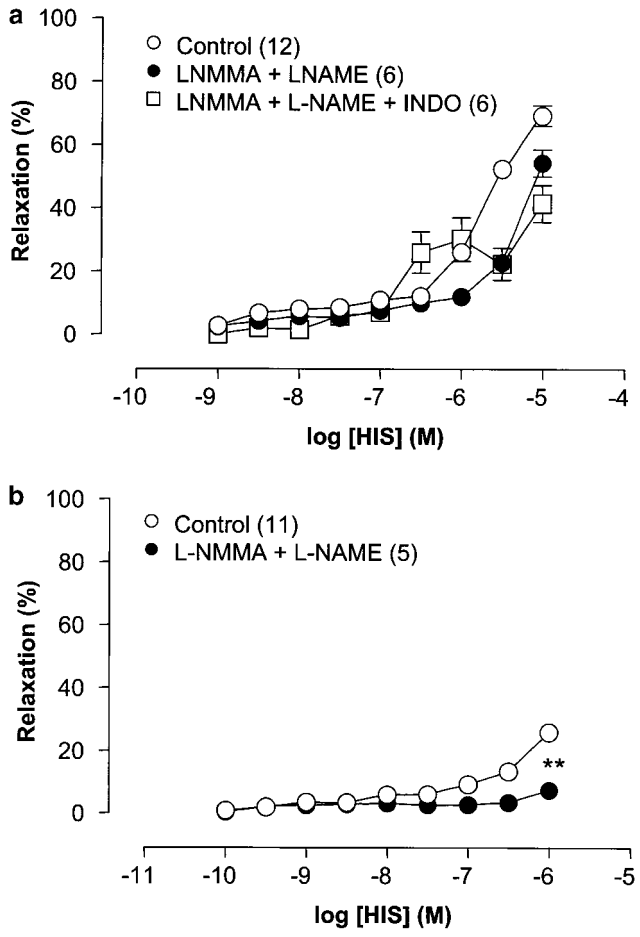

Figure 3 Effect of NO synthase inhibition by L-NMMA and L-NAME in the presence or absence of INDO on relaxation in response to HIS in rat mesenteric small arteries contracted with (a) PE, (b) depolarizing potassium solution containing 80 mM KCl. Data are means ± s.e.m. of (*n*) observations. $^{**}P<0.01$ comparing maximum response between control and L-NMMA + L-NAME.

TNF had effects that were similar to that induced by TNF alone.

Previous studies have reported a range of effects of TNF and IL-1 on vascular and endothelial responses. Greenberg *et al.* (1993) reported generally similar findings to ours in bovine intralobar pulmonary vessels, where 30 min exposure to TNF inhibited NO-dependent relaxation to ACH, BK and HIS without affecting responses to nitroprusside. The responses to all these agents in this preparation appear to have been attributable to release of NO, since they were all inhibited by inhibitors of NO synthase. Aoki *et al.* (1989) reported that 2 h perfusion of cat carotid artery with TNF inhibited responses to ACH, but not $NaNO_2$; however, these effects were not seen after perfusion for only 1 h. In contrast, longer exposure (7–15 h) of rabbit carotid or porcine coronary arteries to IL-1 or TNF (the latter in combination with interferon-γ and lipopolysaccharide) reduced endothelium-dependent relaxations to ACH and substance P, but not to the calcium ionophore, A23187 (Kessler *et al.*, 1997). Kessler *et al.* (1997) also reported that brief (15 min) exposure to IL-1 had no effect on responses to ACH, consistent with our findings. Prolonged exposure to cytokines is also associated with reduced contraction, as has been reported by many investigators (Beasley *et al.*, 1989; Robert *et al.*, 1992, 1993; Thorin-Trescases *et al.*, 1995; Kessler *et al.*, 1997) and this may involve activation of inducible NOS (iNOS) (Busse & Mulsch, 1990), or NO-independent effects on guanylate cyclase or other systems in smooth muscle (Beasley & McGuiggin, 1994; Thorin-Trescases *et al.*, 1995; Kessler *et al.*, 1997). *In vivo*, a 30-min infusion of TNF in rats was reported to depress ACH-induced relaxation in isolated pulmonary arteries and aorta (Wang *et al.*, 1994). Similarly, a 5-day infusion of TNF into pregnant rats has also been reported to cause impaired endothelium-dependent relaxation to ACH in aortic strips isolated from pregnant rats (Davis *et al.*, 2002). It is noteworthy that these studies have been performed using larger conduit-type arteries where EDHF may play a smaller role in mediating responses to ACH and other endothelium-dependent agents.

