

Effects of *in vivo* Lipopolysaccharide Infusion on Vasoconstrictor Function of Rat Isolated Mesentery, Kidney and Aorta

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ABSTRACT

Continuous infusion of lipopolysaccharide (LPS) into conscious rats elicits regionally-selective cardiovascular disturbances. The aim of the present study was to assess contractile function in different vascular preparations (renal, mesenteric, thoracic aorta) taken from rats infused with LPS for 2 or 24 h. Sustained responses to continuous infusion of methoxamine, but not to KCl, were reduced in the aorta (at 2 and 24 h LPS) and mesentery (at 24 h LPS), but not in the renal vascular bed. In contrast, transient responses to bolus doses of methoxamine were unchanged in the mesentery. In Ca^{2+} -imaging experiments with Fura-2, challenge with a single concentration of methoxamine (10 μM , which showed an impaired contractile response at 24 h LPS), induced a rise in intracellular Ca^{2+} in the mesenteric artery that was not different from the control. Furthermore, in the aorta, the contractile response to caffeine was attenuated only in the 2 h LPS group. These results show that there is regional heterogeneity in *in vitro* vascular responsiveness in preparations taken from LPS-infused rats. Thus, in mesenteric beds and aortae, but not renal beds, there is hypocontractility to methoxamine that is not due to a generalised inability of the smooth muscle to contract, which is evident with sustained but not transient application of agonist (mesentery) and which, in late endotoxaemia (24 h LPS), does not appear to involve abnormalities in Ca^{2+} mobilization or entry.

Infection by bacteria can result in profound cardiovascular disturbances. In cases of severe infection this can result in septic shock, characterised by unrelenting hypotension, tachycardia and increased cardiac output. These effects can be simulated by the administration of the bacterial toxin, lipopolysaccharide (LPS), in both man (Suffredini et al., 1989) and animals (Waller et al., 1994; Gardiner et al., 1995; Pastor, 1999; Zhou et al., 2001). The effect of endotoxaemia upon the vasculature has been studied by many different groups, *in vitro*, and also, *ex vivo*, in preparations isolated from animals following the administration of LPS. However, the results are highly variable, probably due to differences in the experimental model, the vascular territory (conduit/resistance vessel, and region), as well as the experimental conditions for the *in vitro* experiments.

An experimental model we have characterised *in vivo* differs from many inasmuch as it involves continuous infusion of relatively low doses of LPS ($150 \mu\text{g kg}^{-1} \text{h}^{-1}$) rather than a high single bolus (commonly 20-30 mg kg^{-1}). In that model we have shown regionally-selective changes in cardiovascular status (Waller et al., 1994; Gardiner et al., 1993, 1994, 1995, 1996a,b) associated with reduced mesenteric vasoconstrictor responses to methoxamine (an α_1 -adrenoceptor agonist) *in vivo*, at 2 and 24 h after the start of LPS infusion (Waller et al., 1994; Tarpey et al., 1998). In contrast, the renal vasoconstrictor response to methoxamine *in vivo* was not suppressed (Waller et al., 1994). Interestingly, mesenteric bed preparations taken from animals infused with LPS and investigated *in vitro* did not show reduced vasoconstrictor effects of methoxamine (Tarpey and Randall, 1998), but this may have been due to the mode of administration since others who have investigated mesenteric arteries isolated from LPS-treated animals have noted an impairment in the sustained contractile response, even though the initial response to agonist exposure

was normal (Martinez et al., 1996; Mitolo-Chieppa et al., 1996). The *in vitro* contractile responsiveness of the renal vasculature in this model of endotoxaemia has not been investigated to date.

Thus, we have now carried out a systematic comparison of responses to methoxamine, given as either bolus doses or as sustained concentrations, in perfused mesenteric vascular beds taken from rats infused with LPS for 2 or 24 h. Since the results showed impaired contractions to methoxamine under sustained conditions in the 24 h LPS-treated group, experiments were carried out to assess changes in intracellular Ca^{2+} in mesenteric arteries taken from rats given LPS for 24 h. Furthermore, we investigated, for the first time, *in vitro* contractile responses of the renal vasculature from this model of endotoxaemia. Additionally, in order to investigate whether endotoxaemia may affect resistance and conduit vessels differently, we assessed the vasoconstrictor function of thoracic aortae isolated from rats treated with LPS. Aortae were used to investigate further the possible role of intracellular Ca^{2+} in hypocontractility to methoxamine in endotoxaemia using caffeine, which elicits contraction by acting on the ryanodine receptor on the sarcoplasmic reticulum to release Ca^{2+} (Zucci and Ronca-Testoni, 1997).

To our knowledge, the present study is the first comparison of the contractile function of three different tissues taken from the same groups of LPS-treated animals. A preliminary account of some of these findings has been reported to the British Pharmacological Society (Farmer et al., 2001).

Materials and Methods

All experiments were performed on adult male Sprague-Dawley rats (290 - 430 g, Charles River UK), with Home Office approval under the Animals (Scientific Procedures) Act 1986.

In vivo Administration of Substances. The animals were surgically prepared to receive chronic infusion of either LPS or saline, via catheters implanted in the right jugular vein under surgical anaesthesia with fentanyl and medetomidine ($300 \mu\text{g kg}^{-1}$ of each i.p.). Following surgery, anaesthesia was reversed and analgesia provided with atipamezole and nalbuphine respectively (both given at 1 mg kg^{-1} s.c.). Animals were allowed to recover overnight during which time they received infusion of saline (0.4 ml h^{-1}) to maintain catheter patency. The animals were housed in individual cages and connected to a fluid-filled swivel to allow overnight intravenous infusion into the conscious animal as described previously (Gardiner et al., 1993). During this time animals were allowed food and water *ad libitum*. On the following day the animals were assigned to one of four groups and subjected to either a 2 h or 24 h intravenous infusion of either LPS (*E.Coli*. Serotype 0127: B8; $150 \mu\text{g kg}^{-1} \text{ h}^{-1}$) or saline (0.4 ml h^{-1}) (Gardiner et al., 1995). After 2 h or 24 h infusion of saline or LPS, rats were anaesthetised with sodium pentobarbitone (up to 60 mg i.v. , supplemented as required) for removal of kidneys, and, subsequently, superior mesenteric arteries or mesenteric vascular beds, and thoracic aortae.

Isolated Mesenteric Vascular Bed Preparation. The isolated mesenteric vascular bed preparation, based on the method of McGregor (1965), was prepared as described previously (Ralevic and Burnstock, 1988). Briefly, a midline incision was made and

the gastrointestinal tract lifted out of the abdominal cavity and placed at the side of the animal on a paper towel soaked in Krebs' solution. The superior mesenteric artery was identified and cannulated from where it leaves the aorta with a blunted hypodermic needle. The mesenteric vascular bed was flushed with Krebs' solution and the mesentery carefully cut away from the gastrointestinal tract. It was placed within an organ bath and perfused at a rate of 5 ml min^{-1} with gassed (95% O_2 , 5% CO_2) Krebs' solution of the following composition (mM): NaCl 106.1, KCl 4.7, KH_2PO_4 1.2, NaHCO_3 25, MgSO_4 1.2, CaCl_2 1.9, and glucose 10, maintained at 37°C . Perfusate was pumped by a peristaltic pump (Cole Parmer Ltd). Relaxations or constrictions of the preparation were measured as changes in perfusion pressure, monitored by a pressure transducer (Ohmeda) situated on a side arm proximal to the preparation. A 30 min period of equilibration was observed following preparation.

