Renalase Protects against Ischemic AKI

H. Thomas Lee,* Joo Yun Kim,* Mihwa Kim,* Peili Wang,† Lieqi Tang,† Sara Baroni,† Vivette D. D’Agati,‡ and Gary V. Desir†

Departments of *Anesthesiology and †Pathology, College of Physicians and Surgeons, Columbia University, New York; and ‡Department of Medicine, Yale University, VA Connecticut Healthcare System, West Haven, Connecticut

ABSTRACT
Elevated levels of plasma catecholamines accompany ischemic AKI, possibly contributing the inflammatory response. Renalase, an amine oxidase secreted by the proximal tubule, degrades circulating catecholamines and reduces myocardial necrosis, suggesting that it may protect against renal ischemia reperfusion injury. Here, mice subjected to renal ischemia reperfusion injury had significantly lower levels of renalase in the plasma and kidney compared with sham-operated mice. Consistent with this, plasma NE levels increased significantly after renal ischemia reperfusion injury. Furthermore, renal tubular inflammation, necrosis, and apoptosis were more severe and plasma catecholamine levels were higher in renalase-deficient mice subjected to renal ischemia reperfusion compared with wild-type mice. Administration of recombinant human renalase reduced plasma catecholamine levels and ameliorated ischemic AKI in wild-type mice. Taken together, these data suggest that renalase protects against ischemic AKI by reducing renal tubular necrosis, apoptosis, and inflammation, and that plasma renalase might be a biomarker for AKI. Recombinant renalase therapy may have potential for the prevention and treatment of AKI.


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Correspondence: Dr. H. Thomas Lee, Department of Anesthesiology, Anesthesiology Research Laboratories, College of Physicians and Surgeons, Columbia University, P&S Box 46 (PH-5), 630 West 168th Street, New York, NY 10032-3784. Email: tl128@columbia.edu

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Ischemic AKI is a major problem for patients subjected to major surgical procedures involving the kidney, liver, heart, or aorta.1 Renal ischemia reperfusion (IR) injury is a frequent cause of clinical AKI, with the incidence of AKI exceeding 50% after major cardiac, hepatobiliary, or aortic surgery.2,3 Furthermore, ischemic AKI is frequently complicated by multi-organ dysfunction, systemic inflammation, sepsis, and death.4 Unfortunately, there are no proven therapies to prevent or treat AKI in the perioperative setting.5

Renalase is a 38-kD, flavin adenine dinucleotide–dependent amine oxidase synthesized and secreted by the renal proximal tubules.6 Renalase degrades circulating catecholamines and regulates systemic BP in rodents and humans.7 Plasma catecholamines and systemic BP are elevated in patients with chronic kidney dysfunction or end stage renal insufficiency.8 Recent studies suggest that renalase deficiency in patients with chronic renal insufficiency leads to increased plasma catecholamine levels and systemic BP.7,9–11 However, the effect of ischemic AKI on kidney renalse and plasma catecholamine levels remains to be determined.

In addition to regulating BP, renalase may protect against inflammatory tissue injury by metabolizing catecholamines. Catecholamines via activation of leukocyte α-adrenergic receptors directly cause inflammation in sepsis and multi-organ dysfunction.12,13 Indeed, patients with chronic renal insufficiency show increased markers of inflammation that contribute directly to increased morbidity and mortality.14 In mice, renalase deficiency resulted in exacerbated cardiac IR injury and exogenous renalase administration reduced myocardial necrosis.15

In this study, we hypothesized that ischemic AKI in mice leads to renalase deficiency and this renalase
deficiency directly exacerbates ischemic AKI. We performed experiments to test the following: (1) whether ischemic AKI leads to reduced kidney and plasma renalase levels, (2) whether ischemic AKI-induced renalase deficiency leads to elevated plasma catecholamine (NE) levels, (3) whether renalase-deficient mice exhibit increased renal IR injury, and (4) whether exogenous administration of recombinant human renalase directly protects against ischemic AKI in mice.

