

# Nitric Oxide-Dependent Reduction in Soluble Guanylate Cyclase Functionality Accounts for Early Lipopolysaccharide-Induced Changes in Vascular Reactivity

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## ABSTRACT

We investigated the role of soluble guanylate cyclase in lipopolysaccharide-induced hyporesponsiveness to phenylephrine. The effects of phenylephrine on the blood pressure of female Wistar rats were evaluated at 2, 8, and 24 h after lipopolysaccharide injection (12.5 mg/kg i.p.). Vasoconstrictive responses to phenylephrine were reduced 40 to 50% in all time periods. Methylene blue, a soluble guanylate cyclase inhibitor (15  $\mu$ mol/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 and 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 an NO synthase blocker. Guanylate cyclase protein levels were lower than 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 the treatment of endotoxemia may be beneficial mainly at early stages of this condition.

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 (Karima et al., 1999). Because the mortality

rate after sepsis is in excess of 50%, it is clear that the present pharmacotherapy is inadequate.

Inflammatory stimuli such as lipopolysaccharide activate a pathway that leads to expression, among other proinflammatory 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 (Alderton et al., 2001). It is well established that overproduction of NO in sepsis is one of the main causes of excessive vasodilation and reduced contractile response to vasoconstrictor agents (Titheradge, 1999). Regarding the molecular mechanism of NO-mediated vascular collapse in shock, the role of cGMP-dependent mechanism seems 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

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**ABBREVIATIONS:** NOS-2, inducible nitric-oxide synthase; LPS, lipopolysaccharide; 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<sup>ω</sup>-nitro-L-arginine methyl ester hydrochloride; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; ANOVA, analysis of variance; NOx, nitrate + nitrite.

many aspects of cellular function via interaction with specific kinases, ions channels, and phosphodiesterases (Hobbs and Ignarro, 1996).

In some studies in endotoxemic animals, inhibition of NO synthesis proved to be beneficial because it corrected hypotension (Kilbourn et al., 1990) or restored vascular response to vasoconstrictors agents (Julou-Schaeffer et al., 1990). In patients with septic shock, 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 because it worsened the hemodynamic status and increased mortality (Cobb et al., 1992; Wright 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 *N*<sup>G</sup>-monomethyl-L-arginine (a nonselective 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). Because 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 hemodynamic effects of NO without major side effects (Zingarelli et al., 1999; Kirov et al., 2001; 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 evaluate 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.

## Materials 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) and had free access to water and food. All procedures were approved by our Institutional Ethics Committee and are in accordance with National Institutes of Health 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 polyethylene-20 and polyethylene-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 IU) 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 was maintained at 36 ± 1°C. Drugs were diluted in sterile Dulbecco's phosphate-buffered

saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, and 8.1 mM NaHPO<sub>4</sub>, 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; Digi-Med, Louisville, KY) software running under Windows 98 (Microsoft Corporation, Redmond, WA). Results are expressed as means ± S.E.M. of the peak changes in MAP (measured in millimeters of mercury), relative to baseline, recorded after the administration of a given compound.

**cGMP Assay.** Eight or 24 h after PBS or lipopolysaccharide injection, rats were killed and exsanguinated, and the right lung or thoracic aorta was harvested. A piece of tissue weighing ~100 mg was minced and incubated in vitro with isobutyl-methylxanthine (a nonselective phosphodiesterase inhibitor, 0.1 mM) for 30 min at 37°C in Hanks' balanced salt solution (138 mM NaCl, 5.3 mM KCl, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 0.4 mM MgSO<sub>4</sub>, 0.49 mM MgCl<sub>2</sub>, 1.26 mM CaCl<sub>2</sub>, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 4.2 mM NaHCO<sub>3</sub>, and 5.5 mM D-glucose). Then sodium nitroprusside (SNP; 100 μM) or PBS was added, and the incubation proceeded for 10 min. Minced tissues were then quickly frozen and homogenized in ice-cold 6% trichloroacetic acid (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 enzyme immunoassay (Amersham Pharmacia Biotech, São Paulo, SP, Brazil) according to the manufacturer's instructions. This method allows measuring cGMP in the range of 50 to 12,800 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 picomoles of cGMP per milligram of protein.

