Protective roles of nitric oxide and testosterone in endotoxemia: evidence from NOS-2-deficient mice

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Laubach, Victor E., Patricia L. Foley, Kim S. Shockey, Curtis G. Tribble, and Irving L. Kron. Protective roles of nitric oxide and testosterone in endotoxemia: evidence from NOS-2-deficient mice. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H2211–H2218, 1998.—Lipopolysaccharide (LPS)-induced septic shock, which triggers nitric oxide (NO) overproduction, multiple organ dysfunction, and death, can be affected by gender and sex hormones. We hypothesized that NO is beneficial during endotoxemia and that this beneficial effect is influenced by sex hormones. C57BL/6 wild-type (WT) mice and congenic inducible NO synthase knockout (KO) mice were injected with LPS, and mortality was recorded for 4 days. After 5 mg/kg LPS, female KO mice had significantly higher mortality than WT. After 12.5 mg/kg LPS, both male and female KO mice had significantly higher mortality than WT. Ovariectomy did not alter mortality, but orchietomy dramatically increased mortality in KO mice. After 5 mg/kg LPS, exogenous testosterone completely prevented the increased mortality in KO mice and orchietomized KO male mice. WT survival was not affected by exogenous testosterone. After 12.5 mg/kg LPS, exogenous testosterone significantly improved survival of female KO mice. Serum enzymes and organ edema, which may not correlate with mortality, were significantly and similarly increased in both WT and KO endotoxemic mice; however, edema was not observed in KO hearts. Thus, NO plays a protective role in endotoxemia while having differential effects on different organs. Importantly, testosterone is beneficial in endotoxemia when NO production is deficient, and may be therapeutic in certain septic patients.

HUMAN SEPTIC SHOCK commonly results from a systemic bacterial infection and is characterized by hypotension, inadequate tissue perfusion, and vascular damage, often leading to multiple organ failure and death. Despite current therapeutic approaches such as antibiotics, volume replacement, inotropic and vasopressor support, and other supportive care, septic shock continues to be a major cause of death in intensive care units (38). The pathophysiology of septic shock is very complex. The shock state is caused by the release of endogenous mediators, primarily tumor necrosis factor (TNF)-α and interleukin (IL)-1β, triggered by the presence of lipopolysaccharide (LPS), a gram-negative bacterial cell wall component. These primary mediators in turn stimulate the production of a cascade of secondary mediators such as prostanoids, leukotrienes, complement components, interferons, and nitric oxide (NO). Purified LPS administered to animals and humans (endotoxemia) is capable of initiating the clinical picture of septic shock in the absence of viable bacteria (10, 36). Although there have been numerous studies focusing on single mediators, the particular relevance of any of these mediators to the pathophysiology and treatment of sepsis still remains unclear.

One of the secondary mediators in sepsis, NO has gained much attention. NO is produced by the enzyme NO synthase (NOS), of which there are three isoforms (see Refs. 17 and 27 for reviews). NOS-1 is expressed mainly in neuronal cells and is thought to play a role in neurotransmission. NOS-3 is expressed mainly in endothelial cells and is involved in regulation of vascular tone. These two isoforms are constitutively synthesized and produce small amounts of NO in response to increases in intracellular calcium. NOS-2, however, is very different from the other two isoforms. Normally not expressed, NOS-2 is calcium independent, is synthesized de novo in a variety of cell types in response to a variety of inflammatory mediators, and produces large amounts of NO over prolonged periods of time. Many of the symptoms of septic shock, especially hypotension, have been attributed to the marked increase in NO production by NOS-2, leading to the hypothesis that overproduction of NO is detrimental during sepsis and that NOS inhibitors may be therapeutic. Indeed, NOS inhibition was efficient in the treatment of hypotension in septic patients, and infusion of an NOS inhibitor was found to increase blood pressure in dogs after LPS injection (16).

The predominant concept that NO production is detrimental during sepsis is being challenged by a growing number of reports that support certain protective actions of NO and that show that inhibition of NO production may actually be harmful in sepsis. It was shown that NOS inhibition following endotoxemia increased hepatic damage (1, 12) and intestinal injury (14), while blocking platelet and leukocyte adhesion to endothelial cells (18, 31).