RESULTS

Renalase Is Selectively Expressed in Renal Proximal Tubules
Figure 1A shows coimmunolocalization analyses of pig kidney tissue incubated with antibodies against megalin (a marker for proximal renal tubules; red) or renalase (green). Renalase and megalin stain perfectly overlap indicating that renalase is expressed in renal proximal tubules. We also performed coimmunolocalization studies with renalase (green) and E-cadherin (a marker for distal renal tubules; red) (Figure 1B). Unlike megalin, E-cadherin does not colocalize with renalase in pig kidneys. These data indicate selective expression of renalase in renal proximal tubules. Our renalase antibody also detected renalase in mouse proximal tubules (data not shown).

Plasma NE Levels after Sham Operation or Renal IR in Mice
We show that plasma NE concentration increased 24 hours after renal IR in renalase wild-type (WT) mice (>2-fold compared with sham-operated renalase WT mice) (Figure 2; n=3–5). The increase in plasma NE concentration was even higher in renalase-deficient mice after renal IR (>5-fold compared with sham-operated renalase knockout [KO] mice).

Plasma and Kidney Renalase Expression after Renal IR
Immunoblotting for plasma renalase revealed significant reductions in plasma renalase 5 hours and 24 hours after renal IR (Figure 3A; n=4–5). Consistent with this decrease in plasma renalase, kidney renalase mRNA expression was significantly attenuated 24 hours after renal IR (Figure 3B; n=4).

Renalase-Deficient Mice Have Increased Ischemic AKI after Renal IR
Baseline plasma creatinine values were similar between renalase WT and renalase KO subjected to sham operation (anesthesia, laparotomy, right nephrectomy, and recovery) (Figure 4A). Plasma creatinine increased significantly in renalase WT and renalase KO mice subjected to moderate (20 minutes) or severe (30 minutes) renal IR compared with sham-operated mice (Figure 4A; n=4–6). However, renalase KO mice had significantly increased renal injury indicated by higher plasma creatinine levels compared with renalase WT mice after both moderate and severe renal IR injury.

Renal Protective Effects of Exogenous Human Recombinant Renalase Administration
We next tested whether exogenous human recombinant renalase pretreatment protects against renal IR injury in mice. Plasma creatinine significantly increased in vehicle (saline)–treated mice subjected to 30 minutes of renal IR compared with sham-operated mice (Figure 4B; n=4–6).

Figure 1. Renalase colocalizes with proximal tubules. Pig kidney sections are costained with a renalase antibody (green) and either with (A) a megalin antibody, a proximal tubule marker (red) or (B) with an E-cadherin, a distal tubule marker (red). Nuclear staining is in blue. We observe that renalase is exclusively expressed in proximal tubules. Representative of three experiments. Original magnification, ×100 for megalin costaining; ×40, for E-cadherin costaining.

Figure 2. Plasma NE levels increase in mice after renal IR injury. We measure plasma NE levels in mice subjected to sham operation or to renal IR injury (n=3–5 per group). Plasma NE concentration is significantly increased 24 hours after renal IR in renalase WT mice, and this increase is even higher in renalase-deficient (KO) mice. *P<0.05 versus sham group; †P<0.05 versus WT IR group.
Pretreatment with human recombinant renalse (0.5, 1.5, or 4.5 mg/kg subcutaneous 10 minutes before renal ischemia) significantly attenuated the increases in plasma creatinine in mice. However, a higher dose of human recombinant renalse (4.5 mg/kg) provided reduced renal protection compared with the recombinant renalse dose of 1.5 mg/kg. We also determined that exogenous renalse (1.5 mg/kg) decreased plasma NE levels in mice subjected to renal IR injury (vehicle-injected mice plasma NE, 2.4±0.13 ng/ml, n=4, versus renalse injected mice plasma NE, 1.6±0.2 ng/ml, n=4; P<0.05) consistent with its renal protective effects.

