Acute renal failure: determinants and characteristics of the injury-induced hyperinflammatory response

Richard A. Zager,1,2 Ali C. M. Johnson,2 Steve Lund,2 and Sherry Hanson2

1Department of Medicine, University of Washington, and the 2Fred Hutchinson Cancer Research Center, Seattle, Washington

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Zager, Richard A., Ali C. M. Johnson, Steve Lund, and Sherry Hanson. Acute renal failure: determinants and characteristics of the injury-induced hyperinflammatory response. Am J Physiol Renal Physiol 291: F547–F556, 2006. First published April 25, 2006; doi:10.1152/ajprenal.00072.2006.—Acute renal failure (ARF) markedly sensitizes mice to endotoxin (LPS), as evidenced by exaggerated renal cytokine/chemokine production. This study sought to further characterize this state by testing the following: 1) does anti-inflammatory heme oxygenase-1 (HO-1) upregulation in selected ARF models prevent this response? 2) Is the ARF hyperresponsive state specifically triggered by LPS? 3) Does excess iNOS activity/protein nitrosylation participate in this phenomenon? and 4) are upregulated Toll receptors involved? Mice with either 1) rhabdomyolysis-induced ARF (massive HO-1 overexpression), 2) cisplatin nephrotoxicity, 3) or HO-1 inhibition (Sn protoporphyrin) were challenged with either LPS (a TLR4 ligand), lipoteichoic acid (LTA; a TLR2 ligand), or vehicle. Two hours later, renal and plasma TNF-α/mRNA, MCP-1/mRNA, renal nitrotyrosine/iNOS mRNA, and plasma cytokines were assessed. Renal TLR4 was gauged by mRNA and Western blot analysis. Both ARF models markedly hyperresponded to both LPS and LTA, culminating in exaggerated TNF-α, MCP-1, and iNOS/nitrotyrosine increments. This was despite the fact that HO-1 exerted anti-inflammatory effects. TLR4 levels were either normal (cisplatin), or markedly depressed (~50%); rhabdomyolysis-induced ARF kidneys, despite the LPS hyperresponsive state. 1) The ARF kidney can hyperrespond to chemically dissimilar Toll ligands; 2) HO-1 does not prevent this response; 3) excess NO/protein nitrosylation can result; and 4) this hyperresponsiveness can be expressed with either normal or reduced renal TLR4 expression. This suggests that diverse signaling pathways may be involved.

endotoxin; lipoteichoic acid; iNOS; tumor necrosis factor-α; monocyte chemotactic protein-1

It has become increasingly recognized that acute renal failure (ARF) is an independent risk factor for mortality in critically ill patients (4, 5, 12). This is despite the use of aggressive renal replacement therapy to prevent the adverse consequences of uremia. Indeed, it is now apparent that even mild, transient, acute renal insufficiency, e.g., following radiocontrast administration or cardiac surgery, can negatively impact long-term patient survival (14). The explanation for these remarkable clinical observations remains unknown. However, the data suggest that ARF, in some way, induces extra-renal tissue damage, culminating in poor patient outcomes.

In an effort to gain initial mechanistic insights into these clinical observations, this laboratory recently tested the following hypothesis: that the acutely damaged ARF kidney has greatly heightened sensitivity to systemic stressors (e.g., endotoxin), culminating in exaggerated renal cytokine and chemo-

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induced TLR4 signaling can evoke nitric oxide (NO) production, is increased NO expression an additional provocateur in the ARF-induced hyperinflammatory state? Studies designed to answer each of these questions form the basis of this report.

METHODS

Animal Utilization

All animal protocols employed male CD-1 mice (25–35 g) that were obtained from Charles River Laboratory (Wilmington, MA). They were maintained under routine vivarium conditions with free food and water access. Experimental protocols were approved by/in conjunction with the Institutional Animal Care and Use Committee at the authors’ institution.

LPS-Mediated TNF-α/MCP-1 Responses in the Glycerol Model of ARF

The first goal of these studies was to test whether the glycerol ARF model, which induces profound renal HO-1 overexpression (15, 17, 18, 23), is protected against renal injury-induced hypersensitivity to LPS. Toward this end, 32 mice were lightly anesthetized with isoflurane and injected with 50% hypertonic glycerol (10 ml/kg in equally divided doses into the hind limbs). Sixteen additional mice, briefly anesthetized with isoflurane, served as controls. Approximately 18 h later, they were placed into cylindrical restrainers. Half were briefly anesthetized with isoflurane, served as controls. Approxi-

mately 18 h later, they were placed into cylindrical restrainers. Half received a tail vein injection of E. coli LPS (2 mg/kg; 0111:B4; L2630; Sigma, St. Louis, MO; in 80 µl of saline). The remaining mice received a sham LPS injection (−80 µl of saline). This created the following experimental groups (~18 h between 1st and 2nd interventions): 1) no glycerol → tail vein saline injection (n = 8); 2) glycerol treatment → tail vein saline injection (n = 8); and 3) no glycerol → LPS injection (n = 8); and 4) glycerol injection → LPS injection (n = 8).

