CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

JUN Amino-Terminal Kinase 1 Signaling in the Proximal Tubule Causes Cell Death and Acute Renal Failure in Rat and Mouse Models of Renal Ischemia/Reperfusion Injury

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Activation of the JUN amino-terminal kinase (JNK) pathway is prominent in most forms of acute and progressive tubulointerstitial damage, including acute renal ischemia/reperfusion injury (IRI). Two forms of JNK, JNK1 and JNK2, are expressed in the kidney. Systemic administration of pan-JNK inhibitors suppresses renal IRI; however, the contribution of JNK1 versus JNK2, and the specific role of JNK activation in the proximal tubule in IRI, remains unknown. These questions were addressed in rat and mouse models of acute bilateral renal IRI. Administration of the JNK inhibitor, CC-930, substantially reduced the severity of renal failure, tubular damage, and inflammation at 24 hours in a rat IRI model. Additionally, *Jnk1*^{-/-} mice, but not *Jnk2*^{-/-} mice, were shown to be significantly protected against acute renal failure, tubular damage, and inflammation in the IRI model. Furthermore, mice with conditional *Jnk1* deletion in the proximal tubule also showed considerable protection from IRI-induced renal failure, tubular damage, and inflammation. Finally, primary cultures of *Jnk1*^{-/-}, but not *Jnk2*^{-/-}, tubular epithelial cells were protected from oxidant-induced cell death, in association with preventing phosphorylation of proteins (receptor interacting serine/threonine kinase 3 and mixed lineage kinase domain-like pseudokinase) in the necroptosis pathway. In conclusion, JNK1, but not JNK2, plays a specific role in IRI-induced cell death in the proximal tubule, leading to acute renal failure. (*Am J Pathol* 2021, 191: 817–828; <https://doi.org/10.1016/j.ajpath.2021.02.004>)

Renal ischemia/reperfusion injury (IRI) is a common cause of acute kidney injury (AKI), which involves a partial or complete cessation of blood flow to the kidney with subsequent restoration of the circulation and re-oxygenation. This can occur in various settings, including kidney transplantation, cardiopulmonary bypass surgery, and severe hypotension associated with major blood loss or sepsis.¹ Ischemia results in a mismatch between oxygen supply and demand in local tissues, whereas re-oxygenation leads to excess production of oxygen free radicals. The ensuing hypoxia, oxidative stress, and impaired removal of waste result in tubular injury, inflammation, and, in cases of severe injury, cell death.¹ The pathogenesis of renal IRI is complex

and involves direct tubular epithelial cell damage through hypoxia/re-oxygenation and mitochondrial damage, as well as indirect tubular damage via the recruitment and activation of neutrophils and monocyte/macrophages.^{1,2}

The JUN amino-terminal kinase (JNK) is a stress-activated protein kinase that regulates cellular responses,

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such as inflammation and cell death.³ JNK is extremely sensitive to activation by reactive oxygen species, osmotic stress, and proinflammatory cytokines. Multiple JNK isoforms are encoded by three genes (*Jnk1*, *Jnk2*, and *Jnk3*), of which JNK1 and JNK2 are widely expressed, including in cells of the kidney.^{3,4} JNK facilitates stress-induced mitochondrial-dependent apoptosis and necrosis,⁵ including tubular cell death induced by ATP depletion or reactive oxygen species.^{6,7}

Activation of JNK signaling is evident in proximal tubular epithelial cells within 20 to 30 minutes of reperfusion of human kidney allografts.⁸ In a similar manner, JNK is strongly activated in tubular cells at 20 minutes after renal IRI in the rat.⁸ Prophylactic treatment with pan-JNK inhibitors, CC-401 or SP600125, provides considerable protection against tubular damage, leukocyte infiltration, and acute renal failure following IRI in rats.^{8,9} However, these studies do not indicate whether both JNK1 and JNK2 contribute to this injury, as is the case in renal interstitial fibrosis in the obstructed kidney,⁴ or whether only one JNK isoform is involved. In addition, these studies do not define whether JNK signaling in the proximal tubule is primarily responsible for inducing acute renal failure following IRI, or whether JNK signaling in other cell types may also be involved.

This study addressed two important mechanistic questions regarding the role of JNK signaling in IRI. First, is there redundancy between JNK1 and JNK2 in this response? Second, is JNK activation within the proximal

tubule sufficient to induce tubular cell damage, inflammation, and acute renal failure?