We also tested whether recombinant renalse treatment after renal reperfusion (after completion of renal ischemia) protected against renal IR injury. Figure 4C shows that recombinant renalse (1.5 mg/kg) given 30 minutes after reperfusion was protective against renal IR injury (n=4–6). Administration of recombinant renalse 60 minutes did not provide renal protection against IR injury.

**Renal Protective Effects of α-Adrenergic Receptor Blockade**

We also tested whether blocking α-adrenergic receptors mimics the renal protective effects of human recombinant renalse administration. Phentolamine, a nonspecific but selective α-adrenergic receptor antagonist (5 mg/kg, intraperitoneally), produced significant renal protection in renalse WT mice subjected to renal 30 minutes of IR injury (Figure 4D). Furthermore, phentolamine also protected renalse KO mice against 30 minutes of renal IR injury (Figure 4D).

**Renalase Modulates Renal Tubular Necrosis after IR**

Renalse-deficient mice subjected to moderate renal IR injury (20 minutes of renal ischemia) developed exacerbated renal histologic injury compared with the renalse WT mice, including increased tubular necrosis and/or proteinaceous casts with increased congestion (Figure 5A, top panels, representative of four to six experiments). In contrast to and consistent with the plasma creatinine data, renalse WT mice treated with human recombinant renalse
(1.5 mg/kg) had dramatically reduced injury compared with vehicle-treated renalase WT mice (Figure 5A, bottom panels). The Jablonski scale renal injury score (0–4) was used to grade renal tubular necrosis 24 hours after renal IR (Figure 5B; n=4–6). Renalase KO mice subjected to moderate renal IR injury (20 minutes of renal ischemia) showed severe acute tubular necrosis (with renal injury scores >3) unlike renalase WT mice subjected to 20 minutes of renal IR injury. In contrast, renalase WT mice treated with human recombinant renalase had significantly lower renal injury scores compared with vehicle-treated renalase WT mice subjected to 30 minutes of renal IR injury.

**Exogenous Renalase Decreases Renal Apoptosis, Neutrophil Infiltration, and Macrophage Infiltration after IR**

Terminal deoxynucleotidyl transferase–mediated digoxigenin–deoxyuridine nick-end labeling (TUNEL) staining detected apoptotic renal cells in kidney of mice subjected to renal IR with predominant proximal tubule cell apoptosis (Figure 6A; magnification, ×100; representative of four experiments). Unlike the kidneys of sham-operated mice, 30 minutes of renal ischemia and 24 hours of reperfusion resulted in severe apoptosis in the kidneys of vehicle (saline)–treated mice (Figure 6A). Recombinant renalase (1.5 mg/kg, subcutaneously) given 10 minutes before renal ischemia significantly reduced the number of apoptotic TUNEL-positive cells in the kidney (Figure 6B; n=4).

Figure 7A shows representative images of neutrophil immunohistochemistry of kidneys (magnification, ×200; representative of four experiments) from mice subjected to 30 minutes of renal ischemia and 24 hours of reperfusion or to sham operation. There was significant neutrophil infiltration (dark brown) in the kidneys of mice treated with saline and subjected to 24 hours of renal IR. In sham-operated mice, we were unable to detect any neutrophils in the kidney. Mice treated with recombinant renalase before renal ischemia had a significantly reduced number of neutrophils in infiltrating the kidney after IR (Figure 7B; n=4).

