Ischemic proximal tubular injury primes mice to endotoxin-induced TNF-α generation and systemic release

R. A. Zager, Ali C. M. Johnson, Sherry Y. Hanson, and Steve Lund

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

Submitted 21 January 2005; accepted in final form 22 March 2005

Zager, R. A., Ali C. M. Johnson, Sherry Y. Hanson, and Steve Lund. Ischemic proximal tubular injury primes mice to endotoxin-induced TNF-α generation and systemic release. Am J Physiol Renal Physiol 289: F289–F297, 2005—Endotoxiaemia (LPS) can exacerbate ischemic tubular injury and acute renal failure (ARF). The present study tested the following hypothesis: that acute ischemic damage sensitizes the kidney to LPS-mediated TNF-α generation, a process that can worsen inflammation and cytotoxicity. CD-1 mice underwent 15 min of unilateral renal ischemia. LPS (10 mg/kg iv), or its vehicle, was injected either 45 min before, or 18 h after, the ischemic event. TNF-α responses were gauged 2 h post-LPS injection by measuring plasma/renal cortical TNF-α and renal cortical TNF-α mRNA. Values were contrasted to those obtained in sham-operated mice or in contralateral, nonischemic kidneys. TNF-α generation by isolated mouse proximal tubules (PTs), and by cultured proximal tubule (HK-2) cells, in response to hypoxia-reoxygenation (H/R), oxidant stress, antimycin A (AA), or LPS was also assessed. Ischemia-reperfusion (I/R), by itself, did not raise plasma or renal cortical TNF-α or its mRNA. However, this same ischemic insult dramatically sensitized mice to LPS-mediated TNF-α increases in both plasma and kidney (~2-fold). During late reperfusion, increased TNF-α mRNA levels also resulted. PTs generated TNF-α in response to injury. Neither AA nor LPS alone induced an HK-2 cell TNF-α response. However, when present together, AA + LPS induced approximately two- to fivefold increases in TNF-α/TNF-α mRNA. We conclude that modest I/R injury, and in vitro HK-2 cell mitochondrial inhibition (AA), can dramatically sensitize the kidney/PTs to LPS-mediated TNF-α generation and increases in TNF-α mRNA. That ischemia can “prime” tubules to LPS response(s) could have potentially important implications for sepsis syndrome, concomitant renal ischemia, and for the induction of ARF.

tumor necrosis factor; HK-2 cells; proximal tubules; sepsis syndrome; antimycin A

PREVIOUS STUDIES FROM THIS laboratory have demonstrated that endotoxiaemia can potentiate both ischemic (27) and nephrotoxic (32) acute renal failure (ARF). The pathophysiological basis of these findings is likely multifactorial but may include endotoxin (lipopolysaccharide; LPS)-mediated renal vasoconstriction and increased proximal tubular cell nephrotoxin uptake (21, 27, 28, 32). An alternative hypothesis, not previously addressed, is whether the presence of acute renal damage might sensitize the kidney, or proximal tubular cells, to LPS-induced cytotoxicity. LPS toxicity is widely assumed to be initiated by TNF-α generation, a process that can initiate a cascade of adverse reactions, culminating in inflammation and either apoptotic or necrotic cell death (9, 12, 16, 24, 26, 34). Thus if acute tubular injury were to increase LPS-mediated TNF-α generation, an exacerbation of that injury might result. Alternatively, an increase in LPS-driven renal TNF-α production, with systemic TNF-α release, might exert extrarenal tissue damage. Indeed, recent observations provide support for this hypothesis, given that acute experimental ischemic renal injury induced myocardial apoptosis and inflammatory cell accumulation in liver and lung via TNF-α-dependent pathways (8, 10).

In light of these considerations, the goals of the present investigation were fourfold: 1) ascertain whether modest renal ischemic-reperfusion (I/R) injury induces TNF-α generation; 2) determine whether that injury heightens LPS-initiated increases in plasma and/or renal TNF-α levels; 3) test whether proximal tubular cells directly participate in this process; and 4) gain some initial insights as to whether alterations in renal mRNA expression might occur under these conditions, suggesting a potential contribution to a heightened LPS-TNF-α response.

METHODS

General Animal Procedures

Male CD-1 mice (25–35 g; Charles River Laboratories, Wilmington, MA) were used for all experiments. They were maintained under routine vivarium conditions with free food and water access throughout. Procedures were performed in accordance with animal care guidelines from the authors’ institution. All surgeries were conducted under deep anesthesia (pentobarbital sodium; 2–3 mg/mouse ip). During anesthesia and recovery, body temperature was maintained at 36–37°C with heating lamps (during surgery), or by keeping the mice in a temperature-controlled apparatus during recovery.

Renal I/R Protocol

Following induction of anesthesia, mice were subjected to a midline abdominal incision. The left renal pedicle was identified, and a smooth vascular clamp was applied. The induction of uniform ischemia was confirmed by the appearance of whole kidney cyanosis. The right kidney was left unperturbed. After the completion of 15 min of ischemia, the vascular clamp was removed and reperfusion was confirmed by the disappearance of organ cyanosis. Tissue harvesting was conducted after 15–60 min (i.e., “early” reperfusion) or at 18 h of the “late” reperfusion period. In the first case, the abdominal cavity was temporarily closed with a hemostat. At the completion of the reperfusion period, a blood sample was withdrawn from the inferior vena cava (IVC) into a heparinized syringe, and the plasma was saved for TNF-α analysis (performed by ELISA; see below). Following blood withdrawal, both the post-I/R left kidney and the contralateral right kidney were resected and saved for further analysis. The contralateral kidneys served as one set of controls, given that they were exposed to the same systemic factors as the postischemic kidneys (including circulating TNF-α in response to systemic LPS challenges; see below) but without having been subjected to I/R.