Materials and Methods

Reagents

The following primary antibodies were used: rabbit antibodies against phosphorylated JUN Ser63, cleaved caspase 3, phosphorylated receptor interacting serine/threonine kinase 3 (RIP3) Thr231/Ser232 and phosphorylated mixed lineage kinase domain-like pseudokinase (MLKL) Ser345 (Cell Signaling, San Diego, CA), rabbit anti- γ -glutamyl transferase, rat anti-mouse neutrophils (Ly6G), mouse anti-rat neutrophils (RP1), and mouse anti- α -tubulin (Abcam, Melbourne, VIC, Australia). CC-930 is a pan-JNK inhibitor and was provided by Celgene Corp. (San Diego, CA).^{10,11}

Animals

Outbred male Sprague-Dawley rats and inbred male C57BL/6J mice were obtained from the Monash Animal Research Platform (Clayton, VIC, Australia). *Mapk8/Jnk1*^{-/-} and *Mapk9/Jnk2*^{-/-} mice on the C57BL/6J background were imported from Jackson Laboratories (Bar Harbor, ME), and validation of the gene knockout is presented in [Supplemental Figure S1](#). *Jnk1*^{ff} and γ -glutamyltransferase (γ GT)—Cre recombinase expressing mice

Figure 1 JNK inhibitor treatment reduces acute kidney injury. Groups of vehicle (veh)— and CC-930—treated rats underwent bilateral ischemic/reperfusion injury (IRI), with sham controls. **A:** Serum creatinine. **B:** Blood urea nitrogen (BUN). **C:** Percentage of tubules showing injury. **D–F:** Periodic acid–Schiff staining of kidney sections. Representative images showing renal histology in **D:** Sham control with normal renal histology. **E:** Vehicle-treated IRI with loss of brush border, nuclear loss, sloughing of cells into the lumen, and cast formation, and. **F:** CC-930—treated IRI with substantial, but reduced, tubular damage. One-way analysis of variance with the Tukey multiple-comparisons test was performed. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars = 100 μ m (**D–F**).

Figure 2 JNK inhibitor treatment reduces tubular cell death and inflammation. Groups of vehicle (veh)— and CC-930—treated rats underwent bilateral ischemic/reperfusion injury (IRI), with sham controls. **A–C:** Images for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. **A:** Sham control. **B:** Vehicle-treated IRI, showing many TUNEL⁺ tubular cells. **C:** CC-930—treated IRI shows a reduction in TUNEL⁺ cells. **D:** Quantification of TUNEL staining. **E–I:** Real-time PCR analysis of kidney mRNA levels for *Kim1/Havcr1* (**E**), *Elane* (**F**), *Cd68* (**G**), *Nos2* (**H**), and *Tnf* (**I**). One-way analysis of variance with the Tukey multiple-comparisons test was performed. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale bars = 100 μ m (**A–C**).

have been described previously,^{12,13} and were backcrossed at least 12 times onto the C57BL/6J background. Animal studies were approved by the Monash Medical Center Animal Ethics Committee and performed according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Bilateral IRI

Rats underwent bilateral IRI, as previously described.^{14,15} Male rats were anesthetized with ketamine and xylazine. A heating blanket connected to a rectal thermometer was used to maintain body temperature at 37°C. Following a midline abdominal incision, both renal pedicles were clamped using non-traumatic vascular clamps for 25 minutes, during which the abdomen was temporarily sutured. Clamps were removed, and kidney reperfusion was confirmed visually. The abdomen was sutured in two layers, and saline was provided by s.c. injection. Analgesia involved s.c. injection of 0.05 mg/kg buprenorphine and 2 to 3 drops of bupivacaine onto the sutures at the end of surgery. Male mice underwent

the same bilateral IRI surgical procedure, with a 17-minute (*Jnk1*^{−/−} study) or a 19-minute (*Jnk2*^{−/−} and *Jnk1*^{fl/fl} γ GT-Cre study) ischemic time.

Groups of 9 or 10 rats were treated with 60 mg/kg b.i.d. CC-930 or vehicle alone (0.5% carboxymethylcellulose and 0.25% Tween-20) by oral gavage, starting 1 hour before surgery and continuing until animals were sacrificed 24 hours after reperfusion. Sham control animals underwent the same surgical procedure, except that the renal pedicles were not clamped. Serum creatinine and blood urea nitrogen levels were measured using a Dupont ARL Analyser (Wilmington, DE) by the Department of Biochemistry, Monash Health.

