British Journal of Pharmacology (2003) 139, 1487–1497
www.nature.com/bjp

Extracellular L-arginine is required for optimal NO synthesis by eNOS and iNOS in the rat mesenteric artery wall

[1,2]Andrew MacKenzie & *,[1]Roger M. Wadsworth

[1]Department of Physiology & Pharmacology, University of Strathclyde, Arbuthnott Building, Glasgow G4 0NR, Scotland

1 The formation of NO from endothelial nitric oxide synthase (eNOS) in rat superior mesenteric artery rings was dependent on extracellular L-arginine, and was optimal at a concentration of L-arginine close to the plasma level (carbachol-stimulated NO: control 15.7 ± 0.9, L-arginine 100 μM 22.8 ± 1.3 nM).

2 Enhancement of NO output by L-arginine was stereospecific, required the cationic amino-acid transporter and was dependent on caveolin.

3 Induction of inducible nitric oxide synthase (iNOS) impaired the stimulated NO synthesis from eNOS (100 nM carbachol-stimulated NO: control 5.7 ± 0.6, iNOS 0.3 ± 0.3 nM).

4 The interaction between iNOS and eNOS was reversed by the superoxide scavenger MnTMPyP. Impairment of eNOS by iNOS was also prevented by L-arginine 100 μM administered simultaneously with carbachol, but not by L-arginine administered during incubation with lipopolysaccharide.

5 These data provide functional evidence that supplementing L-arginine from the extracellular medium optimises the formation of NO from eNOS and suggests that the impairment of eNOS by iNOS is caused by excess formation of superoxide by NO synthase, which can be prevented by L-arginine. These results provide an explanation for the observations that extracellular L-arginine can enhance endothelium function only when the endothelium is impaired or when iNOS has been induced.

British Journal of Pharmacology (2003) **139,** 1487–1497. doi: 10.1038/sj.bjp.0705380

Keywords: Nitric oxide; L-arginine; iNOS

Abbreviations: eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; PSS, physiological salt solution

Introduction

Administration of L-arginine has been found to improve endothelium-dependent vasodilation in patients with coronary artery disease (Duboisrande *et al*., 1992; Adams *et al*., 1997; Chowienczyk *et al*., 1998; Micieli *et al*., 1999; Tousoulis *et al*., 2001). L-arginine also corrects the impairment of endothelium-dependent vasodilation in experimental hypertension (Kitazono *et al*., 1996) and experimental hyperlipidaemia (Brandes *et al*., 2000). The beneficial effects of L-arginine appear to be due to an improvement of endothelial nitric oxide (NO) formation; however, the mechanism through which this could occur is not clear. Extracellular L-arginine is considered to be incapable of stimulating NO formation from endothelial NO synthase (eNOS), since the normal physiological intracellular L-arginine concentration is sufficient to saturate substrate binding of eNOS (Palmer & Moncada, 1989; Su *et al*., 1997). Addition of L-arginine to artery rings, provided there has been no induction of iNOS and provided there is no cardiovascular disease, causes no relaxation (rat aorta – Schott *et al*., 1993; rat mesenteric artery – Martinez *et al*., 1996). By contrast, inducible NO synthase (iNOS) can be stimulated by increasing extracellular L-arginine to generate additional NO; however, iNOS is not significantly elevated in experimental hypertension.

Sepsis and endotoxaemia induce the formation of large amounts of iNOS protein in the artery wall, specifically in the fibroblasts, endothelial and smooth muscle cells. Potentially, iNOS has the capacity to generate large quantities of NO, and it is postulated that overproduction of NO in the artery wall leads to many of the key indicators of endotoxaemia, including depressed vasoconstriction, a hypodynamic vascular syndrome and refractory hypotension, which may contribute ultimately to multiple organ failure. Thus, contractile responsiveness was unaffected in iNOS-deficient mice exposed to endotoxin (Gunnett *et al*., 1998), and L-NIL, a NOS inhibitor selective for the iNOS isoform, prevented the harmful actions of endotoxin (Schwartz *et al*., 1997; Fischer *et al*., 1999). In endotoxaemia, there is also inhibition of soluble guanylate cyclase (Tsuchida *et al*., 1994; Papapetropoulos *et al*., 1996; Scott & Nakayama, 1998), and downregulation of eNOS (Arriero *et al*., 2002; Ermert *et al*., 2002), and it has been suggested that these enzymes are negatively regulated by exposure to exceptional concentrations of NO. Considering these changes together, induction of iNOS and downregulation of eNOS, it is not clear whether the generation of NO in the artery wall by agonists acting on the endothelium will be inhibited or augmented by endotoxaemia.

*Author for correspondence; E-mail: r.m.wadsworth@strath.ac.uk
[2]Current address: School of Science and Technology, Bell College, Almada St, Hamilton ML3 0JB, Scotland, U.K.
Advance online publication: 14 July 2003

There have been no measurements of the NO concentration in the artery wall following addition of L-arginine, and thus the quantitative relationship between extracellular L-arginine supply and NO formation in an intact artery is unknown. In the present study, the effect of L-arginine addition on NO formation by the endothelium, in response to the endothelial cell membrane receptor agonist carbachol, has been compared for eNOS alone, and eNOS plus iNOS.

Methods

Tissue preparation

Male Sprague–Dawley rats (200–300 g) were killed by cervical dislocation. The mesenteric vascular bed was removed and placed in physiological salt solution (PSS) of the following composition (mM): NaCl 118; $NaHCO_3$ 25; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 2.5 and glucose 11; aerated with 95% O_2 and 5% CO_2 (pH 7.4). Segments (length 2 mm) of superior mesenteric artery were mounted on 100 μm diameter wires in a small vessel myograph for isometric tension recording and maintained in PSS at 37°C, as described previously (Simonsen *et al*., 1999). The vessels were normalised to their optimal internal circumference (Mulvany & Halpern, 1977). In some experiments, the endothelium was partially removed by passing a human scalp hair through the vessel lumen and then rubbing it back and forth across the lumen several times.

Calibration of NO microsensor

Each NO microsensor (ISONOP30, World Precision Instruments, Stevenage, U.K.) was calibrated daily with a solution of NO gas in stirred PSS solution maintained at 37°C and bubbled with 5% CO_2 in oxygen (Simonsen *et al*., 1999). Briefly, the microelectrode (a carbon fibre with a polymer coating; ISONOP30, World Precision Instruments, Stevenage, U.K.) was calibrated with a solution of NO gas. This solution was prepared from deoxygenated (argon-bubbled) distilled water, contained in glass vials with a septum closure. The argon gas was led through a 10 mM pyrogallol solution to remove traces of oxygen. Pure NO gas, bubbled through sodium hydroxide (10 mM) to remove higher nitrogen oxides, was then bubbled through the deoxygenated water for 15 min at room temperature (23°C), giving a solution of 1.7 mM (Geventman, 1995), which was then diluted to 0.17 mM. Using gas-tight Hamilton microlitre syringes, known concentrations of NO were added to the microelectrode which was held in a stirred PSS solution maintained at 37°C and bubbled with 5% CO_2 in oxygen. The electrode responded with increases in current to low nanomolar concentrations of NO. The output current of the probes correlated linearly with the concentration of NO. Each sensor was individually calibrated daily with fresh NO solution. Sensors were recalibrated at the completion of the experiment, and no change in sensitivity was found.

The ISONOP30 sensor was found to be selective for NO when the polymer coating was intact, and no response was obtained with sodium nitrite 100 μM, carbachol 10 μM or phenylephrine 1 μM. L-arginine (100 μM or 10 mM) did not alter the sensitivity of the NO electrode to measure low (<20 nM) concentrations of NO as delivered from a NO-saturated solution. Sensors were unresponsive to superoxide anion, as generated by the xanthine/xanthine oxidase system, and to superoxide dismutase. Frequent checks were made of the integrity of the polymer coating, and when it became damaged, a new sensor was used.

