Met Activation Is Required for Early Cytoprotection after Ischemic Kidney Injury

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ABSTRACT
Renal proximal tubule epithelial cells express high levels of the hepatocyte growth factor receptor Met, and both the receptor and ligand are upregulated after ischemic injury. Activation of the Met receptor after hepatocyte growth factor stimulation in vitro promotes activities involved in kidney repair, including cell survival, migration, and proliferation. However, characterizing the in vivo role of these signaling events in proximal tubule responses to kidney injury has been difficult because global Met knockout results in embryonic lethality due to placental and liver abnormalities. Here, we used γLT-Cre to knockout Met receptor expression selectively in the proximal tubules of mice (γLT-Cre;Metfl/fl). The kidneys of these mice developed normally, but exhibited increased initial tubular injury, tubular cell apoptosis, and serum creatinine after ischemia/reperfusion compared with γLT-Cre;Met+/+ kidneys. These changes in γLT-Cre;Metfl/fl mice correlated with a selective reduction in PI3K/Akt activation in response to injury and subsequent decreases in inhibitory phosphorylation of the proapoptotic factor Bad and activating phosphorylation of the ribosomal regulatory protein p70-S6 kinase. Moreover, tubular cell proliferation after ischemia/reperfusion was delayed in γLT-Cre;Metfl/fl mice. In conclusion, this study identifies Met-dependent phosphoinositide 3-kinase activation in proximal tubules as a critical determinant of initial tubular cell survival and reparative proliferation after ischemic injury.


The cells of the renal proximal tubule have a large metabolic demand due to their role in bulk reabsorption of glomerular filtrate. The S3 portion of the proximal tubule lies in the outer medulla of the kidney, a region that normally receives proportionally less blood flow than the cortex, making epithelial cells lining this segment highly susceptible to injury during ischemia/reperfusion (I/R) of the kidney.1–3 Tubular epithelial cell responses to severe ischemia include sublethal injury with shedding of the brush border or cell death due to either necrosis or apoptosis.4,5 The endothelial injury that occurs in this setting initiates an innate inflammatory response of polymorphonuclear cells and macrophages that contributes to tubular cell death by promoting local reactive oxygen species generation and enhanced tubular cell apoptosis.6–9 Functional recovery of tubular architecture and glomerular filtration after such an event requires repopulation of the tubule with healthy segment-appropriate tubular cells, a process that is mediated by migration and proliferation of the surviving tubular cells.10

The hepatocyte growth factor (HGF) receptor Met is expressed by multiple cell types, including the renal proximal tubule. Binding of HGF to Met activates downstream signaling via multiple effectors, including the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase
(MAPK) pathways, leading to diverse biologic events in culture systems including cell survival, differentiation, proliferation, and motility.11–14 These same phenotypic responses are predicted to be important for tubule repair, and previous studies have demonstrated upregulation of message levels for both Hgf and the Met receptor in rodent models of ischemic and nephrotoxic injury.15–18

Consistent with an important physiologic role for HGF-Met signaling in kidney repair, studies utilizing exogenously added Hgf, transgenically expressed Hgf, or neutralizing antibodies to Hgf in models of kidney injury all demonstrate a role for this pathway in the restoration of renal function.8,17,19 Finally, immunofluorescence staining of kidney sections revealed loss of basolateral Met expression in proximal tubular cells of γGT-Cre;Metfl/fl mice (Figure 2D). Renal histology revealed no apparent abnormalities in the cortical or medullary architecture in either group (Supplemental Figure 1A). There were no detectable differences in BUN, serum creatinine, or serum electrolytes in either group of mice at 8–10 weeks of age (Supplemental Figure 1B).

**RESULTS**

**Met Receptor, PI3K, and MAPK Are Rapidly Activated after I/R Injury**

Activation of growth factor receptors such as Met results in intracellular signal transduction via the PI3K and MAPK pathways known to induce both antiapoptotic and proliferative responses in cell culture systems.12,19,28 To define the role of these pathways in vivo, wild-type mice were subjected to I/R injury followed by kidney harvest and Western blot analysis of Met receptor activation and the PI3K and MAPK effectors Akt and Erk. In this setting, Met activation was seen within 6 hours of reperfusion and persisted at days 1, 2, and 3 (Figure 1A). Similarly, Akt and Erk activation peaked between 6 and 24 hours after injury, and both remained elevated on days 2 and 3 compared with sham operation (Figure 1, B and C).

