Abstract Hypotension in septic shock is a reflection of unregulated nitric oxide (NO) production and vascular smooth muscle guanylyl cyclase activation. We examined the effect of methylene blue on lipopolysaccharide (LPS)-induced shock in anesthetized rabbits. Shock was induced with 150 μg/kg LPS after measurement of mean arterial pressure, platelet cGMP, and total plasma NO (nitrogen monoxide + S-nitrosothiols) content. Measurements were repeated before and after the intravenous administration of 1, 5, and 10 mg/kg methylene blue in response to a 55% reduction in mean arterial pressure. At baseline, mean±SEM arterial pressure was 88±3 mm Hg, which fell to 51±3 mm Hg after LPS (P<.05). Methylene blue at doses of 1, 5, and 10 mg/kg produced a prompt dose-dependent increase in mean arterial pressure to 69±2, 77±3, and 81±2 mm Hg, respectively (P<.05 versus mean arterial pressure after LPS) in association with normalization of plasma total NO content (P<.05); however, methylene blue did not significantly affect intraplatelet cGMP levels. Thus, methylene blue restores normal arterial pressure in rabbits with septic shock. This effect is associated with persistent elevation of intraplatelet cGMP levels and normalization of total plasma NO content. These data are consistent with methylene blue–mediated inhibition of NO synthase and/or degradation of NO in this model and suggest a novel therapeutic approach to the treatment of septic shock. (Circ Res. 1994;74:1121-1125.)

Key Words • nitric oxide • lipopolysaccharide • cGMP • S-nitrosothiols • methylene blue

Severe infection with gram-negative bacteria results in increased production of cytokines through the action of endotoxin (lipopolysaccharide [LPS]), a component of the gram-negative bacterial cell wall. In fact, the administration of LPS to experimental animals and humans results in a septic shock–like syndrome characterized by hypotension, increased vascular permeability, and resistance to vasoconstricting agents. Recent evidence has implicated the overproduction of nitric oxide (NO) as central to the development of septic shock.

NO is synthesized enzymatically from L-arginine, O2, and NADPH by a family of flavin adenine dinucleotide–containing and flavin mononucleotide–containing enzymes, the nitric oxide synthases (NOSs). NOS has been identified in a number of biologic tissues and exists in two main functional forms: (1) Constitutive calcium/calmodulin-dependent forms of the enzyme are found in endothelial cells and neuronal cells. (2) Inducible calcium-independent forms are found in a variety of activated cell types, including macrophages, vascular smooth muscle cells, endothelial cells, and hepatocytes. The induction of NOS has been demonstrated in response to a number of stimuli including LPS, interleukin-1, and tumor necrosis factor. The inducible form of NOS, once synthesized, produces NO in an unregulated fashion, and its production from vascular smooth muscle cells results in the hemodynamic manifestations of septic shock.

Several strategies have been applied to the treatment of septic shock that focus on ameliorating the overproduction of NO. Guanidino-substituted L-arginine analogues are inhibitors of inducible NOS and promptly reverse the hypotension associated with LPS and cytokine-induced septic shock. The inducible form(s) of NO synthase requires tetrahydrobiopterin as a cofactor, and inhibition of tetrahydrobiopterin synthesis abolishes LPS-induced NO synthesis in rat aortic smooth muscle cells. Quiescent vascular smooth muscle cells demonstrate little NO activity. The overproduction of NO in LPS-induced shock is thus dependent on new protein synthesis. The induction of NO by these cells can be inhibited by pretreatment with dexamethasone or cycloheximide.

NO-mediated vasodilation and enhanced vascular permeability are dependent on the activation of soluble guanylyl cyclase and the intracellular accumulation of cGMP. The vital stain, methylene blue (MB), has generally been described as an inhibitor of soluble guanylyl cyclase both in vitro and in vivo, and more recent evidence suggests that MB also possesses direct NO inhibitory activity. Notwithstanding these well-recognized properties, the utility of MB in the treatment of LPS-induced shock has not been evaluated. In the present study, we sought to examine the effect of MB on LPS-induced septic shock, with particular attention to the relative contribution of MB in inhibiting guanylyl cyclase or NO.