Figure 7C shows representative images of macrophage (F4/80) immunohistochemistry of kidneys (magnification, ×400; representative of three to four experiments) from mice subjected to 30 minutes of renal ischemia and 24 hours of reperfusion or to sham operation. There was significantly increased macrophage infiltration (brown stain) in the kidneys of mice treated with saline and subjected to 24 hours of renal IR. Mice treated with recombinant renalase before renal ischemia had a

Figure 4. Renalase modulates ischemic AKI in mice. Plasma creatinine levels from mice subjected to sham operation or to renal IR. (A) Renalase WT or renalase-deficient (KO) mice are subjected to 20 minutes (moderate ischemia) or 30 minutes (severe ischemia) of renal ischemia and 24 hours of reperfusion (n=4–6 per group). Renalase deficiency exacerbates renal IR injury in mice. (B) Renalase WT mice are subjected to sham operation or to 30 minutes of renal IR. For mice subjected to renal IR, human recombinant renalase or vehicle (saline) is injected 10 minutes before renal ischemia. Recombinant human renalase produces significant renal protection in renalase WT mice (n=4–6 per group). (C) Preischemic or posts ischemic human recombinant renalase rescues renal function after IR in mice (n=4–6 per group). Human recombinant given 30 minutes after completion of renal ischemia protects against IR injury. (D) Phentolamine, a selective α-adrenergic receptor antagonist (5 mg/kg), protects both renalase WT and renalase-deficient (KO) mice subjected to 30 minutes of renal ischemia and 24 hours of reperfusion (n=4). *P<0.05 versus vehicle-treated mice subjected to sham operation. #P<0.05 versus vehicle-treated WT mice subjected to renal IR. Error bars represent 1 SEM.
significantly reduced number of macrophages infiltrating the kidney after IR (Figure 7D).

**Renalase Deficiency Increases Proinflammatory Gene Expression in the Kidney after IR**

We measured the expression of proinflammatory cytokine mRNAs in the kidney 24 hours after renal IR with RT-PCR: TNF-α, intercellular adhesion molecule 1 (ICAM-1), monocyte chemoattractive protein 1 (MCP-1), and macrophage inflammatory protein 2 (MIP-2). The primer sequences are listed in Table 1. Renalase WT mice significantly increased the expression of all proinflammatory mRNAs examined compared with the sham-operated renalase WT mice (Figure 8). Moreover, renalase-deficient mice had even greater increases in TNF-α, MCP-1, and MIP-2 expression without any changes in ICAM-1 expression compared with renalase WT mice.

**DISCUSSION**

Ischemic AKI is complicated by intrarenal recruitment of proinflammatory leukocytes and systemic inflammation. Recent studies have demonstrated that renal IR is not a single organ disease but involves multiple extrarenal organs including the liver, intestine, and lung. Although many advances have been made detailing the mechanisms of renal tubular cell death after ischemic AKI, the trigger that orchestrates renal and systemic inflammation after ischemic AKI remains unknown. Extrarenal effects of ischemic AKI may explain the disproportionately high mortality in patients with AKI. Therefore, ways to prevent these systemic, extrarenal complications from AKI would contribute greatly to improved patient care and survival.

Renalase is a renal tube–secreted, flavin adenine dinucleotide–dependent amine oxidase that degrades catecholamines including epinephrine, NE, and dopamine without effects against other physiologic amines including serotonin, tyramine, or spermidine. Administration of recombinant renalase decreases cardiac output and BP by regulating plasma catecholamine levels. In contrast, plasma renalase levels are reduced in patients with chronic renal insufficiency. Deficiencies in plasma renalase in these patients are most likely due to the direct reduction in renal tubular renalase synthesis. In rats, plasma and kidney renalase levels are decreased after subtotal nephrectomy. Moreover, unilateral renal artery stenosis causes drastic reductions in renalase expression and secretion compared with the nonstenotic kidney. These studies strongly suggest that the kidney is the major source of steady state renalase secretion to plasma.

We show in this study that renal tubular cell death and acute reduction in renal function after ischemic AKI led to drastic
reductions in renal and plasma reninase levels with a resultant increase in plasma NE. These findings are consistent with the hypothesis that kidney proximal tubule is a major source of circulating reninase. Moreover, our data suggest that secreted reninase is degraded rapidly in plasma and constant new reninase synthesis and release by the kidney must occur to maintain normal plasma reninase levels. Furthermore, because kidney and plasma reninase levels rapidly decreased after ischemic AKI in mice, urine and plasma reninase may also serve as a novel and sensitive biomarker for the early detection of ischemic AKI.

