Late, but Not Early, Inhibition of Soluble Guanylate Cyclase Decreases Mortality in a Rat Sepsis Model

Daniel Fernandes, Regina Sordi, Leticia Kramer Pacheco, Geisson Marcos Nardi, Bettina Tomio Heckert, Christina Gaspar Villela, Amanda Revoredo Lobo, Christina Barja-Fidalgo, and Jamil Assreuy

Department of Pharmaceutical Sciences, Universidade Estadual de Ponta Grossa, Paraná, Brazil (D.F.); Department of Pharmacology, Universidade Federal de Santa Catarina, Santa Catarina, Brazil (R.S., L.K.P., B.T.H., J.A.); Areal de Ciências Biológicas e da Saúde, Universidade do Oeste de Santa Catarina, Joaçaba, Santa Catarina, Brazil (G.M.N.); and Department of Pharmacology, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil (C.G.V., A.R.L., C.B.-F.)

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ABSTRACT

Overproduction of nitric oxide and activation of soluble guanylate cyclase (sGC) are important in sepsis-induced hypotension and hyporesponsiveness to vasoconstrictors. A time course of the expression and activity of sGC in a sepsis model (cecal ligation and puncture [CLP]) was evaluated in rats. Soluble GC α-1 and β-1 subunit mRNA levels increased in the lungs, but not in the aorta. However, in both tissues, the protein levels increased 24 h after sepsis and remained high for up to 48 h. Sodium nitroprusside-stimulated cGMP accumulation was higher 48 h after CLP in the lung and aorta. NOS-2 protein expression peaked 24 h after CLP, decreasing thereafter. The impact of inhibiting the expression of sGC early (8 h) or late (20 h) on vascular reactivity and the indexes of organ damage and mortality were also studied. Late administration of methylene blue (MB) or ODQ (1H-[1,2,4]-oxadiazole[4,3-a]quinoxalin-1-one) restored the blood pressure and vascular responsiveness to vasoconstrictors to normal levels but was ineffective in early sepsis. Late MB injection reduced the plasma levels of urea, creatinine, and lactate. MB improved the survival if administered late, but it increased the mortality when administered early after sepsis onset. The increased sGC expression/activity may be relevant for the late hypotension and hyporesponsiveness to vasoconstrictors in sepsis. In accordance, MB increased survival if administered in late sepsis, but not in early sepsis. Therefore, differential responsiveness to sGC during the course of sepsis may determine the success or failure of treatment with sGC inhibitors.

Severe sepsis and septic shock are major causes of mortality in intensive care units (Vincent et al., 2006). The number of patients with severe sepsis and septic shock is increasing because of increased life expectancy and the rise in the number of immunocompromised patients, among other reasons (Bonde et al., 2006).

Large amounts of nitric oxide (NO) are produced by NO synthase (NOS) 2 (inducible NOS) isoform in response to an injury in the vascular endothelium (Chin-Wei et al., 2008), vascular smooth muscle (Rees et al., 1990), and myocardium (Niu et al., 2008). Increased NO production contributes to some of the key features of septic shock, such as severe hypotension, vascular hyporesponsiveness toward vasoconstrictors, and myocardial dysfunction. NOS inhibition studies in sepsis are mostly based on in vitro or animal studies (for review, see Assreuy, 2006). To date, all the NOS inhibitors that have been tested in the clinical setting are nonselective for the three NOS isoforms, which causes undesired side effects, such as excessive vasoconstriction, which was the most likely reason for a phase III study with a NOS inhibitor in human sepsis to be interrupted (López et al., 2004).