**Generation of Mice with Renal Proximal Tubule–Specific Deletion of the Met Receptor**

The predominant injury after I/R occurs in the S3 segment of the proximal tubule and thick ascending limb. To determine whether Met receptor activation is required for PI3K and MAPK activation after I/R, proximal tubule–specific deletion of the Met receptor was accomplished by mating Metfl/fl mice with γGT-Cre mice to produce γGT-Cre;Metfl/fl mice. Metfl/fl mice contained LoxP sites flanking exon 16 of the Met gene,25 the ATP-binding site required for activation of Met signaling (Figure 2A). Progeny heterozygous for the floxed Met allele (γGT-Cre;Metfl/+ ) were mated to produce γGT-Cre;Metfl/fl and γGT-Cre;Metfl/fl mice used for these studies. Tail genotyping identified mice with the wild-type allele and mice heterozygous and homozygous for the floxed allele with all animals containing recombinase Cre (Figure 2B). Western blot analysis of lysates from renal cortex and medulla demonstrated a significant reduction in Met protein expression in the cortex of γGT-Cre;Metfl/fl mice, whereas renal proximal tubule epithelial cells (PTECs) isolated from these mice demonstrated absence of Met expression (Figure 2C). Finally, immunofluorescence staining of kidney sections revealed loss of basolateral Met expression in proximal tubular cells of γGT-Cre;Metfl/fl mice (Figure 2D). Renal histology revealed no apparent abnormalities in the cortical or medullary architecture in either group (Supplemental Figure 1A). There were no detectable differences in BUN, serum creatinine, or serum electrolytes in either group of mice at 8–10 weeks of age (Supplemental Figure 1B).

**Proximal Tubule–Specific Deletion of Met Results in Greater Functional Impairment and Structural Injury after I/R Surgery**

The importance of Met signaling in proximal tubule injury was assessed in γGT-Cre;Metfl/fl and γGT-Cre;Metfl/+ mice exposed to I/R. On day 1 after I/R, serum creatinine was elevated 2-fold more in γGT-Cre;Metfl/fl mice than in γGT-Cre;Metfl/+ mice (Figure 3A). Creatinine values remained significantly higher in γGT-Cre;Metfl/fl mice on day 3, but were indistinguishable at later time points. Initial BUN levels also tended to be higher in γGT-Cre;Metfl/fl mice, but the difference did not reach statistical significance (Supplemental Figure 1C). Consistent with the creatinine values, blinded tissue injury scoring on day 2 after I/R revealed increased tubular cell death and cast formation in γGT-Cre;Metfl/fl mice (Figure 3B, quantified in C). Of note, the mRNA for the Met ligand Hgf was equally upregulated after I/R in both groups of mice (Supplemental Figure 1D).

**Increased Apoptosis with Marked Reduction in PI3K/Akt Signaling in γGT-Cre;Metfl/fl Mice after I/R**

Tubular cell death seen after I/R occurs as a result of immediate necrosis and subsequent apoptosis.4 In light of the increased tubular cell death seen histologically in γGT-Cre;Metfl/fl mice, tubular cell apoptosis was quantified by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining. On day 2 after I/R, outer medullary proximal tubular cell apoptosis was significantly greater in γGT-Cre;Metfl/fl mice (Figure 4A, quantified in B). The observation that apoptosis was worse in those tubular cells lacking Met expression led us to investigate the possibility that antiapoptotic signaling downstream of Met might be diminished in the γGT-Cre;Metfl/fl mice after I/R. Of the known Met signaling pathways, PI3K/Akt activation has
been the one that is most clearly associated with antiapoptotic responses. Similar to the observation in wild-type mice (Figure 1B), there was a robust activation of Akt in γGT-Cre;Met+/+ mice at both 6 and 24 hours after I/R (Figure 4C, quantified in D). In contrast, γGT-Cre;Met+/+ mice exhibited a marked reduction of p70S6K activation at both time points. Analysis of tubular cell proliferation, assessed by Ki-67 staining, revealed the expected burst of proliferation on day 3 after I/R in γGT-Cre;Met+/+ mice (Figure 5C, quantified in D). However, this early reparative response was significantly reduced in the γGT-Cre;Met+/+ mice. Interestingly, there was a significant increase in Erk activation (the prototypic signal for epithelial cell proliferation in response to growth factors) on day 1 after I/R in γGT-Cre;Met+/+ mice (Figure 5E, quantified in F), suggesting that the reduction in Akt-dependent protein synthesis was sufficient to prevent successful Erk-dependent entry into the cell cycle.

