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The role of induction of nitric oxide synthesis in the altered responses of jugular veins from endotoxaemic rabbits

[1]Patrick Vallance, Richard M.J. Palmer & [2]Salvador Moncada

Wellcome Research Laboratories, Beckenham, Kent

1 Endotoxaemia is characterized by hypotension, peripheral vasodilatation and a reduced response to vasoconstrictors. Clinical studies have indicated that venodilatation contributes to the haemodynamic changes, although there is no direct evidence for abnormal venous reactivity. In the present study, the role of nitric oxide (NO) in modifying the responses of rabbit isolated jugular veins was examined *in vitro*, 4 h after intravenous injection of endotoxin.

2 Treatment with endotoxin reduced the contractile response to the thromboxane-mimetic, 9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U-46619). This affect was endothelium-independent. The response was partially restored by the NO synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA 300 μM).

3 Jugular veins from control animals did not contract to L-NMMA whereas those from endotoxin-treated animals showed concentration-dependent contractions to L-NMMA. The contractions produced by L-NMMA were reversed by L-arginine but not by D-arginine. Treatment of the animals with dexamethasone (4 mg kg^{-1}) 1 h prior to administration of endotoxin significantly attenuated the response to L-NMMA.

4 The response to sodium nitroprusside did not differ significantly between veins from control and endotoxin-treated animals. Endothelial denudation did not alter the sensitivity of the veins to sodium nitroprusside. Acetylcholine produced endothelium-dependent relaxations which were similar in veins from control and endotoxin-treated animals.

5 The results of this study demonstrate that intravenous administration of endotoxin induces hyporesponsiveness to U-46619 in jugular veins. This effect is mediated, at least in part, by the induction of NO synthesis in smooth muscle. The induction is prevented by prior treatment with dexamethasone.

Keywords: Nitric oxide; arginine; endotoxin; septic shock; veins

Introduction

Endotoxaemia is a major clinical problem; the mortality rate is high and treatment is often ineffective. The syndrome is characterized by vasodilatation, a diminished response to vasoconstrictors, and hypotension (Suffredini *et al.*, 1989; Parillo *et al.*, 1990). Tissue perfusion is eventually compromised by the low blood pressure and this contributes to end organ damage and, ultimately, to death (Ziegler *et al.*, 1991).

Recently, it has been shown that the hypotension (Julou-Schaeffer *et al.*, 1990; Thiemermann & Vane, 1990) and arterial hyporeactivity (Julou-Schaeffer *et al.*, 1990; Rees *et al.*, 1990) produced by endotoxin can be accounted for by increased vascular synthesis of the potent vasodilator nitric oxide (NO). Endotoxin and certain cytokines lead to the induction of a calcium-independent NO synthase in vascular endothelium (Radomski *et al.*, 1990) and smooth muscle (Rees *et al.*, 1990; Busse & Mulsch, 1990; Fleming *et al.*, 1991). This induction can be prevented by dexamethasone or cycloheximide (Rees *et al.*, 1990; Radomski *et al.*, 1990) and, once established, the effects can be reversed with N^G-monomethyl-L-arginine (L-NMMA), a specific inhibitor of NO synthase (Rees *et al.*, 1990; Julou-Schaeffer *et al.*, 1990).

Whether there are changes in the reactivity of veins in sepsis is less clear. Clinical observations suggest that, despite increased levels of vasoconstrictors, the veins are inappropriately dilated in sepsis (Bradley *et al.*, 1945), and initial treatment consists of rapid expansion of intravascular volume to maintain right atrial filling pressure (Parillo *et al.*, 1990; Anon., 1990). In this study we have examined the effects of endotoxaemia on the responses of rabbit jugular veins to the vasoconstrictor thromboxane-mimetic, U-46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$), and have sought to establish whether NO contributes to the observed changes.

Methods

Studies were performed on New Zealand White rabbits of either sex weighing 2–2.5 kg. Internal jugular veins were removed after induction of terminal anaesthesia with sodium pentobarbitone. Some animals received intravenous (i.v.) endotoxin (500 μg) 4 h before removal of tissues; some received dexamethasone (4 mg kg^{-1}, i.v.) 1 h prior to endotoxin. The veins were placed in ice-cold Krebs solution containing indomethacin (5 μM) and dissected free of surrounding fat and connective tissue. In some experiments the endothelium was removed by inserting a roughened catheter into the lumen of the vessel and gently rotating the vessel between thumb and forefinger. Absence of functional endothelium was confirmed by failure to relax to acetylcholine.

