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Potentiation of Anandamide Effects in Mesenteric Beds Isolated from Endotoxemic Rats

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ABSTRACT

The aim of the present experiments was to study the effects of exogenously added anandamide on transient norepinephrine (NE)-induced contractions in mesenteric beds isolated from adult male Sprague-Dawley rats 6 h after the i.p. administration of 5 mg kg^{-1} lipopolysaccharide (LPS). LPS treatment induced a 3-fold increase in total nitric-oxide synthase (NOS) activity without modifying either the systolic blood pressure or the vascular reactivity to NE of the isolated mesenteric bed. The endocannabinoid anandamide (0.01–10 μM) caused concentration-dependent reductions of the contractile responses to NE in the isolated mesenteric bed. This effect was significantly potentiated after LPS treatment compared with the controls. Anandamide-induced reductions of the contractile responses to NE in mesenteric beds isolated from LPS-treated rats were unmodified by endothelium removal but significantly diminished by either the anandamide amidase inhibitor phenylmethylsulfonyl fluoride (200 μM) or the vanilloid receptor antagonist capsazepine (1 μM). The vanilloid receptor agonist capsaicin (0.01–100 nM) also caused concentration-dependent relaxations that were potentiated in mesenteric beds from LPS-treated rats. Nevertheless, they were unmodified by 1 μM capsazepine. It is concluded that the potentiation of anandamide relaxations after LPS treatment, which are evident at early stages of endotoxic shock, could involve the participation of an anandamide metabolite and might be mediated, at least in part, through a vanilloid receptor.

Endotoxic shock is a life-threatening circulatory disorder with high mortality rate. Its evolution is characterized by a host systemic inflammatory response that usually leads to a pronounced decrease in blood pressure (Parrillo, 1993). The initiating event of the endotoxic shock is linked to the release of endotoxin (lipopolysaccharide, LPS), a cell wall component ubiquitous to Gram negative bacteria. The in vivo administration of LPS has extensively been used to mimic Gram negative sepsis.

It has been suggested that the LPS-induced increase in nitric oxide (NO) synthesis may be involved in the drop in blood pressure during septic shock (Stoclet et al., 1993). Nevertheless, NO production in sepsis does not seem to be a primary cause of systemic hypotension and other factors are likely to have a major role (Pedoto et al., 1998). In this regard, an increased production of the endocannabinoid anandamide by macrophages has been reported to contribute to the endotoxin-induced hypotension via the activation of peripheral CB1 receptors (Varga et al., 1998).

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The aim of the present study was to test whether the reductions caused by exogenous anandamide on transient norepinephrine (NE)-induced contractions (Mendizábal et al., 2001) are modified in mesenteric beds isolated from LPS-treated rats. The participation of vanilloid receptors in anandamide-relaxations was also analyzed in the mesenteric beds isolated from LPS-treated rats, on the basis that the activation of vanilloid VR1 receptors has been proposed as a predominant mechanism for anandamide-induced relaxation in this vasculature (Zygmunt et al., 1999; Mendizábal et al., 2001). Because clinical trials show that early goal-directed therapy provides significant benefits for the outcome of patients with severe sepsis and septic shock (Rivers et al., 2001), a model of early endotoxemia, at a time when blood pressure was maintained, was selected on the hypothesis that eventual pharmacological manipulations should be performed at the very beginning of septic shock.

Materials and Methods

Animal Treatment and Blood Pressure Measurements. Male Sprague-Dawley rats weighing between 230 and 350 g were used in these studies. Rats were bred in the facilities of the School of Pharmacy and Biochemistry (University of Buenos Aires, Buenos Aires, Argentina). Experiments were conducted in accordance with the

ABBREVIATIONS: LPS, lipopolysaccharide; NE, norepinephrine; NO, nitric oxide; NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; PMSF, phenylmethylsulfonyl fluoride.

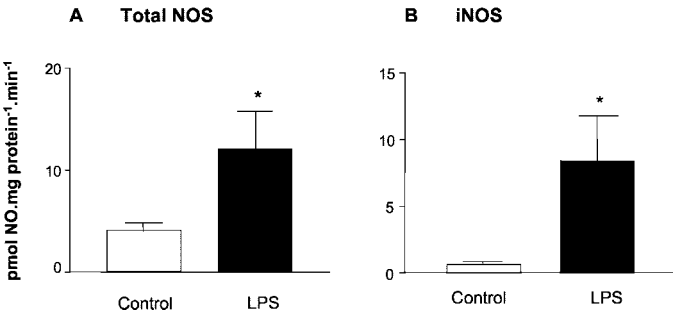

Fig. 1. Effects of LPS treatment on NOS activity in the rat mesenteric bed. NOS activity was measured in homogenates from mesenteric beds isolated 6 h after the intraperitoneal administration of either saline (control) or 5 mg/kg LPS. Total NOS activity was determined in the presence of calcium (A) and specific iNOS activity in the absence of calcium and plus EGTA (B). Results are the mean ± S.E.M. of five to six experiments per group. *, $P < 0.001$ compared with the corresponding controls.

