

Endotoxemic renal failure in mice: Role of tumor necrosis factor independent of inducible nitric oxide synthase

MLADEN KNOTEK, BORIS ROGACHEV, WEI WANG, TEVFIK ECDER, VYACHESLAV MELNIKOV, PATRICIA E. GENGARO, MATTHEW ESSON, CHARLES L. EDELSTEIN, CHARLES A. DINARELLO, and ROBERT W. SCHRIER

Department of Medicine, Divisions of Renal Diseases and Hypertension and Infectious Diseases, University of Colorado Health Sciences Center, Denver, Colorado, USA

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Background. Renal failure is a frequent complication of sepsis with a high mortality. Tumor necrosis factor (TNF) has been suggested to be a factor in the acute renal failure in sepsis or endotoxemia. Recent studies also suggest involvement of nitric oxide (NO), generated by inducible NO synthase (iNOS), in the pathogenesis of endotoxin-induced renal failure. The present study tested the hypothesis that the role of TNF in endotoxemic renal failure is mediated by iNOS-derived NO.

Methods. Renal function was evaluated in endotoxemic [*Escherichia coli* lipopolysaccharide (LPS), 5 mg/kg IP] wild-type and iNOS knockout mice. The effect of TNF neutralization on renal function during endotoxemia in mice was assessed by a TNF-soluble receptor (TNFRp55).

Results. An injection of LPS to wild-type mice resulted in a 70% decrease in glomerular filtration rate (GFR) and in a 40% reduction in renal plasma flow (RPF) 16 hours after the injection. The results occurred independent of hypotension, morphological changes, apoptosis, and leukocyte accumulation. In mice pretreated with TNFRp55, only a 30% decrease in GFR without a significant change in RPF in response to LPS, as compared with vehicle-treated mice, was observed. Also, the serum NO concentration was significantly lower in endotoxemic wild-type mice pretreated with TNFRp55, as compared with untreated endotoxemic wild-type mice (260 ± 52 vs. 673 ± 112 $\mu\text{mol/L}$, $P < 0.01$). In LPS-injected iNOS knockout mice and wild-type mice treated with a selective iNOS inhibitor, 1400W, the development of renal failure was similar to that in wild-type mice. As in wild-type mice, TNFRp55 significantly attenuated the decrease in GFR (a 33% decline, as compared with 75% without TNFRp55) without a significant change in RPF in iNOS knockout mice given LPS.

Conclusions. These results demonstrate a role of TNF in the early renal dysfunction (16 h) in a septic mouse model independent of iNOS, hypotension, apoptosis, leukocyte accumulation,

and morphological alterations, thus suggesting renal hypoperfusion secondary to an imbalance between, as yet to be defined, renal vasoconstrictors and vasodilators.

The course of sepsis is frequently complicated by the development of septic shock and multiorgan failure, with a 50% mortality [1]. In a recent prospective study, acute renal failure was present in approximately 20% of patients with severe sepsis and in 50% of patients with septic shock [1]. Many of the pathophysiological abnormalities of sepsis can be reproduced in experimental animals by the administration of endotoxin, a lipopolysaccharide (LPS) constituent of gram-negative bacteria wall [2]. In experimental endotoxemia, renal failure involves excessive intrarenal vasoconstriction [3; reviewed in 4]. Endotoxin induces several proinflammatory cytokines, among which tumor necrosis factor (TNF) is thought to be of major importance [5]. Accordingly, passive immunization by an anti-TNF- α antibody protected against septic shock in baboons [6] and mice [7]. The pathogenic role of TNF in endotoxemic renal failure has been suggested. For example, an intravenous infusion of TNF- α in rabbits or perfusion of isolated rat kidney with TNF- α led to a decrease in glomerular filtration rate (GFR) [8, 9].

Several mechanisms have been suggested for the effects of LPS/TNF on organ function during endotoxemia. These include reduced organ perfusion due to systemic hypotension, apoptosis, leukocyte accumulation and inflammation, and fibrin deposition [10–16]. Nitric oxide (NO) has also attracted attention as a mediator of hypotension and organ failure in experimental endotoxemia and sepsis in patients and laboratory animals. NO is synthesized by the three NO synthase (NOS) isoenzymes: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) [17]. Constitutive generation of NO by eNOS participates in the maintenance of basal vascular tone.

