IRF-1 Promotes Inflammation Early after Ischemic Acute Kidney Injury

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ABSTRACT

Acute renal ischemia elicits an inflammatory response that may exacerbate acute kidney injury, but the regulation of the initial signals that recruit leukocytes is not well understood. Here, we found that IFN regulatory factor 1 (IRF-1) was a critical, early proinflammatory signal released during ischemic injury in vitro and in vivo. Within 15 min of reperfusion, proximal tubular cells of the S3 segment produced IRF-1, which is a transcription factor that activates proinflammatory genes. Transgenic knockout of IRF-1 ameliorated the impairment of renal function, morphologic injury, and inflammation after acute ischemia. Bone marrow chimera experiments determined that maximal ischemic injury required IRF-1 expression by both leukocytes and radioresistant renal cells, the latter identified as S3 proximal tubule cells in the outer medulla by in situ hybridization and immunohistochemistry. In vitro, reactive oxygen species, generated during ischemia/reperfusion injury, stimulated expression of IRF-1 in an S3 proximal tubular cell line. Taken together, these data suggest that IRF-1 gene activation by reactive oxygen species is an early signal that promotes inflammation after ischemic renal injury.


Ischemia elicits an inflammatory response that may exacerbate acute kidney injury (AKI) in both rodents and humans.1–5 Such ischemic injury also occurs during the process of renal transplantation; this process includes injury during cold storage of the kidney during its transit ex vivo from donor to recipient, warm ischemia during creation of the vascular anastomoses between donor kidney and recipient, and, when a deceased-donor kidney, renal hypoperfusion accompanying the severe trauma or illness that caused brain-death of the donor. This ischemic AKI of transplantation elicits an inflammatory response that exacerbates rejection.6–12 Thus, how ischemic injury is translated into inflammation is a fundamental problem in modern nephrology.

Progress has been made in understanding this problem. Production of cytokines, chemokines, and complement and activation of endothelial adhesion molecules results in the recruitment of neutrophils, macrophages, T cells, B cells, NK cells, and NK-T cells into the ischemic kidney1–4,13–16; however, the first critical signals, which are produced by injured renal tubules and which initiate the inflammation, remain obscure. We now report that IFN regulatory factor 1 (IRF-1) is an early critical proinflammatory signal during ischemic AKI and is pro-
duced within S3 proximal tubule cells. These cells are among the most sensitive cells in the kidney to ischemia\(^1\) and are located in the outer medulla, which is a major site of inflammation during ischemic injury. We show these tubule cells are stimulated by reactive oxygen species (ROS) produced during ischemia/reperfusion.\(^ {19-21} \) In response to ROS in vitro, these tubule cells active their gene for IRF-1. IRF-1 is a transcription factor known to activate proinflammatory genes, including interferons and chemokines, and is itself activated by radiation stress.\(^ {22,23} \) A role for IRF-1 in ischemic AKI has not previously been reported.

**RESULTS**

Less Functional Impairment, Injury, and Inflammation after AKI in Mice with Transgenic Knockout of IRF-1

To test the importance of IRF-1 in ischemic AKI, we compared injury in IRF-1 knockout (\(-/-\)) versus congenic wild-type (\(+/+\)) mice. Transgenic knockout of IRF-1 resulted in less functional impairment at 24 h of reperfusion assessed by serum creatinine in one experiment involving 15 mice per group (Figure 1A) and assessed by blood urea nitrogen in a different experiment involving five mice per group (Figure 1B).

Transgenic knockout also resulted in less structural injury to the cortex and medulla at 24 h. Figure 2A summarizes the findings in four kidneys per group. The slides were read by our pathologist, who was blinded to the experimental groups. Injury was scored on a scale of 0 to 5, with 0 being no tubular injury and 5 being maximal tubular injury, as detailed in the Concise Methods section and previously reported.\(^ {24} \) Representative sections are shown in Figure 3. The IRF-1 (-/-) had less cortical and medullary damage than the IRF-1 (+/+). Figure 3A shows many necrotic tubules, indicated by “N,” in a typical IRF-1 (+/+). In contrast, Figure 3B shows few necrotic tubules in a typical IRF-1 (-/-) ischemic kidney. Some tubules had lost their brush borders, as indicated by “E.”

