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The role of nitric oxide in the responses of the ovine digital artery to vasoactive agents and modification of these responses by endotoxin and cytokines

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1 Laminitis, an important cause of lameness in domestic ungulates, occurs as a result of altered digital perfusion. Endotoxin and cytokines may mediate the vascular derangements observed through alterations in nitric oxide production. In this study, the vascular responses of the isolated ovine digital artery were examined and the influence of endotoxin and cytokines investigated.

2 Neither removal of the endothelium nor incubation with N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) altered the response to phenylephrine (PE, 1 nM to 300 μM). Indomethacin (10 μM) decreased PE log EC_{50} from -6.22 ± 0.08 to -6.55 ± 0.07. Acetylcholine (1 nM to 1 mM) and bradykinin (BK, 100 pM to 3 μM) induced endothelium-dependent relaxation. Bradykinin-induced relaxation was reduced by L-NAME, E_{max} falling from -61.7 ± 7.4 to $-34.0\pm2.1\%$. Addition of indomethacin further reduced BK E_{max} to $-9.6\pm2.8\%$. Sodium nitroprusside (1 nM to 300 μM) produced endothelium-independent relaxation that was unaffected by L-NAME or indomethacin.

3 Following a 6 h incubation with endotoxin (3 μg ml^{-1}), arterial responses to PE and BK did not differ from polymyxin B-treated controls (10 μg ml^{-1}). Arteries incubated for 6 h with interferon-γ (IFN-γ, 10 ng ml^{-1}) and tumour necrosis factor-α (TNF-α, 5 ng ml^{-1}) exhibited greater relaxation to BK ($E_{max}-50.0\pm5.1\%$) than polymyxin B-treated controls ($E_{max}-33.1\pm4.0\%$), but did not differ in their response to PE.

4 Prolonged incubation (16 h) with endotoxin (3 μg ml^{-1}) did not alter the response to PE, however incubation with IFN-γ (10 ng ml^{-1}), TNF-α (5 ng ml^{-1}) and interleukin-1β (20 ng ml^{-1}) for 16 h increased PE log EC_{50} from -6.44 ± 0.09 to -6.10 ± 0.11.

5 Nitric oxide is an important mediator of endothelium-dependent relaxation in ovine digital arteries but does not modulate PE-induced vasoconstriction. Incubation with cytokines decreased the sensitivity of digital arteries to PE.

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Keywords: Endothelium; nitric oxide; digital artery; cyclo-oxygenase; endotoxin; tumour necrosis factor-α; interferon-γ; interleukin-1β; laminitis

Abbreviations: ACh, acetylcholine; BK, bradykinin; $CaCl_2$, calcium chloride; CO_2, carbon dioxide; COX, cyclo-oxygenase; EC_{50}, effective concentration 50%; E_{max}, maximum response; EU, endotoxin units; IFN-γ, interferon-γ; IL-1β, interleukin 1β; KCl, potassium chloride; KH_2PO_4, potassium dihydrogen phosphate; L-NAME, N^G-nitro-L-arginine methyl ester; M, moles per litre; $MgSO_4$, magnesium sulphate; mRNA, messenger ribonucleic acid; NaCl, sodium chloride; $NaHCO_3$, sodium hydrogen carbonate; NO, nitric oxide; NOS, nitric oxide synthase; PE, phenylephrine; s.e.mean, standard error of the mean; SN, sodium nitroprusside; TNF-α, tumour necrosis factor-α

Introduction

Laminitis is an important cause of lameness in domestic ungulates. In such species, the third phalanx is supported within the hoof by a system of interdigitating dermal and epidermal laminae (Pollitt, 1992). Disruption of this interdigitation occurs in laminitis, allowing downward rotation of the third phalanx in severe cases. Histopathological studies show changes consistent with laminar ischaemia (Roberts *et al*., 1980), therefore it is generally accepted that laminitis is a consequence of altered blood flow to the distal limb (Hood *et al*., 1993), although the precise mechanism has yet to be elucidated. Factors such as intense vasoconstriction, arteriovenous shunting, microvascular thrombosis and oedema have been proposed as causes of ischaemia (Moore & Allen, 1996). Those that favour the vasoconstriction hypothesis have likened laminitis to Raynaud's disease in man (Hood *et al*., 1990), in which arterial spasm causes digital ischaemia. In Raynaud's disease, a period of reactive hyperaemia follows the ischaemic phase and is associated with pain, warmth and throbbing of the digits. Similar clinical signs, i.e. warm, painful hooves and pulsating digital arteries, are seen in laminitic horses and it has been suggested that this represents the reperfusion phase, the preceding ischaemic episode going undetected clinically (Hood *et al*., 1990). More meaningful comparison requires a detailed knowledge of the pathogenesis of both conditions, a knowledge that is at present incomplete.

Nitric oxide (NO) has been shown to regulate blood flow to a number of regional vascular beds, including the limbs (Vallance *et al*., 1989; White *et al*., 1993) and alterations in its production could have a significant impact on digital blood flow. Therefore, the initial aim of this study was to investigate the role of NO in the modulation of digital artery tone. In this

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study, the sheep was chosen as an ungulate model and the responses of the ovine digital artery were investigated.

Endotoxaemia appears to predispose to the development of clinical laminitis and so has been implicated in the pathogenesis of the condition (Blanchard *et al*., 1987). Baxter (1995) demonstrated potentiation of noradrenaline-induced vasoconstriction in digital arteries removed from endotoxin-treated horses. The author suggested that this enhanced vasoconstriction was a consequence of reduced NO production and furthermore, that it could account for the reduced perfusion of the digit observed in laminitis. However, studies of the effect of endotoxin on equine digital vessels *in vitro*, have generally failed to demonstrate any change in vascular reactivity (Baxter *et al*., 1989; Bailey & Elliott, 1996). Many of the deleterious effects of endotoxin are mediated by proinflammatory cytokines (Tracey *et al*., 1986; Okusawa *et al*., 1988). In another study, Baxter (1994) exposed equine digital arteries to tumour necrosis factor-α and documented similar changes to those observed in arteries from endotoxaemic horses.