Helsinki Declaration on research involving animals and human beings.

Six hours before the beginning of the functional and biochemical studies, a single injection of LPS from *Escherichia coli* (5 mg/kg i.p.) was administered in 0.25 ml of saline/100 g of body weight. Controls received the same volume of saline. During this 6-h period, food and water were available ad libitum.

The systolic arterial blood pressure, which consisted of the mean of four determinations per rat, was measured by using the tail-cuff method, before as well as 6 h after the LPS injection.

Nitric-Oxide Synthase Activity. Determination of NOS activity was performed by a modification of the [^{14}C]citrulline method described by Bredt and Snyder (1990). Briefly, the mesenteric beds were homogenized in 20 mM HEPES, pH 7.4, containing 1 mM DL-dithiothreitol and 25 mM valine, which was the concentration of the amino acid required to completely inhibit the production of citrulline derived from arginase activity (Mendizábal et al., 2000). The homogenates were centrifuged at 15,000*g* for 15 min and the supernatants were used for both enzymatic and protein determinations. Each sample tube consisted of 250 μl of the supernatant that was diluted with either 250 μl of HEPES containing 1.25 mM $CaCl_2$ or 250 μl of HEPES without $CaCl_2$ plus 1 mM EGTA, to distinguish between total and inducible NOS activity, respectively. Both 120 μM NADPH and 200,000 dpm of [^{14}C]arginine (292 mCi/mmol; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) were added to each sample tube and incubated for 15 min in a Dubnoff incubation bath (50 cycles/min) at 37°C in an atmosphere of 95% O_2, 5% CO_2. At the end of this 15-min period, the samples were immediately applied to individual columns of Dowex AG 50W-X8 200 to 400 mesh sodium form (Bio-Rad, Hercules, CA) and washed with 2 ml of double distilled water. The totality of the collected fluid from each column was counted for [^{14}C]citrulline radioactivity in a liquid scintillation counter. Because NOS converts arginine into equimolar quantities of NO and citrulline, the data were expressed as picomoles of NO produced per milligram of protein. Aliquots of the supernatants were used to determine protein concentration by the method of Lowry et al. (1951).

Vascular Reactivity. Six hours after the i.p. administration of 5 mg/kg LPS, the animals were anesthetized with ether, the abdomen was opened, and the mesenteric bed was cannulated and removed according to the method described by McGregor (1965). The isolated mesenteric bed was transferred to a perspex chamber at 37°C and perfused with Krebs' solution bubbled with 95% O_2, 5% CO_2 (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 2.6 mM $CaCl_2$, and 25.0 mM $NaHCO_3$, and 11.1 mM glucose; final pH 7.4) at a constant flow of 2 ml/min maintained by a peristaltic pump. The rate of perfusion was selected on the basis of previous studies that showed that this experimental approach allowed to reproduce anandamide-induced relaxations on the consecutive contractions elicited by bolus injections of NE (Mendizábal et al., 2001). Changes in vascular resistance were measured as changes in perfusion pressure and recorded by a Statham transducer connected to a Grass polygraph. The mesenteric bed was allowed a settling period of 60 min after mounting, before starting the experiments. The basal perfusion pressure of the mesenteric beds was, throughout the entire study, between 20 and 25 mm Hg for both control and LPS-treated rats.

Experimental Protocols. After an equilibration period of 60 min at 37°C the mesenteric beds isolated from LPS-treated as well as from age-matched control rats were constricted with bolus injections of NE. The dose of NE was selected so as to produce a pressure response of 40 to 60 mm Hg, usually attained with 3 to 10 nmol of NE. When an entire concentration-response curve was performed, no differences in maximal responses were observed between LPS-treated and age-matched control rats, as shown further under *Results* (Fig. 2). Because short-lasting contractile responses were highly reproducible throughout the experiment, consecutive injections of NE were performed up to nine times in each preparation, 20 min apart, as reported previously (Mendizábal et al., 2001).

