Lipopolysaccharide Pretreatment Protects from Renal Ischemia/Reperfusion Injury

Possible Connection to an Interleukin-6-Dependent Pathway

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In vivo administration of low doses of lipopolysaccharide (LPS) to rodents can protect these animals from subsequently administrated, usually lethal doses of endotoxin or LPS. In this study we tested the effects of LPS pretreatment on ischemia/reperfusion injury in the kidney. Male C57/B1 mice were pretreated with different doses of LPS or phosphate-buffered saline on days −4 and −3. The right kidney was removed, and the vessels of the left kidney were clamped for 30 or 45 minutes on day 0. Creatinine levels and survival of animals were monitored. To test the involvement of cytokines, additional animals were harvested before (“time 0”) and 15 minutes, 1, 2, 8, and 16 hours after reperfusion for histology, immunohistochemistry, terminal deoxynucleotidyltransferase-mediated UTP end-labeling assay, and reverse transcriptase-polymerase chain reaction analysis (including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, inducible nitric oxide synthase (iNOS), and interferon (IFN)-γ messenger RNA (mRNA)). In controls, renal ischemia of 30 minutes was nonlethal, whereas 73% of the animals died within 48 ± 18 hours, after 45 minutes of ischemia. All different doses of LPS protected the animals from lethal renal ischemia/reperfusion injury. Starting at similar levels, serum creatinine increased significantly in controls but not in LPS-pretreated animals over time. As early as 2 hours after reperfusion, tubular cell damage was significantly more pronounced in controls than in LPS-treated mice. In controls, tubules deteriorated progressively until 8 hours of reperfusion. At this time, more than 50% of tubular cells were destroyed. This destruction was accompanied by a pronounced leukocytic infiltration, predominantly by macrophages. In contrast, LPS pretreatment prevented the destruction of kidney tissue and infiltration by leukocytes. The terminal deoxynucleotidyltransferase-mediated UTP end-labeling assay revealed significantly more apoptotic cells in controls compared with LPS-pretreated animals. IL-1, IFN-γ, and iNOS mRNA expression did not differ between the groups throughout the time points examined. However, the expression of TNF-α mRNA was significantly increased at 2 hours and IL-6 mRNA was significantly down-regulated before ischemia and shortly after reperfusion in the LPS-pretreated kidneys. Therefore, we found that sublethal doses of LPS induced cross-tolerance to renal ischemia/reperfusion injury. Our data suggest that increased TNF-α and reduced IL-6 mRNA expression might be responsible. However, more studies are needed to decipher the exact mechanism. (Am J Pathol 2000, 156:287–293)
fail to initiate the relaxation of vascular smooth muscle cells, release potent vasoconstrictors, and swell; the permeability is increased, and finally, leukocytes and platelets are trapped and accumulate in the microcirculation and the tissue. Eventually this results in a progressive loss of perfusion and further tissue damage.7,8

Both sepsis and endotoxemia are generally regarded as destructive processes.9–12 Preconditioning with endotoxin results in adaptation or tolerance, which is characterized by a reduced systemic response to a subsequent challenge with a large dose of homologous or heterologous endotoxin.9–12 Although endotoxin has been used to induce resistance against a subsequent identical insult,13 it has also been demonstrated that endotoxin provokes cross-tolerance against other forms of injury. Some authors reported endotoxin-derived protection against ischemia/reperfusion injury in myocardium14 and liver.15

In this study, we established a new model for endotoxin-induced cross-tolerance to renal ischemia/reperfusion injury. To gain some insights into the underlying processes, we evaluated cellular infiltration and cytokine production in a second set of experiments.

### Materials and Methods

#### Animals

Male CD57/Bl mice (weight, 20–30 g) were used as experimental animals, maintained on a standard diet, and given water ad libitum. The animal protocol was reviewed and approved by a governmental animal care and research committee.

#### Experimental Design and Operation

Operative procedures were performed under general anesthesia induced by 5.3 mg/100 g nembutal and 0.02 mg/100 g atropin-sulfate administrated intraperitoneally. After a midline laparatomy incision, renal artery and vein of the left kidney were isolated and occluded with a clamp. After ischemia, the clamp was withdrawn, the right kidney was removed, the laparatomy incision was closed, and the animals were allowed to wake up.

In the first step, lethal renal ischemic time was determined in the mice. Two different times of ischemia were examined: 30 minutes and 45 minutes (n = 26/group).

In the second step, we established a model for endotoxin-induced cross-tolerance to lethal renal ischemia/reperfusion injury. Animals were treated according to three different protocols for lipopolysaccharide (LPS) administration (*Escherichia coli*, serotype 0111:B4; Sigma Chemical Co., St. Louis, MO) (Table 1) (n = 12/group).