Isolated Renal Vascular Bed Preparation. A midline incision was made and the gastrointestinal tract lifted out of the abdominal cavity and placed at the side of the animal on a paper towel soaked in Krebs' solution. The right renal artery was identified, and dissected away from connective tissue along its length, from the aorta to the kidney. A loose ligature was then placed around the renal artery. Using blunt dissection the kidney was separated from the surrounding fatty connective tissue. During this time the kidney was kept moist with Krebs' solution. The animal was then killed by decapitation, and the renal artery cannulated with a blunted hypodermic needle. Once the needle was securely in the artery, the preparation was flushed with 1 ml Krebs' solution, containing heparin (500 U), until the kidney was blanched. The ligature was then tightened to secure the needle in the artery, and the kidney was lifted away with any remaining connective tissue cut away. The preparation was then placed

on a metal grid within an organ bath (37°C) and perfused at a rate of 8 ml min⁻¹ with gassed (95% O₂, 5% CO₂) Krebs' solution of the following composition (mM): NaCl 106.1, KCl 4.7, KH₂PO₄ 1.2, NaHCO₃ 25, MgSO₄ 1.2, CaCl₂ 1.9, and glucose 10, maintained at 37°C. Perfusate was pumped by a peristaltic pump (Cole Parmer Ltd) and responses of the preparation were measured as changes in perfusion pressure, monitored by a pressure transducer (Ohmeda) situated on a side arm proximal to the preparation. A 30 min period of equilibration was observed following preparation.

Isolation of Thoracic Aortae. A midline cut was made along the sternum, the lungs and heart removed, and the thoracic aorta exposed. While the aorta remained *in situ*, the vessel was cleaned of connective tissue on its anterior surface. A cut was made through the vessel just above the diaphragm and the vessel carefully cut away from its posterior connections. Following removal, the vessel was placed into a beaker of cold Krebs' solution, to remove any excess blood, before being placed in cold Krebs' solution in a petri dish. A ring of thoracic aorta, 1.5 cm long, was cut from the centre of the excised vessel and suspended, under 1g of tension, in an organ bath (at 37°C) containing gassed (95% O₂, 5% CO₂) Krebs' solution of the following composition (mM): NaCl 106.1, KCl 4.7, KH₂PO₄ 1.2, NaHCO₃ 25, MgSO₄ 1.2, CaCl₂ 1.9, and glucose 10. The preparations were allowed a 1h period of equilibration prior to the start of experimentation.

Experimental Protocol – Mesenteric Arterial Beds. Following the equilibration period, dose response curves were performed for methoxamine (500 pmol-5 µmol), an α₁-adrenoceptor agonist. All doses were given as 50 µl bolus injections into an injection port, sited proximal to the preparation. The dose interval was determined by

the time to recovery of the response to baseline. In a separate series of experiments, vasoconstrictor responses to cumulative concentrations of methoxamine were compared in the isolated mesenteric arterial vascular beds from the four groups. Methoxamine was added to the perfusate in cumulative concentrations between 1 and 100 μ M. In a separate series of experiments, vasoconstrictor responses to cumulative concentrations of KCl (10 mM–300 mM) were compared. Responses to U46619 were not investigated in the mesenteric arterial bed as in this preparation U46619 is a weak vasoconstrictor (Warner, 1990).

Experimental Protocol – Renal Arterial Beds. In the isolated perfused kidneys, following the equilibration period, a cumulative concentration-response curve was produced for methoxamine by addition of methoxamine to the perfusing Krebs' solution (10 nM–100 μ M), with each addition being given when a plateau to the previous response had been achieved (usually within 5 min of administration). Methoxamine was then washed out over a 30 min period until baseline perfusion pressure was restored. Finally, a cumulative concentration-response curve was produced for KCl by addition of KCl to the perfusing Krebs' solution (10 mM–300 mM), with each addition being given once a plateau to the previous evoked response was obtained.

Experimental Protocol - Aortae. Following the equilibration period, vessels were challenged with cumulative concentrations of methoxamine (1 μ M - 1 mM). The methoxamine was washed out and a 30 min rest period was allowed prior to cumulative concentrations of KCl (10 mM - 300 mM) being added to the organ bath. In a separate series of preparations, aortae were challenged by cumulative

concentrations of the thromboxane A₂ mimetic U46619 (1 nM - 1 μ M). A single concentration (20 mM) of caffeine was added to the organ bath in a separate series of preparations. Tension was measured at 5 s time points up to 1 min, and then at 10 s time points up to 2 min.

Ca²⁺-Imaging of Mesenteric Arteries. These experiments were carried out in superior mesenteric arteries isolated from rats following infusion of either LPS or saline for 24 h. Changes in the level of intracellular Ca²⁺ during challenge with methoxamine and KCl were investigated. Superior mesenteric arteries were dissected out of the animal. They were then carefully cleaned of fat and connective tissue, and dissected into 5 mm segments. These segments were incubated with 5 μ M fura-2 AM in the presence of 0.02% Pluronic F-127, 0.1% Cremophor FL, and 1% dimethylsulphoxide in pre-gassed Krebs-Henseleit buffer for 3 h at room temperature. After this incubation period, the segments were cut open and placed lumen side down in a heated culture dish (Biopetech, Inc) containing Krebs-Henseleit buffer. Two metal supports held the tissue flat against the bottom of the culture dish. The culture dish was then mounted on an inverted microscope (Leica) equipped for dual excitation wavelength fluorescent measurements. The objective was a Nikon CF Fluor (10 x 0.5). The light source was a 75W Xenon lamp. The Krebs-Henseleit buffer bathing the tissue was maintained at 37°C and constantly gassed with 95% O₂/5% CO₂. Tissues were allowed to recover for 20 min prior to Ca²⁺ measurements. Changes in intracellular Ca²⁺ levels were assessed by alternatively exciting the preparation with 340 nm and 380 nm wavelength light with a 3 s delay between exposures. Emitted light (measured at 510 nm) was collected by a photomultiplier (Photonic Science, Tunbridge Wells, UK). The system was controlled by an Apple

Macintosh Power PC using IonVision software (Improvision, Coventry). In its free form fura-2 produces a high fluorescence at 380 nm, and a low fluorescence at 340 nm. When fura-2 binds to Ca^{2+} the opposite is true such that the amount of fluorescence at 380 nm decreases, and the amount of fluorescence at 340 nm increases. The ratio of fluorescence at these wavelengths (340 nm/380 nm) is an index of Ca^{2+} concentration. Therefore, changes in this ratio are recorded as an index of intracellular Ca^{2+} . The Grynkiewicz equation (Grynkiewicz et al., 1985) can be used to calculate the absolute Ca^{2+} concentration from the ratio values. However, the constants required for the equation (the dissociation constant for fura-2, R_{\max} and R_{\min}) are difficult to determine accurately. Therefore, as this study is concerned purely with the changes in intracellular Ca^{2+} levels, we have quoted the 340 nm/380 nm fluorescence ratios, which are directly proportional to the absolute values.

Following 20 min of equilibration, the arteries were challenged with a single 10 μM concentration of methoxamine, and the response recorded until a plateau had been reached (3-5 min). The preparations were then washed out and a further 30 min allowed for equilibration. A single, 60 mM, concentration of KCl was added to the perfusate, and the evoked response recorded until a plateau had been reached (3-5 min).