Concentration-response curves to either anandamide (0.01–10 μM), methanandamide (0.01–10 μM), or capsaicin (0.01–100 nM) were performed by evaluation of the reductions on the contractile responses to NE. Increasing concentrations of the drugs tested were perfused 20 min before and during the bolus injections of NE. Results were expressed as the percentage of reduction of the pressor effect of NE before drug perfusion. The basal tone was unmodified by the concentrations of drugs tested.

To study the participation of anandamide metabolites on the reduction of the contractile responses to NE, the anandamide amidase inhibitor phenylmethylsulfonyl fluoride (PMSF, 200 μM) (Wagner et al., 1999) was perfused 20 min before and simultaneously with the increasing concentrations of anandamide. Methanandamide and capsaicin concentration-response curves were also performed in the presence of 200 μM PMSF.

In some experiments, concentration-response curves to anandamide were performed 45 min after endothelium removal, carried out through perfusion with saponin 0.1% (v/v) for 45 s (Peredo and

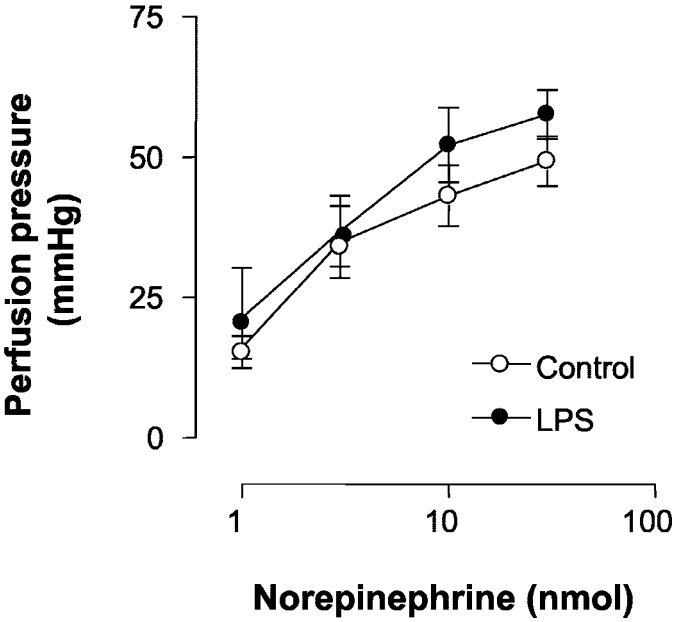

Fig. 2. Effects of LPS treatment on the vascular contractile responses to norepinephrine in the rat isolated mesenteric bed. Dose-response curves to bolus injections of NE (1–100 nmol) were performed in mesenteric beds isolated from either controls (open circles) or LPS-treated rats (5 mg/kg i.p., filled circles). Results are the mean ± S.E.M. of eight experiments per group.

Enero, 1993). The effectiveness of this procedure was evaluated at the end of the experiment by the lack of relaxant effects of an infusion of 0.1 μM acetylcholine on the mesenteric bed previously contracted by infusion of 1 μM NE. This concentration of acetylcholine produced approximately a 50% reduction in the contraction induced by 10 μM NE in endothelium-intact mesenteric beds obtained from control as well as from LPS-treated rats.

To study the involvement of vanilloid receptors on anandamide effects, the vanilloid receptor antagonist capsazepine (1 μM) was perfused 40 min before and simultaneously with the increasing concentrations of anandamide. Capsaicin concentration-response curves were also performed in the presence of 1 μM capsazepine.

Drugs. Lipopolysaccharides from *E. coli* serotype 055:B5, (−)-norepinephrine bitartrate, NADPH, L-citruline, L-arginine, L-valine, DL-dithiothreitol, EGTA, HEPES, saponin, and PMSF were obtained from Sigma-Aldrich (St. Louis, MO). Anandamide, *R*-(+)-methanandamide, capsaicin, and capsazepine were purchased from Tocris Cookson (St. Louis, MO). Anandamide, methanandamide, capsaicin, and PMSF were dissolved in ethanol. Capsazepine was dissolved in dimethyl sulfoxide. The remaining drugs were dissolved in distilled water. Neither capsazepine, PMSF, nor the maximal concentrations used of ethanol (0.1%) and dimethyl sulfoxide (0.1%) had any effect per se on the basal perfusion pressures as well as on the contractile responses elicited by NE in the mesenteric beds.

Statistics. Results are expressed as the mean ± S.E.M. Statistical comparisons were made by either analysis of variance followed by Bonferroni's post hoc *t* test or Student's *t* test for paired and unpaired data. A *P* value less than 0.05 was considered significant.