Two hours post-LPS or -saline injection, the mice were deeply anesthetized with pentobarbital sodium (3–4 mg ip). The abdominal cavity of each mouse was opened, and the right kidney was rapidly excised. A heparinized blood sample was collected immediately at the site of the severed right renal pedicle. The left kidney was then excised. Both kidneys were immediately iced, and the cortices were dissected. Right renal cortical samples were used for total RNA extraction (35); the left renal cortices were extracted for protein in the presence of protease inhibitors (35). [Note: the purpose of resecting the right kidney and then collecting plasma, rather than first phoebo-
notoriously alter mRNA levels). Plasma and left renal protein extracts were assayed for TNF-α and MCP-1 using commercially available ELISA kits (TNF-α, R&D Systems, Minneapolis, MN; MCP-1, BD Biosciences, San Diego, CA). Renal RNA samples were used to assess TNF-α, MCP-1, and GAPDH mRNAs by RT-PCR (36, 37). The TNF-α/MCP-1 results were expressed as ratios to the GAPDH product. The severity of ARF was assessed by blood urea nitrogen (BUN) concentrations. [Note: the employed glycerol ARF protocol has previously been confirmed in this laboratory (35) to induce massive HO-1 protein overexpression, as noted by others.]

Sn Protoporphyrin-Induced HO-1 Inhibition: Renal Responses to LPS

The following experiment was conducted to directly test whether HO-1 impacts renal responses to LPS. Eight mice were lightly anesthetized with isoflurane and injected intraperitoneally with the HO-1 inhibitor Sn protoporphyrin (SnPP; 50 µmol/kg; stock solution, 5 µmol per ml of 0.01 N NaOH/saline). This regimen completely inhibits mouse kidney HO-1 (personal communication, Nath KA; Mayo Clinic, Rochester, MN). An additional eight mice served as controls (injected intraperitoneally with vehicle). Approximately 18 h later, each mouse was injected with LPS, as noted above. Two hours later, renal tissues were extracted and assayed for TNF-α and TNF-α mRNA. A blood sample was simultaneously obtained for plasma TNF-α assessments. To determine SnPP effects independent of LPS injection, mice were injected with SnPP or its vehicle (n = 4 each); 18 h later, plasma and renal tissue samples were obtained for the above TNF-α assessments. (SnPP effects on renal cortical TLR4 abundance/TLR4 mRNA were also determined.)

Glycerol and Cisplatin-Induced ARF: Responses to Lipoteichoic Acid

Glycerol model of ARF. Lipoteichoic acid (LTA) is a cytotoxic/proinflammatory constituent of gram-positive bacteria that is released from cell walls (i.e., a gram-positive bacterial “equivalent” to gram-negative bacterial LPS). However, unlike LPS, LTA is thought to signal via the TLR2, rather than the TLR4, pathway (e.g., Refs. 20 and 26). In light of these considerations, the following experiment was conducted to ascertain: 1) whether LTA, like LPS, induces renal TNF-α/MCP-1 generation and 2) whether the ARF kidney hyperre-

sponds to LTA in a fashion analogous to LPS. To these ends, 10 mice were injected with glycerol, and 10 mice served as controls. Approximately 18 h later, half of the control mice and half of the postglyc-

cerol-treated mice were subjected to tail vein injections of LTA (from S. aureus; 10 mg/kg; L2515; Sigma) or its vehicle (−80 µl of saline). Two hours posttail vein injection, the mice were deeply anesthetized, followed by blood and kidney tissue extractions, as above. TNF-α, MCP-1, and their respective renal cortical mRNA levels were assessed.

Cisplatin model of ARF. It was previously established that by either 18 or 72 h after mice have received cisplatin (CP; 30 mg/kg), their kidneys hyperrespond to LPS (as gauged by TNF-α, MCP-1, IL-10, and each of their respective mRNAs) (37). The following experiment was undertaken to ascertain whether a similar hyperresponsive state exists when the challenge is LTA. Sixteen mice were briefly anesthe-

tized with isoflurane to permit intraperitoneal CP injection (30 mg/kg; stock solution, 1 mg/ml saline). Sixteen additional mice served as controls, receiving an equal volume intraperitoneal injection of saline. Approximately 18 h later, half of the CP-injected mice and half the control mice received either tail vein LTA or LTA vehicle (saline) injection. Two hours post-LTA/vehicle injections, the mice were anesthetized with pentobarbital sodium, followed by renal extraction and blood collection, as previously detailed. Plasma and renal protein/ mRNA samples were assayed for TNF-α, MCP-1, and their respective renal cortical mRNAs (36, 37).

