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Alteration by lipopolysaccharide of the relationship between intracellular calcium levels and contraction in rat mesenteric artery

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1 The aim of this work was to investigate the effect of lipopolysaccharide (LPS) treatment on the relationship between the cytosolic Ca^{2+} ion concentration ($[Ca^{2+}]_i$) and contraction in rat resistance arteries, and the involvement of the L-arginine-nitric oxide (NO)-guanosine 3'-5' cyclic monophosphate (cyclic GMP) pathway in these effects.

2 $[Ca^{2+}]_i$ and tension were simultaneously recorded in small mesenteric arteries removed from rats 4 h after intraperitoneal injection of *E. coli* LPS (30 mg kg^{-1}) or solvent. Cyclic GMP was assayed in vessels submitted to identical treatments.

3 Basal $[Ca^{2+}]_i$ was higher in vessels from LPS-treated rats compared to controls. LPS did not modify the concentration-contraction curve of noradrenaline. However, the increase in basal $[Ca^{2+}]_i$ produced by LPS resulted in a shift of the noradrenaline $[Ca^{2+}]_i$-contraction curve to higher $[Ca^{2+}]_i$ concentrations.

4 L-Arginine (300 μM) relaxed noradrenaline (10 μM) pre-contracted arteries from LPS-treated but not from control rats. This effect of L-arginine was reversed by two inhibitors of NO synthase: N^{ω}-nitro-L-arginine-methyl-ester (L-NAME, 1 mM) and S-methyl-isothiourea (SMT, 0.1 mM). Both the relaxing effect of L-arginine and its reversal by L-NAME or SMT occurred without any change in $[Ca^{2+}]_i$.

5 LPS treatment did not modify the cyclic GMP content of the small mesenteric arteries. In arteries removed from LPS-treated rats but not from controls, addition of L-arginine (300 μM) was associated with a significant increase in cyclic GMP content, an effect which was prevented by both L-NAME (1 mM) and SMT (0.1 mM).

6 L-NAME (1 mM) produced a greater reduction in cyclic GMP content than SMT (0.1 mM) in control vessels exposed to L-arginine (300 μM). Under the same conditions, SMT produced a larger decrease in cyclic GMP level than L-NAME in arteries taken from LPS-treated rats, consistent with selective inhibition by SMT of the inducible NO-synthase after LPS.

7 These results show that LPS produced two effects in small mesenteric arteries: (i) alterations in Ca^{2+} handling and a decreased sensitivity of myofilaments to Ca^{2+}, (ii) induction of NO-synthase activity resulting in exogenous L-arginine-dependent production of NO and cyclic GMP accumulation. Both effects are likely to be involved in the hyporeactivity induced by LPS in resistance arteries.

Keywords: Calcium; endotoxin shock; resistance arteries

Introduction

Lipopolysaccharide (LPS)-administration decreases vascular resistance, produces vascular hyporeactivity to different vasoconstrictors and promotes the overproduction of nitric oxide (NO) through activation of the inducible isoform of NO synthase (iNOS) (see Stoclet *et al.*, 1993). The effect of LPS on vascular reactivity and the involvement of NO in this effect have been well documented in large conductance arteries such as the aorta (Julou-Schaeffer *et al.*, 1990). However, the sensitivity to noradrenaline was only slightly decreased in the femoral artery (Schneider *et al.*, 1994) and was not significantly impaired in small mesenteric arteries (Schneider *et al.*, 1992; Mitchell *et al.*, 1993) from LPS-treated rats, despite the expression of iNOS in these vessels (Mitchell *et al.*, 1993). The expression of iNOS was probably implicated in the exogenous L-arginine-induced relaxation in small resistance arteries (Schneider *et al.*, 1992; 1994).