Results

As shown in Fig. 1, in homogenates of rat mesenteric beds isolated 6 h after the i.p. administration of 5 mg/kg LPS there was a 3-fold increase in total NOS activity (Fig. 1A) and almost a 10-fold enhancement in iNOS activity (Fig. 1B). The calculated calcium-dependent NOS activity, which is considered as a measure of the constitutive isoforms of NOS, was found to be unaltered (controls, 3.5 ± 0.4; LPS, 3.7 ± 0.5 pmol of NO mg protein^{-1} min^{-1}). Despite NOS induction, no differences were found in the systolic arterial blood pressure when measured either before (114 ± 9 mm Hg) or after the LPS treatment (103 ± 6 mm Hg; $n = 5$). At the same time, no changes in NE-induced contractions were observed in mesenteric beds isolated from LPS-treated rats compared with controls (Fig. 2).

As reported previously (Mendizábal et al., 2001), the endocannabinoid anandamide induced a concentration-dependent reduction of the contractile responses to NE (Fig. 3). Anandamide-induced relaxations were significantly potentiated in mesenteric beds isolated 6 h after LPS administration (Fig. 3).

To test whether vanilloid receptors were involved in the potentiation of anandamide effects observed after LPS administration, as described for anandamide relaxations in control tissues (Mendizábal et al., 2001), some experiments were performed in the presence of the vanilloid receptor antagonist capsazepine. As shown in Fig. 4, 1 μM capsazepine significantly attenuated the anandamide-induced reduction of the contractile responses to NE in mesenteric beds isolated from LPS-treated rats. On the other hand, the vanilloid receptor agonist capsaicin induced a concentration-dependent reduction of NE-induced contractions that was also potentiated after LPS administration (Fig. 5). Nevertheless, 1 μM capsazepine failed to antagonize capsaicin-induced relaxations in LPS-treated rats (Fig. 6).

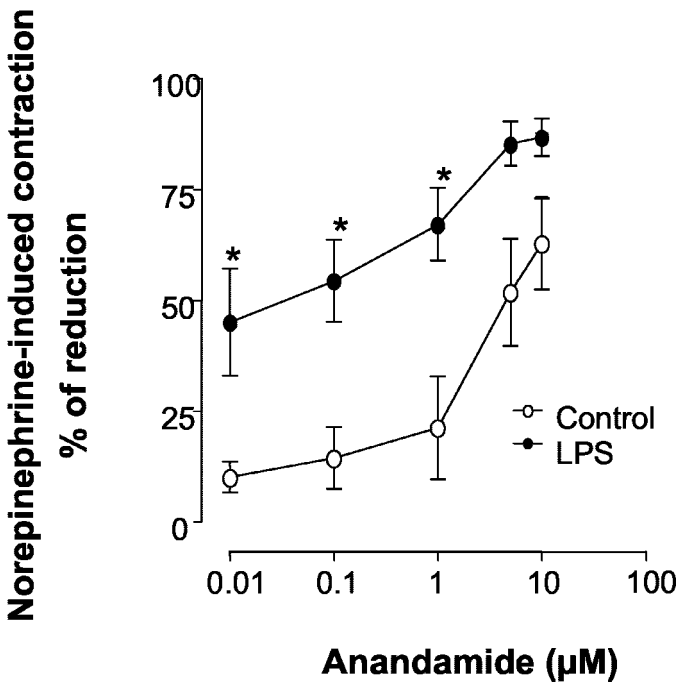

Fig. 3. Effects of anandamide on the contractile responses to norepinephrine in the rat isolated mesenteric bed. Increasing concentrations of anandamide were perfused 20 min before and during a bolus injection of a dose of NE (3–10 nmol) that caused an increase in the perfusion pressure of 40 to 60 mm Hg. Results are expressed as the percentage of reductions caused by anandamide on the initial contractile response to NE. Mesenteric beds were isolated from either controls (open circles) or LPS-treated rats (filled circles). Results are the mean ± S.E.M. of six experiments per group. *, $P < 0.05$ compared with the corresponding control values.

To study whether the vascular endothelium was involved in the greater relaxations caused by anandamide in the LPS-treated rats, concentration-response curves to anandamide after LPS treatment were performed in endothelium-denuded mesenteric beds. As shown in Fig. 7, no differences were observed in the anandamide-induced reductions of the contractile responses to NE after endothelium removal compared with the endothelium-intact preparations.