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Address for reprint requests and other correspondence: R. A. Zager, Fred Hutchinson Cancer Research Ctr., 1100 Fairview Ave. N, Rm. D2–190, Seattle, WA 98109 (e-mail: dzager@fhcrc.org).
In the case of mice subjected to unilateral left ischemia and 18 h of reflow, following completion of the ischemic insult and early reperfusion, the abdominal cavities were closed by separately suturing the abdominal muscle incision and the cutaneous incision using 4-0 sutures. After recovery from anesthesia, they were returned to their cages and allowed free food and water access. After 18 h of reperfusion, they were reanesthetized, the abdominal incisions were opened, a heparinized blood sample was withdrawn from the IVC, and the postischemic and contralateral kidneys were removed.

**Sham Surgical Protocols**

In each case, the postischemic kidney results from any given mouse were contrasted with those obtained from the contralateral kidney. However, as an additional control, mice were subjected to sham unilateral ischemia, using the same protocols outlined above, but without application of the vascular clamp to the left renal pedicle. After the completion of either 15–60 min of sham I/R, or an 18-h sham I/R protocol, plasma and kidney samples were collected as noted above. In the case of the sham-operated mice, only a single kidney was extracted per animal. Sham surgical protocols were temporarily conducted along with the unilateral ischemia protocol.

**Early (15–60 min) Reperfusion Experiments**

*Ischemia-early reperfusion: effects on plasma and renal cortical TNF-α levels.* Ten mice were subjected to the above-described unilateral I/R protocol. After completion of 30 or 60 min of reperfusion (n = 6 and n = 4, respectively), a blood sample was obtained from the IVC, followed by bilateral renal resection. The kidneys were iced, and the renal cortices were resected. The latter were then subjected to homogenization at 4°C in the presence of protease inhibitors (30). After centrifugation, the protein extract was assayed for TNF-α using a commercially available ELISA kit (~250 μg of protein/well, R&D Systems, Minneapolis, MN). A TNF-α standard curve, performed with the TNF-α standard supplied by the manufacturer, was run with each assay (31). Plasma samples were also assayed for TNF-α using the same method (50 μl of plasma/well). Renal cortical and plasma samples obtained from 10-time-matched, sham-operated controls (30 min, n = 6; 60 min, n = 4) were assayed simultaneously. The results from postischemic kidneys were contrasted to those from contralateral kidneys and with kidneys from sham-operated controls. Tissue TNF-α values were expressed as picograms per milligram tissue protein extract.

*Ischemia-early reperfusion: effects on renal cortical TNF-α mRNA levels.* Nine mice were subjected to the unilateral I/R protocol. After 15, 30, or 60 min of reflow (n = 3 at each time point), both kidneys were removed and iced. Renal cortical tissues underwent total RNA extraction via the TRizol method (31), followed by analysis for mouse TNF-α mRNA by RT-PCR as previously described, by factoring results by a simultaneously obtained GAPDH product (31). The results were contrasted to those observed in nine kidneys removed from mice subjected to sham surgery.

**Late (18 h) Reperfusion Experiments**

*Plasma and renal cortical TNF-α expression during late reperfusion.* Four mice were subjected to unilateral left renal ischemia, as noted above. Four additional mice were subjected to sham surgery. The mice were allowed to recover from anesthesia and returned to their cages with free food and water access. Eighteen hours later, they were reanesthetized, and plasma and renal cortical tissue samples were obtained for subsequent TNF-α assay, as noted above. Renal cortical TNF-α mRNA levels during late reperfusion. Four mice were subjected to the unilateral ischemia-18-h reperfusion protocol. At its completion, both kidneys were resected for subsequent TNF-α mRNA analysis. The results from the postischemic kidneys were contrasted to both contralateral nonischemic kidney results and to results obtained from six sham-operated controls.

**Impact of Ischemic Damage on Renal Cortical Responsiveness to LPS Injection**

Renal cortical TNF-α mRNA assessments: 1 h postischemia. The following experiment was undertaken to ascertain whether acute I/R alters renal cortical sensitivity to LPS injection. To this end, four mice were individually placed into cylindrical restrainers and injected via the tail vein with 10 mg/kg of purified *Escherichia coli* LPS (011:B4; L-2630; stock solution, 4 mg/ml saline; Sigma, St. Louis, MO). Approximately 45 min later, they were anesthetized and subjected to the left renal ischemia protocol. At its completion, the abdominal incision was temporarily closed with surgical clamps. After 1 h of reperfusion, the abdomen was opened, the postischemic left kidneys and contralateral right kidneys were excised, and cortical RNA was extracted. In addition, renal cortical RNA was obtained from time-matched, LPS-injected, sham-operated mice (n = 5) to provide further control values.