Our observation that serum creatinine levels do improve in γGT-Cre;Met+/+ mice, albeit more slowly than normal, suggested that Met-independent growth pathways might promote late tubular cell proliferation in these mice. Consistent with this, Ki-67 staining on day 7 after I/R revealed a significant increase in proximal tubular cell proliferation in γGT-Cre;Met+/+ mice compared with the γGT-Cre;Met+/+ controls (Figure 5G).

**DISCUSSION**

Signal transduction through the HGF/scatter factor receptor, Met, has been shown to activate a variety of in vitro physiologic responses essential to cell survival and repair after tissue injury. Most of these responses require activation of PI3K-Akt and MAPK-Erk signaling pathways when assessed in vitro. Our current analysis of these pathways in vivo confirmed that the Met receptor is phosphorylated and activated early after I/R coinciding with previous descriptions of a biphasic rise in plasma and renal HGF levels at 1 hour and 24 hours after similar injury in mice.8 This timing of Met receptor activation aligns well with effector signaling through pAkt and pErk, both of which peaked at 6 and 24 hours after I/R. It is also important to note that although these effects diminished on the second and third day after I/R surgery, they were not completely abolished, suggesting a role for sustained Met

**Figure 1.** Activation of Met, Akt, and Erk in wild-type mice after renal ischemia. (A) Western blot analysis of Met receptor phosphorylation at the activation site in wild-type mice at 6 hours, and 1, 2, and 3 days after I/R compared with sham operation. The graph shows quantification of pMet normalized to β-actin from four separate mice. (B) Kidney whole cell lysates of wild-type mice at the indicated times after I/R injury immunoblotted with pAkt and pErk (p42/44). The graph shows quantification from four separate mice as in A. (C) Lysates as in B immunoblotted and quantified for pErk. *P<0.05 versus sham; **P<0.01 versus sham; ***P<0.001 versus sham. n=4–6 per group.

A separate target for activated Akt is p70-S6 kinase (p70S6K). This protein phosphorylates the S6 ribosomal protein and thus
activation and Akt and Erk signaling during the reparative phase after injury to the renal epithelium.

To elucidate the role of Met receptor activation in regulating these signaling responses to injury, we performed selective knockout of Met expression in the renal proximal tubule. These mice were viable and demonstrated normal physiology and renal histology at baseline. Specifically, the kidney size of \( \gamma GT-Cre; Met^{+/+} \) mice was normal with normal tubule architecture and no evidence for glomerular hypertrophy or reduced numbers of nephrons. This is different than mice with Met knock-out in the collecting duct, where we found small kidneys with reduced nephron numbers but normal architecture of the nephrons that did form.\(^\text{33}\) Cumulatively, these two studies conclusively demonstrate that Met is not required for the formation and elongation of the renal tubules during development, whereas it is required for maximal ureteric bud branching. Interestingly, the studies of Met function in the collecting duct demonstrated that Met normally acts in a cooperative manner with EGF-EGF receptor (EGFR) signaling to maximize ureteric bud branching.

Despite the finding that proximal tubules of \( \gamma GT-Cre; Met^{+/+} \) mice developed normally, we found marked differences in the response of these mice to ischemic injury of the proximal tubule. After I/R, \( \gamma GT-Cre; Met^{+/+} \) mice demonstrated a greater degree of renal dysfunction and tubular damage with elevated tubular injury scoring within the corticomedullary region of the kidney and increased rates of tubular cell apoptosis. Prior studies of renal I/R have revealed two waves of apoptosis present at 12–24 hours and then 2 days after reperfusion.\(^\text{34}\) Our study demonstrates that early activation of the Met receptor is critical in suppressing that second wave of apoptosis.

The PI3K/Akt pathway has been well characterized \(\textit{in vivo}\) and \(\textit{in vitro}\) for its ability to promote cell survival by suppressing apoptosis in various cell types, including renal tubular epithelial cells.\(^\text{35–37}\) In this study, we show that the PI3K/Akt pathway is markedly stimulated at 6–24 hours after reperfusion injury, and that Met receptor signaling is the dominant stimulus for this early response. Additional evidence in support of the role of Met-PI3K activation in normally suppressing apoptogenic responses after I/R is shown by the analysis of Bad inactivation.\(^\text{32}\) Bad is a proapoptotic member of the Bcl-2 family that is inactivated after Akt phosphorylation at serine 136. At 24 hours after reperfusion injury, the level of phosphorylation at this inhibitory site is significantly decreased in mice lacking the proximal tubule Met receptor. These results demonstrate that activation of the Met-PI3K-Akt signaling cascade is critical for promoting survival of sub-lethally injured proximal tubule cells after I/R injury, thus providing a sufficient reservoir of cells for subsequent repair.