Key words: acute renal failure, sepsis, renal vasoconstriction, lipopolysaccharide, hypertension, blood pressure.

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On the other hand, iNOS is induced in various cell types (for example, vascular smooth muscle cells and macrophages) in response to stimulation by LPS, cytokines [TNF- α , interferon- γ (IFN- γ), interleukin-1 β (IL-1 β)] [17], or hypoxia [18], whereby it mediates NO overproduction. Although NO has been proposed as a mediator of renal dysfunction in endotoxemia, the use of nonselective NOS inhibition leads to further deterioration of renal function [19]. Selective inhibition of iNOS, which may be an alternative to avoid endothelial dysfunction, resulted in conflicting data with either improvement [19, 20] or lack of an effect on LPS-induced renal failure [21, 22].

A majority of the studies described previously in this article were performed using models of established septic shock with severe systemic hypotension. Moreover, the mechanisms proposed were reported to occur at later time points (greater than 24 hours) in the course of endotoxemia. The purpose of the present study, therefore, was to examine the potential mechanisms involved in endotoxic renal failure at earlier time points (less than 24 hours) using a normotensive model of endotoxemia. The role of TNF was tested by measuring renal function in the presence of TNF neutralization. In order to examine whether the effect of TNF on renal function is mediated by iNOS induction, renal function was also assessed in endotoxemic iNOS knockout mice. Furthermore, based on previous reports, we examined renal tissue for evidence of early apoptosis, leukocyte accumulation, or morphological changes that could contribute to the early reduction in GFR in endotoxemia.

METHODS

Mice and reagents

Experiments were carried out using male B6,129F2/J and iNOS knockout B6129 mice weighing 20 to 30 g, purchased from Jackson Laboratories, (Bar Harbor, ME, USA). Studies of renal function were conducted in mice injected intraperitoneally with 5 mg/kg of *Escherichia coli* LPS (Difco, Detroit, MI, USA). A saline solution of LPS (0.75 mg/mL) was freshly prepared before each experiment. The soluble TNF receptor TNFRp55 that was used in this study was kindly supplied by Dr. Carl K. Edwards (Amgen, Inc., Thousand Oaks, CA, USA). TNFRp55 is a construct of the extracellular four domains of the TNFRp55 in which two chains of the extracellular domains are linked by a polyethylene bridge [23]. The soluble receptor was kept at -70°C until thawed once, diluted in saline, and used in experimental studies as previously described [24]. TNFRp55 (10 mg/kg) was administered intraperitoneally one hour before an intraperitoneal injection of LPS. 1400W, a selective iNOS inhibitor (purchased from Alexis Corp., San Diego, CA, USA) [25] was freshly dissolved in saline (3 mg/mL)

before each experiment. It was administered in a dose of 20 mg/kg intraperitoneally at 1 and 12 hours after LPS. If not stated otherwise, chemicals were purchased from Sigma (St. Louis, MO, USA).

Experimental model

Twelve hours following injection of LPS, mice were anesthetized with an intraperitoneal injection of 2,2,2-tribromoethanol (Avertin; Aldrich, Milwaukee, WI, USA). Supplemental injections of the anesthetic were administered as needed to maintain the mice in a sedated state throughout the procedure, and the animals were kept on a heating pad. Right internal jugular vein and carotid artery were catheterized using a PE10 catheter connected to a PE50 catheter. The urinary bladder was catheterized with a PE50 catheter, after incision of the bladder fundus. Mice were infused intravenously with 6% albumin solution in isotonic saline at a rate of 3.5 $\mu\text{L}/\text{min}$ during the entire procedure.

Mean arterial pressure measurement

Intracarotid mean arterial pressure (MAP) was measured using a Spectramed Statham transducer connected to a model 7P1K low-level DC preamplifier and was read on a model 79 Grass polygraph.