Materials and Methods

Xylazine was purchased from Mobay Corp., and ketamine hydrochloride was obtained from Aveco Co., Inc. Sodium pentobarbital was purchased from Anthony Products Co.
Citrate-phosphate-dextrose (CPD) solution contained (mmol/L) citric acid 15.6, sodium citrate 90, NaH₂PO₄·H₂O 15, and dextrose 142, pH 7.35. LPS (Escherichia coli strain 0111:B₄), MB, and all other reagent grade chemicals were purchased from Sigma Chemical Co.

Animal Preparation

Twenty-three New Zealand White rabbits (2.8 to 3.8 kg) were sedated with ketamine hydrochloride (50 mg/kg IM) and anesthetized with sodium pentobarbital (30 mg/kg IV). Anesthesia was maintained by supplemental intravenous pentobarbital as needed. A tracheostomy was performed, and the trachea was intubated with a specifically designed endotracheal tube. Body temperature was maintained with a homeothermic blanket system (Harvard Apparatus Ltd), and the femoral artery and vein were cannulated with polyethylene tubing (PE-90, Clay Adams). Arterial pressure was monitored with a pressure transducer (Cobe Inc) and recorded on a Gould physiograph (model RS-3800, Gould Electronics Inc). Rabbits were monitored for 20 minutes after surgery before beginning experimental protocols. All procedures were approved by the Brockton/West Roxbury VA Institutional Animal Care and Use Committee.

Experimental Protocol

The effects of LPS on arterial pressure, platelet cGMP content, and total plasma NO content (free NO+S-nitrosothiol) were examined in six rabbits. Animals were observed for 20 minutes to establish a stable hemodynamic state, and blood (4 mL) was obtained for total plasma NO content and platelet cGMP levels. Animals were administered LPS (150 μg/kg IV for 1 minute); LPS administration was followed by blood sampling and arterial pressure recording at hourly intervals for a 6-hour period. Each blood sample was replaced with an equal volume of 0.9% saline to maintain volume status.

The effect of MB on LPS-induced septic shock was examined in 12 rabbits. After the establishment of a stable hemodynamic state, blood (4 mL) was obtained for determination of platelet cGMP content and total plasma NO content. LPS (150 μg/kg) was administered as an intravenous bolus for 1 minute, and animals were observed for 3 hours or until mean arterial pressure fell to 55% of the baseline value. On achieving either end point, MB was serially administered intravenously at 20-minute intervals at doses of 1, 5, and 10 mg/kg. Before the administration of MB and 20 minutes after each dose, blood (4 mL) was obtained for platelet cGMP content and determination of total plasma NO content. Intravascular volume was maintained by replacing each blood sample with an equal volume of normal saline. In a separate set of seven rabbits, we evaluated the effect of MB on mean arterial pressure, total plasma NO content, and platelet cGMP levels. These seven animals were treated as described above with the exception that saline placebo replaced LPS.

Determination of Platelet cGMP Content and Total Plasma NO

Plasma was collected with 10% (vol/vol) CPD, and platelet-rich plasma (PRP) was prepared by centrifugation at 150g for 11 minutes (25°C). Platelet counts were determined with a Coulter Counter (model ZM, Coulter Electronics Co), and 0.5 mL of PRP was precipitated with an equal volume of ice-cold 10% trichloroacetic acid and stored at −70°C for subsequent analysis of platelet cGMP content. Samples for platelet cGMP content were analyzed by a commercially available immunoassay (Cayman Chemical Co). Platelet-poor plasma was prepared by centrifugation of whole blood (with 10% [vol/vol] CPD) at 800g for 11 minutes (25°C), diluted fivefold, and analyzed immediately for total plasma NO content by a photoysis chemiluminescence detection system as described previously. The details of this system have been described, and this method allows for the detection of free NO as well as protein and low-molecular-weight S-nitrosothiols in plasma. The data were analyzed using repeated-measures ANOVA or Friedman repeated measures on ranks with post hoc Dunn’s or Dunnett’s multiple range tests where appropriate. The relation between total plasma NO, arterial pressure, and platelet cGMP was evaluated using linear regression (Spearman). Statistical significance was accepted if the null hypothesis was rejected at the .05 level.