Endothelium-dependent relaxation to a particular agent may be mediated by a number of factors, including NO (Moncada *et al.*, 1991), cyclooxygenase products such as

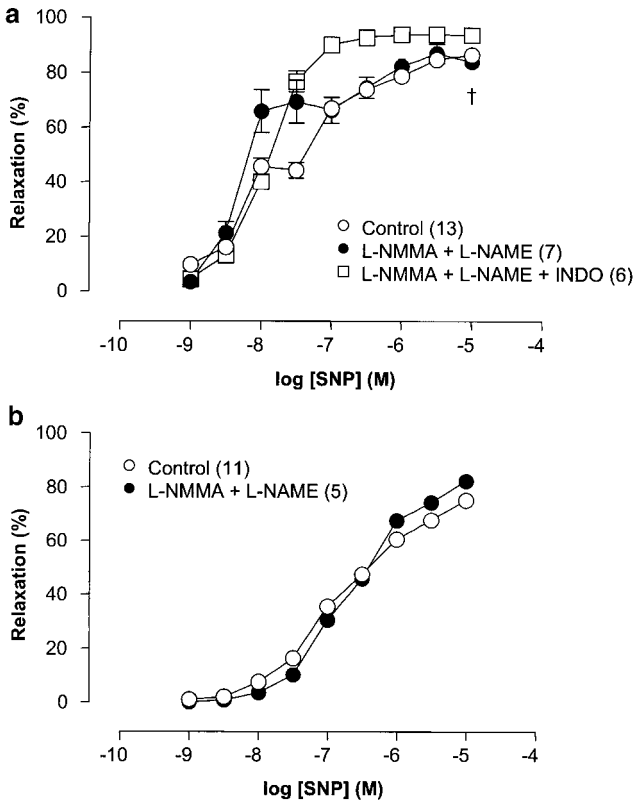

Figure 4 Effect of NO synthase inhibition by L-NMMA and L-NAME in the presence or absence of INDO on relaxation in response to SNP in rat mesenteric small arteries contracted with (a) PE, (b) depolarizing potassium solution containing 80 mM KCl. Data are means ± s.e.m. of (n) observations. $^{\dagger}P<0.05$ comparing maximum response between control and L-NMMA + L-NAME + INDO.

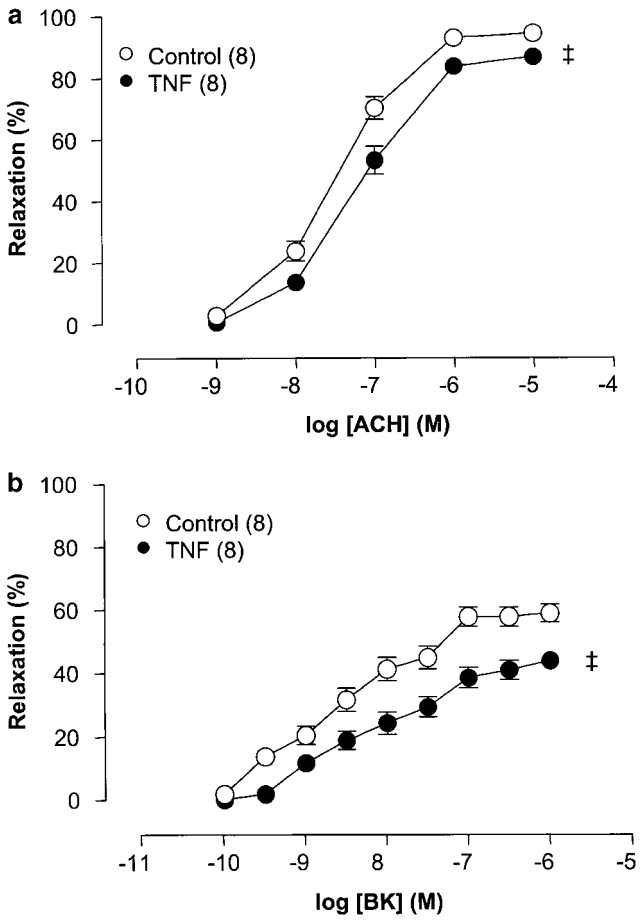

Figure 5 Effect of TNF on relaxation in response to (a) ACH and (b) BK in rat mesenteric small arteries contracted with PE. Data are means ± s.e.m. of (n) observations. $^{\ddagger}P<0.05$ comparing $\log EC_{50}$ response between control and TNF-treated arteries.

prostacyclin (PGI_2) (Vane *et al*., 1987) and EDHF (Triggle & Ding, 2002). The identity of EDHF remains uncertain, but it is accepted that its action involves an increase in K conductance and can therefore be inhibited by abolishing the electrochemical gradient for K^+ions (Chen & Suzuki, 1989). This was conducted in these studies by using a solution containing 80 mM potassium to induce tone. This technique has been reported previously not to affect responses to NO (Fukao *et al*., 1995; Chen & Cheung, 1997); however, we observed that SNP was less potent in K_{80}- than PE-contracted arteries and it is possible that responses to NO may also be affected by depolarization with high potassium solutions in our preparation.

