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Nitric oxide-dependent reduction in soluble guanylate cyclase funcionality accounts for early lipopolysaccharide-induced changes in vascular reactivity

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Running Title: Loss in guanylate cyclase response in endotoxemic shock

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Abbreviations: LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; sGC, soluble guanylate cyclase; GTN, glyceryl trinitrate; SNP, sodium nitroprusside; SNAP, S-nitroso-N acetyl-DL-penicillamine; MAP, mean arterial pressure; L-NAME, N^{ϖ}-nitro-L-arginine methyl ester hydrochloride.

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Abstract

We investigated the role of soluble guanylate cyclase (sGC) in lipopolysaccharide-induced hyporesponsiveness to phenylephrine. The effects of phenylephrine on female Wistar rats blood pressure were evaluated at 2, 8 and 24 h after lipopolysaccharide injection (12.5 mg/kg, i.p.). Vasoconstrictive responses to phenylephrine were reduced 40-50% in all time periods. Methylene blue, a soluble guanylate cyclase inhibitor, (15 µmol/kg, i.v.) restored the reactivity to phenylephrine in animals injected with lipopolysaccharide 2 and 24 h earlier. However, it failed to do so in animals injected with lipopolysaccharide 8 h earlier. Incubation with sodium nitroprusside (SNP) increased lung and aorta cGMP levels in control animals, as well as in tissues of rats treated with lipopolysaccharide 24 h earlier. However, SNP failed to increase tissue cGMP in rats injected 8 h earlier. Lipopolysaccharide reduced the vasodilatory response to NO donors 8 h after injection. This effect and the decreased lung cGMP accumulation in response to SNP were reversed by a NO synthase blocker. Guanylate cyclase protein levels were below controls in lungs harvested from rats injected 8 h earlier, and were back to normal values in lungs of rats injected 24 h earlier with lipopolysaccharide. Thus, data indicate that there is a temporal window of 8 h after lipopolysaccharide injection in which soluble guanylate cyclase is not functional and that this loss of function is NO dependent. Thus, the putative use of soluble guanylate cyclase inhibitors in endotoxemia may be beneficial mainly at early stages of this condition.

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Introduction

Septic shock, the most severe complication of sepsis, is a serious disorder with significant morbidity and mortality even after the appropriate antibiotic and supportive therapy are initiated. The poor outcome is considered to be a consequence of an overactive systemic inflammatory response elicited by microbial products, mainly lipopolysaccharide. The disease state is characterized by hypotension, hyporeactivity to vasoconstrictor agents, vascular damage and disseminated intravascular coagulation, which leads to multiple organ failure and death (for a review see Karima et al., 1999). As the mortality rate following sepsis is in excess of 50%, it is clear that the present pharmacotherapy is inadequate.

Inflammatory stimuli such as lipopolysaccharide activates a pathway that leads to expression, among other pro-inflammatory proteins, of the inducible nitric oxide synthase (NOS-2). Once expressed, this enzyme is active for several hours and produces large amounts of nitric oxide (NO, for a review see Alderton et al., 2001). It is well established that overproduction of NO in sepsis is one of the main causes for excessive vasodilation and reduced contractile response to vasoconstrictors agents (for a review, see Titheradge, 1999). Regarding the molecular mechanism of NO-mediated vascular collapse in shock, the role of cGMP-dependent mechanism appears to be well established (Fleming et al., 1991; Paya et al., 1993; Keaney et al., 1994; Silva-Santos et al., 2002). NO activates soluble guanylate cyclase which produces the second messenger cGMP, which in turn governs many aspects of cellular function via interaction with specific kinases, ions channels and phosphodiesterases (Hobbs and Ignarro, 1996).

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In some studies in endotoxemic animals, inhibition of NO synthesis proved to be beneficial since it corrected hypotension (Kilbourn et al., 1990), or restored vascular response to vasoconstrictors agents (Julou-Schaeffer et al., 1990). In septic shock patients, NOS inhibition led to increases in blood pressure, an advantageous effect (Petros et al., 1991), whereas other studies in animals and in patients have shown that NOS inhibition was detrimental, since it worsened the hemodynamic status and increased mortality (Wright et al., 1992; Cobb et al., 1992). The concern raised about the safety of NOS inhibition in septic shock (Cobb et al., 1992; Wright et al., 1992) was confirmed by the interruption of a phase III trial of the use of L-NMMA (a non-selective NOS inhibitor) as an adjuvant treatment in septic shock because the increased mortality (Lopez et al., 2004). This apparent paradox is probably related to the fact that blockage of the production of NO, a molecule with multiple physiological and pathological activities, may be hazardous to the host. For example, NO release during sepsis may have beneficial effects to the host by increasing blood flow to ischemic areas, scavenging oxygen free radicals and exerting microbicidal properties (Moncada et al., 1991; Rubanyi et al., 1991; Malawista et al., 1992). Since highly selective NOS-2 inhibitors are not yet clinically available, one interesting possibility would be interfering with events downstream of NO release. In this regard, inhibition of the main NO target enzyme, soluble guanylate cyclase may represent a safer option capable of counteracting hemodinamic effects of NO without major side effects (Kirov et al., 2001; Zingarelli et al., 1999; Zacharowski et al., 2001).

Thus, if soluble guanylate cyclase inhibitors are to become an effective therapeutic strategy for septic shock treatment, a better understanding of

soluble guanylate cyclase involvement in the detrimental roles of NO in this condition is clearly warranted. Therefore, the objective of the present study was to evaluated the involvement of soluble guanylate cyclase in the vascular hyporesponsiveness to vasoconstrictors seen at different time points after lipopolysaccharide injection, and to evaluate the efficacy of treatment with soluble guanylate cyclase inhibitors.

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Material and methods

Animals

Female Wistar rats (weighing 280-350 g) used in this study were housed in a temperature- and light-controlled room (23 ± 2°C; 12 h light/dark cycle), with free access to water and food. All procedures were approved by our Institutional Ethics Committee and are in accordance with NIH Animal Care Guidelines.

Blood pressure measurement

Under anesthesia with ketamine and xylazine (90 and 15 mg/kg, respectively, supplemented at 45- to 60-min intervals), heparinized PE-20 and PE-50 polyethylene catheters were inserted, respectively, into the left femoral vein for drug injections and into the right carotid artery for recording of mean arterial pressure (MAP), and blood withdrawal. To prevent clotting, a bolus dose of heparin (300 I.U.) was injected immediately after vein cannulation. Animals were allowed to breathe spontaneously via a tracheal cannula. Body temperature was monitored by a rectal thermometer and maintained at 36° ± 1°C. Drugs were diluted in sterile Dulbecco's phosphate-buffered saline (PBS, in mM 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4, and 8.1 $NaHPO_4$; pH 7.4). At the end of the experiment, animals were sacrificed with a pentobarbitone overdose. Blood pressure data were recorded (at a 10-s sampling rate) with a Digi-Med Blood Pressure Analyzer system (Model 190) interfaced to Digi-Med System Integrator (Model 200; Louisville, KY, USA) software, running under Windows 98™ (Microsoft Corporation, Redmond, WA, USA). Results are expressed as means ± SEM of the peak changes in MAP (as mmHg), relative to baseline, recorded following administration of a given compound.