Renal iNOS Responses to LPS Injection: Impact of Prior Renal Injury

The following experiment was undertaken to ascertain whether heightened NO expression is another component of the ARF-induced renal cytokine/chemokine hyperresponsive state. To this end, the following experimental groups (n = 6–8 mice per group) were established: groups 1 and 2); control mice ± LPS challenge; groups 3 and 4): 18 h postglycercor mice ± LPS challenge; groups 5 and 6): 18 h post-CP mice ± LPS challenge; and groups 7 and 8): 72 h post-CP mice ± LPS challenge. Two hours post-LPS/vehicle injec-

tions, the mice were anesthetized, and the kidneys were extracted for both protein and RNA. The following assessments were made: iNOS mRNA assessments: Right renal cortical RNA from each mouse was assayed for inOS and GAPDH mRNAs by RT-PCR, using general methods as previously described (36, 37), and employing the condi-
tions/primers presented in Table 1. Results were expressed as ratios to the GAPDH product.

Nitrotyrosine assessments. Renal cortical protein nitrosylation was assessed by Western blotting (34). Protein samples (75 µg/lane) were electrophoresed into 4–12% precast gels (from Bio-Rad, Hercules,
CA; catalog no. 345–0124). Nitrotirosine was probes with a rabbit polyclonal antinitrotirosine antibody (Abcam; cat. no. 23704 Cambridge, MA; 1:500 dilution). Secondary detection was performed with a horseradish peroxidase (HRP)-labeled antirabbit IgG (from donkey; Amersham Biosciences; 1:12,000) and enhanced chemiluminescence (ECL). Two approaches were used: 1) proteins were electrophoresed into the gels for just 15 min to allow protein entry, but not sufficiently long to permit wide separation of the protein bands (which would hinder overall quantification of degrees of nitrosylation of the entire protein extract by densitometric analysis); and 2) full electrophoresis (>60 min) to assess which protein band(s) were predominately affected by the nitrosylation process. (Note: a 70-kDa band was the dominant nitrotyrosylated product. Thus it, as well as total lane densities, was assessed.) Equal protein transfer was confirmed by subsequent India ink staining.

**TLR4 Expression in Response to Glycerol- or CP-Induced Renal Injury**

The following experiments were undertaken to determine whether alterations in renal TLR4 expression correlate with the ARF-induced LPS hypersensitive state. Renal tissues from 18-h glycerol mice, 18-h CP mice, and their controls (n = 6 each) were subjected to the following assessments.

**TLR4 mRNA expression.** TLR4 mRNA was determined in the above kidneys by RT-PCR (36, 37) employing primers and conditions presented in Table 1. Results were expressed as ratios to simultaneously determined GAPDH product.

**Western blotting for renal cortical TLR4 expression.** Renal cortical protein extracts (50 μg) were electrophoresed into 4–12% polyacrylamide gels (60-minute electrophoresis) and then probed for TLR4 with a rabbit antihuman TLR4 antibody (Imgenex, San Diego, CA; Cat. no. 578A; dilution, 1:200). Anti-TLR4 detection was performed with HRP-labeled donkey antirabbit IgG (1:12,000 dilution) followed by ECL reaction and quantitation by densitometry. To confirm equal protein transfer, two approaches were taken: first, protein staining with India ink was staining; and second, in representative runs, a dominant nitrotyrosylated product. Thus it, as well as total lane densities, was assessed.) Equal protein transfer was confirmed by subsequent India ink staining.

### Calculations and Statistics

All values are given as means ± SE. Statistical comparisons were performed by unpaired Student’s t-test, unless stated otherwise. If multiple statistical comparisons were made between groups, the Bonferroni correction was applied. Significance was judged by a P value of <0.05.

### RESULTS

**Glycerol ARF: Responses to LPS**

**Renal cortical TNF-α protein/mRNA assessments.** Glycerol injection induced severe ARF, as assessed by BUN concentrations (129 ± 11 vs. controls, 26 ± 1 mg/dl; P < 0.001). Despite this injury, no increase in renal cortical TNF-α (Fig. 1, left) or TNF-α mRNA resulted (right) compared with control mouse kidney values.

LPS injection into control mice significantly increased renal cortical TNF-α and TNF-α mRNA (*P* < 0.01 vs. controls). When injected into glycerol ARF mice, two- to threefold greater TNF-α and TNF-α mRNA increases were observed vs. those seen in LPS-injected controls. Thus, although glycerol injection did not independently alter TNF-α/TNF-α mRNA, it sensitized the mice to LPS-induced TNF-α cytokine/mRNA increases.

**Renal cortical MCP-1 protein/mRNA assessments.** The glycerol model slightly increased renal cortical MCP-1 (Fig. 2, left). A corresponding increase in MCP-1 mRNA was also observed (Fig. 2, right).