Recombinant reninase therapy may provide a novel therapeutic tool for the prevention and treatment of AKI, because we showed powerful protective effects of recombinant reninase against renal IR injury in mice. Specifically, exogenous recombinant reninase attenuated renal tubular necrosis (Jablonski renal injury score). Furthermore, we demonstrate reduced influx of proinflammatory neutrophils and macrophages into the kidney and renal tubular apoptosis after renal IR in recombinant reninase–treated mouse kidneys. Therefore, we conclude that exogenous administration of human recombinant reninase provides powerful renal protection against ischemic AKI by targeting all three pathways (necrosis, apoptosis, and inflammation) of renal cell injury. Similar to our findings, exogenous human recombinant reninase significantly protected against myocardial necrosis and decreased plasma catecholamine levels in reninase-deficient mice.15

We noted that recombinant reninase (1.5 mg/kg) provided significant but partial renal protection (creatinine decreasing from approximately 2.4 mg/dl to 1.4 mg/dl), most likely due to the severity of our ischemic AKI model (30 minutes of warm ischemia). Thirty minutes of renal ischemia would have caused significant renal tubular necrosis during ischemia that may not be rescued with reninase treatment. We also discovered that reninase does not provide dose-dependent protection and there was some reversal of protection at doses of 4.5 mg/kg. We believe that the high dose (4.5 mg/kg) failed to provide increased renal protection because reninase causes dose-dependent reduction in systemic BP.6 A previous study showed that a 4 mg/kg reninase dose reduced mean arterial pressure by approximately 40%.6 This reduction in systemic BP may have negated the renal protective effects of high-dose recombinant reninase.

Recombinant reninase therapy was also partially protective when administered 30 minutes after renal ischemia. This is highly exciting as recombinant reninase therapy may be effective for a diverse group of patients at risk for ischemic AKI. Although renal ischemia can be anticipated in many surgical procedures, a significant number of patients present to the hospital after renal ischemic injury has already occurred. Postischemic therapy for AKI will increase the translational as well as clinical significance because not all ischemic AKI can be anticipated in advance. However, we noted significant differences in the efficacy of reninase administered 10 minutes before renal ischemia and 30 minutes after reperfusion. We believe
that this is because of the severity of early reperfusion injury that occurs after 30 minutes of warm kidney ischemia. It appears that recombinant renalse must be present in the circulation to counteract the significant renal injury that occurs during 30 minutes after reperfusion.

We show in this study that increases in plasma NE levels were greater in renalse KO mice compared with the renalse WT mice after renal IR injury. Wu et al. have also demonstrated that plasma levels of catecholamines, including epinephrine, dopamine, and NE, are increased in renalse KO mice. We also showed in this study that renalse KO mice suffered increased renal tubular injury after renal IR. Furthermore, we demonstrate increased TNF-α, MCP-1, and MIP-2 after renal IR in renalse-deficient mice. In particular, MIP-2 is a chemokine involved in inflammation and immunoregulation and is a potent regulator of neutrophil chemotaxis. Consistent with our findings, renalse KO mice have exacerbated myocardial necrosis due to IR. Taken together, renalse deficiency appears to exacerbate ischemic organ injury and results in higher plasma catecholamine levels.