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cGMP assay

Eight or 24 h after PBS or lipopolysaccharide injection, rats were killed, exsanguinated and the right lung or thoracic aorta were harvested. A piece of tissue weighing ~ 100 mg was minced and incubated *in vitro* with isobutyl-methylxanthine (a non-selective phosphodiesterase inhibitor; 0.1 mM) for 30 min at 37^{o}C in Hank's balanced salt solution (in mM, NaCl 138; KCl 5.3; KH_2PO_4 0.44; $MgSO_4$ 0.4; $MgCl_2$ 0.49; $CaCl_2$ 1.26; Na_2HPO_4 0.34; $NaHCO_3$ 4.2; D-glucose 5.5). Then SNP (100 µM) or PBS were added and the incubation proceeded for 10 minutes. Minced tissues were then quickly frozen and homogeneized in ice-cold 6% TCA (1 ml) with a tissue homogenizer. Homogenates were centrifuged and supernatants were extracted four times with water-saturated ethyl ether. cGMP was measured by ELISA using a commercially available EIA (Amersham Pharmacia Biotech, São Paulo, SP, Brazil) according to the manufacturer's instructions. This method allows measuring cGMP in the range of 50-12800 fmol/well, and is based on competition between cGMP present in the sample and peroxidase-labeled cGMP for binding to specific anti-cGMP antibody. Total protein was estimated assuming that 1 mg ~ 1 unit of absorbance at 280 nm. Results were expressed as pmol cGMP/mg protein.

NOx assay

Briefly, zinc sulphate-deproteinized plasma samples were subjected to nitrate conversion. Nitrate was converted to nitrite using *E. coli* nitrate reductase for 2 h at 37°C. Samples were centrifuged for bacteria removal and 100 µL of each sample was mixed with Griess reagent (1% sulfanilamide in 10% phosphoric

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acid/0.1% naphtyl-ethylenediamine in Milli-Q water) in a 96-well plate and read at 540 nm in a plate reader. Standard curves of nitrite and nitrate (0–150 µM) were run simultaneously. As under these conditions nitrate conversion was always greater than 90%, no corrections were made. Values are expressed as µM NOx (nitrate + nitrite).

RNA extraction and PCR analysis

Lungs were harvested 8 and 24 h after rats were injected with lipopolysaccharide (12.5 mg/kg). Control animals received vehicle (PBS). The TRIzol method was used to isolate total RNA. Total RNA was quantified using UV spectrophotometry at 260 nm. After DNase treatment (RQ1 RNase-Free DNase, Promega), total RNA (1.0 µg) was reverse-transcribed into cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo (dT) 15 primer (Promega, São Paulo, SP, Brazil). cDNA were amplified by polymerase chain reaction (PCR) with *Taq* DNA polymerase using a GeneAmp® PCR System 2400 (Perkin- Elmer, USA) and the reaction conditions were as follows: 95°C for 90 s, 35 cycles at 95°C for 30 s, at 45°C for 60 s, and 72°C for 26 s. The following primers were used to amplify *Rattus norvegicus* soluble guanylate cyclase alpha1 subunit cDNA (GeneBank accession no. U60835): forward, 5'-GAAATCTTCAAGGGTTATG-3' (1527–1545), and reverse, 5'-CACAAAGCCAGGACAGTC-3' (2335–2352). The primers used to amplify beta1 subunit cDNA (Gene bank accession no. AB099521): forward, 5'-GGTTTGCCAGAACCTTGTATCCACC-3' (1450-1474) and reverse, 5'-GAGTTTTCTGGGGACATGAGACACC-3' (1709-1733). The expected size of the soluble guanylate cyclase PCR product was 825 bp for the alpha1 subunit and 284 for the beta1 subunit. The housekeeping gene glyceraldehyde 3-

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phosphate dehydrogenase (GAPDH) primers was used to validate the cDNA in each reaction: forward, 5'-GGTGAAGGTCGGAGTCAACGGA-3', and reverse, 5'-GAGGGATCTCGCTCCTGGAAGA-3´. PCR products were electrophoresed on a 2% agarose gel and visualized by UV exposure on a transilluminator. The semiquantitative comparison between the samples was done by measuring the intensities of the bands corresponding to the amplified fragments, using Adobe Photoshop software. The values corresponding to soluble guanylate cyclase mRNA were measured in relation to the mRNA levels of the housekeeping gene GAPDH.

Immunoelectrophoresis for guanylate cyclase beta1 subunit

Lungs were obtained from animals injected 8 or 24 h earlier with lipopolysaccharide (12.5 mg/kg i.p.). Control tissue was obtained from rats injected with vehicle (PBS). Tissue was homogenized in ice-cold buffer (in mM: 50 HEPES, 1 $MgCl_2$, 10 EDTA, and 1% Triton X-100, pH 6.4, containing 1 µg/mL each of aprotinin, leupeptin, soy bean trypsin inhibitor, and 1 mM PMSF). Protein samples (100 µg/lane) were subjected to gel electrophoresis (SDS/PAGE 8% gel). After electrophoresis, the proteins were electrotransferred to PVDF membrane (1 h; 15 V) in Tris-glycine buffer (48 mM Tris-HCl and 39 mM glycine). The membrane was incubated overnight at 4°C with T-PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10.8 mM $Na_2HPO_42H_2O$ and 0.05% Tween-20, pH 7.4) containing 5% skimmed milk followed by incubation with rabbit (polyclonal) anti-soluble guanylate cyclase beta1 antibody (2 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. Following washing, membranes were incubated with alkaline phosphatase

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labelled secondary antibodies (1:3000). Immunocomplexes were visualized by incubation with substrate solution (Tris 100 mM, NaCl 100 mM, $MgCl_2$ 5 mM, pH 9.5, containing nitroblue tetrazolium (NBT) 50 µg/ml and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) 25 µg/ml; Amersham Biosciences, São Paulo, Brazil). The bands were quantified by densitometry using Scion Image software (Scion Co., Frederick, MD, USA).

Effects of guanylate cyclase inhibition on lipopolysaccharide-induced vascular changes to phenylephrine and NO donors

Rats were injected with 12.5 mg/kg of lipopolysaccharide from *E. coli* (i.p.), whereas control animals received sterile PBS (1 ml/kg). Two, 8 or 24 h after PBS or lipopolysaccharide injection, animals were prepared for MAP recording as described above. Two consecutive dose-response curves to phenylephrine (3, 10 and 30 nmol/kg) were obtained. The first curve was obtained 30 min after preparation setup and the second curve was obtained 30 min after intravenous injection of methylene blue (15 µmol/kg) or PBS. In a separate set of experiments, curves for glyceryl trinitrate (GTN; 10, 100 and 1000 nmol/kg), for sodium nitroprusside (SNP; 3, 10 and 30 nmol/kg) or for S-nitroso-N-acetyl-DL-penicillamine (SNAP; 10, 100 and 1000 nmol/kg) were obtained 30 min after preparation setup.

Effect of L-NAME on vascular responses to phenylephrine and glyceryl trinitrate

To evaluate the effects of NO on lipopolysaccharide-induced vascular changes for phenylephrine and glyceryl trinitrate we used N^{ϖ}-nitro-L-arginine methyl

ester hydrochloride (L-NAME), a non-selective NOS inhibitor (Rees et al., 1990). L-NAME (55 µmol/kg, i.p.), or vehicle (PBS, 1 ml/kg) were administered twice, 1 h and 6 h after lipopolysaccharide injection. This protocol was followed to ensure that the plasma levels of L-NAME would be enough to inhibit NOS activity. The choice of this protocol was based on studies showing that L-NAME vasoactive effects *in vivo* may last from 3 to 6 h after a single bolus injection (Conner et al., 2000). Another control group received PBS plus NOS inhibitor to evaluate the influence of L-NAME on normal vasoconstrictor responses to phenylephrine and glyceryl trinitrate. Eight hours after lipopolysaccharide or PBS injection, animals were prepared for MAP recording as described above and a single dose-response curve to phenylephrine or glyceryl trinitrate was obtained 30 min after preparation setup.

Effects of L-NAME on cGMP accumulation

To evaluate this, L-NAME (55 µmol/kg, i.p.) or vehicle (PBS, 1 ml/kg) were administered twice, 1 h and 6 h after lipopolysaccharide or PBS injection. Eight hours after lipopolysaccharide or PBS injection, rats were killed, exsanguinated and lungs were harvested and processed as described above.

