LATE BUT NOT EARLY INHIBITION OF SOLUBLE GUANYLATE CYCLASE
DECREASES MORTALITY IN A RAT SEPSIS MODEL

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Abbreviations: NO, nitric oxide; sGC, soluble guanylate cyclase; MB, methylene blue; CLP, cecal ligation and puncture; MAP, mean arterial pressure; MPO, myeloperoxidase; ODQ, 1H-[1,2,4]-oxadiazole[4,3-a]quinoxalin-1-one; SNP, sodium nitroprusside

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ABSTRACT

Overproduction of nitric oxide (NO) 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 alpha-1 and beta-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 was also studied. Late administration of methylene blue (MB) or ODQ 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 administrated early after sepsis onset. The increased sGC expression/activity may be relevant for the late hypotension and hyporesponsiveness to vasoconstrictors in sepsis. Accordingly, 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.
INTRODUCTION

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 due to increased life expectancy and the rise in the number of immunocompromised patients, among other reasons (Angus et al., 2006).

Large amounts of nitric oxide (NO) are produced by NO synthase type 2 (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., 1999) 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 towards vasoconstrictors and myocardial dysfunction. NOS inhibition studies in sepsis are mostly based on in vitro or animal studies (for a review, see Assreuy, 2006). To date, all the NOS inhibitors that have been tested in the clinical setting are non-selective 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 (Lopez et al., 2004).

Soluble guanylate cyclase (sGC) is a key element in NO signaling. This enzyme is usually a heterodimer of alpha and beta subunits and it is activated by NO binding to its heme prosthetic group. Two alpha and two beta subunits have been cloned and sequenced (for a review see Pyriochou and Papapetropoulos, 2005). In vascular smooth muscle cells, sGC is a heterodimer composed of alpha1 and beta1 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, methylene blue (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 (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 LPS 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, provides an inflammatory source of necrotic tissues that more closely resembles the human condition (Buras et al., 2005).

Activation of sGC is an important mechanism of vascular collapse during septic shock (Paya et al., 1993; Keaney et al., 1994; Donati et al., 2002; Silva-Santos 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.
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 the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cecal ligation and puncture (CLP)

CLP surgery was performed as previously described (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 non-obstructing ligation right above the ileo-cecal valve. The cecum was punctured twenty 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, in mM 137 NaCl, 2.7 KCl, 1.5 KH₂PO₄, and 8.1 NaHPO₄; pH 7.4) subcutaneously.

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, Texas, USA) coupled to a Powerlab 8/30 acquisition system (AD Instruments Pty Ltd., Castle Hill, Australia). Results are expressed as the means ± SEM of the peak changes in MAP (as mmHg) following administration of a given compound, relative to the baseline.

**Cyclic GMP 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 methylxanthine (a non-selective phosphodiesterase inhibitor; 0.1 mM) for 30 min at 37°C in Hank’s balanced salt solution (in mM; NaCl 138, KCl 5.3, KH₂PO₄ 0.44, MgSO₄ 0.4, MgCl₂ 0.49, CaCl₂ 1.26, Na₂HPO₄ 0.34, NaHCO₃ 4.2 and D-glucose 5.5). 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% TCA (1 ml). Cyclic GMP was measured by ELISA (Amersham Pharmacia Biotech, São Paulo, SP, Brazil) according to the manufacturer’s instructions. Total protein was determined by the Bradford method. Results are expressed as pmol cGMP/mg protein. For the plasma cGMP assay, blood was collected in tubes containing 7.5 mM EDTA and the plasma was stored at -80 ºC. Results are expressed as nM cGMP.

**RNA extraction and PCR analysis**

Total RNA was extracted with TRIzol and the concentration was determined using UV spectrophotometry at 260 nm. cDNAs were amplified by polymerase chain reaction with Taq DNA polymerase using a GeneAmp® PCR System 2400 (Perkin-Elmer, USA). Reaction conditions were as follows. Alpha 1 subunit: 95°C for 5 min and 35 cycles comprised of 95°C for 45 sec, 45°C for 45 sec, and 72°C for 60 sec
with a final step of 72°C for 7 min. Beta 1 subunit: 95°C for 5 min and 35 cycles comprised of 95°C for 45 sec, 45°C for 45 sec, and 72°C for 25 sec with a final step of 72°C for 7 min. GAPDH: 95°C for 5 min and 32 cycles comprised of 95°C for 45 sec, 45°C for 45 sec, and 72°C for 25 sec with a final step of 72°C for 7 min. The following primers were used to amplify *Rattus norvegicus* sGC 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) were forward, 5′-GGTTTGCCAGAACCTTGTATCCACC-3′ (1450-1474); and reverse, 5′-GAGTTTTCTGGGGACATGAGACACC-3′ (1709-1733). The expected size of the sGC PCR product was 825 bp for the alpha1 subunit and 284 bp for the beta1 subunit. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were used to validate cDNA in each reaction and primers were forward, 5′-GGTGAAGGTCGGAGTCAACGGA-3′, and reverse, 5′-GAGGGATCTCGCTCCTGGAAGA-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 sGC beta1 subunit**