Soluble guanylate cyclase (sGC) is a key element in NO signaling. This enzyme is usually a heterodimer of α and β subunits, and it is activated by NO binding to its heme

ABBREVIATIONS: NO, nitric oxide; NOS, NO synthase; sGC, soluble guanylate cyclase; MB, methylene blue; ODQ, 1H-[1,2,4]-oxadiazole[4,3-a]-quinoxalin-1-one; CLP, cecal ligation and puncture; PBS, phosphate-buffered saline; MAP, mean arterial pressure; SNP, sodium nitroprusside; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; ANOVA, analysis of variance; MPO, myeloperoxidase; NOx, nitrite + nitrate.
prosthetic group. Two α and two β subunits have been cloned and sequenced (for review, see Pyrriochou and Papapetropoulos, 2005). In vascular smooth muscle cells, sGC is a heterodimer composed of α1 and β1 subunits (Nakane et al., 1990). Oxidation of its ferrous heme by methylene blue (MB) or ODQ, a highly selective sGC inhibitor, inhibits NO-mediated activation of sGC (Schrammel et al., 1996). In the clinical setting, MB has been used to treat methemoglobinemia and malaria (Wainwright and Crossley, 2002). Several uncontrolled studies in patients with septic shock requiring adrenergic support have shown that sGC inhibited by MB restores the mean arterial pressure and improves the myocardial contractility (Schneider et al., 1992; Daemen-Gubbels et al., 1995; Preiser et al., 1995; Andresen et al., 1998). On the other hand, sGC inhibition was found to be deleterious to gas exchange in the lung and myocardial contractility (Gachot et al., 1995; Weingartner et al., 1999). These findings prompted some researchers to question the use of MB in human sepsis (Schneider, 1995), whereas other groups found that the beneficial effects are worth the risk (Donati and Preiser, 2006).

Pro and con studies on the use of methylene blue in sepsis may have neglected a central question: What is the functionality of soluble guanylate cyclase during septic shock? Previous work from our laboratory has shown that ODQ failed to restore vascular responsiveness in response to phenylephrine when administered early (8 h) after lipopolysaccharide (da Silva-Santos et al., 2002). This failure of ODQ to restore vascular responsiveness has been shown to depend, at least in part, on a reduction in the sGC protein content (Fernandes et al., 2006).

Although useful, the lipopolysaccharide model has several pitfalls. Sepsis is a condition that changes with the passage of time and, therefore, may respond quite differently to a given pharmacological approach, depending on when it is performed. In the present report, we used the cecal ligation and puncture (CLP)-induced sepsis model, which establishes a polymicrobial infection, providing an inflammatory source in part, on a reduction in the sGC protein content (Fernandes et al., 2006).

Activation of sGC is an important mechanism of vascular collapse during septic shock (Paya et al., 1993; Keaney et al., 1994; da Silva-Santos et al., 2002; Donati et al., 2002). Thus, the main goal of the present report was to study the expression and activity of sGC in a sepsis model. In addition, we evaluated the impact of inhibiting sGC early (8 h) and late (20 h) after sepsis onset on mortality and some biochemical parameters.

Materials and Methods

Animals

Female Wistar rats (weighing 200–300 g) 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 the investigation conforms to the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

CLP

CLP surgery was performed as described previously (Wichterman et al., 1980), with minor modifications. Rats were anesthetized with ketamine and xylazine (90 and 15 mg/kg, respectively). The cecum lumen was reduced by a nonobstructing ligation right above the ileo-cecal valve. The cecum was punctured 20 times with an 18-gauge needle, and a small amount of cecal content was squeezed through the punctures. It was placed back in the abdominal cavity, and walls were sutured. Sham-operated rats underwent a similar surgical procedure with cecal exposure, but it was neither ligated nor punctured. All animals received 2 ml of sterile Dulbecco’s phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8.1 mM NaHPO4, pH 7.4) subeutaneously.