The cytosolic calcium concentration ($[Ca^{2+}]_i$) is increased in aortae from septic rats (Song *et al.*, 1993), in endotoxin-treated hepatocytes (Portoles *et al.*, 1991) and in peripheral blood cells from patients with sepsis (Zaloga *et al.*, 1993). These studies suggest that an increase in $[Ca^{2+}]_i$ may contribute to the metabolic abnormalities and to the impairment of vascular responsiveness during sepsis. However, it is unknown whether treatment of the rat with LPS, which represents an experimental model of endotoxin shock, alters $[Ca^{2+}]_i$ in resistance vessels. Moreover, to the best of our knowledge, the relationship between $[Ca^{2+}]_i$ and contraction after LPS treatment has never been determined. Therefore, the present work was designed to examine the effect of LPS on $[Ca^{2+}]_i$ in rat small mesenteric arteries. Observations were made recording simultaneously the changes in $[Ca^{2+}]_i$ and contractile force. The involvement of L-arginine-NO-guanosine 3'-5'-monophosphate (cyclic GMP) pathway was assessed by determining cyclic GMP content of the tissues and by using the NO synthase (NOS) inhibitor, N^{ω}-nitro-L-arginine-methyl-ester (L-NAME) and the recently-described selective inhibitor of iNOS (Southan *et al.*, 1995), S-methyl-isothiourea (SMT).

Methods

Arterial preparation and mounting

Male Wistar rats (12-14 wk old) bred in our institute were treated with an intraperitoneal bolus injection of either saline (0.9% NaCl, 0.1 ml 100 g^{-1} body weight) or *Escherichia coli*

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LPS (30 mg kg^{-1} in the same volume of saline). Four hours later the rats were killed by cervical dislocation and exsanguinated by carotid artery transection. The viscera were exposed, and a proximal segment of the small bowel was removed and pinned in a dissecting dish containing physiological solution (PSS) of the following composition (in mM): NaCl 119, KCl 4.7, KH_2PO_4 0.4, $NaHCO_3$ 14.9, $MgSO_4$ 1.17, $CaCl_2$ 2.5, and glucose 5.5. Branch II or III resistance arteries were cleaned of fat and connective tissue and a 2 mm long segment was removed. The segment was mounted in a myograph (Mulvany & Halpern, 1977) filled continuously with PSS at 37°C and gassed with 95% O_2-5% CO_2 (pH 7.4). Briefly, two tungsten wires (30 μm diam) were inserted through the lumen of the vessels. Mechanical activity was recorded isometrically by a force transducer (model DSG BE4, Kistler-Morse) connected to one of the two tungsten wires; the other wire was attached to a support carried by a micromanipulator.

After mounting, the vessel was equilibrated for 30 min before being passively stretched to an internal diameter which yielded a circumference equivalent to 90% of that given by an internal pressure of 100 mmHg; this required a load of about 200 mg. The internal diameter of the vessels used in this study was 150–200 μm. After the vessel was set to its working length, it was challenged twice with 10 μM noradrenaline to elicit reproducible contractile responses. The presence of functional endothelium was assessed in all preparations by the ability of 1 μM acetylcholine to induce >50% relaxation of vessels pre-contracted with 10 μM noradrenaline.

Simultaneous measurement of $[Ca^{2+}]_i$ and contraction

Changes in $[Ca^{2+}]_i$ were determined simultaneously with mechanical activity by measuring the fluorescence of trapped fura 2 with a dual-excitation wavelength fluorometer (Fluorolog II, SPEX, Edison, NJ), as described by Andriantsitohaina *et al.* (1995). The vessel segments were loaded with fura 2 by incubation with the acetoxymethyl ester form of the dye over 2 h. The experiments were then performed in normal PSS at 37°C and continuously gassed with 95% O_2-5% CO_2 (pH 7.4). $[Ca^{2+}]_i$ was calculated by use of the equation described by Grynkiewicz *et al.* (1985).

Concentration-response curves to noradrenaline were constructed by cumulative application of noradrenaline (0.01–10 μM). When the maximal contractile response was developed, L-arginine was added at a maximally active concentration (300 μM) to the bath. When the response to L-arginine reached a steady-state, L-NAME or SMT were added. The NOS inhibitors were used at maximally-active concentrations which were 1 mM and 0.1 mM for L-NAME and SMT, respectively.