Clearances

A saline solution of inulin (50 g/L) and paraaminohippuric acid (PAH; 10 g/L) was intravenously infused intrajugularly for 75 minutes at a rate of 3.5 $\mu\text{L}/\text{min}$ using a KD Scientific model 200 infusion pump before three consecutive 30-minute urine samples were collected. Urine volume was determined by weight. A blood sample was withdrawn after the last urine collection. Urine and serum inulin and PAH concentrations were determined spectrophotometrically. Clearances were calculated as a ratio between mean urinary excretion rates of inulin or PAH and serum concentrations of inulin or PAH per 100 g body wt.

NO measurement

Serum NO was measured by chemiluminescence with NaNO_3 as a standard using a NO analyzer (Sievers Instr., Inc., Boulder, CO, USA).

Tissue analyses

All tissue analyses were conducted 16 hours after LPS injection. Tissue samples were fixed in 4% paraformaldehyde and were paraffin embedded. Samples were examined for evidence of apoptosis using the terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling (TUNEL) method to detect in situ DNA strand breaks (TACS 2 TdT-blue label in situ apoptosis detection kit; Trevigen, Inc., Gaithersburg, MD, USA). Other paraffin-embedded samples were stained with hematoxylin and eosin for morphological examination.

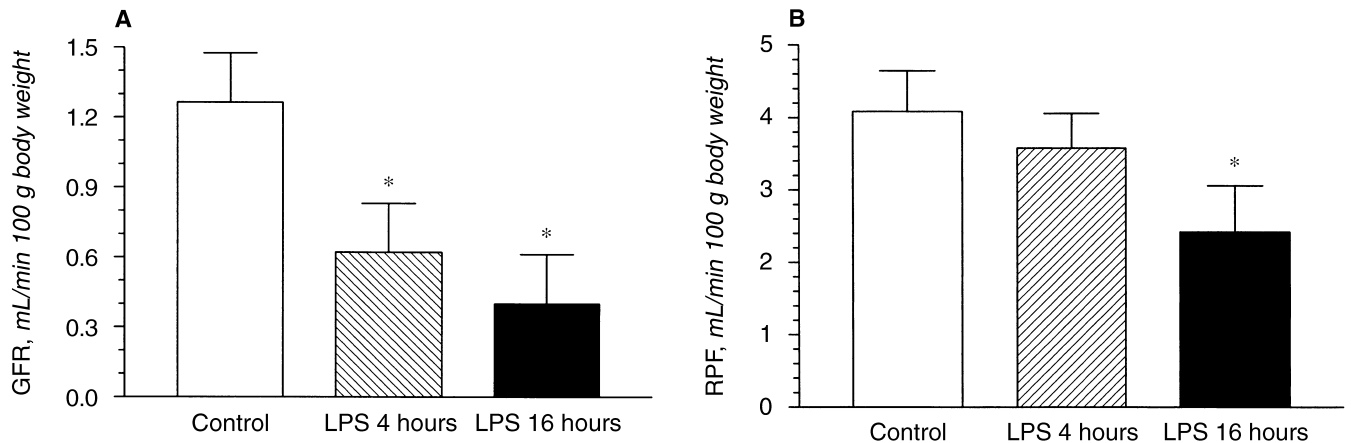


Fig. 1. Endotoxemic renal failure in wild-type mice. Glomerular filtration rate (GFR; A) and renal plasma flow (RPF; B) were determined at 4 and 16 hours after an injection of 5 mg/kg intraperitoneal endotoxin. Data are expressed as mean \pm SEM, $N = 6$. * $P < 0.05$ vs. control by ANOVA.

Neutrophil accumulation was assessed on acetone-fixed tissue sections by an indirect immunofluorescent technique using a rat anti-mouse neutrophil antibody (Serotec Inc., Raleigh, NC, USA). Leukocyte accumulation was also determined by measuring myeloperoxidase activity of snap-frozen kidney samples by the method of Laight et al [26].

Statistical analyses

Values are expressed as mean \pm SEM. Multiple-group comparisons were assessed by analysis of variance (ANOVA) using the post hoc Newman-Keuls test. $P < 0.05$ was considered statistically significant.