Results

Arterial Pressure, Platelet cGMP, and Plasma NO With LPS Administration

We first investigated the effect of LPS on arterial pressure, platelet cGMP, and total plasma NO content in normal rabbits, and these results are presented in Fig 1. Before the administration of LPS, mean arterial pressure was 80±4 mm Hg. After 150 μg/kg IV LPS, mean arterial pressure decreased significantly (𝑃<.05 versus baseline) in a time-dependent manner to a minimum of 42±5 mm Hg 5 hours after the administration of LPS (Fig 1A). The reduction in mean arterial pressure was accompanied by an increase in platelet cGMP levels from 1.89±0.87 pmol/10⁸ platelets at baseline to 6.37±1.50 pmol/10⁸ platelets 5 hours after the administration of LPS (Fig 1B, 𝑃<.05). Likewise, total plasma NO content increased from a baseline value of 146±23 to 281±37 nmol/L 6 hours after LPS administration (Fig 1C, 𝑃<.05). In this same set of experiments, we examined the relation between total plasma NO content and both mean arterial pressure and platelet cGMP levels, and these results are presented in Fig 2. Arterial pressure correlated significantly with total...
plasma NO content ($r = -0.41, P = 0.008$), and similarly, platelet cGMP content was related to total plasma NO content ($r = 0.64, P = 0.001$).

**MB and LPS-Induced Shock**

The effect of MB on mean arterial pressure in LPS-induced shock was next examined. A representative tracing of the mean arterial pressure response to MB in LPS-induced shock is shown in Fig 3. Administration of LPS was associated with a fall in mean arterial pressure that was attenuated in a dose-dependent fashion by the administration of MB. The overall responses to LPS and MB are presented in Fig 4. The baseline mean arterial pressure fell significantly from $88 \pm 3$ to $51 \pm 3$ mm Hg ($P < 0.05$) after LPS administration (Fig 4A). Subsequent administration of normal saline had no significant effect on mean arterial pressure (data not shown); however, intravenous MB at serial doses of 1, 5, and 10 mg/kg produced a dose-dependent increase in mean arterial pressure to $69 \pm 2$, $77 \pm 3$, and $81 \pm 2$ mm Hg, respectively (all $P < 0.05$ versus minimum mean pressure, Fig 4A). This increase in mean arterial pressure was stable for the 20 minutes between each dose of MB. After the cumulative administration of 16 mg/kg MB, mean arterial pressure was not significantly different from the baseline state ($81 \pm 2$ versus $88 \pm 3$ mm Hg, $P = NS$). As shown in Fig 4A, administration of MB in the absence of LPS-induced shock had no significant effect on mean arterial pressure over an equivalent time course.

We evaluated intraplatelet cGMP levels as an index of LPS-induced NO production. Intraplatelet levels of cGMP in response to LPS-induced septic shock and MB administration are shown in Fig 4B. Before the administration of LPS, intraplatelet cGMP content was $2.75 \pm 0.92$ pmol/10^8 platelets. After the administration of LPS, intraplatelet cGMP increased 3.5-fold to $9.09 \pm 1.57$ pmol/10^8 platelets ($P < 0.05$ versus the control value) at the nadir of mean arterial pressure. The administration of intravenous MB at doses of 1, 5, and 10 mg/kg had no significant effect on the platelet content of cGMP despite the profound effect of MB on mean arterial pressure. As shown in Fig 4B, in the absence of LPS-induced shock, MB had little effect on the increase in cGMP content.

**Fig 2.** Scatterplots showing linear regression relating total plasma nitric oxide (NO) content to mean arterial pressure (A) and platelet cGMP (B) in anesthetized rabbits exposed to 150 μg/kg IV lipopolysaccharide. Animals were studied as described in Fig 1, and measurements of total plasma NO, mean arterial pressure, and cGMP were obtained at time 0 and hourly thereafter. Data represent 42 observations obtained from six animals.