Determination of tissue cyclic GMP content

Second- and third-generation mesenteric arteries, taken from either control and LPS-treated rats, were incubated at 37°C in the absence or in the presence of L-arginine (300 μM), L-arginine plus L-NAME (1 mM) or L-arginine plus SMT (0.1 mM) for 30 min in PSS containing isobutylmethylxanthine (IBMX, 10 μM) and gassed with 95% O_2-5% CO_2. IBMX was added in order to inhibit cyclic GMP degradation via cyclic nucleotide phosphodiesterases. The reaction was terminated by transferring the vessels into an ice-cold HCl solution (0.1 N). Following homogenization of the tissues, the cyclic GMP content was determined by radioimmunoassay (Cailla *et al.*, 1976). Cyclic GMP was expressed as picomol μg^{-1} DNA. The DNA content was measured as described by Brunk *et al.*, (1976).

Statistical analysis

For construction of concentration-effect curves, tension values obtained at the peak of the response elicited after each addition

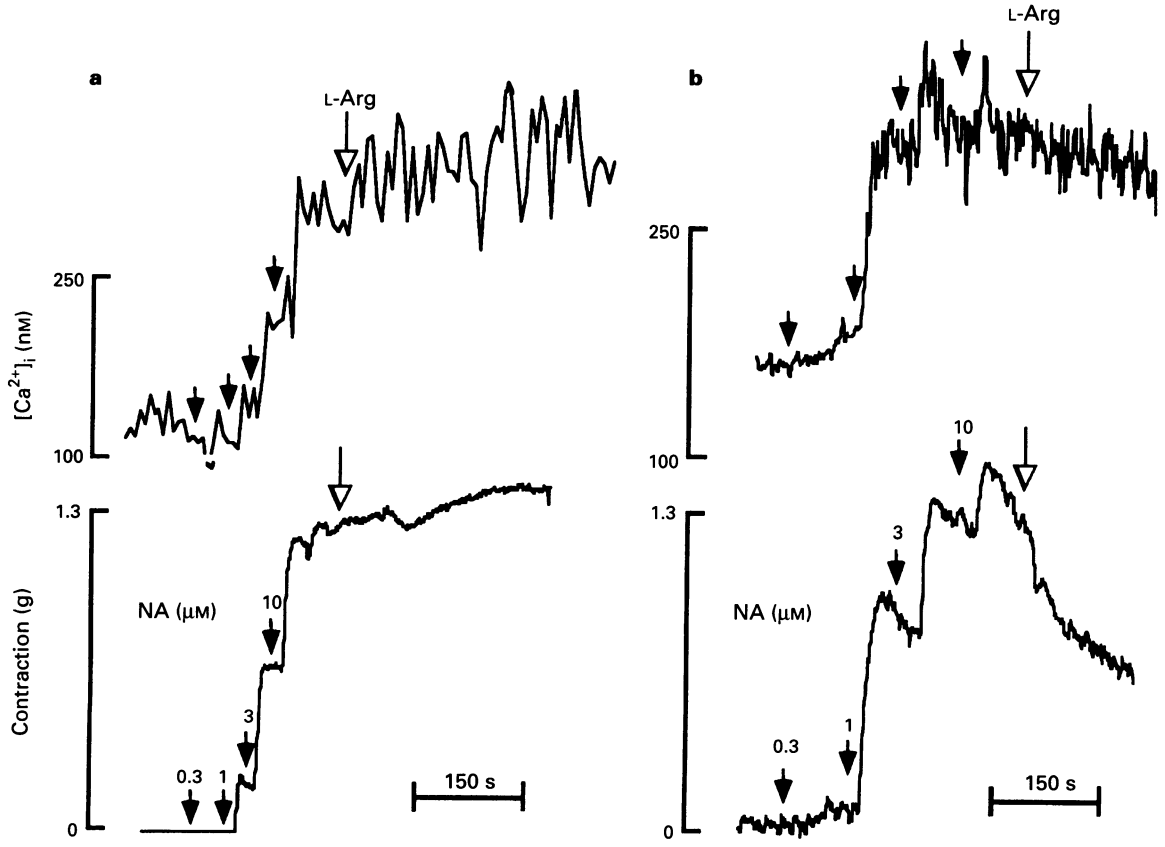

Figure 1 Representative traces showing the effect of noradrenaline (NA) and L-arginine (L-Arg, 300 μM) on $[Ca^{2+}]_i$ (upper panels) and contraction (bottom panels) in small mesenteric arteries from control (a) or lipopolysaccharide (LPS)-treated (b) rats. Similar results were obtained in 5–9 separate experiments.

of noradrenaline were used. EC_{50} values (concentrations of noradrenaline producing a half-maximal response) were calculated and Ca^{2+}-force curves were adjusted by use of a logit-log regression. Analysis of variance (ANOVA) was used for statistical analysis. $P<0.05$ was considered significant.

Drugs and solutions

E. coli LPS (LD_{50}: 32.76 mg kg^{-1}, 055:B5) was purchased from Difco (Detroit, MI), SMT was a generous gift from Dr C. Thiemermann (The William Harvey Research Institute, London) and all other chemicals were purchased from Sigma (Grenoble, France). Fura 2 was dissolved in dimethyl sulphoxide. All other drugs were diluted in de-ionized water (Q10, Millipore).

Results