We hypothesize that the renal protective effects of recombinant renalse are, at least in part, due to increased metabolisms of plasma and tissue catecholamines. Consistent with this hypothesis, we show that recombinant renalse-mediated renal protection also resulted in significantly reduced plasma catecholamine levels. Increased catecholamine levels after ischemic AKI may exacerbate kidney injury by decreasing renal blood flow as well as by direct effects on renal tubules and immune cells. Catecholamines have been implicated in promoting tissue and organ injury in sepsis and systemic inflammatory response syndrome. For example, gut-derived NE has been implicated in causing hepatic injury and systemic inflammation in sepsis. Previous studies have shown that intestine-derived NE activates hepatic Kupffer cell α2-adrenoceptors to increase TNF-α generation and release. In septic rats, α2-adrenergic receptors upregulate in Kupffer cells to potentiate inflammatory response and organ injury. Furthermore, α1-adrenergic receptors increase LPS-mediated induction of proinflammatory cytokines in human monocytes and macrophages. Therefore, both α1- and α2-adrenergic receptors are implicated in proinflammatory effects of increased circulating catecholamines. Supporting a pathogenic role of α-adrenergic receptors against ischemic AKI, we found that blockade of α-adrenergic receptors provided significant renal protection in renalse WT as well as renalse KO mice.

Renal sympathetic nerves may play an important role in regulating ischemic AKI by modulating catecholamines released from the kidney. Indeed, increased renal sympathetic nerve activity during and after ischemic AKI increases renal
Table 1. Primers used to amplify cDNAs based on published GenBank sequences for mice

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<td>5′-CACCACCCCGTGTGATGCC-3′</td>
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ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemotactic protein-1; MIP-2, macrophage inflammatory protein 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

vein catecholamines released to systemic circulation and directly contributes to increased renal injury after IR.\textsuperscript{33} Furthermore, a previous study by Jiang \textit{et al.} showed that renal denervation increased plasma and kidney renalse levels.\textsuperscript{34} Therefore, renal sympathetic nerve activation may negatively modulate kidney and plasma renalse levels. Taken together, we propose that increased renal sympathetic nerve activity during and after renal IR exacerbates ischemic AKI by increasing plasma catecholamine levels and downregulating kidney/plasma renalse levels.

Given the robust protective effects of recombinant renalse, it is possible that renalse may protect against AKI via additional mechanisms beyond degradation of catecholamine. It would be extremely interesting to demonstrate in future studies whether renalse protects against other models of AKI (e.g., cisplatin nephrotoxicity) as well as whether recombinant renalse protects against necrosis and/or apoptosis in proximal tubule cells in culture. These experiments would significantly broaden the translational aspect of recombinant renalse and could provide further mechanistic insight to the observed beneficial properties of renalse against ischemic AKI.

In summary, our findings that exogenous recombinant renalse rescues against ischemic AKI and plasma renalse levels falls rapidly after ischemic AKI represent a novel approach to combat ischemic AKI. Our studies may lead to new therapeutic approaches with a drug that can reduce all three pathways of renal cell death (necrosis, apoptosis, and inflammation) to lessen the clinical perils from AKI and have implications in organ protection strategies beyond the kidney.

**Concise Methods**

**Synthesis of Recombinant Human Renalse**

Human recombinant renalse was synthesized as described.\textsuperscript{35} The complete methods are available in the Supplemental Material.

**Murine Model of Renal IR Injury**

After Columbia University Institutional Animal Care and Use Committee approval, we subjected adult male renalse-deficient (KO) mice\textsuperscript{15} on a C57BL/6 background to renal IR as described.\textsuperscript{36,37} Renalse KO or WT mice (C57BL/6 from Harlan Laboratories, Indianapolis, IN) were subjected to sham operation or to 20 minutes (moderate) or 30 minutes (severe) of renal ischemia and 24 hours of reperfusion. To test the renal protective effects of recombinant human renalse, we pretreated mice with saline (vehicle) or with recombinant renalse (0.5, 1.5, or 4.5 mg/kg, subcutaneously) 10 minutes before 30 minutes of renal ischemia. In addition, we tested whether renalse treatment after completion of renal ischemia also provides renal protection. Separate cohorts of mice were treated with saline or with renalse (1.5 mg/kg, subcutaneously) 30 minutes or 60 minutes after reperfusion of the ischemic kidney. To test whether blocking α-receptors would mimic the renal protective effects of human recombinant renalse administration, we administered phenolamine, an α-receptor antagonist (5 mg/kg, intraperitoneally), in some mice 15 minutes before renal ischemia.