In addition to the role of Met receptor signaling in suppressing tubular cell apoptosis, we also found that tubules lacking Met failed to proliferate normally in the early phase after reperfusion injury. One possible explanation for this is that the increase in apoptosis simply left fewer cells to proliferate. However, the reduction in tubular cell proliferation seen on day 3 is out of proportion to the increase in apoptosis seen on day 2, suggesting that entry into the cell cycle may be delayed in this model even in surviving tubular cells. In light of the clear decrease in Met-stimulated PI3K/Akt activation in the
mice lacking tubular cell Met, we examined the possibility that loss of Akt signaling might impair the initial proliferative response. Once phosphorylated, Akt can induce activation of the mammalian target of rapamycin, leading to p70S6K phosphorylation/activation with subsequent phosphorylation of the S6 ribosomal protein and induction of protein synthesis.38–40 This pathway has been found to be required for proliferation due to both the general need for increased protein synthesis as well as the specific requirement for activated p70S6K in the expression of cyclin D proteins and entry into cell cycle.41–43 Consistent with the role of Met-induced activation of this pathway in the early proliferative response, γGT-Cre;Metfl/fl mice exhibited a >70% reduction in p70S6K expression 1 day after I/R. Therefore, we believe that the combination of greater initial tubular cell apoptosis and the concomitant reduction in p70S6K activation accounts for the marked reduction in the early proliferative phase of tubule repair seen in mice lacking the Met receptor in the proximal tubule.

Surprisingly, despite the dependence of the proximal tubule on Met receptor signaling to suppress early apoptosis and increase reparative proliferation, the GFRs of these young mice did improve at later time points to a level indistinguishable from wild-type controls. Analysis of tubular cell proliferation on day 7 after injury suggests that this recovery was due at least in part to sustained tubular cell proliferation at later time points. This is consistent with the large proliferative reserve seen in tubular cells isolated from young mice,44 and suggests that other growth factor pathways are likely to be important in the composite response to tubular injury, a conclusion that is supported by the finding that Erk was activated to a similar degree in both wild-type and knockout mice.

A plausible explanation for this can be found in the results of a recent study by Chen et al.,45 that examined the effect of pharmacologic inhibition or selective deletion of the renal proximal tubule EGFR in mice subjected to renal ischemia. Renal dysfunction and structural injury were similar at early time points after I/R in all groups of mice but approached a statistical difference within the pharmacologic inhibition group and the proximal tubule–specific deletion group at 6 days postischemia. The authors demonstrated reductions in EGFR-Erk and EGFR-P13K/Akt signaling beginning on day 2 after renal ischemic injury in mice with either pharmacologic inhibition or EGFR deletion in the proximal tubule. In contrast, we find that Met receptor activation alters initial PI3K/Akt signaling and promotes worse initial injury. Jointly, these data suggest that Met receptor activation and EGFR activation act in a complementary and sequential manner after I/R to promote tubular cell survival and proliferative repair, similar to our previous findings in ureteric bud branching.

In summary, we have described a mechanism by which HGF/Met receptor signaling through the PI3K/Akt pathway protects sublethally injured renal tubular cells against early apoptosis. Mice lacking the Met receptor in the proximal tubule have worsened renal function, structural injury, and apoptosis after ischemic injury. We conclude that Met-dependent PI3K activation after I/R is a nodal pathway for cytoprotection and early tubular repair, and thus might serve as a logical therapeutic target to promote recovery from AKI.