In the resting state, basal $[Ca^{2+}]_i$ was increased from 102 ± 16 nM ($n=5$) in control vessels to 170 ± 17 nM ($n=9$) ($P<0.05$) in vessels from LPS-treated rats. As illustrated in Figure 1, noradrenaline induced a concentration-dependent increase in $[Ca^{2+}]_i$ in arteries from control and LPS-treated rats. After addition of each dose of noradrenaline, $[Ca^{2+}]_i$ rapidly increased and oscillated thereafter around a constant plateau level. The mean values of $[Ca^{2+}]_i$ reached in arteries exposed to noradrenaline are shown in Figure 2a. The absolute values of $[Ca^{2+}]_i$ were higher in vessels from LPS-treated than in those from control rats, and the concentration-effect curve of noradrenaline on $[Ca^{2+}]_i$ was shifted upward ($P<0.05$) (Figure 2a). However, the increase in $[Ca^{2+}]_i$ (plateau minus basal values of $[Ca^{2+}]_i$) produced by each concentration of noradrenaline was not different in control and LPS-exposed vessels.

The increases in tension produced by noradrenaline were generally sustained in control vessels, but not in those from LPS-treated rats (Figure 1). The curve describing the relationship between the noradrenaline concentration and the peak tension value reached after each addition of noradrenaline was not altered by LPS (Figure 2b), the EC_{50} values were, respectively, 1.76 ± 0.29 μM versus 1.34 ± 0.29 μM in LPS-treated and control vessels (not significantly different).

Figure 2c shows that LPS shifted the noradrenaline $[Ca^{2+}]_i$-force curve to the right in a parallel manner ($P<0.05$) as a result of the increase in basal $[Ca^{2+}]_i$.

Following completion of the concentration-effect curves to noradrenaline, the addition of L-arginine (300 μM) produced relaxation in the arteries removed from LPS-treated rats but not in those from control. In LPS-treated vessels, the decrease of contraction measured 3 min after addition of L-arginine was $24.6\pm4.3\%$ ($P<0.05$) whereas spontaneous relaxation without L-arginine was not statistically significant (Figure 3). However, the L-arginine-induced relaxation was not associated with a decline in $[Ca^{2+}]_i$ (Figure 1b). Addition of L-NAME (1 mM) or SMT (0.1 mM) restored the contraction to the levels reached before L-arginine addition (Figure 3) without producing any effect on $[Ca^{2+}]_i$ (not shown).