Statistics. Responses of the perfused vascular beds were measured as increases in perfusion pressure above baseline. Aortic contractions were measured as a change in tension (g) over baseline values. Data are given as means \pm s.e.mean. Data were analysed using one- or two-way analysis of variance, with Tukey's multiple comparison post-hoc test, or t-tests as appropriate (Graph Pad Prism, Version 3.0). Differences were only considered significant if $p < 0.05$.

Materials. LPS (from *Escherichia coli* serotype 0127: B8), methoxamine, caffeine and U46619 were obtained from Sigma (UK). LPS was dissolved in sterile saline and prepared to a concentration of $150 \mu\text{g ml}^{-1}$. KCl was obtained from BDH Laboratory Supplies (Poole, UK). Anaesthetic agents: fentanyl citrate (Martindale), medetomidine (Domitor) and sodium pentobarbitone (Sagatal, Rhône Mérieux Ltd, Harlow, UK). Reversing agents: nalbuphine (HCl Nubain, Dupont, Stevenage, UK) and atipamezole (HCl Antisedan, Pfizer Ltd, Sandwich, UK).

Results

The experimental model of endotoxaemia that is used here has been characterised extensively by us, and details of the regionally-selective changes in resting cardiovascular status and responses to vasoconstrictors *in vivo* are available in Gardiner et al., 1993, 1994, 1995, 1996a,b; Waller et al., 1994; Tarpey et al., 1998).

Mesenteric Arterial Beds: Dose- and Concentration-Response Relationships to Methoxamine, and Concentration-Response Relationships to KCl. There were no significant differences in the baseline perfusion pressures between the 2 h saline, 24 h saline, 2 h LPS and 24 h LPS groups, and these were 14 ± 4 mmHg ($n = 6$), 10 ± 5 mmHg ($n = 5$), 7 ± 3 mmHg ($n = 8$), and 16 ± 2 mmHg ($n = 9$), respectively.

Methoxamine, when given as a bolus, elicited dose-dependent increases in perfusion pressure ($p < 0.001$) (Fig. 1). No significant differences existed between the four groups in the maximal elicited rise in perfusion pressure. Interestingly, a plot of the time course of the methoxamine-elicited contraction to a dose of $1.5 \mu\text{mol}$ (Fig. 1b), showed that although there was no significant difference in the maximal attained

rise in perfusion pressure, there was a trend in the 24 h LPS group for the response to decrease more rapidly; although this was not significantly different compared to the 24 h saline group it was significantly different from the 2 h LPS and saline groups ($p < 0.05$).

Cumulative concentrations of methoxamine elicited concentration-dependent increases in perfusion pressure ($p < 0.0001$) (Fig. 2a). These were significantly reduced in mesenteries from rats following 24 h LPS infusion (Fig. 2a). The maximum response was significantly depressed in 24 h LPS rats compared to 24 h saline rats, being 64.4 ± 9.1 mmHg ($n=8$) and 106.7 ± 9.6 mmHg ($n=9$) respectively ($p < 0.05$). No significant differences were noted between the 2 h LPS and 2 h saline groups, with maximum responses being 96.6 ± 15.6 mmHg ($n=8$) and 99.4 ± 9.3 mmHg ($n=9$) respectively. There were no significant differences in EC_{50} values between 2 h saline, 24 h saline, 2 h LPS or 24 h LPS groups, at 14.9 ± 6.1 μ M, 7.3 ± 1.6 μ M, 7.9 ± 2.1 μ M and 10.9 ± 2.0 μ M, respectively.

Cumulative concentrations of KCl elicited significant concentration-dependant increases in perfusion pressure ($p < 0.0001$) (Fig. 2b). There were no significant differences between the 2 and 24h LPS groups and their respective controls in their response to cumulative concentrations of KCl.

Mesenteric Arteries: Methoxamine-Stimulated Ca^{2+} Responses. There was no significant difference in methoxamine-stimulated Ca^{2+} responses between arteries from 24 h saline and 24 h LPS-treated rats, the changes in the 340 nm/380 nm ratio being 0.16 ± 0.05 ($n=5$) and 0.11 ± 0.07 ($n=5$) respectively (Fig. 3a). The responses were also similar when expressed as a percentage of the KCl response (Fig. 3b). The time to maximum response to methoxamine in arteries from 24 h LPS-treated rats

(303 ± 107 s, $n=5$) was not significantly different compared to that in the 24 h saline treated rats (190 ± 98 s, $n=5$) (Fig. 3c). There was no significant difference between the two groups in the change in 340 nm/380 nm ratio induced by 60 mM KCl being 0.15 ± 0.05 ($n=5$) and 0.15 ± 0.04 ($n=5$) in LPS-treated and saline-treated groups respectively (Fig. 3d).

Renal Arterial Beds: Concentration-Response Relationships to Methoxamine and KCl. Experiments were performed on 4 experimental groups, 2 h saline, 24 h saline, 2 h LPS and 24 h LPS. There were no significant differences between any of the groups in the measured basal renal perfusion pressures, these being 52 ± 11 mmHg ($n=7$), 45 ± 5 mmHg ($n=8$), 60 ± 13 mmHg ($n=9$) and 51 ± 4 mmHg ($n=5$), respectively.

Responses to cumulative concentrations of methoxamine in the renal arterial beds displayed concentration-dependency in all four groups ($P<0.0001$) (Fig. 4). There were no significant differences between 2 h saline ($n=6$), 24 h saline ($n=8$), 2 h LPS ($n=9$) or 24 h LPS ($n=5$) groups; R_{Max} values were 133 ± 22 mmHg, 139 ± 18 mmHg, 161 ± 25 mmHg and 190 ± 17 mmHg, respectively and pEC_{50} values were 6.37 ± 0.24 , 6.27 ± 0.30 , 6.24 ± 0.63 and 6.24 ± 0.19 , respectively.

Responses to cumulative concentrations of KCl were concentration-dependent ($p<0.0001$). There were no significant differences between 2 h saline ($n=7$), 24 h saline ($n=8$), 2 h LPS ($n=9$) or 24 h LPS ($n=5$) groups; R_{Max} values were 58 ± 13 mmHg, 64 ± 7 mmHg, 75 ± 11 mmHg and 91 ± 16 mmHg, respectively and pEC_{50} values were 1.51 ± 0.17 , 1.60 ± 0.06 , 1.69 ± 0.05 and 1.84 ± 0.06 , respectively.

Aortae: Concentration-Response Relationships to Methoxamine, KCl and U46619. The increases in tension elicited by cumulative concentrations of methoxamine are shown in Figure 5a. All groups displayed concentration-dependent contraction to methoxamine ($p < 0.0001$). Both the 2 h LPS ($n=6$) and the 24 h LPS ($n=4$) groups showed significantly lower contractility to methoxamine than either 2 h saline ($n=5$) or 24 h saline ($n=6$) groups ($P < 0.05$). This is reflected in the values of maximal response, which were 0.94 ± 0.09 g, 0.88 ± 0.10 g, 0.44 ± 0.05 g and 0.49 ± 0.10 g, for 2 h saline, 24 h saline, 2 h LPS and 24 h LPS groups respectively. With regard to pEC_{50} values, the 2 h LPS group was significantly lower than the 2 h saline groups, being 5.3 ± 0.1 and 5.9 ± 0.1 , respectively ($p < 0.001$). However there was no significant difference between the 24 h LPS group and the 24 h saline groups, being 5.7 ± 0.1 and 5.7 ± 0.1 , respectively.