Blood Pressure Measurement

Under anesthesia, heparinized PE-20 and PE-50 polyethylene catheters were inserted into the left femoral vein for drug injections and into the right carotid artery for recording the mean arterial pressure (MAP) and blood withdrawal. Animals were allowed to breathe spontaneously, and the body temperature was maintained at 36 ± 1°C. Drugs were diluted in sterile PBS. Sacrifice was carried out by pentobarbitone overdose. Blood pressure was recorded with a catheter pressure transducer (Mikro-Tip; Millar Instruments Inc., Houston, TX) coupled to a Powerlab 8/30 acquisition system (ADInstruments Pty Ltd., Castle Hill, Australia). Results are expressed as the means ± S.E.M. of the peak changes in MAP (as millimeters of mercury) after administration of a given compound, relative to the baseline.

cGMP Assay

The lower half of the left lung or the thoracic aorta was rapidly excised. A fragment weighing ~100 mg was minced and incubated in vitro with isobutyl mercaptoethane (a nonspecific 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 KH2PO4, 0.4 mM MgSO4, 0.49 mM MgCl2, 1.26 mM CaCl2, 0.34 mM Na2HPO4, 4.2 mM NaHCO3, and 5.5 mM d-glucose). Sodium nitroprusside (SNP; 100 μM) or PBS was added, and the incubation proceeded for 10 min. Tissue fragments were quickly frozen and homogenized in ice-cold 6% trichloroacetic acid (1 ml). cGMP was measured by enzyme-linked immunosorbent assay (GE Healthcare, São Paulo, SP, Brazil) according to the manufacturer’s instructions. Total protein was determined using UV spectrophotometry at 260 nm. cDNAs were amplified by polymerase chain reaction with Taq DNA polymerase (Promega) under the following conditions: 25 s, with a final step of 72°C for 7 min. The following primers were used to amplify 1 subunit cDNA (GenBank accession no. U60835): forward, 5’-GAAAATCCATCAAGGTATG-3’ (1527–1545); and reverse, 5’-CACAAAGGAGAGCATGTC-3’ (2335–2352). The primers used to amplify β1 subunit cDNA (GenBank accession no. AB099521) were forward, 5’-AGTTGACCCAGAATCTGGTCC-3’ (1450–1474); and reverse, 5’-GATTTTCGTGGAGCAGAAGTGAC-3’ (1709–1733). The expected size of the sGC PCR product was 825 bp for the α1 subunit and 284 bp for the β1 subunit. The housekeeping gene GAPDH primers were used to val-
idate cDNA in each reaction, and primers were forward, 5'-GGTGGAAGGTCTGAGCTGCAAGGA-3'; and reverse, 5'-GGAGGATCTCGGTCTCGTGGAGA-3'. PCR products were electrophoresed on a 1.5% agarose gel and visualized by UV exposure on a transilluminator. The value corresponding to sGC mRNA level was measured using the GAPDH mRNA levels as a reference.

**Immunoelectrophoresis for the sGC β1 Subunit**

Tissues were homogenized in ice-cold buffer (50 mM HEPES, 1 mM MgCl₂, 10 mM EDTA, and 1% Triton X-100, pH 6.4, containing 1 mg/ml each aprotinin, leupeptin, and soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride) and centrifuged. Supernatant protein samples (50 μg/lane) were subjected to gel electrophoresis (SDS-polyacrylamide gel electrophoresis, 8% gel) and transferred to polyvinylidene difluoride membranes [30 min for sGC or 90 min for NOS2; at 0.8 mA/cm² and 15 V in Tris-glycine buffer (48 mM Tris-HCl/39 mM glycine/10% methanol)]. The membrane was incubated for 1 h at room temperature with Tween-PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 10.8 mM Na₂HPO₄·2H₂O, and 0.05% Tween 20, pH 7.4), containing 5% skimmed milk, followed by incubation with a rabbit polyclonal anti-sGC β1 (0.2 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. After washing, membranes were incubated with a biotinylated secondary antibody (1:100; Amersham Biosciences) and then with horseradish peroxidase-conjugated streptavidin (1:100; Amersham Biosciences). Immunocomplexes were visualized by chemiluminescence, and band intensity was quantified by densitometry using Scion Image software (Scion Corporation, Frederick, MD). Loading controls were carried out using an anti-actin antibody under the same conditions.