Another striking difference between the IRF-1 (+/+ and IRF-1 (-/-) ischemic kidneys was the greater inflammation in the former. We stained for esterase found in neutrophils and macrophages.\(^ {25,26} \) Our pathologist, who was blinded to the experimental groups, scored three kidneys per group. The results are shown in Figure 2B; there were few neutrophils/macrophages in nonischemic IRF-1 (+/+ and IRF-1 (-/-) kidneys. There were many more of these leukocytes in ischemic IRF-1 (+/+ compared with ischemic IRF-1 (-/-) kidneys.

We found that the greatest inflammation was in the areas of
greatest ischemic injury. Although the ischemic IRF-1 (−/−) kidneys were much less injured than their IRF-1 (+/+ ) counterparts, they did have rare focal areas of severe injury. To emphasize the decreased inflammation in the IRF-1 (+/+ ) kidneys, we chose to show areas of similar severe injury in the IRF-1 (+/+ ) and IRF-1 (+/+ ) kidneys in Figure 4. These were areas of the outer medulla, where, as we discuss later, IRF-1 is expressed in the IRF-1 (+/+ ) kidney. Note the much larger number of red esterase-positive leukocytes in the IRF-1 (+/+ ) kidney (Figure 4A) compared with the IRF-1 (−/−) kidney (Figure 4B).

Early Increased Abundance of Renal IRF-1 mRNA after Ischemia
If IRF-1 is a critical early proinflammatory signal, then it should be expressed early after ischemic injury in wild-type mice. Our experiments confirm this prediction. Figure 5, A and B, shows a typical gel and densitometry, and Figure 5C summarizes seven experiments. Altogether, they show peak expression within the first hour after reperfusion, followed by lesser, albeit still elevated, expression up to 72 h after reperfusion.

Furthermore, if IRF-1 is a critical early proinflammatory signal, then it should be expressed no later than cytokines known to exacerbate injury and inflammation after renal ischemia. Figure 6 confirms this prediction by focusing on the first 2 h after reperfusion. IRF-1 mRNA abundance increases by 15 min and no later than mRNA for TNF-α, IL-6, and IFN-γ, which have previously been shown to participate in ischemic AKI.27–32

Bone Marrow Transfer Shows Maximal Ischemic Acute Renal Failure Requires IRF-1 Expression by Both Radiosensitive and Radioresistant Renal Cells
As a first step in elucidating which renal cells express IRF-1 during AKI, we used bone marrow chimeras to analyze the contribution of radiosensitive cells, mainly leukocytes, versus radioresistant cells, such as epithelia. We lethally irradiated IRF-1 (+/+ ) or (−/−) mice and rescued them with IRF-1 (+/+ ) or (−/−) bone marrow. Because these were congeneric
mice that differ only at the IRF-1 gene, there was no graft-versus-host or host-versus-graft reaction. Figure 7A shows representative PCR of genomic radiosensitive peripheral blood (P.Blood) leukocytes and radioresistant cells in the mouse tails. We expect that the genotype of the latter is the same as the radioresistant cells in the kidney. These data confirmed the chimerism of the mice used in our experiment. Figure 7B shows that maximal ischemic AKI occurred when IRF-1 was expressed on both radiosensitive renal leukocytes and radioresistant renal cells, such as epithelia. Figure 7C shows the statistical analysis.

IRF-1–Expressing Radioresistant Cells Are Proximal Tubular Cells in the Ischemic Outer Medulla

Although IRF-1 is known to participate in the leukocyte activation and leukocytes are known to participate in ischemic AKI,1–4,13,14,16,33 the requirement for IRF-1–expressing radioresistant renal cells was unexpected. We therefore characterized these cells using in situ hybridization and immunofluorescence.

Figure 8 shows that IRF-1 mRNA was expressed on tubular cells in the ischemic outer medulla. Figure 8A is a low-power darkfield photomicrograph of an ischemic kidney hybridized with S\(^{35}\)-labeled antisense IRF-1 RNA. There was a prominent signal in the outer medulla. Figure 8B shows the absence of signal with the control S\(^{35}\)-labeled sense IRF-1 RNA. Figure 8C shows that the control nonischemic kidney does not stain with S\(^{35}\)-labeled antisense IRF-1 RNA. Figure 8D is a high-power bright-field photomicrograph of the ischemic outer medulla that was hybridized with S\(^{35}\)-labeled antisense IRF-1 RNA. Silver grains are evident over tubular epithelial cells. The immunofluorescence studies identify which tubules express IRF-1.

