Desensitization of Soluble Guanylate Cyclase in Renal Cortex during Endotoxemia in Mice

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Abstract. Acute endotoxemic renal failure involves renal vasoconstriction, which presumably occurs despite increased nitric oxide (NO) generation by inducible NO synthase in the kidney. The present study examined the hypothesis that the renal vasoconstriction during endotoxemia occurs in part because of desensitization of soluble guanylate cyclase (sGC). Endotoxic shock was induced in male B6/129F2/J mice by an intraperitoneal injection of Escherichia coli lipopolysaccharide. The endotoxemia resulted in shock and renal failure as evidenced by a decrease in mean arterial pressure and an increase in serum creatinine and urea nitrogen. Serum NO evidenced by a decrease in mean arterial pressure and an increase in serum creatinine and urea nitrogen. Serum NO increased in a time-dependent manner, reaching the highest increase in serum creatinine and urea nitrogen. Serum NO increased in a time-dependent manner, reaching the highest increase at 24 h, in parallel with induction of inducible NO synthase protein in the renal cortex. In renal cortical slices obtained from endotoxemic mice, cyclic guanosine monophosphate (cGMP) increased significantly at 6 h and 15 h as compared with control but normalized at 24 h after injection of lipopolysaccharide. Incubation of renal cortical slices with 2 mM sodium nitroprusside resulted in a similar accumulation of cGMP in slices taken from control and endotoxemic mice at 6 h and 15 h. However, in slices from 24-h endotoxemic mice, accumulation of cGMP in response to sodium nitroprusside was significantly lower. This lower stimulability of sGC was not paralleled by a decrease in its abundance in renal cortex on immunoblot. Taken together, these results demonstrate a desensitization of sGC in renal cortex during endotoxemia, which may contribute to the associated renal vasoconstriction.

Sepsis is frequently complicated by the development of acute renal failure (1). In experimental endotoxemia, acute renal failure is observed in association with renal vasoconstriction (2,3). This renal vasoconstriction with sepsis occurs in the setting of marked systemic vasodilatation, which is caused in part by the vascular expression of inducible nitric oxide synthase (iNOS) (4,5). Expression of iNOS has also been demonstrated during endotoxemia in the kidney (6). NO induces vasodilatation by stimulation of soluble guanylate cyclase (sGC), which catalyzes the synthesis of cyclic guanosine monophosphate (cGMP) (7). sGC is present in the cytosol as a heterodimer, consisting of an α and a β subunit (8). Besides iNOS, endotoxin also Stimulates several vasocostrictive systems, such as renin-angiotensin (9,10) and endothelin (11,12). Hence, the vasoactive effect of endotoxin results from the balance between vasodilatory and vasoconstrictive mediators. It has recently been shown that sGC undergoes in vitro desensitization in response to prolonged stimulation with NO donors (13) or lipopolysaccharide (LPS) (14) in cultured cells. If this desensitization occurs in the renal vasculature during endotoxemia in vivo, an unopposed vasoconstriction would contribute to the acute renal failure. The present study was therefore designed to examine whether desensitization of sGC occurs in the renal cortex of a mouse model of endotoxic shock and acute renal failure.

Materials and Methods
Experiments were carried out on male B6/129F2/J mice (Jackson Lab., Bar Harbor, MA) weighing 20 to 30 g in accord with the NIH Guide for the Care and Use of Laboratory Animals.

Surgery and Mean Arterial Pressure Measurement
Mice were anesthetized with an intraperitoneal injection of 2.22 tribromoethanol (Aldrich, Milwaukee, WI). During surgery, animals were kept on a heating pad. The right carotid artery was catheterized using a PE10 catheter connected to a PE50 catheter. Intracarotid mean arterial pressure (MAP) was measured using Grass polygraph in conscious, unrestrained animals 24 h after injection of LPS. Mice were injected with LPS (30 mg/kg intraperitoneally) 1 h after surgery.