The LPS treatment did not modify the cyclic GMP content of small mesenteric arteries (Figure 4). In control vessels, the level of cyclic GMP was not altered by L-arginine addition. However, L-arginine addition was associated with a 1.8 fold increase ($P<0.05$) in the cyclic GMP content in vessels from LPS-treated rats. In the presence of L-arginine, both L-NAME (1 mM) and SMT (0.1 mM) decreased the cyclic GMP content in vessels from control and LPS-treated rats. L-NAME produced a greater reduction of cyclic GMP level than SMT in

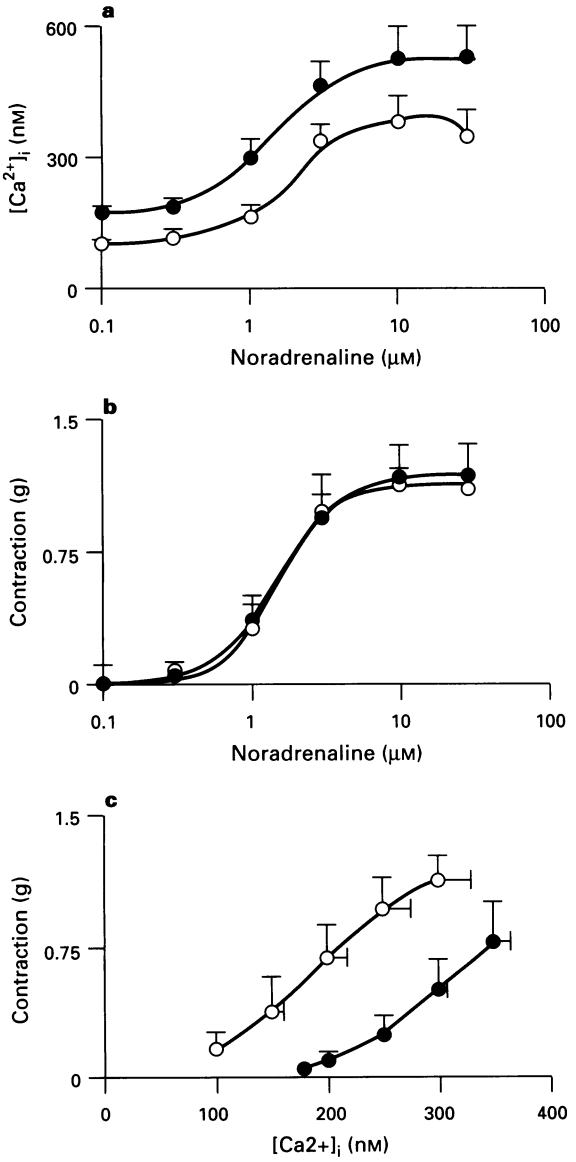

Figure 2 Concentration-response curves for noradrenaline-induced increase in $[Ca^{2+}]_i$ (a) and contraction (b) in small mesenteric arteries from control (○; $n=5$) and lipopolysaccharide (LPS)-treated (●; $n=9$) rats. (c) $[Ca^{2+}]_i$-force relationship in small mesenteric arteries, exposed to noradrenaline, from control (○; $n=5$) and the LPS-treated (●; $n=9$) rats. Values are mean±s.e.mean.