There is also conflicting evidence as to whether inhibition of NOS leads to reduced mortality in animal models of endotoxemia. Recent studies have shown that NOS inhibition can be protective (37), nonprotective (6), or detrimental (26). One explanation for this conflict is that the inhibitors used in these studies were nonselective, having differential effects on each of the NOS isoforms. It is because of this lack of a truly NOS-2-specific inhibitor that NOS-2-deficient mice have...
been recently developed by three independent laboratories (20, 23, 39). These knockout (KO) mice are phenotypically normal but lack the ability to produce increased NO during endotoxemia. We have previously shown that mortality was unaltered in NOS-2-deficient mice after LPS challenge (20). Others have found partial (23) or complete (39) protection in LPS-challenged NOS-2-deficient mice. The basis for these differences is not known but could include differences in strain, LPS preparation, and gender of the mice used.

None of the NOS-2-deficient mouse studies mentioned above were comparable in terms of strain and gender of mice. All used mice of a hybrid strain (C57BL/6 (B6)/129) for Refs. 20 and 23 and MF 1/129) for Ref. 39, which is unavoidable early after generation of the KO animal. Also, possible gender differences were not addressed. Gender differences in the susceptibility to and morbidity from sepsis have been observed in several human studies (2, 24). In animal models, Zellweger et al. (42) showed a higher survival rate of septic female mice over males, and Wichmann et al. (40) showed that immune responsiveness in female mice is enhanced after hemorrhagic shock, as opposed to decreased responsiveness in males.

We hypothesized that NOS-2-deficient mice would be more vulnerable to endotoxic death and that this would be affected by gender and sex hormones. In the current study, NOS-2-deficient mice were used to examine the effects of gender, strain, and sex hormones on LPS-induced mortality.

METHODS

Animals. NOS-2-deficient mice were generated by homologous recombination and shown to lack NOS-2 protein expression as previously described (20). These mice were backcrossed onto the B6 strain for ten generations (F10) to obtain congenic KO mice. Some NOS-2-deficient mice used were backcrossed onto the 129J strain. These were obtained by mating chimeric males with 129J females to produce homozygous NOS-2-deficient 129J mice. All mice used in these studies were of the congenic F10 backcross (B6 background) or 129J background and were 12–16 wk of age. Wild-type (WT) B6 or 129J mice (The Jackson Laboratory, Bar Harbor, ME) were used as controls. Animals were housed under diurnal lighting conditions (12 h light, 12 h dark) and allowed free access to food and water. All procedures were approved by the Institutional Animal Care and Use Committee.

Endotoxemia. Mice were injected intraperitoneally with 5 or 12.5 mg/kg of LPS (Escherichia coli, serotype 026:B6; Difco Laboratories, Detroit, MI) resuspended in sterile saline. These doses of LPS were chosen on the basis of preliminary studies in WT B6 mice in which the lower dose resulted in less than 30% mortality and the higher dose resulted in greater than 70% mortality. The volumes injected ranged from 0.18 to 0.30 ml, depending on the weight of the animal (0.01 ml/g). Deaths were recorded on a daily basis, and any mice that survived out to 4 days were fully recovered and were considered long-term survivors.

Surgical procedures. For orchietomy, mice were anesthetized by intraperitoneal administration of ketamine (80 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (8 mg/kg; Vedco, St. Joseph, MO). After aseptic preparation of the surgical site, a small incision was made at the tip of the scrotum. The tunica was opened and the testes, cauda epididymis, vas deferens, and spermatic blood vessels were exteriorized. The blood vessels and vas deferens were cauterized and the testes and epididymis removed. The remaining tissue was returned into the sac, and the procedure was repeated for the other testis. The skin incision was closed with a wound clip. Animals were allowed to recover for 2 wk before study.

For ovariectomy, mice were anesthetized as above and a small area of the dorsum between the scapulae was prepared for aseptic surgery. A small skin incision was made, and a subcutaneous pocket was created with forceps. The Silastic implant was placed in the pocket, and the skin was closed with a wound clip. To make the implant, testosterone and cholesterol (Sigma Chemical, St. Louis, MO) were mixed in a 1:1 ratio (wt/wt) and packed to 8 mm into Silastic tubing (15 mm in total length, 1.47 mm ID, 1.95 mm OD; Dow Corning, Midland, MI) to provide sufficient testosterone to maintain at least normal physiological blood levels. Animals were allowed to recover for 2 wk before study.