Figure 9 shows that IRF-1 protein is expressed by proximal tubules in the ischemic but not the nonischemic outer medulla. Figure 9A is a black and white photomicrograph that shows staining of the ischemic outer medulla by anti–IRF-1 antibody. Figure 9B shows that the nonischemic kidney is not stained.

We compared the staining of the ischemic kidney by the primary anti–IRF-1 antibody (Figure 10A) versus staining by a rabbit IgG control (Figure 10B). Both sections were also incubated with the secondary goat anti-rabbit-Cy3. There was staining of the tubule cells only with the anti–IRF-1 antibody. This indicates true detection of IRF-1 protein. Furthermore, our observations showed both increased IRF-1 mRNA (Figure 8) and increased protein (Figures 9 through 11) in ischemic tubules of the outer medulla. These observations support each other.

Figure 11 displays color photomicrographs that show staining of the ischemic kidney with anti–IRF-1 (red) and fluorescein-labeled Lotus tetragonolobus lectin (green). This lectin specifically stains proximal tubules.34,35 The strongest staining is for the S3 straight segment of the proximal tubule that is in the outer medulla, and there is lesser staining of the S1 and S2...
segments in the cortex. Note the red and green staining of the proximal tubules in the outer medulla in the low-power view of Figure 11A. Figure 11B is a high-power view showing a proximal tubule that has the typical luminal staining with the lectin (green) as well as basal staining with IRF-1 (red). Using this technology, we are unable to determine with certainty the precise cytoplasmic versus nuclear location of the IRF-1; however, our immunohistology suggests that the IRF-1 has a cytoplasmic location. This cytoplasmic IRF-1 may require a posttranslational modification before it translocates into the nucleus.36

ROS Induce IRF-1 Expression by S3 Tubular Cells In Vitro

Having shown that S3 proximal tubular cells express IRF-1 during ischemic AKI in vivo, we asked whether ROS, suggested to be produced during ischemic AKI,19–21,37 would stimulate these cells to express IRF-1 protein and mRNA in vitro. To generate ROS, we allowed xanthine oxidase to convert hypoxanthine into xanthine and superoxide.31,38 Figure 12 shows that IRF-1 protein increased after 2 h of exposure to ROS (Figure 12A) in a dose-responsive manner (Figure 12B). Although endotoxin may increase IRF-1 expression, we excluded the possibility of significant endotoxin contamination of the xanthine oxidase. Heating at 99°C destroys xanthine oxidase enzyme activity but not endotoxin. As shown in Figure 12C, heating destroyed xanthine oxidase enzymatic activity, prevented ROS production, and also prevented IRF-1 protein production; therefore, IRF-1 expression required intact xanthine oxidase enzymatic activity to produce ROS, and there was no significant endotoxin contamination. Figure 12 also shows that IRF-1 mRNA abundance increased after 1 h and peaked after 2 h of exposure to ROS (Figure 12D). The S3 proximal tubular cells increased IRF-1 mRNA in a dose-responsive manner to ROS (Figure 12E).

DISCUSSION

We now propose IRF-1 as an early critical signal, which is expressed by injured renal tubular epithelia and which translates ischemia renal injury into inflammation. We showed that transgenic knockout of IRF-1 ameliorated functional renal impairment, morphologic injury, and inflammation after acute ischemia (Figures 1 through 4). These experiments demonstrated the importance of IRF-1 in renal ischemic injury. Furthermore, we showed that IRF-1 expression began within 15 min of reperfusion, was maximal at 1 h, and persisted for 72 h
in vivo (Figures 5 and 6). Thus, IRF-1 was not only important but also was an important early signal that exacerbates ischemic injury.

To understand which cells expressed IRF-1 during ischemic AKI, we lethally irradiated IRF-1 (+/+H11001) and (−/−H11002) mice and rescued them with either IRF-1 (+/+H11001) or (−/−H11002) bone marrow. There was no graft-versus-host or host-versus-graft disease in these bone marrow chimeras, because the IRF-1 (+/+H11001) and (−/−H11002) mice differed only at the IRF-1 gene. These experiments showed that both radiosensitive and radioresistant cells must express IRF-1 for maximal ischemic AKI (Figure 7). The requirement for IRF-1—expressing radiosensitive cells was expected because the radiosensitive cells were leukocytes that exacerbate ischemic AKI,1–4,13,14,16,33 and because IRF-1 regulated leukocyte activation22,39; however, the requirement for radioresistant renal cells was unexpected.