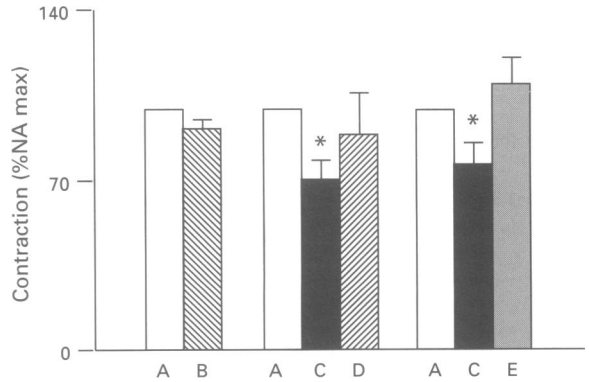

Figure 3 Histograms showing the effect of L-arginine (300 μM, columns C) and subsequent addition of L-NAME (1 mM, column D) or S-methyl-isothiourea (0.1 mM, column E) on tension in small mesenteric arteries from LPS-treated rats which were pre-contracted with a maximally-effective concentration of noradrenaline (NA, 10 μM; columns A). Column B shows the tension in vessels without L-arginine, measured 3 min after the plateau level was reached. Values are the mean of 4 experiments and vertical lines indicate s.e.mean. *Significantly different from 10 μM noradrenaline ($P<0.05$).

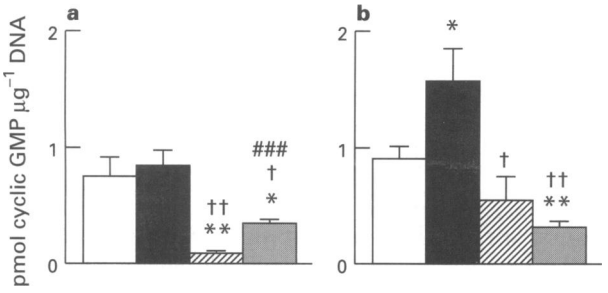

Figure 4 Histograms showing the cyclic GMP content of small mesenteric arteries from control (a, $n=6$) or lipopolysaccharide-treated (b, $n=6$) rats, incubated in the absence (open columns) or in the presence of L-arginine (300 μM, solid columns), L-arginine plus L-NAME (1 mM, hatched columns) or L-arginine plus S-methyl-isothiourea (0.1 mM, stippled columns). Values are mean ± s.e.mean. $*P<0.05$, $**P<0.01$ significantly different compared to values obtained in the absence of L-arginine. $\dagger P<0.05$, $\dagger\dagger P.<0.01$ vs. vessels incubated with L-arginine alone. $\#\#\#P<0.001$ vs. vessels treated with L-arginine plus L-NAME.

vessels from control rats. In contrast, the reduction produced by SMT was greater than that produced by L-NAME in vessels from LPS-treated rats.

Discussion

The results show that LPS treatment produced an increase in basal $[Ca^{2+}]_i$ and modified the relationship between $[Ca^{2+}]_i$ and the contraction elicited by noradrenaline in small mesenteric arteries of the rat.

The increase in basal $[Ca^{2+}]_i$ observed in the present study is in accordance with the results described in the literature in different tissues including the aorta (Song *et al.*, 1993; Zaloga *et al.*, 1993). It was suggested that the elevation in $[Ca^{2+}]_i$ caused by sepsis or endotoxin treatment might be due to an impairment of the calcium storage in intracellular organelles (Song *et al.*, 1993). Indeed, administration of dantrolene, an inhibitor of Ca^{2+} release from the sarcoplasmic reticulum, caused a diminution in $[Ca^{2+}]_i$ in aorta from septic rats (Song *et al.*, 1993) and an improvement in the survival of endotoxin-treated mice (Hotchkiss & Karl, 1994). The present findings suggest that an alteration in calcium handling similar to that seen in the aorta from septic rats probably occurs in resistance arteries of rats treated with LPS.