LPS injection raised both MCP-1 and its mRNA in all mice. However, the increases were ~2× as great in the glycerol ARF mice vs. their LPS-injected controls. Thus the MCP-1 results mirrored the above noted TNF-α results, i.e., glycerol-induced injury hypersensitized the kidney to LPS-mediated cytokine/chemokine increases.

**Plasma cytokine levels in control and glycerol ARF mice.** The glycerol ARF model caused no discernible increase in TNF-α levels (<5 pg/ml for glycerol and control mice; the lower limit of assay detection; Fig. 3). Plasma MCP-1 levels

### Table 1. Primers/conditions used for RT-PCR analyses of iNOS and TLR4 mRNA

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequences</th>
<th>PCR Conditions</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse–TLR4</td>
<td>5’-TAG AAG AGC TGG AGG ACC TGG ATT-3’</td>
<td>94°C–45 s, 58.5°C–45 s, 534 bp</td>
<td></td>
</tr>
<tr>
<td>Mouse–iNOS</td>
<td>5’-ACT GCT TCT GTG CCT GGA GCC ACT-3’</td>
<td>72°C–45 s, 28 cycles, 520 bp</td>
<td></td>
</tr>
<tr>
<td>Mouse–GAPDH</td>
<td>5’-TAG TGC GAT GCA CAA CTG GGT ATT-3’</td>
<td>72°C–45 s, 32 cycles, 437 bp</td>
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</table>

The primers and conditions used to analyze mouse Toll-like receptor 4 (TLR4), mouse inducible nitric oxide synthase (iNOS), and GAPDH (as a housekeeping gene) are presented. The reactions were run as competitive RT-PCR reactions. All values are presented as ratios of either the TLR4 or the iNOS product divided by GAPDH product.
were slightly higher in glycerol ARF mice vs. their controls (0.2 ± 0.02 vs. 0.1 vs. 0.05 ng/ml, respectively; *P < 0.05). However, these absolute values were quantitatively trivial and thus appear as “zero” in Fig. 3.

LPS induced dramatic increases in TNF-α and MCP-1 plasma levels. The degree of the TNF-α increase was ~3× greater in glycerol ARF mice vs. LPS-injected controls (again consistent with increased sensitivity to LPS). Conversely, the LPS-induced plasma MCP-1 increases were comparable for the two groups (consistent with prior observations that renal cortical MCP-1 and MCP-1 mRNA increases in response to LPS do not correspond well to changes in plasma MCP-1 levels) (37).

SnPP-Induced HO-1 Inhibition: Renal Responses to LPS

As shown in Table 2, in the absence of LPS, SnPP caused an approximate 50% increase in renal cortical TNF-α without altering TNF-α mRNA. This occurred without any change in plasma TNF-α levels (undetectable; <5 pg/ml).

SnPP also sensitized to LPS, significantly increasing LPS-mediated TNF-α/TNF-α mRNA levels. An increase in plasma TNF-α levels also occurred (LPS ± SnPP 2,517 ± 45 vs. LPS alone, 1,930 ± 29 pg/ml, *P < 0.01). These changes were not associated with any change in TLR4 mRNA levels (Table 2) or TLR4 protein density, as assessed by Western blotting (controls, 3,345 ± 150 densitometry units; SnPP, 3,154 ± 158; NS).

Glycerol ARF: Responses to LTA

TNF-α protein: renal cortical and plasma assessments. Control values for renal cortical TNF-α/TNF-α mRNA (data from above experiments) are depicted in Fig. 4 by the horizontal dotted lines (upper limit of 95% confidence band). LTA injection significantly increased renal cortical (Fig. 4, left) and plasma TNF-α concentrations (Fig. 4, right) in all mice (*P < 0.01 vs. control values). However, glycerol mice clearly hyperresponded, as evidenced by ~50% greater increases in renal cortical TNF-α, and 20× greater plasma TNF-α increases.
compared with LTA-injected controls. Conversely, LTA induced comparable TNF-α mRNA responses in both groups of animals (Fig. 4, middle).

**MCP-1 protein: renal cortical and plasma assessments.** Control renal cortical MCP-1/MCP-1 mRNA values (data from above LPS experiments) are depicted in Fig. 5 by the horizontal dotted lines (upper limit of 95% confidence band). LTA injection into normal mice induced slight increases in both renal cortical MCP-1 and MCP-1 mRNA (*P < 0.05 vs. controls). The glycerol mice hyperresponded to LTA, approximately doubling renal cortical MCP-1 protein/mRNA values vs. those seen in the LTA-injected controls. Although LTA increased plasma MCP-1 levels in both normal and glycerol ARF mice (*P < 0.05 vs. control values), comparable increases were observed in both sets of animals (again consistent with above noted observations: that plasma MCP-1 levels do not correlate well with renal cortical MCP-1/ MCP-1 mRNA changes).