Our data indicate that, in rat mesenteric arteries, the endothelium-dependent actions of ACH, BK and HIS involve both NO and EDHF, apparently acting in a synergistic manner, but that their individual contribution to relaxation by a particular agent may differ. Responses to ACH and BK were less affected by inhibition of EHDF than responses to HIS, whereas the effect of NO synthase inhibition was more marked for ACH and BK than HIS. These observations regarding the importance of EDHF in mediating responses to HIS are in keeping with a previous study of pressurized rat mesenteric arteries (Lagaud *et al*., 1999). Cyclooxygenase products do not appear to play a major role in responses to ACH, BK or HIS as previously reported for ACH-induced relaxation in this preparation (Wu *et al*., 1994; Lacy *et al*., 2000), although

Table 2 Effect of tumour necrosis factor-α (TNF) on $\log EC_{50}$ and E_{max} to ACH, BK, HIS and SNP in rat mesenteric small arteries contracted with PE or depolarizing potassium solution containing 80 mM KCl (K_{80})

		Control	*TNF*
ACH (PE)	$\log EC_{50}$	−7.5 ± 0.2 (8)	−7.2 ± 0.2 (8)*
	E_{max} (%)	95 ± 2 (8)	86 ± 4 (8)
ACH (K_{80})	$\log EC_{50}$	−6.9 ± 0.2 (8)	NC
	E_{max} (%)	57 ± 8 (8)	15 ± 4 (8)**
BK (PE)	$\log EC_{50}$	−8.4 ± 0.4 (8)	−7.8 ± 0.4 (8)*
	E_{max} (%)	59 ± 8 (8)	44 ± 8 (8)
HIS (PE)	$\log EC_{50}$	−5.8 ± 0.1 (9)	−6.0 ± 0.1 (9)
	E_{max} (%)	84 ± 4 (9)	87 ± 2 (9)
SNP (PE)	$\log EC_{50}$	−7.0 ± 0.3 (8)	−7.0 ± 0.3 (8)
	E_{max} (%)	72 ± 5 (8)	74 ± 6 (8)
SNP (K_{80})	$\log EC_{50}$	−7.1 ± 0.1 (6)	−7.2 ± 0.1 (6)
	E_{max} (%)	83 ± 4 (6)	84 ± 4 (6)

Data are means ± s.e.m. of (n) observations. $^{*}P<0.05$ and $^{**}P<0.01$ comparing responses with control. NC = not calculated (maximum relaxation < 20%).

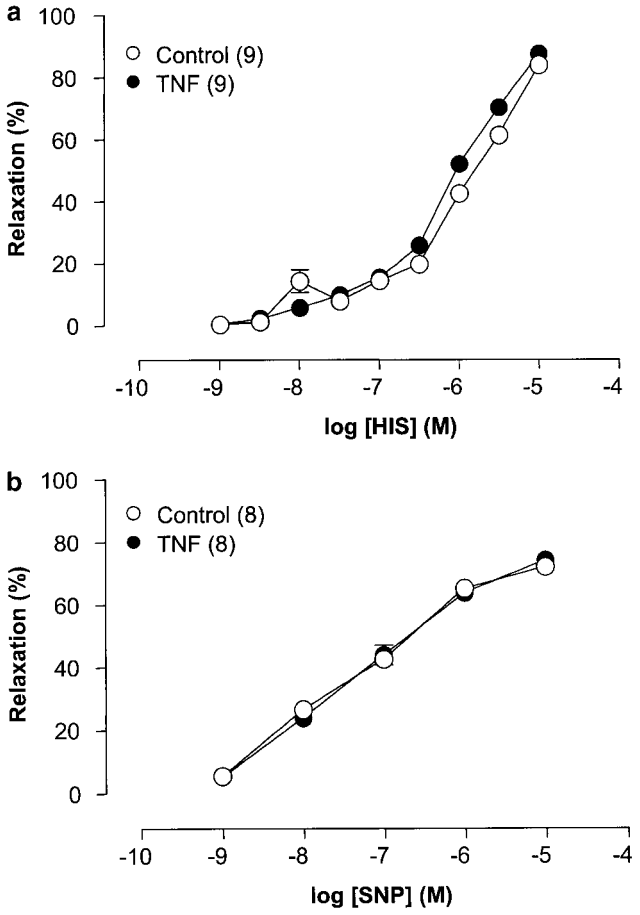

Figure 6 Effect of TNF on relaxation in response to (a) HIS and (b) SNP in rat mesenteric small arteries contracted with PE. Data are means ± s.e.m. of (*n*) observations.

