TNF-α-dependent bilateral renal injury is induced by unilateral renal ischemia-reperfusion

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Meldrum, Kirstan K., Daniel R. Meldrum, Xianzhong Meng, Lihua Ao, and Alden H. Harken. TNF-α-dependent bilateral renal injury is induced by unilateral renal ischemia-reperfusion. Am J Physiol Heart Circ Physiol 282: H540–H546, 2002; 10.1152/ajpheart.00072.2001.—While tumor necrosis factor (TNF)-α is an important mediator of renal ischemia-reperfusion (I/R) injury, its role in contralateral renal injury after isolated renal ischemia remains unknown. We therefore investigated the effect of isolated left renal ischemia on the nonischemic contralateral kidney. To study this, male Sprague-Dawley rats were anesthetized and exposed to varying degrees of left renal I/R injury. Both kidneys were subsequently harvested, serum samples were obtained, and TNF-α protein expression (ELISA), TNF-α mRNA content (RT-PCR), TNF-α immunolocalization, and neutrophil infiltration (myeloperoxidase assay) were determined. The effect of TNF-α on neutrophil infiltration was assessed by neutralizing TNF-α with TNF binding protein (TNF-BP) before left renal I/R injury. TNF-α protein expression, TNF-α mRNA induction, and neutrophil infiltration increased significantly in both kidneys after unilateral renal I/R injury. Furthermore, the administration of TNF-BP before unilateral renal I/R substantially reduced the degree of neutrophil infiltration bilaterally. These results constitute the initial demonstration that unilateral renal I/R induces bilateral TNF-α production and neutrophil infiltration through a TNF-α-dependent mechanism.

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humane care in compliance with the National Institutes of Health’s (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985).

Chemicals and reagents. Recombinant human TNF binding protein (TNF-BP) was kindly supplied by Dr. Carl Edwards (Amgen; Boulder, CO). TNF-BP is expressed in Escherichia coli as the four extracellular domains of the p55 TNF receptor linked together at the Fc portion of IgG. TNF-BP was diluted in normal saline containing 0.25% human serum albumin. Negative control rats received vehicle (0.25% human serum albumin) pretreatment before I/R. Unless specifically mentioned, all other chemicals were obtained from Sigma (St. Louis, MO).

Experimental groups and operative technique. The entire left renal pedicle (artery and vein) was isolated and occluded with anatraumatic snare, and the abdomen was subsequently closed. Sham animals underwent identical surgical treatment, including isolation of the renal pedicle; however, occlusion of the pedicle was not performed. In the reperfusion treatment groups, the renal pedicle snare was removed externally without the need for abdominal reentry. The animals were able to breathe spontaneously and were maintained under a heat lamp throughout the duration of the experiment. After abdominal closure, the animals were allowed to awake spontaneously. Mean arterial pressure (MAP), oxygenation (pulse oximetry), and temperature were monitored and recorded in each animal. Upon completion of the experiment, the animals were reanesthetized, both kidneys were removed and frozen in liquid nitrogen, serum samples were taken, and the animals were subsequently euthanized. The samples were stored at −70°C until further testing could be performed. The animals were divided into the following experimental groups: 1) 1-h sham operation (negative control, n = 4); 2) 2-h sham operation (negative control, n = 4); 3) 3-h sham operation (negative control, n = 4); 4) 5-h sham operation (negative control, n = 4); 5) 15 min of ischemia alone (n = 3); 6) 30 min of ischemia alone (n = 3); 7) 45 min of ischemia alone (n = 3); 8) 1 h of ischemia alone (n = 6); 9) 1 h of ischemia followed by 1 h of reperfusion (n = 6); 10) 1 h of ischemia followed by 2 h of reperfusion (n = 6); and 11) 1 h of ischemia followed by 4 h of reperfusion (n = 6). To determine the effect of TNF-α expression on neutrophil accumulation in both kidneys, TNF-α was neutralized (TNF-BP) before the animals were exposed to 1 h of left renal ischemia and 4 h of reperfusion (n = 6). TNF-BP (160 mg) suspended in 0.5 ml PBS was administered intravenously 30 min before injury. This dose was based on previous experiments demonstrating prevention of I/R-induced renal neutrophil accumulation and injury (10).