*Plasma and renal cortical TNF-α assessments: 1 h postischemia.* The above experiment was repeated exactly as described above (4 unilateral ischemia and 4 sham-operated mice, all subjected to LPS injection). However, at the end of 1 h of reperfusion, plasma samples were obtained and renal cortical protein extracts were prepared (30). These were assayed for TNF-α by ELISA, as noted above.

*Renal cortical TNF-α mRNA assessments: 18 h postischemia.* Ten mice were subjected to unilateral left renal ischemia+18 h of reperfusion. Six additional mice were subjected to sham surgery. At the 18-h time point, the mice were injected with 10 mg/kg of purified *E. coli* LPS, as noted above. Following LPS injection, the mice were returned to their cages. Two hours later, they were anesthetized, and the kidneys were resected. Each of the postischemic kidneys, and their contralateral controls, underwent renal cortical dissection and RNA extraction for subsequent TNF-α mRNA analysis. One kidney from each of the sham-operated mice was removed 2 h post-LPS treatment for mRNA determinations.

*Plasma and renal cortical TNF-α assessments: 18 h postischemia.* Four mice were subjected to unilateral renal ischemia, and four mice underwent sham surgery. Eighteen hours later, they each received an LPS injection (iv). Two hours post-LPS, the mice were anesthetized, blood samples were collected, and the kidneys were resected. Plasma and cortical extracts were analyzed for TNF-α levels.

*Plasma and renal cortical TNF-α levels after LPS injection in the setting of ARF.* In the above experiments, the presence of a normal contralateral kidney prevented the development of azotemia/ARF. The following experiment ascertained whether azotemia alters LPS-mediated TNF-α generation. Five mice were subjected to right nephrectomy, followed by the induction of 15-min left renal ischemia. The animals were then sutured and allowed to recover from anesthesia. After 18 h of reperfusion, they were subjected to LPS injection. Two hours later, they were reanesthetized, and plasma samples were obtained for blood urea nitrogen (BUN) and TNF-α assay. The plasma results were contrasted to those obtained from five time-matched, sham-operated mice injected with LPS, and to those mice previously subjected to the unilateral 18-h reperfusion+LPS injection protocol, as detailed above.

**Isolated Mouse Proximal Tubule Experiments**

Impact of in vivo LPS treatment on isolated tubule TNF-α levels. The following experiment was undertaken to ascertain whether renal cortical TNF-α levels reflect accumulation within proximal tubules (as opposed to other cortical structures, e.g., glomeruli, or contaminating blood). To this end, three mice were injected with LPS, and 2 h later renal cortical proximal tubules (PT) were isolated by a standard method employed in this laboratory (cortical dissection, dicing, sieving, collagenase digestion, and centrifugation through 32% Percoll; Ref. 30). Upon completion of the isolation, the iced tubules were subjected to protein extraction (in Triton X-100 lysis buffer with protease inhibitors; Ref. 30). The protein extracts were used for...
TNF-\(\alpha\) assay, with the values being expressed as picograms per milligram tubule protein (protein by bichinconinic assay method). The results were compared with those found in proximal tubules harvested in the same fashion from three mice subjected to LPS vehicle (saline) injection.

Impact of exogenous TNF-\(\alpha\) administration on proximal tubular TNF-\(\alpha\) levels. The following experiment was undertaken to ascertain whether in vivo proximal tubules can accumulate TNF-\(\alpha\) from the systemic circulation (e.g., via filtration or conceivably via extraction from peritubular blood). Three mice were injected with 200 ng of purified mouse TNF-\(\alpha\) (R&D Systems). Three control mice received the TNF-\(\alpha\) vehicle (saline, containing 1% BSA). Thirty minutes postinjection, the mice were anesthetized, the kidneys were removed, and proximal tubules were isolated. Protein extracts were prepared and assayed for TNF-\(\alpha\) levels. The results were compared with those observed in tubules extracted from three vehicle-injected mice.

The above experiment was repeated in five mice, except that TNF-\(\alpha\) was injected with 25% mannitol (used to evoke a diuresis; 200 \(\mu\)l total volume). Five control mice received 200 \(\mu\)l of mannitol without TNF-\(\alpha\) addition. Approximately 30 min later, they were anesthetized, and the urinary bladder was exposed through an abdominal incision and emptied with gentle pressure. A plasma sample was obtained. Urine (diluted 1:5 in ELISA diluent; 1% BSA in saline) and plasma were assayed for TNF-\(\alpha\).

Effect of ex vivo acute tubule injury on isolated tubule TNF-\(\alpha\) levels. The following experiments were undertaken to ascertain whether proximal tubules can generate TNF-\(\alpha\), thereby directly contributing to stress-initiated renal cortical TNF-\(\alpha\) increments.