Simultaneous measurements of NO concentration and force

Immediately following calibration, the NO-sensitive microelectrode was inserted in the myograph through a hole drilled in one side, and then sealed with grease (high-vacuum grease, Dow Corning, Merck Ltd, U.K.). The electrode (length 2 mm, diameter of 30 μM), visualised with a stereo zoom microscope, was inserted into the artery lumen by means of a micromanipulator and placed close to the endothelial surface. The NO electrode was connected to an amplifier (NO meter, World Precision Instruments), and the amplified signal was registered on a recorder permitting simultaneous measurements of NO and force.

In the rat superior mesentery artery, carbachol-induced relaxation is abolished by endothelial denudation or treatment with the NO scavenger oxyhaemoglobin or the NOS inhibitor L-NOARG (Hwa *et al*., 1994; Simonsen *et al*., 1999), and thus appears to be entirely mediated by NO. For equivalent relaxation, the concentrations of NO recorded using the NO sensor at the surface of the endothelium are similar whether the NO is generated within the endothelial cells in response to carbachol, or in the bulk solution from SNAP or SIN-1 (Simonsen *et al*., 1999). Both the time course and the concentration of NO recorded with the sensor correlate closely with the resulting relaxation. This evidence suggests that the NO concentrations recorded with this technique are a reasonable estimate of the physiologically relevant concentration of NO within the artery wall.

Experimental protocols

Carbachol Cumulative concentration–response curves to carbachol (1 nM–3 μM) were constructed on arterial segments following production of phenylephrine (1 μM)-induced tone. We have previously shown in this artery that NO output and arterial relaxation are abolished by endothelial denudation or treatment with oxyhaemoglobin or L-NOARG (Simonsen *et al*., 1999). After washing, the effects of various drugs (L- and D-arginine, SOD, L-lysine, gramicidin, MnTMPyP, 1400W) were examined. The vessels were incubated for 20 min with the drug specific to the experiment before constricting with phenylephrine and repeating the cumulative concentration–response curve to carbachol. When necessary, the concentration of phenylephrine used to constrict the artery was altered to ensure that the level of tone was similar to that of the control. The effects of carbachol in the presence of the drug treatment were compared to the control responses in the same artery before drug addition.

Effect of L-arginine administration not attributable to iNOS In the absence of endotoxin, two experimental approaches were used to rule out the presence of iNOS. (1) Arterial segments were incubated with 1 μM dexamethasone immediately following the normalising procedure. After 3 h, control responses to carbachol were conducted as described. Following washing, dexamethasone was reintroduced to the

vessel and L-arginine added; 20 min later, the second concentration–response curve to carbachol was constructed. (2) In other experiments, the tissue was incubated for 20 min with 10 μM 1400W which is 1000-fold more potent against iNOS than eNOS in rat (Garvey *et al*., 1977). Augmentation by 100 μM L-arginine of carbachol-induced relaxation was unchanged when induction of iNOS was prevented by incubation throughout the experiment with dexamethasone (1 μM) (relaxation at carbachol 30 nM was enhanced from 23.5 ± 4.7 to 38.5 ± 5.0% of phenylephrine-induced tone in tissues treated with dexamethasone and dexamethasone with L-arginine, respectively, $n=8$, $P<0.01$) or by addition of the selective iNOS inhibitor 1400W (10 μM) (relaxation at carbachol 30 nM was enhanced from 16.1 ± 4.2 to 34.1 ± 5.0% of phenylephrine-induced tone in artery rings treated with 1400W compared with 1400W and L-arginine, respectively, $n=7$, $P<0.01$). L-arginine had no effect in endothelium-denuded artery rings ($n=5$, data not shown).

CAT inhibitors Two separate inhibitors of L-arginine active transport were used. The amino acid L-lysine (a competitive substrate for the transporter) and the cation ionophore gramicidin (which destroys the ion gradient necessary for cationic amino-acid transport) have been previously shown to reduce L-arginine transport through plasma membrane vesicles to 3 ± 2 and 19 ± 5% of control levels, respectively (Zharikov & Block, 1998).

Anti-α-caveolin Anti-α-caveolin has previously been shown to successfully bind to caveolin at 1 μg ml^{-1} (Song *et al*., 1996). Preliminary experiments were conducted to determine the optimal concentration of the anticaveolin antibody and these showed that 20 μg ml^{-1} was the greatest concentration that had no effect on phenylephrine-induced contraction or carbachol-mediated relaxation (pD_2 for relaxation was 7.41 ± 0.09 and 7.30 ± 0.10 in untreated artery rings compared to anti-caveolin antibody-treated artery rings, respectively, $n=4$, $P>0.05$). To optimise the use of the anti-caveolin antibody, arterial segments were incubated with the antibody in a small volume of PSS (500 μl) maintained at 37°C. After 60 min, the artery was removed, placed in fresh PSS and mounted in the myograph. The remainder of the experiment was conducted as normal.

Superoxide dismutase The concentration of superoxide dismutase (SOD) used (100 U ml^{-1}) has previously been shown to maximally inhibit endogenous superoxide formation in artery rings (MacKenzie *et al*., 1999).

iNOS induction with lipopolysaccharide Lipopolysaccharide (LPS) has previously been shown to successfully induce iNOS expression in rat mesenteric arteries (Martinez *et al*., 1996; MitoloChieppa, 1996; O'Brien *et al*., 2001). Preliminary experiments were conducted to determine the optimal concentration and incubation period of LPS, which was found to be administration of LPS at 20 μg ml^{-1} for 2 h, followed by washout and incubation in PSS for a further 2 h. In precontracted arteries, L-arginine caused no relaxation in the absence of LPS; however, following LPS treatment, L-arginine gave a relaxation that was abolished by 1400W (see the Results).

L-arginine administration in conjunction with induction of iNOS The timing of L-arginine's action was assessed in two ways. Firstly, L-arginine was added to the Krebs' solution 20 min before carbachol, but absent during the 4 h incubation period. Secondly, L-arginine was present during the 4 h incubation period, but absent during carbachol administration.

MnTMPyP The concentration of MnTMPyP used (600 μM) has previously been shown to scavenge intracellularly located superoxide in artery rings (MacKenzie & Martin, 1998). MnTMPyP was coincubated along with LPS. Following washout of LPS, MnTMPyP was reintroduced to the vessel for the remaining 2 h period and after the final washout, MnTMPyP was again administered to the tissue 20 min before concentration–responses to carbachol were determined.

1400W The highly selective inhibitor of iNOS, 1400W (Garvey *et al*., 1977), was present throughout the entire 4 h incubation period and also during the challenge with carbachol.

Drugs

L-arginine hydrochloride, D-arginine hydrochloride, carbamylcholine chloride (carbachol), dexamethasone, gramicidin (from *Bacillus brevis*), L-lysine dihydrochloride, phenylephrine hydrochloride and SOD (Cu/Zn-containing enzyme from bovine erythrocytes) were obtained from Sigma (Poole, U.K.). *N*-[3-aminomethyl]benzylacetamidine (1400W) was obtained from Calbiochem (Nottingham, U.K.). Mn(III) tetrakis [1-methyl-4-pyridyl] porphyrin (MnTMPyP) was obtained from Aldrich (Dorset, U.K.). Anti-human caveolin (recognises the α-isoform, crossreactive with rat) was obtained from TCS Biologicals (Buckingham, U.K.) and was delivered as a 1 μg μl^{-1} stock dissolved in a 100 mM Tris-glycine and 0.05% sodium azide solution. All other drugs were dissolved in saline (0.9%) except for dexamethasone (10 mM stock) and gramicidin (1 mM stock), which were both dissolved in ethanol. All subsequent dilutions were made in Krebs' solution. L-arginine hydrochloride at either 100 μM or 10 mM had no effect on the pH of gas-bubbled PSS held at 37°C.

Analysis of data

Relaxation to carbachol is expressed as a percentage of the pre-existing level (100%) of phenylephrine-induced tone. Axes labelled [NO] refer to increases in NO concentration above a baseline level, measured immediately prior to the addition of carbachol. The pD_2 values to carbachol were calculated as the negative logarithm of the molar concentration that produced 50% of the maximal effect. The results are expressed as means $\pm$ s.e.m. n = animal number. Differences between means were analysed using one-way analysis of variance followed by the Bonferroni post-test. Probability levels less than 5% were considered significant. Statistical analysis and manipulation were performed using GraphPad Prism.

