Endotoxin-induced renal tolerance against ischemia and reperfusion injury is removed by iNOS, but not eNOS, gene-deletion

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Endotoxin including lipopolysaccharide (LPS) confers organ tolerance against subsequent challenge by ischemia and reperfusion (I/R) insult. The mechanisms underlying this powerful adaptive defense remain to be defined. Therefore, in this study we attempted to determine whether nitric oxide (NO) and its associated enzymes, inducible NOS (iNOS) and endothelial NOS (eNOS, a constitutive NOS), are associated with LPS-induced renal tolerance against I/R injury, using iNOS (iNOS knock-out) or eNOS (eNOS knock-out) gene-deleted mice. A systemic low dose of LPS pretreatment protected kidney against I/R injury. LPS treatment increased the activity and expression of iNOS, but not eNOS, in kidney tissue. LPS pretreatment in iNOS, but not eNOS, knock-out mice did not protect kidney against I/R injury. In conclusion, the kidney tolerance to I/R injury conferred by pretreatment with LPS is mediated by increased expression and activation of iNOS. [BMB reports 2010; 43(9): 629-634]

INTRODUCTION

Ischemia followed by reperfusion (I/R) results in tissue injury in a number of organs, including the kidney, brain, and heart. In the kidney, I/R causes acute kidney injury (AKI), a high-mortality, high-morbidity condition. Additionally, the high mortality of I/R-induced AKI has seen no significant improvement over the past several decades. Moreover, ischemic-AKI ultimately progresses to chronic renal failure, a hugely expensive and care-intensive condition (1-3).

Stress-exposed organs activate their defense systems to cope with stress. In previous studies, it has been reported that organs exposed to endotoxin are thereafter resistant not only to subsequent identical endotoxin stress, but also to heterogenous insults including I/R (4-9). Several recent studies have demonstrated that endotoxin-induced tolerance is involved in the production of nitric oxide (NO), which performs an important function in I/R injury (6, 8, 10). NO is produced by constitutive nitric oxide synthases (cNOS), including endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). However, the role of NOS isoforms in endotoxin-induced kidney tolerance against I/R insult remains to be clarified (5, 6). Therefore, the principal objective of this study was to determine the following: 1) whether LPS pretreatment protects the kidney against subsequent I/R insults induced at early and delayed time points after the treatment; and 2) whether or not that protection is associated with NOS activation--and if this is the case, which forms of NOS protein contribute to that protection. In this study we determined that LPS-pretreatment protects the kidney against I/R insult, and that iNOS plays a role in that protection, but eNOS does not.

RESULTS

LPS-pretreatment protects kidney function against I/R insult

In order to determine whether or not LPS-pretreatment protects the kidney against I/R insult, and if so to determine how long that protection persists, mice were treated with either LPS or 0.9% saline for 5 or 24 hours, or 8 days, and then subjected to either 30 minutes of bilateral renal ischemia (ischemia) or sham-operation. 30 minutes of bilateral renal ischemia resulted in a dramatic increase in plasma creatinine (PCr) levels 24 hours after the ischemia (Fig. 1). The post-ischemic PCr concentrations in the LPS-pretreated mice at 5 or 24 hours, but not 8 days, before ischemia were significantly lower than those in the mice pretreated with vehicle (Fig. 1); this suggests that pretreatment with LPS does indeed protect the kidneys against I/R insult, and that this kidney protection disappears over time. Sham-operation did not cause any significant changes in PCr concentration in any of the experimental groups (Fig. 1).

LPS-pretreatment increases the activity and expression of iNOS, but not eNOS, in kidney tissues

We determined the activity and expression of iNOS and eNOS
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Fig. 1. Plasma creatinine (PCr) and blood urea nitrogen (BUN) concentrations after ischemia and reperfusion (I/R). C57BL/6 male mice were subjected to either 30 minutes of bilateral renal ischemia or sham-operation at 5 or 24 hours and 8 days after receiving treatment with either lipopolysaccharide (LPS) or 0.9% saline (Vehicle). The concentrations of PCr and BUN were measured 24 hours after either I/R or sham-operation. The values are expressed as the means ± SE (n = 6). *P < 0.05 vs. respective vehicle-ischemia.