The effect that endotoxin and cytokines exert on vascular function and more specifically NO production has a wider significance. Endotoxin-induced expression of nitric oxide synthase (NOS) is believed to mediate, at least in part, the vascular hyporesponsiveness that is a feature of septic shock (Beasley, 1990; Fleming *et al*., 1991). Whilst this phenomenon has been readily demonstrated in isolated rodent vessels or cells (Beasley *et al*., 1991), it has proved difficult to demonstrate in tissues from other species, including man (Beasley & McGuiggin, 1994).

Thus, the primary aim of this study was to investigate the regulation of digital blood flow, with particular reference to factors implicated in the development of laminitis, such as NO and endotoxin. In addition, since the vascular reactivity of digital vessels has not been widely investigated *in vitro*, this work may have relevance as a comparative study of digital vascular physiology.

Some of these results have been presented as abstracts to the British Pharmacological Society (Pawson *et al*., 1997a,b).

Methods

Collection and preparation of tissues

Sheep digital arteries were obtained from a local abattoir. Distal forelimbs, disarticulated at the carpus, were collected from sheep, recently killed by stunning and exsanguination. Prior to transport, the median artery was cannulated and the more distal digital arteries were flushed *in situ* with refrigerated oxygenated Krebs solution. In the laboratory, the skin of the palmar aspect of the distal limb was reflected to reveal the palmar common digital artery, a section of which was dissected free, cleared of loose connective tissue and cut into rings, 2 – 3 mm long. Where required, arterial rings were denuded of endothelium by gently rubbing the internal surface of the artery with a wooden cocktail stick (Elliott *et al*., 1994). The efficiency of this procedure was assessed by examination of arterial sections stained for Von Willebrand's factor, which is associated specifically with endothelial cells (Gariano *et al*., 1996).

Measurement of isometric tension in isolated digital arteries

Digital arterial rings were mounted between stainless steel wires in 10 ml tissue baths containing warm (30°C), oxygenated Krebs. A temperature of less than 37°C was selected to reduce spontaneous activity of the vascular smooth muscle (Elliott *et al*., 1994). The lower wire was fixed in position, whilst the upper wire was attached to an FT03 force-displacement transducer (Grass Instrument Company, MA, U.S.A.), which measured isometric tension. The output from each of the transducers was relayed *via* a pre-amplifier to a four channel *Multitrace* pen recorder (Lectromed U.K. Ltd., Letchworth, Hertfordshire, U.K.). In a small number of experiments a polygraph (*Grass Model 7E*, Grass Instrument Company, MA, U.S.A.) was used in place of the pre-amplifier and pen recorder. Prior to each experiment the transducers were calibrated using a selection of standard weights.

Once suspended, the arterial rings were stretched to a tension of 2 g and allowed to equilibrate for 2 h. This degree of passive stretch was chosen on the basis of preliminary experiments, which showed phenylephrine (PE) responses to be optimal at a resting tension of 2 g. After equilibration, vessel viability was assessed by adding PE (1 μM), those vessels that produced less than 2 g of tension in response to this dose were not used. The vessels were then washed with fresh Krebs solution to allow tension to fall to baseline before beginning an experiment. On completion of an experiment, all arterial rings were removed from the tissue bath and weighed whilst still wet.

Effect of endothelium removal

Cumulative concentration-response curves to PE (1 nM to 300 μM) were constructed in intact and endothelium-denuded digital arterial rings. Once the dose-response curves were completed the vessels were washed several times with fresh Krebs solution and tension was allowed to fall to baseline. All contractile responses were expressed as tension in g g^{-1} tissue wet weight.

To investigate vasodilator responses, the arterial rings were first contracted using PE (600 nM). This dose gave approximately 50% of the maximum response, as determined above. Once the contraction stabilized, cumulative concentration-response curves to bradykinin (BK, 100 pM to 3 μM), acetycholine (ACh, 1 nM to 1 mM) and sodium nitroprusside (SN, 1 nM to 300 μM) were constructed in intact and endothelium-denuded arterial rings. As for PE, tension was allowed to stabilize before each new dose of drug was given. The relaxant response to BK failed to stabilize and transient relaxation gave way to contraction, making construction of a cumulative dose-response curve difficult. Dose-response curves for BK were therefore repeated in a non-cumulative fashion, each dose of BK being added to a stable contraction produced by PE, 600 nM. All relaxant responses were expressed as the percentage change in tension, i.e. the tension change (g g^{-1}) as a percentage of the PE-induced tension (g g^{-1}).

Effect of nitric oxide synthase and/or cyclo-oxygenase inhibition

These experiments were conducted in intact arterial rings. The integrity of the endothelium was checked using BK (10 nM), only vessels that relaxed by 10% or more were used further. Arterial rings were pre-incubated for 20 min with (i) no additions; (ii) the non-selective cyclo-oxygenase (COX) inhibitor indomethacin (10 μM); (iii) the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) or (iv) both indomethacin (10 μM) and L-NAME (300 μM). Concentration-response curves to PE (1 nM to 300 μM), BK (300 pM to 100 nM) and SN (1 nM to 30 μM) were then constructed, as described previously.

In a second experiment, BK dose-response curves were compared in digital arteries pre-incubated with (i) no additions; (ii) L-arginine (10 mM); (iii) L-NAME (300 μM) or (iv) both L-arginine (10 mM) and L-NAME (300 μM).