RESULTS

Endotoxemic renal failure in wild-type mice

To induce renal failure, mice were injected intraperitoneally with 5 mg/kg LPS, a relatively low, nonlethal dose of LPS that permitted surgery and physiological measurements without an excessive mortality. In addition, with this dose of LPS, it was possible to evaluate renal function in the absence of severe hypotension (MAP, 110 ± 9 mm Hg in vehicle-injected vs. 101 ± 8 mm Hg in treated with LPS, $N = 5$, $P = \text{NS}$). After 16 hours, endotoxemia resulted in an elevation in serum NO concentration, as compared with vehicle-injected mice (647 ± 178 $\mu\text{mol/L}$ vs. 24 ± 4 $\mu\text{mol/L}$, $N = 4$, $P < 0.05$). Renal clearances of PAH and inulin were determined at 4 and 16 hours following an injection of LPS. As shown in Figure 1A, GFR decreased significantly four hours after the administration of LPS. At 16 hours, GFR in LPS-treated mice decreased to 30% of control value (Fig. 1A). Changes in renal plasma flow (RPF) followed a similar pattern (Fig. 1B). There were no morphological changes in the kidneys of LPS treated mice compared with controls. Moreover, in other studies in which the

mice were treated with higher doses of LPS, the urinary sodium concentration, measured 24 hours after LPS, was lower in the LPS-treated mice (data not shown). These findings are compatible with renal hypoperfusion and intact tubular function.

Effect of TNF neutralization on renal function and serum NO in wild-type mice

In these experiments, renal function was evaluated at 16 hours after administration of LPS. Pretreatment of mice with TNFRp55 (10 mg/kg IP, 1 hour before LPS) resulted in less of a decrease in GFR (30%) as compared with LPS-treated mice given vehicle instead of TNFRp55, in which GFR fell by 75% (Fig. 2A). After TNFRp55 pretreatment, no decrease in RPF from control levels was observed (Fig. 2B). At the same time, the serum NO level in endotoxemic mice was reduced with TNFRp55, as compared with LPS-treated mice given vehicle (673 ± 112 vs. 260 ± 52 $\mu\text{mol/L}$, $N = 6$, $P < 0.01$), indicating suppressed induction of iNOS. There was no significant difference in MAP between the three groups 16 hours following injection of vehicle or LPS (102 ± 5 vs. 92 ± 6 vs. 94 ± 4 mm Hg; $N = 4$ to 6, $P = \text{NS}$; control vs. LPS vs. LPS + TNFRp55).

Renal function in iNOS knockout mice

Endotoxemia did not change the serum NO concentration measured after 16 hours in iNOS knockout mice in response to LPS (28.2 ± 7.2 vs. 40.9 ± 5.2 $\mu\text{mol/L}$, $P = \text{NS}$). However, in iNOS knockout mice, GFR decreased in response to LPS by 70% and RPF by 65% (Fig. 3). There was no change in MAP in response to LPS (107 ± 5 vs. 116 ± 8 mm Hg, $N = 4$, $P = \text{NS}$; control vs. LPS). Similarly, treatment of wild-type mice with a selective iNOS inhibitor, 1400W, did not prevent decrease in GFR in response to LPS (0.25 ± 0.04 mL/min 100 g body weight vs. 0.25 ± 0.06 mL/min 100 g body weight, $N =$