Analysis of Kidney Damage

Tubular damage was assessed in the outer medulla on periodic acid–Schiff–stained sections (2 μ m thick) of formalin-fixed tissue. The percentage of tubular cross-sections exhibiting damage was scored under high power ($\times 400$): damage was characterized as loss of the brush border, nuclear loss, and

Figure 3 JNK signaling in ischemic/reperfusion injury (IRI). Groups of vehicle (veh)— and CC-930—treated rats underwent bilateral IRI, with sham controls. **A–C:** Immunostaining of phosphorylated JUN (p-JUN) Ser63. **A:** Sham control with occasional stained cells. **B:** Vehicle-treated IRI, showing p-JUN staining in many tubular cells. **C:** CC-930—treated IRI shows a dramatic reduction in p-JUN—stained cells. **D:** Western blot analysis for p-JUN Ser63 in kidney lysates with TUBA1A/tubulin loading control. **E:** Quantification of Western blot analysis. One-way analysis of variance with the Tukey multiple-comparisons test was performed. *** $P < 0.001$. Scale bars = 100 μ m (**A–C**).

sloughing of cells into the lumen. The average number of tubular cross-sections scored in the rat model was 1293 per case, and the average number was 1155 per case in the mouse model. In the rat model, cell death was assessed in sections (4 μ m thick) of formalin-fixed tissue by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining with the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore-Chemicon, Ryde, NSW, Australia). The number of TUNEL⁺ tubular cells in the outer medulla was counted in high-power ($\times 400$) fields. All scoring was performed on blinded slides (F.Y.M., K.G.).

Immunohistochemistry

Immunostaining for phosphorylated JUN (p-JUN), cleaved caspase 3, and γ GT was performed on formalin-fixed sections (4 μ m thick) using antigen retrieval with 0.1 mol/L sodium citrate, pH 6.0, and a three-layer avidin-biotin peroxidase complex staining method, as previously described.¹⁶ Immunostaining for neutrophils was performed on cryostat sections of tissues fixed in 2% paraformaldehyde-lysine-periodate using a peroxidase-conjugated anti-peroxidase method.

The number of neutrophils and cleaved caspase 3 stained cells was counted in the outer medulla under high power ($\times 400$). All scoring was performed on blinded slides (K.G., F.Y.M.).

Western Blot Analysis

Cultured cells or frozen kidney tissue was homogenized in 0.5 mL of lysis buffer, sonicated, centrifuged, and processed for Western blot analysis, as previously described.¹⁷ Blots

were incubated with antibodies to phosphorylated JUN, phosphorylated RIP3, phosphorylated MLKL, or α -tubulin antibodies, which were detected with goat anti-rabbit Alexa Fluor 680 or donkey anti-mouse IRDye 800 secondary antibodies (Thermo Fisher Scientific, Scoresby, VIC, Australia) using the Odyssey Infrared Image Detecting System (LI-COR, Lincoln, NE). Densitometry analysis used ImageJ software version 1.51r (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>).

Real-Time PCR

RNA was extracted from frozen kidney samples with the Ambion RiboPure Kit (Thermo Fisher Scientific) and reverse transcribed with random primers using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). The PCR was performed using a StepOne Real-Time PCR system (Thermo Fisher Scientific) using TaqMan probes. The primer/probes for *Nos2* and *Tnfr* have been described previously,¹⁸ and the other primer/probes were purchased from Thermo Fisher Scientific. All amplicons were normalized against 18S in the same reaction, and the relative amount of mRNA was determined using the comparative cycle threshold (Δ Ct) method.

Primary Tubular Epithelial Cell Culture

Tubular cells were isolated from the kidney of normal *Jnk1*^{−/−}, *Jnk2*^{−/−}, and wild-type (WT) mice and cultured on collagen-coated plates, as previously described.¹⁹ To measure cell death, cells were starved in 1% fetal calf serum for 18 hours, and then varying concentrations of hydrogen peroxide were added for 24 hours. Cells then were analyzed