Effect of incubating digital arterial rings with endotoxin

For these investigations, vessels were not discarded if they failed to produce 2 g of tension in response to 10^{-6} M PE, or failed to relax to 10 nM BK. In a preliminary experiment, the effect of the endotoxin-binding agent polymyxin B on digital artery vascular responses was investigated. Arterial rings were pre-incubated for 20 min with (i) no additions or (ii) 10 μg ml^{-1} polymyxin B and dose-response curves to PE (1 nM to 300 μM) and BK (100 pM to 100 nM) were determined.

Subsequently, digital arterial rings were suspended in the tissue bath and bathed in warm (30°C), oxygenated Krebs solution, containing 10 μg ml^{-1} polymyxin B or 3 μg ml^{-1} endotoxin, for 6 h. Following incubation, dose-response curves to PE (1 nM to 300 μM) and BK (300 pM to 100 nM) were obtained. Two samples of Krebs solution, one taken from a control tissue bath (minus polymyxin B) and a second from a tissue bath to which 3 μg ml^{-1} endotoxin had been added, were stored at −20°C prior to being assayed for endotoxin content.

In a second experiment, vessels were stored in refrigerated oxygenated Krebs for approximately 7 h before being transferred to 10 ml *Medium 199* containing either endotoxin (3 μg ml^{-1}) or polymyxin B (10 μg ml^{-1}). The arterial rings were incubated at 37°C for approximately 16 h in an incubator (95% air and 5% CO_2), before being suspended in the tissue bath and equilibrated as previously described. Cumulative concentration-response curves to PE were constructed in both endotoxin (3 μg ml^{-1}) and polymyxin B-treated (10 μg ml^{-1}) arteries.

Effect of incubating digital arterial rings with cytokines

The two experiments used to investigate endotoxin effects were repeated here, substituting cytokines for endotoxin. Thus, dose-response curves to PE (1 nM to 300 μM) and BK (300 pM to 100 nM) were compared in arterial rings pre-incubated for 6 h in warm (30°C), oxygenated Krebs with 10 μg ml^{-1} polymyxin B or interferon-γ (IFN-γ, 10 ng ml^{-1}) and tumour necrosis factor-α (TNF-α, 5 ng ml^{-1}). Also, the PE dose-response curve was examined after a 16 h incubation in *Medium 199* at 37°C, containing either polymyxin B (10 μg ml^{-1}) or the cytokine mixture IFN-γ (10 ng ml^{-1}), TNF-α (5 ng ml^{-1}) and interleukin-1β (IL-1β, 20 ng ml^{-1}).

Drugs and solutions

Acetylcholine chloride, L-arginine hydrochloride, bradykinin acetate, indomethacin, N^G-nitro-L-arginine methyl ester hydrochloride, phenylephrine hydrochloride, polymyxin B sulphate and sodium nitroprusside dihydrate were all purchased from Sigma Chemical Company (Poole, Dorset, U.K.). Solutions of ACh, L-NAME, PE, polymyxin B and SN were prepared on the morning of each experiment by dissolving the drug in sterile phosphate buffered saline (pH 7.4). Solutions were stored on ice, prior to, and during the experiment. Aliquots of SN were wrapped in tin foil to prevent inactivation by sunlight. Stock solutions of the remaining drugs were stored at −20°C and were thawed prior to use. Stock solutions of BK (1 μM) and L-arginine (1 M) were prepared in phosphate buffered saline, whilst indomethacin was initially dissolved in 100% ethanol to give a 10 mM stock solution. This was further diluted to a 1 μM solution in phosphate buffered saline before freezing.

Lipopolysaccharide, extracted from *Escherichia coli* serotype 055:B5, human recombinant IFN-γ, IL-1β and TNF-α were also purchased from Sigma Chemical Company. Lipopolysaccharide was dissolved in phosphate buffered saline on the morning of the experiment and stored in the refrigerator in polystyrene tubes, prior to use. Cytokines were reconstituted, according to manufacturer's instructions, in sterile phosphate buffered saline with or without bovine serum albumin. Solutions (10 μg ml^{-1}) were then divided into aliquots and stored at −20°C. An L-arginine supplemented culture medium was used for the 16 h incubations. *Medium 199* with Earle's salts, glutamine and 25 mM HEPES buffer was obtained from GibcoBRL, Life Technologies Ltd., Paisley, Scotland, U.K.

The Krebs solution had the following composition (mM): NaCl 118, KCl 4.57, $CaCl_2$ 1.27, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25 and glucose 11.1. All ingredients were *BDH AnalaR* reagents obtained from Merck Ltd. (Poole, Dorset, U.K.). Where necessary, it was oxygenated by bubbling a mixture of 95% oxygen and 5% carbon dioxide (Special Gases Group, Air Products, Crewe, U.K.) through the solution for at least 10 min.

Phosphate buffered saline was prepared by dissolving phosphate buffered saline tablets (Unipath Ltd., Basingstoke, Hampshire, U.K.) in distilled water and where necessary, adjusting pH to 7.4 by addition of 2 M hydrochloric acid or 2 M sodium hydroxide (Merck Ltd.). The solution was then autoclaved.

Endotoxin assay

Two samples of Krebs solution, one control and one containing added endotoxin, and a sample of distilled water were screened for endotoxin contamination using an *E-Toxate* kit (Sigma Chemical Company, Poole, Dorset, U.K.), according to the manufacturer's instructions (Sigma Technical Bulletin 210). All samples and dilutions were assayed in duplicate and where one positive and one negative result were obtained a third test was performed. Negative controls, i.e. endotoxin free water plus lysate, were included in each batch of assayed samples.