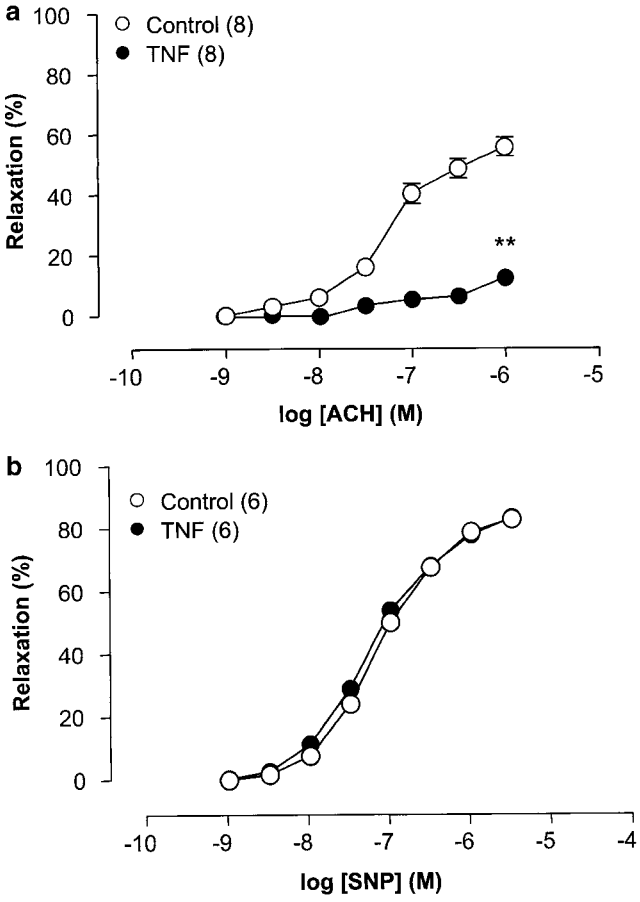

Figure 7 Effect of TNF on relaxation in response to (a) ACH and (b) SNP in rat mesenteric small arteries contracted with depolarizing potassium solution containing 80 mM KCl. Data are means ± s.e.m. of (*n*) observations. $^{**}P<0.01$ comparing maximum response between control and TNF-treated arteries.

bradykinin-dependent contraction in pressurized rat mesenteric arteries has been reported to be inhibited by INDO (Fasciolo *et al.*, 1990).

Unlike ACH and BK, responses to HIS were not inhibited by TNF. This could be explained by TNF selectively inhibiting NO, as opposed to EDHF. This proposal is supported by the inability of TNF to inhibit ACH-induced relaxation when NO synthase was inhibited, or when superoxide oxide formation was reduced by SOD. Contractile responses to PE or K_{80}, or relaxation to SNP were unaffected by TNF and this suggests that changes in smooth muscle contractility, or responsiveness to cyclic guanosine monophosphate (cGMP) do not play a part in the effect of TNF after a brief duration of exposure as used in this study.

TNF may affect endothelial function through a number of mechanisms (reviewed in Madge & Pober, 2001). In particular, it has been reported to downregulate endothelial nitric oxide synthase (eNOS) mRNA expression (Yoshizumi *et al.*, 1993) and protein (de Frutos *et al.*, 1999). However, given the relatively short exposure times used in this study, a mechanism involving altered levels of protein expression does not appear to be particularly likely. Our observations with SOD suggest that generation of O_2^- may underlie the inhibitory effects of TNF on NO-dependent relaxation seen in our study. TNF has previously been reported to induce production of O_2^- in cultured endothelial cells (Murphy *et al.*, 1992), probably via activation of a phagocyte-type NADPH oxidase complex (Li *et al.*, 2002).

Although extrapolation of animal data to clinical scenarios must be made with caution, it is interesting that the magnitude of the impairment of response to ACH and BK was similar to that previously reported in subcutaneous arteries from pre-eclamptic women compared to normal pregnant women (McCarthy *et al.*, 1993; Knock & Poston, 1996). Inhibitory effects of TNF on endothelium-dependent relaxation have been observed *in vivo* in humans. Infusion of TNF into the brachial artery has been reported to impair forearm vasodilation to ACH (Nakamura *et al.*, 2000), and anti-TNF therapy

Table 3 Effect of interleukin 1β (IL-1) alone or in the presence of TNF on log EC_{50} and E_{max} to ACH and SNP in rat mesenteric small arteries contracted with PE

		Control	*IL-1*	*IL-1+TNF*
ACH	log EC_{50}	−7.5 ± 0.1 (12)	−7.5 ± 0.2 (5)	−7.1 ± 0.2 (8)**
	E_{max} (%)	90 ± 5 (12)	74 ± 8 (5)	84 ± 7.7 (8)
SNP	log EC_{50}	−7.4 ± 0.2 (12)	−7.5 ± 0.2 (4)	−7.4 ± 0.3 (8)
	E_{max} (%)	82 ± 3 (12)	73 ± 10 (4)	79 ± 5 (8)

Data are means ± s.e.m. of (*n*) observations. $^{**}P<0.01$ comparing responses with control.