cGMP Studies
Mice were injected intraperitoneally with 40 mg/kg LPS and were killed at 6 h, 15 h, or 24 h after injection of LPS. Renal cortical slices were obtained on ice using a Stadie-Riggs microtome (Thomas Scientific, Swedesboro, NJ). For ex vivo cGMP measurement, slices were placed in 0.1 N HCl and were frozen in liquid nitrogen. For in vitro experiments, slices were placed into Erlenmeyer flasks in buffer containing 110 mM NaCl, 18 mM NaHCO3, 5 mM KCl, 1 mM CaCl2,
2 mM Na$_2$HPO$_4$, 1 mM MgSO$_4$, 5 mM glucose, 2 mM glutamine, 10 mM Na butyrate, 4 mM Na lactate, and HEPES (pH 7.4 at 37°C and saturated with 95% O$_2$/5% CO$_2$) and 5 mM isobutylmethylxanthine (IBMX), on ice. The flask were then transferred to a water bath at 37°C and preincubated for 5 min, after which an additional 15-min incubation was carried out in the presence or absence of 2 mM sodium nitroprusside (SNP). Incubation was terminated by adding to samples an excessive amount of ice-cold phosphate-buffered saline. The slices were subsequently homogenized using glass-Teflon homogenizer (20 strokes/2000 rpm) and spun at 4500 rpm/15 min. Protein concentration of the supernatant was determined using the Bradford method with bovine serum albumin as standard. Further processing of the samples for determination of cGMP was carried out according to the manufacturer’s instructions using a commercially available RIA kit (Amersham, Arlington Heights, IL).

**Serum Creatinine, Blood Urea Nitrogen, and NO Measurement**

Serum creatinine and blood urea nitrogen (BUN) were determined using a creatinine analyzer and BUN analyzer, respectively (Beckman Instruments, Inc., Fullerton, CA). Serum NO was measured using an NO chemiluminescence analyzer (Sievers Instruments, Inc., Boulder, CO).

**Western Blot for iNOS and sGC**

Renal cortex was homogenized using glass-Teflon homogenizer (20 strokes, 2000 rpm) in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetate, 1% Triton-100 [pH 7.2]) plus protease inhibitors (20 µM leupeptin, 20 µM pepstatin A, and 200 µM phenylmethylsulfonylfluoride). The homogenate was centrifuged at 4500 rpm for 15 min. Supernatant protein was measured by the Bradford method using bovine serum albumin as a standard. Supernatant samples were mixed with sample buffer containing 50 mM Tris, 0.5% glycerol, 0.01% bromophenol blue, and 0.75% sodium dodecyl sulfate (pH 6.8). Samples containing 200 µg (iNOS) or 50 µg (sGC) of protein were fractionated by Tris/glycine/sodium dodecyl sulfate 7.5% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Bedford, MA). Nonspecific binding was reduced by the incubation of membranes for 1 h at room temperature with 5% low-fat milk in 50 mM Tris, 150 mM NaCl, 0.1% Tween20 (pH 7.5) (TTBS). Membranes were subsequently incubated for 1 h with a polyclonal anti-iNOS antibody (a gift from Dr. Bruce Kone, University of Texas-Houston Trauma Research Center) or polyclonal anti-sGC antibody (Calbiochem, San Diego, CA). An additional 1-h incubation was performed with a secondary antibody, anti-rabbit IgG coupled to horseradish peroxidase (Amersham), at 1:1000 dilution in TTBS. Detection of the protein bands was carried out using enhanced chemiluminescence (Amersham). Positive control for sGC purified from bovine lung was obtained from Alexis Biochemicals (San Diego, CA).

**Statistical Analyses**

Values are expressed as mean ± SEM. Multiple group comparisons were done using the ANOVA, with a post hoc Newman-Keuls test. A P value of less than 0.05 was considered significant.

**Results**

**Endotoxic Shock and Renal Failure**

Administration of endotoxin resulted in a decrease of MAP from 106.7 ± 4.9 to 61.7 ± 8.8 mmHg (P < 0.05) at 24 h.

![Figure 1](image-url). (A) Endotoxic shock in mice 24 h after administration of lipopolysaccharide (LPS). Mean arterial pressure (MAP) was measured in conscious mice. Each data point represents mean ± SEM from four animals. * P < 0.05 versus control. (B, C) Acute renal failure 24 h after administration of LPS. Each data point represents mean ± SEM from 15 animals. *, P < 0.05; **, P < 0.01 versus control.
The 24-h mortality in response to LPS was 26.5%. Development of acute renal failure was evidenced by an elevation of serum creatinine and BUN, measured 24 h after administration of LPS (Figure 1, B and C).

Induction of iNOS and Changes in cGMP in Renal Cortex during Endotoxemia

To document induction of iNOS, serum NO and iNOS protein expression in renal cortex were determined in control mice and endotoxemic mice at 6 h, 15 h, and 24 h after injection of LPS. Serum NO increased in a time-dependent manner, reaching maximum at 24 h after LPS (Figure 2A). A similar time course of iNOS protein expression was observed on Western blot of renal cortex obtained from endotoxemic mice (Figure 2B). To determine whether changes in cGMP parallel those of NO and iNOS protein expression, cGMP was measured in renal cortical slices obtained from control or endotoxemic mice after different periods of endotoxemia. Renal cortical cGMP was increased at 6 h and 15 h, and return to control levels occurred at 24 h of endotoxemia (Figure 3). Stimulation of sGC