Tissue homogenization. A portion of each kidney was homogenized for the TNF-α ELISA assay. Homogenization was performed after the samples had been diluted in 4 volumes of homogenate buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EGTA, 1 mM dithiothreitol, and Complete Protease Inhibitor tabs (Boehringer-Mannheim; Indianapolis, IN)) using a vertishear tissue homogenizer. Renal homogenates were centrifuged at 3,000 g for 15 min, supernatant total protein concentration was quantified using the Lowry assay, and supernatents were stored at −70°C until the TNF-α ELISA assay could be performed.

TNF-α protein expression. Renal homogenate and serum TNF-α protein content were determined using ELISA. ELISA was performed by adding 100 μl of each sample to wells in a 96-well plate of a commercially available ELISA kit (R&D Systems). The samples were tested in duplicate. According to the manufacturer, this ELISA is highly specific for TNF-α, with a detection limit of 15 pg/ml. TNF-α ELISA was performed according to the manufacturer’s instructions. Final results were expressed as picograms of TNF-α per milligram of protein (tissue) or per milliliter (serum).

RT-PCR. Semi-quantitative RT-PCR was used to assess renal TNF-α gene expression. Renal tissue was obtained from sham-operated controls and both the injured and contralateral kidney after an early time course of graded left renal ischemia and reperfusion (three samples per time point). Total RNA was extracted from the tissue by homogenization in TRIzol (GIBCO-BRL; Gaithersburg, MD) and then isolated by precipitation with chloroform and isopropyl alcohol. Two micrograms of the isolated RNA were subjected to RT-PCR with reverse transcriptase using random hexadecamers as primers (Promega; Madison, WI). The samples were incubated for 10 min at 70°C, chilled for 5 min, and, after the addition of SuperScript II RT (Life Technologies; Gaithersburg, MD), incubated at 37°C for 1 h. PCR was performed by adding 1 μl of RT product to PCR SuperMix containing Taq DNA polymerase (GIBCO-BRL). For each RT sample, PCR for TNF-α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 60 pmol primer) were performed. Thirty picomoles of each TNF-α primer sequence (sense: 5′-TTC CTC ACT CAC ACC ATC AGC C-3′; antisense: 5′-TGC CCA GAT TCA GCA AAG TCC-3′) were used, yielding a 224-bp product. The samples were loaded in a thermocycler and run for 3 min at 94°C, 34 cycles of 94°C for 1 min, 55°C for 2 min, and then 72°C for 2 min. The samples were run for an additional 7 min at 72°C and held at 4°C until loaded onto the gel. The amplified products were separated in a 2% agarose gel containing 1× Tris-borate-EDTA; pH 8.3. PCR amplification products were quantified by staining the gel with ethidium bromide and determining the density of each band using NIH Image analysis software (version 1.62). The data are presented as the ratio of the densitometric units of the TNF-α mRNA band to the densitometric units of the GAPDH mRNA band.