Contractions elicited in response to cumulative concentrations of U46619 displayed concentration-dependency ($p < 0.0001$) (Fig. 5b). There were no significant differences between the experimental groups in the contractions elicited by U46619 at any of the concentrations tested. Maximal attained contractions were 1.03 ± 0.17 g ($n=6$), 1.16 ± 0.17 g ($n=7$), 1.13 ± 0.10 g ($n=6$) and 1.03 ± 0.14 g ($n=7$), for 2 h saline, 24 h saline, 2 h LPS and 24 h LPS groups respectively. With regard to pEC_{50} values there were no significant differences between 2 h saline, and 2 h LPS or between 24 h saline and 24 h LPS groups, being 8.1 ± 0.1 , 8.2 ± 0.2 , 8.2 ± 0.2 and 7.8 ± 0.1 , respectively.

Contractions elicited by cumulative concentrations of KCl were not significantly different between the experimental groups, but did display concentration-dependency (Fig. 6). Maximal contractions were 0.56 ± 0.12 g ($n=5$), 0.53 ± 0.13 g ($n=6$), 0.54 ± 0.08 g ($n=6$) and 0.70 ± 0.16 g ($n=4$), for 2 h saline, 24 h

saline, 2 h LPS and 24 h LPS groups respectively. In addition, pEC_{50} were not significantly different between the groups, being 1.68 ± 0.26 , 1.43 ± 0.27 , 1.54 ± 0.04 and 1.94 ± 0.13 , respectively.

Aortae: Contractile Response to Caffeine. The time-course of contractions elicited by 20 mM caffeine is illustrated in Figure 7. In the 2 h LPS group there was a significant decrease ($p < 0.05$) in tension elicited by caffeine, between 15 s and 40 s, compared to the corresponding saline-treated group. However, in aortae taken after 24 h of LPS-infusion there were no significant differences in the response to caffeine compared to either control group.

Discussion

We have previously observed regional and temporal heterogeneity *in vivo* with respect to vascular responsiveness in a model of endotoxaemia which involves continuous LPS infusion (Waller et al., 1994). The aim of the present study was to investigate, in the same experimental model, whether or not such regional heterogeneity is also evident *in vitro* and whether or not endotoxaemia differentially affects the vasocontractile function of conduit and resistance vessels. Furthermore, we sought to investigate the possible role of Ca^{2+} in the hypocontractility to methoxamine (an α_1 -adrenoceptor agonist) that we observed.

Hypocontractility to methoxamine in the mesenteric arterial bed, but not the renal arterial bed, in endotoxaemia. The present results in the mesenteric arterial beds are consistent with previous studies (Tarpey and Randall, 1998; Mitchell et al., 1993) in

that there was no difference in responses to methoxamine given as bolus doses at either 2 h or 24 h. However, we now clearly show that there is hypocontractility of the mesenteric arterial beds at 24 h after the onset of LPS infusion, if methoxamine is applied as cumulative concentrations. This was not due to a generalised inability of the mesenteric arterial smooth muscle to contract, as responses to KCl were unimpaired. These results are, in some respects, similar to those of others, who showed a waning of the contractile response to noradrenaline in the mesenteric arterial bed (Mitolo-Chieppa et al., 1996) and mesenteric resistance arteries (Martinez et al., 1996) isolated from rats treated with LPS.

It is interesting that hypocontractility was apparent when methoxamine was applied as sustained concentrations, but not when applied as bolus doses. One possible explanation for this could be the way Ca^{2+} is utilised during the course of a contraction. Specifically, the transient contractions evoked by bolus doses of methoxamine may depend primarily on mobilization of Ca^{2+} from intracellular stores, whilst the sustained contractions elicited by prolonged exposure to methoxamine may involve, to a greater extent, entry of extracellular Ca^{2+} into the smooth muscle cells and Ca^{2+} sensitization. The possible role of Ca^{2+} in impaired responses to methoxamine was investigated in isolated superior mesenteric arteries and aortae (the choice of preparations for these experiments was largely dictated by the nature of the experiments) as discussed below.

In contrast to the impairment of vascular contractility to methoxamine in both the mesenteric arterial bed and the aorta, isolated from endotoxaemic rats, the renal vascular bed isolated from these animals displayed no significant attenuation of the contractile response to cumulative concentrations of methoxamine or KCl. The lack of change in the renal response to methoxamine in endotoxaemia is consistent with

findings in the renal vasculature *in vivo* (Waller et al., 1994), although the renal vascular bed *in vivo* displays a greater degree of hyperaemic vasodilatation after 24 h LPS than the mesentery (Waller et al., 1994). The present results indicate that renal vasodilatation is not likely to be due to a loss of α -adrenoceptor-mediated vasoconstrictor tone. In a number of tissues (including the mesentery and aorta) induction of inducible NO synthase (iNOS) is maximal at 6 h after the start of LPS infusion, but this returns to control levels at 24 h (Gardiner et al., 1995; Mitchell et al., 1995) and kidney iNOS does not change during LPS infusion (Gardiner et al., 1995), therefore, other factors must be responsible for the vasodilatation seen at that stage. It remains to be determined what mechanisms are responsible for protecting the renal vascular bed under these conditions.

Hypocontractility to methoxamine in thoracic aortae in endotoxaemia. The aortic preparations also showed hypocontractility to methoxamine, but here the difference was apparent in preparations isolated at both 2 and 24 h after the onset of LPS infusion. Thus, endotoxaemia can cause hypocontractility to a given vasoconstrictor (methoxamine) in both conduit (aorta) and resistance (mesenteric arterial bed) vessels, although temporal differences in the susceptibility of these two vasculatures to impaired responsiveness were observed. In neither case was the hypocontractility to methoxamine due to a generalised impairment of smooth muscle contractile function as responses to KCl were not affected. Thus, hypocontractility to methoxamine in this model of endotoxaemia could involve one or more of the steps including and/or subsequent to stimulation of α_1 -adrenoceptors.

Interestingly, in contrast to the pronounced impairment of contractions to methoxamine, contractions to U46619 in the aortae were unaffected by LPS infusion,

at either 2 h or 24 h. Similarly, U46619-induced contractions were reported to be largely maintained in rat mesenteric arteries in an *in vitro* model of endotoxaemia, whilst those to phenylephrine were attenuated (O'Brien et al., 2001; Wylam et al., 2001). In rats rendered tolerant to lethal doses of endotoxin by repeated sublethal doses of endotoxin, the pressor response to phenylephrine was attenuated, but a higher peak response to U46619 was observed compared to controls (Coffee et al., 1991), although in the same model the sensitivity of the response to U46619 in isolated aortic rings was reduced (Temple et al., 2001). The reason for these differences between the contractile agents is unclear, but one possibility is differences between their signalling pathways. For example, RhoA, a signalling molecule involved in sensitization of the smooth muscle contractile machinery to Ca^{2+} , is activated to a greater extent by U46619 than by noradrenaline in rabbit aortic smooth muscle (Sakurada et al., 2001).