Tissue collection. Tissues (lungs, heart, and liver) were collected from anesthetized mice 12 h after saline or LPS injection, blotted, and weighed. The 12-h time point was chosen because NOS-2 expression normally peaks at 6–12 h after induction in most cell types (8, 21, 22, 34). The tissues were dried for 24 h at 60°C and weighed again to obtain wet-to-dry weight ratios. Wet-to-dry weight ratio, which measures accumulation of tissue fluid, has been used as a standard in many studies and is one of the most common indexes of tissue edema without correction for blood content (11) and has been shown to correlate with the permeability index as measured by 125I-labeled albumin accumulation in tissues (11, 35). Blood was collected by cardiac puncture, and serum was obtained using Microtainer serum separator tubes (Becton-Dickinson, Franklin Lakes, NJ) and stored at −80°C. Serum testosterone levels were measured using the ImmuChem Testosterone radioimmunnoassay kit as instructed by the manufacturer (ICN Biomedicals, Costa Mesa, CA). Analysis of serum chemistry was performed by AniLytics (Gaithersburg, MD) with the use of a Hitachi 717 Chemistry Analyzer. Alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) levels were measured for indications of liver injury, creatine phosphokinase (CPK) levels were measured as an indication of cardiac and skeletal muscle injury, and blood urea nitrogen (BUN) levels were measured as an indication of kidney injury.

Statistics. Survival data were analyzed by a log-rank test to compare Kaplan-Meier survival curves. ANOVA was used to determine whether significant differences existed between groups, and measurements are reported as means ± SE. A P value of 0.05 or less was used to indicate significant differences between measurements.

RESULTS

LPS-induced mortality in B6-backcrossed NOS-2-deficient mice. WT B6 and congenic B6-backcrossed NOS-2-deficient KO mice were injected intraperitone-
ally with either 5 or 12.5 mg/kg LPS, and survival was recorded daily (Fig. 1). High-dose LPS (12.5 mg/kg) resulted in significantly higher mortality than low dose (5 mg/kg) in all groups. No significant differences in survival were seen between WT males and WT females after high-dose LPS. However, after low-dose LPS, WT males had lower survival than WT females (P < 0.05). At 5 mg/kg LPS, male WT and KO mice had similar survival curves, whereas female KO mice had significantly higher mortality than WT females (P < 0.01). At 12.5 mg/kg, KO mice had significantly higher mortality than corresponding WT mice (P < 0.05 for females, P < 0.01 for males). No deaths occurred in groups of WT or KO mice injected with saline, the vehicle control (data not shown).

As described previously with F2 KO mice (20), the congenic KO mice used in this study lacked any increase in serum nitrite and nitrate levels after LPS injection (data not shown), indicating that the NOS-2 gene is defective and unable to lead to NO production.

LPS-induced mortality in 129J backcrossed NOS-2-deficient mice. To evaluate possible differences between B6 and 129J strains in susceptibility to endotoxemia, 129J WT and congenic 129J backcrossed NOS-2-deficient mice (129J KO) were injected intraperitoneally with 5 mg/kg LPS, and survival was recorded daily (Fig. 2). No differences in survival occurred between 129J WT and 129J KO male mice. Survival of female 129J KO mice was significantly reduced compared with 129J WT (P < 0.05). These results were very similar to those seen in the B6 strain (Fig. 1). No deaths occurred in groups of WT or KO mice injected with saline, the vehicle control (data not shown).

Wet-to-dry weight ratios after LPS administration. As an indicator of organ injury (edema), wet-to-dry weight ratios of hearts, lungs, and livers were measured in male mice 12 h after saline or 5 mg/kg LPS injection. In WT mice, wet-to-dry weight ratios significantly increased in lung, heart, and liver after LPS treatment (P < 0.05) (Fig. 3). In KO mice, wet-to-dry weight ratios significantly increased in lung and liver (P < 0.05), with no alteration in heart wet-to-dry weight ratio (Fig. 3).

Serum chemistry after LPS administration. As another indicator of tissue injury, serum enzymes were evaluated in male WT and KO mice 12 h after saline or 5 mg/kg LPS injection (Fig. 4). ALT, SDH, BUN, and CPK levels were similar in saline-treated WT and KO mice, and were all significantly increased after LPS injection in both WT and KO mice (P < 0.0001), indicating multiple organ injury. Although significantly increased over saline-treated mice, ALT and CPK levels in KO mice were not increased to the extent seen in WT mice after LPS injection (P < 0.01, Fig. 4). This experiment was performed with female mice as well. Here, as with the male mice, groups of ten female mice (WT and KO) were injected with either saline or 5 mg/kg LPS. Twelve hours later serum enzyme levels were measured and found to be increased significantly and similarly in both WT and KO mice treated with LPS (data not shown). Importantly, the serum enzyme

Fig. 1. Lipopolysaccharide (LPS)-induced mortality in C57BL/6 (B6) wild-type (WT) and B6-backcrossed nitric oxide synthase isoform 2 (NOS-2)-deficient knockout (KO) mice. Groups (n = 20) of female (top) and male (bottom) mice were injected intraperitoneally with either 5 or 12.5 mg/kg LPS, and survival was monitored daily for 4 days. Squares, 5 mg/kg LPS; triangles, 12.5 mg/kg LPS; open symbols, WT mice; closed symbols, KO mice. *P < 0.01 and **P < 0.05 vs. corresponding WT controls. *P < 0.05 vs. WT female at 5 mg/kg LPS by log-rank test.