Immunolocalization of TNF-α. Immunolocalization of renal TNF-α production was determined using sections of renal tissue obtained from sham-operated animals and the ipsilateral and contralateral kidney in animals exposed to 1 h of left renal ischemia followed by 2 h of reperfusion (time point of maximal TNF-α production). Transverse 5-μm cryosections were prepared with a cryostat (2800 Frigocut E. Reichert-Jung) and collected on poly-L-lysine-coated slides. All sections were fixed for 10 min in 70% acetone-30% methanol at −20°C. Normal goat serum was applied as a blocking agent, and the slides were washed in PBS three times for 3 min. Sections were then incubated with diluted primary antibody (rabbit anti-rat TNF-α polyclonal antibody, 1:200 dilution, Genzyme; Cambridge, MA) for 1 h. The sections were washed with PBS and incubated with Cy-3-conjugated goat anti-rabbit IgG for 45 min. After a wash with PBS, the nuclei were stained with bis-benzimide (10 μg/ml in PBS) for 30 s, and the slides were washed with PBS three times for 2 min. Cell membranes were counterstained with Oregon green 488-labeled wheat germ agglutinin (1:100 dilution, Molecular Probes) for 30 min and washed with PBS three times. The slides were mounted with a glycerol-based anti-quinching agent, o-phenylene diamine-d-HCl, and stored at −4°C. The specificity of the TNF-α antibody was assessed by incubating adjacent sections from each experimental group with goat anti-rat TNF-α antibody before incubation with the secondary antibody and confirming elimination of the TNF-α signal. To test for nonspecific fluorescence, adjacent sections from each experimental group were incubated with nonimmune purified rabbit IgG instead of the primary antibody. Nonspe-
specific fluorescence was digitally subtracted, and the sections were photographed with a confocal microscope.

**Renal tissue myeloperoxidase.** Myeloperoxidase (MPO) is an enzyme specific for neutrophils and is an accepted index of neutrophil accumulation. Renal samples were obtained from sham-operated animals and the injured and contralateral kidney in animals undergoing 1 h of left renal ischemia, followed by 4 h of reperfusion (with or without pretreatment with TNF-BP). The tissue was homogenized for 30 s in 4 ml of 20 mM potassium phosphate buffer; pH 7.4. The samples were centrifuged for 30 min at 40,000 g at 4°C (Beckman L-80 ultracentrifuge, Beckman Instruments; Palo Alto, CA). The supernatant was discarded, and the pellet was resuspended in 4 ml of 50 mM potassium phosphate buffer (pH 6.0) with 0.5 g/dl cetrimonium bromide. The samples were then sonicated for 90 s (ultrasonic homogenizer, Cole-Parmer Instruments; Chicago, IL) and incubated for 2 h at 60°C. Homogenates were centrifuged at 14,000 g for 10 min. The supernatant was decanted, and 25 µl were added to 725 µl of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine and 5 × 10⁻⁴ M hydrogen peroxide. The change in absorbance was measured spectrophotometrically (Beckman DU/7 spectrophotometer, Beckman Instruments; Irvine, CA) at 460 nm. One unit of MPO activity was defined as the quantity of enzyme degrading 1 µmol of peroxide per minute at 25°C.

**Statistical analysis.** Data are presented as mean values ± SE. Differences at the 95% confidence level were considered significant. The experimental groups were compared using ANOVA with a post hoc Bonferroni-Dunn test (StatView 4.0).

**RESULTS**

**Vital parameters.** The vital parameters collected and recorded during each experiment are listed in Table 1. In all treatment groups, MAP remained above 60 mmHg, oxygen saturation remained above 93%, and core temperature remained above 36°C.

**Time course of bilateral renal TNF-α production after renal ischemia and reperfusion.** The time course of bilateral renal TNF-α production after varying lengths of unilateral renal ischemia and reperfusion is shown in Fig. 1. The values are represented as picograms of TNF-α per milligram of protein. Sham-treated animals demonstrated low levels of TNF-α expression (20.6 ± 1.3, 22.5 ± 0.87, 22.8 ± 1.7, and 18 ± 0.9 pg TNF-α/mg protein for 1-, 2-, 3-, and 5-h shams, respectively). At 1 h of isolated ischemia, TNF-α production increased in both the ipsilateral and contralateral kidney compared with the 1-h sham (26.3 ± 1.5 pg TNF-α/mg protein, P < 0.05 vs. sham, and 30.3 ± 3.3 pg TNF-α/mg protein, P < 0.05 vs. sham, respectively). TNF-α expression increased further in both kidneys after 1 h of ischemia and 1 h of reperfusion (33 ± 3.3 pg TNF-α/mg protein, P < 0.05 vs. 2-h sham (ipsilateral), and 37 ± 3.6 pg TNF-α/mg protein, P < 0.05 vs. 2-h sham (contralateral), and TNF-α expression peaked in both kidneys after 1 h of ischemia and 2 h of reperfusion (37 ± 5 pg TNF-α/mg protein, P < 0.05 vs. 3-h sham (ipsilateral), and 39 ± 3 TNF-α pg/mg protein, P < 0.05 vs. 3-h sham (contralateral)). After 4 h of reperfusion, TNF-α levels decreased toward baseline in both kidneys (20 ± 2.7 (ipsilateral) and 25 ± 2.0 pg TNF-α/mg protein, P < 0.05 vs. 5-h sham (contralateral)).