Role of Ca^{2+} in hypocontractility in endotoxaemia. We attempted to explore some of the underlying mechanisms of the changes involved. Ca^{2+} measurements in the mesenteric artery were restricted to 24 h, since that was the time when significant changes in contractile responses were observed. The results showed a tendency for smaller changes in Ca^{2+} after LPS treatment, but these were not significant so it would appear that, as shown by Martinez et al. (1996), a failure to release Ca^{2+} does not underly hypocontractility to methoxamine. In the aortae, preparations taken at 24 h after LPS treatment also showed normal responses to caffeine, indicating that a change in mobilization of intracellular Ca^{2+} also does not underly hypocontractility to methoxamine, pointing to changes in Ca^{2+} -sensitization of contractile proteins (which can take place without large changes in Ca^{2+}) or to the involvement of another factor. Others have shown an important contribution of L-arginine availability and the nitric

oxide (NO) pathway in regulating the extent of mesenteric hypocontractility produced by LPS (Mitolo-Chieppa et al., 1996). We did not investigate the extent to which that was involved in the changes observed here.

In the aorta, responses to methoxamine were reduced at 2 h and 24 h LPS, but the response to caffeine was reduced only at 2 h LPS. Thus, this indicates that there may be an impairment of Ca^{2+} release from internal stores at 2 h, but not at 24 h, after LPS infusion. Previous studies have shown elevated basal levels of intracellular Ca^{2+} in rat aorta caused by sepsis or endotoxin treatment, which may result from an impairment of Ca^{2+} storage in intracellular organelles (Song et al., 1993; Martinez et al., 1996). Impaired storage, and by implication release, of Ca^{2+} in intracellular organelles could explain the decreased response to caffeine observed at 2 h. Inducible NOS activity in the aorta in this model of endotoxaemia has been shown to increase between 2 and 6 h, and then to be undetectable at 24 h, after the onset of LPS infusion (Gardiner et al., 1995), which is, in some respects, similar to the temporal change in the response to caffeine. In this regard it is interesting that NO has been shown to reduce the rate of Ca^{2+} release from skeletal muscle sarcoplasmic reticulum and open probability of ryanodine receptors in lipid bilayers (Meszaros et al., 1996).

If changes in Ca^{2+} mobilization or entry (present study) and NO (Gardiner et al., 1995; Mitchell et al., 1995) do not underlie the mesenteric and aortic hypocontractility to methoxamine that we have observed, at least at 24 h LPS, this raises the question of what mechanisms are involved. One possibility is an involvement of K^{+} channels, as these have been shown to be upregulated by LPS treatment (Czaika et al., 2000), and are involved in relaxation to LPS and in *ex vivo* aortic hypocontractility to phenylephrine and noradrenaline in LPS-treated rats (Hall et al., 1996; Sorrentino et al., 1999; Chen et al., 2000).

Conclusion. In conclusion, the present study has demonstrated that in mesenteric arterial beds and in aortae, but not in the renal arterial bed, isolated from rats following infusion of LPS, there is an attenuation of the contractile response to methoxamine. This is not due to a generalised inability of the smooth muscle to contract and, at least in late endotoxaemia (24 h LPS), does not appear to involve abnormalities in Ca^{2+} mobilization or entry. Moreover, in the mesenteric arterial beds from the endotoxaemic animals there clearly is an impairment of sustained contractile responses to continuous activation, but not to transient activation, of α_1 -adrenoceptors, which may be significant for different patterns of stimulation used by sympathetic nerves and/or circulating catecholamines.

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Footnotes

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b) Reprint requests to: Dr Vera Ralevic, School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Clifton Boulevard, Nottingham NG7 2UH, United Kingdom.

Figure Legends

Figure 1. a) The dose-response relationship to methoxamine in the isolated mesenteric arterial bed. Increases in perfusion pressure of mesenteric arterial beds isolated from rats following intravenous infusion of 2 h saline (n=6), 24 h saline (n=5), 2 h lipopolysaccharide (LPS; n=8) or 24 h LPS (n=9) to injections of bolus doses of methoxamine (500 pmol-50 μ mol).

b) Time course of response to a single dose of methoxamine in the isolated mesenteric arterial bed. Change in perfusion pressure with time of mesenteric arterial beds isolated from rats following intravenous infusion of 2 h saline (n=6), 24 h saline (n=5), 2 h lipopolysaccharide (LPS; n=8) or 24 h LPS (n=9) in response to single injections of a 1.5 μ mol dose of methoxamine.

Figure 2. a) The concentration-response relationship to methoxamine in the isolated mesenteric arterial bed. Increases in perfusion pressure of mesenteric arterial beds isolated from rats following intravenous infusion of 2 h saline (n=9), 24 h saline (n=9), 2 h lipopolysaccharide (LPS; n=8) or 24 h LPS (n=8) to cumulative concentrations of methoxamine (1-100 μ M). * $p < 0.05$ (24 h LPS vs 24 h saline).

b) The concentration-response relationship to KCl in the isolated mesenteric arterial bed. The increases in perfusion pressure of mesenteric arterial beds isolated from rats following intravenous infusion of 2 h saline (n=7), 24 h saline (n=7), 2 h lipopolysaccharide (LPS; n=6) or 24 h LPS (n=6) to cumulative concentrations of KCl (10 mM - 300 mM). * $p < 0.05$ (24 h saline vs 2 h LPS).

Figure 3. Ca^{2+} -imaging experiments with Fura-2 during challenges with methoxamine and KCl. Experiments utilising Fura-2 Ca^{2+} -imaging in superior

mesenteric arteries isolated from rats following 24 h of intravenous infusion with either saline (n=5) or lipopolysaccharide (LPS; n=5). **a.** Change in 340/380 ratio upon challenge with methoxamine (10 μ M); **b.** Change in 340/380 ratio upon challenge with methoxamine (10 μ M), expressed as a percentage of the response to KCl (60 mM); **c.** Time taken to the maximum contraction to methoxamine (10 μ M) (s); **d.** Change in 340/380 ratio upon challenge with KCl (60 mM).

Figure 4. Concentration-response relationship to methoxamine in the isolated renal arterial bed of endotoxaemic rats. The response to cumulative concentrations of methoxamine (10 nM - 100 μ M) in kidneys isolated from rats following infusion of either 2 h saline (n=6), 24 h saline (n=8), 2 h LPS (n=9) or 24 h LPS (n=5). Data are means with vertical bars indicating s.e.mean.

Figure 5. a) Cumulative concentration-response curves to methoxamine (1 μ M - 1 mM) in isolated aortae. The tension (g) elicited upon contraction, in response to cumulative concentrations of methoxamine in aortae, isolated from rats, following infusion of either 2 h saline (n=5), 24 h saline (n=5), 2 h lipopolysaccharide (LPS; n=6) or 24 h LPS (n=4). Data are means with vertical bars indicating s.e.mean. * p <0.05 (vs control), ** p <0.01 (vs control), † p <0.001 (vs control).

b) Cumulative concentration-response curves to U46619 (1 nM-1 μ M) in isolated aortae. The tension (g) elicited upon contraction, in response to cumulative concentrations of U46619 in aortae, isolated from rats, following infusion of either 2 h saline (n=6), 24 h saline (n=7), 2 h lipopolysaccharide (LPS; n=6) or 24 h LPS (n=7). Data are means with vertical bars indicating s.e.mean.

Figure 6. Cumulative concentration-response curves to KCl (10 mM - 300 mM) in isolated aortae. The tension (g) elicited upon contraction, in response to cumulative concentrations of KCl in aortae, isolated from rats, following infusion of either 2 h saline (n=5), 24 h saline (n=6), 2 h lipopolysaccharide (LPS; n=6) or 24 h LPS (n=4). Data are means with vertical bars indicating s.e.mean.