Fig. 2. LPS-induced mortality in 129J WT and 129J KO mice. Groups (n = 12) of female (triangles) and male (squares) mice were injected intraperitoneally with 5 mg/kg LPS, and survival was monitored daily for 4 days. Open symbols, WT mice; closed symbols, KO mice. *P < 0.05 vs. WT female by log-rank test.
increases in females were also similar to what was measured in the male mice (Fig. 1). This indicates that, at least at the 12 h time point, serum enzyme levels do not correlate with mortality.

Low-dose LPS-induced mortality after gonadectomy and exogenous testosterone. The large difference in survival observed between female WT and KO mice at 5 mg/kg, and the lack of a difference in male mice, suggests that sex hormones may be involved. Thus this dose of LPS was chosen to evaluate survival after gonadectomy in endotoxemic mice (Fig. 5). Female mice underwent ovariectomy followed by LPS challenge 2 wk later to determine the effect of estrogen depletion on mortality. As shown in Fig. 5, left, ovariectomy did not alter the survival of female WT or KO mice. Male mice underwent orchietomy followed by LPS challenge 2 wk later to determine the effect of testosterone depletion on mortality. As shown in Fig. 5, right, orchietomy did not alter the survival of male WT mice but dramatically lowered survival of KO mice (P < 0.05).

Survival after gonadectomy suggested that testosterone may be somewhat protective, at least in male KO mice. To test this, mice underwent gonadectomy as before with the addition of a Silastic testosterone implant at the time of surgery. Mice were then challenged with 5 mg/kg LPS 2 wk later. Addition of exogenous testosterone to ovariectomized female WT mice did not alter survival; however, exogenous testosterone significantly increased survival in ovariectomized female KO mice to a level similar to that seen in the WT mice (P < 0.05) (Fig. 5, left). In male mice, exogenous testosterone significantly increased survival in orchietomized KO mice to a level similar to that seen in WT mice (P < 0.05) (Fig. 5, right).

High-dose LPS-induced mortality after exogenous testosterone. Because testosterone offered protection in the KO mice after 5 mg/kg LPS challenge, a similar experiment was performed at the more lethal, higher dose of LPS to evaluate whether exogenous testosterone is protective in WT mice as well. Mice were given testosterone implants (with no gonadectomy) followed by 12.5 mg/kg LPS 2 wk later. Mortality was then monitored on a daily basis. At this dose of LPS, exogenous testosterone offered no protection in either WT or KO male mice (not shown). In female mice, however, exogenous testosterone significantly increased
survival of KO mice (P < 0.05) while not affecting survival of WT mice (Fig. 6).

Serum testosterone levels. To measure the effect of endotoxemia on endogenous testosterone, serum levels of testosterone were measured in WT and KO male mice 12 h after saline or 5 mg/kg LPS injection (Fig. 7). Basal levels of testosterone were similar between WT and KO saline-treated mice (0.665 ng/ml and 0.759 ng/ml, respectively). After LPS administration, testosterone levels dropped to nearly undetectable levels in both WT and KO male mice (0.014 ng/ml and 0.007 ng/ml, respectively). This is consistent with studies showing reduced testosterone in male sepsis patients (5) and in endotoxemic mice (3). To confirm loss or gain of testosterone after surgery, testosterone was measured in orchiectomized mice with or without implants.

Orchiectomy resulted in nearly undetectable testosterone levels, and testosterone implants in orchiectomized mice resulted in levels over twice that of normal (1.919 ng/ml) (Fig. 7). Silastic implants were designed to provide serum testosterone of at least normal levels.