Our results provide evidence that in vessels from LPS-treated rats the contractile response to noradrenaline was not modified despite the increase in basal $[Ca^{2+}]_i$, suggesting a dissociation between $[Ca^{2+}]_i$ and contraction after LPS treatment. Since the $[Ca^{2+}]_i$-force relationship to noradrenaline was shifted to the right in vessels from LPS-treated animals when compared to those of control rats, it is most likely that LPS produced a decrease in the sensitivity of contractile myofilaments to Ca^{2+}. Indeed, it has been hypothesized that large increases in $[Ca^{2+}]_i$ may decrease the sensitivity of the contractile apparatus to $[Ca^{2+}]_i$ (Himpens *et al.*, 1989). However, the mechanism involved in the reduced Ca^{2+} sensitivity remains to be established. One possible mechanism might be the increase of cyclic GMP content in smooth muscle induced by LPS treatment. It has been shown that the LPS-induced increase in the production of NO results in an elevated level of cyclic GMP via activation of soluble guanylyl cyclase (Fleming *et al.*, 1991). Moreover, an elevation of cyclic GMP can influence myocardial contraction by reducing the relative response of myofilaments to Ca^{2+}, probably via a cyclic GMP-dependent protein kinase (Shah *et al.*, 1994). The mechanism triggering activation of contractile protein is different in cardiac and smooth muscle cells (Stern & Lakatta, 1992; Osol, 1995). Since LPS did not induce any detectable increase in the cyclic GMP content of small resistance arteries, the results presented here do not favour the hypothesis that the increase in basal $[Ca^{2+}]_i$ and the dissociation between $[Ca^{2+}]_i$ and contraction resulted from a cyclic GMP-dependent mechanism. However, we cannot exclude the possibility that the large amounts of NO produced by iNOS activation after LPS treatment (see Moncada & Higgs, 1993) can activate another system through a cyclic GMP-independent mechanism (Bolotina *et al.*, 1994). In addition, LPS could release other mediators which produce more complex effects on vascular responsiveness than those mediated by NO (Beutler & Kruys, 1995).

L-Arginine produced an increase in cyclic GMP associated with relaxation of the small mesenteric arteries from LPS-treated but not from control rats. Both relaxation and elevation of cyclic GMP content induced by L-arginine were reversed by two NO-synthase inhibitors, L-NAME and SMT. Furthermore, in vessels from the LPS-treated rats, SMT, a relatively-selective iNOS inhibitor (Southan *et al.*, 1995), produced a greater effect on the cyclic GMP level than L-NAME. In contrast, L-NAME produced a greater effect than SMT in control vessels. These findings are in agreement with previously published results showing that after induction of iNOS by LPS, L-arginine is a limiting factor in NO production and activation of soluble guanylyl cyclase (Schneider *et al.*, 1992; 1994). The relaxation and enhanced cyclic GMP level induced by L-arginine in vessels from LPS-treated rats was not associated with any variation in $[Ca^{2+}]_i$, suggesting that under these conditions overproduction of cyclic GMP enhanced the desensitization of contractile myofilaments by LPS. Such findings were unexpected in view of previous results showing that cyclic GMP induced a decrease in $[Ca^{2+}]_i$ but no alteration in sensitivity to Ca^{2+} of contractile machinery in the same arteries removed from control rats (Andriantsitohaina *et al.*, 1995). One possible explanation is that LPS treatment blunted the decline of $[Ca^{2+}]_i$ produced by cyclic GMP but decreased the sensitivity of the contractile proteins to Ca^{2+} by unmasking a cyclic GMP-dependent mechanism. Indeed, Ca^{2+} handling was found to be altered by LPS in the present study. Furthermore, LPS can activate many systems, such as arachidonic metabolism, complement, thrombin, reactive oxygen intermediates and produce more complex effects (Beutler & Kruys, 1995) which may alter the sensitivity of contractile proteins to Ca^{2+}. Additional studies are required for a more complete understanding of this phenomenon induced by LPS treatment.

In conclusion, the present work shows that LPS, in addition to inducing iNOS, produces an increase in basal $[Ca^{2+}]_i$ and a dissociation between $[Ca^{2+}]_i$ and contractions in small mesenteric arteries of the rat, an effect which may be involved in the vascular hyporeactivity observed during endotoxin shock. Enhanced production of cyclic GMP subsequent to the expression of iNOS potentiates the dissociation between $[Ca^{2+}]_i$ and contraction which is induced by LPS. A major finding of this study is that LPS modifies the relationships between cyclic GMP and Ca^{2+} handling and its subsequent effect in rat resistance arteries.

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