Tissues were homogenized in ice-cold buffer (in mM: 50 HEPES, 1 MgCl₂, 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 phenylmethanesulphonyl fluoride) and centrifuged. Supernatant protein samples (50 µg/lane) were subjected to gel electrophoresis (SDS/PAGE, 8% gel) and transferred to PVDF 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 hour at room temperature with T-PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 10.8 mM Na$_2$HPO$_4$.2H$_2$O and 0.05% Tween-20, pH 7.4) containing 5% skimmed milk, followed by incubation with a rabbit polyclonal anti-sGC beta1 (0.2 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4ºC. Following washing, membranes were incubated with a biotinylated secondary antibody (1:1000, Amersham Biosciences, England, UK) and then with horseradish peroxidase conjugated streptavidin (1:1000, Amersham Biosciences, England, UK). Immunocomplexes were visualized by chemiluminescence and band intensity was quantified by densitometry using Scion Image software (Scion Co., Frederick, MD, USA). Loading controls were carried out using an anti-actin antibody under the same conditions.

**NOx assay**

Briefly, zinc sulphate-deproteinized plasma samples were subjected to conversion of nitrate to nitrite using *E. coli* nitrate reductase for 3 h at 37°C as previously described (Silva-Santos et al., 2002). Values are expressed as µM NOx (nitrate + nitrite).

**Myeloperoxidase (MPO) assay**

Lungs were homogenized in hexadecyltrimethylammonium bromide buffer (HTAB, 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 per mg of protein.

**Urea, creatinine and lactate assays**

Urea nitrogen and creatinine levels were measured using commercially available clinical assay kits (Gold Analisa Diagnostica, Belo Horizonte, MG, Brazil).
Lactate was measured as previously described (MacQueen and Plaut, 1979). Briefly, 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 (2-amino-2-methyl-1,3-propanediol 75 mM, NAD+ 15 mM, phenazine methosulfate 0.5 mM, nitroblue tetrazolium chloride 1.8 mM, Brij-35 0.3% and 130 IU 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, Bio-Tek Instruments, Inc., USA). Standard curves of lactate (0–800 µg/ml) were run simultaneously. Values are expressed as µg/ml plasma.

**Leukocyte count**

Total leukocyte counts were determined in Neubauer chambers and are expressed as cells/mm³ x 10⁵.

**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 vasoconstrictors**

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 PBS (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 hours after surgery. ODQ was dissolved in dimethylsulphoxide (DMSO) and injected in a volume not exceeding 0.5 ml/kg. Sham and CLP control animals received vehicle (PBS or DMSO) at the same time. The mortality rate was recorded over a five-day period. Doses of MB and ODQ were chosen based on our own experience (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 hours after surgery. Twelve to sixteen hours 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 ± SEM of the peak changes in heart rate (as bpm) 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, since 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 (1H-[1,2,4]-oxadiazole[4,3-a]quinoxalin-1-one; Schrammel et al., 1996), phenylephrine chloride, angiotensin II, sodium nitroprusside, isobutyl-methylxanthine (IBMX), 2-amino-2-methyl-1, 3-propanediol, NAD+, phenazine methosulfate, nitroblue tetrazolium chloride, Brij-35 and lactate
dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO, USA).
Heparin was kind gift of Cristalia Laboratories (São Paulo, SP, Brazil).

**Statistical analysis**

Data are expressed as the means ± SEM of n animals. Statistical significance was analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA followed by an appropriate post-hoc 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 Inc., Tulsa, OK, USA) and the other tests were performed using GraphPad Prism software (San Diego, CA, USA).
RESULTS

Figure 2 shows a composition of typical recordings obtained from sham-operated and septic animals. Sham-operated animals had a mean arterial pressure (MAP) of ~100 mmHg. On the other hand, CLP animals presented a progressive hypotension that attained the lower value (60 mmHg) 48 hours after surgery. The quantitative results of these experiments are shown in Figures 3 and 7.