Results

Absence of endotoxin

NO output and arterial relaxation in the absence of extracellular L-*arginine* In endothelium-containing segments of rat superior mesenteric artery, submaximally contracted with phenylephrine (1 μM), carbachol (1 nM–10 μM) stimulated NO formation in the endothelium correlating with relaxation (Figure 1a). Luminal NO concentration with carbachol 10 μM was 15.6$\pm$0.5 nM corresponding to a relaxation of 96.4$\pm$1.1%, and the pD_2 for increase in NO concentration and relaxation were 6.67$\pm$0.04 and 7.27$\pm$0.13 (n = 20), respectively.

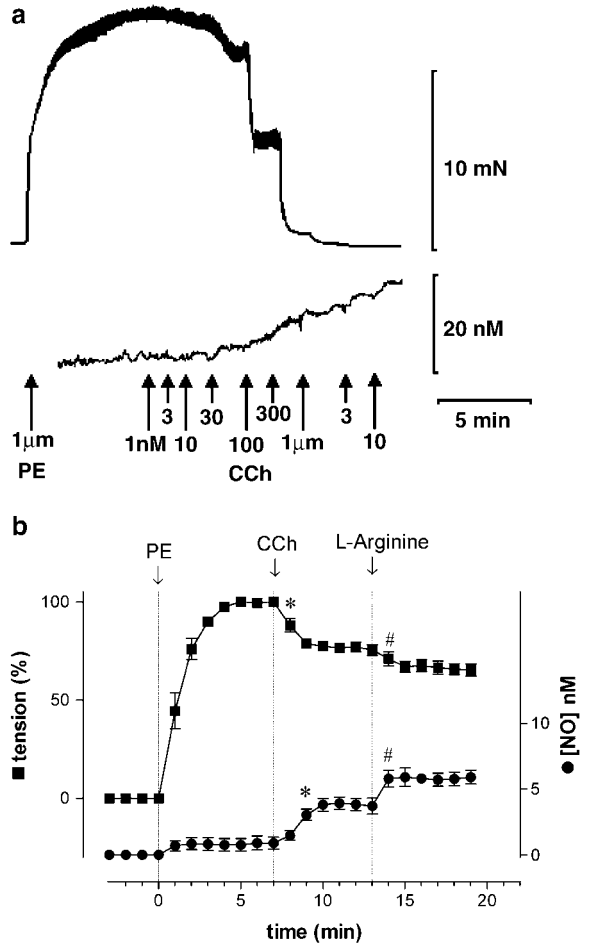

Figure 1 Simultaneous measurements of force (upper trace) and NO concentration (lower trace) in an endothelium-intact segment of rat superior mesentery artery. (a) Effect of carbachol. Following contraction with phenylephrine (PE; 1 μM), carbachol (CCh; 1 nM–10 μM) produced a concentration-dependent increase in NO concentration and arterial relaxation. (b) Effect of L-arginine. The contraction induced by phenylephrine was associated with a small rise in NO concentration. The subsequent addition of carbachol (30 nM) simultaneously increased NO concentration and produced a relaxation. Addition of 100 μM L-arginine produced a further rise in NO concentration and enhanced relaxation. The points are means $\pm$s.e.m., n = 4. $^*P<0.05$ and $^{\#}P<0.05$ indicate the first response that is significantly different for the measurement immediately prior to the addition of carbachol and L-arginine, respectively.

Readmission of L-*arginine* Administration of L-arginine alone to rat superior mesenteric artery did not cause any relaxation nor any stimulation of NO formation. However, in the presence of a low concentration of carbachol (giving less than 50% relaxation), L-arginine caused a further increase in NO concentration and a matching further relaxation (Figure 1b). Addition of 100 μM L-arginine induced a simultaneous increase in NO concentration and relaxation of 2.8$\pm$0.4 nM and 11.7$\pm$2.3%, respectively. The effects of L-arginine on NO formation were maximal and steady after approximately 3 min.

When the PSS was supplemented with 100 μM L-arginine, carbachol generated more NO and also induced greater relaxation (Figure 2b). L-arginine increased maximal NO concentration by 45% from 15.7$\pm$0.9 to 22.8$\pm$1.3 nM. The increased NO was associated with enhanced arterial relaxation at carbachol 30 nM (relaxation was increased by 228% from 14.8$\pm$4.5 to 33.8$\pm$6.0% of phenylephrine-induced tone.) Enhancement by L-arginine was highly concentration-dependent; thus at the lower concentration of 10 μM, L-arginine had no effect on carbachol-induced relaxation or NO formation (Figure 2a). At the higher concentration of 1 mM, L-arginine had no effect on relaxation, but did increase luminal NO output from 16.9$\pm$0.8 to 19.8$\pm$1.2 nM (at carbachol 10 μM) (Figure 2c). Increasing L-arginine to 10 mM produced, in contrast, an impairment of carbachol-induced NO formation (NO concentration at carbachol 10 μM was reduced from 15.3$\pm$0.9 to 11.7$\pm$0.9 nM) and an impairment of relaxation (relaxation at carbachol 0.1 μM was reduced from 79.0$\pm$6.3 to 49.0$\pm$6.9% of phenylephrine-induced tone) (Figure 2d). Both the enhancement (by 100 μM L-arginine) and the impairment (by 10 mM L-arginine) of carbachol-induced relaxation were specific to the L-isoform of the amino acid. A 20 min incubation of D-arginine at either 100 μM or 10 mM had no effect on the ability of carbachol to induce arterial relaxation (pD_2 were 7.35$\pm$0.04, 7.33$\pm$0.02 and 7.29$\pm$0.02 in untreated tissues and in tissues treated with 100 μM and 10 mM D-arginine, respectively, n = 6, $P>0.05$).

Effect of 100 μM L-*arginine on artery with damaged endothelium* In artery rings where the endothelium had been partly removed, the action of 100 μM L-arginine was more prominent and carbachol-induced relaxation was enhanced over the range 0.1–1 μM carbachol (Figure 3a). Relaxation at 1 μM carbachol was significantly increased from 52.7$\pm$7.2 to 66.7$\pm$5.7% of phenylephrine-induced tone.

L-*arginine transporter* The inhibitors of cellular arginine uptake, 200 μM L-lysine or 10 μM gramicidin (Zharikov & Block, 1998), each abolished the augmentation of carbachol relaxation produced by 100 μM L-arginine (relaxation at carbachol 30 nM was 25.8$\pm$8.1 and 21.6$\pm$5.4% of phenylephrine-induced tone in tissues treated with L-lysine, compared with L-lysine plus L-arginine, respectively, n = 5, $P>0.05$, whereas relaxation was 19.0$\pm$4.8 and 16.6$\pm$3.9% in tissues treated with gramicidin compared to gramicidin plus L-arginine, respectively, n = 5, $P>0.05$). Treatment of the artery with the anti-caveolin antibody (20 μg ml^{-1}, 60 min) prevented L-arginine (100 μM) enhancement of carbachol-induced relaxation (relaxation at carbachol 30 nM was 16.1$\pm$4.2 and 16.9$\pm$5.9% of phenylephrine-induced tone in tissues treated