**Nitrate + Nitrite Assay.** In brief, zinc sulfate-deproteinized plasma samples were subjected to nitrate conversion. Nitrate was converted to nitrite using *Escherichia 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 acid/0.1% naphthyl-ethylenediamine in Milli-Q water; Millipore Corporation, Billerica, MA) 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. Because nitrate conversion was always greater than 90% under these conditions, no corrections were made. Values are expressed as micromoles of NO<sub>x</sub> (nitrate + nitrite).

**RNA Extraction and Polymerase Chain Reaction 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, São Paulo, SP, Brazil), total RNA (1.0 μg) was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase and oligo(dT) 15 primer (Promega). cDNA were amplified by polymerase chain reaction (PCR) with TaqDNA polymerase using a GeneAmp PCR System 2400 (PerkinElmer Life and Analytical Sciences, Boston, MA) and the reaction conditions were as follows: 95°C for 90 s, 35 cycles at 95°C for 30 s, 45°C for 60 s, and 72°C for 26 s. The following primers were used to amplify *Rattus norvegicus* soluble guanylate cyclase α1 subunit cDNA (GenBank accession no. U60835): forward, 5'-GAAATCTTCAAGGGTTATG-3' (1527–1545), and reverse, 5'-CAAAGCCAGGACAGTC-3' (2335–2352). The primers used to amplify β1 subunit cDNA (GenBank accession no. AB099521): forward, 5'-GGTTTGCCAGAACCCTTGTATCCACC-3' (1450–1474), and reverse, 5'-GAGTTTTCTGGGGACATGAGACACC-3' (1709–1733). The expected size of the soluble guanylate cyclase PCR product was 825 base pairs for the α1 subunit and 284 for the β1 subunit. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers was used to validate the cDNA in each reaction:

forward, 5'-GGTGAAGGTCGGAGTCAACGGA-3', and reverse, 5'-GAGGGATCTCGCTCTGGAAGA-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 Photoshop software (Adobe Systems, Mountain View, CA). 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 $\beta 1$ 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 (50 mM HEPES, 1 mM  $MgCl_2$ , 10 mM EDTA, and 1% Triton X-100, pH 6.4, containing 1  $\mu g/ml$  each of aprotinin, leupeptin, soy bean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride). Protein samples (100  $\mu g/lane$ ) were subjected to gel electrophoresis (SDS-polyacrylamide gel electrophoresis 8% gel). After electrophoresis, the proteins were electrotransferred to polyvinylidene difluoride 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 137 mM NaCl, 2.7 mM KCl, 1.5 mM  $KH_2PO_4$ , 10.8 mM  $Na_2HPO_4 \cdot 2H_2O$ , and 0.05% Tween 20, pH 7.4, containing 5% nonfat milk followed by incubation with rabbit (polyclonal) anti-soluble guanylate cyclase  $\beta 1$  antibody (2  $\mu g/ml$ , Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature. After washing, membranes were incubated with alkaline phosphatase-labeled secondary antibodies (1:3000). Immunocomplexes were visualized by incubation with substrate solution (100 mM Tris, 100 mM NaCl, 5 mM  $MgCl_2$ , pH 9.5, containing nitro blue tetrazolium 50  $\mu g/ml$ , and 5-bromo-4-chloro-3-indolyl phosphate 25  $\mu g/ml$ ; Amersham Biosciences, São Paulo, Brazil). The bands were quantified by densitometry using Scion Image software (Scion Co., Frederick, MD).