Data analysis

Concentration-response curves were constructed by plotting either tension or percentage change in tension against the logarithm of drug concentration. A non-linear curve-fitting package (*Origin 4.1*, Microcal Software Inc., MA, U.S.A.) was used to fit the curves to the Boltzman equation:

$$y = \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}} + A_2 \quad (1)$$

where A_1 = initial y value, A_2 = final y value, x_o = centre x value, and dx = width. This permitted derivation of the maximum response (E_{max}), which is equal to A_2 and the EC_{50}, which is given by x_o. Mean E_{max} and mean EC_{50} values were calculated for each treatment group and parallel control and these parameters were compared by one-way analysis of variance (*Minitab for Windows 10.2*, Minitab Inc., PA, U.S.A.). A *P*-value of less than 0.05 was taken to indicate a statistically significant difference. Where necessary, results

were further analysed by Tukey's pairwise comparisons (*Minitab for Windows 10.2*). All results are expressed as mean values ± s.e.mean.

Results

Effect of endothelium removal

Removal of the endothelium in rubbed arterial rings was confirmed by histological examination. This intervention did not significantly alter the PE dose-response curve. Dose-response curve parameters, i.e. log EC_{50} and E_{max} values, are given in Table 1.

In the presence of an intact endothelium, BK induced transient relaxation followed by contraction. This contraction interfered with the construction of a cumulative dose-response curve and necessitated repeating the dose-response curves in a non-cumulative fashion. Removal of the endothelium abolished the relaxant response to BK, however contraction still occurred (Figure 1). The resultant increases in E_{max} and log EC_{50} are given in Table 1.

Acetylcholine induced a variable relaxant response in unrubbed arteries and curve fitting was only possible in 15 out of 18 dose-response curves. This yielded an E_{max} of $-16.1 \pm 12.1\%$ and a log EC_{50} of -5.52 ± 0.24. No response was recorded in arteries from which the endothelium had been removed.

Removal of the endothelium did not significantly alter the dose-response curve to SN. The log EC_{50} values for unrubbed and rubbed arteries were -6.56 ± 0.19 ($n=7$) and -6.68 ± 0.15 ($n=7$), respectively. Maximum relaxant responses were also similar, i.e. $-103.6 \pm 1.2\%$ ($n=7$) and $-103.8 \pm 0.5\%$ ($n=7$).

Effect of nitric oxide synthase and/or cyclo-oxygenase inhibition

Dose-response curve parameters to PE were not significantly altered by L-NAME. However, indomethacin both alone and in combination with L-NAME, significantly reduced the log EC_{50} (Figure 2, Table 1). No significant changes in E_{max} were recorded.

Maximum relaxation to BK was significantly decreased by treatment with L-NAME (Figure 3, Table 1). Whilst indomethacin alone had no significant effect, when combined with L-NAME it produced a further significant decrease in E_{max}. Neither treatment significantly altered the log EC_{50}. In a second experiment, arteries incubated with L-arginine and L-NAME had E_{max} and log EC_{50} values that did not differ significantly from controls, thus L-arginine was able to counteract the effect of L-NAME (Table 1). Incubation with L-arginine alone did not significantly alter dose-response curve parameters.

Pre-incubation with L-NAME and/or indomethacin did not significantly alter SN dose-response curves. Maximum relaxation to SN was $-106.3 \pm 0.9\%$ ($n=6$) for control arteries,

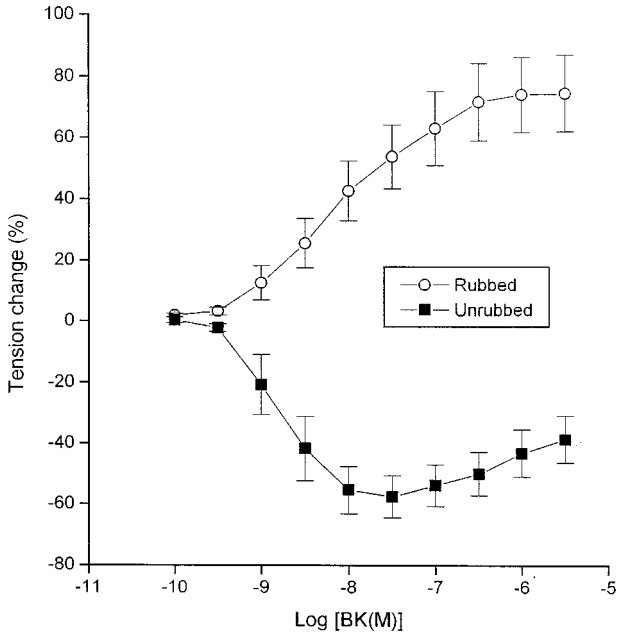

Figure 1 Non-cumulative bradykinin (BK) dose-response curves in rubbed ($n=8$) and unrubbed ($n=8$) ovine digital arteries pre-contracted with phenylephrine (PE, 600 nM). Data are expressed as means ± s.e.mean of n observations.