**Cisplatin ARF: Responses to LTA**

**Renal cortical TNF-α responses.** By 18 h post-CP treatment, modest renal insufficiency had developed (BUNs, 42 ± 5 vs. 26 ± 2 mg/dl for controls; *P < 0.01). This injury was associated with a significant increase in TNF-α mRNA (Fig. 6, right), but not in renal cortical TNF-α protein levels (Fig. 6, left).

LTA injection raised both TNF-α and TNF-α mRNA. The CP-treated mice hyperresponded to LTA injection, as denoted by approximately twofold greater increases in both TNF-α cytokine and its mRNA, vs. values seen in LTA-injected controls.

**Renal cortical MCP-1 responses.** CP treatment alone induced quantitatively trivial, but statistically significant, increases in renal cortical MCP-1 (Fig. 7, left) and MCP-1 mRNA (Fig. 7, right).

All mice responded to LTA injection with significant increases in both MCP-1 protein and MCP-1 mRNA (<0.01 vs. control values). Once again, the CP-treated mice hyperresponded to LTA, with approximately two- to threefold greater increases in both MCP-1 and MCP-1 mRNA levels being observed, vs. LTA-injected controls. Thus the results depicted in Figs. 6 and 7 indicate that by 18 h post-CP treatment, mice hyperrespond to LTA, as denoted by exaggerated increases in renal cortical TNF-α mRNA and MCP-1/mRNA vs. their LTA-injected controls.

**Plasma cytokine responses.** Both the 18-h CP-pretreated mice and the LTA-injected control mice had virtually no detectable TNF-α in plasma (<5 pg/ml; appearing as zero/near zero in the figure). However, when injected into CP-pretreated mice, LTA strikingly increased plasma TNF-α levels, rising to ~800 pg/ml (Fig. 8, left).

LTA injection into control mice raised plasma MCP-1 levels (*P < 0.01 vs. normal values). The CP-pretreated mice hyperresponded to LTA injection (2× increase over values observed in LTA-injected controls; Fig. 8, right).

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### Table 2. SnPP effects on TNF-α, MCP-1, and TLR4 ± LPS

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SnPP (45 ± 2)</th>
<th>LPS (85 ± 4)</th>
<th>LPS + SnPP (106 ± 8)</th>
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<tbody>
<tr>
<td>TNF-α (cortex)</td>
<td>33 ± 0.2</td>
<td>45 ± 2 (P &lt; 0.005)</td>
<td>85 ± 4</td>
<td>106 ± 8 (P &lt; 0.05)</td>
</tr>
<tr>
<td>TNF-α mRNA</td>
<td>0.14 ± 0.05</td>
<td>0.14 ± 0.05 (NS)</td>
<td>0.54 ± 0.1</td>
<td>1.1 ± 0.02 (P &lt; 0.03)</td>
</tr>
<tr>
<td>TLR4 mRNA</td>
<td>1.04 ± 0.9</td>
<td>0.97 ± 0.09 (NS)</td>
<td>1.3 ± 0.1</td>
<td>1.5 ± 0.1 (NS)</td>
</tr>
</tbody>
</table>

Values are means ± SE. TNF-α cytokine and TNF-α mRNA levels in renal cortex obtained from mice under control conditions, with heme oxygenase-1 (HO-1) inhibition [with Sn protoporphyrin (SnPP)], both under basal conditions and 2 h post-IV LPS exposure. Additionally, TLR4 mRNA and TLR4 protein were assessed. SnPP caused a doubling of TNF-α levels in the absence of LPS. SnPP also increased renal cortical TNF-α levels during the LPS challenge. However, SnPP did not impact TLR4 mRNA or TLR4 protein expression, as assessed by Western blot. NS, not significant.
Assessments of iNOS mRNA.

Despite substantial renal injury, neither glycerol alone nor CP treatment alone increased renal cortical iNOS mRNA levels (controls, 0.5 ± 0.005; 18 h postglycerol, 0.5 ± 0.1; 18 h CP, 0.2 ± 0.02; 72 h CP, 0.5 ± 0.2). BUNs were controls 26 ± 1 mg/dl, 18 h glycerol mice 128 ± 12, 18 h CP mice 42 ± 4, and 72 h CP mice 134 ± 34 mg/dl.

The control mice responded to LPS injections with ~3× increases in renal cortical iNOS mRNA (Fig. 9, left). The 18-h CP mice also manifested a 3× increase in iNOS mRNA with the LPS challenge, mirroring values in the controls. Conversely, the glycerol ARF mice manifested a profound TNF-α increase in response to LTA injection. Middle: above noted preferential increases in renal cortical and plasma TNF-α levels in the glycerol ARF mice were expressed in the absence of a difference in renal TNF-α mRNA expression. LTA significantly increased TNF-α mRNA in both groups (*P < 0.01) compared with normal mean values.