Fig. 2. Expressions of inducible nitric oxide synthase (iNOS, A) and endothelial NOS (eNOS, B) in kidney after systemic LPS-treatment. C57BL/6 male mice were treated with lipopolysaccharide (LPS) or 0.9% saline (Veh.); the kidneys were harvested 5 or 24 hours, or 8 days after treatment. The expressions of enzymes were measured via Western blot analysis. Antibody against GAPDH was used as an equal protein-loading marker. The values are expressed as the means ± SE. *P < 0.05 vs. vehicle.

Fig. 3. Activity of calcium-dependent NOS (A) and calcium-independent NOS (B) in kidney tissue after either LPS-treatment or 0.9% saline (Vehicle). C57BL/6 male mice were subjected to either 30 minutes of bilateral renal ischemia or sham-operation 5 or 24 hours after either lipopolysaccharide (LPS)-treatment or 0.9% saline. NOS activities were measured in the kidney tissue after treatment with either LPS for 5 (LPS 5 hrs) or 24 (LPS 24 hrs) hours or Vehicle for 5 hours (Vehicle 5 hrs). Values are expressed as fold increases over vehicle for 5 hours (n = 4). The values are expressed as the means ± SE. * and #P < 0.05 and 0.01 vs. vehicle, respectively.

in the kidney tissues of mice treated with either LPS or vehicle. The level of iNOS expression increased significantly 5 hours after LPS-treatment, and then decreased gradually over time (Fig. 2A). However, eNOS levels were not significantly altered by systemic LPS treatment (Fig. 2B). We then measured the activity of the NOS isoforms. Calcium-independent NOS, i.e., iNOS, activity increased significantly 5 hours after LPS treatment and then declined over time (Fig. 3A). Although the activity of calcium-dependent NOS, which includes eNOS and nNOS, in the kidney tissues after systemic LPS treatment showed the trend of slight increase, however, it was not statistical significance as compared to treatment with vehicle (Fig. 3B).

iNOS, but not eNOS, gene deletion removes the kidney tolerance afforded by LPS pretreatment

We tested the involvement of the iNOS and eNOS proteins in the protection afforded by pretreatment with LPS, using iNOS or eNOS knock-out mice. We confirmed the genotypes of mice via Western blot analysis on the LPS-treated mouse kidneys using anti-iNOS and -eNOS antibodies (Fig. 4A).
hours after initial pretreatment, the concentration of ischemia plasma creatinine in the LPS-pretreated eNOS knock-out mice was significantly lower than that in the vehicle-treated eNOS knock-out mice (Fig. 4B), thus indicating that eNOS is not a critical factor in LPS-induced kidney tolerance. 24 hours after LPS treatment, ischemia plasma creatinine concentrations in the LPS-treated iNOS knock-out mice did not differ significantly from that detected in the vehicle-treated iNOS knock-out mice (Fig. 4B). These data demonstrate that iNOS, but not eNOS, is a critical factor in LPS-induced kidney tolerance. Sham-operation did not increase PCr levels in any of the experimental animals (Fig. 4B). The level of post-I/R PCr in the vehicle-treated iNOS knock-out mice was higher than that in the vehicle-treated eNOS knock-out mice (Fig. 4B), thereby indicating that iNOS gene deletion enhances kidney susceptibility to I/R injury.

**DISCUSSION**

In this study, we found that systemic low-dosage LPS-pretreatment protects kidneys against subsequent I/R insult and that this protective effect was observed in iNOS, but not eNOS, knock-out mice. Furthermore, systemic LPS-pretreatment induced increases in iNOS activity and expression in kidney tissues, but did not cause any detectable increases in eNOS activity or expression. In conclusion, systemic LPS-pretreatment renders the kidneys tolerant against I/R injury, and that this tolerance is mediated by increased activation of iNOS in kidney tissues.