Reagents

Methylene blue, phenylephrine chloride, lipopolysaccharide (from *E. coli* serotype 026:B6), sodium nitroprusside, isobutyl-methylxanthine (IBMX), N^{ϖ}-nitro-L-arginine methyl ester hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). SNAP was prepared in our laboratory by a

published method (Field et al., 1978). Glyceryl trinitrate (Tridil™) and heparin were a kind gift of Cristália (São Paulo, SP, Brazil).

Statistical Analysis

Data are expressed as mean ± SEM of n animals. Statistical significance was analysed by one-way analysis of variance (ANOVA) followed by t-test subjected to the Bonferroni correction or using Student's non-paired "t" test. A *P* value of less than 0.05 was considered significant. Statistical analysis was performed using Graph-Pad Prism (San Diego, CA, USA) software.

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Results

Effects of methylene blue on vasoconstrictive responses of lipopolysaccharide-injected rats

In the present study, lipopolysaccharide injection did not decrease mean arterial pressure values either 8 or 24 h after injection (98.6 ± 2.1 mmHg in control animals *versus* 104.7 ± 2.5 and 106.7 ± 2.7 mmHg, 8 and 24 h after lipopolysaccharide, respectively; n=8). However, lipopolysaccharide did increase plasma nitrate + nitrite (NOx) levels, an indication of increased NO production (Fig. 1), since it was prevented by L-NAME (see below). The increase of NOx levels was significant after 6 h and peaked 16 h after lipopolysaccharide injection (Fig. 1). As can be seen in Fig. 2, animals injected with lipopolysaccharide 2, 8 or 24 h before MAP recording had their constrictive responses to phenylephrine reduced by ~ 50% when compared to control animals. When methylene blue was injected shortly before phenylephrine in animals which received lipopolysaccharide 2 or 24 h earlier, the responsiveness was restored to values similar to those obtained in control animals (Figs. 2A and 2C). On the other hand, methylene blue failed to restore vascular responsiveness in animals treated with lipopolysaccharide 8 h earlier (Fig. 2B). As previously shown by our group (Silva-Santos et al., 2002), the selective inhibitor of guanylate cyclase ODQ (Garthwaite et al., 1995) displayed the same profile of action. Methylene blue, as well as ODQ, did not change phenylephrine response in PBS-treated animals (data not shown).

Effects of lipopolysaccharide on the vasodilatory response to NO donors

To evaluate soluble guanylate cyclase functionality after lipopolysaccharide injection, we used NO donors as pharmacological tools to activate the enzyme and increase cGMP production, with the consequent vasodilation and reduction in MAP. The vasodilatory response to glyceryl trinitrate and sodium nitroprusside were reduced in animals injected with lipopolysaccharide 8 h earlier (Fig. 3B), but were similar to control values in rats injected with lipopolysaccharide 2 or 24 h earlier (Figs. 3A and 3C). The same response profile was observed when another NO donor, S-nitroso-acetyl-DL-penicillamine (SNAP), was used (data not shown).

Cyclic GMP accumulation

In vitro incubation of lungs (Fig. 4A) or aorta (Fig. 4B) harvested from control animals with SNP, led to a 10- and 20-fold increase in cGMP levels, respectively. However, in tissues from animals injected with lipopolysaccharide 8 h earlier, SNP failed to change cGMP levels. In contrast, SNP-induced cGMP accumulation in tissues of animals injected with lipopolysaccharide 24 h earlier was similar to control animals (Figs. 4A and 4B).

Effects of L-NAME on lipopolysaccharide-induced changes in vascular responses to phenylephrine and glyceryl trinitrate

L-NAME prevented the onset of hyporesponsiveness to glyceryl trinitrate (Fig. 5A) and to phenylephrine (Fig. 5B), 8 h after lipopolysaccharide injection. The two-injection pre-treatment with L-NAME caused a small increase in MAP of control animals (98.6 ± 2.1 and 116 ± 4.2 mmHg, for PBS and PBS + L-NAME

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groups, respectively, n=8). However, L-NAME did not increase MAP of animals injected with lipopolysaccharide (104.7 ± 2.5 and 95.6 ± 4.1 mmHg, for lipopolysaccharide and lipopolysaccharide + L-NAME groups, respectively, n=8). In control animals, L-NAME did not change the vascular response to phenylephrine or GTN (data not shown). L-NAME effectively prevented the increase in plasma NOx levels (control 46 ± 12 µM; lipopolysaccharide 187 ± 19 µM and lipopolysaccharide + L-NAME 55 ± 14 µM, n=4).

Effects of L-NAME on cGMP accumulation

As shown in Fig. 6, in vitro stimulation of lung tissue with SNP increased cGMP levels. However, this stimulatory effect of SNP did not appear in lungs obtained from animals injected with lipopolysaccharide 8 h earlier. This effect of SNP in stimulating guanylate cyclase was restored by pre-treatment of animals with L-NAME (Fig. 6).

Effects of lipopolysaccharide injection on lung guanylate cyclase mRNA and protein levels

In lungs obtained from rats injected with lipopolysaccharide 8 h earlier, soluble guanylate cyclase alpha1 subunit mRNA levels remained unchanged compared to control animals, but were increased 24 h after lipopolysaccharide (Fig. 7A). Beta1 subunit mRNA levels were increased 8 h and 24 h after lipopolysaccharide (Fig. 7B). With regard to guanylate cyclase protein levels, lipopolysaccharide caused a reduction 8 h after its injection, whereas 24 h later, enzyme levels were similar to those from control animals (Fig. 7C).

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Discussion

We have previously shown that the soluble guanylate cyclase inhibitor ODQ failed to bring phenylephrine response back to normal levels in rats injected with lipopolysaccharide 8 h earlier but completely reversed this hyporesponsiveness in animals injected with lipopolysaccharide 24 h earlier (Silva-Santos et al., 2002). We now show that the failure of soluble guanylate cyclase inhibitors in restoring the vascular response to phenylephrine in animals injected 8 h earlier with lipopolysaccharide is caused by a substantial reduction in enzyme response. In addition, this decreased functionality depends on reductions of both enzyme protein levels and activity.

Excessive NO production has been shown to play a pivotal and largely detrimental role in septic shock (for a review see Titheradge, 1999). NO is one of major causes of diminished responsiveness to vasoconstrictors (Julou-Schaeffer et al., 1990; Petros et al., 1991), a central event of septic shock and contributes to the high mortality rate associated with this disorder. After injection of bacterial lipopolysaccharide, plasma levels of NO stable metabolites nitrate + nitrite (NOx), an indicator of whole body NO production, progressively rise within a few hours. Moreover, lipopolysaccharide injection is associated with a marked depression in vascular reactivity to vasoconstrictors, thus reproducing in animals an important consequence of clinical septic shock.

We have shown that hyporesponsiveness to vasoconstrictors has an early onset (2 h) after lipopolysaccharide injection, a time when plasma NOx levels were identical to control levels. Previous reports showed that the loss in the response to vasoconstrictors occurs 60 min after lipopolysaccharide injection in the anaesthetized rats, while NOS-2 (iNOS) increased expression

can only be demonstrated 4 h after lipopolysaccharide injection (Julou-Schaeffer et al., 1990; Szabo et al., 1993). Whereas the initial loss in vasoconstrictor responses seems to be associated with NO derived from constitutive NOS (Szabo et al., 1993), the hyporesponsiveness to vasoconstrictors is unequivocally associated with NOS-2 expression at later times after lipopolysaccharide injection (Julou-Schaeffer et al., 1990). Closer inspection of Figure 2 (black bars) reveals that the hyporesponsiveness to phenylephrine in animals injected with lipopolysaccharide 2 or 8 h earlier is identical to that observed in rats that received lipopolysaccharide 24 h earlier, indicating that the refractoriness to phenylephrine persisted and remained constant, at least within 24 h after lipopolysaccharide injection.