In the third step, one group of animals was treated with 2 mg/kg LPS on day −4, and 10 mg/kg LPS on day −3, whereas the other animals received vehicle (0.9% NaCl) and served as controls. To determine the role of several cytokines, animals were narcotized and bled, and the kidneys were removed and stored in 4% buffered formalin or liquid nitrogen before ischemia (time 0) or 15 minutes, 1, 2, 8, and 16 hours after reperfusion (n = eight/group/time point).

#### Functional Parameters

Serum creatinine concentrations were determined photometrically with a commercially available test kit (Boehringer Ingelheim, Ingelheim, Germany).

#### Histological Analysis

Paraffin sections of kidneys fixed in 4% neutral buffered formalin were stained with hematoxylin and eosin and periodic acid-Schiff reagent. Samples were coded and examined in a blinded fashion. Tubular damage and leukocyte infiltration were semiquantitatively evaluated on a scale from 0 to 3 (0 = none, 1 = mild, 2 = moderate, 3 = severe). Additionally, neutrophils were manually counted as cells per field of view at ×400 magnification.

#### Immunohistological Analysis

Fresh frozen sections were stained with antibodies against lymphocytes (CD4 and RM45, Pharmingen, San Diego, CA) and macrophages (CD11b, M1/70, Pharmingen) to establish the involvement lymphocytes. Furthermore, we evaluated apoptosis with the TUNEL assay based on the description of the manufacturer (Boehringer Mannheim, Mannheim, Germany).

Positively stained cells were counted at ×400 magnification and described as cells per field of view.

#### Reverse Transcriptase-Polymerase Chain Reaction

**Total RNA Isolation**

Total RNA was extracted and used for reverse transcriptase-polymerase chain reaction (RT-PCR). A part of the kidney was stored in 500 μl of cold lysis solution,
containing 4 mol/L guanidine isothiocyanate (Sigma), 25 mmol/L sodium citrate (pH 7.0), 0.1 mol/L β-mercapto-ethanol, and 0.5% sarcosyl, and frozen in liquid nitrogen. Total RNA was extracted by the modified guanidine-isothiocyanate preparation method. Briefly described, frozen tissues were mixed with 4 ml guanidine isothiocyanate buffer (4 mol/L guanidine isothiocyanate; Sigma) and acid phenol-chloroform (pH 4; Sigma), and homogenized. The samples were centrifuged at 1500 g for 10 minutes at 20°C. The supernatant was treated with an equal volume of isopropanol. The mixture was centrifuged, and the RNA was washed with RNeasy Total RNA Isolations Kit (Qiagen GmbH, Germany) and stored at −80°C until further processing. RNA concentration was measured spectrophotometrically.

Reverse Transcription

RNA was amplified by reverse transcription (RT) with an oligo(dT)12-18 primer (Life Technologies, Inc., Grand Island, NY). Total RNA (1 μg) was added to 0.5 μg of primer. A reaction mixture was added containing buffer solution (50 mmol/L Tris-hydrochloride buffer, pH 8.3, 75 mmol/L potassium chloride, 5 mmol/L magnesium dichloride, 5 mmol/L dithiothreitol; Life Technologies, Inc.); 1 mmol/L each of adenosine triphosphate, thymidine triphosphate, guanosine triphosphate, and cytosine triphosphate (deoxynucleoside triphosphates from Boehringer Mannheim GmbH); 40 U/μl of recombinant ribonuclease inhibitor (Promega), and 0.5 μl of 200-U/μl Maloney-murine leukemia virus reverse transcriptase (Life Technologies), and the first chain reaction was allowed to proceed (36°C, 1 h). The reaction was halted by heating to 95°C for 5 minutes followed by cooling on ice.

Amplification of Specific Complementary DNA

Specific complementary-DNA products corresponding to mRNA for TNF-α, IFN-γ, IL-1, IL-6, inducible nitric oxide synthase (iNOS), and β-actin were amplified by PCR. A 1-μl sample was taken from the RT reaction for PCR, which was performed in PCR buffer (750 mmol/L Tris-hydrochloride, pH 9.0, 200 mmol/L (NH₄)₂SO₄, 0.1% (w/v) Tween, 20 mmol/L magnesium dichloride; Dianova, Hamburg, Germany), using 0.2 mmol/L of each deoxynucleoside triphosphate, 1 μmol/L of both primers (Eurogentec, Belgium), and 2.5 U Thermus aquaticus (Taq) DNA polymerase (Dianova). A Perkin-Elmer Thermal Cycler (Model 2400, Perkin-Elmer, Norwalk, CT) was used for amplification with the following sequence profile: initial denaturation at 94°C for 3 minutes followed by 30–40 cycles of three-temperature PCR (denaturing, 94°C for 30 seconds; annealing, 55°C for 30 seconds; extension, 72°C for 30 seconds) and ending with a final extension at 72°C for 7 minutes and cooling to 4°C.