**Time course of serum TNF-α protein expression.** The time course of serum TNF-α protein expression after varying lengths of unilateral renal I/R injury is shown in Fig. 2. The values are represented as picograms of TNF-α per milliliter serum. Sham-treated animals demonstrated low serum levels of TNF-α (2.8 ± 2.8, 0 ± 0, 0 ± 0, and 0 ± 0 pg TNF-α/ml serum for 1-, 2-, 3-, and 5-h shams, respectively) as did animals exposed to 1 h of isolated left renal ischemia (0.2 ± 0.2 pg TNF-α/ml serum), 1 h of left renal ischemia followed by 2 h of reperfusion (0 ± 0 pg TNF-α/ml serum), and 1 h of left renal ischemia followed by 4 h of reperfusion (3.6 ± 3.6 pg TNF-α/ml serum). In contrast, serum TNF-α levels increased significantly after 1 h of left renal ischemia and 1 h of reperfusion (56 ± 20 pg TNF-α/ml serum, P < 0.05 vs. sham).

**Time course of bilateral renal TNF-α mRNA induction after ipsilateral renal ischemia.** Tissue samples from both kidneys were obtained after an early time

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**Table 1. Vital parameters collected and recorded during each experiment**

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>MAP, mmHg</th>
<th>Oxygen Saturation, %</th>
<th>Temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1 h</td>
<td>70 ± 6</td>
<td>96 ± 1.5</td>
<td>37 ± 0</td>
</tr>
<tr>
<td>2 h</td>
<td>60 ± 6</td>
<td>98 ± 1</td>
<td>37 ± 0</td>
</tr>
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<td>3 h</td>
<td>83 ± 12</td>
<td>95 ± 0.5</td>
<td>37 ± 0.5</td>
</tr>
<tr>
<td>5 h</td>
<td>95 ± 10</td>
<td>93 ± 1</td>
<td>37 ± 0</td>
</tr>
<tr>
<td>1-h Isolated ischemia</td>
<td>64 ± 1</td>
<td>94 ± 2</td>
<td>37 ± 0</td>
</tr>
<tr>
<td>1-h Ischemia/1-h reperfusion</td>
<td>88 ± 13</td>
<td>95 ± 1</td>
<td>37 ± 0.5</td>
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<td>1-h Ischemia/2-h reperfusion</td>
<td>85 ± 25</td>
<td>94 ± 0.5</td>
<td>37 ± 0</td>
</tr>
<tr>
<td>1-h Ischemia/4-h reperfusion</td>
<td>95 ± 10</td>
<td>95 ± 2.5</td>
<td>36 ± 0</td>
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Values are means ± SE. MAP, mean arterial pressure.
course of graded left renal ischemia. Sham-operated animals did not demonstrate any TNF-α mRNA induction (Fig. 3, A and B). TNF-α mRNA increased significantly in both the normal contralateral and injured kidney after 30 min of isolated left renal ischemia. Densitometric analysis of TNF-α mRNA expression as a percentage of GAPDH mRNA is shown in Fig. 3B. After 15 min of ischemia, the TNF-α mRNA expressed represented 55 ± 27% and 104 ± 42% of GAPDH mRNA in the ipsilateral and contralateral kidney, respectively. After 30 min of ischemia, the TNF-α mRNA expressed represented 119 ± 12% and 120 ± 30% of GAPDH mRNA in the ipsilateral (P < 0.05 vs. sham) and contralateral kidney (P < 0.05 vs. sham), respectively. TNF-α mRNA expression was not significantly increased over the sham in either kidney after 45 min (44 ± 8.6% of GAPDH mRNA (ipsilateral) and 41 ± 4.1% of GAPDH mRNA (contralateral)) or 1 h (46 ± 21% of GAPDH mRNA (ipsilateral) and 48 ± 22% of GAPDH mRNA (contralateral)) of ipsilateral ischemia.