HYPOXIA-REOXYGENATION AND FE-MEDIATED OXIDATIVE STRESS. Four sets of proximal tubules were isolated from four individual mice, suspended in experimentation buffer (30), warmed to 37°C, and divided into four equal aliquots in 10-ml Erlenmeyer flasks (~3 mg tubule protein/flask). The flasks were incubated in a 37°C shaking water bath as follows: 1) control incubation \(\times 1\) h (95% \(O_2\)-5% \(CO_2\)); 2) incubation \(\times 1\) h in the presence of 25 \(\mu\)M ferrous ammonium sulfate (Fe), complexed to an equimolar amount of hydroxyquinoline (HQ) to permit intracellular access and oxidant stress (30); 3) induction of hypoxia \(\times 7.5\) min (gassing with 95% \(N_2\)-5% \(CO_2\)), followed by 52.5 min of reoxygenation (regassing with 95% \(O_2\)-5% \(CO_2\)); and 4) a second control incubation. After completion of the 1-h incubations, lethal cell injury was gauged by \%lactate dehydrogenase (LDH) release (30). The tubules were then pelleted, and protein extracts were prepared (30) and assayed for TNF-\(\alpha\) levels.

TUBULE LPS EXPOSURE. Four sets of tubules were prepared and divided into four aliquots, as noted above. Two aliquots were incubated \(\times 1\) h under control conditions and two were incubated \(\times 1\) h with 100 \(\mu\)g/ml of \(E.\ coli\) LPS. \%LDH release and tubule TNF-\(\alpha\) levels were then assessed, as noted above.

Renal Histology Following 15 min of Unilateral Ischemia+18 h of Reflow

To confirm that the 15-min I/R protocol, as utilized for all in vivo experiments, induces substantial renal histological damage, three mice were subjected to unilateral ischemia+18-h reperfusion. The postischemic kidneys were resected, cut lengthwise, fixed in 10% buffered formalin, and embedded in paraffin. Four-micrometer sections were cut and stained with hematoxylin/eosin and examined by light microscopy.

Plasma and Renal Cortical TNF-\(\alpha\) Levels Following Prolonged Renal Ischemia

The following experiment was undertaken to ascertain whether a more prolonged, bilateral, ischemic insult would result in increased plasma or renal TNF-\(\alpha\) generation. To this end, five mice were subjected to 32 min of bilateral renal pedicle occlusion and 60 min of vascular reflow using the surgical protocols outline above. Five sham-operated mice served as controls. At the end of the reperfusion/sham reperfusion period, plasma and renal cortical samples were obtained for TNF-\(\alpha\) assay, as discussed above.

Cultured Proximal Tubular Cell Experiments

The following experiment was undertaken to complement the above-described in vivo and isolated tubules studies. Specifically, two questions were asked: 1) Do proximal tubules mount an LPS-TNF-\(\alpha\) response to purified LPS in the absence of any other insults (e.g., circulating cytokines, chemokines, white cells, etc); and 2) Does the presence of simulated oxygen-deprivation injury [mitochondrial blockade with antimycin A (AA)] augment proximal tubule cell TNF-\(\alpha\) production in response to LPS. To these ends, 32 T75 Costar flasks of HK-2 cells (a human proximal cell line; e.g., Ref. 33), maintained in culture with keratinocyte serum-free medium (K-SFM; Refs. 29 and 33) were studied at near confluence under the following incubation conditions: 1) control incubation \(\times 18\) h; 2) control conditions \(\times 6\) h, followed by the addition of 100 \(\mu\)g/ml of LPS \(\times 12\) h; 3) addition of AA (7.5 \(\mu\)M; Ref. 33) \(\times 18\) h; or 4) combined AA+LPS addition (6 h AA alone and then 12 h of combined LPS+AA treatment). Each of the four treatment groups consisted of eight flasks. At the end of the incubations, the media was removed, the cells were washed in HBSS, and then recovered with a rubber policeman (33). One-half of the flasks in each group underwent protein extraction (33) for subsequent TNF-\(\alpha\) assay. The remaining flasks underwent RNA extraction for TNF-\(\alpha\) mRNA analysis (33). Thus there were four determinations with each treatment for each of these two end points, performed at different times. Human TNF-\(\alpha\) levels were measured by the Cytokine Shared Resource Laboratory at the authors’ institution (Fred Hutchinson Cancer Research Center). Human TNF-\(\alpha\) and GADDH message levels were determined using commercially available primers (R&D Products), employed in RT-PCR assay, as previously described for mice (31). Cell injury in each of the experimental groups was determined by \%LDH release (performed under identical conditions, but in two 24-well cluster Costar plates). [Technical note: AA stops mitochondrial, but not glycolytic, ATP production. Hence, only modest (~35%) ATP reductions and mild cell injury are induced (Ref. 29; and see RESULTS)].

Calculations and Statistics

All values are presented as means \pm SE. Comparisons were performed by either paired or unpaired Student’s \(t\)-test, unless stated otherwise. Left vs. right kidney results were compared by paired \(t\)-test. Results between different animals, isolated tubules from different animals, or different flasks of HK-2 cells, were analyzed by unpaired \(t\)-test. If multiple comparisons were made, the Bonferroni correction was applied. Significance was judged by a \(P\) value of \(<0.05\).

RESULTS

Plasma TNF-\(\alpha\) levels Following 15-min I/R \pm LPS Injection

Plasma TNF-\(\alpha\) levels in the absence of LPS. As shown in Fig. 1, left, I/R failed to raise plasma TNF-\(\alpha\) levels into a detectable range (i.e., above 1–2 pg/ml). This was true whether the plasma assessments were made during the early (30 or 60 min) reperfusion or the late (18 h) reperfusion period. TNF-\(\alpha\) could also not be detected in plasma samples obtained from sham-operated animals.