When challenged with increasing doses of phenylephrine, sham-operated animals responded with a dose-dependent increase in blood pressure (Fig. 2). In contrast, twelve hours 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 Figure 8 (compare open and closed bars). Figure 8 also shows that the ability to respond to angiotensin II was also compromised during sepsis.

Lung mRNA levels of sGC alpha1 and beta1 subunits increased over time reaching their peak at 24 h and returning to basal levels by 48 h after CLP (Fig. 4A and 4B). 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. 5A and 5B). 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. 5C). 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 sodium nitroprusside (SNP) led to a ten-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 hours earlier (Fig. 8A). However, the inhibitor was effective in restoring both phenylephrine and angiotensin II effects when injected 24 or 48 hours after the CLP surgery (Fig. 8B and 8C). 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 hours (Fig. 9A and 9B). Rats injected with methylene blue 20 hours after the CLP surgery survived significantly longer than the CLP group (55% versus 20%, respectively). On the other hand, the animals given methylene blue eight hours 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 MPO activity and induced leucopenia (Table 1). All parameters increased over time in sepsis (compare Tables 1 and 2). Treatment with methylene blue eight hours after CLP did not change any of the values that were increased by CLP. However, treatment with methylene blue 20 hours 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 eight hours after CLP worsened the loss in the isoprenaline effect. On the other hand, the late injection (20 hours after CLP) of methylene blue did not change the sepsis-induced decrease in the isoprenaline effect.
DISCUSSION

Sepsis has a profound impact on the blood pressure and on the response to vasoconstrictors. Hypotension increased progressively and the cardiovascular derangement were 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. Beta1 subunit was chosen as an index of total sGC protein level, since 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, since 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 upregulation 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 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 indeed be relevant to vascular deterioration seen in sepsis. Of note is the observation that increased 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 decreasing thereafter (data not shown). This pattern is identical to that shown in previous reports (Okamoto et al., 2000; Silva-Santos et al., 2002). Interestingly, 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; Preiser et al., 1995; Daemen-Gubbels et al., 1995; Andresen et al., 1998; Gachot et al., 1995; 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 due to improvements in hemodynamic, tissue perfusion status and organ damage, as suggested by the reduced plasma lactate, creatinine and urea. Of note, 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 hours after surgery) substantially decreased the mortality, suggesting that the sGC/cGMP pathway contributes to death. Interestingly, methylene blue increased the mortality when injected eight hours after the CLP surgery. One possible explanation for these contrasting effects would be related to the non-specific 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, since 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 appears to be beneficial to maintain myocardial contractile capacity. However, large amounts of NO and cGMP produced later appear 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. In spite 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, in 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 due to 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 levels, associated with the reduction of NOS-2-derived NO, may turn the former protective event into detrimental consequences for the host, which may explain the protective effect of the late sGC inhibition by MB and ODQ. Late inhibition of sGC improved several markers of tissue damage indicating that indeed this enzyme and its product is 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, co-morbidities 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 anti-bacterial 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, since 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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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Timeline for the protocol designed to study the effects of sGC inhibition in different periods after sepsis onset. Treatment with methylene blue (or PBS; 10 mg/kg and 200 µl, respectively) was performed 8 or 20 hours after surgery. Twelve or sixteen hours after treatment, the rats were instrumented for heart rate measurement. Immediately after, animals were then sacrificed and the blood and tissues were obtained for assays.

Fig. 2. Trace recording of a typical experiment showing the CLP-induced hypotension and hyporesponsiveness to phenylephrine. Following preparation for the mean arterial pressure (MAP) recording, increasing bolus doses (3, 10, and 30 nmol/kg) of phenylephrine were injected i.v. The first trace recording depicts a sham-operated animal 24 h after surgery. The following records show CLP-operated animals at the indicated time points after the surgery.

Fig. 3. Time-course of sepsis-induced hypotension. As 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. Each bar represents the mean data from eight animals and the vertical lines are SEM. Statistical analysis: one-way ANOVA followed by Dunnett post hoc t test. * p < 0.05 compared to the sham-operated animals.

Fig. 4. Effects of the cecal ligation and puncture (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 in “Methods” section. Panel A: RT-PCR products and the ratio of the sGC alpha1 subunit/GAPDH obtained by densitometry. Panel B: RT-PCR products and the sGC beta1 subunit/GAPDH ratio. Panel C: representative immunoelectrophoresis for soluble guanylate cyclase beta1 subunit and densitometry. As 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 hours 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 SEM. Statistical analysis: one-way ANOVA followed by Dunnett post hoc t test. * P < 0.05 compared to the respective sham group.