**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* (administered intraperitoneally), whereas control animals received sterile PBS (1 ml/kg). Then 2, 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  $\mu mol/kg$ ) or PBS. In a separate set of experiments, curves for glyceryl trinitrate (GTN; 10, 100, and 1000 nmol/kg), SNP (3, 10, and 30 nmol/kg), or *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP; 10, 100, and 1000 nmol/kg) were obtained 30 min after preparation setup.

**Effect of *N*<sup>ω</sup>-Nitro-L-arginine Methyl Ester Hydrochloride 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*<sup>ω</sup>-nitro-L-arginine methyl ester hydrochloride (L-NAME), a nonselective NOS inhibitor (Rees et al., 1990). L-NAME (55  $\mu mol/kg$  i.p.), or vehicle (PBS, 1 ml/kg) were administered twice, 1 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  $\mu mol/kg$  i.p.) or vehicle (PBS, 1 ml/kg) were administered twice, 1 and 6 h after lipopolysaccharide or PBS injection.

Eight hours after lipopolysaccharide or PBS injection, rats were killed and 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, and *N*<sup>ω</sup>-nitro-L-arginine methyl ester hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). 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  $\pm$  S.E.M. of *n* animals. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by *t* test subjected to the Bonferroni correction or using Student's nonpaired *t* test. A *P* value of less than 0.05 was considered significant. Statistical analysis was performed using Prism software (GraphPad Software Inc., San Diego, CA).

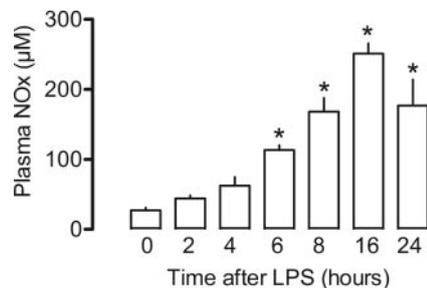
## Results

### 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 ( $98.6 \pm 2.1$  mm Hg in control animals versus  $104.7 \pm 2.5$  and  $106.7 \pm 2.7$  mm Hg 8 and 24 h after lipopolysaccharide, respectively; *n* = 8). However, lipopolysaccharide did increase plasma NO<sub>x</sub> levels, an indication of increased NO production (Fig. 1), because it was prevented by L-NAME (see below). The increase of NO<sub>x</sub> 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% compared with control animals. When methylene blue was injected shortly before phenylephrine in animals that received lipopolysaccharide 2 or 24 h earlier, the responsiveness was restored to values similar to those obtained in control animals (Fig. 2, A and C). On the other hand, methylene blue failed to restore vascular responsiveness in animals treated with lipopolysaccharide 8 h earlier (Fig. 2B). As shown previously 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 and 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



**Fig. 1.** Time-dependent increases in serum NO<sub>x</sub> levels during endotoxemia. Rats were given lipopolysaccharide (12.5 mg/kg i.p.), and at indicated periods, blood was collected and assayed for NO<sub>x</sub> levels as detailed under *Materials and Methods*. Each bar represents the mean of six animals, and vertical lines are the S.E.M. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc *t* test. \*, *p* < 0.01 compared with the control group (time, 0 h).

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 was reduced in animals injected with lipopolysaccharide 8 h earlier (Fig. 3B) but was similar to control values in rats injected with lipopolysaccharide 2 or 24 h earlier (Fig. 3, A and C). The same response profile was observed when another NO donor, SNAP, was used (data not shown).

**cGMP 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 that in control animals (Fig. 4, A and B).

**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 pretreatment with L-NAME caused a small in-

crease in MAP of control animals ( $98.6 \pm 2.1$  and  $116 \pm 4.2$  mm Hg for PBS and PBS + L-NAME groups, respectively;  $n = 8$ ). However, L-NAME did not increase the MAP of animals injected with lipopolysaccharide ( $104.7 \pm 2.5$  and  $95.6 \pm 4.1$  mm Hg 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,  $46 \pm 12 \mu\text{M}$ ; lipopolysaccharide,  $187 \pm 19 \mu\text{M}$ ; and lipopolysaccharide + L-NAME,  $55 \pm 14 \mu\text{M}$ ,  $n = 4$ ).