To understand what regulated IRF-1 expression, we compared the kinetics of IRF-1 expression with the expression of IL-6, TNF-α, and IFN-γ. These cytokines are produced by the kidney during ischemic AKI and exacerbate injury.27–32 Although in some cells under some circumstances these cytokines stimulate IRF-1 gene expression in vitro,41–43 our data indicated that these cytokines were not produced before IRF-1 and therefore did not stimulate IRF-1 expression in vivo (Figure 6). Furthermore, the literature is consistent with our conclusion. The relationship of IFN-γ and IRF-1 has previously been examined, not in the context of renal function after ischemic AKI but in the context of renal MHC class II expression after ischemia. These studies used IFN-γR knockout mice and showed that IRF-1 expression did not require IFN-γ.44 Additional studies45 showed that IFN-γ expression did not occur in vivo (Figures 5 and 6). Thus, IRF-1 was not only important but also was an important early signal that exacerbates ischemic injury.

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To characterize the radioresistant renal cells, we performed in situ hybridization for IRF-1 mRNA and immunostaining for IRF-1 proteins (Figures 8 through 11). Both of these techniques showed IRF-1 expression by tubules in the outer medulla. Simultaneous immunostaining with anti–IRF-1 anti-

Figure 9. IRF-1 immunohistology of wild-type ischemic kidneys. (A) Ischemic kidney. This shows a black and white photomicrograph of an ischemic kidney at 4 h of reperfusion. The kidney has been stained with rabbit anti–IRF-1 followed by Cy3 goat anti-rabbit antibody. White arrow points to several of many IRF-1–positive tubules in the outer medulla. (B) Nonischemic kidney. This shows a black and white photomicrograph of a nonischemic kidney that has been immunostained for IRF-1. Note absence of IRF. Magnification, ×25.

Figure 10. Anti–IRF-1 immunohistology of wild-type ischemic kidneys. (A) IRF-1 on ischemic tubules of the outer medulla. Red fluorescence indicates staining with anti–IRF-1 antibody. 100x. (B) No staining on ischemic tubules with rabbit IgG control. Magnification, ×100.
techniques, we did not find differences between ischemic IRF (+/+ or −/−) kidneys at 4 h of reperfusion in the cytokines previously described to regulate ischemic AKI: TNF-α, IL-6, and IFN-γ. Instead, we found that the most differentially expressed proinflammatory gene in IRF (+/+ versus −/−) ischemic kidneys was increased type I IFN-α7 in the former. This result was confirmed by quantitative reverse transcriptase-PCR. Furthermore, type I IFN is important in the pathogenesis of ischemic AKI because transgenic knockout of the type I IFN receptor ameliorates injury. In addition, we found that ROS stimulates IFN-α7 production by renal tubular cells in vitro and that this production is prevented by siRNA knockdown of IRF-1. Altogether, these data suggest that ROS activates the IRF-1 gene, that IRF-1 stimulates IFN-α7 production, and that this type I IFN exacerbates ischemic AKI via the type I IFN receptor. We expect that diminished IFN-α7 in IRF-1 (−/−) kidneys would result in less inflammation because type I interferons are known to stimulate chemokine production. Manuscript in preparation (Y. Wang, J. Chen, Q. Wu, J. Hartono, and C. Lu).

Figure 7 shows that irradiated IRF-1 (−/−) mice reconstituted with either IRF-1 (+/+) or IRF-1 (−/−) bone marrow were equally protected from ischemic injury. This suggests molecules produced by ischemic IRF-1 (+/+) tubules are necessary for maximal injury; perhaps this molecule is the type I IFN-α7 as we speculate.

Our chimera experiments showed that maximal injury also required a contribution from the IRF-1 gene in radiosensitive cells, most likely leukocytes; however, we did not find leukocyte IRF-1 protein by immunohistology (Figures 9 through 11) or mRNA by in situ hybridization (Figure 8) at 4 h of reperfusion. One possibility is that the IRF-1–expressing injurious leukocytes may not be present in the kidney at 4 h of reperfusion. For example, T cells are found transiently in the ischemic kidney, their function may not require IRF-1 gene expression before they arrive in the ischemic kidney. Thus, IRF-1 is not required for maximal injury; perhaps this molecule is the type I IFN-α7 as we speculate.