It is widely accepted that soluble guanylate cyclase activation is one of the most important effectors of NO, mainly in the cardiovascular system. Thus, in the last decade, several studies have pointed towards soluble guanylate cyclase as a potential target for drug development. Inhibition of enzyme activity with methylene blue increased blood pressure in anesthetized endotoxemic rats (Cheng and Pang, 1998; Paya et al., 1993) and rabbits (Keaney et al., 1994). However, the conclusions derived from studies with methylene blue should be drawn with caution, since this compound is not a specific inhibitor of soluble guanylate cyclase. Methylene blue also inhibits NO synthase activity (Mayer et al., 1993) and generates oxygen species (Visarius et al., 1997). Despite this caveat and at least as far vascular responsiveness is concerned, the main target of methylene blue is soluble guanylate cyclase since ODQ, a highly selective inhibitor of the enzyme (Garthwaite et al., 1995), also restored the responsiveness to vasoconstrictors in endotoxemic animals (Silva-Santos et al.,

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2002), increased survival in lipopolysaccharide-treated mice (Zingarelli et al., 1999), and reduced lipopolysaccharide-induced multiple organ dysfunction (Zacharowski et al., 2001). These findings clearly show that soluble guanylate cyclase is a key mechanism in the regulation of vascular tone during endotoxemia and sepsis.

Here we have demonstrated that methylene blue restored the vasoconstrictive responses to phenylephrine that were reduced by lipopolysaccharide injection 2 and 24 h earlier, but failed to do so in rats injected with lipopolysaccharide 8 h earlier. This lack of effect of methylene blue 8 h after lipopolysaccharide prompted us to study in greater detail the soluble guanylate cyclase functional response during endotoxic shock. The vasodilatory response to nitric oxide donors (GTN and SNP) was reduced by 40-50% 8 h after lipopolysaccharide injection. On the other hand, the response to nitric oxide donors was not changed in rats injected 2 and 24 h earlier with lipopolysaccharide. Therefore, the failure of soluble guanylate cyclase inhibitors in restoring vasoconstrictive response to phenylephrine in rats injected with lipopolysaccharide injection 8 h earlier was temporally mirrored by a loss in the vasodilatory response to nitric oxide donors in the same period. Together, the data suggest that after 8 h of lipopolysaccharide injection a decrease may be occurring in soluble guanylate cyclase funcionality. These results are in line with other reports showing that exposure of bovine isolated mesenteric arterial rings to interferon-γ (De Kimpe et al., 1994) or rat aortic rings to lipopolysaccharide (Tsuchida et al., 1994) inhibits sodium nitroprusside-induced vasodilation and cGMP accumulation. The reduced effect for glyceryl trinitrite observed 8 h after lipopolysaccharide cannot be attributed to an altered biotransformation of this

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compound, since sodium nitroprusside and SNAP (a nitrosothiol), which generate NO non-enzymatically, showed the same profile of response. The finding that the response to the highest GTN dose (1000 nmol/kg) was similar in control rats and in animals injected with lipopolysaccharide 8 h earlier is suggestive that high NO concentrations may be affecting other targets besides soluble guanylate cyclase, such as potassium channels (Bolotina et al., 1994).

The hypothesis of a reduced soluble guanylate cyclase functionality was strenghtened by the finding that sodium nitroprusside induced several-fold increases in cGMP levels in tissues (lung and aorta) taken from control rats and from animals injected 24 earlier with lipopolysaccharide, but failed to stimulate soluble guanylate cyclase in tissues taken from rats injected 8 earlier. It is noteworthy that the reduction of soluble guanylate cyclase activity 8 h after lipopolysaccharide is coincident with the peak of NOS-2 expression and NO production in the same experimental model (Silva-Santos et al., 2002). The exact mechanism by which soluble guanylate cyclase activity may be inhibited by NO is still an open issue. Our results strongly suggest that overproduction of NO following lipopolysaccharide injection has a relevant role in the decreased soluble guanylate cyclase functionality, since treatment with L-NAME prevented endotoxin-induced loss in vasodilatory response to glyceryl trinitrite and in the hyporesponsiveness to phenylephrine, and also reversed the failure of sodium nitroprusside in increasing lung cGMP accumulation. Our findings are in line with reports showing that exposure of crude or purified soluble guanylate cyclase to NO leads to development of tolerance to NO effects (Braughler, 1983) and exposure of bovine coronary arterial rings, rat aorta or cultured vascular smooth muscle cells to NO donors leads to impairments in relaxation

and cGMP accumulation (Henry et al., 1989; Tsuchida et al., 1994; Papapetropoulos et al., 1996a). Enhanced degradation of cGMP in lungs of lipopolysaccharide-treated animals due to an increased phosphodiesterase activity is not likely to explain the lack of sodium nitroprusside stimulation, since our experiments were performed in the presence of a phosphodiesterase inhibitor. The potential reduction in concentrations of the guanylate cyclase substrate GTP caused by LPS is not also likely to explain our results since L-NAME, which inhibits only NOS activity and presumably does not interfere with other LPS effects, restored guanylate cyclase activity.

Endogenously produced NO has been implicated in the down-regulation of enzyme mRNA levels that follows exposure of cultured smooth muscle cells to lipopolysaccharide (Papapetropoulos et al., 1996b; Scott and Nakayama, 1998; Takata et al., 2001). This is in contrast to our in vivo finding that guanylate cyclase mRNA levels are either normal for the alpha1 subunit or increased for the beta1 subunit, 8 h after lipopolysaccharide. At later times (24 h) both mRNA are substantially higher than in control tissue. We do not have a good explanation for this discrepancy but it may well be attributed to far more complex events that take place in in vivo models.

Concerning protein levels, it has been shown that prolonged incubation with NO donors decreases soluble guanylate cyclase protein expression (Filippov et al., 1997). Our results of decreased protein level 8 h after endotoxin are in agreement with those reports. However, it is difficult to reconciled protein levels with subunit mRNA pattern at this time. This discrepancy may indicate that steady-state mRNA levels of soluble guanylate cyclase should not be taken as direct indicators of protein levels, since NO and cGMP have been shown to

regulate soluble guanylate cyclase mRNA stability/translation (Filippov et al., 1997; Takata et al., 2001). The higher-than normal protein levels observed 24 h after lipopolysaccharide probably reflects the higher mRNA level found in endotoxemic rats and may be a compensatory response to the earlier loss in guanylate cyclase function. In any event, it is important to note that cGMP accumulation in the presence of a phophodiesterase inhibitor (which reflect more accurately enzyme activity than RT-PCR or immunoelectrophoresis) show that enzyme activity is decreased and returns to normal values, 8 and 24 h after lipopolysaccharide injection, respectively.

We have chosen the beta1 subunit to monitor enzyme levels based on previous works showing that in cell culture, this subunit appears to be more rapidly and robustly regulated after an inflammatory stimuli (Takada et al., 2001). Additionally, since the beta1 subunit is an obligate partner in active soluble guanylate cyclase heterodimers and contains the major heme binding domain, reduction in beta1 subunit amount would be enough to explain the decrease in NO-stimulated soluble guanylate cyclase activity (Hobbs, 1997).

The exact mechanism by which there is a reduction in protein levels is not clear. As mRNA levels for both subunits increased following LPS injection, the reasons for the decreased protein levels may include two mechanisms, namely impairment of translation process or increased degradation rate of the protein. As for the first, NO decreases sGC subunit mRNA stability via a translation-dependent mechanism (Fillipov et al., 1997). As for the second mechanism, it has been recentely demonstrated that soluble guanylate cyclase is associated to heat shock protein-90, and that this heterocomplex might be important for guanylate cyclase stability (Papapetropoulos et al., 2005). Thus

LPS and/or NO-induced disruption of this protein complex, promoting ubiquitination and increased proteolytic degradation of sGC would explain our findings. Although the consequences of sGC down-regulation are not clear, it may be a protective mechanism avoiding the excessive hypotension consequent to the high NO production, which would then be safely directed towards killing of invading microorganisms in sepsis. Although speculative at this moment, this hypothesis warrants further investigation.