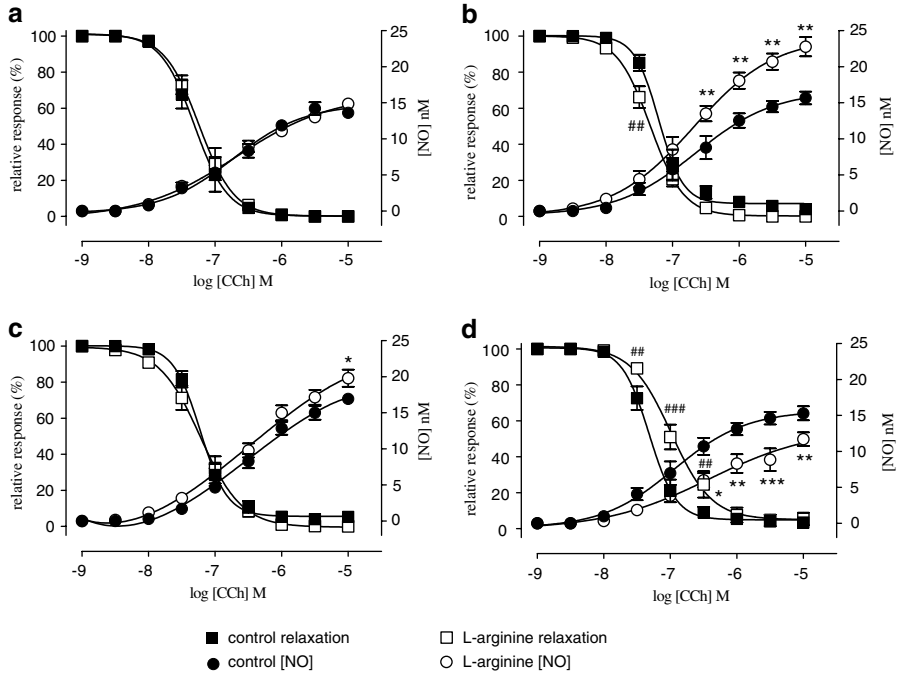

Figure 2 Average relaxations and increases in NO concentration induced by increasing concentrations of carbachol (CCh) in rat superior mesenteric arterial segments in the absence and the presence of exogenously applied L-arginine at 10 (a) and 100 μM (b), and 1 (c) and 10 mM (d). L-arginine was added 20 min before carbachol. Relaxations are relative to the initial contraction induced by phenylephrine and NO concentrations are given as increases above the level in the absence of carbachol. The points are means$\pm$s.e.m., $n=3$–6. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ indicate significant differences in NO concentration following treatment with L-arginine. $P<0.01$ and $P<0.001$ indicate significant differences in relaxation following treatment with L-arginine.

with anti-caveolin antibody compared to anti-caveolin antibody with L-arginine, respectively, $n=6$, $P>0.05$).

Role of superoxide in inhibitory but not augmenting action of L-*arginine* Arterial segments were incubated with a saturating concentration of SOD, (100 U ml^{-1}), which on its own enhanced carbachol-induced relaxation, demonstrating significant superoxide generation within the artery wall. L-arginine 100 μM significantly enhanced carbachol-induced relaxation in the presence of SOD (Figure 3b). In contrast, the inhibitory effect of 10 mM L-arginine was prevented by a coincubation with 100 U ml^{-1} SOD (Figure 3c). Indeed, with 10 mM L-arginine the resulting relaxation was enhanced beyond control levels and was similar to the effects observed in the presence of SOD alone.

Treatment with endotoxin

Evidence for induction of iNOS by the endotoxin pretreatment schedule L-arginine caused neither NO generation nor relaxation when added to untreated rat superior mesenteric artery rings, that is, artery rings that had not received endotoxin (Figure 4a). However, in arteries that had been incubated with endotoxin, addition of 100 μM L-arginine caused substantial NO formation, which was accompanied by relaxation (Figure 4a). These actions of L-arginine are characteristic of the presence of iNOS (Schott *et al*., 1993) and confirm that iNOS has been induced in the endotoxin-treated arteries but is absent in the control arteries.

Effect of endotoxin treatment on NO generated by eNOS Induction of iNOS by endotoxin treatment led to impairment of NO formation and relaxation in response to stimulation of eNOS by carbachol in rat mesenteric artery rings. The carbachol-mediated NO concentration–response curve was displaced to the right in endotoxin-treated rings, showing an alteration in sensitivity but not in maximum permitted output of NO (Figure 4b). Relaxation was also substantially reduced, but maximal relaxation was retained at a high concentration of carbachol, resulting in a parallel shift in the relaxation curve (Figure 4b). Inspection of Figure 4b shows that the concentration of NO generated by carbachol 100 nM in control arteries was the same as the concentration of NO generated by carbachol 300 nM in endotoxin-treated arteries (5.7 ± 0.6 and 6.3 ± 0.8 nM, respectively, $n\geqslant5$, $P>0.05$); however, the degree of relaxation generated was markedly different (74.1 ± 3.5 and 48.4 ± 11.0%, respectively, $P<0.01$). Thus, in addition to reducing NO formation, endotoxin also impaired the sensitivity of the vascular smooth muscle to NO.

Coincubation with the selective inhibitor of iNOS, 1400W (10 μM), completely prevented the impairment of carbachol-induced relaxation following endotoxin incubation (pD_2 were 7.25 ± 0.04 and 7.29 ± 0.02 in tissues treated with 1400W compared with 1400W plus endotoxin, respectively, $n=5$, $P>0.05$), demonstrating that induction iNOS is necessary for the depression of endothelium function following endotoxin administration.

Modulation of iNOS activity by addition of L-*arginine* Addition of 100 μM L-arginine to the Krebs' solution 20 min

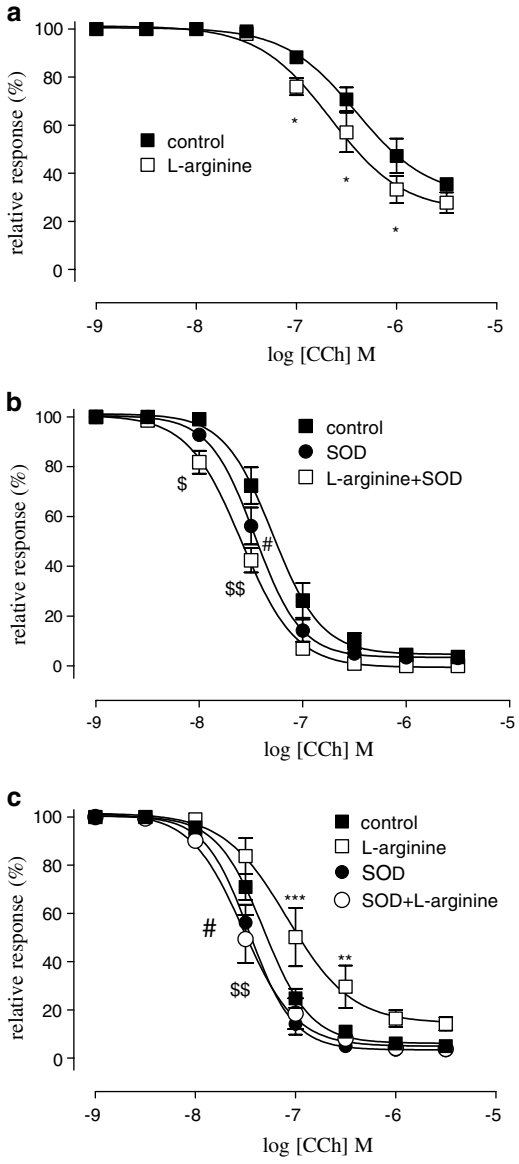

Figure 3 (a) L-arginine (100 μM) enhanced the relaxation induced by carbachol (CCh) in endothelium-impaired arteries. Rat superior mesenteric artery rings were partly endothelium-denuded to reduce maximal relaxation to ~70%. (b) The relaxation induced by carbachol (CCh) of phenylephrine-contracted, endothelium-containing segments of rat superior mesenteric artery was enhanced following treatment with SOD (100 U ml^{-1}). Coincubation of SOD and L-arginine (100 μM) enhanced relaxation beyond the influence of SOD alone. (c) Concentration–response curves showing that the ability of L-arginine (10 mM) to impair carbachol (CCh)-induced relaxation was abolished by pretreatment with SOD (100 U ml^{-1}). Coincubation of SOD and L-arginine (10 mM) enhanced relaxation beyond the control values. The points are means ± s.e.m., $n=4-5$. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ indicate a significant difference following treatment with L-arginine. $^{\#}P<0.05$ indicates a significant difference following treatment with SOD. $^{\$}P<0.05$ and $^{\$\$}P<0.01$ indicate a significant difference following the combined treatment with SOD and L-arginine with respect to control relaxation.