**NOX Assay**

In brief, zinc sulfate-deproteinized plasma samples were subjected to conversion of nitrate to nitrite using Escherichia coli nitrate reductase for 3 h at 37°C as described previously (da Silva-Santos et al., 2002). Values are expressed as micromolar NOX (nitrate + nitrite).

**Myeloperoxidase Assay**

Lungs were homogenized in hexadecyltrimethylammonium bromide buffer (0.5% in 80 mM sodium phosphate buffer, pH 5.4). The supernatant was assayed for enzyme activity, and results were expressed as the optical density at 450 nm/mg protein.

**Urea, Creatinine, and Lactate Assays**

Urea nitrogen and creatinine levels were measured using commercially available clinical assay kits (Gold Analisa Diagnostica Ltda, Belo Horizonte, Minas Gerais, Brazil). Lactate was measured as described previously (MacQueen and Plaut, 1979). In brief, 5 μl of deproteinized plasma, standards, and the blank were pipetted into microplate wells. The reaction was started by adding 100 μl of the reagent mixture (75 mM 2-amino-2-methyl-1,3-propanediol, 15 mM NAD⁺, 0.5 mM phenazine methosulfate, 1.8 mM nitroblue tetrazolium chloride, 0.3% Brij-35, and 130 IU of lactate dehydrogenase), and the mixture was incubated at 37°C for 10 min. After incubation, 150 μl of 0.1 N HCl was added to each well to stop the reaction, and the absorbance was read against the reagent blank at 550 nm using a microplate reader (Ultra Microplate Reader 808; BioTek Instruments, Winooski, VT). Standard curves of lactate (0–800 μg/ml) were run simultaneously. Values are expressed as micrograms per milliliter of plasma.

**Leukocyte Count**

Total leukocyte counts were determined in Neubauer chambers and are expressed as cells per cubic millimeter × 10⁹.

**Experimental Protocols**

**Protocol 1: Time Course of sGC during CLP.** Twelve, 24, or 48 h after CLP or sham surgery, the rats were sacrificed, and lungs and thoracic aortas were harvested for cGMP assay, RT-PCR, and immunoelectrophoresis.

**Protocol 2: Effects of sGC Inhibition on CLP-Induced Hypotension and Hyporesponsiveness to Vasconstrictors.** Twelve, 24, or 48 h after CLP or sham surgery, the animals were prepared for MAP recording as described above. Two consecutive dose-response curves to phenylephrine (3, 10, and 30 nmol/kg) or angiotensin II (3, 10, and 30 pmol/kg) were obtained before and 30 min after methylene blue (10 mg/kg) or ODQ (200 μl) injection.

**Protocol 3: Effects of sGC Inhibition on Survival.** Sham or CLP rats were randomized to receive MB (10 mg/kg s.c.) or ODQ (2 mg/kg s.c.) 8 or 20 h after surgery. ODQ was dissolved in dimethyl sulfoxide and injected in a volume not exceeding 0.5 ml/kg. Sham and CLP control animals received vehicle (PBS or dimethyl sulfoxide) at the same time. The mortality rate was recorded over a 5-day period. Doses of MB and ODQ were chosen based on our own experience (da Silva-Santos et al., 2002; Fernandes et al., 2006) and from other published studies (Keaney et al., 1994; Zacharowski et al., 2001).

**Protocol 4: Effects of sGC Inhibition in Different Periods after Sepsis Onset.** Treatment with MB (or PBS; 10 mg/kg and 200 μl, respectively) was performed 8 or 20 h after surgery. Twelve to 16 h after treatment (as illustrated in Fig. 1), the rats were instrumented, isoprenaline (1 nmol/kg i.v.) was injected, and the heart rate was measured. Results are expressed as means ± S.E.M. of the peak changes in heart rate (as beats per minute) relative to the baseline. Animals were then sacrificed, and the blood and tissues were obtained for assays. The choice of the time for MB injections was based on two considerations. The first was to avoid (or at least minimize) the physical presence of MB at the time of analysis because its half-life is 6 h (Peter et al., 2000). The second consideration was to study the influence of MB at two widely spaced moments in the course of sepsis.