**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 pretreatment of animals with L-NAME (Fig. 6).

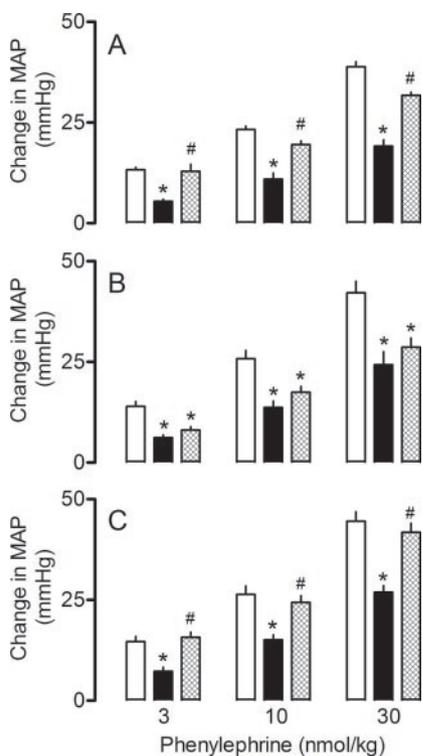
**Effects of Lipopolysaccharide Injection on Lung Guanylate Cyclase mRNA and Protein Levels.** In lungs obtained from rats injected with lipopolysaccharide 8 h earlier, soluble guanylate cyclase  $\alpha 1$  subunit mRNA levels remained unchanged compared with control animals but were increased 24 h after lipopolysaccharide (Fig. 7A).  $\beta 1$  Subunit mRNA levels were increased 8 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 of control animals (Fig. 7C).

## Discussion

We have shown previously 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 (Titheradge, 1999). NO is one of the 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 the NO-stable metabolites 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 with control levels. Previous reports showed that the loss in the response to vasoconstrictors occurs 60 min after lipopolysac-



**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 MAP recording 2 (A), 8 (B), or 24 h (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. □, rats injected with PBS; ■, rats injected with lipopolysaccharide; ▨, rats injected with lipopolysaccharide and an intravenous bolus injection of methylene blue (15  $\mu\text{mol/kg}$ ) 30 min before the first dose of phenylephrine. Each bar represents the mean of eight animals, and vertical lines are the S.E.M. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc *t* test. \*,  $p < 0.05$  compared with the control group (PBS); #,  $p < 0.05$  compared with lipopolysaccharide-injected animals.