Comparison of data shown in Figures 4 and 7 indicate that the decrease in sodium nitroprusside stimulatory effect is higher than the decrease in guanylate cyclase protein levels. High concentrations of NO can react with heme groups and cysteine residues (Davis et al., 2001). Since soluble guanylate cyclase has both targets, it may be that high concentrations of NO produced in the first hours of endotoxemia would disrupt enzyme activity by reaction with both tartgets. This hypothesis also remains to be proven.

It has been suggested that downregulation in cGMP accumulation following lipopolysaccharide injection could be a part of a homeostatic mechanism to counteract the massive hypotension seen in shock (Papapetropoulos et al., 1996b). Although it is widely accepted that the development of septic shock occurs in different phases with different characteristics, most of the therapeutic interventions are directed at treating the refractory hypotension. These interventions, however, have not been consistently successful (Baumgartner and Calandra, 1999). Notwithstanding that lipopolysaccharide is not a good model for human sepsis and that appropriate caution should be taken when interpreting the results and conclusions from the present study, the results shown here suggest that

methylene blue therapy for sepsis-induced hypotension and vascular refractoriness may be critically dependent on the time after shock onset.

In summary, we have shown that depending on the time after endotoxemia onset soluble guanylate cyclase inhibition may or may not restore the responsiveness to vasoconstrictors. This failure of soluble guanylate cyclase inhibitors appears to be a consequence of NO-dependent inhibition of soluble guanylate cyclase, which recovery in funcionality seems to depend on *de novo* enzyme protein synthesis. Therefore, differential responsiveness to soluble guanylate cyclase during the course of endotoxemic shock may determine the success or the failure of treatment with soluble guanylate cyclase inhibitors. These findings may support new studies and new approaches on the role of soluble guanylate cyclase in endotoxemia and sepsis.

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Legends to Figures

Fig. 1. Time-dependent increases in serum nitrate + nitrite (NOx) levels during endotoxemia. Rats were given lipopolysaccharide (12.5 mg/kg, i.p.), and at indicated periods blood was collected and assayed for NOx levels as detailed in the *Materials and Methods* section. Each bar represents the mean of 6 animals and vertical lines are the SEM. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc t test. * $p<0.01$ compared to the control group (time 0 h).

Fig. 2. Effects of methylene blue on lipopolysaccharide-induced vascular hyporesponsiveness to phenylephrine. Lipopolysaccharide (12.5 mg/kg, i.p.) was injected and animals were prepared for mean arterial pressure (MAP) recording 2 h (Panel A), 8 h (Panel B) or 24 h (Panel C) after lipopolysaccharide injection. Control rats received PBS (1 ml/kg, i.p.). Increasing doses of phenylephrine (3, 10 and 30 nmol/kg,i.v.) were injected and changes in MAP were recorded. Open bars represent rats injected with PBS, black bars rats injected with lipopolysaccharide and cross-hatched bars rats injected with lipopolysaccharide and an i.v. bolus injection of methylene blue (15 µmol/kg) 30 min before the first dose of phenylephrine. Each bar represents the mean of 8 animals and vertical lines are the SEM. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc t test. * $p<0.05$ compared to the control group (PBS) and $^{\#}$ $P < 0.05$ compared to lipopolysaccharide-injected animals.

Fig. 3. Effect of lipopolysaccharide on vasodilatory response to nitric oxide donors. Lipopolysaccharide (12.5 mg/kg, i.p.) was injected and animals were prepared for mean arterial pressure (MAP) recording 2 h (Panel A), 8 h (Panel B) or 24 h (Panel C) after lipopolysaccharide injection. Control rats received PBS (1 ml/kg, i.p.). Increasing doses of glyceryl trinitrate or sodium nitroprusside were injected (i.v.) and changes in MAP were recorded. Open bars represent rats injected with PBS and black bars rats injected with lipopolysaccharide. Each bar represents the mean of 8 animals and vertical lines the SEM. Statistical analysis was performed using Student's "t" test for non-paired samples. * $P < 0.05$ compared to the respective control group (PBS).

Fig. 4. Cyclic GMP (cGMP) accumulation in response to sodium nitroprusside (SNP) stimulation in lungs or aorta from vehicle- (PBS) or lipopolysaccharide-treated animals. Lipopolysaccharide (12.5 mg/kg, i.p.) was injected and 8 h or 24 h later, rats were sacrificed by exsanguination, lungs (Panel A) or thoracic aorta (Panel B) were harvested, minced and incubated *in vitro* with sodium nitroprusside (SNP; 100 µM; 10 min, black bars) or PBS (open bars) in the presence of isobutyl-methylxanthine (0.1 mM). Tissues were quickly frozen and homogeneized in ice-cold trichloroacetic acid and cGMP was measured by ELISA. Each bar represents the mean of 3 animals and vertical lines the SEM. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc t test. * $P < 0.05$ compared to the control (PBS/SNP).

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Fig. 5. Effect of L-NAME on lipopolysaccharide-induced mean arterial pressure changes in response to vasoactive compounds. Lipopolysaccharide (12.5 mg/kg, i.p.) was injected and animals were prepared for mean arterial pressure (MAP) recording 8 h after lipopolysaccharide injection. Control rats received PBS (1 ml/kg, i.p.). Vasodilatory responses to glyceryl trinitrate (Panel A) and vasoconstrictive responses to phenylephrine (Panel B) are shown. Open bars represent rats injected with PBS, black bars rats injected with lipopolysaccharide and cross-hatched bars rats injected with lipopolysaccharide and L-NAME (55 µmol/kg, i.p.), 1 and 6 h after lipopolysaccharide. Each bar represents the mean of 6-8 animals and vertical lines the SEM. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc t test. * $P < 0.05$ compared to the control group (PBS) and $^{\#}$ $P < 0.05$ compared to lipopolysaccharide group.

Fig. 6. Effects of L-NAME on cyclic GMP (cGMP) accumulation in response to sodium nitroprusside (SNP) stimulation in lipopolysaccharide-treated animals. Lipopolysaccharide (12.5 mg/kg, i.p.) was injected and 8 h after lipopolysaccharide injection, lungs were harvested, minced and incubated *in vitro* with sodium nitroprusside (SNP; 100 µM; 10 min, black bars) or PBS (open bars) in the presence of isobutyl-methylxanthine (0.1 mM). Lungs were quickly frozen and homogenized in ice-cold trichloroacetic acid and cGMP was measured by ELISA. L-NAME (55 µmol/kg, i.p.) was injected 1 and 6 h after lipopolysaccharide. Each bar represents the mean of 3 animals and vertical lines the SEM. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc t test. * $P < 0.05$ compared to PBS/SNP group.

Fig. 7. Effect of lipopolysaccharide (LPS) on lung soluble guanylate cyclase (sGC) mRNA and protein levels. Lipopolysaccharide (12.5 mg/kg, i.p.) was injected and 8 h or 24 h after injection and lungs were harvested. Control animals (C) received vehicle (PBS). Total RNA was isolated, and RT-PCR was performed using primers as described in *Material and Methods* section. Panel A, RT-PCR products and ratio sGC alpha1 subunit/GAPDH obtained by densitometry. Panel B, RT-PCR products and ratio sGC beta1 subunit/GAPDH. Panel C, representative immunoelectrophoresis for soluble guanylate cyclase beta1 subunit and densitometry. Each bar represents the mean of 3 animals and vertical lines the SEM. Statistical analysis was performed using Student's "t" test for non-paired samples. * $P < 0.05$ compared to the respective control group.