**Fig. 5.** Effects of the cecal ligation and puncture (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 in “Methods” section. Panel A: RT-PCR products and the ratio of sGC alpha1 subunit/GAPDH obtained by densitometry. Panel B: RT-PCR products and the sGC beta1 subunit/GAPDH ratio. Panel C: a representative immunoelectrophoresis for soluble guanylate cyclase beta1 subunit and densitometry. As 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 hours 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 SEM. Statistical analysis: one-way ANOVA followed by Dunnett post hoc t test. * P < 0.05 compared to the respective sham group.

**Fig. 6.** Basal and sodium nitroprusside-stimulated cyclic GMP accumulation in the lung and aorta tissues. Lung (Panel A) or thoracic aorta (Panel 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 sodium nitroprusside (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 Figure 3. Each bar represents the mean of the data from four to six animals and the vertical lines are the SEM. Statistical analysis: one-way ANOVA followed by Dunnett post hoc t test. * p < 0.05 compared to the sham tissue stimulated with SNP. # p < 0.05 compared to the unstimulated sham tissue (incubated with PBS).

**Fig. 7.** Effect of methylene blue on the CLP-induced hypotension. Open bars represent the sham, black bars the CLP, crosshatched and striped bars are the 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 SEM. Statistical analysis: two-way ANOVA followed by Bonferroni’s post hoc t test. * p < 0.05 compared to the control group (sham) and # p < 0.05 compared to the CLP rats.

**Fig. 8.** Effects of methylene blue on the cecal ligation and puncture (CLP)-induced vascular hyporesponsiveness. Twelve (Panel A), 24 (Panel B) or 48 h (Panel C) after...
the CLP or sham surgery, the animals were prepared for the mean arterial pressure recording. Open bars represent the sham, black bars the CLP, crosshatched and striped bars are the sham and CLP rats, respectively, injected with methylene blue (10 mg/kg) 30 minutes before the vasoconstrictors. Each bar represents the mean of the data from eight animals and the vertical lines are the SEM. Statistical analysis: two-way ANOVA followed by Bonferroni’s post hoc t test. * p < 0.05 compared to the control group (sham) and # p < 0.05 compared to the CLP rats.

**Fig. 9.** Effect of methylene blue or ODQ on survival. CLP animals (squares) were randomly assigned to receive a single injection of methylene blue (10 mg/kg, sc; Panel A) or ODQ (2 mg/kg, sc; Panel B) at 8 hours (triangles) or 20 hours (circles) after the CLP procedure. The sham animals received vehicle 8 or 20 hours after the surgery (diamonds). The mortality rate was recorded over a five-day period. A logrank test was used for the comparison of the survival curves (n = 20 per group). * P < 0.05 compared to the CLP group.
TABLES

TABLE 1

Effect of early treatment with methylene blue (8 hours after the CLP procedure) on several variables.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham/PBS</th>
<th>Sham/MB</th>
<th>CLP/PBS</th>
<th>CLP/MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO response</td>
<td>153.7 ± 11.4</td>
<td>154.2 ± 11.8</td>
<td>108.0 ± 4.6*</td>
<td>73.7 ± 5.2*#</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>11.2 ± 2.9</td>
<td>10.3 ± 3.8</td>
<td>4.2 ± 2.5 *</td>
<td>3.8 ± 1.9*</td>
</tr>
<tr>
<td>MPO activity</td>
<td>19.4 ± 2.4</td>
<td>18.4 ± 2.3</td>
<td>77.1 ± 6.2*</td>
<td>69.1 ± 4.4 *</td>
</tr>
<tr>
<td>Lactate</td>
<td>134.5 ± 39.4</td>
<td>176.7 ± 23.7</td>
<td>323.8 ± 52.1*</td>
<td>295.5 ± 22.2*</td>
</tr>
<tr>
<td>Urea</td>
<td>20.9 ± 2.9</td>
<td>19.1 ± 1.2</td>
<td>60.6 ± 6.0*</td>
<td>52.8 ± 7.1*</td>
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<tr>
<td>Creatinine</td>
<td>0.29 ± 0.06</td>
<td>0.25 ± 0.04</td>
<td>0.56 ± 0.05*</td>
<td>0.51 ± 0.03*</td>
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<tr>
<td>Plasma NOx</td>
<td>21.2 ± 2.0</td>
<td>22.0 ± 8.8</td>
<td>164.5 ± 24.6*</td>
<td>156.1 ± 16.3*</td>
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<tr>
<td>Plasma cGMP</td>
<td>15.1 ± 0.1</td>
<td>11.7 ± 0.5</td>
<td>10.8 ± 2.14</td>
<td>7.1 ± 0.8*</td>
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</tbody>
</table>

Rats were injected with methylene blue (MB, 10 mg/Kg, sc) 8 hours after CLP.