The possibility that the potentiation of anandamide-induced relaxations in the LPS group could have been related to an alteration in the metabolism of anandamide was tested by studying the effects of the anandamide amidase inhibitor PMSF. As shown in Fig. 8, 200 μM PMSF did not modify anandamide-induced relaxations in control mesenteric beds (Fig. 8A) but caused a significant reduction on the anandamide effects in mesenteric beds isolated from LPS-treated rats (Fig. 8B). To rule out a nonspecific effect of PMSF, the drug was also tested on the relaxant responses elicited by both methanandamide and the vanilloid receptor agonist capsaicin in mesenteric beds isolated from LPS-treated rats. As shown in Fig. 9, neither methanandamide (Fig. 9A) nor capsaicin effects (Fig. 9B) was modified when assayed in the presence of the anandamide amidase inhibitor PMSF (200 μM).

Discussion

According to previous evidence, the endocannabinoid anandamide can induce, in addition to reductions on vascular tone (Randall et al., 1996; Wagner et al., 1999), a concentration-

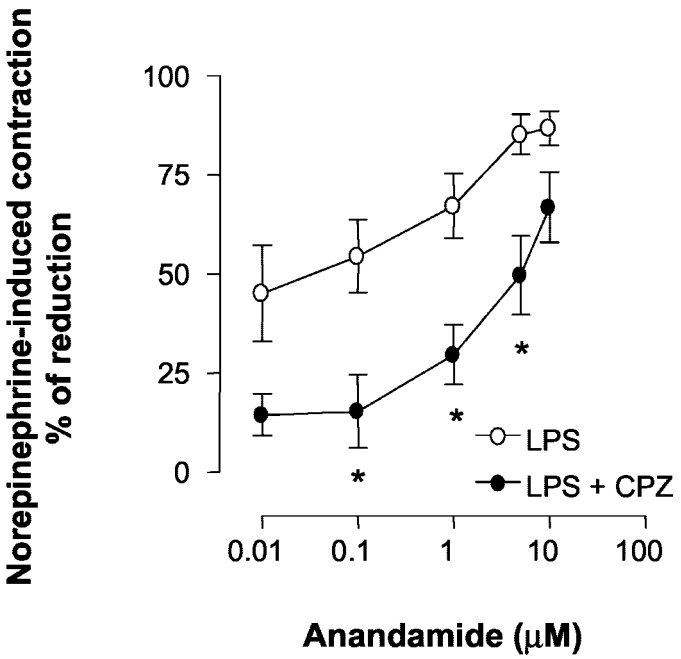

Fig. 4. Effects of the vanilloid receptor antagonist capsazepine on the anandamide-induced reduction of the contractile responses to norepinephrine in LPS-treated rats. The isolated mesenteric beds were perfused with the vanilloid receptor antagonist capsazepine (CPZ; 1 μM), 40 min before and simultaneously with increasing concentrations of anandamide. The initial contraction to NE was induced 40 min after capsazepine perfusion. Results are the mean ± S.E.M. in the LPS-treated (open circles; $n = 6$) and in LPS-treated plus 1 μM capsazepine groups (filled circles; $n = 7$). *, $P < 0.05$ compared with the corresponding control values.