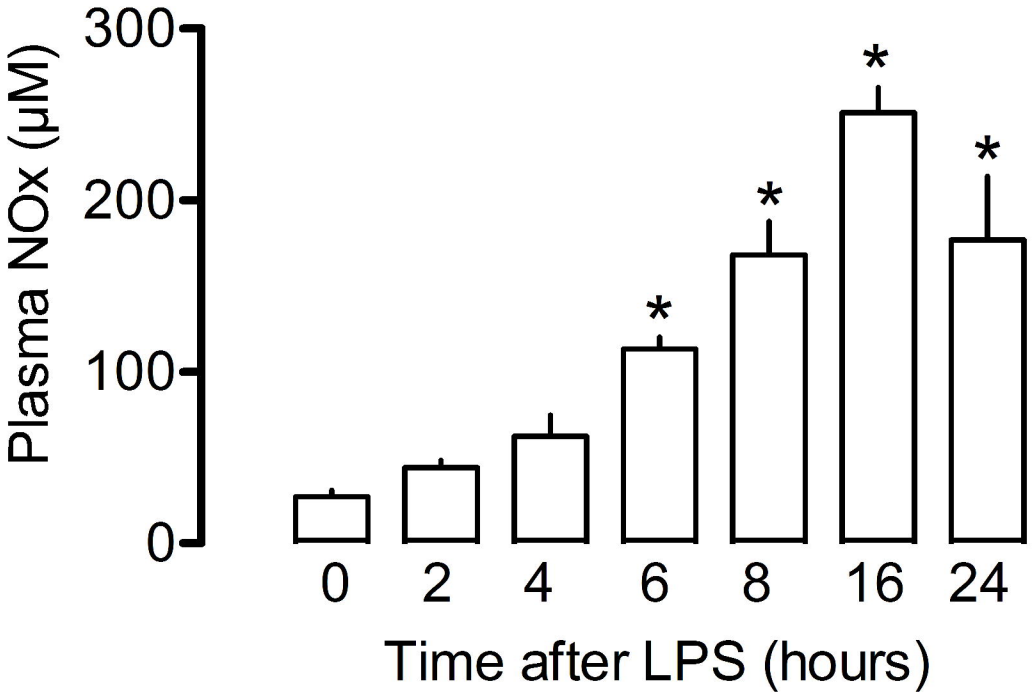

Figure 1

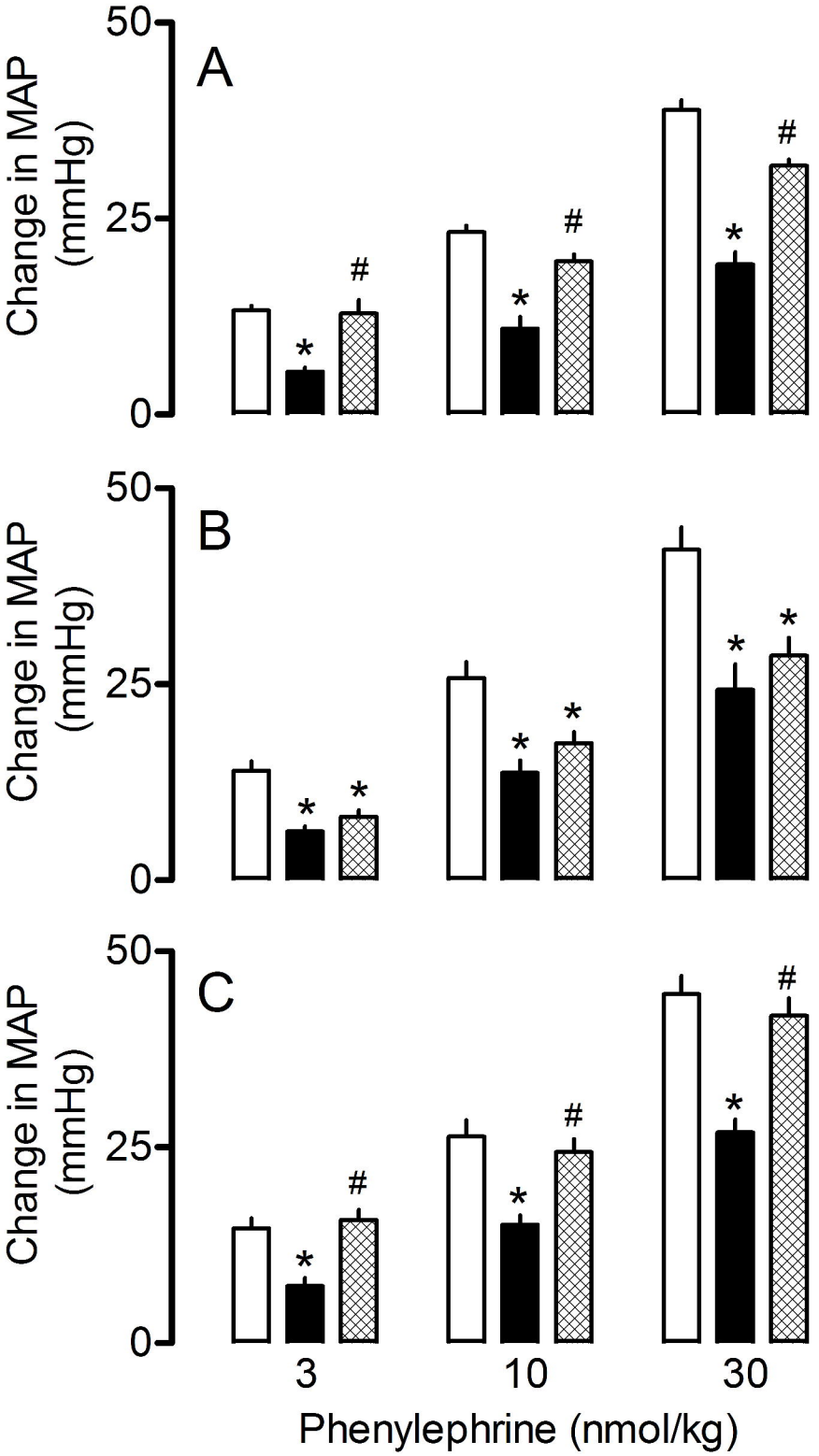

Figure 2

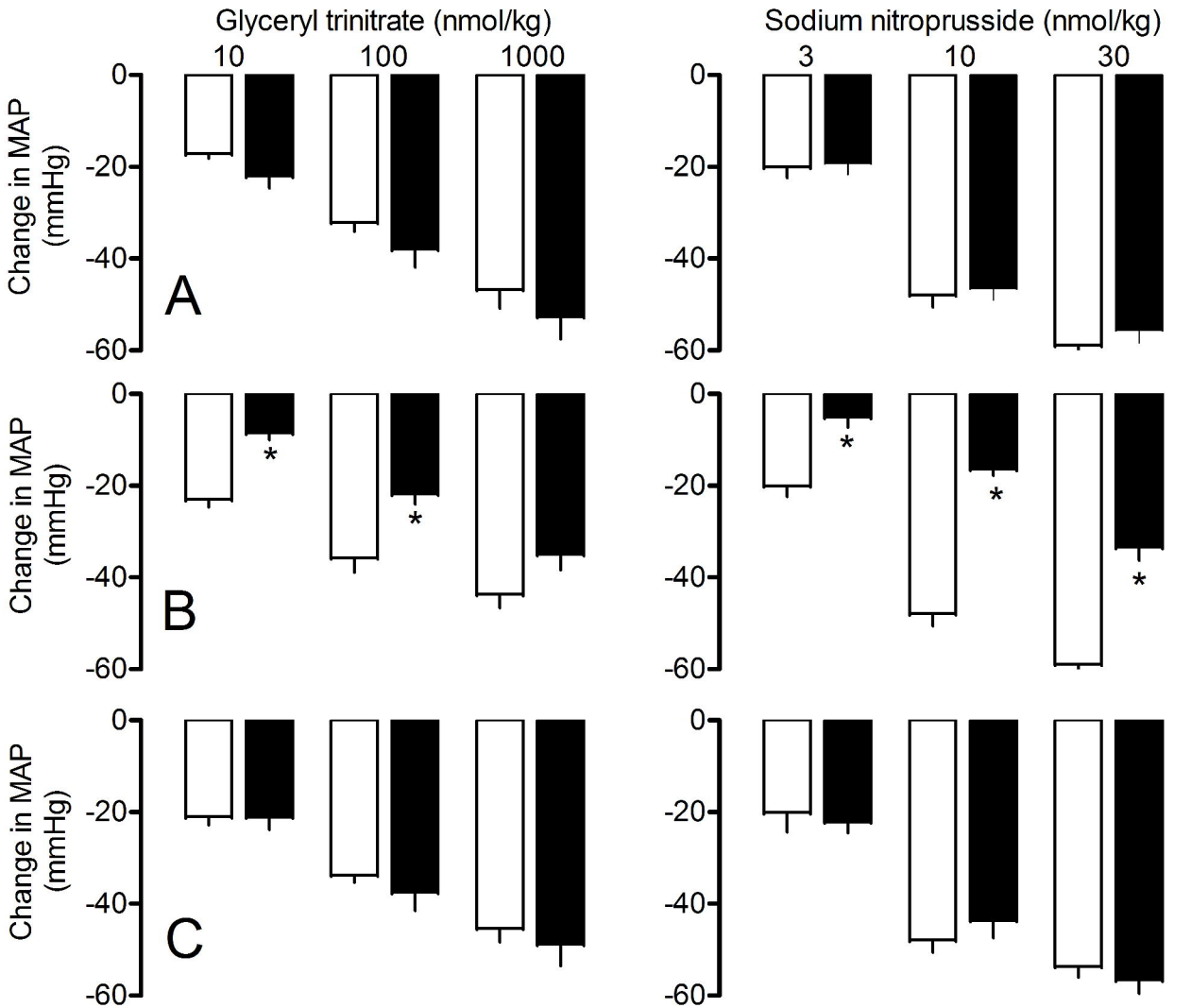
Glyceryl trinitrate (nmol/kg)
10
100
1000
Sodium nitroprusside (nmol/kg)
3
10
30