**Reagents**

Methylene blue, ODQ (Schrammel et al., 1996), phenylephrine chloride, angiotensin II, sodium nitroprusside, 3-isobutyl-1-methylxanthine, 2-amino-2-methyl-1, 3-propanediol, NAD⁺, phenazine methosulfate, nitroblue tetrazolium chloride, Brij-35, and lactate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). Heparin was a kind gift of Cristália Laboratories (São Paulo, Brazil).

**Statistical Analysis**

Data are expressed as the means ± S.E.M. of n animals. Statistical significance was analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA followed by an appropriate post hoc analysis.
test as indicated in the figure legends. Differences in the survival study were determined with a log rank test. A $p$ value of less than 0.05 was considered significant. Two-way ANOVA analysis was performed using the Statistica software package (StatSoft, Tulsa, OK), and the other tests were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

**Results**

Figure 2 shows a composition of typical recordings obtained from sham-operated and septic animals. Sham-operated animals had an MAP of ~100 mm Hg. On the other hand, CLP animals presented a progressive hypotension that attained the lower value (60 mm Hg) 48 h after surgery. The quantitative results of these experiments are shown in Figs. 3 and 8.

When challenged with increasing doses of phenylephrine, sham-operated animals responded with a dose-dependent increase in blood pressure (Fig. 2). In contrast, 12 h after CLP, the vasoconstrictive response to phenylephrine declined sharply. This profound hyporesponsiveness to phenylephrine remained essentially the same throughout the rest of the evaluation period. The quantitative results of these experiments are shown in Fig. 8 (compare open with closed bars). Figure 8 also shows that the ability to respond to angiotensin II was also compromised during sepsis.

Lung mRNA levels of sGC $\alpha_1$ and $\beta_1$ subunits increased over time, reaching their peak at 24 h and returning to basal levels by 48 h after CLP (Fig. 4, A and B). Soluble GC protein levels were high at 24 h after CLP, remained elevated at 36 h, and started to decline by 48 h after sepsis onset (Fig. 4C). The pattern of change in the sGC mRNA level in the aorta was different from the lung (Fig. 5, A and B). However, similar to what was found in the lung tissue, sGC protein levels increased by 24 h after CLP and remained elevated up to 48 h after sepsis onset (Fig. 4C). As shown in the figure legend, results obtained with the time-matched sham animals (12, 24, and 48 h after surgery) were similar at the different time points. Therefore, we pooled these results and represented the three time-matched sham groups as a single bar for the sake of clarity. Samples appearing in typical gels are from the sham animals operated on 24 h before tissue harvesting.

In vitro incubation of lung tissue obtained from the sham-operated animals with SNP led to a 10-fold increase in cGMP accumulation. However, SNP failed to induce cGMP accumulation in lung tissue obtained from rats submitted to the CLP surgery 12 h earlier. In contrast, the stimulatory effect of SNP was present in the lung tissue of CLP animals operated on 48 h earlier (Fig. 6A). A very similar pattern was observed in the aorta (Fig. 6B).

As shown before, CLP led to a significant and progressive fall in blood pressure. Methylene blue injection normalized the blood pressure only in animals submitted to the CLP procedure 48 h earlier, leaving the sepsis hypotension unaffected at earlier periods. In agreement with previous work (Cheng and Pang, 1998), methylene blue did not change MAP in the sham animals (Fig. 7).