Organs exposed to stress tend to be resistant not only to subsequent identical or local stresses, but also to heterogenous or systemic stresses (4-9). LPS induces the production of cytokines such as TNF-α and interleukins which results in cell damages. It might be expected that organs previously exposed LPS would be more susceptible to a second insult, I/R insult which results in the induction of cytokines and chemokines. It has been reported in animals and human, however, that prior stresses by endotoxins and I/R can confer resistance to subsequent insults (11, 12). Several studies have demonstrated that prior LPS-stress produces protective factors such as NO as well as reduces post-I/R induction of cytokines (11, 13). Hiasa et al reported that LPS-pretreatment attenuates NF-κB activation and gene expression of inflammatory cytokines in rat heart (11). In this study, we determined that a systemic injection of low-dose LPS (1 mg/kg BW)-pretreatment protected kidneys against I/R insults induced 5 and 24 hours after treatment, and that this protective effect had disappeared by 8 days after the LPS treatment. This result shows that the protection is associated with a temporal activation of protective factors and a reduction of post-I/R harmful factors. In a previous study, Heemann et al. reported that high-dose LPS pretreatment protected the kidney almost completely against I/R injury induced 4 days after treatment (5). The protective effect described by Heemann et al. was substantially greater in magnitude and persisted for far longer than was observed in the present study (5). The less profound renal tolerance observed in the present study may, then, be attributable to the level of LPS used relative to the levels used in the study of Heemann et al. (5). With regard to the effects of ischemic preconditioning in rendering the kidney resistant to subsequent I/R injury, we determined that stronger ischemic preconditioning, which induced tissue damage, conferred a longer-lasting and stronger kidney tolerance when compared with weaker ischemic preconditioning, which did not induce tissue damage (12, 14). In the heart,
has been previously reported that momophosphoryl A (MPA), an LPS derivative, provides a cardioprotective effect against I/R injury, and that this protective effect was dependent on the amount of MPA (6, 15). This suggests that the duration and strength of endotoxin-induced tolerance depends on the amount of endotoxin and the pretreatment time; in this regard, it is similar to ischemic preconditioning.

Currently, new evidence is emerging to suggest an association between endotoxin-induced tolerance and increased transcription, novel gene expression, and de novo protein synthesis (16, 17). In the heart, NO/iNOS has been implicated as a candidate for endotoxin-induced organ tolerance (6), but in the kidney this remains unclear. In the present study, we determined that systemic LPS-treatment significantly increased iNOS expression levels in kidney tissues, but induced no increase in eNOS levels. In accordance with the levels of NOS expression, LPS treatment increased calcium-independent NOS (iNOS) activity in kidney tissue, but did not increase calcium-dependent constitutive NOS (eNOS) activity. Furthermore, in studies employing NOS-gene-deleted mice, we determined that LPS pretreatment in iNOS knock-out mice did not protect kidneys against I/R injury, whereas LPS pretreatment applied to eNOS knock-out mice protects the kidneys against I/R injury. This suggests that iNOS mediates LPS-induced kidney tolerance, whereas eNOS does not. In a finding somewhat contradictory to the results of this study, Heemann et al. identified iNOS as a minor factor in LPS-induced kidney tolerance, as they found no significant changes in the expression of iNOS mRNA 3 days after high-dose LPS treatment (5). In our study we detected an increase in iNOS expression within 24 hours after low-dose LPS-treatment, but not 8 days after LPS-treatment. This suggests that iNOS protein activation is transient, and the activation is the deciding factor in kidney tolerance appearing in the early stages after LPS-treatment; however, iNOS is not a critical factor in the delayed protection noted in later stages after LPS-treatment. Wang et al. reported that, in the heart, systemic low-dose LPS treatment increased the expression of iNOS mRNA and proteins at 4 and 6 hours after treatment, but not at 24 hours after treatment, and that treatment with the selective iNOS inhibitor, aminoguanidine, eliminated the cardioprotection afforded by LPS treatment; this suggests that iNOS is the mediator of that cardioprotection (6).