To examine the cause of the decreased cGMP concentration at 24 h, we determined stimulation of sGC by SNP in vitro in renal cortical slices obtained from control and endotoxemic mice. There was no difference in the increase in cGMP content after incubation with SNP of renal cortical slices obtained from control mice and mice after 6 h and 15 h of endotoxemia. However, the response of cGMP to SNP in slices from mice after 24 h of endotoxemia was significantly decreased, as compared with control and 6 h and 15 h (Figure 4A). In the absence of SNP, cGMP in renal cortical slices followed a similar pattern as ex vivo (Figure 3); specifically, an increase in cGMP occurred at 6 h and 15 h, and return to control levels occurred at 24 h of endotoxemia (Figure 4B). Expression of sGC, determined by the Western blot of renal cortex, was not significantly different at 24 h as compared with 12 h (Figure 5).

Discussion

Acute renal failure occurs in almost 50% of patients with septic shock (1). The main pathophysiological mechanism is intense renal vasoconstriction (2,3), which occurs in the presence of systemic arterial vasodilation. The NO-cGMP system is considered to be the main vasodilatory mechanism in experimental endotoxemia or sepsis, mainly because of induction of iNOS (4,5). In parallel to the induction of iNOS, there is activation of several vasoconstrictor systems, which counteract the vasodilatory and hypotensive effects of NO. Among the vasoconstrictors, angiotensin II and endothelin play a prominent role (9–12). It has been shown, for example, that angiotensin-converting enzyme inhibitors (15) or nonselective endothelin receptor antagonists decrease systemic vascular resistance and/or increase hypotension in endotoxemic animals (15–18). There is also evidence for the involvement of the vasoconstrictors angiotensin II (15) and endothelin (15,17) in renal vascular responses to endotoxemia. That renal vasoconstriction occurs with sepsis despite increased iNOS in the kidney (6) led to our hypothesis that desensitization of sGC may occur during the course of endotoxemia.

Mice strains may differ with respect to their sensitivity to

Figure 2. Induction of inducible nitric oxide synthase (iNOS) during endotoxemia. (A) Time-dependent increase in serum NO during endotoxemia. Each data point represents mean ± SEM from four to six animals. *, P < 0.05; **, P < 0.01 versus control. (B) Expression of iNOS protein in renal cortex during endotoxemia. Renal cortex (200 μg protein/lane) was blotted with a polyclonal anti-iNOS antibody, as detailed in the Materials and Methods section. +, positive control.

Figure 3. Cyclic guanosine monophosphate (cGMP) concentration in renal cortex during endotoxemia. Renal cortical slices were obtained from mice after indicated periods of endotoxemia. cGMP was determined by RIA, as described in the Materials and Methods section. Each data point represents mean ± SEM from 6 to 10 animals. *, P < 0.05 versus control.
LPS, and a relatively high dose of LPS was needed to induce shock and renal failure in B6/129 mice in the present study. In these mice, a time-dependent increase in serum NO, with a concomitant increase in iNOS protein expression in renal cortex, was demonstrated. However, after an increase in renal cortical cGMP at 6 h and 15 h after LPS, cGMP decreased to control level at 24 h. This observation could be due to desensitization and/or downregulation of sGC or to increased activity of phosphodiesterases. Subsequent in vitro experiments using buffer containing IBMX, a phosphodiesterase inhibitor, excluded an increased degradation of cGMP at 24 h of endotoxemia. Rather, stimulation of sGC was diminished, because the NO donor, SNP, evoked a much smaller increase in cGMP in slices obtained at 24 h, as compared with slices from earlier time points. It has been shown in some in vitro studies that desensitization of sGC by pretreatment of cultured cells with NO donors or LPS is mediated at least partly by a decreased abundance in sGC α or β subunit (13,14). However, we did not demonstrate decreased protein expression of sGC in our mouse model of sepsis using an antibody generated against both subunits of sGC. In the case of particulate GC, the role of dephosphorylation in its desensitization has been demonstrated (19), and this possibility needs to be studied with sGC.

In summary, desensitization of sGC may have important functional consequences for the kidney in endotoxemia, because the resultant diminished vasodilatory influence of NO would allow an unopposed action of renal vasoconstrictors, such as angiotensin and endothelin. This sGC desensitization could then mimic the results observed with nonselective inhibition of NO synthase, which is known to increase renal vasoconstriction and worsen renal failure in rat endotoxemia (20). Thus, the present results demonstrate desensitization of sGC in renal cortex of endotoxemic mice, which may contribute to the maintenance of vasoconstrictor-mediated acute renal failure during endotoxemia.

References
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