Table 1 The effect of endothelium removal or treatment with indomethacin (INDO), N^G-nitro-L-arginine methyl ester (L-NAME) or L-arginine (L-ARG) on ovine digital artery responses to phenylephrine (PE) and bradykinin (BK)

	Phenylephrine					*Bradykinin*				
		EC_{50}		E_{max}			EC_{50}		E_{max}	
	n	*log[PHE(M)]*	P	*g g^{-1}*	P	n	*log[BK(M)]*	P	*%*	P
Unrubbed	12	-6.13 ± 0.07	0.087	2107 ± 76	0.591	8	-8.68 ± 0.13	0.022	-61.8 ± 7.4	< 0.001
Rubbed	12	-6.29 ± 0.05		2188 ± 129		8	-7.91 ± 0.27*		76.0 ± 12.1*	
Control	8	-6.22 ± 0.08	0.005	2479 ± 213	0.871	6	-8.89 ± 0.15	0.035	-61.7 ± 5.6	< 0.001
INDO	7	-6.55 ± 0.07*		2603 ± 349		7	-8.92 ± 0.16		-51.0 ± 5.5	
L-NAME	7	-6.37 ± 0.12		2732 ± 251		7	-8.56 ± 0.20		-34.0 ± 2.1*	
INDO ± L-NAME	6	-6.68 ± 0.05*		2731 ± 149		7	-8.18 ± 0.22		-9.6 ± 2.8*†	
Control						6	-8.80 ± 0.12	0.004	-71.3 ± 6.0	0.004
L-ARG						6	-8.78 ± 0.15		-61.6 ± 8.9	
L-NAME						7	-8.14 ± 0.13*		-30.7 ± 8.0*	
L-ARG ± L-NAME						6	-8.49 ± 0.12		-55.3 ± 4.2	

The effects of incubation with INDO 10 μM, L-NAME 300 μM and L-ARG 10 mM were investigated in intact arterial rings. Data are expressed as mean values ± s.e.mean for n observations. *indicates that results are significantly different from the unrubbed or control group, whilst †indicates that results are significantly different from the L-NAME group.

$-104.2\pm1.7\%$ ($n=6$) for indomethacin-treated arteries, $-103.4\pm0.6\%$ ($n=6$) for L-NAME-treated arteries and $-105.8\pm1.1\%$ ($n=6$) for arteries treated with both indomethacin and L-NAME. Similarly, log EC_{50} values were -6.46 ± 0.12, -6.72 ± 0.06, -6.70 ± 0.15 and -6.80 ± 0.05.

Effect of incubation with endotoxin

Polymyxin B did not significantly alter dose-response curve parameters to PE or BK. The two control arteries produced a maximum tension of 2501 and 2561 g g^{-1} in response to PE, whilst log EC_{50} values were -5.89 and -6.09, respectively. These parameters were not different in polymyxin B-treated arteries (2081 and 2556 g g^{-1}, -5.61 and -6.28, $n=2$). Likewise control (-34.2 and -39.8%, -7.75 and -7.99, $n=2$) and polymyxin B-treated (-34.4 and -43.9%, -7.51 and -8.37, $n=2$) dose-response curves to BK did not differ.

Incubation with endotoxin for 6 h produced an increase in PE E_{max}, which failed to reach statistical significance ($P=0.10$, Table 2). Phenylephrine log EC_{50} value was unchanged. Following a 16 h incubation with endotoxin, PE E_{max} tended to fall, but this change was not significant ($P=0.36$). The s.e.mean for the maximum contraction of endotoxin-incubated arteries was high, reflecting the wide variation in response (Table 2). In addition, PE log EC_{50} tended to increase although this change was not significant at the 5% level ($P=0.06$).

Incubation with endotoxin for 6 h did not significantly alter the BK dose-response curve. These results are summarized in Table 2.

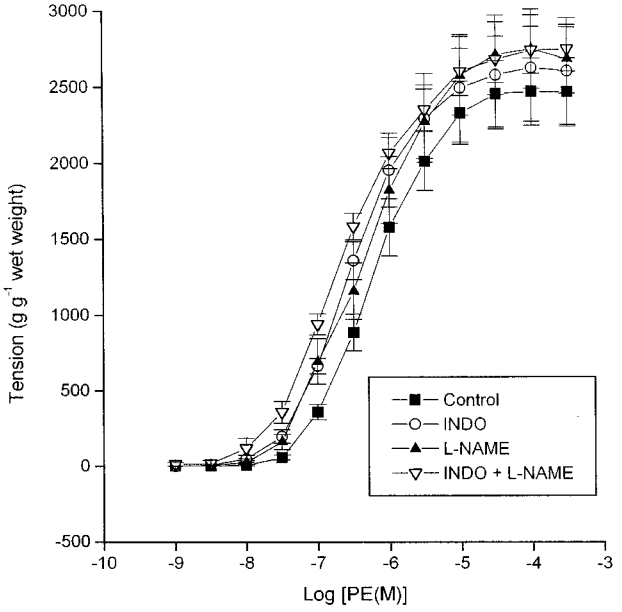

Figure 2 Cumulative phenylephrine (PE) dose-response curves in ovine digital arteries incubated with either no additions ($n=8$), with indomethacin (INDO, 10 μM, $n=7$), with N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM, $n=7$) or with both INDO (10 μM) and L-NAME (300 μM, $n=6$). Data are expressed as means$\pm$s.e.mean of n observations.

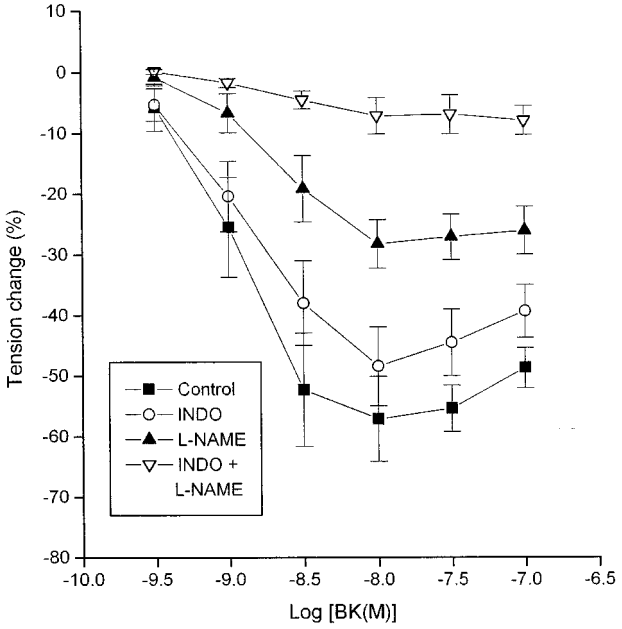

Figure 3 Non-cumulative bradykinin (BK) dose-response curves in ovine digital arteries incubated with either no additions ($n=6$), with indomethacin (INDO, 10 μM, $n=7$), with N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM, $n=7$) or with both INDO (10 μM) and L-NAME (300 μM, $n=7$). Data are expressed as means$\pm$s.e.mean of n observations.