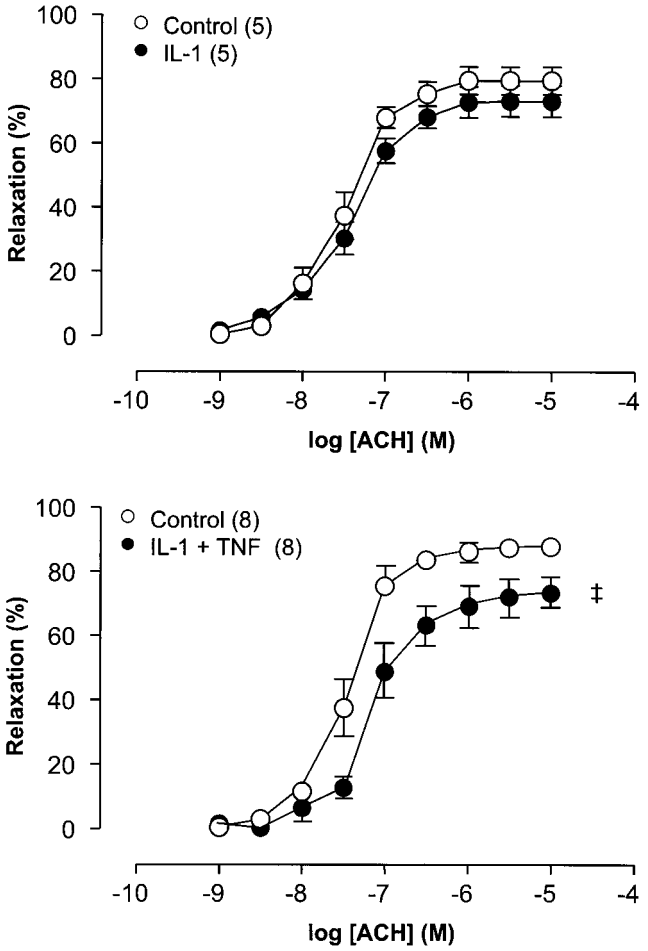

Figure 8 Effect of (a) interleukin 1-β (IL-1), (b) IL-1 and TNF on relaxation in response to ACH in rat mesenteric small arteries contracted with PE. Data are means$\pm$s.e.m. of (n) observations. $^{\ddagger}P<0.05$ comparing log EC_{50} response between control, and IL-1 and TNF-treated arteries.

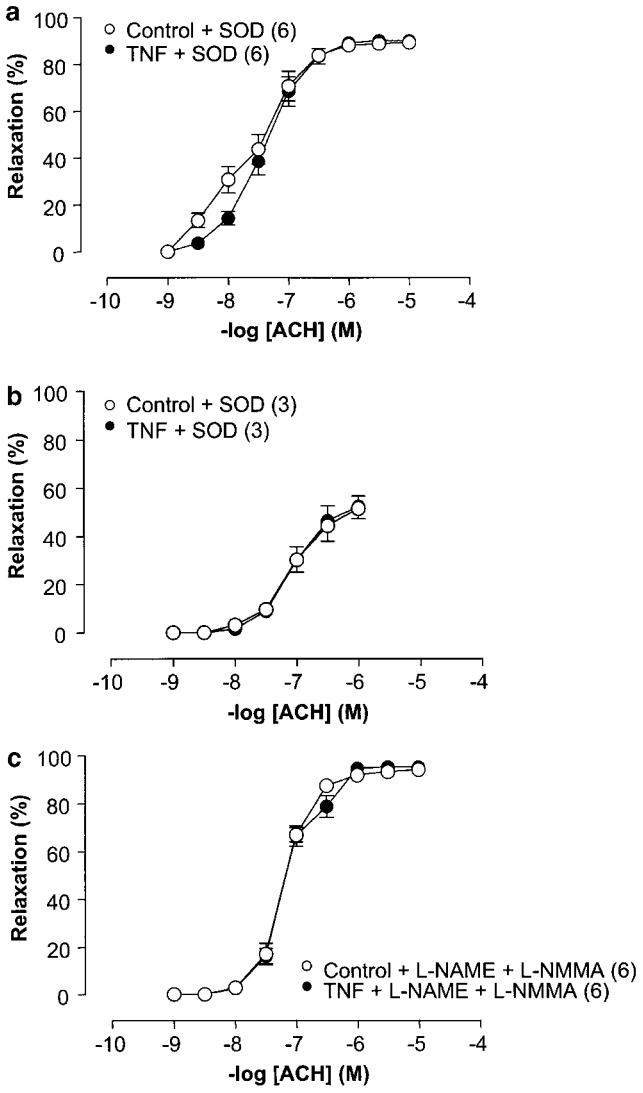

Figure 9 Effect of (a) SOD on inhibition of acetylcholine-induced relaxation by TNF in rat mesenteric small arteries contracted with PE, (b) SOD on inhibition of ACH-induced relaxation by TNF in rat mesenteric small arteries contracted with depolarizing potassium solution containing 80 mM KCl, (c) L-NMMA and L-NAME on inhibition of ACH-induced relaxation by TNF in rat mesenteric small arteries contracted with PE. Data are means$\pm$s.e.m. of (n) observations.

with etanercept, a recombinant TNF receptor that binds to, and functionally inactivates TNF, improves endothelium-dependent relaxation in patients with heart failure (Fichtlscherer *et al*., 2001). It is possible that the elevated TNF levels, resulting from the excessive maternal immune response or placental hypoxia could contribute to the endothelial dysfunction seen in pre-eclampsia through increased production of O_2^-. Such a mechanism could explain a recent study reporting that antioxidant treatment with vitamin E and vitamin C has a beneficial effect on the incidence of pre-eclampsia (Chappell *et al*., 1999).