Each vein was cut into 2 rings of 3 mm length. Rings were suspended between 2 hooks connected to a transducer for the measurement of isometric force. The preparations were suspended in a 20 ml organ bath filled with warmed (37°C) oxygenated (95% O_2, 5% CO_2) Krebs solution containing indomethacin (5 μM) and, in some experiments, cycloheximide (100 μM). This concentration of cycloheximide inhibits protein synthesis in the rabbit aorta by 85% (Deblois *et al.*, 1988). Basal tension was initially set at 1.5 g and adjusted to 0.75 g after 1 h equilibration. This tension gives optimal contractile responses in these vessels (Leff *et al.*, 1987).

[1] Permanent address: Department of Pharmacology & Clinical Pharmacology, St George's Hospital Medical School, London SW17 0RE.
[2] Author for correspondence.

Contraction studies

Concentration-response curves were constructed for U-46619 (10^{-10}–10^{-6} M) in the presence or absence of L-NMMA (300 μM added 10 min before addition of U-46619). In all experiments basal tension was again adjusted to 0.75 g before adding U-46619.

In a separate series of experiments the effects of L-NMMA or D-NMMA (0.5×10^{-5}–3.2×10^{-4}M) were tested on vessels pre-contracted with U-46619 (10^{-9} M). Induction of a small amount of tone with a contractile agonist enhances the response of vessels to L-NMMA (Rees *et al.*, 1989).

Relaxation studies

Concentration-response curves were constructed for acetylcholine (0.5×10^{-9}–10^{-6} M), sodium nitroprusside (10^{-9}–10^{-5} M) and L-arginine (10^{-4}–10^{-3} M) in rings precontracted by U-46619 (2×10^{-8} M; EC_{90}).

In a separate series of experiments the effects of L-arginine and D-arginine (10^{-4}–10^{-3} M) were tested on rings pre-contracted with L-NMMA (300 μM).

Drugs

The following drugs were used: acetylcholine bromide (Sigma), L- and D-arginine hydrochloride (Sigma), bacterial endotoxin (*S. Typhosa*; Difco Ltd), cycloheximide (Sigma), dexamethasone sodium phosphate (Decadron Shock-Pak, Merck Sharp & Dohme), indomethacin (Sigma), L- and D-N^G-monomethyl-L-arginine (synthesized by Dr H.F. Hodson), sodium nitroprusside (Sigma), U-44619 (Cayman Chemicals). U-44619 was dissolved in ethanol and diluted in saline. All other drugs were dissolved in saline immediately prior to use.

Statistics

Results are expressed as mean ± s.e.mean and compared by Student's *t* test for paired or unpaired observations as appropriate, where $P<0.05$ is considered significant. For each experiment, values for EC_{30}, EC_{50} and EC_{90} were obtained from sigmoid logistic curves.

Results

After 1–2 h, animals treated with endotoxin became apathetic, respiratory rate increased and diarrhoea was common. Animals treated with dexamethasone appeared healthy after endotoxin. One animal treated with endotoxin died after 2 h and was excluded from analysis.

Contraction studies

The potency of, and maximum response to U-46619 was reduced significantly in veins from endotoxin-treated animals irrespective of endothelial integrity (Figure 1a,b). In veins with functionally intact endothelium the EC_{30} increased approximately fourfold, from $5.8 \pm 1.34 \times 10^{-10}$ M (control animals; $n=8$) to $2.35 \pm 0.58 \times 10^{-9}$ M (endotoxin-treated animals; $n=7$; $P<0.01$). This effect of endotoxin was attenuated by L-NMMA (300 μM): in veins with functionally intact endothelium L-NMMA decreased the EC_{30} for U-46619 from $2.53 \pm 0.44 \times 10^{-9}$ M ($n=11$) to $8.41 \pm 2.48 \times 10^{-10}$ M ($P<0.05$; Figure 2a) and in veins without functionally intact endothelium, L-NMMA decreased the EC_{30} for U-46619 from $2.85 \pm 0.7 \times 10^{-9}$ M ($n=7$) to $7.34 \pm 1.65 \times 10^{-10}$ M ($P<0.05$; Figure 2b). In keeping with previous studies (Martin *et al.*, 1992) inhibition of NO synthase did not increase the potency of U-46619 in veins from control animals ($n=5$ endothelium functionally intact; data not shown).