Gel Electrophoresis

The amplified PCR product was identified by electrophoresis of 10-μl sample aliquots on 1.5% agarose gel stained with 0.5 μg/ml of ethidium bromide. The sample products were visualized by UV transillumination, and the gel was photographed. Specific products were identified by size in relation to a known 1-kb oligonucleotide DNA ladder (Life Technologies) run with each gel. Cytokine complementary DNA was semiquantitated by densitometric comparison with β-actin (internal control) from the same sample after the positive image was digitized by video for computerized densitometry. The results are given as the ratio of intensity of cytokines and β-actin mRNA ± SEM.

Statistical Analysis

Data were expressed as mean ± SEM. Parametric data were compared using Student’s t-test. Nonparametric data were tested using Mann-Whitney analysis of ranks. P < 0.05 was accepted as statistically significant.

Results

Survival after Different Times of Ischemia

After 30 minutes of renal ischemia, all animals (n = 26) survived until the end of the follow-up (15 days). After 45 minutes of renal ischemia (n = 21), 20 animals died after 48 ± 24 hours (74%). One animal survived for 13 days (Figure 1).

Effect of Pretreatment with LPS on Ischemia/Reperfusion Injury

After pretreatment with LPS, all animals (n = 35) survived for more than 50 days, independent of the administration protocol. Therefore, 45 minutes of ischemia was chosen for the following experiments.

Whereas the body weight of vehicle-treated animals remained constant during the period observed, it decreased in LPS-treated mice after the first administration by approximately 10% and recovered thereafter.

Serum creatinine was similar before and 15 minutes after perfusion in both groups (controls: 1.2 ± 0.06 and 0.76 ± 0.07; versus LPS: 0.94 ± 0.11 and 0.65 ± 0.06 mg/dl). One hour after ischemia, creatinine had in-
increased in both groups (controls: 1.69 ± 0.19; versus LPS: 1.11 ± 0.17 mg/dl). However, this increase was more pronounced in controls. Thereafter creatinine remained constant in the LPS group (1.21 ± 0.16 mg/dl), whereas it increased in controls (3.03 ± 0.18 mg/dl). One hour after ischemia, the differences between the groups reached statistical significance (Figure 2).

**Histology and Immunohistochemistry**

Immediately before ischemia, minor tubular damage was observed in the LPS group, although kidneys from controls had no apparent morphological changes. At 15 minutes after reperfusion, tubular damage was similar in LPS- and vehicle-treated animals. However, the damage progressed slowly in LPS-pretreated animals up to 2 hours after reperfusion and returned to a normal appearance after 8 hours. The degree of leukocyte infiltration was low in these animals. Kidneys of vehicle-treated animals deteriorated dramatically over time with an accompanying strong infiltration of leukocytes. The infiltrate consisted predominantly of neutrophils. By 8 hours after reperfusion, almost half of the kidney was destroyed in these animals (Table 2 and Figure 3). Furthermore, we observed more cells undergoing apoptosis in controls than in LPS-pretreated animals. As revealed by the TUNEL assay, 1.80 ± 0.64 cells per field of view stained positive in LPS-pretreated animals compared with 0.53 ± 0.17 in controls at 16 h after ischemia (P < 0.01).

**RT-PCR**

At 2 hours, the expression of TNF-α mRNA was significantly higher in the LPS group than in controls. Furthermore, in LPS-pretreated animals, macrophage-associated IL-6 mRNA levels were threefold lower before ischemia than in controls and remained significantly lower at 15 minutes after reperfusion, but these levels increased to higher levels than in controls thereafter. On the other hand, in both groups, IL-1 was up-regulated at 15 minutes, low until 2 hours, and up-regulated at 8 and 16 hours. The mRNA patterns of IFN-γ, IL-1, and iNOS did not differ between the groups (Figure 5).