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Table 4 Effect of TNF on log EC_{50} and E_{max} to ACH in rat mesenteric small arteries contracted with PE in the presence of superoxide dismutase (SOD) or L-NMMA and L-NAME

		Control+SOD	*TNF+SOD*
	log EC_{50}	-7.4 ± 0.2 (6)	-7.4 ± 0.2 (6)
ACH (PE)	E_{max} (%)	90 ± 4 (6)	90 ± 5 (6)
	log EC_{50}	-6.9 ± 0.2 (3)	-6.9 ± 0.2 (3)
ACH (K_{80})	E_{max} (%)	52 ± 8 (3)	52 ± 8 (3)
		CONTROL+l-NAME+l-NMMA	*TNF+l-NAME+l-NMMA*
	log EC_{50}	-7.2 ± 0.1 (6)	-7.1 ± 0.2 (6)
ACH (PE)	E_{max} (%)	93 ± 4 (6)	92 ± 4 (6)

Data are means$\pm$s.e.m. of (n) observations.

References

AOKI, N., SIEGFRIED, M. & LEFER, A.M. (1989). Anti-EDRF effect of tumor necrosis factor in isolated, perfused cat carotid arteries. *Am. J. Physiol,* **256,** H1509–H1512.

ASHWORTH, J.R., BAKER, P.N., WARREN, A.Y. & JOHNSON, I.R. (1999). Mechanisms of endothelium-dependent relaxation in myometrial resistance vessels and their alteration in preeclampsia. *Hypertens. Pregnancy,* **18,** 57–71.

BEASLEY, D., COHEN, R.A. & LEVINSKY, N.G. (1989). Interleukin 1 inhibits contraction of vascular smooth muscle. *J. Clin. Invest.,* **83,** 331–335.

BEASLEY, D. & MCGUIGGIN, M. (1994). Interleukin 1 activates soluble guanylate cyclase in human vascular smooth muscle cells through a novel nitric oxide-independent pathway. *J. Exp. Med.,* **179,** 71–80.

BENYO, D.F., SMARASON, A., REDMAN, C.W., SIMS, C. & CONRAD, K.P. (2001). Expression of inflammatory cytokines in placentas from women with preeclampsia. *J. Clin. Endocrinol. Metab.,* **86,** 2505–2512.

BUSSE, R. & MULSCH, A. (1990). Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett.,* **275,** 87–90.

CHAPPELL, L.C., SEED, P.T., BRILEY, A.L., KELLY, F.J., LEE, R., HUNT, B.J., PARMAR, K., BEWLEY, S.J., SHENNAN, A.H., STEER, P.J. & POSTON, L. (1999). Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomised trial. *Lancet,* **354,** 810–816.

CHEN, G. & CHEUNG, D.W. (1997). Effect of K(+)-channel blockers on ACh-induced hyperpolarization and relaxation in mesenteric arteries. *Am. J. Physiol.,* **272,** 2306–2312.

CHEN, G. & SUZUKI, H. (1989). Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. *J. Physiol.,* **410,** 91–106.

COCKELL, A.P. & POSTON, L. (1997). Flow-mediated vasodilatation is enhanced in normal pregnancy but reduced in preeclampsia. *Hypertension,* **30,** 247–251.

CONRAD, K.P., MILES, T.M. & BENYO, D.F. (1998). Circulating levels of immunoreactive cytokines in women with preeclampsia. *Am. J. Reprod. Immunol.,* **40,** 102–111.

DANIEL, Y., KUPFERMINC, M.J., BARAM, A., JAFFA, A.J., FAIT, G., WOLMAN, I. & LESSING, J.B. (1998). Plasma interleukin-12 is elevated in patients with preeclampsia. *Am. J. Reprod. Immunol.,* **39,** 376–380.

DAVIS, J.R., GIARDINA, J.B., GREEN, G.M., ALEXANDER, B.T., GRANGER, J.P. & KHALIL, R.A. (2002). Reduced endothelial NO-cGMP vascular relaxation pathway during TNF-alpha-induced hypertension in pregnant rats. *Am. J. Physiol.,* **282,** R390–R399.

DE FRUTOS, T., DE MIGUEL, L.S., GARCIA-DURAN, M., GONZALEZ-FERNANDEZ, F., RODRIGUEZ-FEO, J.A., MONTON, M., GUERRA, J., FARRE, J., CASADO, S. & LOPEZ-FARRE, A. (1999). NO from smooth muscle cells decreases NOS expression in endothelial cells: role of TNF-alpha. *Am. J. Physiol.,* **277,** H1317–H1325.

FASCIOLO, J.C., VARGAS, L., LAMA, M.C. & NOLLY, H. (1990). Bradykinin-induced vasoconstriction of rat mesenteric arteries precontracted with noradrenaline. *Br. J. Pharmacol.,* **101,** 344–348.