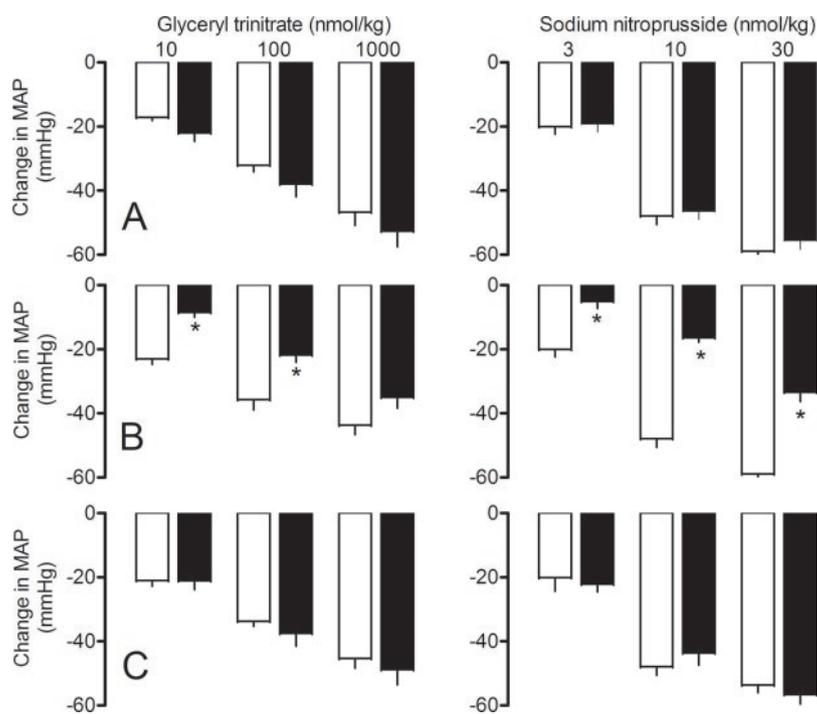
charide injection in the anesthetized rats, whereas NOS-2-increased expression can only be demonstrated 4 h after lipopolysaccharide injection (Julou-Schaeffer et al., 1990; Szabo et al., 1993). Whereas the initial loss in vasoconstrictor responses seems to be associated with NO derived from constitutive 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 Fig. 2 reveals that the hyporesponsiveness to phenylephrine in animals injected with lipopolysaccharide 2 or 8 h earlier is identical with 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 toward soluble guanylate cyclase as a potential target for drug development. Inhibition of enzyme activity with methylene blue increased blood pressure in anesthetized endotoxemic rats (Paya et al., 1993; Cheng and Pang, 1998) and rabbits (Keane et al., 1994). However, the conclusions derived from studies with methylene blue should be drawn with caution, because 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 as vascular responsiveness is concerned, the main target of methylene blue is soluble guanylate cyclase because 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., 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.

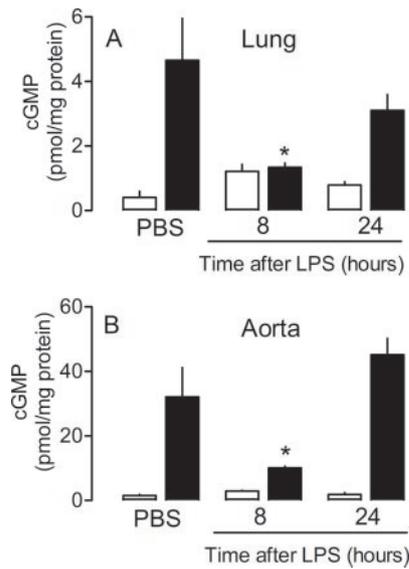
Here we have demonstrated that methylene blue restored the vasoconstrictive responses to phenylephrine that were reduced by lipopolysaccharide injection 2 and 24 h earlier but failed to do so in rats injected with lipopolysaccharide 8 h earlier. This lack of effect of methylene blue 8 h after lipopolysaccharide 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 to 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 occur in soluble guanylate cyclase functionality. These results are in line with other reports showing that exposure of bovine isolated mesenteric arterial rings to interferon- $\gamma$  (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 trinitrate observed 8 h after lipopolysaccharide cannot be attributed to an altered biotransformation of this compound, because sodium nitroprusside and SNAP (a nitrosothiol), which generate NO nonenzymatically, 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 targets besides soluble guanylate cyclase, such as potassium channels (Bolotina et al., 1994).