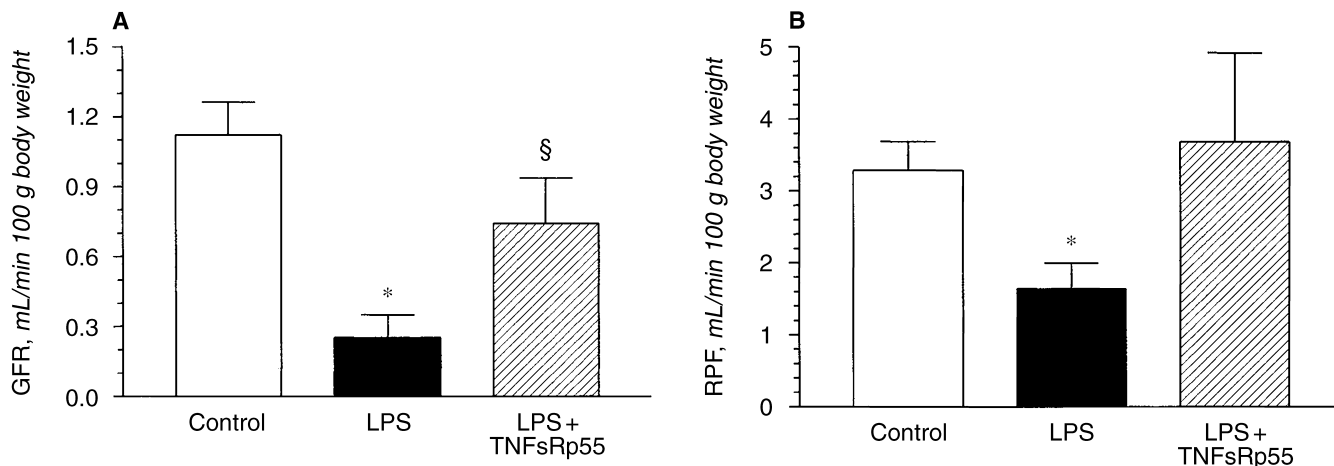


Fig. 2. Effect of TNFRp55 (10 mg/kg IP) on renal function in wild-type mice. Mice were pretreated with TNFRp55 for one hour before the administration of 5 mg/kg intraperitoneal endotoxin. GFR (A) and RPF (B) were determined 16 hours thereafter. Data are expressed as mean \pm SEM, $N = 6$. * $P < 0.05$ vs. Control; § $P < 0.05$ vs. LPS, by ANOVA.

4, $P = \text{NS}$; LPS vs. LPS + 1400W), although the serum NO concentration decreased significantly (149 ± 27 vs. 355 ± 65 $\mu\text{mol/L}$, $N = 4$, $P < 0.05$) with 1400W treatment. MAP was not significantly influenced by 1400W (97 ± 5 vs. 105 ± 6 mm Hg, $N = 4$, $P = \text{NS}$; LPS vs. LPS + 1400W).

Effect of TNF neutralization on renal function in iNOS knockout mice

To check whether the protective effect of TNF neutralization is preserved in LPS-treated iNOS knockout mice, renal function was evaluated in mice given TNFRp55 one hour before LPS. Similar to wild-type mice, pretreatment of iNOS knockout mice with TNFRp55 attenuated renal dysfunction in response to LPS (Fig. 4A). Endotoxemic iNOS knockout mice pretreated with 10 mg/kg TNFRp55 had less of a decrease in GFR (35%) as compared with LPS-treated iNOS knockout mice given vehicle instead of TNFRp55, in which GFR fell by 75%. Again, RPF was not different than controls in the TNFRp55-treated animals (Fig. 4B). In addition, MAP did not change in response to LPS and TNF blockade (98 ± 7 vs. 103 ± 7 vs. 99 ± 8 mm Hg, $N = 4$ to 6, NS, control vs. LPS vs. LPS + TNFRp55).

Tissue analyses

No evidence of apoptosis was observed as assessed by TUNEL staining at 16 hours after LPS treatment in wild-type mice. Morphologically, the kidneys appeared unchanged by light microscopy. In addition, no significant leukocyte accumulation could be detected at 16 hours by either neutrophil staining or measurement of myeloperoxidase activity.

DISCUSSION

In the present study, a model of LPS-induced renal failure in mice was developed. Use of a relatively low dose of LPS permitted the study of renal function in the absence of hypotension and mortality. The administration of LPS to wild-type mice decreased renal function in a time-dependent manner. Thus, renal failure in the present study occurred in the absence of systemic hypotension, similar to previous work in rats [3]. Since TNF- α is a proinflammatory cytokine in endotoxemia and sepsis [5], the role of this cytokine in the renal failure was examined. Importantly, neutralization of TNF, using TNFRp55, afforded protection against LPS-induced renal failure in wild-type mice. With the pretreatment with TNFRp55, GFR decreased only by 30%, as compared with a 75% decrease without TNF neutralization. Moreover, TNFRp55 prevented the fall in RPF in response to LPS. These findings suggested a functional role of TNF in the pathogenesis of endotoxin-associated renal failure, as proposed in previous studies [8, 9]. In rabbits, an intravenous infusion of TNF- α induced glomerular fibrin deposition and renal failure [9]. Similarly, in the perfused rat kidney, TNF- α led to a decrease in GFR [8].