**Immunolocalization of renal TNF-α production.** Renal samples were obtained from sham-operated animals and the ipsilateral and contralateral kidney in animals exposed to 1 h of ipsilateral ischemia followed by 2 h of reperfusion. Sections of each renal sample were stained for TNF-α using immunohistochemical techniques to determine the cellular localization of TNF-α production in each treatment group. Only trace amounts of TNF-α were detected in samples obtained from sham-operated animals (Fig. 4A). In contrast, easily visible TNF-α (red stain) was present in both the ipsilateral and contralateral kidney after left renal I/R injury (Fig. 4, B and C). TNF-α production localized primarily to renal tubular cells in the injured kidney after I/R. The nonischemic contralateral kidney exhibited TNF-α staining in both tubular and glomerular cells.

**Renal tissue neutrophil accumulation.** The MPO assay was performed on renal samples obtained from sham-operated animals and animals exposed to 1 h of left renal ischemia followed by 4 h of reperfusion. Animals undergoing renal I/R were treated with either vehicle or TNF-BP 30 min before injury. MPO levels were elevated in both kidneys (Fig. 5) after left I/R (23 ± 3 U/g in ipsilateral vs. 4 ± 1.4 U/g in sham, P < 0.05; 17 ± 1.5 U/g in contralateral vs. 3 ± 1 U/g in sham, P < 0.05). As expected, MPO levels were higher in the ischemic kidney than the nonischemic contralateral kidney. Interestingly, pretreatment with TNF-BP decreased (P < 0.05 vs. sham) the MPO levels in both kidneys (11 ± 2 U/g in ipsilateral and 9 ± 1.7 U/g in contralateral) to a similar degree.

**DISCUSSION**

This study constitutes the initial demonstration that unilateral renal ischemia induces bilateral renal TNF-α production and TNF-α-dependent neutrophil infiltration. We (10) have previously shown that TNF-α is an important mediator of renal I/R injury and dem-

![Fig. 2. Time course of serum TNF-α levels (ELISA) during unilateral ischemia-reperfusion. Serum TNF-α levels increased significantly after 1 h of unilateral renal ischemia and 1 h of reperfusion.](http://ajpheart.physiology.org/)
onstrated that TNF-α induces neutrophil sequestration and renal dysfunction after I/R. TNF-α produced in response to ischemia causes local cellular injury through a variety of mechanisms. In addition to recruiting and activating various cells within the immune system, TNF-α is a proinflammatory agent that stimulates the production of other inflammatory mediators. Furthermore, TNF-α is directly cytotoxic to many cells (3, 4, 6, 8, 11, 13, 21, 28, 30, 34). While TNF-α is clearly an important mediator of local I/R injury, its role in remote organ damage after isolated ischemia is just beginning to be elucidated. Investigators have demonstrated TNF-α production from remote sites (lung) after I/R and linked TNF-α production to neutrophil-mediated pulmonary injury (32, 38). Indeed, TNF-α production may contribute to the occasional development of adult respiratory distress syndrome and multiorgan injury after single organ ischemia and reperfusion.