The hypothesis of a reduced soluble guanylate cyclase func-

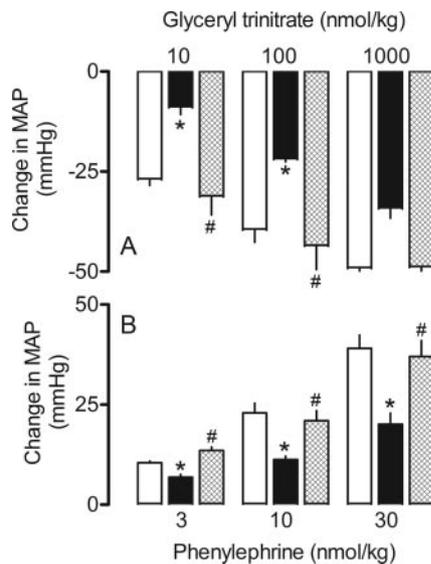


**Fig. 3.** Effect of lipopolysaccharide on vasodilatory response to nitric oxide donors. Lipopolysaccharide (12.5 mg/kg i.p.) was injected, and animals were prepared for MAP recording 2 (A), 8 (B), or 24 h (C) after lipopolysaccharide injection. Control rats received PBS (1 ml/kg i.p.). Increasing doses of glyceryl trinitrate or sodium nitroprusside were injected intravenously, and changes in MAP were recorded. □, rats injected with PBS; ■, rats injected with lipopolysaccharide. Each bar represents the mean of eight animals, and vertical lines the S.E.M. Statistical analysis was performed using Student's *t* test for nonpaired samples. \*, *p* < 0.05 compared with the respective control group (PBS).

tionalities were strengthened by the finding that sodium nitroprusside induced severalfold increases in cGMP levels in tissues (lung and aorta) taken from control rats and from

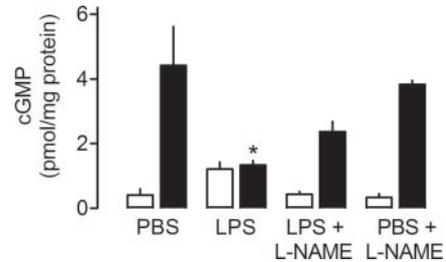


**Fig. 4.** cGMP accumulation in response to SNP stimulation in lungs or aorta from vehicle (PBS)- or lipopolysaccharide-treated animals. Lipopolysaccharide (12.5 mg/kg i.p.) was injected, and 8 or 24 h later, rats were killed by exsanguination, and lungs (A) or thoracic aorta (B) was harvested, minced, and incubated in vitro with SNP (100  $\mu$ M, 10 min; ■) or PBS (□) in the presence of isobutyl-methylxanthine (0.1 mM). Tissues were quickly frozen and homogenized in ice-cold trichloroacetic acid, and cGMP was measured by ELISA. Each bar represents the mean of three animals and vertical lines the S.E.M. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc *t* test. \*, *p* < 0.05 compared with the control (PBS/SNP).

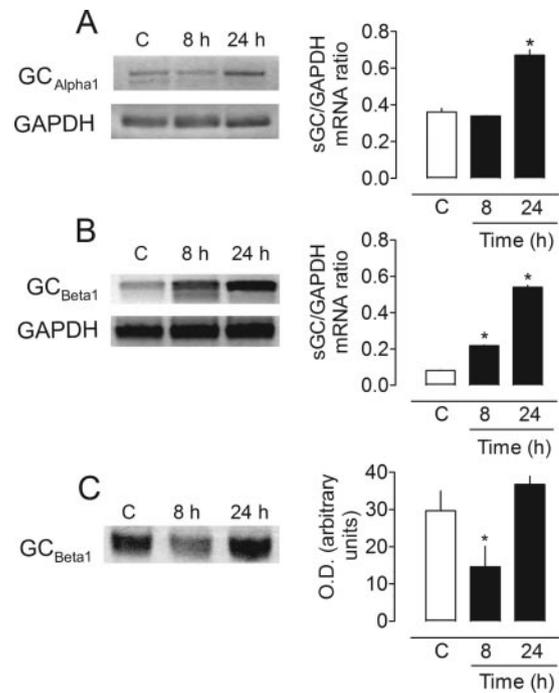


**Fig. 5.** Effect of L-NAME on lipopolysaccharide-induced mean arterial pressure changes in response to vasoactive compounds. Lipopolysaccharide (12.5 mg/kg i.p.) was injected, and animals were prepared for MAP recording 8 h after lipopolysaccharide injection. Control rats received PBS (1 ml/kg i.p.). Vasodilatory responses to glyceryl trinitrate (A) and vasoconstrictive responses to phenylephrine (B) are shown. □, rats injected with PBS; ■, rats injected with lipopolysaccharide; ▨, rats injected with lipopolysaccharide and L-NAME (55  $\mu$ mol/kg i.p.) 1 and 6 h after lipopolysaccharide. Each bar represents the mean of six to eight animals and vertical lines the S.E.M. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc *t* test. \*, *p* < 0.05 compared with the control group (PBS); #, *p* < 0.05 compared with the lipopolysaccharide group.