In pre-contracted (U-46619 10^{-9} M) veins from endotoxin-treated animals, L-NMMA ($EC_{90} = 2.1 \pm 0.4 \times 10^{-5}$ M), but not D-NMMA (data not shown), caused further contractions (Figure 3a) which were endothelium-independent. *Ex vivo* incubation with cycloheximide (100 μM for 2 h before dose-response curves and throughout the remainder of study) did not alter the contractile response to L-NMMA ($n=3$; data not shown). Pretreatment of the animals with dexamethasone (4 mg kg^{-1} i.v. given 1 h before endotoxin) significantly inhibited the contractile response to L-NMMA ($n=4$; Figure 3). L-NMMA (300 μM) also caused slowly-developing endothelium-independent contractions of veins from endotoxin-treated animals which were not pre-contracted with U-46619 ($n=8$). In veins taken from control animals, L-NMMA did not cause significant contractions ($n=7$).

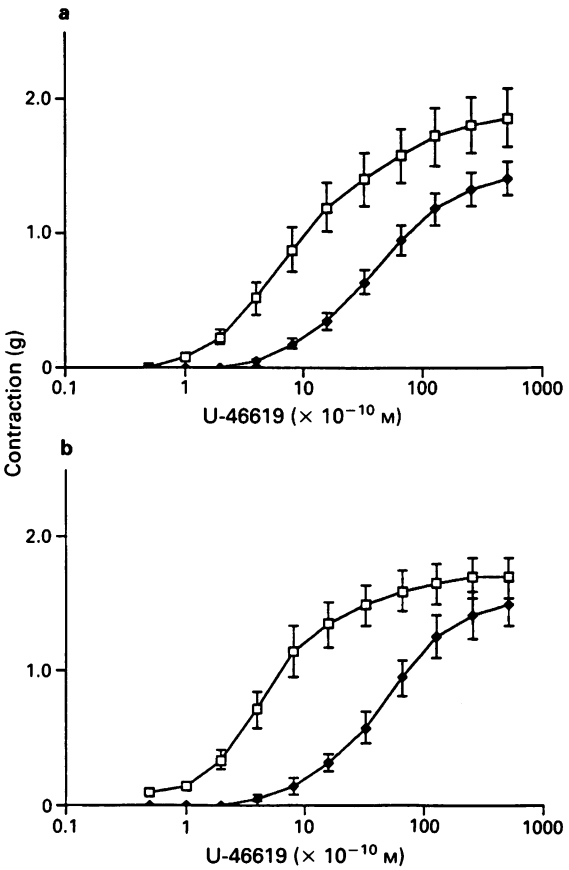

Figure 1 (a) Contraction to U-46619 in endothelium-intact veins taken from endotoxin-treated (◆; $n=7$) and control (□; $n=8$) rabbits; (b) contraction to U-46619 in endothelium-denuded veins taken from endotoxin-treated (◆; $n=7$) and control (□; $n=4$) rabbits.

Relaxation studies

Acetylcholine caused endothelium-dependent relaxation which did not differ significantly between veins taken from endotoxin-treated ($EC_{50} = 6.1 \pm 2.1 \times 10^{-9}$ M) and control animals ($EC_{50} = 4.2 \pm 0.7 \times 10^{-9}$ M; Figure 4).

Sodium nitroprusside caused concentration-dependent relaxations which were not significantly different in endothelium-intact and endothelium-denuded vessels. The potency of sodium nitroprusside did not differ significantly between veins taken from control (EC_{50} intact vein $= 9.11 \pm 1.1 \times 10^{-8}$ M; EC_{50} denuded vein $= 9.87 \pm 4.3 \times 10^{-8}$ M) and endotoxin-treated animals (EC_{50} intact vein $= 9.65 \pm 6.2 \times 10^{-8}$ M; EC_{50} denuded vein $= 2.71 \pm 1.3 \times 10^{-7}$ M; Figure 4).