Clinically, renal ischemia/infarction is managed with observation. Several studies (1, 9, 17) have demonstrated that conservative management of this condition is apparently safe and not associated with life-threatening complications. In light of mounting evidence implicating TNF-α production after single organ I/R in remote organ damage, we investigated the effect of unilateral renal ischemia on the nonischemic contralateral kidney. Our results indicate that unilateral renal ischemia induces TNF-α production in both the ipsilateral (injured) and nonischemic contralateral kidney after 0, 1, or 2 h of reperfusion. After 4 h of reperfusion, TNF-α levels decline toward baseline in both the ipsilateral and contralateral kidney. Serum levels of TNF-α reflect tissue levels, with peak serum TNF-α expression occurring after 1 h of left renal ischemia and 1 h of reperfusion. Given these observations, RT-PCR was performed on renal samples to confirm that the observed elevation in contralateral kidney TNF-α was due to cellular production of, and not circulating, TNF-α. TNF-α mRNA induction occurred in both the normal contralateral and injured kidney after 30 min of isolated left renal ischemia but
became undetectable after 45 min or 1 h of isolated left renal ischemia. Interestingly, contralateral TNF-α mRNA induction occurred before reperfusion of the ischemic ipsilateral kidney. This pattern of mRNA induction supports the observed time course of TNF-α protein expression and suggests that contralateral renal TNF-α production during isolated ipsilateral renal ischemia may represent, in part, a “stress” phenomenon.

To provide further evidence of TNF-α production and to localize the intrarenal source of TNF-α in the contralateral kidney, immunohistochemistry was performed. Indeed, we detected intracellular TNF-α in both the ipsilateral and contralateral kidney. Interestingly, the pattern of TNF-α production differed between the two kidneys. In the nonischemic kidney, TNF-α production was more uniformly distributed between glomerular and tubular cells. In contrast, the ischemic kidney exhibited a predominance of tubular cell TNF-α production. The increased glomerular cell production of TNF-α in the contralateral kidney may indicate that circulating factors are an important source of remote cellular injury. In contrast, the predominance of tubular cell TNF-α production in the ipsilateral kidney may reflect the well-recognized sensitivity of these cells to ischemic injury (14, 20, 31). The pathophysiologic and clinical implications of these findings are not entirely clear. It has been well established that reperfusion of ischemic tissue leads to the production of reactive oxygen species and cytokines, such as TNF-α. These factors may circulate to remote sites, including the contralateral kidney, and induce further cytokine production and inflammation. Interestingly, our results demonstrate that the contralateral kidney is affected before reperfusion of the ischemic kidney, suggesting that some other mechanism of injury (i.e., stress) is also involved.

The MPO assay was used to assess the neutrophil response of both kidneys to unilateral renal I/R. An increase in neutrophil accumulation was detected in both kidneys after 1 h of ischemia and 4 h of reperfusion. While the measured inflammatory response was somewhat less in the nonischemic contralateral kidney than in the injured kidney, the observed increase in neutrophil infiltration demonstrates that the contralateral kidney suffers a biologically significant injury after ipsilateral renal I/R. Furthermore, deletion of the TNF-α signal by administration of TNF-BP diminished the inflammatory response in both kidneys to a similar degree. While the observed inhibition of neutrophil accumulation may be partially related to indirect effects of TNF-BP, our data demonstrate that the reduction in neutrophil accumulation is a direct result of specific TNF-α bioactivity inhibition by TNF-BP (10). This finding supports our previous observations (10) and suggests that TNF-α is an important mediator of neutrophil-induced remote organ injury after unilateral renal I/R.

These results also support much of the current investigative work in MOF. Recently, a “two hit” model of inflammatory injury has been proposed in the pathophysiology of MOF (22, 27). This paradigm is based on observations that neutrophils become “primed” after an initial noxious stimulus (i.e., trauma, ischemia, or sepsis) such that their response to a subsequent insult is altered (12, 19, 26, 27). In this manner, neutrophils may become sequestered and primed in remote organs after unilateral renal ischemia; however, they are not activated (releasing oxygen radicals and proteases and causing tissue damage) until they have received a second, sometimes seemingly insignificant, insult.

The current literature implies that unilateral renal ischemia/infarction is a benign condition, which may safely be ignored therapeutically. We have demonstrated, however, that ipsilateral renal I/R causes inflammatory injury to the contralateral kidney and possibly to other remote organs. The degree of injury to the contralateral kidney may be insufficient to cause detectable functional impairment; nevertheless, it may make the contralateral kidney susceptible to further injury during states of additional physiological stress.

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