Change in MAP (mmHg)
0
-20
-40
-60
A
B
C
*

Figure 3

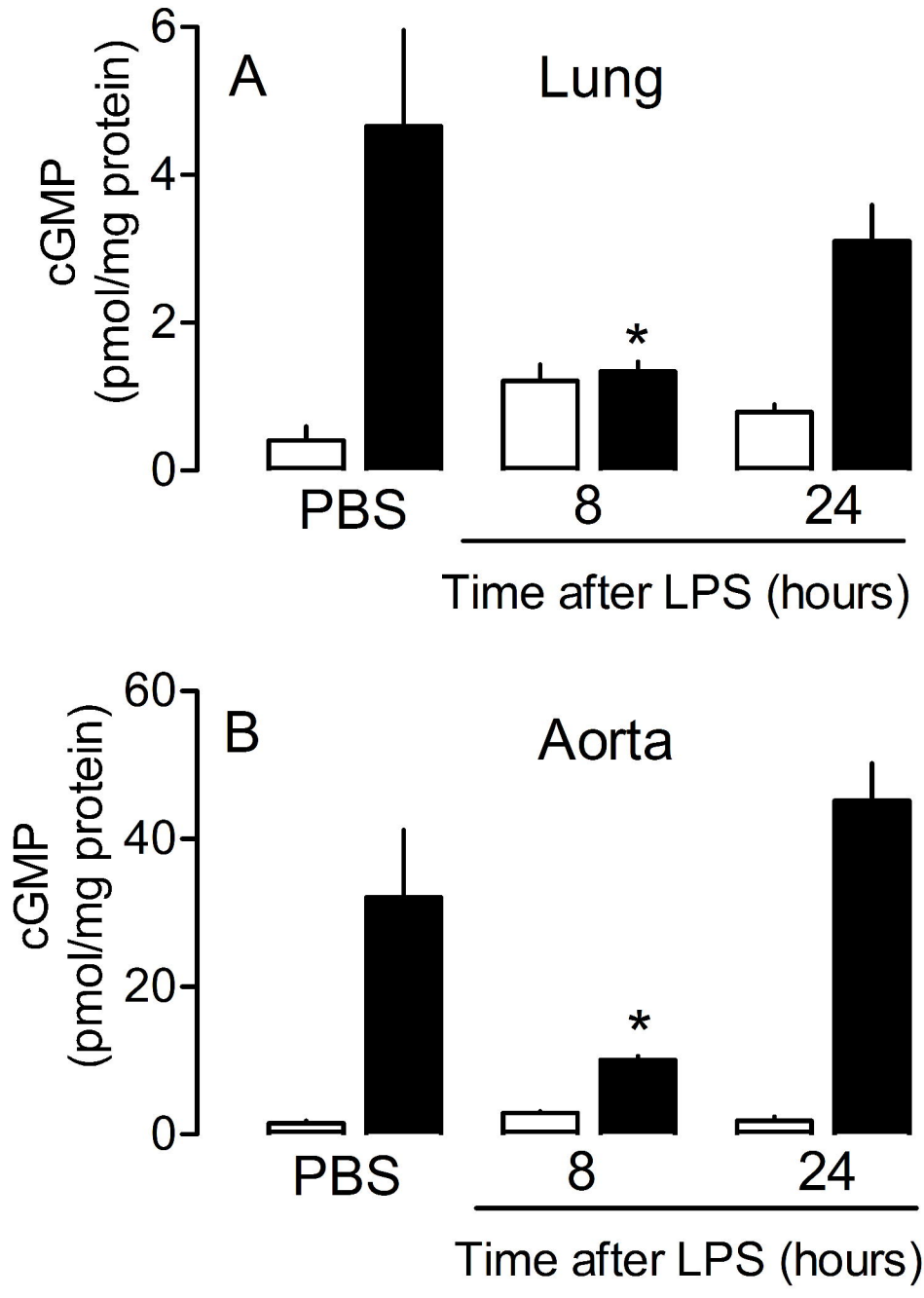
A
Lung
cGMP
(pmol/mg protein)
6
4
2
0
*
PBS
8
24
Time after LPS (hours)
B
Aorta
cGMP
(pmol/mg protein)
60
40
20
0
*
PBS
8
24
Time after LPS (hours)