Figure 7. Contractions elicited by caffeine in isolated aortae. The tension (g) elicited upon contraction, in response to caffeine (20 mM), in aortae, isolated from rats at either 2h (a), following infusion of either saline (n=6) or lipopolysaccharide (LPS; n=7), or at 24h (b) following infusion of either saline (n=7), or LPS (n=5). Data are means with vertical bars indicating s.e.mean. * $p < 0.05$.

Figure 1

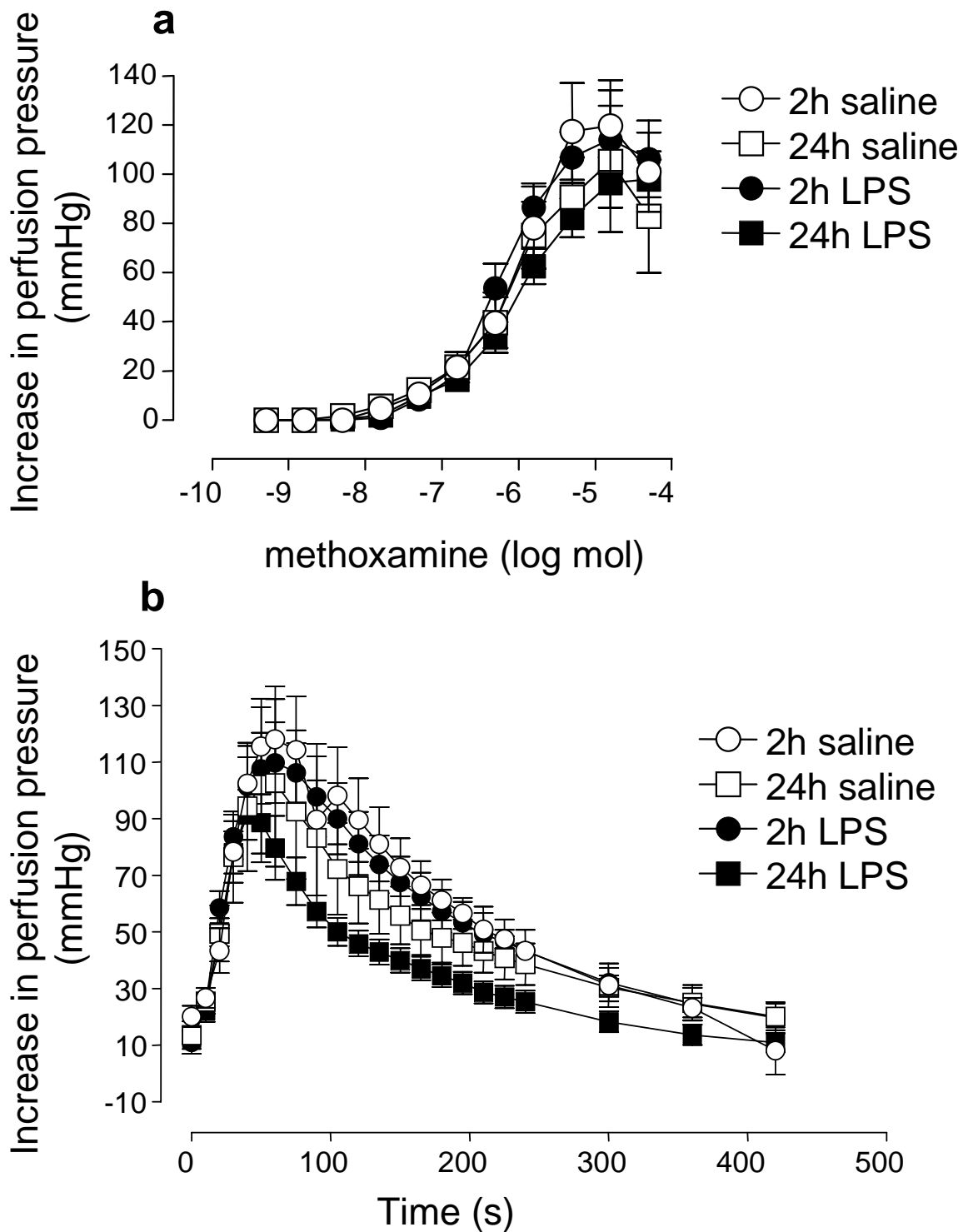


Figure 2