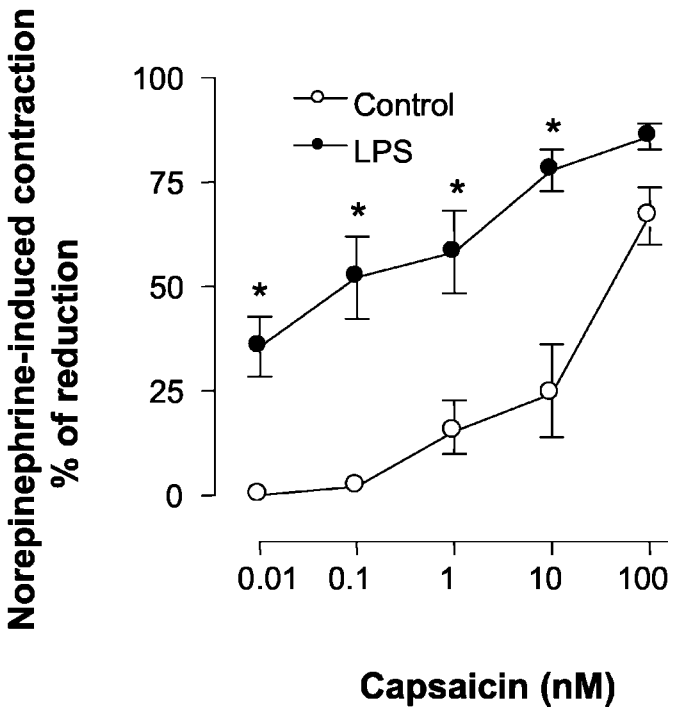

Fig. 5. Effects of the vanilloid receptor agonist capsaicin on the contractile responses to norepinephrine in rat isolated mesenteric beds. Increasing concentrations of the vanilloid receptor agonist capsaicin were perfused 20 min before and during a bolus injection of a dose of NE (3–10 nmol) that caused an increase in the perfusion pressure of 40 to 60 mm Hg in mesenteric beds isolated from either control (open circles) or LPS-treated rats (filled circles). Results are the mean ± S.E.M. ($n = 4$–5) of the percentage of reductions of the initial contraction to NE. *, $P < 0.05$ compared with the corresponding control values.

dependent inhibition of the transient contractions elicited by bolus injections of NE in the rat mesenteric bed (Mendizábal et al., 2001). The main finding of the present work, performed in a phase of endotoxemia when blood pressure was maintained, is that anandamide-induced reductions of the contractile responses to NE are significantly potentiated in rat mesenteric beds isolated 6 h after the i.p. administration of 5 mg/kg LPS. The possible involvement of vanilloid receptors as well as the participation of anandamide metabolites in the latter effect also arises from the present results. On the other hand, the fact that after endothelium removal no changes were observed on the anandamide-induced reductions of the contractile responses to NE precludes the possible involvement of endothelium-derived factors in the potentiation of anandamide effects found during endotoxemia in the mesenteric bed after LPS treatment.

Lack of alterations in the mesenteric vascular contractility to NE after LPS administration, at a time when iNOS activity was indeed increased, is in accordance with previous studies with adrenergic agonists (Mitchell et al., 1993; Fatehi-Hassanabad et al., 1995; Eerdmans et al., 1996). Nevertheless, hyporeactivity to NE in perfused mesenteric beds after LPS administration was described previously (Mitolo-Chieppa et al., 1996) and lower responses to methoxamine were observed when this agonist was administered as an infusion instead of as a bolus (Farmer et al., 2001).

In the rat mesenteric bed, the vasorelaxant effects of anandamide are likely to be mediated through vanilloid receptors (Zygmunt et al., 1999; Mendizábal et al., 2001) and coupled to the release of the vasodilator calcitonin gene-related peptide from perivascular sensitive nerve terminals (Zygmunt et al., 1999; Ralevic et al., 2001). The present results, which show that after LPS administration the potentiation of the relaxant effects of anandamide is mimicked by the vanilloid receptor agonist capsaicin and antagonized by the vanilloid receptor antagonist capsazepine, would suggest that vanilloid receptors are supersensitive during endotoxemia. In this regard, it has been proposed that peripheral terminals of sensory nerves may be an important target for the action of LPS, via generation of cytokines, that in turn sensitize the nerve terminals and facilitate calcitonin gene-related peptide release (Hua et al., 1996). Moreover, it has been described that vanilloid receptor sensitivity can be facilitated in response to various forms of stimulus such as low pH (McLatchie and Bevan, 2001) and prostanoids (Lopshire and Nicol, 1998). Taking into account that endotoxemia is associated with tissue acidification (West and Wilson, 1996) and increased production of several eicosanoids, including anandamide (Makhlouf et al., 1997; Wagner et al., 1998), it is possible that vanilloid receptors somehow linked to the control of vascular reactivity could be overstimulated during endotoxemia, as proposed for vanilloid receptors linked to pain transmission in certain pathologies (Olah et al., 2001). Hence, it seems that although specific CB1 receptors have been reported to trigger the profound and long-lasting hypotension elicited by endocannabinoids (Jarai et al., 1999) and to mediate the extreme hypotension associated with various forms of shock (Wagner et al., 1997, 2001; Varga et al., 1998), vanilloid receptor-mediated effects of anandamide might be