FICHTLSCHERER, S., ROSSIG, L., BREUER, S., VASA, M., DIMMELER, S. & ZEIHER, A.M. (2001). Tumor necrosis factor antagonism with etanercept improves systemic endothelial vasoreactivity in patients with advanced heart failure. *Circulation,* **104,** 3023–3025.

FUKAO, M., HATTORI, Y., KANNO, M., SAKUMA, I. & KITABATAKE, A. (1995). Thapsigargin- and cyclopiazonic acid-induced endothelium-dependent hyperpolarization in rat mesenteric artery. *Br. J. Pharmacol.,* **115,** 987–992.

GREENBERG, S., XIE, J., WANG, Y., CAI, B., KOLLS, J., NELSON, S., HYMAN, A., SUMMER, W.R. & LIPPTON, H. (1993). Tumor necrosis factor-alpha inhibits endothelium-dependent relaxation. *J. Appl. Physiol.,* **74,** 2394–2403.

HAMAI, Y., FUJII, T., YAMASHITA, T., NISHINA, H., KOZUMA, S., MIKAMI, Y. & TAKETANI, Y. (1997). Evidence for an elevation in serum interleukin-2 and tumor necrosis factor-alpha levels before the clinical manifestations of preeclampsia. *Am. J. Reprod. Immunol.,* **38,** 89–93.

HIGGINS, J.R. & DE SWIET, M. (2001). Blood-pressure measurement and classification in pregnancy. *Lancet,* **357,** 131–135.

KESSLER, P., BAUERSACHS, J., BUSSE, R. & SCHINI-KERTH, V.B. (1997). Inhibition of inducible nitric oxide synthase restores endothelium-dependent relaxations in proinflammatory mediator-induced blood vessels. *Arterioscler. Thromb. Vasc. Biol.,* **17,** 1746–1755.

KNOCK, G.A. & POSTON, L. (1996). Bradykinin-mediated relaxation of isolated maternal resistance arteries in normal pregnancy and preeclampsia. *Am. J. Obstet. Gynecol.,* **175,** 1668–1674.

KUPFERMINC, M.J., PEACEMAN, A.M., WIGTON, T.R., REHNBERG, K.A. & SOCOL, M.L. (1994). Tumor necrosis factor-alpha is elevated in plasma and amniotic fluid of patients with severe preeclampsia. *Am. J. Obstet. Gynecol.,* **170,** 1752–1757.

LACY, P.S., PILKINGTON, G., HANVESAKUL, R., FISH, H.J., BOYLE, J.P. & THURSTON, H. (2000). Evidence against potassium as an endothelium-derived hyperpolarizing factor in rat mesenteric small arteries. *Br. J. Pharmacol.,* **129,** 605–611.

LAGAUD, G.J., SKARSGARD, P.L., LAHER, I. & VAN BREEMEN, C. (1999). Heterogeneity of endothelium-dependent vasodilation in pressurized cerebral and small mesenteric resistance arteries of the rat. *J. Pharmacol. Exp. Ther.,* **290,** 832–839.

LI, J.M., MULLEN, A.M., YUN, S., WIENTJES, F., BROUNS, G.Y., THRASHER, A.J. & SHAH, A.M. (2002). Essential role of the NADPH oxidase subunit p47(phox) in endothelial cell superoxide production in response to phorbol ester and tumor necrosis factor-alpha. *Circ. Res.,* **90,** 143–150.

MADGE, L.A. & POBER, J.S. (2001). TNF signaling in vascular endothelial cells. *Exp. Mol. Pathol.,* **70,** 317–325.

MANTOVANI, A., GARLANDA, C., INTRONA, M. & VECCHI, A. (1998). Regulation of endothelial cell function by pro- and anti-inflammatory cytokines. *Transplant. Proc.,* **30,** 4239–4243.

MCCARTHY, A.L., WOOLFSON, R.G., RAJU, S.K. & POSTON, L. (1993). Abnormal endothelial cell function of resistance arteries from women with preeclampsia. *Am. J. Obstet. Gynecol.,* **168,** 1323–1330.

MONCADA, S., PALMER, R.M. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.,* **43,** 109–142.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.,* **41,** 19–26.

MURPHY, H.S., SHAYMAN, J.A., TILL, G.O., MAHROUGUI, M., OWENS, C.B., RYAN, U.S. & WARD, P.A. (1992). Superoxide responses of endothelial cells to C5a and TNF-alpha: divergent signal transduction pathways. *Am. J. Physiol.,* **263,** L51–L59.

NAKAMURA, M., YOSHIDA, H., ARAKAWA, N., SAITOH, S., SATOH, M. & HIRAMORI, K. (2000). Effects of tumor necrosis factor-alpha on basal and stimulated endothelium-dependent vasomotion in human resistance vessel. *J. Cardiovasc. Pharmacol.,* **36,** 487–492.