**Fig 4.** Graphs showing mean arterial pressure (A), platelet cGMP content (B), and total plasma nitric oxide (NO) content (C) in response to intravenous LPS (150 μg/kg) or placebo and observed for 3 hours or until mean arterial pressure fell to 55% of the control (CTL) value. After the observation period, intravenous methylene blue was administered at 20-minute intervals at doses of 1, 5, and 10 mg/kg. Plasma samples were obtained before LPS or placebo administration, before methylene blue administration, and 20 minutes after each dose of methylene blue. Intraplatelet cGMP and total plasma NO content were determined from platelet-rich and platelet-poor plasma, respectively, as described in “Materials and Methods.” Data are presented as mean±SEM (n=12). Pits indicates platelets. *$P < 0.05$ vs baseline; $^{*}P < 0.05$ vs LPS by Dunn’s or Dunnett’s multiple range test.

**Fig 3.** Representative tracings of mean arterial pressure (MAP) in response to lipopolysaccharide (LPS)-induced shock and methylene blue administration in an anesthetized rabbit. LPS administration was associated with a 30 mm Hg fall in MAP. Intravenous methylene blue, given at 20-minute intervals at doses of 1, 5, and 10 mg/kg, produced a dose-dependent increase in MAP. Methylene blue-mediated increases in MAP were evident immediately after dye administration (indicated by arrow) and stable for the 20-minute observation period between doses. CTL indicates the control condition; IVB, intravenous bolus.
platelet cGMP content, indicating that MB does not independently activate platelet guanylyl cyclase at the doses used in the present in vivo study.

**Plasma NO and S-Nitrosothiol Content**

We investigated the effect of MB on total plasma NO content in LPS-induced septic shock. Changes in plasma NO content in response to LPS and MB administration are shown in Fig 4C. At baseline, total plasma NO content was 137±30 nmol/L. After the administration of LPS, total plasma NO content increased 2.5-fold to 324±74 nmol/L (P<.05 versus baseline), corresponding to the nadir of blood pressure. Total plasma NO content returned to baseline values, namely, 180±25, 108±24, and 126±42 nmol/L after the administration of 1, 5, and 10 mg/kg MB, respectively (all P=NS versus baseline value). In the absence of LPS (saline control), total plasma NO content did not change significantly (136±12 versus 137±37 nmol/L), whereas administration of 1, 5, and 10 mg/kg MB produced a modest reduction in total plasma NO content to 120±21, 122±32, and 118±18 nmol/L, respectively (all P=NS versus baseline value).

**Discussion**

The results of the present study indicate that MB reverses hypotension in rabbits with LPS-induced shock. Reversal of LPS-induced hypotension on administration of MB was prompt and associated with normalization of total plasma NO content. In contrast, intravenous MB did not significantly alter platelet levels of cGMP despite effective normalization of arterial pressure and total plasma NO content.

In the present study, the administration of LPS (150 μg/kg) as an intravenous bolus resulted in a sustained reduction of arterial pressure that evolved over 2 hours and remained stable for 6 hours. The temporal development of hypotension in our rabbit model of LPS-induced septic shock is consistent with previous observations in dogs,10 rats,27 and rabbits.19 Moreover, total plasma NO content correlated significantly with mean arterial pressure and platelet cGMP in response to parenteral LPS (Fig 2), providing further confirmation that NO is central to the pathogenesis of septic shock.

In animals with LPS-induced shock, we observed a prompt, dose-dependent increase in mean arterial pressure after the administration of MB. These results are consistent with the observations of Paya et al.28 These investigators administered 3 mg/kg IV MB to endotoxemic rats and observed prompt reversal of hypotension and restoration of vascular responses to norepinephrine.28 This effect of MB on arterial pressure and vascular responses to norepinephrine persisted for at least 30 minutes after MB administration.