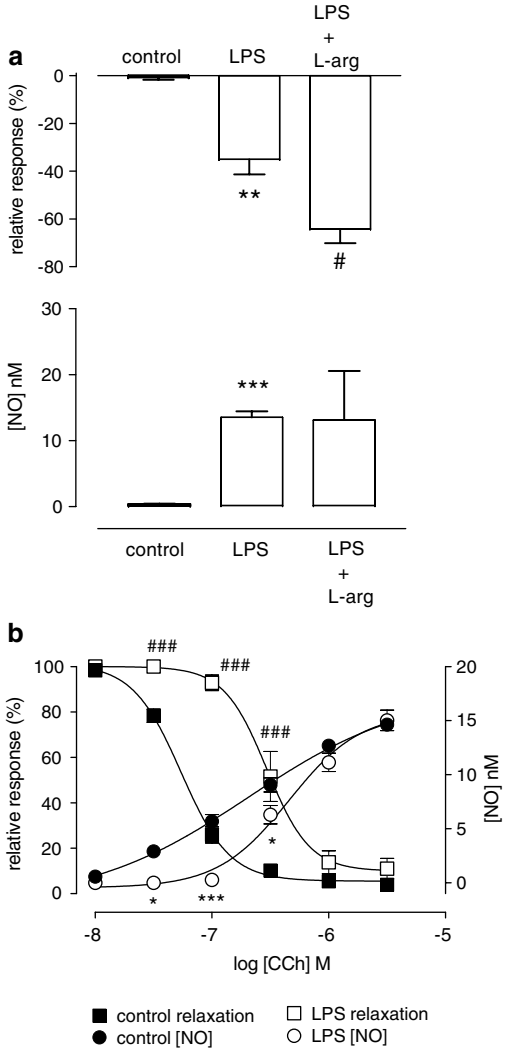

Figure 4 (a) Simultaneous measurement of force and NO concentration in response to application of L-arginine (100 μM) in rat superior mesenteric segments pretreated with LPS and with LPS combined with L-arginine (followed by washout). Application of L-arginine generated significant relaxation and NO output in LPS-treated tissues, providing evidence for the induction of iNOS. The points are means ± s.e.m., $n=3-6$. $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences in relaxation or NO output following treatment with LPS. $^{\#}P<0.05$ indicates a significant difference in relaxation in LPS plus L-arginine pretreated vessels with respect to LPS alone. (b) Average relaxations and increases in NO concentration induced by increasing concentrations of carbachol (CCh) in rat superior mesenteric arterial segments in the absence and presence of LPS. Induction of iNOS with LPS significantly impairs both NO formation and arterial relaxation in response to carbachol. The points are means ± s.e.m., $n=3-6$. $^{\#\#\#}P<0.001$ indicates a significant difference in relaxation following treatment with LPS. $^{*}P<0.05$ and $^{***}P<0.001$ indicate significant differences in NO concentration following treatment with LPS.

before carbachol (but absent during endotoxin administration), restored carbachol-induced NO to normal (Figure 5a). Carbachol-induced relaxation, although enhanced by L-arginine, was not restored to control level (Figure 5a). In contrast, addition of L-arginine during incubation with endotoxin (but absent during carbachol administration) did not restore NO formation. However, there was some restoration of relaxation in response to carbachol, but relaxation was not restored to control levels (Figure 5b). These experiments show that L-arginine administration can protect eNOS against damage resulting from induction of iNOS, and that the critical time is during activation of eNOS by carbachol.

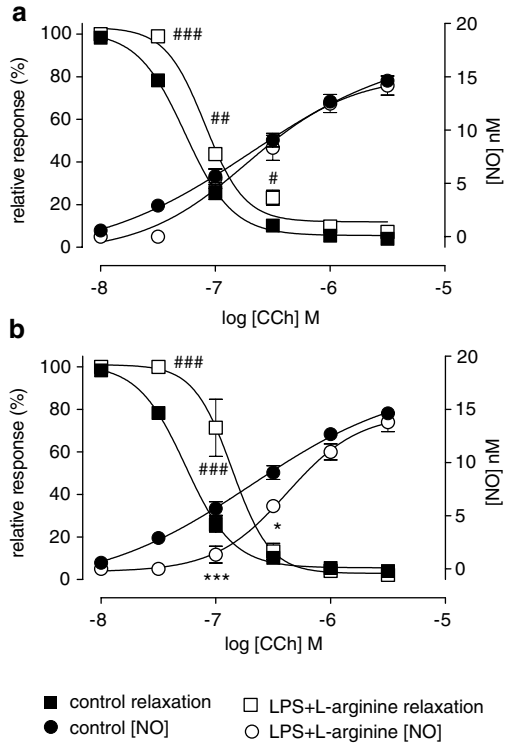

Figure 5 Concentration–respone curves showing relaxations and increases in NO concentration induced by increasing concentrations of carbachol (CCh) in rat superior mesenteric arterial segments in the absence and presence of lipopolysaccharide and L-arginine. L-arginine was administered either (a) 20 min prior to carbachol-induced relaxation or (b) throughout incubation with endotoxin, but absent during carbachol administration. The points are means $\pm$s.e.m., $n \geqslant 5$. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ indicate significant differences in relaxation following treatment with LPS and L-arginine. $^{*}P<0.05$ and $^{***}P<0.001$ indicate significant differences in NO concentration following treatment with LPS and L-arginine.

In contrast to the latter experiments, L-arginine coadministration did not affect NO formation by iNOS, as determined by NO formation in response to a test addition of L-arginine (Figure 4a). However, relaxation was enhanced in the L-arginine-treated group (Figure 4a).

Role of superoxide scavenger – MnTMPyP The membrane-permeant SOD mimetic, MnTMPyP (600 μM, present during both endotoxin treatment and during carbachol challenge), partly protected carbachol-induced NO. MnTMPyP also caused some restoration of carbachol-induced relaxation, though relaxation remained depressed in endotoxin-treated compared to untreated arteries (Figure 6). MnTMPyP on its own had no effect on NO formation or on relaxation in response to carbachol (pD_2 for NO concentration was 6.69 ± 0.09 compared to 6.71 ± 0.08, while the pD_2 for relaxation was 7.26 ± 0.03 compared to 7.30 ± 0.06, respectively, in untreated arteries compared to arteries treated with MnTMPyP, $n=4$, $P>0.05$).

Responses to an NO donor Spermine NONOate, which spontaneously generates NO in solution, was used as an NO donor acting independently of the endothelium. Relaxation of the rat mesenteric artery induced by spermine NONOate was impaired in endotoxin-treated tissues (Figure 7a). However, the effect of endotoxin was prevented by incubation with MnTMPyP (Figure 7b).

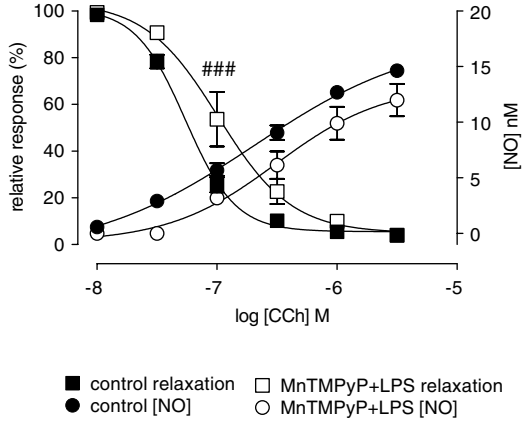

Figure 6 Average relaxations and increases in NO concentration induced by increasing concentrations of carbachol (CCh) in rat superior mesenteric arterial segments in the absence and presence of LPS and MnTMPyP. The points are means $\pm$s.e.m., $n \geqslant 3$. $^{\#\#\#}P<0.001$ indicates a significant difference in relaxation following treatment with LPS and MnTMPyP.