Veins taken from endotoxin-treated animals which were precontracted with L-NMMA (300 μM) showed dose-depen-

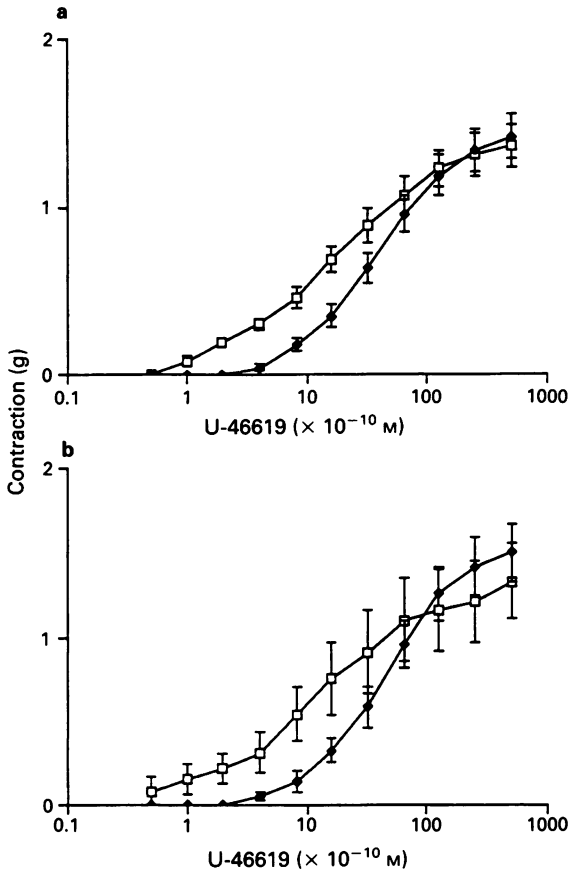

Figure 2 (a) Contraction to U-46619 in endothelium-intact veins from endotoxin-treated animals in the presence (□) and absence (◆) of N^G-monomethyl-L-arginine (L-NMMA, 300 μM); (b) contraction to U-46619 in endothelium-denuded veins from endotoxin-treated animals in the presence (□) and absence (◆) of L-NMMA (300 μM).

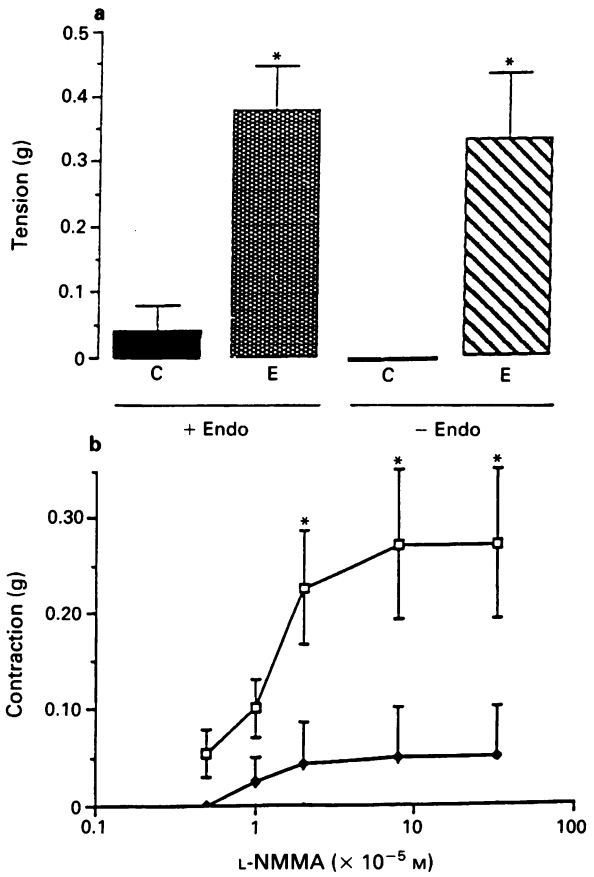

Figure 3 (a) The effects of N^G monomethyl-L-arginine (L-NMMA, 300 μM) on basal tone in endothelium-intact (+ Endo) and endothelium-denuded (− Endo) veins from endotoxic (E; $n = 8$) and control (C; $n = 7$) rabbits. $^*P < 0.01$. Responses shown are maxima. (b) Concentration-response to L-NMMA in endothelium-intact veins taken from endotoxic rabbits (□; $n = 8$), and endotoxic rabbits pretreated with dexamethasone (◆; $n = 4$). The veins were partially contracted with U-46619 (10^{-9} M) prior to addition of L-NMMA. Significant difference from baseline $^*P < 0.05$.

dent relaxations to L-arginine, but not D-arginine ($n = 5$; data not shown). Full relaxation was seen in response to 400 μM L-arginine. In contrast, no significant relaxation to L-arginine was seen when veins from endotoxin-treated animals were precontracted with U-46619 ($n = 3$ endothelium functionally intact; $n = 3$ endothelium functionally absent; data not shown).