A number of previous studies have reported beneficial and harmful effects of NO arising under pathophysiological conditions (2, 14, 18). It has been recognized that low amounts of NO generated by constitutive NOS (eNOS and nNOS) are beneficial (18). There have also been some reports that eNOS overexpression reduces I/R and hypoxic injury in a variety of tissues and cells (19-22). Therefore, we determined the role of eNOS in the involvement of the eNOS protein in LPS-induced kidney tolerance. In the present study we detected no significant changes in eNOS activation after LPS-pretreatment, and eNOS gene deletion did not abolish the LPS-induced protection. These findings indicate that inducible NOS, but not constitutive NOS, is a critical factor in LPS-induced kidney tolerance. In an ischemic preconditioning mouse model, we also determined that eNOS gene deletion did not remove the kidney tolerance acquired by ischemic preconditioning (14). Recently, we reported that eNOS knock-out did not increase the susceptibility of the kidney to I/R injury (2, 14). In agreement with these studies, we determined in this study that eNOS gene deletion did not affect the susceptibility of the kidney to I/R insult, whereas iNOS did.

Finally, the NO generated by increased iNOS activation after LPS-treatment induces the reduction of inflammatory reactions and the acceleration of blood flow in ischemic sites via blood vessel dilation (2, 14, 23, 24), consequently protecting organs against I/R insult. In fact, there have been some reports suggesting that NO donors, such as L-arginine, may protect the kidneys against I/R injury (2), whereas NO inhibitors may exacerbate kidney I/R injury (14). It was also recently reported that peroxynitrite, a product of nitric oxide and superoxide, is a known potent free radical metabolite that induces oxidative stress, functions as a vasodilator, and inhibits platelet aggregation and inflammatory cell adherence, thus ameliorating or reducing I/R injury (16, 25-28).

MATERIALS AND METHODS

Animals

Experiments were conducted with 8-week-old C57BL/6j (wild-type), eNOS knock-out (eNOS -/-), and iNOS knock-out (iNOS -/-) male mice obtained from Jackson Laboratories. The mice were permitted free access to water and standard mouse chow. In all cases, studies were conducted in accordance with the animal experimental procedures of the Animal Care and Use Committee of Kyungpook National University. The kidneys were harvested in liquid nitrogen for biochemical studies.

Ischemia and reperfusion

Mice were anesthetized with pentobarbital sodium (60 mg/kg body weight, BW; intraperitoneally) prior to surgery. Body temperature was maintained at 36-38°C throughout surgery and recovery. The kidney was exposed through a flank incision and then the renal pedicle was clamped with a non-traumatic microaneurysm clamp (Roboz) for 30 minutes. The incision was temporarily closed during ischemia. After the removal of the clamp, reperfusion of the kidneys was confirmed visually. Control mice were subjected to sham-operation. Some mice were treated intraperitoneally with either lipopolysaccharide (LPS, 1 mg/kg body weight, Sigma) or 0.9% saline (vehicle) at 5 or 24 hours, or 8 days prior to ischemia. Each experimental group consisted of more than 4 mice (n = 4-6).

Renal functional parameters

Seventy microliters of blood were extracted from the retro-orbital vein plexus at the times indicated in the figures. Plasma
creatinine (PCR) concentrations were measured using a Beckman Analyzer II (Beckman, Fullerton, CA).

**NOS catalytic assay**

Calcium-dependent or calcium-independent NOS activity was measured with a commercial NOS activity assay kit (Cayman Chemistry Company, Ann Arbor, MI), as described previously (2). The values were normalized to the protein level and expressed in terms of the fold increase over the controls.

**Western blot analysis**

Proteins were extracted from the kidneys as described previously (12). Protein samples were separated on 8% SDS-PAGE gels and then transferred to Immobilon membrane (Millipore, Bedford, MA). The membranes were then incubated with mouse monoclonal anti-iNOS, -eNOS (BD Transduction Laboratories), or GAPDH (Santa Cruz Biotechnology) antibodies, and then with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Signals were visualized and detected using an ECL system (Amersham Biosciences). Band densities were measured using Lab Works 4.5 (n = 4).

**Statistics**

The results were expressed as the means ± SE. Statistical differences among groups were calculated using Student’s t-test. Differences between groups were considered statistically significant at a P value of < 0.05.

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