CLP reduced the response to phenylephrine and angiotensin II by ~50% (Fig. 8). Methylene blue failed to modify the CLP-induced hyporesponsiveness to both vasoconstrictors when administered to rats made septic 12 h earlier (Fig. 8A). However, the inhibitor was effective in restoring both phenylephrine and angiotensin II effects when injected 24 or 48 h after the CLP surgery (Fig. 8, B and C). Methylene blue did not change phenylephrine or angiotensin II responses in the sham animals (Fig. 8).

Survival of the CLP rats declined sharply in the first 24 h and then progressively up to 120 h (Fig. 9, A and B). Rats injected with methylene blue 20 h after the CLP surgery survived significantly longer than the CLP group (55 versus 20%, respectively). On the other hand, the animals given methylene blue 8 h after the CLP surgery presented with an increased mortality (Fig. 9A). Essentially the same results were obtained with a highly selective sGC inhibitor, ODQ (Fig. 9B).

CLP increased the levels of NOx, creatinine, urea, lactate, lung myeloperoxidase (MPO) activity, and induced leucopenia (Table 1). All parameters increased over time in sepsis (compare Table 1 with Table 2). Treatment with methylene blue 8 h after CLP did not change any of the values that were increased by CLP. However, treatment with methylene blue 20 h after CLP reduced some of the variables (lactate, urea,
and creatinine) but not others (MPO activity, leucopenia, or plasma NOx). CLP also caused impairment in isoprenaline-induced increases in heart rate, and this effect was also aggravated with the passage of time. Treatment with methylene blue 8 h after CLP worsened the loss in the isoprenaline effect. On the other hand, the late injection (20 h after CLP) of methylene blue did not change the sepsis-induced decrease in the isoprenaline effect.

**Fig. 4.** Effects of the CLP on the soluble guanylate cyclase mRNA and protein levels in the lung. Twelve, 24, 36, or 48 h after the surgery, the animals were sacrificed, and the lungs were harvested. Total RNA was isolated, and RT-PCR was performed using primers as described under Materials and Methods. A, RT-PCR products and the ratio of the sGC α1 subunit/GAPDH obtained by densitometry. B, RT-PCR products and the sGC β1 subunit/GAPDH ratio. C, representative immunoelectrophoresis for soluble guanylate cyclase β1 subunit and densitometry. Because the results obtained in the time-matched sham animals were very similar, they were pooled and represented as a single bar for the sake of clarity. Samples appearing in the gels were harvested from the sham animals that were operated on 24 h before. All experiments were repeated twice. Each bar represents the mean of the data from three to six animals, and the vertical lines are the S.E.M. Statistical analysis: one-way ANOVA followed by Dunnett post hoc t test. *, p < 0.05 compared with the respective sham group.

**Fig. 5.** Effects of the CLP on the soluble guanylate cyclase mRNA and protein levels in the aorta. Twelve, 24, 36, or 48 h after the surgery, the animals were sacrificed, and the thoracic aortas were harvested. Total RNA was isolated, and RT-PCR was performed using primers as described under Materials and Methods. A, RT-PCR products and the ratio of sGC α1 subunit/GAPDH obtained by densitometry. B, RT-PCR products and the sGC β1 subunit/GAPDH ratio. C, representative immunoelectrophoresis for soluble guanylate cyclase β1 subunit and densitometry. Because the results obtained in the time-matched sham animals were very similar, they were pooled and represented as a single bar for the sake of clarity. Samples appearing in the gels were harvested from the sham animals that were operated on 24 h before. All experiments were repeated twice. Each bar represents the mean of the data from three to five animals, and the vertical lines are the S.E.M. Statistical analysis: one-way ANOVA followed by Dunnett post hoc t test. *, p < 0.05 compared with the respective sham group.
Fig. 6. Basal and sodium nitroprusside-stimulated cGMP accumulation in the lung and aorta tissues. Lung (A) or thoracic aorta (B) were harvested 12, 24, or 48 h after the CLP or sham surgery, minced, and ~100 mg of each tissue was incubated in vitro with SNP (100 μM, 10 min, black bars) or PBS (open bars) in the presence of isobutyl-methylxanthine (0.1 mM). Results from the time-matched sham animals were pooled as detailed in Fig. 3. Each bar represents the mean of the data from four to six animals, and the vertical lines are the S.E.M. Statistical analysis: one-way ANOVA followed by Dunnett post hoc t test.