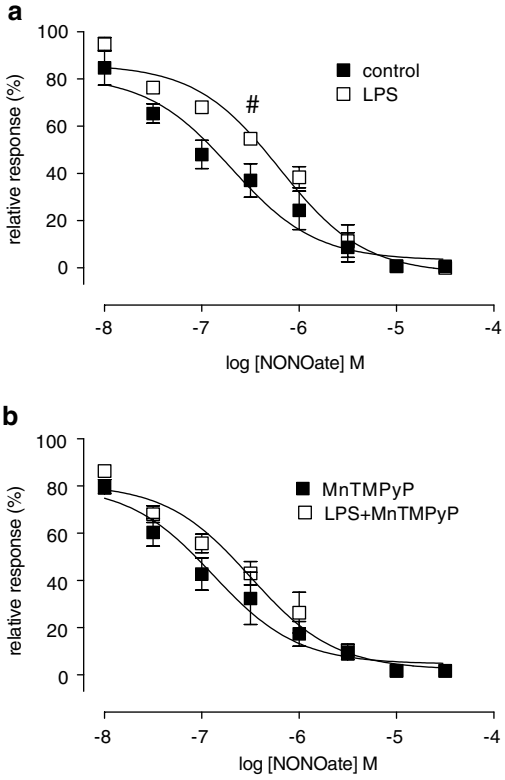

Figure 7 Concentration–respone curves showing that the relaxation induced by the NO donor spermine NONOate (NONOate) in rat superior mesenteric arterial segments was (a) impaired following treatment with LPS, and that (b) coincubation with MnTMPyP prevented this inhibition. The points are means $\pm$s.e.m., $n \geqslant 3$. $^{\#}P<0.05$ indicates significant differences in relaxation following treatment with LPS.

Discussion

Effect of 100 μM extracellular L-arginine on NO formation by eNOS

In the absence of extracellular L-arginine, carbachol was able to release a large amount of NO and induce full relaxation of the precontracted superior mesenteric artery ring. Nevertheless, addition of 100 μM L-arginine to the Krebs' solution substantially enhanced carbachol-induced NO formation. This additional NO was functionally significant, since carbachol-induced relaxation at its EC_{50} was more than doubled. Since this artery produces an excess of NO leading to complete relaxation in L-arginine-free Krebs' solution, further boosting the NO production by addition of 100 μM L-arginine did not change the *maximal* relaxation. However, when arterial segments were partly endothelium-denuded, L-arginine was observed to enhance carbachol relaxation over the concentration range for carbachol. Thus, the functional effects of increasing NO formation are more important when NO output is impaired. This is likely to be the explanation for the reports in the literature that L-arginine enhances the vasodilation in arteries only when the endothelium is damaged or dysfunctional (Drexler *et al*., 1991; Schwarzacher *et al*., 1997; Böger *et al*., 1998; Brandes *et al*., 2000; Tousoulis *et al*., 2001).

The present study is the first to demonstrate that the addition of L-arginine to a carbachol-stimulated arterial segment causes a direct increase in NO concentration and further enhances relaxation. The time course of L-arginine's enhancement of NO (3 min) is consistent with an enzymatic conversion and argues against an instantaneous chemical reaction.

The effect of incubation with SOD suggests that the inhibitory action of 10 mM L-arginine may be caused by superoxide generation. However, the augmenting action of 100 μM L-arginine is not the result of superoxide scavenging.

Requirement of cationic transporter and caveolin for extracellular L-arginine access to eNOS

The augmentation of carbachol's action by 100 μM L-arginine was blocked by two inhibitors of the cationic amino-acid transporter (L-lysine and gramicidin) and by incubation with a caveolin antibody. Thus, it is likely that the additional NO formed when L-arginine is administered to the Krebs' solution, requires uptake of L-arginine into endothelial cells using the cationic amino-acid transporter. There is evidence that some eNOS is located within caveolae, possibly isolating it from the large internal store of L-arginine (Anderson, 1993). Caveolins are specific membrane proteins located in caveolae that may regulate eNOS (Garcia-Cardena *et al*., 1996) and may link it to the cationic amino-acid transporter. The effect of anticaveolin in the present experiments suggests that some functional eNOS is located at the caveoli. Anticaveolin may act by uncoupling caveolin from the cationic amino-acid transporter, or may prevent access of L-arginine to the transporter site or may perhaps prevent a necessary conformational change in caveolin/transporter complex. In the absence of extracellular L-arginine, none of the interventions (L-lysine, gramicidin or anti-caveolin) had any effect on carbachol-induced relaxation, suggesting that adequate NO output can be maintained without reuptake or transmembrane cycling of endogenous L-arginine.

Evidence for two functional groups of eNOS in vascular endothelial cells

The present experiments are consistent with the existence of two functional groups of eNOS in the vascular endothelium. In the absence of extracellular L-arginine, NO was produced in large quantities to give maximal relaxation for the duration of a long *in vitro* experiment, in agreement with data showing that cellular L-arginine concentration (0.1–1 mM) is in excess of the K_m for eNOS (1–10 μM) (Palmer & Moncada, 1989). The accessory pathway can accept extracellular L-arginine and is dependent on caveolin and the cationic amino-acid transporter. This postulated accessory pathway may boost the production of NO, which would be particularly important if NO output is compromised. These results suggest a functional correlate for the observation that eNOS, caveolin and CAT all colocate within endothelial cells and that eNOS and CAT form a complex within the caveolae (McDonald *et al*., 1997). Another study found two locations for eNOS, segregating either with fractions of the Golgi membrane or with caveolin-enriched plasma membrane (Fulton *et al*., 2002).

The concentration of L-arginine (100 μM) that was effective in enhancing NO output in this study is very similar to the plasma L-arginine concentration of Sprague–Dawley rats, 150–200 μM (Pieper & Dondlingere, 1997). This may suggest that extracellular L-arginine is physiologically important, such that there is a requirement of plasma concentrations of L-arginine for maximal NO production. Most studies on isolated preparations do not supplement the extracellular medium with L-arginine and therefore NO release and subsequent relaxation may be suboptimal. However, caution must be observed following treatment with high concentrations of L-arginine since this may impair NO activity *via* a superoxide-dependent mechanism.

Role of superoxide in the depression of eNOS output by induction of iNOS

Several studies have hypothesised that the interaction between iNOS and eNOS depends on excessive formation of NO by iNOS, which then either downregulates or inactivates eNOS (Schwartz *et al*., 1997; Da Silva-Santos *et al*., 2002). However, in the present study, it was found that addition of L-arginine, 20 min before carbachol, was able to restore NO concentration to normal. This result argues against an impairment developing during the long exposure to endotoxin, as would be required for downregulation of eNOS protein synthesis. It also suggests that the endotoxin-mediated impairment of arterial function is not due to excess NO.

An alternative explanation for the effect of iNOS induction on eNOS function is provided by the observation that addition of L-arginine induced formation of NO in the artery wall (and relaxation), showing that endogenous L-arginine is insufficient to feed the available pool of iNOS. Previous work has shown that when NOS (including iNOS) is starved of saturating concentrations of L-arginine, the enzyme can produce superoxide anion (Xia *et al*., 1996). Thus, we may postulate that the iNOS, induced by endotoxin incubation, generates significant levels of superoxide in the artery wall, and this superoxide

excess is then able to degrade NO chemically. An observation that is consistent with this hypothesis is the finding that overexpression of eNOS protected against the fall in blood pressure and lung injury caused by endotoxin (Yamashita *et al*., 2000). In the latter study, the additional eNOS may be forming enhanced levels of NO that can overcome the superoxide formed from iNOS.

Evidence that superoxide mediates the interaction between iNOS and eNOS in the artery wall is provided by our results with the superoxide-quenching agent, MnTMPyP, which limited the impairment of NO formation and relaxation caused by iNOS induction. Since MnTMPyP is able to cross the cell membrane (unlike exogenously applied authentic SOD), it will be able to quench superoxide formed within the endothelial cell from iNOS. In certain redox environments, MnTMPyP can actually generate superoxide (Mackenzie *et al*., 1999); however, in the protocol employed here, it would appear to have only SOD mimetic properties and indeed has been used successfully to scavenge intracellular superoxide in other mesenteric arteries of the rat (Miller *et al*., 2000).

These conclusions are supported by many published studies. Endotoxaemia has been shown to cause superoxide generation (Yin *et al*., 1998) and accumulation of lipid peroxidation products (Zhang *et al*., 2000). Moreover, nitrotyrosine, a marker for peroxynitrite, which is generated from the combination of superoxide and NO, accumulated in patients with septic shock (Strand *et al*., 2000). Administration of antioxidants has been found to reduce organ dysfunction following endotoxin shock (Zacharowski *et al*., 2000) and to protect the aorta against hyporeactivity and endothelium dysfunction (Zingarelli *et al*., 1997). In endotoxaemia, the attenuated response to the NO donor sodium nitroprusside was normalised by the NO synthase inhibitor L-NAME (Tsuchida *et al*., 1994; Bogle *et al*., 2000). LPS can upregulate other enzymes that are capable of generating superoxide, such as NADPH oxidase (Brandes *et al*., 1999), and this could contribute to superoxide formation following LPS administration.