**Discussion**

Tissue adaptation to repeated stress has long been described. However, the observation that cells can acutely adapt to an insult and then revert back to a resting or

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**Table 2.** Quantification of Kidney Damage Before and After Ischemia/Reperfusion Injury

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Groups</th>
<th>Leukocyte infiltration</th>
<th>Nucleus atypia</th>
<th>Vacuolization in the tubular cells</th>
<th>Hyalinization in the tubular cells</th>
<th>Dissolve of the tubular cells</th>
<th>Coming off of the tubular cells</th>
<th>Lack of the tubular cells</th>
<th>Hyaline in the tubules</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>0.0 ± 0.0</td>
<td>0.00 ± 0.0</td>
<td>0.00 ± 0.0</td>
<td>0.00 ± 0.0</td>
<td>0.00 ± 0.0</td>
<td>0.00 ± 0.0</td>
<td>0.00 ± 0.0</td>
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<td></td>
<td>LPS</td>
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<td>0.58 ± 0.20</td>
<td>0.14 ± 0.14</td>
<td>0.00 ± 0.0</td>
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<tr>
<td>0.25</td>
<td>Controls</td>
<td>0.5 ± 0.15</td>
<td>0.40 ± 0.16</td>
<td>0.10 ± 0.01</td>
<td>0.00 ± 0.0</td>
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<tr>
<td></td>
<td>LPS</td>
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<td>0.88 ± 0.22</td>
<td>0.50 ± 0.19</td>
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<td>1</td>
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<td>0.66 ± 0.24</td>
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<td>2.22 ± 0.15**</td>
<td>1.67 ± 0.17</td>
<td>1.22 ± 0.15**</td>
<td>1.76 ± 0.15**</td>
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<tr>
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<td>0.44 ± 0.17</td>
<td>0.78 ± 0.13</td>
<td>0.22 ± 0.13</td>
<td>0.89 ± 0.11</td>
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<td>0.56 ± 0.24</td>
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<tr>
<td>16</td>
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<td>2.44 ± 0.17**</td>
<td>2.00 ± 0.16**</td>
<td>1.33 ± 0.16**</td>
<td>1.78 ± 0.15**</td>
<td>2.44 ± 0.17**</td>
<td>1.78 ± 0.15**</td>
<td>2.11 ± 0.26**</td>
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<tr>
<td></td>
<td>LPS</td>
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<td>0.56 ± 0.24</td>
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</table>

* P < 0.05; ** P < 0.01.
steady-state phenotype has only recently been elucidated. One of the first descriptions of acute adaptation was reported in HeLa cells,\textsuperscript{18} in which sublethal hyperthermic stress conferred tolerance to a subsequent hyperthermic insult. Conditioning the cells against thermal insult does not only seem to be conserved across many cell types but may also induce cross-tolerance to other forms of injury. In this study, we demonstrated for the first time that repeated LPS pretreatment protected mice from otherwise lethal renal ischemia/reperfusion injury. Brown et al\textsuperscript{10} and Colletti et al\textsuperscript{15} have previously reported that endotoxins protect from myocardial and hepatic ischemia/reperfusion. However, they did not analyze cytotoxic patterns, nor did they study a similar model of ischemia/reperfusion injury.

Despite experiments, the underlying mechanisms and cellular mediators responsible for the endotoxin-related tolerance to ischemia/reperfusion injury remain elusive. It is known that polymorphonuclear cells are involved in this process. The results of in vitro and in vivo experiments have supported the conclusion that macrophage-associated cytokines, such as TNF-\(\alpha\), IL-1, IL-6, and IFN-\(\gamma\), may play a major role in this process.\textsuperscript{19} The most widely examined cytokine in the development of endotoxin tolerance is TNF-\(\alpha\). We demonstrated an increased level of TNF-\(\alpha\) mRNA expression after renal ischemia in LPS-pretreated animals. However, despite an increased TNF-\(\alpha\) mRNA level, renal injury was reduced. These results indicate an involvement of TNF-\(\alpha\) and particularly a missing response to TNF-\(\alpha\). It is known that TNF-\(\alpha\) appears almost immediately on LPS injection in mice. However, repeated injections of LPS resulted in unresponsiveness, and no further TNF-\(\alpha\) production was detectable for at least days.\textsuperscript{7} In contrast to our experiment, these results indicated a direct involvement to TNF-\(\alpha\) because the level of TNF-\(\alpha\) correlated to LPS unresponsiveness.

However, the TNF-\(\alpha\) level also increased in another experiment after endotoxin pretreatment and hepatic ischemia/reperfusion injury, and again this elevation correlated to a protection against lung injury after hepatic ischemia/reperfusion.
The mechanism for the enhanced production of TNF-α in animals treated with LPS and subsequently subjected to ischemia is difficult to explain. The most likely hypothesis for the protective effects induced by nonlethal LPS administration is a transient induction of TNF-α. This early increase of TNF-α will then provide protection against the subsequent organ damage by a down-regulation of TNF receptors. Otherwise, it is still hard to explain why the damages were reduced by LPS pretreatment despite a higher TNF-α mRNA expression.