DISCUSSION

Nitric oxide can be both cytoprotective and cytotoxic. This fact is paramount in attempting to understand the role of NO in septic shock, especially because septic shock is complex, can occur in multiple degrees of severity, and may or may not be accompanied by multiple organ dysfunction. It is known that septic shock is associated with an increase in NO formation.
these results indicate several important conclusions. 129J strain (129J KO). In this case, survival was the survival of KO mice that were backcrossed onto the B6 background, the mice in this study were thus congenic and could be directly compared, eliminating any genetic contributions from the 129J strain. In addition, because studies have shown a sexual dimorphism in mortality from sepsis (2, 24, 42), we conducted experiments in groups based on gender. Thus the opportunity exists to evaluate the role of NO in endotoxemia without the confounding effects of strain and gender. We are aware that, although overcoming the problem of nonselective NOS inhibitors, the NOS-2-deficient mice could have unknown compensatory mechanisms or effects resulting from the chronic deficiency of NOS-2 during growth and development. This is an inherent outcome of current knockout technology. The NOS-2-deficient mice, however, have a normal phenotype with normal growth, reproduction, and histology of all major organs and tissues (20).

Clinical manifestations of septic shock include hypotension, circulatory failure, organ injury, and often death. However, animal models of endotoxemia utilize purified LPS to mimic the systemic inflammation that occurs during bacterial sepsis, and death is a quantitative endpoint at specific doses of LPS in these models. This study focused on mortality in WT versus KO mice. After high- and low-dose LPS, mortality was higher in female KO mice compared with WT controls, whereas mortality was higher in male KO mice after high-dose LPS only. WT males had a significant increase in mortality over WT females at 5 mg/kg LPS, which is consistent with studies indicating increased mortality in human males (2, 24) and in male rodents (42). The high dose of 12.5 mg/kg LPS may be too overwhelming to allow gender differences to occur. We also tested the survival of KO mice that were backcrossed onto the 129J strain (129J KO). In this case, survival was equivalent to what was seen in the B6 mice. Overall, these results indicate several important conclusions.

First, B6 WT and 129J WT mice had similar survival after 5 mg/kg LPS injection. Thus, as separate inbred strains, B6 and 129J mice appear to have comparable susceptibilities to endotoxemia. Both of these strains are relatively resistant to LPS-induced lethality compared with other strains of mice, hence the necessity for the use of 12.5 mg/kg LPS to achieve greater than 70% mortality in WT mice. Second, because survival was also similar between B6 KO and 129J KO mice, the particular background (129J or B6) is probably not a major factor in endotoxemic mortality in NOS-2-deficient mice. However, it is apparent that 129J/B6 hybrid mice, as used in our initial studies (20), do not respond in the same manner as either inbred strain. Third, KO female mice had higher mortality than both WT females and WT males. Perhaps one factor contributing to the increased resistance seen in female septic patients is the level of NO production. It has been shown that estrogen stimulates NO synthesis in endothelial cells (19), in rat arterioles (13), and in the human circulation (32). Finally, NO appears to be protective during endotoxemia because mortality was higher in the KO mice in all instances except after 5 mg/kg LPS in the males. Even in this group, KO mice did not survive any better than WT. It should be remembered that NO is only one of many endogenous mediators that orchestrate the pathophysiology of endotoxemia. NO can regulate or be regulated by other mediators. Although not measured in this study, MacMicking et al. (23) reported that levels of IL-1α, IL-1β, TNF-α, and IL-6 were similar between WT and NOS-2-deficient mice following LPS injection. Thus it appears that NOS-2 deficiency does not alter the cytokine response in LPS-challenged mice.

To better understand the state of tissue injury during endotoxemia, wet-to-dry weight ratios and serum chemistries were measured. As expected, LPS significantly increased wet-to-dry weight ratios and serum enzymes in WT mice, indicating LPS-induced multiple organ injury. Wet-to-dry weight ratio increases in KO lung and liver were similar to WT; however, heart wet-to-dry weight ratio was not increased in the KO mice. The level of LPS-induced serum SDH and BUN increase was similar in WT and KO mice, indicating similar levels of liver and kidney injury, respectively. However, ALT and CPK increases in the KO mice were not as high as observed in WT. Overall, these results imply multiple organ injury in endotoxemic WT and KO mice. The indications that certain organs may be less injured in KO mice while others are not highlight possible differential effects of NO in different organs. In other words, NO may be protective in some organs and cytotoxic in others. It should be pointed out that these measurements, which are not functional measurements, were done at a single time point (12 h after LPS), which may not reflect the level of injury at other time points. It is important to note that, by these experiments, we were not attempting to correlate organ injury with mortality, and our data indicate that this correlation may not even exist. Although endotoxemia results in multiple organ injury, we measured injury...
only in the heart, lung, and liver, and thus the contribution of other organ injuries to mortality is not known in our model. Although some organs may be protected by the lack of NO, failure of just a single organ can result in death.