Plasma TNF-\(\alpha\) levels with LPS challenge. In contrast to the above, LPS injection caused massive plasma TNF-\(\alpha\) elevations, raising values to \(-1,000\) pg/ml in sham-operated mice by 2 h postinjection (Fig. 1, right). When LPS was injected into mice which had been subjected to unilateral ischemia, it
induced an approximate 2× greater increase in plasma TNF-α levels compared with sham-operated, LPS-treated controls. This was true whether the assessments were made in mice which had been subjected to the LPS injection/early I/R protocol (P < 0.03) or the 18-h reperfusion/LPS injection protocol (P < 0.015). Thus, in composite, these assessments indicated that (1) ischemia + early or late reperfusion, by itself, did not raise plasma TNF-α levels; 2) LPS, by itself, markedly increased plasma TNF-α in all mice; and 3) the presence of either early or late I/R injury sensitized mice to an exaggerated (~2×) LPS-initiated plasma TNF-α response.

**Plasma TNF-α Levels with Unilateral Ischemia + Uninephrectomy + LPS Treatment**

Inducing right nephrectomy + left renal ischemia (15 min) caused severe ARF, as assessed by BUN concentrations 18 h later (133 ± 20 vs. normal BUN concentrations in mice of 20–30 mg/dl). When these 18 h posts ischemic mice were injected with LPS, plasma TNF-α levels rose to 1,624 ± 158 compared with 914 ± 68 pg/ml in sham-operated mice (P < 0.025). The values observed in these bilateral ischemia/18-h reperfusion/LPS mice did not significantly differ from results in the previous unilateral ischemia/18-h reperfusion/LPS mice. Thus, in concert, these results indicate that azotemia/ARF did not diminish the LPS-TNF-α response.

**Renal cortical TNF-α Levels Following I/R ± LPS Injection**

Renal TNF-α levels following I/R without LPS. As shown in Fig. 2, left, TNF-α could be detected in all renal cortical homogenates. However, I/R failed to increase these levels, whether the assessments were made at 30 min, 60 min, or 18 h of reflow. For ease of presentation, the 30- and 60-min data are combined in Fig. 2, as no significant differences were noted at these 2 time points. This lack of difference with I/R was true whether the results in the postischemic (left) kidney were compared with those in either contralateral, nonischemic kidneys or with values in the sham-operated controls.

Renal cortical TNF-α levels following I/R with LPS exposure. LPS raised renal cortical TNF-α levels to values of ~180 pg/mg protein (Fig. 2, right). This represented an approximate 6× increase over values noted in the non-LPS-exposed kidneys (~30 pg/mg protein), as discussed above. LPS induced ~1.5–2× greater renal cortical TNF-α increases in those kidneys that had sustained I/R damage (Fig. 2, right). This was true whether the assessments were made in the early or late (18 h) reperfusion period. The greater LPS-induced renal cortical TNF-α increases in the postischemic kidneys could be directly attributed to the presence of tissue damage, given that the contralateral (nonischemic) kidneys from these mice failed to develop statistically greater TNF-α elevations than did the
Isolated Proximal Tubule Experiments

TNF-α levels following purified TNF-α injection. Isolated tubules harvested from mice injected with purified TNF-α demonstrated an approximate doubling of renal tubular TNF-α levels compared with coisolated control tubules (Fig. 4). TNF-α could also be detected in plasma (450 ± 75 pg/ml) and in urine (700 ± 345 pg/ml) 30 min after intravenous injection. Conversely, no TNF-α was found in plasma or urine from control mice (P < 0.01; Wilcoxon rank sum test vs. TNF-α injection). Thus circulating TNF-α undergoes renal uptake/excretion.

Isolated proximal tubule TNF-α levels following in vitro injury. Control incubations. Proximal tubules, subjected only to 60 min of control oxygenated incubation manifested strikingly higher TNF-α levels than did tubules that were isolated from renal cortex and kept at 4°C without in vitro incubation (i.e., the “control tubules” harvested in the above-described experiments). This is illustrated in Fig. 5: the upper limit of the 95% confidence band for TNF-α levels in normal, nonincubated (4°C) tubules was 30 pg/mg protein (depicted by the horizontal bar). In contrast, control tubules subjected to 60 min of oxygenated incubation demonstrated ~4–5× higher TNF-α levels. This indicates that simple isolation injury and/or the process of rewarming from isolation temperature to 37°C for subsequent ex vivo incubation is sufficient to cause dramatic tubule TNF-α generation. This principal was confirmed in an additional experiment: four tubule aliquots, isolated from two additional normal mice, had TNF-α levels measured both before and after 60 min of oxygenated incubation: with incubation, tubule TNF-α rose approximately fourfold over basal values (P < 0.01).

TNF-α levels following hypoxia-reoxygenation or Fe-mediated oxidant stress. As shown in Fig. 5, both hypoxia-reoxygenation (H/R) and Fe-mediated oxidant stress caused even greater TNF-α increases than were observed simply with oxygenation. These increments were associated with substantial lethal cell injury, as denoted by %LDH release (controls, 11 ± 0.2%; H/R, 66 ± 3%; FeHQ, 58 ± 2%; both P < 0.001 vs. controls).