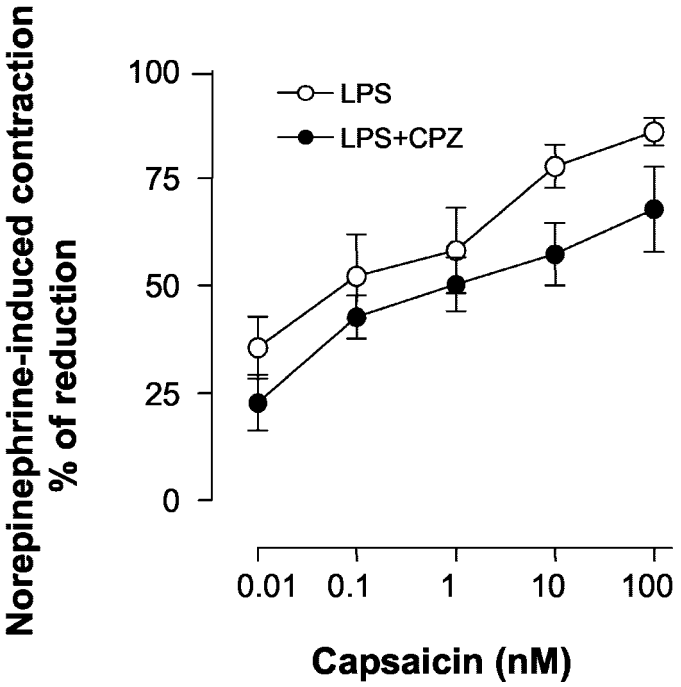

Fig. 6. Effects of the vanilloid receptor antagonist capsazepine on the capsaicin-induced reduction of the contractile responses to norepinephrine in rat mesenteric beds isolated from LPS-treated rats. The isolated mesenteric beds were perfused with the vanilloid receptor antagonist capsazepine (CPZ; 1 μM), 40 min before and simultaneously with the increasing concentrations of capsaicin. The initial contraction to NE was induced 40 min after capsazepine perfusion. Results are the mean ± S.E.M. in LPS-treated (open circles; $n = 5$) and in LPS-treated plus 1 μM capsazepine groups (filled circles; $n = 5$). *, $P < 0.05$ compared with the corresponding control values.

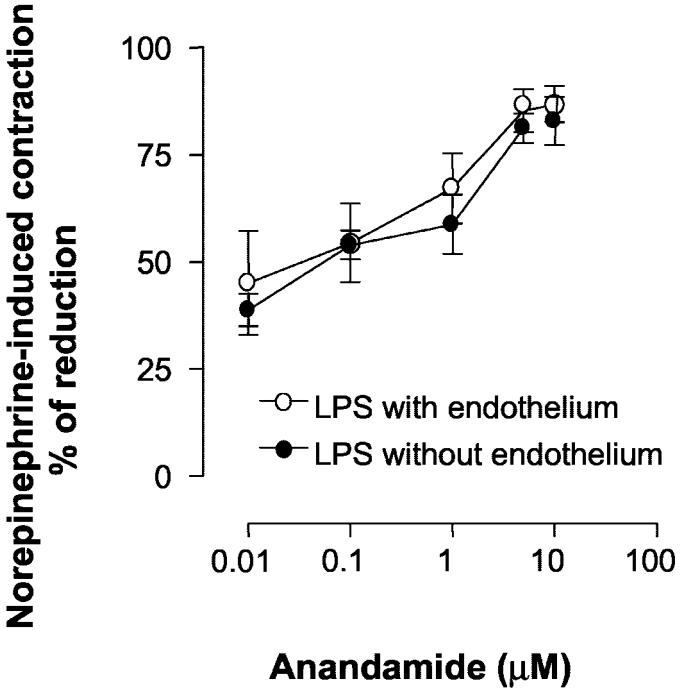

Fig. 7. Effects of endothelium removal on anandamide- induced reductions of contractile responses to norepinephrine in rat mesenteric beds isolated from LPS-treated rats. Anandamide was perfused 20 min before and during a bolus injection of a dose of NE (3–10 nmol) that caused an increase in the perfusion pressure of 40 to 60 mm Hg. Endothelium was removed through a 45-s perfusion with 0.1% saponin. Results are the mean ± S.E.M. of the percentage of reduction of an initial contraction to NE, performed 60 min after the perfusion with Krebs' solution on either intact endothelium mesenteric beds (open circles; $n = 6$) or endothelium-denuded preparations (filled circles; $n = 4$).

important in regulating regional blood flow in early stages of endotoxemia. Nevertheless, no information is available in septic shock about the effects of vanilloid receptor blockade on blood pressure.

Although a single population of vanilloid receptors has been reported in mesenteric beds from guinea pig (Andersson et al., 2002), the finding that capsazepine, at the concentration used in our study, had antagonized the effects of anandamide, but not those of capsaicin, is in accordance with the proposal of different vanilloid receptor subtypes (Szallasi and Blumberg, 1996) with different susceptibilities to blockade by capsazepine (Griffiths et al., 1996; Liu et al., 1998).