Table 2 The effect of incubation with endotoxin or cytokines, including interferon-γ (IFN-γ), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), on ovine digital artery responses to phenylephrine (PE) and bradykinin (BK)

	Phenylephrine					*Bradykinin*				
	n	EC_{50} *log[PHE(M)]*	P	E_{max} *g g^{-1}*	P	n	EC_{50} *log[BK(M)]*	P	E_{max} *%*	P
Control	7	-6.37 ± 0.07	0.671	2286 ± 82	0.097	6	-8.51 ± 0.09	0.210	-54.1 ± 2.7	0.183
Endotoxin (6 h)	7	-6.42 ± 0.08		2544 ± 118		6	-8.70 ± 0.11		-44.2 ± 6.4	
Control	7	-6.49 ± 0.11	0.061	2066 ± 92	0.360					
Endotoxin (16 h)	7	-6.09 ± 0.16		1698 ± 376						
Control	6	-6.43 ± 0.09	0.253	2130 ± 120	0.352	6	-8.42 ± 0.17	0.264	-33.1 ± 4.0	0.021
IFN-$\gamma\pm$TNF-α (6 h)	6	-6.29 ± 0.08		1946 ± 145		6	-8.70 ± 0.17		-50.9 ± 5.1*	
Control	6	-6.44 ± 0.09	0.033	2318 ± 110	0.422					
IFN-$\gamma\pm$IL-1$\beta\pm$ TNF-α (16 h)	6	-6.10 ± 0.11*		2134 ± 190						

The following concentrations of endotoxin and cytokines were used, endotoxin 3 μg ml^{-1}, IFN-γ 10 ng ml^{-1}, IL-1β 20 ng ml^{-1} and TNF-α 5 ng ml^{-1}. Control arteries were incubated with polymyxin (10 μg ml^{-1}). Data are expressed as mean values$\pm$s.e.mean for n observations and * indicates results that are significantly different from the control group.

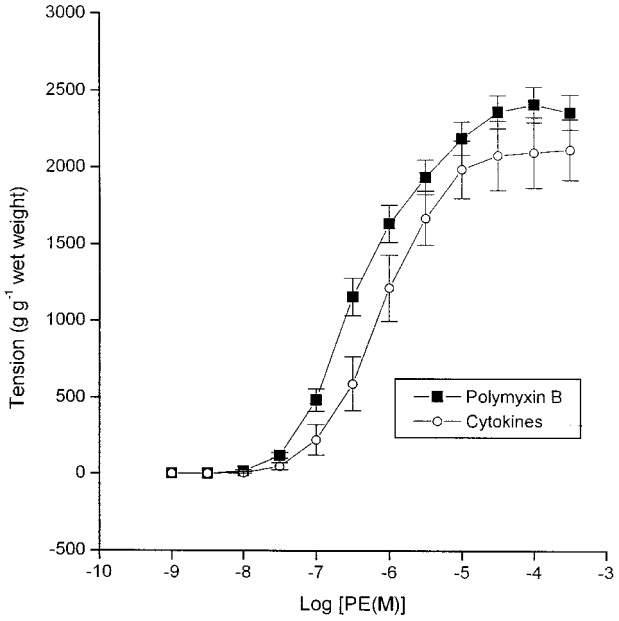

Figure 4 Cumulative phenylephrine (PE) dose-response curves in ovine digital arteries incubated with either polymyxin B (10 μg ml^{-1}, $n=6$) or a mixture of cytokines ($n=6$), containing tumour necrosis factor-α (5 ng ml^{-1}), interleukin-1β (20 ng ml^{-1}) and interferon-γ (10 ng ml^{-1}). Data are expressed as means$\pm$s.e.mean of n observations.

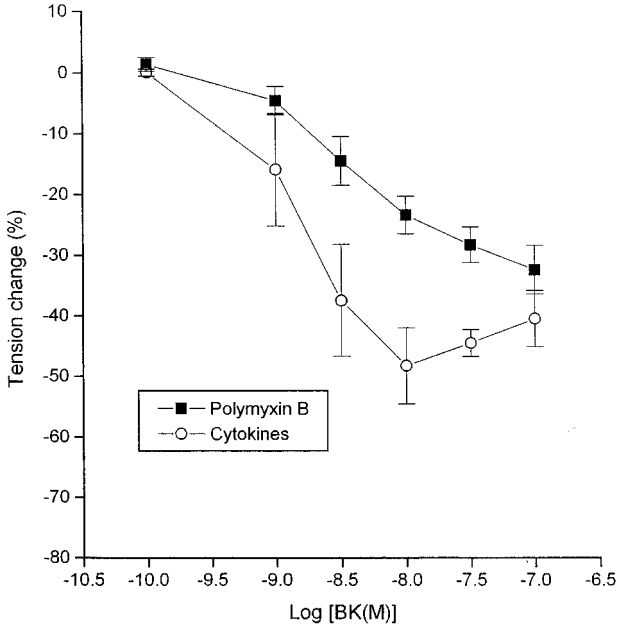

Figure 5 Non-cumulative bradykinin (BK) dose-response curves in ovine digital arteries incubated with either polymyxin B (10 μg ml^{-1}, $n=6$) or a mixture of cytokines ($n=6$), containing tumour necrosis factor-α (5 ng ml^{-1}) and interferon-γ (10 ng ml^{-1}). Data are expressed as means$\pm$s.e.mean of n observations.