Since the discovery that hypotensive effects of TNF- α and endotoxin could be blocked by inhibition of NOS [27, 28], the role of NO in the hypotension and organ failure in endotoxemia or sepsis has become the subject of several studies. NO is generated by the three NOS isoenzymes. The constitutively expressed NOSs (nNOS and eNOS) are regulated by changes in intracellular calcium, whereas the inducible isoform, iNOS, does not require calcium and is primarily regulated at the transcription level [17]. The eNOS-mediation of NO production participates in the regulation of normal vascular

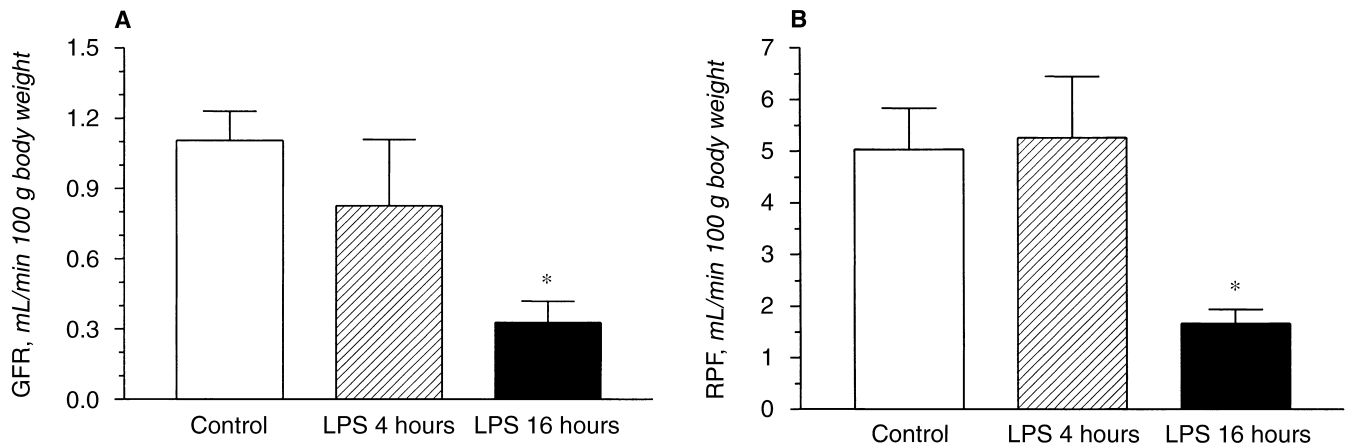


Fig. 3. Endotoxic renal failure in inducible nitric oxide synthase (iNOS) knockout mice. GFR (A) and RPF (B) were determined at 4 and 16 hours after injection of 5 mg/kg intraperitoneal endotoxin. Data are expressed as mean \pm SEM, $N = 6$. * $P < 0.05$ vs. Control, by ANOVA.

tone. Endotoxin is a potent inducer of iNOS directly and through its cytokine mediators TNF- α , IFN- γ , and IL-1 β [17]. iNOS-derived NO has been implicated as a mediator of renal failure in endotoxemia [19–22]. Experiments using nonselective NOS blockade frequently resulted in a worsening of renal function due to increased renal vasoconstriction [19]. In addition, nonselective NOS blockade was shown to promote glomerular thrombosis in endotoxemic rats [29]. Thus, selective inhibition of iNOS could be an alternative strategy to avoid endothelial dysfunction after nonselective NOS inhibition. We hypothesized that suppression of iNOS by TNF neutralization may reduce LPS-induced renal failure. Indeed, pretreatment of mice with TNFRp55 reduced the increase in serum NO in response to LPS. Similar results have been reported in cultured rat brain cells in which either TNFRp55 or an IL-1 receptor antagonist produced a dose-dependent partial inhibition of the NO response to LPS [30]. Although TNFRp55 binds directly to TNF, it is possible that other downstream cytokines could be affected. However, as indicated by only partial inhibition of LPS-induced NO in wild-type mice treated with TNFRp55, all mediators of iNOS induction were clearly not blocked in our studies. Moreover, Faggioni et al have shown that IFN- γ levels are not reduced by TNFRp55 in a model of ConA-induced hepatotoxicity [24].