The control mice responded to LPS injections with ~3× increases in renal cortical iNOS mRNA (Fig. 9, left). The 18-h CP mice also manifested a 3× increase in iNOS mRNA with the LPS challenge, mirroring values in the controls. However, both the 18-h glycerol mice and the 72-h CP mice massively overresponded to LPS, with iNOS mRNAs reaching values that were ~5× (glycerol) and 10× (CP) higher than those seen in the LPS-injected controls.
Renal nitrotyrosine analyses. Nitrotyrosine was observed in all renal cortical samples. By 2 h post-LPS injection, no increase in nitrotyrosine levels was seen in control kidneys (Fig. 9, right).

Total baseline nitrotyrosine expression was significantly depressed (by ~50%) in both the 18-h glycerol and in the 72-h CP kidney extracts (vs. normal kidney values). However, unlike the control kidneys, both the glycerol and 72-h CP kidneys manifested dramatic responses to LPS injection, doubling their total nitrotyrosine levels (Fig. 9, right). The bulk of the nitrotyrosine increase appeared as an approximate 70-kDa band (Fig. 10; rows 4 and 5; *). When the Western blots were run with a longer separation time to further isolate this 70-kDa band (depicted in line 6 of Fig. 10), statistically greater levels were observed in the LPS ± glycerol and LPS ± CP kidneys vs. any other groups [controls, 202 ± 82 vs. LPS, 253 ± 63 (NS); glycerol, 292 ± 57 vs. glycerol ± LPS 482 ± 45; P < 0.01; CP 161 ± 40 vs. CP ± LPS 443 ± 104; P < 0.01]. Thus a correlate of the preferential increase in iNOS mRNA in the LPS-challenged injured kidneys was an approximate doubling of nitrotyrosine levels (as assessed by either total values or 70-kDa values). This was compared with no changes for LPS-injected controls.

TLR4 Analyses

Renal cortical Western blot analyses. Representative blots are presented in Fig. 10 (rows 1 and 2). TLR4 appeared as a
doublet (reflecting differing degrees of glycosylation) at the relevant molecular size of ~95 kDa. By 18 h postglycerol injection, an approximate 50% reduction in TLR4 expression was apparent, reflecting decreases in both bands (row 1). CP toxicity, assessed at 18 h postinjection, did not significantly alter TLR4 expression (Fig. 10, row 2). Statistical comparisons of the Western blot TLR4 densities are provided in Fig. 11, left. Glycerol induced a statistically significant (~50%) TLR4 reduction. CP treatment had no effect.

Renal cortical TLR4 mRNA expression. By 18 h postglycerol or CP injection, ~2× increases in TLR4 mRNA were observed vs. time-matched control values (Fig. 11, right).

HK-2 cell TLR4 Western blot assessments. As in renal cortex, HK-2 cell TLR4 was again detected as a doublet at ~95 kDa (see line 3, Fig. 10). Fe-mediated oxidative stress caused an approximate 40% reduction in TLR4 (density units: controls, 701 ± 52; Fe, 414 ± 51; P < 0.005). Thus this was highly analogous to the ~50% glycerol ARF-mediated TLR4 reductions, noted above. Conversely, antimycin A (AA), known to predispose to LPS-mediated inflammation (37), did not significantly alter HK-2 cell TLR4 levels (721 ± 53; NS vs. the above controls).

DISCUSSION

TLRs are plasma membrane glycoproteins that recognize and bind pathogen-associated molecules. Via recruitment of two proximate “downstream” adaptor proteins, MyD88 and TRIF, they signal the production of inflammatory molecules that are part of the innate immune response (1, 2, 28, 29). Although these pathways may confer survival advantages, when uncontrolled, an exaggerated inflammatory state may result, potentially culminating in multiorgan dysfunction and cell death. It is through the TLR4 pathway that LPS is thought to exert its toxic effects. Factors that protect against LPS-mediated TLR4 signaling remain poorly defined. However, recent observations suggest that HO-1, a potent cytoprotectant with antioxidant, vasodilator, and anti-inflammatory activities (reviewed in Ref. 16) can curtail LPS/TLR-initiated toxicity. This conclusion is based on observations that HO-1 “knock-out” mice have increased vulnerability to LPS (32) and that HO-1 overexpression, e.g., as induced via heme protein injection (21) or genetic manipulation (38), mitigates LPS-triggered TNF-α production and lethality.