Effect of incubation with cytokines

Following a 6 h exposure to IFN-γ and TNF-α, the PE dose-response curve was not significantly altered (Table 2). However, when the incubation period was extended to 16 h and IL-1β was added to the cytokine mixture, a significant increase in log EC_{50} was recorded (Figure 4, Table 2). Maximum contraction to PE was not significantly different ($P=0.42$). Incubation with IFN-γ and TNF-α produced an apparent increase in BK E_{max} (Figure 5), however BK-induced relaxation of the control vessels was less than previously recorded (Table 2).

Endotoxin assay

The most dilute concentration of endotoxin standard to test positive was a solution containing 0.125 endotoxin units ml^{-1} (EU ml^{-1}). All three samples, i.e. control Krebs, endotoxin-containing Krebs and distilled water, tested positive for endotoxin. The estimated endotoxin concentrations for the three samples were 128 EU ml^{-1}, 2048 EU ml^{-1} and 2 EU ml^{-1} respectively. The endotoxin used in this study contained not less than 500 EU μg^{-1}. If it is assumed that any contaminant endotoxin had similar activity, the endotoxin concentrations can be expressed as not more than 0.26, 4.10 and 0.004 μg ml^{-1}.

Discussion and conclusions

Contractile responses may be modified by basal release of NO from the endothelium. Abolition of this influence, by removing the endothelium or incubating vessels with a NOS inhibitor has been shown to enhance the contractile response to adrenergic agonists in rat arteries and rabbit veins (Martin *et al*., 1986; McGrath *et al*., 1990). However in this study, neither removal of the endothelium nor incubation with L-NAME, significantly altered the response to PE, implying that basal release of NO does not significantly modify the contractile response. This finding is not unprecedented. McGrath *et al*. (1990) showed that whilst noradrenaline-induced contraction of rabbit saphenous vein was enhanced by endothelium removal, the response of rabbit saphenous artery was unaltered. Cogswell *et al*. (1995) demonstrated a similar lack of effect in equine digital arteries. Interestingly, log EC_{50} to PE was significantly decreased by 10 μM indomethacin, a dose sufficient to inhibit COX activity (Radomski *et al*., 1987). Thus in ovine digital arteries vasodilatory prostaglandins appear to modify the α-adrenergic response, as reported in a number of systemic arteries from the dog, cat and rabbit (Cherry *et al*., 1982).

The response of ovine digital arteries to BK was biphasic, transient relaxation being followed by contraction. A similar response has been described in the sheep femoral artery (Félétou *et al*., 1994). The mechanism of BK-induced relaxation appears to vary in vessels from different species (Furchgott, 1984). In cat and rabbit mesenteric arteries it is endothelium-independent and mediated by COX products, whilst in a variety of canine and human arteries BK-induced relaxation has been attributed to endothelium-dependent relaxing factor (Cherry *et al*., 1982). In this study, BK-induced relaxation of ovine digital artery proved to be endothelium-dependent and was significantly reduced by L-NAME, confirming the role of NO in this response. Whilst inhibition of COX alone had no significant effect, it did enhance the inhibition produced by L-NAME, implying an additional role for COX products. The contractile phase of the BK response, which was endothelium-independent, was not investigated further.

Acetylcholine-induced endothelium-dependent relaxation, first demonstrated in rabbit aorta (Furchgott & Zawadzki,

1980), occurs in vessels from a variety of species. In this study, ACh-induced relaxation was inconsistent, a finding previously reported in sheep pulmonary artery (30% reduction in serotonin-induced tone, Kemp *et al*., 1997), although earlier studies of the same vessel reported greater relaxation of PE-induced tone (Abman *et al*., 1991; Toga *et al*., 1996), 51 and 70%, respectively.

Sodium nitroprusside, an endothelium-independent vasodilator, relaxes vascular smooth muscle through release of NO (Schultz *et al*., 1977; Feelisch & Noack, 1991). Studies of rat and rabbit arteries and veins have demonstrated enhanced relaxation to nitrovasodilators following removal of the endothelium or inhibition of NOS (McGrath *et al*., 1990; Moncada *et al*., 1991), possibly as a consequence of desensitization of guanylate cyclase by basal release of NO from the endothelium, which suppresses the response to exogenous NO. However, no such effect was demonstrated here and neither endothelium-removal, L-NAME nor indomethacin significantly altered the response to SN.

In order to study the effect of endotoxin on isolated digital arteries it was necessary to incubate control arteries in an endotoxin-free solution. The endotoxin assay indicated that the Krebs solution contained detectable amounts of endotoxin. To counteract this, polymyxin B, 10 μg ml^{-1}, which has been shown to bind the lipid A portion of lipopolysaccharide (Morrison & Jacobs, 1976), was added to control solutions. Concentrations of 1–100 μg ml^{-1} polymyxin B have been reported to block the effects of endotoxin on vascular smooth muscle (Rees *et al*., 1990; Schott *et al*., 1993; Moritoki *et al*., 1994).