Change in the action of NO following induction of iNOS

In addition to the impairment of carbachol-induced eNOS activation, the present experiments provide evidence for changes in the action of NO (either generated by carbachol and measured with the microsensor or supplied by the NO donor spermine NONOate). Thus, L-arginine administration (present 20 min before carbachol) restored NO output to control levels but relaxation was still impaired, suggesting a dysfunction of the NO-signalling pathway. However, when L-arginine was present during both the incubation and test periods, the NO output induced a greater relaxation than when the L-arginine was added 20 min before carbachol, from which it can be concluded that impairment of NO signalling occurred during the long period of endotoxin administration. This was confirmed by the addition of L-arginine during incubation with endotoxin (but absent during the carbachol test), which also reduced the effect of endotoxin on NO action, but did not change NO formation. It is possible that L-arginine, which is known to have antioxidant action, may in part produce this effect by protecting against detrimental consequences of superoxide anion generation. Thus, our results suggest a close parallelism between NO synthesis and NO action in the artery wall, both of which were reduced as a result of superoxide formation (not as a result of excess NO formation) from iNOS. However, there is a difference in the time course since inhibition of NO action built up during the 4 h incubation with endotoxin, whereas the inhibition of eNOS could be rapidly reversed.

Conclusions

The present experiments are the first to report measurements of NO concentration in the artery wall following extracellular L-arginine administration, and following induction of iNOS. The experiments show that in the healthy rat superior mesenteric artery, the stimulated release of NO and arterial relaxation is sensitive to extracellular L-arginine concentration. At concentrations close to plasma levels, L-arginine enhances NO output and relaxation in a manner that is stereospecific, independent of antioxidant action, reliant on cellular uptake, dependent on intact caveolin and may be mediated through the activity of an accessory eNOS pathway. In contrast, higher concentrations of L-arginine act to inhibit NO activity and relaxation *via* a superoxide-dependent mechanism. Thus, it may be concluded that alteration of extracellular L-arginine is a regulatory point in the NO pathway in healthy tissue, which validates the rationale for extending L-arginine administration as a therapy in diseased arteries. Induction of iNOS in the artery wall markedly reduced NO formation by eNOS in response to carbachol, which appeared to be mediated by superoxide. The finding that L-arginine restored carbachol-induced NO formation suggests that endothelium depression is due to functional impairment, not overactivity, of NO synthesis.

This work was supported by the British Heart Foundation (PG/1999177).

References

ADAMS, M.R., MCCREDIE, R., JESSUP, W., ROBINSON, J., SULLIVAN, D. & CELERMAJER, D.S. (1997). Oral L-arginine improves endothelium-dependent dilatation and reduces monocyte adhesion to endothelial cells in young men with coronary artery disease. *Atherosclerosis,* **129,** 261–269.

ANDERSON, R.G. (1993). Caveolae: where incoming and outgoing messengers meet. *Proc. Natl. Acad. Sci. U.S.A.,* **1993,** 10909–10913.

ARRIERO, M.M., DE LA PINTA, J.C., ESCRIBANO, M., CELDRAN, A., MUNOZ-ALMEDA, L., GARCIA-CANETE, J., JIMENEZ, A.M., CASADO, S., FARRE, J. & LOPEZ-FARRE, A. (2002). Aspirin prevents *Escherischia coli* lipopolysaccharide- and *Staphylococcus aureus*-induced downregulation of endothelial nitric oxide synthase expression in guinea-pig pericardial tissue. *Circ. Res.,* **90,** 719–727.

BÖGER, R.H., BODE-BÖGER, S.M., THIELE, W., CREUTZIG, A., ALEXANDER, K. & FROLICH, J.C. (1998). Restoring vascular nitric oxide formation by L-arginine improves the symptoms of intermittent claudication in patients with peripheral arterial occlusive disease. *J. Am. Coll. Cardiol.,* **32,** 1336–1344.

BOGLE, R.G., MCLEAN, P.G., AHLUWALIA, A. & VALANCE, P. (2000). Impaired vascular sensitivity to nitric oxide in the coronary microvasculature after endotoxaemia. *Br. J. Pharmac.,* **130,** 118–124.

BRANDES, R.P., BRANDES, S., BOGER, R.H., BODE-BOGER, S.M. & MUGGE, A. (2000). L-arginine supplementation in hypercholesterolemic rabbits normalises leukocyte adhesion to non-endothelial matrix. *Life Sci.,* **66,** 1519–1524.

BRANDES, R.P., KODDENBERG, G., GWINNER, W., KIM, D.Y., KRUSE, H.J., BUSSE, R. & MUGGE, A. (1999). Role of increased production of superoxide anions by NAD(P)H oxidase and xanthine oxidase in prolonged endotoxemia. *Hypertension,* **33,** 1243–1249.

CHOWIENCZYK, P.J., KNEALE, B.J., BRETT, S.E., PAGANGA, G., JENKINS, B.S. & RITTER, J.M. (1998). Lack of effect of vitamin E on L-arginine-responsive endothelial dysfunction in patients with mild hypercholesterolaemia and coronary artery disease. *Clin. Sci.,* **94,** 129–134.

DA SILVA-SANTOS, J.E., TERLUK, M.R. & ASSREUY, J. (2002). Differential involvement of guanylate cyclase and potassium channels in nitric oxide-induced hyporesponsiveness to phenylephrine in endotoxaemic rats. *Shock,* **17,** 70–76.

DREXLER, H., ZEIHER, A.M., MEINZER, K. & JUST, H. (1991). Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet,* **338,** 1546–1550.

DUBOISRANDE, J.L., ZELINSKY, R., ROUDOT, F., CHABRIER, P.E., CASTAIGNE, A., GESCHWIND, H. & ADNOT, S. (1992). Effects of infusion of L-arginine into the left anterior descending coronary artery on acetylcholine-induced vasoconstriction of human atheromatous coronary arteries. *Am. J. Cardiol.,* **70,** 1269–1275.

ERMERT, M., RUPPERT, C., GUNTHER, A., DUNCKER, H.R., SEEGER, W. & EMERT, L. (2002). Cell-specific nitric oxide synthase–isoenzyme expression and regulation in response to endotoxin in intact rat lungs. *Lab. Invest.,* **82,** 425–441.

FISCHER, L.G., HORSTMAN, D.J., HAHNENKAMP, K., KECHNER, N.E. & RICH, G.F. (1999). Selective iNOS inhibition attenuates acetylcholine- and bradykinin-induced vasoconstriction in lipo-polysaccharide-exposed rat lungs. *Anaesthesiology,* **91,** 1724–1732.

FULTON, D., FONTANA, J., SOWA, G., GRATTON, J.P., LIN, M., LI, K.X., MICHELL, B., KEMP, B.E., RODMAN, D. & SESSA, W.C. (2002). Localization of endothelial nitric oxide synthase phosphorylated on serine 1179 and nitric oxide in Golgi and plasma membrane defines the existence of two pools of active enzyme. *J. Biol. Chem.,* **277,** 4277–4284.

GARCIA-CARDENA, G., FAN, R., STERN, D.F., LIU, J. & SESSA, W.C. (1996). Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J. Biol. Chem.,* **271,** 27237–27240.

GARVEY, E.P., OPLINGER, J.A., FURFINE, E.S., KIFF, R.J., LASZLO, F., WHITTLE, B.J.R. & KNOWLES, R.G. (1977). 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase *in vitro* and *in vivo*. *J. Biol. Chem.,* **272,** 4959–4963.

GEVANTMAN, L.H. (1995). In *Handbook of Chemistry and Physics*, ed. Lide, D. p. 6, Boca Raton, FL, USA: CRC Press.

GUNNETT, C.A., CHU, Y., HEISTAD, D.D., LOIHL, A. & FARACI, F.M. (1998). Vascular effects of LPS in mice deficient in expression of the gene for inducible nitric oxide synthase. *Am. J. Physiol.,* **44,** H416–H421.