Exogenous LPS Challenge. In contrast to the H/R or Fe challenge, exogenous LPS addition failed to raise isolated tubule TNF-α levels above values observed in the coincubated

Renal Cortical TNF-α mRNA Expression Postischemia ± LPS Injection

No significant increases in TNF-α mRNA levels could be discerned in the early reperfusion period (15, 30, or 60 min) compared with contralateral kidneys or sham controls. All values within these three early postischemic groups were highly comparable and, hence, were combined for ease of data presentation (see Fig. 3, left). Even after 18 h of reflow, no increase in renal cortical TNF-α message could be observed (Fig. 3, left).

In contrast to the postischemic kidneys, kidneys harvested 2 h post-LPS injection showed dramatic TNF-α mRNA increases (compare data in Fig. 3, right, vs. Fig. 3, left). During early reperfusion (1-h postischemia/2 h post-LPS injection), the postischemic and contralateral kidneys had the same degree of LPS-driven TNF-α mRNA elevations as did the sham-operated/LPS-injected controls (Fig. 3, right, first 3 bars). However, after 18 h of reperfusion, the postischemic kidneys demonstrated ~2× greater LPS-mediated TNF-α mRNA increases compared with either contralateral kidneys or kidneys from LPS-treated, sham-operated mice (Fig. 3, right, last 3 bars). In sum, these experiments indicated that 1) the employed I/R protocols alone did not increase renal cortical TNF-α mRNA, as assessed during either the early reperfusion or the late (18 h) reperfusion period; 2) LPS induced dramatic renal cortical TNF-α mRNA increases in all mice; and 3) postischemic kidneys were sensitized to LPS-mediated TNF-α mRNA generation but only during the late (18 h), not the early (15–60 min) reperfusion phase.

Isolated Proximal Tubule Experiments

TNF-α levels in renal tubules isolated from control and LPS-injected mice. Isolated proximal tubules harvested from LPS-injected mice demonstrated approximately twofold higher TNF-α levels than control tubules (Fig. 4). This implies that renal cortical TNF-α elevations following LPS injection reflect, at least in part, changes in proximal tubules.
controls (Fig. 5). LPS also did not alter LDH release (controls, 14 ± 0.2%; LPS, 12 ± 0.2%).

Renal Histology

Each of the kidneys subjected to 15 min of ischemia + 18 h of reflow demonstrated marked histological damage. This consisted of extensive outer medullary stripe proximal tubular necrosis, patchy tubule necrosis in renal cortex, sloughing of tubular debris into tubular lumina, and cast formation (Fig. 6). Thus the absence of plasma or renal cortical TNF-α elevations with the 15-min I/R experiment could not be ascribed to a lack of ischemic tissue damage.

Prolonged I/R Protocol

Induction of 32 min of ischemia + 60 min of reperfusion induced no increase in plasma TNF-α levels (no detectable TNF-α levels in the I/R or the sham-operated groups). Furthermore, no increases in renal cortical TNF-α values following I/R could also be observed (30 ± 3 vs. 28 ± 2 pg/ml in sham-operated controls).

Cell Culture Experiments

mRNA. As shown in Fig. 7, left, TNF-α mRNA levels were identical in HK-2 cells maintained under control conditions or with addition of LPS. AA treatment caused a small, but significant, increase in TNF-α message (P < 0.01 vs. controls). However, when LPS was added to AA-treated HK-2 cells, there was a greatly exaggerated TNF-α mRNA response. Thus AA had sensitized to LPS-mediated increases in TNF-α mRNA.

Cytokine levels. TNF-α cytokine levels corresponding to the above message levels are presented in Fig. 7, right. Neither LPS alone nor AA alone caused any increase in TNF-α levels. However, when LPS was added to AA-treated cells, an approximate fivefold increase in HK-2 cell TNF-α levels resulted. Thus these experiments were consistent with several basic findings from the above in vivo, and isolated proximal tubule, experiments: 1) AA, like I/R, did not increase TNF-α cytokine levels; 2) AA, like I/R, dramatically sensitized to LPS-mediated TNF-α generation; 3) a correlate of this 18-h increase in TNF-α cytokine expression induced by AA + LPS was an increase in TNF-α mRNA (as seen with the 18-h I/R experiments); and 4) as with isolated tubules, LPS alone did not induce HK-2 cells to generate TNF-α. (This suggests that some systemic cofactor, e.g., another cytokine, chemokine, white cells etc., may be required for LPS to induce a renal tubular TNF-α response.)

LDH release. AA but itself induced only slight lethal cell injury (9 ± 0.5% LDH release vs. 5 ± 0.5% for controls; P < 0.001). This is consistent with the fact that glycolytic metabolism maintains ~70% of normal ATP during HK-2 cell mitochondrial blockade (Ref. 29 and as previously discussed). LPS induced no lethal injury (5 ± 0.5% LDH release). With LPS + AA, 10 ± 0.5% LDH release resulted (not significant vs. AA alone).
DISCUSSION

In recent years, there has been increasing recognition that systemic inflammation can contribute to the induction of ARF (e.g., Refs. 1, 6, 7, 22, 25). Supporting this link are clinical studies that have correlated plasma and urinary cytokine levels with degrees of tubular dysfunction, the risk of developing ARF, and subsequent patient outcomes (6, 7, 22). Animal models of ARF have provided evidence for direct mechanistic links, with the most compelling case coming from studies of cisplatin-induced ARF (18 –20, 23). Following cisplatin administration, increases in both plasma and intrarenal TNF-α concentrations result (18). The pathogenic relevance of these changes is underscored by two complementary pieces of information: 1) TNF-α-deficient (knockout) mice are relatively resistant to cisplatin nephrotoxicity (18); and 2) this protection can be recapitulated in wild-type mice with TNF-α inhibitors (18).