In the present study, the effects of MB on arterial pressure were associated with normalization of total plasma NO content. This finding was surprising in that MB has classically been viewed as a “selective” guanylyl cyclase inhibitor.24,29,30 MB is generally believed to inhibit soluble guanylyl cyclase through oxidation of the ferrous heme group integral to the apoenzyme and/or critical thiols essential for activity.31,32 Thus, one might have expected total plasma NO content to remain elevated if MB were to act through the inhibition of soluble guanylyl cyclase.

There is developing appreciation that MB may inhibit endothelium-derived relaxing factor (EDRF)-mediated phenomena through mechanisms independent of guanylyl cyclase inhibition. Under physiological conditions, the reduced form of MB is readily oxidized by molecular oxygen leading to the generation of superoxide anion.33 Superoxide has been shown to inactivate EDRF,34 and previous studies in cultured smooth muscle cells35 and in cerebral36 and skeletal muscle37 arterioles have implicated superoxide anion in MB-mediated inhibition of endothelium-dependent vasodilation. Therefore, one must consider that our results demonstrating the reversal of LPS-induced hypotension may be a consequence of MB-mediated superoxide production. MB also affects other EDRFs that may contribute to reversing hypotension. Studies in porcine aortic endothelial cells38 and canine coronary arteries39 have also demonstrated MB-mediated inhibition of prostacyclin production; these findings may also have contributed, in part, to the results reported here.

Hypotension in LPS-induced shock results from the induction of NOS14 and unregulated NO production17 by vascular smooth muscle cells.15 Recent studies suggest that NOS contains bound or heme iron,40 and MB is known to affect iron-containing enzymes.21,32 Recently, Mayer et al.40 have demonstrated inhibition of brain cerebellar NOS by MB with an apparent IC50 of 9.2 μmol/L. Taken together, these observations suggest that our results demonstrating reversal of LPS-induced hypotension with MB in conjunction with reduced total plasma NO content may be a consequence of MB-mediated inhibition of both NOS and bioactive NO equivalents.

In the present study, reversal of LPS-induced hypotension was not associated with a reduction in platelet content of cGMP. Possible explanations for this observation include direct activation of platelet guanylyl cyclase in response to the doses of MB used or a dependence in biologic half-lives between platelet cGMP and free NO and/or S-nitrosothiols in plasma. We consider the former possibility unlikely, since animals given LPS placebo (saline) failed to demonstrate elevations in platelet cGMP content in response to 1, 5, and 10 mg/kg MB (Fig 4B). With regard to the latter possibility, in vitro studies using short-acting NO donors, such as sodium nitrite, nitroprusside, and nitroglycerin, have demonstrated persistent elevations of tissue42 and platelet41 cGMP levels up to 5 minutes after exposure to the NO donor. Therefore, it is possible that our results reflect only the difference in the biologic half-life between plasma EDRF and platelet cGMP.

In summary, the results presented here indicate that the vital stain MB restores normal arterial pressure in a rabbit model of LPS-induced septic shock. The restoration of normal arterial pressure was associated with a normalization of total plasma NO content and persistent elevation of platelet cGMP content. These data are consistent with MB-mediated inhibition of NOS and/or inactivation of NO and suggest a novel therapeutic approach to the treatment of septic shock.

**Acknowledgments**

This study was supported in part by National Institutes of Health (NIH) grants HL-40411, HL-43344, HL-48743, and HL-07604 and a Merit Review Award from the Veterans Administration. Dr Keaney is the recipient of an NIH National Research Service Award (F32 HL-08635). Dr Stamler is a Pew
Scholar in the Biomedical Sciences and the recipient of an NIH Clinical Investigator Award (K08 HL-02582), and Dr Locolzo is the recipient of an NIH Research Career Development Award (K04 HL-02773).

References
Methylene blue reverses endotoxin-induced hypotension.

J F Keaney, Jr, J C Puyana, S Francis, J F Loscalzo, J S Stamler and J Loscalzo

Circ Res. 1994;74:1121-1125
doi: 10.1161/01.RES.74.6.1121

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/74/6/1121