**Measurement of Renal Function**

Plasma creatinine was measured as described, with an enzymatic creatinine reagent kit according to the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA).\textsuperscript{38}

**Measurement of Plasma NE**

Plasma NE in mice subjected to sham operation or to renal IR was measured with a commercial ELISA kit according to the manufacturer’s instructions (Rocky Mountain Diagnostics, Colorado Springs, CO).

**Histologic Detection of Necrosis, Apoptosis, and Neutrophil Infiltration**

An established grading scale of necrotic injury (renal injury score: 0–4) to the proximal tubules was used for the histopathological assessment of IR-induced damage as outlined by Jablonski \textit{et al.}\textsuperscript{16} and as described previously in our studies.\textsuperscript{39,40} We detected apoptosis after
renal IR with TUNEL staining as described using a commercially available in situ cell death detection kit (Roche, Indianapolis, IN) according to the instructions provided by the manufacturer. Kidney neutrophil and macrophage infiltrations were assessed with immunohistochemistry 24 hours after IR as described previously.19 Neutrophils and macrophages infiltrating the kidney were quantified in five to seven randomly chosen ×200 (neutrophils) or ×400 (macrophages) microscope image fields in the corticomedullary junction and results were expressed as neutrophils counted per ×200–400 field.

RT-PCR and Immunoblotting Analyses for Mouse Renalase
We measured mRNA encoding mouse renalase with RT-PCR as described. Glyceraldehyde 3-phosphate dehydrogenase mRNA was also measured to control for equal RNA input. In addition, mouse kidney cortex was also collected for immunoblotting analyses of renalase (Abcam, Cambridge, MA) and β-actin (internal protein loading control; Sigma) as described previously.

Measurement of Proinflammatory mRNA Expression after Intestinal IR
Kidney inflammation after renal IR in mice were additionally determined by measuring mRNA encoding markers of inflammation, including IL-17A, ICAM-1, MCP-1, MIP-2, and TNF-α and IL-6 (liver and kidney only) (Table 1). RT-PCR was performed as described.

Coimmunolocalization of Endogenous Renalase with E-Cadherin or Megalin in Pig Kidney
Our renalase antibody was synthesized against human renalase sequence. We performed renalase immunohistochemistry in pig kidneys because we felt that the human renalase sequence would be best conserved in pigs. Pig kidney slices were fixed, permeabilized, and incubated with anti-renalase 28–40 (raised against renalase peptide EAGTKIDVPWAGQYITSNPC) and with either anti-E-cadherin (BD Biosciences) or anti-megalin primary antibody (kind gift of Dr. D. Biemesderfer, Yale University School of Medicine, New Haven, CT) for 2 hours. Secondary antibodies (Alexa 488-goat anti-rabbit for detecting renalase) and Alexa 555-goat anti-mouse (Molecular Probes, for detecting E-cadherin or megalin) were then applied. Slides were imaged with a fluorescence microscope (Carl Zeiss Inc) and photographed using SPOT camera software (Diagnostic Instruments Inc).

Statistical Analyses
The data were analyzed with the t test when comparing means between two groups or with one-way ANOVA plus the Tukey’s post hoc multiple comparison test when comparing multiple groups. Two-way ANOVA plus Bonferroni post-test was used to test the effects of sham operation or renal IR injury on different mouse strains or treatment groups. The ordinal values of the renal injury scores were analyzed by the Mann–Whitney nonparametric test. In all cases, P<0.05 was
taken to indicate significance. All data are expressed throughout the text as mean ± SEM.

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DISCLOSURES

G.V.D. has two issued patents on renalase discovery.

REFERENCES


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