Figure 4

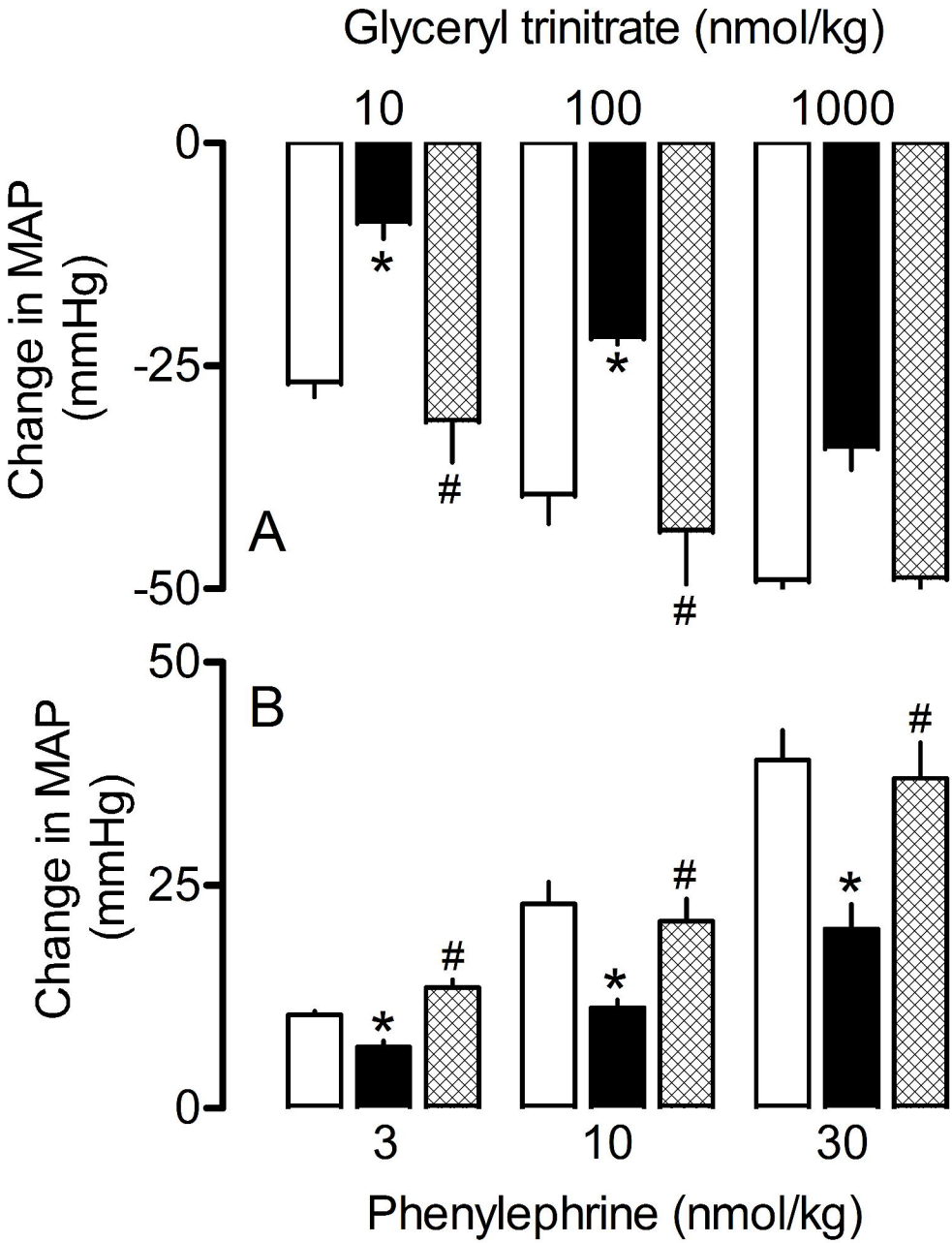
Glyceryl trinitrate (nmol/kg)
10
100
1000
0
-25
-50
Change in MAP (mmHg)
A
*
#
*
#
B
50
25
0
Change in MAP (mmHg)
*
#
*
#
*
#
3
10
30
Phenylephrine (nmol/kg)

Figure 5

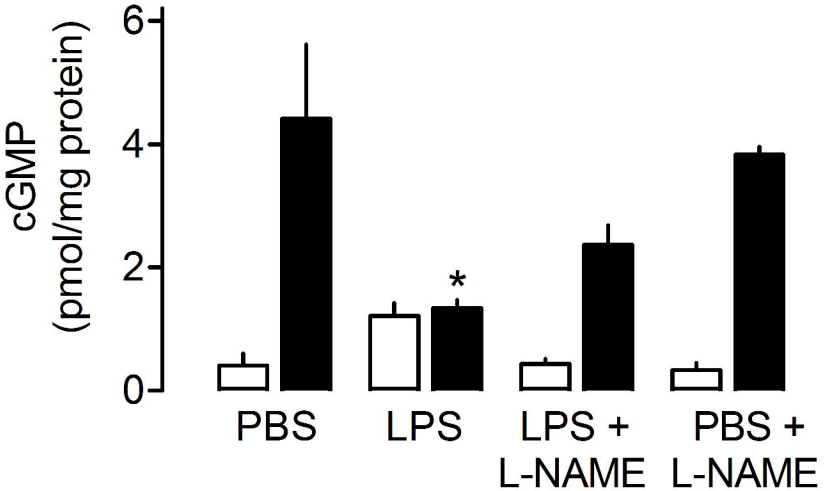

Figure 6

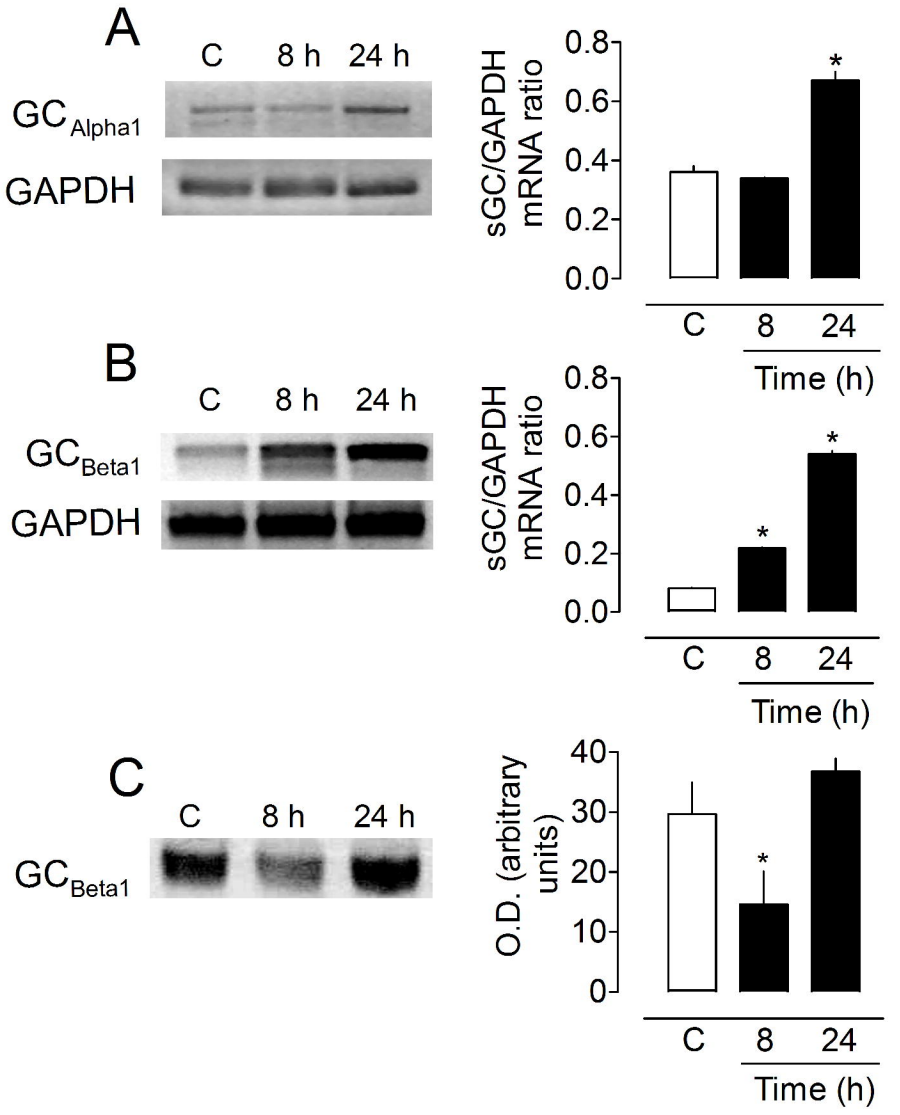
A
C
8 h
24 h
GC Alpha1
GAPDH
sGC/GAPDH mRNA ratio
0.8
0.6
0.4
0.2
0.0
*
C
8
24
Time (h)
B
C
8 h
24 h
GC Beta1
GAPDH
sGC/GAPDH mRNA ratio
0.8
0.6
0.4
0.2
0.0
*
*
C
8
24
Time (h)
C
C
8 h
24 h
GC Beta1
O.D. (arbitrary units)
40
30
20
10
0
*
C
8
24
Time (h)

Figure 7