**CONCISE METHODS**

**Reagents and Antibodies**

Antibodies against Met, phospho-Met, phospho-Erk, phospho-Akt, phospho-p70S6K, and phospho-Bad were purchased from Cell Signaling Technology (Beverly, MA). Met and β-actin antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA). Anti-megalin antibody was obtained as a kind gift from Dr. Daniel C. Biemesderfer (Yale School of Medicine, New Haven, CT).
Tail genotyping was performed using primers for approved by the Yale Institutional Animal Care and Use Committee. All mouse experiments were conducted under a protocol for mice containing Cre;Met334 mice containing (red stain) for identification of proximal tubules. DAPI is used for counterstaining. (B) Percentage of apoptotic proximal tubular cells quantified as seen in A. ***P<0.001 versus γGT-Cre;Met-/+; n=4 per group. (C) Kidney lysates prepared from sham-operated mice, at indicated times after I/R surgery in γGT-Cre;Met-/- and γGT-Cre;Met-/+ mice and immunoblotted with pAkt antibody with β-actin as a loading control. (D) Quantification of pAkt as in C. ****P<0.0001 versus γGT-Cre;Met-/+ by two-way ANOVA. n=6 per group. (E) Western blot analysis of kidney lysates from γGT-Cre;Met-/- and γGT-Cre;Met-/+ mice obtained at day 1 after injury and immunoblotted with pBad. (F) Quantification of pBad as in E normalized to β-actin. *P<0.05 versus γGT-Cre;Met-/+; n=5 per group.

Generation of Conditional Met Knockout Mice

Met-/+ mice on a mixed 129SV/C57Bl/6 background were developed as previously described.35 These mice were mated with transgenic mice containing γGT-Cre on the same 129SV/C57Bl/6 background. Tail genotyping was performed using primers for Met and Cre expression as previously described.33 Age-matched homozygous γGT-Cre;Met-/+ and wild-type control mice γGT-Cre;Met-/- were used in all experiments. All mouse experiments were conducted under a protocol approved by the Yale Institutional Animal Care and Use Committee.

I/R Surgery

γGT-Cre;Met-/+ mice aged 8–10 weeks and their γGT-Cre;Met-/- control were anesthetized using xylazine and ketamine. Exposure of bilateral renal pedicles was accomplished after using a midline ventral incision. The right renal pedicle was ligated with sutures and nephrectomized, whereas the left renal pedicle was clamped for 25 minutes with visually confirmed ischemia. Reperfusion of the left kidney was visualized after clamp release. Sham animals underwent midline ventral incision without clamping. To prevent dehydration, mice were administered 1 ml of prewarmed normal saline intraperitoneally before closure of muscle and skin layers.

Renal Functional Measurement

Mouse blood collection occurred at 3 days before and 1, 3, 7, 14, 21, and 28 days after I/R surgery. BUN levels and serum creatinine were analyzed using the Yale University School of Medicine Core Mouse Metabolic Phenotyping Center.

Isolation of PTECs

Mouse PTECs were isolated in a modified previously described protocol.29 In brief, uninjured mouse kidneys were harvested after cardiac perfusion with 0.125% collagenase type 1 (Worthington Biochemical Corporation, Lakewood, NJ) in M199 Hanks’ solution (Lonza Group, Basel, Switzerland). Isolated renal cortex was minced, placed in collagenase solution, and incubated in 37°C while 5% CO2 balanced with room air gas was infused into the solution for 40 minutes. Collagenase solution was removed and remnant tissues and cells were resuspended in renal epithelial growth medium (Lonza) containing penicillin/streptomycin and 2.5% FBS. Solution was passed through a 40-μm cell strainer, plated, and grown to confluence before protein isolation and immunoblotting.

Protein Isolation and Western Blot Analyses

Mice were anesthetized by intraperitoneal injection of xylazine and ketamine. Kidneys were harvested at 6 hours and 1, 2, and 3 days after surgery and immediately flash frozen in liquid nitrogen. Tissue was homogenized in radioimmunoprecipitation assay buffer (Teknova, Hollister, CA) containing EDTA-free protease and phosphatase inhibitors (Halt Inhibitor Cocktail; Thermo Scientific, Rockford, IL) using a Dounce homogenizer and centrifuged at 10,000 × g for 15 minutes at 4°C. Equal amounts of protein lysate were separated by SDS-PAGE, transferred onto Immobilon-P membranes (Millipore, Bedford, MA), probed with the appropriate antibody, and visualized by enhanced chemiluminescence (Amersham Biosciences, Pittsburgh, PA) and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Quantification of signals was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