In parallel to these results, we were not able to detect any differences in the kinetics of IL-1 and IFN-γ mRNA expression between the groups. However, the level of IL-6 mRNA was significantly reduced before ischemia and shortly after reperfusion in LPS-pretreated animals. In an in vivo study, Hewitt et al demonstrated that intermittent ischemia (preconditioning) resulted in significantly decreased IL-6 and TNF-α expression as compared with continuous ischemia (without preconditioning). In addition, it has been reported that daily injections of LPS in rats resulted in a measurable release of TNF-α and IL-6 within the first few days only and was abrogated thereafter. Our data indicate that repeated treatment of endotoxin can inhibit IL-6 mRNA expression in the injured organ. We think that reduced IL-6 expression may reflect a state of unresponsiveness of macrophages. This may be related to down-regulation of the TNF receptors. On the other hand, IL-6 by itself is a strong chemoattractant and may lead to the infiltration and consequent activation of leukocytes. This hypothesis would explain the reduced infiltration observed in the LPS group. The reduced infiltration by these leukocytes could further explain the lower degree of damage and apoptosis in these animals.

However, another hypothesis can be postulated. Hypoxia is one of the most pronounced promoters of necrosis/apoptosis by itself. As a result of necrosis/apoptosis, leukocytes infiltrate to phagocytose the damaged tissue. If LPS pretreatment reduces the susceptibility of tissue cells to hypoxia-induced cell death per se, the degree of damaged tissue would be reduced, and fewer leukocytes would infiltrate the affected organs.

Cytokines induce iNOS gene expression and thus increase NO levels. After ischemia and reperfusion, injured vascular cells and adherent leukocytes produce free radicals and inactivate NO, which may promote vasocostriction and increase permeability, local edema, and leukocyte adhesion. Because we did not find any differences in iNOS mRNA expression between our groups, we would conclude that NO is of minor importance for ischemia/reperfusion in our model. However, we examined mRNA expression of cytokines only in organs and not in the blood or specifically in the endothelium. Thus, it is possible that differences related to certain cells, eg, endothelial cells, were not detected. Additionally, we used RT-PCR to detect the mRNA levels of cytokines, while others examined the protein level in the blood or, for endotoxin tolerance, in the peritoneal macrophages. Whole-blood assays are certainly reasonable in septicemia, but, based on the short half-life of the mediators involved, these assays seem to poorly reflect processes in a single organ such as the kidney. Therefore, we did not perform such assays. Furthermore, although peritoneal macrophages are undoubtedly a good model for LPS-tolerance, their function and relation to renal injury are unknown and hard to predict. Additionally, most experiments were performed in vitro and not in vivo.

How LPS pretreatment confers protection against renal ischemia/reperfusion injury remained unclear but is undoubtedly multifactorial. Early investigators believed that low-dose LPS stimulates the reticulo-endothelial system such that subsequent larger doses of LPS are more actively cleared. More recently, the predominant mechanism emphasized is that low-dose LPS alters monocyte secretion of a variety of inflammatory mediators including proteolytic enzymes, arachidonic acid metabolites, reactive oxygen species, and cytokines. On the other hand, low-dose LPS could directly down-regulate the expression of endothelial-cell adhesion receptors, which could account for the decrease in tissue sequestration of neutrophils. Key molecules involved in this endothelial cell-neutrophil adhesion are intracellular adhesion molecule-1 and E-selectin. Both are an ideal molecular indicator of endothelial cell activation in response to ischemia/reperfusion injury. In vitro models, hypoxia/reoxygenation stimulated endothelial cells to express adhesion molecules and increased adhesion of neutrophils to the endothelial surface. Neutrophil depletion has been shown to protect rats against ischemic renal injury in some studies. Thus, given the complexity of events preceding neutrophil invasion into the tissues, one could postulate that LPS pretreatment may inhibit the activation of endothelial cells.

Although renal failure after ischemia and reperfusion can be managed by dialysis, such injury influences the outcome of transplantation, revascularization procedures, and episodes of hypoperfusion. In this experiment, we developed a new model for endotoxin-induced cross-tolerance to renal ischemia/reperfusion injury. Although this model may seem quite extreme, similar damages may occur in the clinical setting for non-heart-beating donors. TNF-α and IL-6 may play a central role in this process. However, future studies are needed to decipher the exact causes of the increased survival in this model.

References