Fig. 7. Effect of methylene blue on the CLP-induced hypotension. Open bars, sham; black bars, CLP; cross-hatched and striped bars, sham and CLP rats, respectively, injected with methylene blue (10 mg/kg). Each bar represents the mean of the data from eight animals, and the vertical lines are the S.E.M. Statistical analysis: two-way ANOVA followed by Bonferroni’s post hoc t test. *, p < 0.05 compared with the sham tissue stimulated with SNP. #, p < 0.05 compared with the unstimulated sham tissue (incubated with PBS).

Discussion

Sepsis has a profound impact on the blood pressure and on the response to vasoconstrictors. Hypotension increased progressively, and the cardiovascular derangement was intensified by the failures in vascular and cardiac responsiveness.

One of the main findings of the present report is that the sGC protein levels and the consequent increase in NO-induced stimulation of its activity are augmented during sepsis. The β1 subunit was chosen as an index of total sGC protein level because it is regulated faster after an inflammatory stimulus (Takata et al., 2001); it is an obligate partner in active sGC heterodimers, and it contains the heme-binding domain (Pyriochou and Papapetropoulos, 2005). The newly synthesized sGC is fully functional because cGMP accumulation in response to SNP correlates very well with the increased enzyme content. This is suggestive that the increased sGC expression/activity in late sepsis may be relevant to the observed vascular abnormalities. To our knowledge, this is the first demonstration of the sGC up-regulation in a sepsis animal model. Egression of cGMP from the intracellular compartment has a slow time course that, together with a relatively fast urinary excretion, may explain why cGMP did not increase during sepsis (reviewed in Sager, 2004). This may also explain the apparent lack of correlation between plasma NOx and cGMP levels.

Chronic hypoxia increases the sGC protein levels in the lung (Li et al., 2001). One of the sepsis hallmarks is a reduced tissue oxygenation and increased lactate levels, as our results have confirmed. Therefore, sepsis-induced tissue hypoxia may be one of the reasons for the higher sGC content found in late sepsis. Our finding that shows that the same pattern was found in the aorta is suggestive that increases in the sGC content may be relevant to vascular deterioration seen in sepsis. Of note is the observation that increasing the sGC protein content correlated with the increase in its mRNA in the lung, but not in the aorta. We do not have an explanation for this finding at present. Although chronic exposure of rat pulmonary artery smooth muscle cells to NO donor compounds decreased the sGC subunit mRNA, protein levels, and enzyme activity (Filippov et al., 1997), this study was conducted in cultured cells, whereas ours was in live animals. In any event, the stimulatory effect of SNP on the sGC of both aorta and lung correlates very well with the higher enzyme content in late sepsis.

NOS-2 protein content reached a maximum 24 h after CLP and decreased thereafter (data not shown). This pattern is identical to that shown in previous reports (Okamoto et al., 2000; da Silva-Santos et al., 2002). It is interesting that although NO and cGMP are critically involved in a very important signaling pathway, the pattern of expression of their producing enzymes was not entirely coincident, at least in sepsis. Thus, it may be that the augmented level of the target (sGC) may compensate for the decreasing agonist (NO) production in such a way that the hypotension and the hyporesponsiveness to vasoconstrictors are more evident late in sepsis.