The final possibility is that IRF-1 changes leukocyte populations before they arrive in the ischemic kidney. Thus, IRF-1 is required for the differentiation of some T cell subpopulations, some dendritic cell/macrophage subpopulations, and NK cells from bone marrow; however, after these leukocytes arrive in the ischemic kidney, their function may not require IRF-1 gene activation to produce mRNA and protein. These leukocytes would be absent from the IRF-1 (−/−) mice, and their absence may contribute to the lesser ischemic AKI in these mice. Our experiments are consistent with literature that one or more of these leukocyte populations contribute to ischemic AKI.

Our observations may have implications for ischemic disease in organs in addition to the kidney. Transgenic knockout of IRF-1 ameliorates ischemic central nervous system and hepatic disease; however, the expression of IRF-1 by parenchymal cells, most likely leukocytes; however, we did not find leukocyte IRF-1 protein by immunohistology (Figures 9 through 11) or mRNA by in situ hybridization (Figure 8) at 4 h of reperfusion. One possibility is that the IRF-1–expressing injurious leukocytes may not be present in the kidney at 4 h of reperfusion. For example, T cells are found transiently in the ischemic kidney, their function may not require IRF-1 gene expression before they arrive in the ischemic kidney. Thus, IRF-1 is not required for maximal injury; perhaps this molecule is the type I IFN-α7 as we speculate.

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Our experiments in this study were initially motivated by our observations that IRF-1 gene activation was inhibited by docosahexaenoic acid in an infection model in vitro and that docosahexaenoic acid ameliorated ischemic AKI in vivo. However, we now know that docosahexaenoic acid does not ameliorate AKI via inhibition of IRF-1, because IRF-1 is expressed within 15 min after reperfusion, whereas docosahexaenoic acid has beneficial effects when administered many hours after ischemia.

In conclusion, the inflammatory response to ischemic injury exacerbates damage in AKI. A fundamental unanswered question is which signals translate ischemic injury of renal tubule cells into inflammation? We now propose that an early signal is the ROS-induced renal tubular expression of the proinflammatory transcription factor IRF-1.

**CONCISE METHODS**

**Mice**

IRF-1 (-/-) mice were B6.129-Irf1S2tm1Mak from the Jackson Laboratories (stock no. 002762; Bar Harbor, ME). This strain was originally developed by Mak and colleagues and has been backcrossed more than 27 generations to the C57BL/6J mouse that is therefore the appropriate congenic control (http://jaxmice.jax.org/strain/002762.html). The C57BL/6J IRF-1 (+/+) mice were also obtained from the Jackson Laboratories. We used our 6-wk-old male mice according to guidelines of the University of Texas, Southwestern Medical Center, and the National Institutes of Health.

**Model of Murine Ischemic Acute Renal Failure**

Mice were anesthetized using inhaled isoflurane and maintained at 37°C using a heating pad and a temperature controlling system with a rectal probe (TR-100 Temperature controller; Fine Science Tools, Foster City, CA). The right kidney was first removed, and the left renal pedicle was clamped for 17 min. Sham surgery consisted of laparotomy and dissection but not clamping of the left renal pedicle. Peripheral serum was assayed for creatinine and blood urea nitrogen using a kinetic method by the Refletron automated system (Roche Diagnostics, Indianapolis, IN). During the first 72 h after murine renal ischemia, the serum creatinine measured by the Jaffe method correlates well with the GFR measured by inulin clearance. Thus, serum creatinine by the Jaffe method is a valid tool for our experiments; however, the Jaffe method consistently overestimates the serum creatinine when compared with measurements made by
HPLC, but the comparisons between groups using the Jaffe method are still valid.58

Histopathology
Mice were killed at various times after reperfusion. The kidneys were removed, bisected, fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. The slides were examined by our pathologist, X.I.Z., who was blinded to genotypes and treatment groups. To score injury, at least 10 fields (×400) of the cortex and the outer medulla were assessed for epithelial necrosis, loss of brush border, tubular dilation, and cast formation. At least 10 fields (×400) were reviewed for each slide, and the percentage of tubules displaying these findings was estimated and an “injury score” determined as follows: 0, none; 1, 1 to 10%; 2, 11 to 25%; 3, 26 to 45%; 4, 46 to 75%; and 5, >75%. This is a widely used scoring system initially used by Thurman et al.14

In addition, slides from each kidney were stained for esterase by the naphthol AS-D chloroacetate esterase method using a kit purchased from Sigma (St. Louis, MO).25 Esterase is found in large amounts in neutrophils and in smaller amounts in macrophages; furthermore, fibroblasts, renal tubule cells, and endothelia do not contain esterase.26 X.I.Z. counted the number of esterase-expressing cells (neutrophils or macrophages) in a blinded manner.