HWA, J.J., GHIBAUDI, L., WILLIAMS, P. & CHATTERJEE, M. (1994). Comparison of acetylcholine-dependent relaxation in large and small arteries of rat mesenteric vascular bed. *Am. J. Physiol.,* **266,** H952–H958.

KITAZONO, T., FARACI, F.M. & HEISTAD, D.D. (1996). L-arginine restores dilator responses of the basilar artery to acetylcholine during chronic hypertension. *Hypertension,* **27,** 893–896.

MACKENZIE, A., FILIPPINI, S. & MARTIN, W. (1999). Effects of superoxide dismutase mimetics on the activity of nitric oxide in rat aorta. *Br. J. Pharmacol.,* **127,** 1159–1164.

MACKENZIE, A. & MARTIN, W. (1998). Loss of endothelium-derived nitric oxide in rabbit aorta by oxidant stress: restoration by superoxide dismutase mimetics. *Br. J. Pharmacol.,* **124,** 719–728.

MARTINEZ, M.C., MULLER, B., STOCLET, J.C. & ANDRIANTSITOHAINA, R. (1996). Alteration in lipopolysaccharide of the relationship between intracellular calcium levels and contraction in rat mesenteric artery. *Br. J. Pharmacol.,* **118,** 1218–1222.

MCDONALD, K.K., ZHARIKOV, S., BLOCK, E.R. & KILBERG, M.S. (1997). A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the 'arginine paradox'. *J. Biol. Chem.,* **272,** 31213–31216.

MICIELI, G., BOSONE, D., ZAPPOLI, F., MARCHESELLI, S., ARGENTERI, A. & NAPPI, G. (1999). Vasomotor response to CO_2 and L-arginine in patients with severe internal carotid artery stenosis; pre- and post-surgical evaluation with transcranial Doppler. *J. Neurol. Sci.,* **163,** 153–158.

MILLER, A.A., MEGSON, I.L. & GRAY, G.A. (2000). Inducible nitric oxide synthase-derived superoxide contributes to hyperactivity in small mesenteric arteries from a rat model of chronic heart failure. *Br. J. Pharmac.,* **131,** 29–36.

MITOLOCHIEPPA, D., SERIO, M., POTENZA, M.A., MONTAGNANI, M., MANSI, G., PECE, S., JIRILLO, E. & STOCLET, J.C. (1996). Hyporeactivity of mesenteric vascular bed in endotoxin-treated rats. *Eur. J. Pharmacol.,* **309,** 175–182.

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

O'BRIEN, A.J., WILSON, A.J., SIBBALD, R., SINGER, M. & CLAPP, L.H. (2001). Temporal variation in endotoxin-induced vascular hyporeactivity in a rat mesenteric artery organ culture model. *Br. J. Pharmacol.,* **133,** 351–360.

PALMER, R.M. & MONCADA, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.,* **158,** 348–352.

PAPAPETROPOULOS, A., ABOUMOHAMED, G., MARCZIN, N., MURAD, F., CALDWELL, R.W. & CATRAVAS, J.D. (1996). Downregulation of nitrovasodilator-induced cyclic GMP accumulation in cells exposed to endotoxin or interleukin-1 beta. *Br. J. Pharmacol.,* **118,** 1359–1366.

PIEPER, G.M. & DONDLINGERE, L.A. (1997). Plasma and vascular tissue arginine are decreased in diabetes: acute arginine supplementation restores endothelium-dependent relaxation by augmenting cGMP production. *J. Pharmacol. Exp. Ther.,* **283,** 684–691.

SCHOTT, C.A., GRAY, G.A. & STOCLET, J.C. (1993). Dependence of endotoxin-induced vascular hyporeactivity on extracellular L-arginine. *Br. J. Pharmacol.,* **108,** 38–43.

SCHWARTZ, D., MENDONCA, M., SCHWARTZ, I., XIA, YY., SATRIANO, J., WILSON, CB. & BLANTZ, R.C. (1997). Inhibition of constitutive nitric oxide synthase (NOS) by nitric oxide generated by inducible NOS after lipopolysaccharide administration provokes renal dysfunction in mice. *J. Clin. Invest.,* **100,** 439–448.

SCHWARZACHER, S.P., LIM, T.T., WANG, B., KERNOFF, R.S., NIEBAUER, J., COOKE, J.P. & YEUNG, A.C. (1997). Local intramural delivery of L-arginine enhances nitric oxide generation and inhibits lesion formation after balloon angioplasty. *Circulation,* **95,** 1863–1869.

SCOTT, W.S. & NAKAYAMA, D.K. (1998). *Escherichia coli* lipopolysaccharide downregulates soluble guanylate cyclase in pulmonary artery smooth muscle. *J. Surg. Res.,* **80,** 309–314.

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. (Lond.),* **516,** 271–282.

SONG, K.S., SCHERER, P.E., TANG, Z., OKAMOTO, T., LI, S., CHAFEL, M., CHU, C., STAVE KOHTZ, D. & LISANTI, M.P. (1996). Expression of caveolin-3 in skeletal, cardiac, and smooth muscle cells. Caveolin-3 is a component of the sarcolemma and co-fractionates with dystrophin and dystrophin-associated glycoproteins. *J. Biol. Chem.,* **271,** 15160–15165.

STRAND, O.A., LEONE, A., GIERCKSKY, K.E. & KIRKEBOEN, K.A. (2000). Nitric oxide indices in human septic shock. *Crit. Care Med.,* **28,** 2779–2785.

SU, Y., COUCH, M. & BLOCK, E.R. (1997). Substrate inhibition of nitric oxide synthase in pulmonary artery endothelial cells in culture. *Nitric Oxide,* **1,** 469–475.

TOUSOULIS, D., DAVIES, G.J., TENTOLOURIS, C., GOUMAS, G., STEFANADIS, C. & TOUTOUZAS, P. (2001). Vasomotor effects of L- and D-arginine in stenotic atheromatous coronary plaque. *Heart,* **86,** 296–301.

TSUCHIDA, S., HIRAOKA, M., SUDO, M., KIGOSHI, S. & MURAMATSU, I. (1994). Attenuation of sodium nitroprusside responses after prolonged incubation of rat aorta with endotoxin. *Am. J. Physiol.,* **36,** H2305–H2310.

XIA, Y., DAWSON, V.L., DAWSON, T.M., SNYDER, S.H. & ZWEIER, J.L. (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 6770–6774.

YAMASHITA, T., KAWASHIMA, S., OHASHI, Y., OZAKI, M., UEYAMA, T., ISHIDA, T., INOUE, N., HIRATA, K., AKITA, H. & YOKOYAMA, M. (2000). Resistance to endotoxin shock in transgenic mice overexpressing endothelial nitric oxide synthase. *Circulation,* **101,** 931–937.

YIN, K., HOCK, C.E., TAHAMONT, M. & WONG, P.Y.K. (1998). Time-dependent cardiovascular and inflammatory changes in acute endotoxaemia. *Shock,* **9,** 434–442.

ZACHAROWSKI, K., OLBRICH, A., CUZZOCREA, S., FOSTER, S.J. & THIEMERMANN, C. (2000). Membrane-permeable radical scavenger, tempol, reduces multiple organ injury in a rodent model of gram-positive shock. *Crit. Care Med.,* **28,** 1953–1961.

ZHANG, C.J., WALKER, L.M. & MAYEUX, P.R. (2000). Role of nitric oxide in lipopolysaccharide-induced oxidant stress in the rat kidney. *Biochem. Pharmacol.,* **59,** 203–209.

ZHARIKOV, S.I. & BLOCK, E.R. (1998). Characterization of L-arginine uptake by plasma membrane vesicles isolated from cultured pulmonary artery endothelial cells. *Biochem. Biophys. Acta,* **1369,** 173–183.

ZINGARELLI, B., DAY, B.J., CRAPO, J.D., SALZMAN, A.L. & SZABO, C. (1997). The potential role of peroxynitrite in the vascular contractile and cellular energetic failure in endotoxaemic shock. *Br. J. Pharmac.,* **120,** 259–267.

(Received April 11, 2003
Revised May 12, 2003
Accepted May 13, 2003)