Methylene blue has been used in several studies in human sepsis (Schneider et al., 1992; Daemen-Gubbels et al., 1995; Gachot et al., 1995; Preiser et al., 1995; Andresen et al., 1998; Weingartner et al., 1999; Donati et al., 2002). MB was effective in increasing MAP of septic rats only when injected 48 h after the CLP procedure. At earlier times, the sepsis-induced hypotension was unaffected by methylene blue. This finding suggests that the late increase in sGC expression/activity may be the likely reason that explains the higher efficacy of methylene blue in late sepsis. Methylene blue was effective in reducing mortality when administered late in sepsis, coincident with improved hemodynamic parameters. Thus, it may be that this beneficial effect of methylene blue should be because of improvements in hemodynamic, tissue perfusion...
status, and organ damage, as suggested by the reduced plasma lactate, creatinine, and urea. It is noteworthy that methylene blue did not change the inflammatory parameters (leucopenia and increased MPO activity). These results are in accordance with findings that methylene blue infusion did not change cytokine levels in patients with severe sepsis (Memis et al., 2002).

Administration of methylene blue late (20 h after surgery) substantially decreased the mortality, suggesting that the sGC/cGMP pathway contributes to death. It is interesting that methylene blue increased the mortality when injected 8 h after the CLP surgery. One possible explanation for these contrasting effects would be related to the nonspecific actions of MB. This compound has additional pharmacological actions besides sGC inhibition, such as generation of oxygen radicals, NOS-2 inhibition, and interference with potassium channel function (Mayer et al., 1993; Stockand and Sansom, 1996). However, the lack of selectivity explanation seems unlikely because ODQ very closely reproduced both the detrimental and beneficial effects of the early and late injections of methylene blue on survival. Another possible explanation is based on recent work showing that in early endotoxemia, a small amount of cGMP is produced in the heart, and it seems to be beneficial to maintain myocardial contractile capacity. However, large amounts of NO and cGMP produced later seem to mediate heart depression (Cohen et al., 2006). In this line of thought, we have shown that early methylene blue treatment worsens myocardial function in the CLP model as assessed by the heart-stimulatory effect of isoprenaline. Despite a clear understanding of the time-dependent and contrasting effects of MB and ODQ, it seems that both outcomes depend on sGC inhibition.

One point to be taken into account is that at the early time point in our CLP model, the NOS-2 protein level (and, conceivably, its enzymatic activity) is high, whereas at a later stage of sepsis, the enzyme protein levels have returned to basal levels (data not shown). NOS-2-derived NO may, contrary to the general belief, be protective (Cauwels et al., 2000). Along this line of thought, NO seems to be essential to...
maintain organ blood flow during early endotoxic shock (Zhang et al., 1996). Thus, the detrimental effects of methylene blue and ODQ in early sepsis may be because of an inhibition of a critically important fraction of sGC that mediates the NO (produced by either NOS-2 or NOS-3) protective effects. Late in sepsis, it may be that the increase in sGC improves several markers of tissue damage, indicating that this enzyme and its product are detrimental late in sepsis.

In the present study, we demonstrated that the timing of sGC inhibition might significantly affect survival in a rat sepsis model. However, septic shock is a highly complex pathophysiological condition. The course of sepsis has different stages with different characteristics, which also depend on gender, age, comorbidities, and the infectious agent. Animal models have been developed in an effort to create reproducible systems for studying sepsis pathogenesis and for the purpose of preliminary testing of potential therapeutic agents. However, demonstrated benefits from a therapeutic agent in animal models have rarely been translated into success in human clinical trials. Therefore, results obtained in animal models may not be directly applicable to the clinical setting.

The antibacterial effects of NO produced by defense cells do not depend on the sGC activity (Nathan, 1992). Our data suggest that inhibition of sGC may be a better option than inhibiting NOS-2 because it would preserve cGMP-independent NO effects, particularly at the late stages of sepsis. Thus, sGC inhibition may be a useful therapeutic strategy if administered at the proper window of opportunity. Sepsis is a condition that changes with the passage of time. If a patient’s stage of sepsis could be determined at any given time, it may allow for better patient specific tailoring of the therapeutic approach.

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