Discussion

Bacterial endotoxin, and certain cytokines, diminish arterial contractility (McKenna *et al.*, 1989; Beasley *et al.*, 1990). Recently, it has been demonstrated that this effect is mediated by induction of a calcium-independent NO synthase within vascular endothelial (Radomski *et al.*, 1990) and smooth muscle cells (Rees *et al.*, 1990; Busse & Mulsch, 1990; Joulou-Schaeffer *et al.*, 1990; Fleming *et al.*, 1991). The results of the present study, using rabbit isolated jugular veins, demonstrate that the contractile responses of veins taken from endotoxin-treated rabbits are also impaired and that this impairment is associated with induction of NO synthesis.

Evidence for reduced contractile responses in veins taken from endotoxaemic rabbits is provided by the experiments using U-46619, a thromboxane-mimetic. Veins removed 4 h after intravenous injection of bacterial endotoxin showed diminished contraction to U-46619. The functional abnormality appears to lie in the vascular smooth muscle since the attenuation of the contractile response was the same in endothelium-intact and endothelium-denuded preparations.

The experiments using L-NMMA, a stereospecific inhibitor of NO synthesis (Palmer *et al.*, 1988a,b; Rees *et al.*, 1989) demonstrate that the diminished contraction to U-46619 is associated with induction of continuous NO synthesis. Veins from endotoxin-treated animals showed a dose-dependent contraction to L-NMMA, whereas those from control animals did not. In addition, L-NMMA partially restored the potency of U-46619 in veins from endotoxin-treated animals.

Our finding that L-NMMA, but not D-NMMA, caused dose-dependent contraction of veins from endotoxin-treated animals suggests that under certain pathological conditions, such as endotoxic shock, veins may synthesize NO continuously and that this modifies venous tone. The observation that L-NMMA did not contract veins from control animals is in keeping with previous reports (Leff *et al.*, 1987; Giles *et al.*, 1990; Martin *et al.*, 1992) suggesting that these veins, like many others (Vallance *et al.*, 1989; Ekelund & Mellander, 1990; Yang *et al.*, 1991), do not normally synthesize NO continuously.

The appearance of continuous synthesis of NO in response to endotoxin and the inhibition of this by pretreatment with dexamethasone, suggests induction of NO synthase (Radomski *et al.*, 1990; Rees *et al.*, 1990). The effects of L-NMMA were endothelium-independent, indicating that the induction is occurring in the venous smooth muscle. However, the precise nature of the NO synthase induced in the venous smooth muscle remains to be determined and we do not yet