The present results, however, do not support a major role of iNOS in endotoxemic renal failure. Specifically, the degree of renal failure (a 70% decrease in GFR) was similar in iNOS knockout or 1400W-treated mice as compared with endotoxemic wild-type mice. iNOS knockout mice did not increase serum NO in response to LPS, while endotoxemic wild-type mice treated with 1400W significantly decreased the serum NO concentration, as compared with untreated endotoxemic wild-type mice. These findings suggest that inhibition of iNOS does

not provide protection against endotoxemic renal failure in this nonhypotensive endotoxemic model in mice. In previous studies, iNOS inhibitors improved glomerular filtration [19, 20], whereas there was no effect in others [21, 22]. In these studies, renal function was evaluated in anesthetized animals after a relatively short period of endotoxemia. In the present study, the effect of anesthesia on renal function was avoided by using sedated mice. Also, our experiments started at 12 hours after LPS, when iNOS was clearly induced in wild-type mice, as serum NO increased markedly at that time. In contrast to the failure to incriminate NO, TNF neutralization by a soluble receptor attenuated renal failure in iNOS knockout mice. Similar to wild-type mice, in endotoxemic iNOS knockout mice pretreated with TNFRp55, GFR fell by only 33%, without a reduction in RPF, as compared with a 75% decrease in GFR and a 40% decrease in RPF without TNFRp55 pretreatment.

There are numerous mechanisms whereby TNF- α may contribute to renal failure in endotoxemia. Many of the changes that have previously been reported to occur and contribute to the resultant organ failure of endotoxemia were observed in models of established septic shock with severe systemic hypotension. Moreover, these events occurred mainly at later time points (later than 24 hours) in the course of endotoxemia. In the present study, however, as discussed previously in this article, we developed a normotensive model of endotoxemia and examined time points earlier in the course of endotoxemia (4 and 16 hours). We could therefore eliminate the role of reduced renal perfusion pressure as a potential mechanism of the LPS/TNF-induced renal failure in our study. Additionally, as discussed previously in this article, the results of our study eliminated iNOS-mediated NO production as a mechanism of the early renal dysfunction since (1) the endotoxemic iNOS knockout mice had similar renal