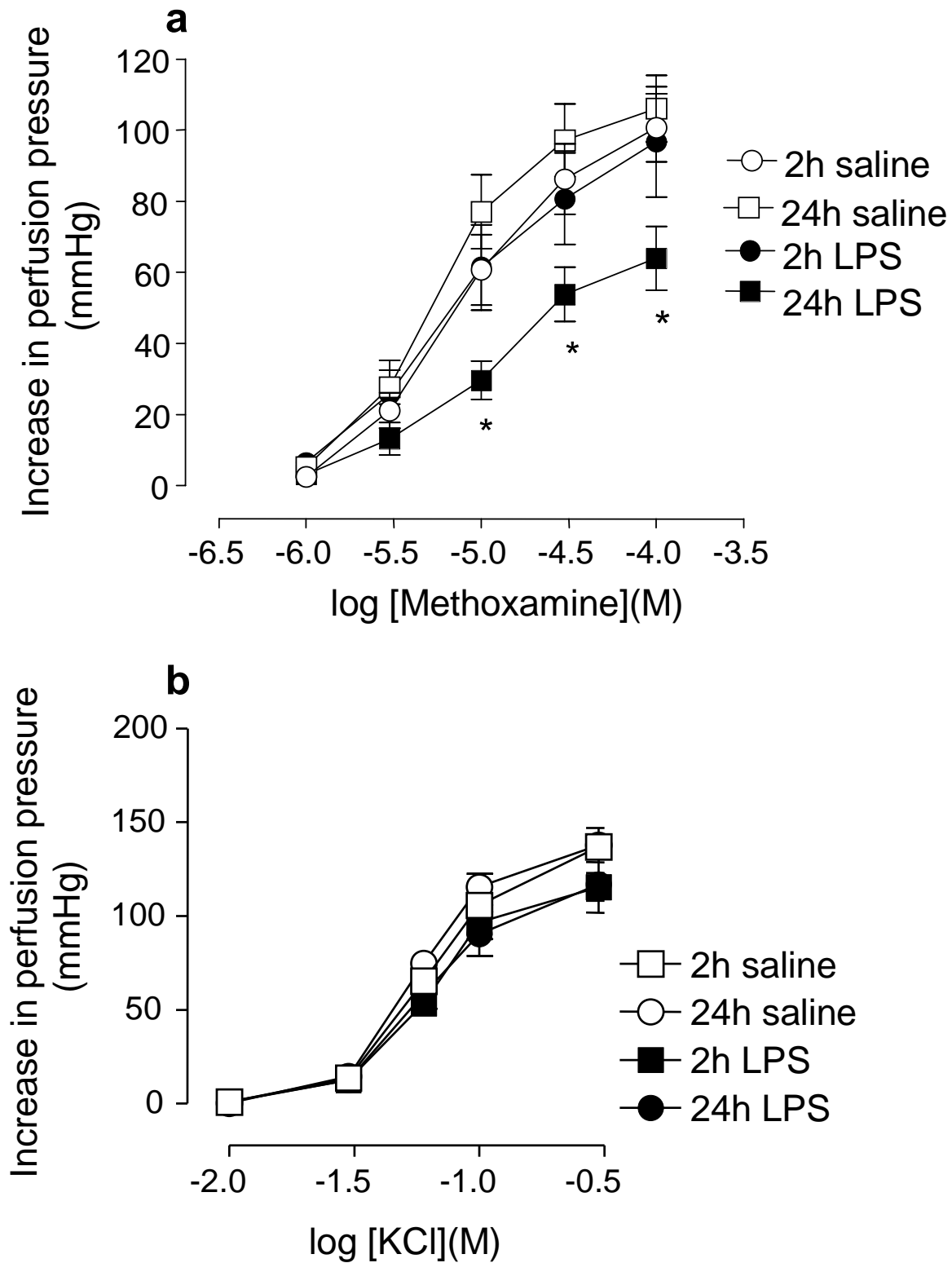


Figure 3

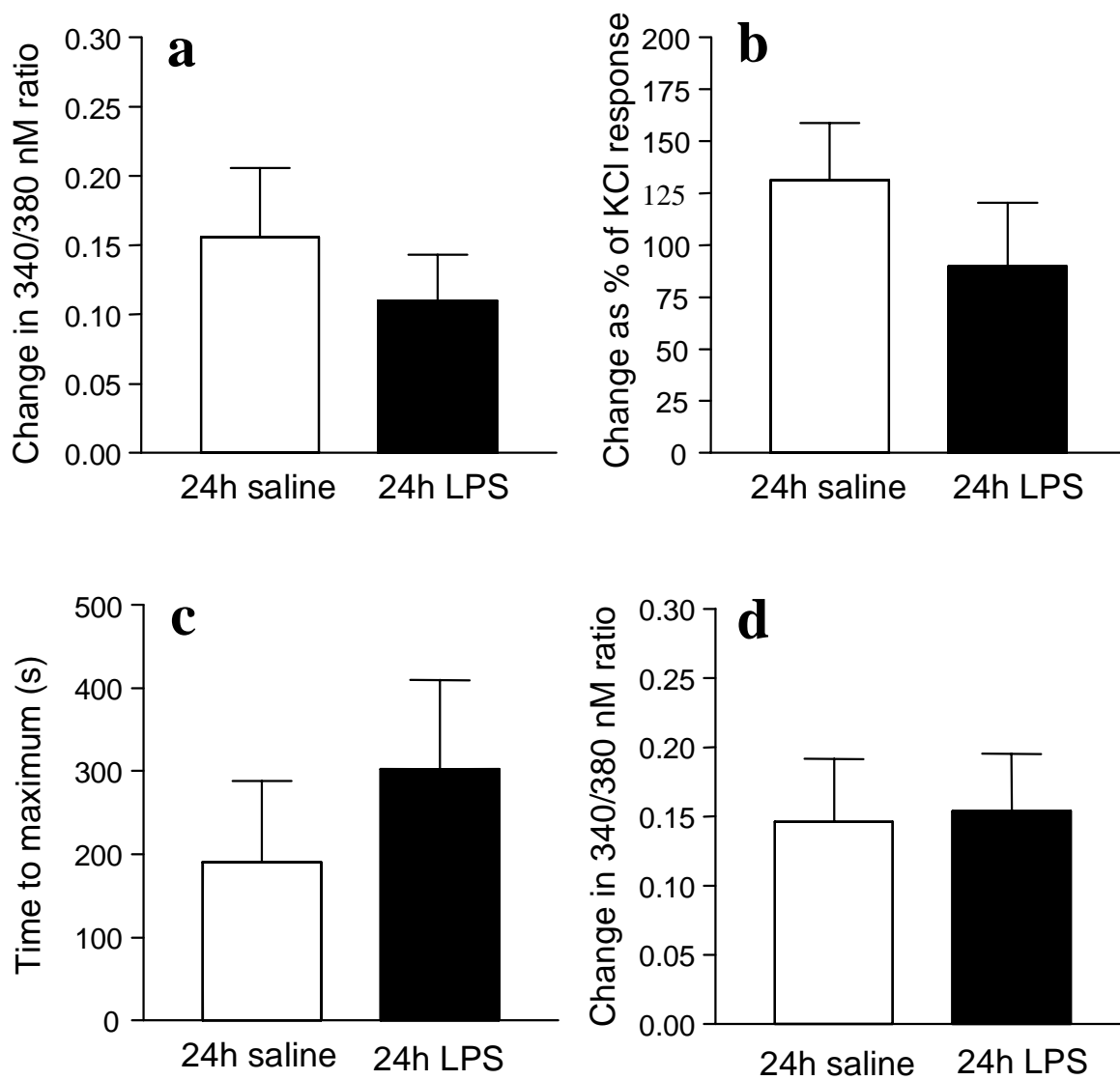


Figure 4

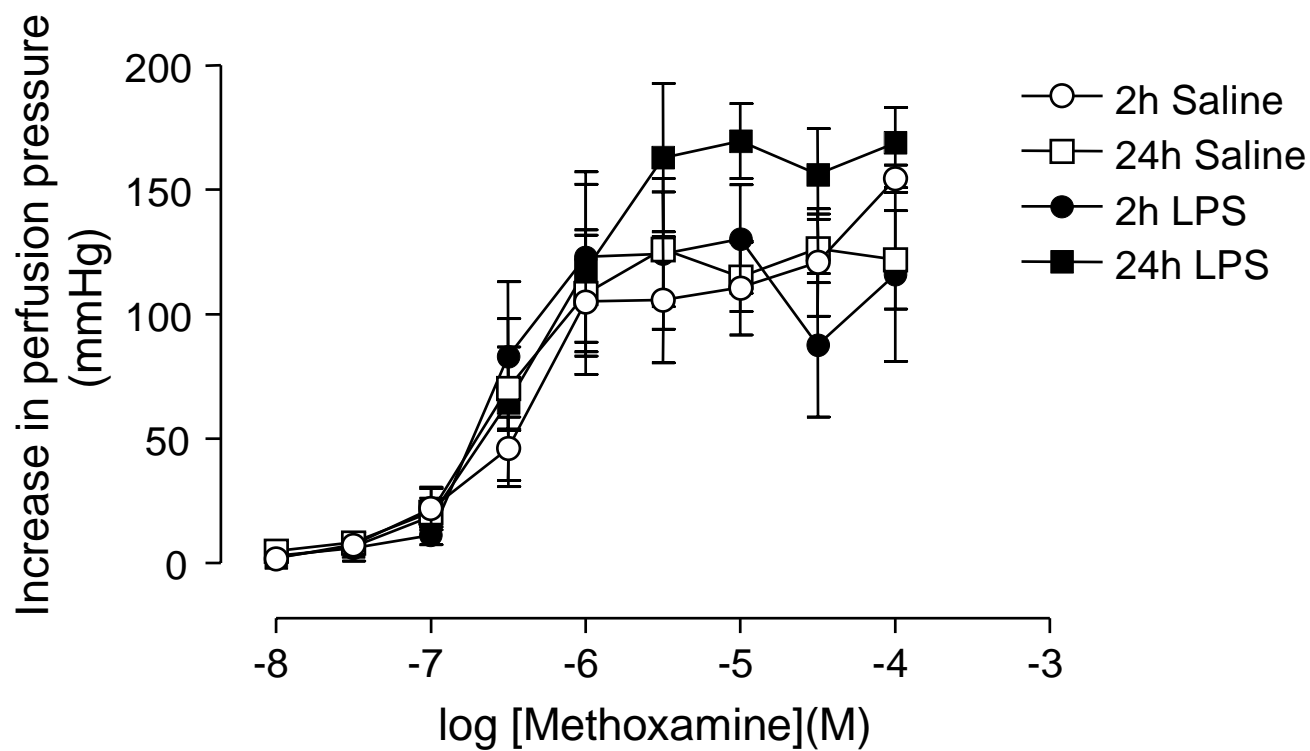


Figure 5

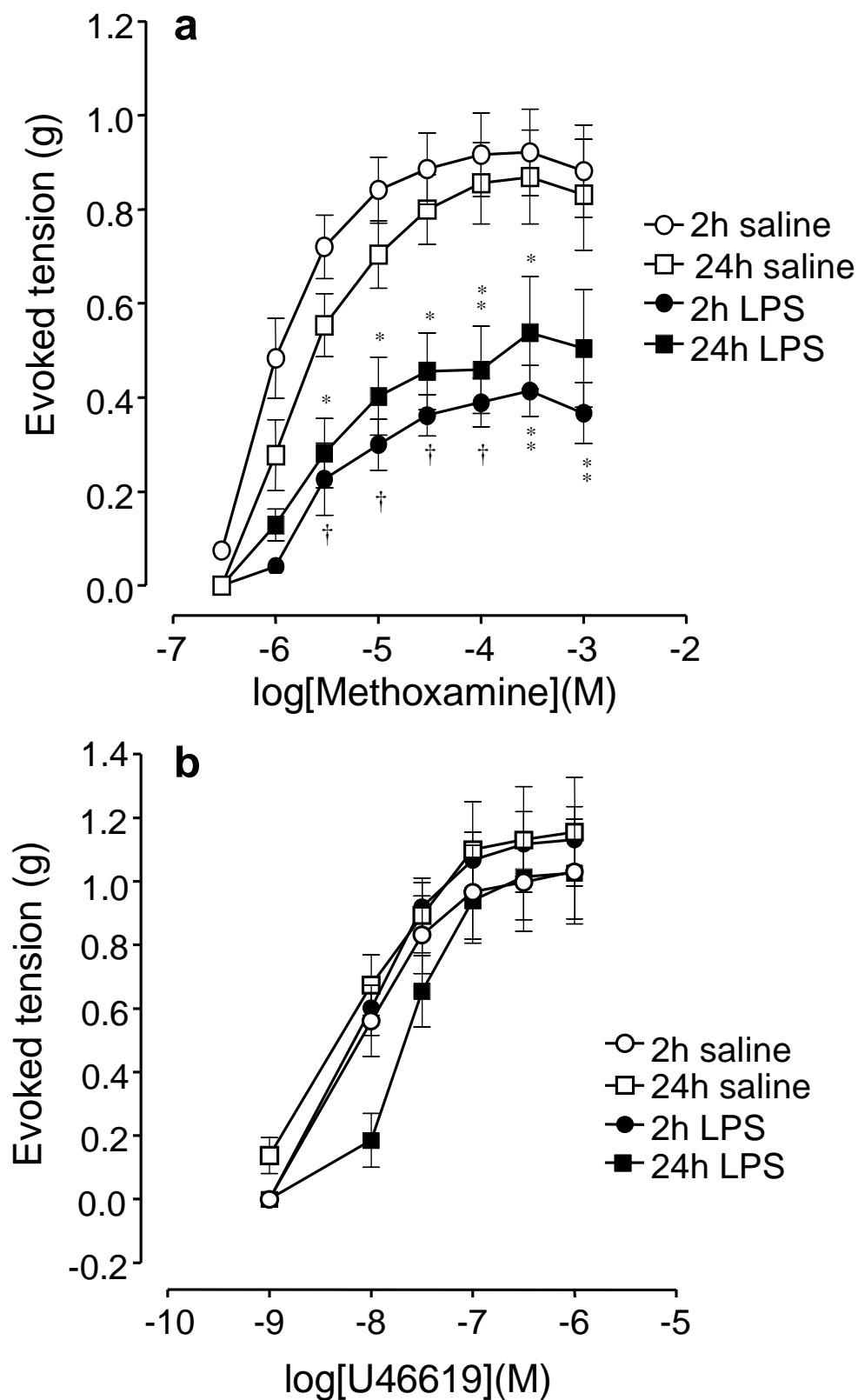


Figure 6

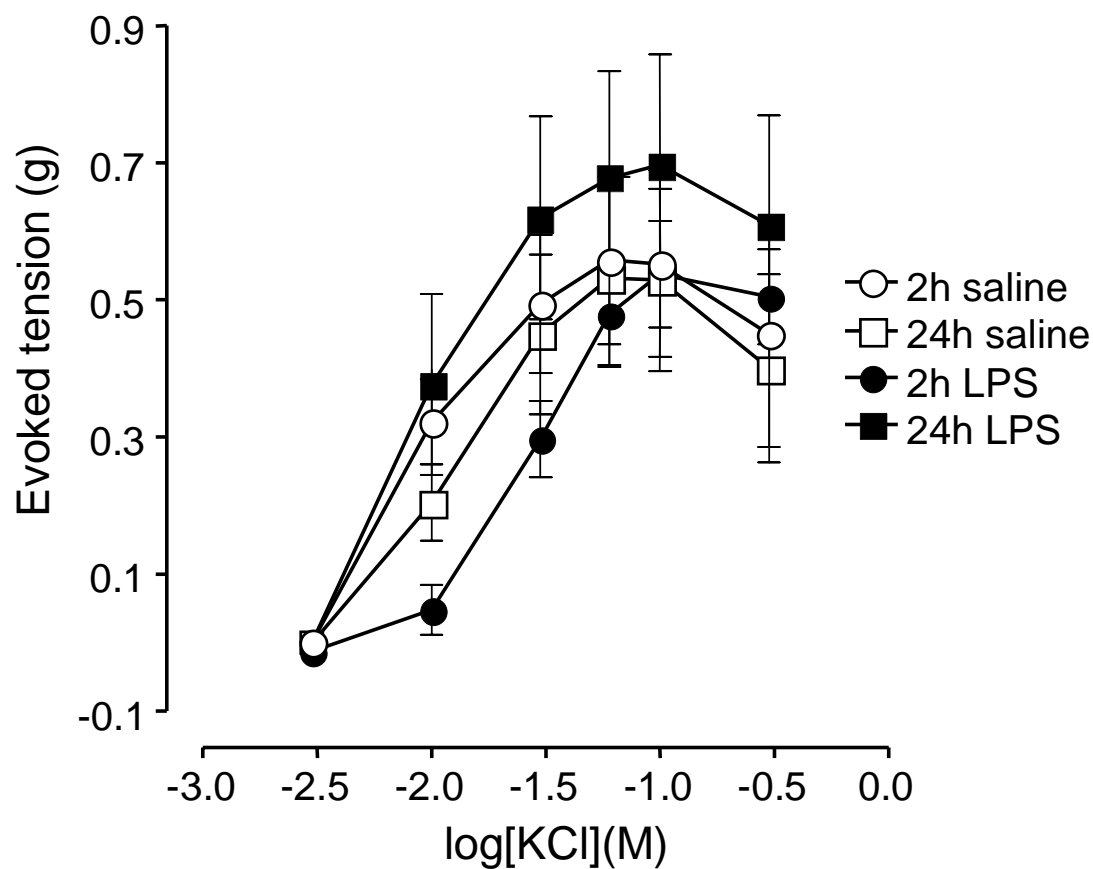


Figure 7