It has been suggested that gender differences in endothelial function are due to increases in NO release in females, perhaps related to sex hormones (4, 25). Because of the sexual dimorphism observed in survival of KO mice after 5 mg/kg LPS, the role of sex hormones was evaluated at this dose. Ovariectomy did not alter survival of either WT or KO female mice. Although orchietomy did not alter survival of male WT mice, it significantly reduced survival of the male KO mice to a level similar to that of female KO mice. This suggests that testosterone may be protective during endotoxemia. Moreover, this suggests an as-yet-uncharacterized interaction of testosterone and NO. To test this, exogenous testosterone was given to orchietomized male mice as well as to ovariectomized female mice to maintain serum levels of testosterone. The testosterone implants resulted in testosterone levels over twice the normal level (Fig. 7). This difference between pharmacological and physiological testosterone levels complicates the interpretation of the results; however, the implants did prevent the decline in testosterone level that occurs in endotoxemic mice. Although other concentrations of testosterone were not evaluated, the exact concentration of serum testosterone that is achieved is probably secondary to the fact that the decline in testosterone during endotoxemia is prevented.

Exogenous testosterone was found to dramatically improve survival of male and female KO mice to levels comparable to WT mice. Because WT mice already had relatively high survival after 5 mg/kg LPS, we did not test for further improvements in survival of these mice due to exogenous testosterone. Thus the effects of testosterone were tested in mice after high-dose LPS (12.5 mg/kg), when all mice, regardless of gender or NOS-2 expression, had low survival. Here, exogenous testosterone again significantly improved survival, but only in female KO mice. At the high dose of LPS, exogenous testosterone was not tested in gonadectomized mice. We believe that, in the male KO mice, orchietomy would result in a mortality too great to show any benefit of exogenous testosterone because the female KO mice benefited less from exogenous testosterone after 12.5 mg/kg LPS than after 5 mg/kg LPS (compare Figs. 5 and 6).

Serum levels of sex steroid hormones are known to be altered in sepsis. Previous studies found that estrogen levels were elevated in male and female patients with sepsis while testosterone levels dropped dramatically in males (5, 7). In rodent studies, endotoxin caused dramatic reductions in serum testosterone (3, 33). Our results corroborate these studies by showing large reductions in serum testosterone in WT and KO mice following LPS injection. It is not understood why hormonal variations exist during sepsis, nor is it known whether changes in estrogen or testosterone levels play positive or negative roles in sepsis. There is little information on the use of testosterone as a therapeutic tool for septic shock. Although we did not find any positive effects of exogenous testosterone in WT mice, the female KO mice did benefit from testosterone treatment. These results, along with the observation that male KO mice survival dropped dramatically after orchietomy and greatly improved after testosterone treatment, suggest that exogenous testosterone is beneficial during endotoxemia when NO is underproduced. In addition, there may be an unknown interaction that normally occurs between testosterone and NO. Although the vascular effects of testosterone remain undefined, Yue et al. (41) found that testosterone induced endothelium-independent relaxation in isolated rabbit coronary artery and aorta by means independent of prostaglandin I2 and cGMP. One plausible explanation for our results lies in the possible vasodilatory effects of testosterone, which may compensate for the lack of NO. It may be that prevention of the initial decline in testosterone level, which occurs rapidly after the onset of endotoxemia, is important in determining mortality in endotoxemia. It would be interesting to see whether exogenous testosterone, added after the onset of endotoxemia, would prove to be therapeutic in KO mice.

If testosterone offers protection from endotoxemic lethality in mice, then how could testosterone be protective in male mice following LPS if LPS completely inhibits testosterone production? This question is important but cannot be answered completely at this time. Our results indicate that it is the prevention of the decline in testosterone production during endotoxemia that offers protection in male mice, but this was observed only in the KO male mice. It seems that what is most deleterious is the combined lack of both inducible NO and testosterone. If either one of these is present during endotoxemia, then survival is significantly improved.

In summary, our study used a gene-knockout model of NOS-2 deficiency to determine the role of NOS-2-generated NO in endotoxemia. The results indicate that NO plays a protective role in endotoxemia; however, NOS-2 may or may not be protective in certain organs. In addition, gender affects the outcome in WT as well as NOS-2-deficient mice. Most importantly, exogenous testosterone plays a protective role in endotoxemia where NO production is deficient, and perhaps could also be beneficial in certain septic patients.

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