Anandamide is degraded by the enzyme anandamide amidase into arachidonic acid and ethanolamine (Deutsch and Chin, 1993). The finding that anandamide-induced relaxations were reduced by the anandamide amidase inhibitor PMSF in mesenteric beds isolated from LPS-treated but not from control rats could be due either to an increased anandamide amidase activity or to an enhanced effect of arachidonic acid metabolites during septic shock. In this regard, several products of arachidonic acid formed through lipoxygenase pathways can activate vanilloid receptors under certain inflammatory states (Hwang et al., 2000; Olah et al., 2001). Moreover, the possibility of a nonspecific effect of PMSF on vascular responses to NE during septic shock is precluded on the basis of the lack of effect of PMSF on the relaxations induced by either the vanilloid receptor agonist capsaicin or the nonmetabolizable anandamide analog methanandamide.

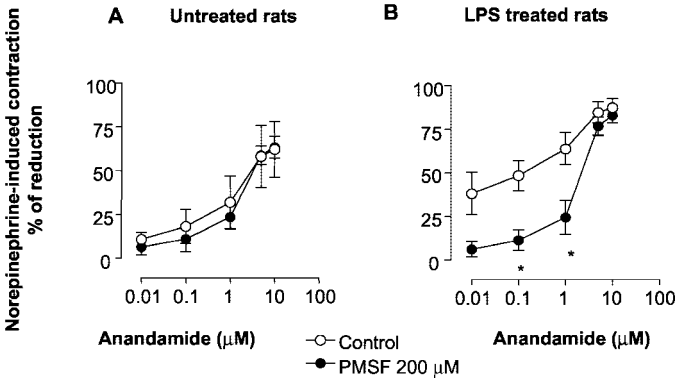

Fig. 8. Effects of the anandamide amidase inhibitor PMSF on anandamide-induced reductions of the contractile responses to norepinephrine in rat mesenteric beds isolated from control and LPS-treated rats. PMSF (200 μM) was perfused 20 min before and simultaneously with increasing concentrations of anandamide in mesenteric beds isolated from either control (A) or LPS-treated rats (B). The initial contraction to NE was induced 20 min after PMSF perfusion. Results are the mean ± S.E.M. of six experiments per group in the absence (open circles) and in the presence (filled circles) of 200 μM PMSF. *, $P < 0.01$ compared with the corresponding control values.

In summary, the present study shows that the relaxant effects of the endocannabinoid anandamide in the mesenteric bed are potentiated at early stages after LPS treatment. This potentiation is not dependent on the endothelium, is probably mediated through vanilloid receptors, and is possibly involved the participation of anandamide metabolites. Although no conclusions can be drawn regarding a link between

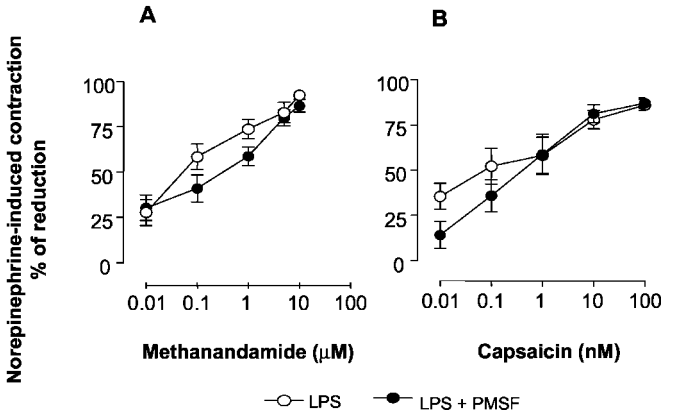

Fig. 9. Effects of the anandamide amidase inhibitor PMSF on methanandamide and capsaicin-induced reductions of the contractile responses to norepinephrine in rat mesenteric beds isolated from LPS-treated rats. PMSF (200 μM) was perfused 20 min before and simultaneously with increasing concentrations of either methanandamide (A) or capsaicin (B) in mesenteric beds isolated from LPS-treated rats. The initial contraction to NE was induced 20 min after PMSF perfusion. Results are the mean ± S.E.M. of four to six experiments per group in the absence (open circles) and in the presence (filled circles) of 200 μM PMSF. $*$, $P < 0.01$ compared with the corresponding control values.

the decrease in blood pressure in advanced stages of septic shock and the observed potentiation of anandamide effects at early stages of endotoxemia, this latter finding could eventually contribute to the understanding of the pathophysiology of septic shock. Further studies, such as those addressed to evaluate the expression of vanilloid receptors after LPS administration, are undoubtedly necessary to fully understand the intimate mechanisms underlying anandamide effects during septic shock.

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