In our previous studies (36, 37), we demonstrated that the ARF kidney hyperresponds to LPS, with exaggerated cytokine and chemokine production being the result. However, because HO-1 confers protection against LPS-initiated TLR signaling (e.g., Ref. 30–32), we hypothesized that an ARF model with robust HO-1 overexpression should be resistant to an LPS-initiated hyperinflammatory response. To test this hypothesis, we selected the glycerol ARF model because it evokes the most profound renal HO-1 increases documented to date (e.g., Refs. 33, 34).
15, 17, 23, 35). Thus HO-1 upregulation might block LPS hyperresponsiveness in this particular ARF model. Despite this theoretical consideration, we observed that LPS induced approximately two- to threefold greater cytokine/chemokine increases in glycerol ARF mice compared with their LPS-treated controls. Indeed, the levels of these increases are highly comparable to those previously noted in our prior CP and postischemic ARF studies (36, 37). To prove that HO-1 can, indeed, protect the kidney against LPS-induced cytokine generation, mice were treated with SnPP to inhibit HO-1, and then they were challenged with LPS. SnPP significantly increased renal cortical TNF-α under basal conditions, and it enhanced renal TNF-α and renal TNF-α mRNA responses to LPS. It also exaggerated LPS-induced plasma TNF-α increases. Thus, despite HO-1’s protective influence, confirmed herein, it is clearly insufficient to override a glycerol-induced, ARF-initiated, proinflammatory state.

In our prior studies, ARF-induced cytokine hyperresponsiveness was defined solely by responses to LPS. Therefore, the second goal of the present study was to ascertain whether acute renal injury might also sensitize the kidney to a non-LPS stressor that signals via an alternative, non-TLR4, pathway. Toward this end, mice with either glycerol-induced ARF or CP nephrotoxicity were challenged with S. aureus LTA, a TLR2 ligand (20, 26). As with LPS, both CP and glycerol mouse mounted a TNF-α response that was observed at ~70 kDa (denoted by *). Row 6: separate analyses of this 70-kDa band demonstrated its existence almost exclusively in the CP ± LPS and the glycerol ± LPS groups (see RESULTS for values).

Fig. 10. Representative Western blots of TLR4 and nitrotyrosine expression. Row 1: TLR4 in glycerol (G) and control (C) kidneys. TLR4 appeared as a doublet at ~95 kDa (reflecting different degrees of glycosylation; Ref. 6). There was an approximate 50% reduction in its expression in the postglycerol kidney samples (see Fig. 11). This affected both of the protein bands. Row 2: TLR4 in 18 h CP and control kidneys. No significant differences were observed between the groups. Row 3: TLR4 expression in culture proximal tubular (HK-2) cells following 18-h treatments with either Fe gluconate treatment (“Fe”; used to simulate rhabdomyolysis-induced, Fe-mediated oxidant stress) or antimycin A (AA). As with the in vivo blots of TLR4, a doublet appearance was observed. AA had no effect on either TLR4 band. Conversely, Fe treatment caused a statistically significant TLR4 reduction, principally involving the lower molecular wt. band. Rows 4 and 5: total nitrotyrosine (NT) expression in control kidneys (C), 72 h post-CP kidneys (CP), and postglycerol (G) kidneys. LPS failed to raise NT levels in control mice. NT levels were suppressed in both CP and glycerol mice in the absence of LPS (compared with controls). However, with LPS, both CP and glycerol mouse mounted a NT response that was observed at ~70 kDa (denoted by *). Row 6: separate analyses of this 70-kDa band demonstrated its existence almost exclusively in the CP ± LPS and the glycerol ± LPS groups (see RESULTS for values).

Fig. 11. Renal TLR4 quantitation, as assessed by Western blotting and mRNA analyses. Left: by 18 h postglycerol injection, renal cortical TLR4 levels were reduced by ~50%, as gauged by Western blotting. Conversely, no significant change in TLR4 was observed in 18 h post-CP-treated kidneys. Right: both the glycerol and CP-treated mice manifested an approximate doubling of renal cortical TLR4 mRNA compared with normal mice.
resulted (~800 pg/ml). Supporting the notion that the kidney induces/participates in this response is that LTA-injected ARF mice manifested about twofold greater increases in renal cortical TNF-α/mRNA and MCP-1/mRNA than did LTA-injected controls. Indeed, these LTA results could have substantial clinical relevance: they imply that more than one stressor (i.e., beyond LPS) can initiate excessive cytokine/chemokine production in the ARF kidney. Of note, multiple bacterial products, degraded tissue constituents (e.g., RNA, DNA), and stress proteins (e.g., heat shock proteins) can act as TLR ligands (2, 16, 28, 29). This raises the possibility that patients who sustain extrarenal tissue injury might release such ligands into the systemic circulation, and with renal access, an exaggerated TLR-based inflammatory response could result. Indeed, such factors might also induce extrarenal TLR4 signaling, thereby contributing to increases in circulating as well as intrarenal cytokine levels. To the degree that cytokines are excreted in the urine (e.g., Ref. 36), ARF per se might further contribute to elevated plasma cytokine levels.