OGUOGHO, A., ALOAMAKA, C.P. & EBEIGBE, A.B. (1996). Depressed endothelium-dependent relaxation responses to acetylcholine and histamine in isolated human epigastric arteries from pre-eclamptic women. *Clin. Auton. Res.,* **6,** 153–155.

PASCOAL, I.F., LINDHEIMER, M.D., NALBANTIAN-BRANDT, C. & UMANS, J.G. (1998). Preeclampsia selectively impairs endothelium-dependent relaxation and leads to oscillatory activity in small omental arteries. *J. Clin. Invest.,* **101,** 464–470.

POSTON, L. & CHAPPELL, L.C. (2001). Is oxidative stress involved in the aetiology of pre-eclampsia? *Acta Paediatr.* (Suppl.), **90,** 3–5.

PRIETO, D., BUUS, C., MULVANY, M.J. & NILSSON, H. (1997). Interactions between neuropeptide Y and the adenylate cyclase pathway in rat mesenteric small arteries: role of membrane potential. *J. Physiol.,* **502(Pt 2),** 281–292.

REDMAN, C.W., SACKS, G.P. & SARGENT, I.L. (1999). Preeclampsia: an excessive maternal inflammatory response to pregnancy. *Am. J. Obstet. Gynecol.,* **180,** 499–506.

RINEHART, B.K., TERRONE, D.A., LAGOO-DEENADAYALAN, S., BARBER, W.H., HALE, E.A., MARTIN Jr. J.N. & BENNETT, W.A. (1999). Expression of the placental cytokines tumor necrosis factor alpha, interleukin 1beta, and interleukin 10 is increased in preeclampsia. *Am. J. Obstet. Gynecol.,* **181,** 915–920.

ROBERT, R., CHAPELAIN, B., JEAN, T. & NELIAT, G. (1992). Interleukin-1 impairs both vascular contraction and relaxation in

rabbit isolated aorta. *Biochem. Biophys. Res. Commun.,* **182,** 733–739.

ROBERT, R., CHAPELAIN, B. & NELIAT, G. (1993). Different effects of interleukin-1 on reactivity of arterial vessels isolated from various vascular beds in the rabbit. *Circ. Shock,* **40,** 139–143.

SIMONSEN, U., WADSWORTH, R.M., BUUS, N.H. & MULVANY, M.J. (1999). *In vitro* simultaneous measurements of relaxation and nitric oxide concentration in rat superior mesenteric artery. *J. Physiol.,* **516,** 271–282.

SUZUKI, Y., YAMAMOTO, T., SUZUMORI, K., KAJIKURI, J. & ITOH, T. (2000). Modified histamine-induced NO-mediated relaxation in resistance arteries in pre-eclampsia. *Eur. J. Pharmacol.,* **410,** 7–13.

THORIN-TRESCASES, N., HAMILTON, C.A., REID, J.L., MCPHERSON, K.L., JARDINE, E., BERG, G., BOHR, D. & DOMINICZAK, A.F. (1995). Inducible L-arginine/nitric oxide pathway in human internal mammary artery and saphenous vein. *Am. J. Physiol.,* **268,** H1122–H1132.

TRIGGLE, C.R. & DING, H. (2002). Endothelium-derived hyperpolarizing factor: is there a novel chemical mediator? *Clin. Exp. Pharmacol. Physiol.,* **29,** 153–160.

VANE, J.R., GRYGLEWSKI, R.J. & BOTTING, R.M. (1987). The endothelial cell as a metabolic and endocrine organ. *Trends Pharmacol. Sci.,* **8,** 491–501.

VANHEEL, B. & VAN DE VOORDE, J. (2000). EDHF and residual NO: different factors. *Cardiovasc. Res.,* **46,** 370–375.

VINCE, G.S., STARKEY, P.M., AUSTGULEN, R., KWIATKOWSKI, D. & REDMAN, C.W. (1995). Interleukin-6, tumour necrosis factor and soluble tumour necrosis factor receptors in women with pre-eclampsia. *Br. J. Obstet. Gynaecol.,* **102,** 20–25.

VISSER, W., BECKMANN, I., BREMER, H.A., LIM, H.L. & WALLENBURG, H.C. (1994). Bioactive tumour necrosis factor alpha in pre-eclamptic patients with and without the HELLP syndrome. *Br. J. Obstet. Gynaecol.,* **101,** 1081–1082.

WANG, P., BA, Z.F. & CHAUDRY, I.H. (1994). Administration of tumor necrosis factor-alpha *in vivo* depresses endothelium-dependent relaxation. *Am. J. Physiol.,* **266,** H2535–H2541.

WU, X.C., JOHNS, E., MICHAEL, J. & RICHARDS, N.T. (1994). Interdependence of contractile responses of rat small mesenteric arteries on nitric oxide and cyclo-oxygenase and lipoxygenase products of arachidonic acid. *Br. J. Pharmacol.,* **112,** 360–368.

YOSHIZUMI, M., PERRELLA, M.A., BURNETT Jr. J.C. & LEE, M.E. (1993). Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ. Res.,* **73,** 205–209.

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