Kidney Immunofluorescence, Immunohistochemistry, and Histology

Mice were anesthetized by intraperitoneal injection of xylazine and ketamine followed by in vivo perfusion fixation with 4%
Figure 5. Met receptor inactivation leads to a reduction in early p70S6K phosphorylation and delayed tubular proliferation. (A) Kidney lysates prepared from sham-operated mice at 6 hours and 1 day after I/R surgery in γGT-Cre;Met+/+ and γGT-Cre;Metfl/fl mice. Lysates are immunoblotted with phosphorylated p70S6K antibody using β-actin as a loading control. (B) Quantification of phosphorylated p70S6K as in A. **P<0.01; ***P<0.001 versus γGT-Cre;Met+/+ by two-way ANOVA. n=5 per group. (C) Immunostaining of the renal outer medulla of γGT-Cre;Metfl/fl and γGT-Cre;Met+/+ mice with Ki-67 (nuclei stained brown) to assess tubular cell proliferation. (D) Quantification of proliferation as in C. ***P<0.001 versus γGT-Cre;Met+/+. n=4 per group. (E) Whole kidney lysates of γGT-Cre;Met+/+ and γGT-Cre;Metfl/fl mice probed with pErk (42/44) antibody after sham operation or I/R surgery at the indicated times with β-actin as the loading control. (F) Quantification of Erk activation as in E. ***P<0.001 versus γGT-Cre;Met+/+, by two-way ANOVA. n=6 in each group. (G) Quantification of tubular cell proliferation on day 7 after I/R injury. **P<0.01 versus γGT-Cre;Met+/+. n=4 per group.
paraformaldehyde and processed for histology (hematoxylin and eosin, unstained sections from paraffin blocks). Sections were subjected to antigen retrieval (Retrievagen; BD, Franklin Lakes, NJ) and then blocked at room temperature using saline containing 0.1% BSA/10% goat serum for 1 hour. Immunostaining was performed with the appropriate primary antibody overnight at 4°C followed by Alexa Fluor goat anti-rabbit 594 or goat anti-mouse 488 secondary antibodies (Invitrogen, Carlsbad, CA) for visualization. Ki-67 staining was performed by Yale Mouse Research Pathology. The TUNEL assay (Roche, Indianapolis, IN) was completed for identification of apoptotic cells, as per manufacturer protocols. DAPI (4′,6-diamidino-2-phenylindole) was used as a counterstain (Vector Laboratories, Burlingame, CA). Quantification of cells expressing the specified marker was conducted by manual count of positive cells/DAPI nuclei in outer medullary proximal tubule cells in 10 randomly chosen ×400 fields using a Nikon microscopy system (Nikon, Inc., Melville, NY).

Morphometric Evaluation of Kidney Injury
Kidney sections stained with hematoxylin and eosin were examined and quantified for histologic changes associated with tubular injury by a single-blinded renal pathologist (G.M.). Scoring was carried out by calculating the percentage of tubules in the corticomedullary junction that displayed cell necrosis, loss of brush border, cast formation, and tubule dilation as follows: 0, none; 1, 10%; 2, 11%–25%; 3, 26%–45%; 4, 46%–75%; and 5, ≥76%. Ten randomly chosen fields (×200) were examined for each slide.

Quantitative PCR
Kidneys were obtained at baseline, 1 day, and 7 days after surgery from GT-Cre;Met+/− and GT-Cre;Met−/− mice with total RNA isolated using TRIzol reagent (Life Technologies, Carlsbad, CA). One microgram of RNA was reverse transcribed using random hexamer primers (SuperScript II; Invitrogen) and gene expression analysis was determined by quantitative PCR using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Berkeley, CA). Primers used for PCR were chosen for efficiency of 90%–100% (details available upon request). Normalization was to hypoxanthine guanine phosphoribosyltransferase expression from the same PCR reaction (ΔCt).

Statistical Analyses
All data were expressed as means ± SEMs for separate experiments. The two-tailed t test was used for statistical analysis, with a P value <0.05 compared with controls considered statistically significant. Between-groups comparison among postischemia time points was analyzed by two-way ANOVA for repeated measures and by multiple comparisons, with P<0.05 considered statistically significant.

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DISCLOSURES
None.

REFERENCES


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Supplemental Figure. (A,B) Histology and serum values of γGT-Cre;Met<sup>fl/fl</sup> mice are similar to γGT-Cre;Met<sup>+/+</sup> mice at baseline. (C) Blood urea nitrogen (BUN) values for the two groups at the indicated times following I/R. n=3 to 7 per group per time point. (D) HGF message by quantitative real-time PCR at baseline and day 1 after I/R in γGT-Cre;Met<sup>fl/fl</sup> and γGT-Cre;Met<sup>+/+</sup> mice.