To date, only TNF-α, MCP-1, and IL-10 have been documented to participate in the ARF-initiated renal “hyperresponsive” state (36, 37). Each is thought to be a downstream product of the MyD88 adaptor arm of the TLR signaling cascade. Conversely, LPS-mediated NO generation is thought to arise via the TLR-TRIF adaptor pathway (27, 39). Therefore, to more fully define the biological scope, and signaling pathways, of the ARF-initiated proinflammatory state, NO expression by the ARF kidney and its responsiveness to LPS were assessed. Three notable results arose from these investigations. First, despite the induction of substantial, if not massive, renal injury, neither rhabdomyolysis nor CP toxicity increased baseline renal iNOS mRNA. Second, despite essentially identical baseline iNOS mRNAs for the control, post-glycerol, and post-CP kidneys, the latter two massively overresponded to LPS, with their iNOS mRNA levels rising 5 to 10 times more than LPS-challenged controls. Third, a correlate of this LPS-initiated iNOS mRNA hyperresponsiveness was an increase in renal nitrotyrosine formation. Whereas LPS failed to increase nitrotyrosine levels in control kidneys, it doubled total, as well as 70-kDa, renal nitrotyrosine in the setting of glycerol- or CP-induced ARF. This was despite the fact that baseline total nitrotyrosine levels in the glycerol and CP kidneys were actually suppressed vs. controls. Clearly then, LPS-mediated NO hyperproduction, with increased protein nitrosylation, appears to be concomitants of the ARF-initiated hyperresponsive state. These data also imply that the TRIF pathway likely participates in this phenomenon.

The final goal of the present study was to ascertain whether increased TLR4 expression might be a simple explanation for why acute renal injury sensitizes to LPS. Noteworthy in this regard are prior studies that have reported increased TLR4 abundance in response to chronic cyclosporine administration and during the late recovery stage of postschismic ARF (assessed by immunohistochemistry or Western blots) (8, 11, 13, 33). Because LPS hyperresponsiveness is present at 18 h post-glycerol or CP injection, this time point was chosen for our TLR4 assessments. Surprisingly, glycerol (Fe mediated) ARF caused a 50% reduction in renal cortical TLR4 expression, as assessed by Western blotting. To confirm that these results reflected, at least in part, a proximal tubule event, cultured HK-2 cells were challenged with Fe, and a 40% TLR4 reduction resulted. Conversely, neither in vivo CP toxicity nor in vitro AA toxicity (which also increase LPS responsiveness) (36, 37) increased TLR4 expression. Clearly then, these results indicate that ARF-induced LPS hyperresponsiveness cannot simply be explained by TLR4 upregulation (at least during the early injury vs. the late injury phase) (8, 11, 13, 33). It is notable that TLR4 mRNA was increased in both glycerol- and CP-treated kidneys, despite reduced or normal TLR4 protein expression. This underscores that TLR4 mRNA levels cannot be used as a surrogate marker for TLR4 protein levels. Indeed, the TLR4 mRNA increases might simply represent a compensatory response to injury-associated TLR4 protein destruction.

In conclusion, the present results expand on our previous observations of ARF-induced cytokine hyperresponsiveness (36, 37) in several important ways. First, we provided the first demonstration that the glycerol ARF kidney markedly hyperresponds to LPS, despite the fact that robust HO-1 induction, a hallmark of this ARF model, has been proven to dampen LPS renal cytokine signaling. This indicates that acute renal injury is clearly sufficient to override HO-1’s anti-inflammatory effects, culminating in an exaggerated cytokine/chemokine production state. Second, the present study indicates that ARF-induced cytokine hyperresponsiveness is not limited to LPS as the inciting agent. That S. aureus LTA, a TLR2 ligand, can recapitulate LPS actions raises the distinct possibility that multiple stressors/Toll ligands may be capable of triggering the ARF-associated inflammatory response. Third, this study provides the first evidence that iNOS is an active participant in the “effector arm” of the ARF cytokine hyperresponsive state, presumably by acting via the TRIF (as opposed to the MyD88) adaptor protein pathway. The biological relevance of this finding is underscored by the results of the nitrotyrosine assays, given that both ARF models, but not controls, increased renal nitrotyrosine levels in response to LPS; and fourth, ARF-induced hyperresponsiveness is not determined by, or predicated on, increased TLR4 expression. Indeed, exaggerated LPS responsiveness can be expressed even in the setting of markedly reduced TLR4 levels (e.g., in glycerol ARF). However, these findings do not exclude increased TLR signaling, as it remains possible that the activity of residual TLR units in ARF kidneys could be enhanced. Alternatively, LPS and LTA could be mounting exaggerated cytokine/chemokine responses via non-TLR pathways. Exploration of this issue remains an important subject for future study, in part, because new therapeutic approaches for blocking these presumed maladaptive cytokine responses could result.

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GRANTS

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