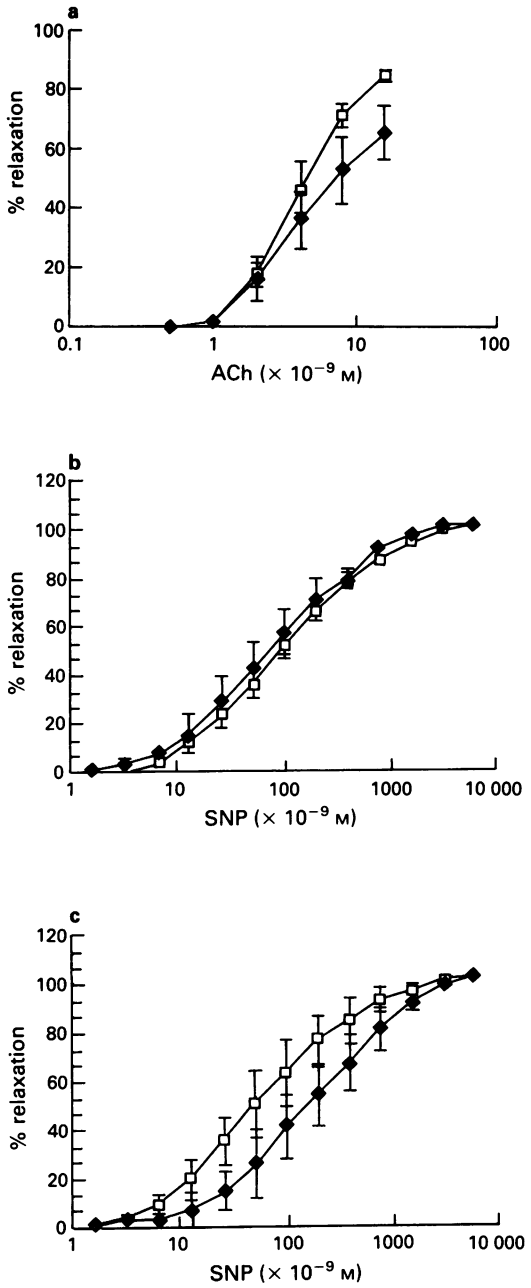

Figure 4 (a) Response to acetylcholine (ACh) in veins from control (□) and endotoxic (◆) rabbits ($n = 4$); (b) response to sodium nitroprusside (SNP) in endothelium-intact (□) and denuded (◆) veins from control rabbits ($n = 4$); (c) response to sodium nitroprusside (SNP) in endothelium-intact (□) and denuded (◆) veins from endotoxin-treated rabbits ($n = 4$).

know whether endotoxin also causes induction of NO synthase in venous endothelium. Incubation of the veins with cycloheximide (100 μM for 2 h *in vitro*), when protein synthesis would be expected to be inhibited by 85% (Deblois *et al.*, 1988), did not alter the responses, indicating that, once induced, the enzyme remains active for several hours without the need for further protein synthesis.

The diminished response to U-46619 seen in veins from endotoxin-treated animals was only partially restored by L-NMMA. This might be due to incomplete inhibition of NO synthesis, and, in support of this, it has been shown that inhibitors of NO synthase do not fully inhibit the NO-mediated relaxant response to acetylcholine in these veins (Martin *et al.*, 1992). Alternatively, additional mechanisms may also contribute to the diminished contractile response. These might include tachyphylaxis to thromboxane A_2 (TXA_2), levels of which rise in endotoxaemia (Cook *et al.*, 1980), induction of other local vasoactive mediators in the wall of the vein, or the onset of irreversible tissue damage. Any contribution by cyclo-oxygenase products to the responses seen was excluded by adding indomethacin to the Krebs solution.

No relaxation was seen when L-arginine was added to vessels from endotoxin-treated rabbits precontracted by U-46619. This is in contrast to studies in arteries which have shown a relaxant effect of L-arginine once the calcium-independent NO synthase has been induced (Rees *et al.*, 1990; Julou-Schaeffer *et al.*, 1990). The reasons for this discrepancy are not yet known but our results suggest that even when NO synthase has been induced, levels of arginine are not rate-limiting for NO synthesis in these veins. It is possible that there are arterio-venous differences in the uptake, storage or utilization of arginine.

In arteries, basal release of NO from the constitutive endothelial NO synthase appears to down-regulate the guanylate cyclase and diminish the sensitivity of smooth muscle to NO and exogenous nitrovasodilators (Moncada *et al.*, 1991). Lack of basal synthesis of NO by venous endothelia may explain the relative venoselectivity of the nitrovasodilators (Collier *et al.*, 1978; Moncada *et al.*, 1991; Benjamin & Vallance, 1991). It might be expected that once continuous NO synthesis has been induced in the veins, their sensitivity to nitrovasodilators would decrease. However, induction of basal synthesis of NO for 4 h did not alter the sensitivity of these vessels to sodium nitroprusside or to acetylcholine, suggesting that longer exposure to NO might be necessary to down-regulate the guanylate cyclase in these vessels.

Circulating levels of a variety of vasoconstrictors, including TXA_2, are increased in endotoxic shock (Cook *et al.*, 1980) and yet there is inappropriately low venous tone (Bradley *et al.*, 1945). Our findings suggest that this may be due to local changes in the blood vessel wall leading to venodilatation and hypo-responsiveness to vasoconstrictors. Induction of NO synthase by endotoxin decreases venous tone and the response to U-46619. If other veins behave similarly to the rabbit jugular veins, significant venous pooling and a postural fall in arterial pressure would follow induction of NO synthase.

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