RNase Protection Assay
Total RNA was isolated from frozen kidneys using RNA-Easy Midi Kits (cat. no. 75144; Qiagen, Santa Clara, CA) according to the manufacturer’s instructions and quantified using a spectrophotometer at a wavelength of 260. This total RNA was hybridized with 32P-labeled probes for TNF-α, IL-6, and IFN-γ. These probes were synthesized using In Vitro Transcription Kit (cat. no. 45004K) and mCK-3b templates from Pharmingen (Franklin Lakes, NJ). The RNase protection probe for IRF-1 was also used for in situ hybridization (see next section). RNase treatment was performed using the Riboquant RPA kit (cat. no. 45014K; Pharmingen). After purification of RNA hybrids, the samples were run on a polyacrylamide gel (8 M urea/6% acrylamide:bis-acrylamide) and imaged and analyzed with Molecular Dynamics Storm 820 PhosphorImager and ImageQuant software.

In Situ Hybridization for IRF-1 mRNA; Immunostaining for IRF-1 Protein on Proximal Tubules
After 1 h of reperfusion or sham surgery, kidneys were fixed via transcardiac perfusion. Anesthetized animals were first perfused with heparinized normal saline until the kidney was cleared of blood. The perfusate was then switched to 4% paraformaldehyde at room temperature. Kidneys were removed and cleaned of adherent connective tissue. Trimmed kidney portions were immersed in chilled 4% paraformaldehyde and fixed overnight at 4°C. The following day, they were rinsed in normal saline/DEPC solution. The tissues were then paraffin embedded, sectioned at 5-μm thickness, and mounted on silanated microscope slides (Vector Laboratories, Burlingame, CA).

For in situ hybridization, we first prepared cDNA from a murine ischemic kidney by reverse transcription. A 381-bp fragment of IRF-1 was then amplified by reverse transcriptase–PCR using the following primers: 5‘-CATCTCCACACAGCTTCTCT-3’ (sense primer) and 5‘-CGGATCCCCACACGGCGATACAGC-3’ (antisense primer; Biosyn, Lewisville, TX). This fragment was subcloned into pDrive cloning Vector (Qiagen PCR Cloning Kit). DHS-α competent cells were transformed by heat-shock procedures. Subsequently, clones containing plasmids with the IRF-1 insert were selected, isolated, and purified using the plasmid midi kit (Qiagen). S35-labeled sense probes were prepared after linearization of the plasmid with BamHI by transcription with T3 polymerase; antisense probes were prepared from the plasmid after linearization with NotI by transcription with T7 polymerase. Radioactive in situ hybridization was performed on paraffin-embedded sections using S-35–radiolabeled RNA probes as described previously by our group.59,60 Briefly, after hybridization and washing, the slides were dipped in K5 nuclear emulsion (Ilford, UK), exposed, and developed in Kodak D19. Photomicrographs were taken with a Leica Laborlux-S microscope equipped with a darkfield condenser.

For immunohistology, paraffin sections were heated at 58°C for 30 min followed by dewaxing and rehydration in xylene and ethanol, respectively, using a robotic slide stainer (Sakura, Torrance, CA). Antigen retrieval was performed by microwave boiling the tissues (MWP 800 microwave Processor, Zeiss, Germany) in citrate-based buffer (Biogenex, San Ramon, CA) for 3 × 5 min, after which slides were allowed to cool in solution for 20 min. Sections were then washed in TBST (0.1 M Tris, 0.9% NaCl, and 0.3% Triton X-100) for 3 × 5 min and incubated in 1% normal goat serum in TBST to block nonspecific binding sites. Diluted primary antibody, rabbit anti-mouse IRF-1 at 1:100 (cat. no. sc-640; Santa Cruz Biotechnology, Santa Cruz, CA) and normal rabbit IgG (cat. no. sc-2027; Santa Cruz Biotechnology) at 1:100 were applied to the samples, and the sections were incubated overnight at 4°C. Unbound primary antibody was removed from slides in TBST wash, and the slides were incubated with secondary antibody, goat anti-rabbit-Cy3 at 1:500 (cat. no. 111165144; Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature in the dark for 1 h and rinsed with TBST. The samples were then incubated with fluorescein-labeled Lotus tetragonolobus lectin (cat. no. FL-1321; Vector Laboratories) for 2 h in the dark. After additional rinses in TBST, sections were mounted in Vectashield (Vector Laboratories) and viewed by fluorescence microscopy. This lectin specifically stains the luminal surface of proximal tubules, especially the S3 straight segment in the outer medulla.34,35