TNF-α has also been implicated in the pathogenesis of postischemic ARF. Donnahoo et al. (3) reported significant intrarenal TNF-α elevations following 1 h of ischemia in the rat. However, 30 min of ischemia did not recapitulate this result. Thus it was concluded that increased renal TNF-α generation requires a prolonged ischemic event (3, 4). In contrast, Kelly (10), studying a rat model of ARF, obtained somewhat different results, noting very slight increases in plasma TNF-α (from 0 to 2.5 pg/ml) with a 32-min ischemic challenge. Conversely, using an inbred strain of mice, the latter investigators found a more robust TNF-α response (rising from 0 to ~150 pg/ml with the same 32-min ischemic challenge; Ref. 11). Thus, in composite, these results indicate that with severe renal I/R injury (32–60 min), TNF-α generation can result.

To gain further insights into this issue, the present study has assessed both plasma and intrarenal TNF-α levels in mice subjected to 15 min of unilateral renal ischemia. Given that surgical stress can induce a systemic inflammatory response, a unilateral ischemia model was used to permit each postischemic kidney to be compared with its own contralateral, nonischemic, control. In addition, sham-operated mice were employed. It is notable that this 15-min ischemic challenge is much shorter than what was used in the studies noted above. However, in our hands, it still induces significant histological (Fig. 6) and functional renal damage (e.g., as denoted by BUNs of ~130 mg/dl when combined with contralateral nephrec-

Fig. 6. Renal histology following 15 min of unilateral ischemia+18 h reperfusion. Hematoxylin and eosin-stained sections revealed extensive outer medullary, and patchy cortical, proximal tubular necrosis. Tubular cell sloughing with cast formation resulted. This confirms that the 15-min ischemia protocol, as employed in the in vivo studies discussed above, induced marked renal damage.

Fig. 7. TNF-α mRNA and cytokine expression in cultured human proximal tubular (HK-2) cells in response to mitochondrial inhibition with antimycin A (AA), LPS, or combined AA+LPS treatment. Left: LPS did not independently alter HK-2 cell TNF-α message levels, whereas AA induced a minor mRNA response. However, when LPS and AA were present together, a marked increase in TNF-α RNA resulted. Right: neither LPS nor AA alone raised TNF-α cytokine levels. However, the combination of AA+LPS evoked a dramatic increase in HK-2 cell TNF-α levels (P < 0.01 compared with AA alone or controls). NS, not significant.
tomy). Nevertheless, despite inducing considerable ischemic injury, no increase in TNF-α or TNF-α message resulted, as assessed by plasma and intrarenal TNF-α levels during either the early (≤60 min) or late (18 h) reperfusion periods. Furthermore, when a more prolonged ischemic insult (32 min of ischemia/60 min of reflow) was tested, no increases in renal cortical or plasma TNF-α concentrations were observed. These results do not negate those obtained by other workers, as described above. However, the present results do indicate that I/R need not always dictate a TNF-α response.

In sharp contrast to renal I/R, LPS injection elicited dramatic TNF-α increases in both plasma and renal cortex. When this LPS challenge was imposed on mice subjected to the unilateral ischemia protocols, approximately twofold greater LPS-initiated plasma and renal TNF-α elevations resulted compared with LPS alone. This was true whether the LPS was administered just before the ischemic challenge or after an 18-h reperfusion period. Given that 15 min of renal ischemia, by itself, did not alter TNF-α levels at either time point, it is clear that concomitant, or preexistent, ischemic damage primed the mouse to an exaggerated LPS-initiated TNF-α response. The postischemic kidney, per se, was undoubtedly responsible for this result, given that heightened TNF-α accumulation was observed only in the postischemic kidney and not in its contralateral control. Finally, it should be noted that immune responses may be blunted by the presence of renal failure (2, 5, 13). However, this did not appear to be the case with LPS-mediated TNF-α generation, as comparable TNF-α increments were observed in mice with unilateral ischemia ≥ contralateral nephrectomy (i.e., renal ischemia ± azotemia/ARF).

TNF-α levels are controlled by both transcriptional and translational events (24). To gain some potential initial insights into mechanisms by which ischemic renal injury sensitizes the kidney to LPS-triggered TNF-α generation, TNF-α mRNA levels were assessed in renal cortex. I/R alone caused no increase in renal cortical TNF-α mRNA, consistent with the lack of increase in TNF-α cytokine levels. In contrast, LPS alone induced dramatic increments in renal cortical TNF-α message, implying that the resulting increase in renal TNF-α may have stemmed, at least in part, from transcriptional events. During early I/R, the heightened LPS-initiated renal TNF-α production could be dissociated from any changes in TNF-α mRNA. Conversely, at 18 h of reflow, the postischemic kidney mounted an exaggerated TNF-α mRNA response (vs. LPS treatment alone). This suggests that different mechanisms for LPS-driven TNF-α production (e.g., transcription vs. translation) may have been operative in the late vs. the early I/R protocols. The reason(s) for these differences remains unknown. Whether it might in some way stem from the fact that LPS was administered just before ischemia (in the early I/R experiments) rather than after ischemia (in the late reperfusion experiments) is one potential possibility that could be explored.