Twenty hours after surgery, the rats were instrumented for heart rate measurement. An intravenous dose of isoprenaline (ISO, 1 nmol/kg) was injected and the increase in heart rate was recorded (bpm). At the end of heart rate measurement, the animals were sacrificed and blood and tissues were obtained. Variables are leukocyte count (cells/mm^3 x 10^3), lung myeloperoxidase (MPO) activity (450 nm optical density/mg protein), lactate (µg/ml), urea (mg/dL), creatinine (mg/dL), NOx (µmol/L), and cGMP (nM). Data are expressed as mean ± SEM of 6-8 animals per group. Statistical analysis was performed using two-way ANOVA followed by Bonferroni’s post hoc t test. * P< 0.05 compared to sham PBS-operated group.
TABLE 2

Effect of late treatment with methylene blue (20 hours after the CLP procedure) on several variables.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham/PBS</th>
<th>Sham/MB</th>
<th>CLP/PBS</th>
<th>CLP/MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>ISO response</td>
<td>148.7 ± 14.4</td>
<td>152.2 ± 12.6</td>
<td>84.5 ± 15.1*</td>
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<tr>
<td></td>
<td>Leukocytes</td>
<td>14.2 ± 3.2</td>
<td>15.3 ± 4.1</td>
<td>6.2 ± 2.7 *</td>
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<tr>
<td></td>
<td>MPO activity</td>
<td>41.4 ± 5.1</td>
<td>39.4 ± 3.4</td>
<td>89.0 ± 2.8 *</td>
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<td></td>
<td>Lactate</td>
<td>268.6 ± 15.7</td>
<td>282.7 ± 31.2</td>
<td>490.1 ± 68.2*</td>
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<tr>
<td></td>
<td>Urea</td>
<td>17.6 ± 1.8</td>
<td>19.3 ± 1.5</td>
<td>43.6 ± 7.0*</td>
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<td></td>
<td>Creatinine</td>
<td>0.18 ± 0.01</td>
<td>0.20 ± 0.01</td>
<td>0.36 ± 0.03*</td>
</tr>
<tr>
<td></td>
<td>Plasma NOx</td>
<td>15.2 ± 4.4</td>
<td>19.3 ± 4.8</td>
<td>138.8 ± 15.6*</td>
</tr>
<tr>
<td></td>
<td>Plasma cGMP</td>
<td>12.0 ± 0.2</td>
<td>12.3 ± 1.0</td>
<td>18.7 ± 0.3*</td>
</tr>
</tbody>
</table>

Rats were injected with PBS or methylene blue (MB, 10 mg/Kg, s.c.) 20 hours after CLP. Thirty-six hours after surgery, the rats were instrumented for heart rate measurement followed by blood and tissue harvesting as described in Table 1. Variables are as in Table 1. Data are expressed as mean ± SEM of 6-8 animals per group. Statistical analysis was performed using two-way ANOVA followed by Bonferroni’s post hoc t test. * P< 0.05 compared to sham-PBS group and # p < 0.05 compared to CLP-PBS group.
Figure 1
Figure 2
Figure 3

Mean arterial pressure (mmHg)

<table>
<thead>
<tr>
<th>Time after CLP (hours)</th>
<th>Sham</th>
<th>12</th>
<th>24</th>
<th>48</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>
Figure 4

A) Time (h)  
\[\text{S} \quad 12 \quad 24 \quad 48\]  
\(\text{sGC}_{\alpha 1}\)  
\(\text{GAPDH}\)

B) Time (h)  
\[\text{S} \quad 12 \quad 24 \quad 48\]  
\(\text{sGC}_{\beta 1}\)  
\(\text{GAPDH}\)

C) Time (h)  
\[\text{S} \quad 12 \quad 24 \quad 36 \quad 48\]  
\(\text{sGC}\)  
\(\text{Actin}\)
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9