Impaired endothelium-dependent relaxation following exposure to endotoxin has been documented in isolated rat aorta (Beasley *et al*., 1990) and in arteries obtained from endotoxin-treated dogs (Wylam *et al*., 1990). It is not clear how endotoxin impairs endothelium-dependent relaxation, decreased stability of endothelial NOS mRNA (Yoshizumi *et al*., 1993) and altered receptor-effector coupling have been proposed (Daniel-Issakani *et al*., 1989). Bhagat *et al*. (1996) showed that endothelial dysfunction occurs in the absence of structural damage to the endothelium and used the term 'endothelial stunning' to describe the effect. If endothelial stunning occurs in digital vessels, it could be important in the pathogenesis of laminitis, providing a possible link between endotoxaemia and digital hypoperfusion. Baxter (1995) demonstrated reduced relaxation to BK in equine digital arteries removed from horses treated with endotoxin *in vivo*. However, in this study incubation with endotoxin failed to alter BK-induced relaxation.

Incubation with cytokines might also be expected to impair endothelium-dependent relaxation. This has been confirmed in a range of arteries from different species, including cat carotid artery (Aoki *et al*., 1989), bovine pulmonary artery (Greenberg *et al*., 1993) and equine digital arteries incubated for just 10 min with TNF-α (Baxter, 1994). In the current study, the relaxant response of digital arteries to BK was not inhibited by cytokines, in fact cytokine-treated arteries exhibited greater relaxation than polymyxin B-treated controls. However in comparison to earlier results, BK-induced relaxation of control vessels was reduced in this experiment. The reason for this discrepancy is unclear. It is possible that polymyxin B influenced the response to BK, but this seems unlikely since no such influence was apparent in previous investigations comparing polymyxin B and endotoxin-treated arteries.

Endotoxin and cytokines have also been shown to depress contractile responses. Marked depression of vasoconstriction has been observed in rat aorta following exposure to concentrations of endotoxin ranging from 1 ng to 10 μg ml^{-1} (Beasley *et al*., 1990; Fleming *et al*., 1990; McKenna, 1990; Rees *et al*., 1990). In this study however, incubation with endotoxin did not alter the contractile response of the ovine digital artery. Results from studies conducted in species other than the rat have also demonstrated a less consistent response to endotoxin. For example, overnight incubation with endotoxin failed to alter the response of equine digital veins to PE, although serotonin-induced contractions were depressed (Bailey & Elliott, 1996). Similarly, Dixon *et al*. (1990) reported that endotoxin did not inhibit noradrenaline-induced contraction in porcine carotid arteries, whilst serotonin-induced contraction of porcine coronary arteries was reduced (Shibano & Vanhoutte, 1993).

Whilst endotoxin failed to alter the contractile response significantly, prolonged incubation with a mixture of cytokines, IFN-γ, TNF-α and IL-1β, reduced the sensitivity of ovine digital arteries to PE. Maximal contraction was unchanged. Cytokine-mediated depression of contraction has been demonstrated in rat (Beasley *et al*., 1989; Beasley, 1990; French *et al*., 1991) and rabbit aorta (Robert *et al*., 1992) incubated with IL-1β and porcine coronary artery incubated with TNF-α (Shibano & Vanhoutte, 1993). However, these studies demonstrated a greater degree of cytokine-mediated inhibition, with maximal contractions being decreased by up to 66% (Robert *et al*., 1992). In the rat, endotoxin or cytokine-induced vascular hyporeactivity has been attributed to the expression of inducible NOS in vascular smooth muscle, which in turn increases NO production (Beasley, 1990; Beasley *et al*., 1991). Thus the limited response to incubation with endotoxin or cytokines observed in this study could reflect a reduced capacity for NOS induction in sheep. There is some evidence to support this view. Bogdan *et al*. (1997) found that ovine macrophages exposed to endotoxin (0.004–4 μg ml^{-1}) and/or IFN-γ (300 units ml^{-1}) did not readily express inducible NOS. Within a species, regional differences in NOS expression are possible, for example whilst IL-1β significantly depresses noradrenaline-induced contractions of rabbit aorta, contraction of rabbit femoral artery is enhanced (Robert *et al*., 1993).

Experimental conditions may be critical in determining the expression and activity of inducible NOS *in vitro*. Enzyme induction is not an immediate effect and extended exposure to endotoxin or cytokines may be required. Beasley (1990) reported that exposure to endotoxin for 2–3 h was necessary to induce NOS in rat aorta, whilst in bovine arteries more prolonged exposure, up to 20 h, may be needed (Dekimpe *et al*., 1994). The concentration of endotoxin does not appear to be crucial and concentrations from 1 ng ml^{-1} to 10 μg ml^{-1} have been used to induce NOS in the rat aorta (Beasley, 1990; McKenna, 1990). However, the optimum concentration of cytokines has not been determined and may vary in different species (Lamas *et al*., 1992).

To avoid the problems of *in vitro* incubation, arteries can be obtained from endotoxin-treated animals. Nelson *et al*. (1991) demonstrated a reduced response to noradrenaline in sheep femoral arteries, 8–12 h after endotoxin administration. The EC_{50} was increased, whilst maximal contraction was unchanged, findings that are consistent with the results of this study. In contrast, noradrenaline-induced contraction was enhanced in digital arteries removed from endotoxin-treated horses (Baxter, 1995). To reconcile these differing results it is important to appreciate that endotoxin and cytokines can have opposing effects on NO synthesis. The net effect on vascular tone will depend on the balance between endothelial stunning and expression of inducible NOS. In the study by Baxter,

vessels were harvested just 1–2 h after endotoxin administration and this may have been too soon to observe the effects of NOS induction.

In conclusion, whilst NO was clearly an important mediator of endothelium-dependent relaxation in ovine digital arteries, it did not significantly modify the response to the vasoconstrictor PE. Following incubation with cytokines sensitivity to PE was decreased, however, no evidence of endotoxin or cytokine-mediated impaired endothelium-dependent relaxation was found. These findings do not support the hypothesis that endotoxin-induced endothelial dysfunction may enhance vascular tone in ungulate digital arteries, contributing to the pathogenesis of laminitis.

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