animals injected 24 earlier with lipopolysaccharide but failed to stimulate soluble guanylate cyclase in tissues taken from rats injected 8 h 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 after lipopolysaccharide injection has a relevant role in the de-



**Fig. 6.** Effects of L-NAME on cGMP accumulation in response to 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 SNP (100  $\mu$ M for 10 min; ■) or PBS (□) 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  $\mu$ mol/kg i.p.) was injected 1 and 6 h after lipopolysaccharide. Each bar represents the mean of three animals and vertical lines the S.E.M. Statistical analysis was performed using ANOVA followed by Bonferroni's post hoc *t* test. \*, *p* < 0.05 compared with PBS/SNP group.



**Fig. 7.** Effect of LPS on lung sGC mRNA and protein levels. Lipopolysaccharide (12.5 mg/kg i.p.) was injected, and 8 h or 24 h after injection, lungs were harvested. Control animals (C) received vehicle (PBS). Total RNA was isolated, and RT-PCR was performed using primers as described under *Materials and Methods*. A, RT-PCR products and ratio sGC  $\alpha$ 1 subunit/GAPDH obtained by densitometry. B, RT-PCR products and ratio sGC  $\beta$ 1 subunit/GAPDH. C, representative immunoelectrophoresis for soluble guanylate cyclase  $\beta$ 1 subunit and densitometry. Each bar represents the mean of three animals and vertical lines the S.E.M. Statistical analysis was performed using Student's *t* test for nonpaired samples. \*, *p* < 0.05 compared with the respective control group.

creased soluble guanylate cyclase functionality, because treatment with L-NAME prevented endotoxin-induced loss in vasodilatory response to glyceryl trinitrate and in the hyporesponsiveness to phenylephrine and 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 the development of tolerance to NO effects (Braugher, 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., 1996b). 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, because 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, because 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 the exposure of cultured smooth muscle cells to lipopolysaccharide (Papapetropoulos et al., 1996a; 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  $\alpha 1$  subunit or increased for the  $\beta 1$  subunit 8 h after lipopolysaccharide. At later times (24 h), both mRNA levels 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 reconcile 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, because 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 phosphodiesterase inhibitor (which reflects more accurately enzyme activity than RT-PCR or immunoelectrophoresis) shows that enzyme activity is decreased and returns to normal values 8 and 24 h after lipopolysaccharide injection, respectively.

We have chosen the  $\beta 1$  subunit to monitor enzyme levels based on previous works showing that in cell culture, this subunit seems to be more rapidly and robustly regulated after an inflammatory stimuli (Takata et al., 2001). In addition, because the  $\beta 1$  subunit is an obligate partner in active soluble guanylate cyclase heterodimers and contains the major heme binding domain, reduction in  $\beta 1$  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. Because mRNA levels for both subunits increased after 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 (Filippov et al., 1997). As for the second mechanism, it has been recently demonstrated that soluble guanylate cyclase is associated with 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 toward the killing of invading micro-organisms in sepsis. Although speculative at this moment, this hypothesis warrants further investigation.

Comparison of data shown in Figs. 4 and 7 indicates 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). Because 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 targets. This hypothesis also remains to be proven.

It has been suggested that down-regulation in cGMP accumulation after lipopolysaccharide injection could be a part of a homeostatic mechanism to counteract the massive hypotension seen in shock (Papapetropoulos et al., 1996a). 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 seems to be a consequence of NO-dependent inhibition of soluble guanylate cyclase, which recovery in functionality 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 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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