A considerable problem with interpreting renal cortical cytokine levels is that systemically generated cytokines, e.g., from monocytes or distant organs, can potentially reach the kidney and hence be assayed along with it. Furthermore, finding increased levels within the renal cortex does not permit assessment of whether a particular cytokine resides within proximal tubular cells, the prime target of ischemic ARF. To help clarify these issues, experiments were performed in proximal tubular segments harvested from 1) LPS-treated mice; 2) TNF-α-injected mice; or 3) sham-operated controls. These assessments provided valuable new insights, as follows. First, when normal mice were injected with LPS, an increase in TNF-α could be detected in isolated proximal tubules. This indicates that the LPS-mediated increases in renal cortical TNF-α levels reflected, at least in part, proximal tubular cell events. Second, injection of purified TNF-α into the systemic circulation led to its accumulation in isolated proximal tubules, presumably via filtration, or possibly peritubular uptake. To gain some support for filtration, TNF-α was sought in mouse urine 30 min after its intravenous injection. Whereas normal mice demonstrated no urinary TNF-α, high levels were rapidly detected in urine after its injection. Given the magnitude and rapidity of this result, it seems likely that TNF-α filtration occurred. Indeed, given TNF-α’s size of ~26 kDa (i.e., the fraction unbound to soluble receptors; Ref. 23), glomerular filtration would be predicted. However, a component of tubular secretion cannot be excluded at this time.

Given the above findings, one cannot distinguish whether the observed increases in renal cortical TNF-α with the combined LPS + ischemia protocol necessarily stemmed from systemic uptake and/or intrarenal generation. However, given that LPS dramatically increased renal cortical TNF-α mRNA levels, renal production may be inferred. To gain more definitive evidence for injury-induced TNF-α generation, isolated tubules were subjected to in vitro hypoxic or oxidant injury, followed by TNF-α assessments. These experiments demonstrated that severe I/R injury (66% LDH release) increased tubule TNF-α levels. (Indeed, this is consistent with prior in vivo observations that severe ischemic injury can elicit a TNF-α response, as discussed above; Refs. 3, 4, 10, 11.) FeHQ also evinced a TNF-α response. Perhaps most strikingly, just isolation-rearowing injury was sufficient to evoke a dramatic TNF-α increase. Thus, in composite, each of these findings indicates that freshly isolated proximal tubules can, indeed, respond to severe stress with increases in TNF-α production and thus contribute to increases in renal cortical TNF-α concentrations. What was surprising, however, was that LPS addition alone did not elicit an isolated tubule TNF-α response.

To further explore the latter observation, additional experiments were performed in cultured HK-2 cells, permitting assessments of proximal tubule LPS responsiveness in the absence of tubule isolation-rearowing damage. Once again, LPS by itself failed to increase TNF-α levels. However, when the cells were pretreated with AA to evoke mitochondrial blockade, LPS induced profound TNF-α, and TNF-α message, responses. Why LPS alone did not elicit TNF-α is unknown at this time. However, the results of these HK-2 cell experiments are most intriguing because they seemingly underscore the major finding of the in vivo experiments: that even mild “ischemic” renal injury, insufficient on its own to raise TNF-α levels, is clearly capable of sensitizing tubular cells to LPS, thereby augmenting renal cortical and plasma TNF-α concentrations.

In conclusion, the present study indicates that moderate ischemia renal injury, which by itself causes no TNF-α generation, can dramatically prime the kidney for an exaggerated LPS-mediated TNF-α response. If a similar situation were to exist in humans, then even mild ischemic renal damage could potentially exacerbate endotoxemia/sepsis and possibly act in concert to precipitate severe ARF (27). The mechanism by
which mild ischemia sensitizes the kidney to LPS remains unknown. However, the available data suggest that both transcriptional (increases in TNF-α mRNA) and nontranscriptional mechanisms may be involved. The present results also indicate that the kidney is able to rapidly take up and excrete TNF-α from the circulation. Thus postinjury renal cortical TNF-α increases can potentially be impacted by not only in situ intrarenal production but also uptake from the circulation. Finally, the current studies provide a potential new insight into mechanisms by which LPS may exacerbate experimental ARF (27, 32): given that ischemic injury “primes” the kidney to LPS-initiated TNF-α generation, augmented TNF-α-mediated cytotoxicity, as well as increased intrarenal inflammation, may result. This scenario is consistent with the growing concept that most forms of ARF likely have a multifactorial basis, rather than arising from a pure ischemic or nephrotoxic event (14, 15). Indeed, this principle has recently been underscored by demonstrations that sepsis syndrome, induced in aged mice by cecal ligation, acting in concert with intravascular volume depletion, can induce a clinically relevant model of “ischemic” ARF (17). The present results provide one potential underlying mechanism for this result.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R37-DK-38432–18 and R01-DK-68520–01.

REFERENCES