Bone Marrow Chimeras
Bone marrow chimeras were generated according to our previously reported protocols.31 Briefly, IRF-1 (+/+) or IRF-1 (−/−) mice were lethally irradiated with 2 s of 5 Gy and then rescued by injections of 8 × 106 cells. These mice were housed in a sterile facility and fed irradiated food and chlorinated water to prevent infection during engraftment. Eight weeks after transplantation, peripheral leukocytes and tail from each mouse were genotyped using PCR. Mice were bled by retro-orbital plexus puncture using a small-diameter heparin-coated microhematocrit capillary tube, and tail biopsies measuring 0.5-cm lengths were cut. The genotype of the IRF-1 (−/−) and IRF-1 (+/+) mice was confirmed by PCR of genomic DNA from tail snips and peripheral blood according to the protocol obtained from the Jackson Lab Tech support site (http://jaxmice.jax.org/pub-cgi/proto-
inactivates the enzyme but not any endotoxin contamination. In some experiments, xanthine oxidase was heated at 90°C for 2 h; this treated with different dosage of hypoxanthine and xanthine oxidase.

SV40 large T antigen transgenic mouse.62 

sected from the S3 segment of the proximal tubule of the kidney of an J Am Soc Nephrol

according to the manufacturer’s instructions. The primers used to am-

scription Kit (Applied Biosystems). Real-time PCR reactions were carried

RNA was prepared from S3 cells using TRIzol (Invitrogen, Carlsbad, CA)

Western Blot Analysis

After treatment with various stimuli, whole-cell extracts were pre-

pared from S3 cells using a lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and a cocktail of protease inhibitors (Roche). Crude extracts were passed through a 23-G needle 10 times to disrupt cells completely and centrifuged to remove debris. Supernatants were subjected to 4 to 20% SDS-PAGE, transferred to NC membrane (Millipore, Billerica, MA) and blocked with 5% nonfat milk overnight at 4°C, and incubated with rabbit anti-mouse IRF-1 antibody at 1:2000 (Santa Cruz Biotechnology, Canvers, MA) for 2 h at room temperature. Chemiluminescent signals were generated by the addition of the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and detected on a radiographic film.

Real-Time PCR

RNA was prepared from S3 cells using TRizol (Invitrogen, Carlsbad, CA) and was quantified by ultraviolet spectrometry at 260 nm. Reverse transcrip-
tion was carried out using the High Capacity cDNA Reverse Transcrip-
tion Kit (Applied Biosystems). Real-time PCR reactions were carried out using the SYBR Green reagents (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The primers used to am-

plify the cDNAs of interest were the following: IRF-1 forward primer was 5’-CACACGGTGACAGTGGCTG-3’, and the reverse primer was 5’-TACAGGCCCTACAAAACAGGAGA-3’; the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer was 5’-AACTTTG-GGATTGTGGAAGG-3’, and the reverse primer was 5’-ACACATTGGGTTAGGAAACA-3’. For minimization and control of the sample variations, mRNA expression of the target gene were normalized relative to the expression of the housekeeping gene GAPDH. Two-step real-time PCR denaturing, annealing, and extension reactions was performed for 40 cycles of 30 s at 95°C and 1 min at 60°C.

Statistical Analysis

Results are presented as means ± SE. The data were compared by ANOVA and the unpaired t test as appropriate. Significant differences were accepted at P < 0.05. Sigma Plot 2000 software (SPSS, Chicago, IL) was used for the graphs and statistical analysis.

ACKNOWLEDGMENTS

This study was supported by the Beecherl Foundation, National In-
stitutes of Health grant R01DK0696303, and the University of Texas Southwestern O’Brien Kidney Research Core Center (National Institu-
tes of Health grant P30DK079328).

Some of the data were presented as a poster at the annual meeting of the American Society of Nephrology, Philadelphia, PA; November 4 through 9, 2008.

We are grateful to Kathy Truean for assistance in the preparation of the figures and the manuscript and to Drs. Mariuz Kielar and Ro-
han Jeyarajah for helpful discussions.

DISCLOSURES

None.

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