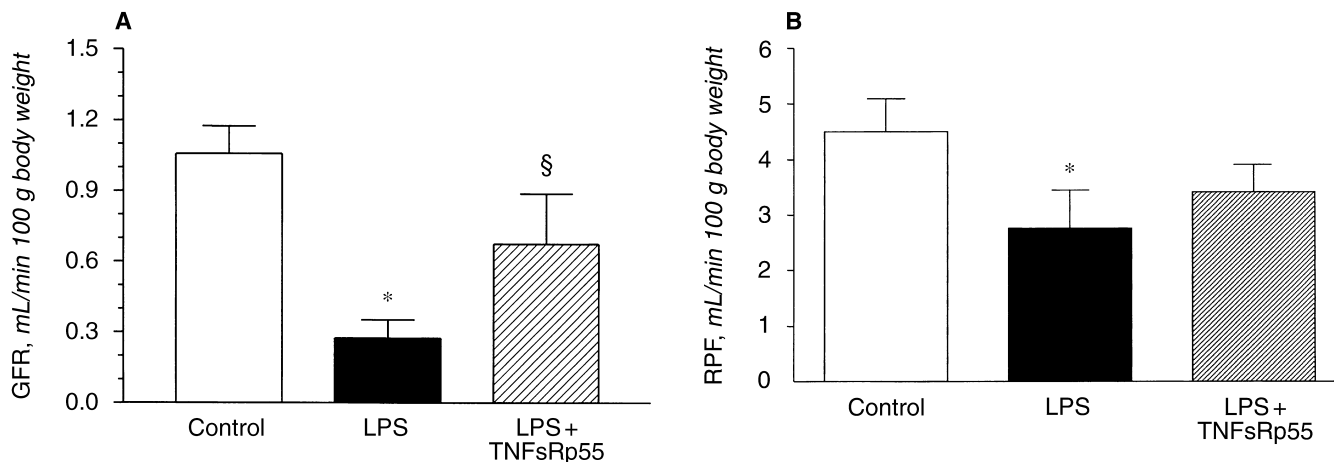


Fig. 4. Effect of TNFsRp55 (10 mg/kg IP) on renal function in iNOS knockout mice. Mice were pretreated with TNFsRp55 one hour before the administration of 5 mg/kg intraperitoneal endotoxin. GFR (A) and RPF (B) were determined 16 hours thereafter. Data are expressed as mean \pm SEM, $N = 4$ to 6. * $P < 0.05$ vs. Control; § $P < 0.05$ vs. Control and LPS, by ANOVA.

dysfunction as the endotoxemic wild-type mice, and (2) the wild-type and iNOS knockout mice were similarly protected by TNF neutralization. Other mechanisms by which TNF- α has been suggested to induce renal injury and dysfunction include glomerular fibrin deposition [9], and renal endothelial, mesangial, or tubular cell apoptosis due to up-regulation of Fas and activation of caspase 3 [11, 12]. In our model, however, morphological examination showed no evidence of renal fibrin deposition or pathology in either the wild-type or iNOS knockout mice. Similarly, no apoptosis was observed in kidneys from LPS-treated wild-type mice as detected by TUNEL staining. Furthermore, renal leukocyte accumulation was not observed in our study as assessed by neutrophil-specific staining or myeloperoxidase activity. In this regard, Ysebaert, DeGreef, and Vercauteren have recently reported that myeloperoxidase activity reflects not only neutrophil accumulation/activation, but also the presence of monocytes or macrophages [31]. Therefore, the lack of any increase in myeloperoxidase activity in our study indicates that no significant accumulation or activation of either neutrophils or monocytes/macrophages occurred. Thus, any role of apoptosis, cellular accumulation, and fibrin deposition in this mouse model of sepsis must occur later in the course of the disease.

Based on these and the present results, we conclude that the early renal dysfunction of endotoxemia results from renal hypoperfusion caused mainly by an imbalance in renal vasoconstrictors and vasodilators. Several vasoconstrictors have been reported to participate in the renal dysfunction of endotoxemia [4]. Studies using currently available specific blockers are difficult or impossible to interpret since these compounds alter systemic hemodynamics. Therefore, future studies to explore these mech-

anisms will require the use of kidney-selective blockers that preserve systemic blood pressure.

The present results have important clinical implications. Mortality of patients with septic shock and renal failure remains high [1]. Although a nonselective NOS inhibition may exert a beneficial effect on MAP in patients with septic shock, a recent randomized, placebo-controlled trial with a nonselective NOS inhibitor, L-NMMA, in patients with septic shock was discontinued early because of an increased mortality in the treated group [32]. The present results suggest that a more promising approach may involve the blockade of the action of TNF.

In summary, the present study demonstrates a protective effect of TNF neutralization on renal function in non-hypotensive endotoxemia in wild-type and iNOS knockout mice. The deleterious effect of TNF at 4 and 16 hours after LPS administration to impair renal function was demonstrated to be independent of diminished renal arterial pressure, apoptosis, leukocyte accumulation, and iNOS. These results suggest that early in mouse sepsis an imbalance between as yet unidentified renal vasoconstrictors and vasodilators mediates the renal dysfunction.

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Reprint requests to Robert W. Schrier, M.D., Department of Medicine, University of Colorado Health Sciences Center, Box B178, Denver, Colorado 80262, USA.
E-mail: robert.schrier@uchsc.edu

APPENDIX

Abbreviations used in this article are: eNOS, endothelial nitric oxide synthase; GFR, glomerular filtration rate; IFN- γ , interferon- γ ; IL-1 β ;

interleukin-1 β ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAP, mean arterial pressure; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; PAH, puroaminohippuric acid; RPF, renal plasma flow; TdT-TUNEL, terminal deoxynucleotidyl transferase-mediated nick-end labeling; TNF, tumor necrosis factor; TNFRp55, tumor necrosis factor soluble receptor; 1400W, selective iNOS inhibitor.

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