Figure 4 JNK1 promotes acute kidney injury. Groups of wild-type (WT) and *Jnk1*^{-/-} mice underwent bilateral ischemic/reperfusion injury (IRI), or a sham procedure. **A:** Serum creatinine. **B:** Blood urea nitrogen (BUN). **C–F:** Periodic acid–Schiff staining of kidney sections. **C:** WT sham control, showing normal renal histology. **D:** WT IRI, showing considerable tubular damage with loss of brush border, nuclear loss, sloughing of cells into the lumen, and cast formation. **E:** Higher-power field of **D**. **F:** *Jnk1*^{-/-} IRI shows significant, but reduced, tubular damage. **G–I:** Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (examples of stained cells shown by arrows). **G:** WT sham control lacks TUNEL staining. **H:** WT IRI shows multiple TUNEL-stained cells. **I:** *Jnk1*^{-/-} IRI shows a reduction in TUNEL-stained cells. **J–L:** Cleaved caspase 3 staining (examples of stained cells shown by arrows). **J:** WT sham control lacks staining. **K:** WT IRI shows multiple cells stained for cleaved caspase 3 (Casp-3). **L:** *Jnk1*^{-/-} IRI shows a reduction in stained cells. **M:** Percentage of tubules showing injury. **N:** Quantification of TUNEL staining. **O:** Quantification of cleaved caspase 3–stained cells. One-way analysis of variance with the Tukey multiple-comparisons test was performed. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 100 μm (**C**, **D**, and **F**); 20 μm (**E**); 40 μm (**G–L**).

using the Cell Death Detection ELISA Kit (Roche, Mannheim, Germany), with results normalized to the DNA content in cell lysates using a Quant-iT DNA Assay Kit (Molecular Probes, Eugene, OR) and expressed as the ratio of OD/DNA content.

Statistical Analysis

Data are shown as means $\pm$ SD. Data were analyzed by one-way analysis of variance with the Tukey multiple

comparison test using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA).

Results

Pharmacologic JNK Inhibition Prevents IRI-Induced AKI

Vehicle-treated rats developed severe acute kidney injury at 24 hours after bilateral renal IRI based on a marked

Figure 5 JNK1 promotes neutrophil infiltration. Groups of wild-type (WT) and *Jnk1*^{-/-} mice underwent bilateral ischemic/reperfusion injury (IRI), or a sham procedure. **A–C:** Immunostaining for neutrophils in the outer medulla. **A:** WT sham control, showing occasional neutrophils. **B:** WT IRI, showing many infiltrating neutrophils around damaged tubules. **C:** *Jnk1*^{-/-} IRI shows significant, but reduced, neutrophil infiltration. **D:** Quantification of neutrophil infiltration. One-way analysis of variance with the Tukey multiple-comparisons test was performed. **P < 0.01, ***P < 0.001. Scale bars = 100 μ m (**A–C**).

increase in serum creatinine and blood urea nitrogen levels (Figure 1, A and B). This was associated with pronounced histologic damage, including extensive tubular necrosis (Figure 1, C–E). Tubular damage was also evident by up-regulation of the tubular damage marker, *Kim1/Havcr1* (Figure 2E), and considerable tubular cell death, as shown by TUNEL staining (Figure 2, A, B and D). Vehicle-treated IRI showed a substantial neutrophil infiltrate based on immunostaining and increased *Elane* mRNA levels (Supplemental Figure S2, A and B, and Figure 2F), as well as a significant infiltrate of macrophages (Supplemental Figure S2, D and E) with increased mRNA levels of *Cd68* and the macrophage activation marker, *Nos2* (Figure 2, G and H). There was also up-regulation of the inflammatory cytokine, *Tnf* (Figure 2I).

Because CC-930 does not prevent JNK phosphorylation,¹¹ phosphorylation of JUN at Ser63 was examined as a readout of JNK activity. Only occasional cells exhibited p-JUN staining in the sham control. By contrast, many tubular epithelial cells exhibited p-JUN staining in the vehicle-treated IRI group (Figure 3, A and B). By comparison, CC-930 treatment substantially reduced the number of p-JUN⁺ cells, as shown by immunostaining and Western blot analysis (Figure 3, C–E). CC-930 treatment provided substantial protection against acute kidney injury, as shown by much lower serum creatinine and blood urea nitrogen levels (Figure 1, A and B), less histologic damage (Figure 1,

C and F), reduced *Kim1/Havcr1* mRNA levels (Figure 2E), and fewer TUNEL⁺ cells (Figure 2, C and D). In addition, CC-930 treatment significantly reduced the neutrophil and macrophage infiltration (Supplemental Figure S2, C and F) and expression of *Nos2* and *Tnf* (Figure 2, E–I).

Jnk1^{-/-} Mice Are Protected from IRI-Induced AKI

Because CC-930 inhibited virtually all JNK activity in the IRI model, a genetic approach was used to examine the relative contribution of JNK1 versus JNK2. The response of WT and *Jnk1*^{-/-} mice was compared in a 24-hour model of bilateral IRI-induced acute kidney injury. WT mice developed acute kidney injury 24 hours after IRI, as shown by increased levels of serum creatinine and blood urea nitrogen, histologic tubular damage, and tubular cell death, as identified by TUNEL and cleaved caspase 3 staining (Figure 4, A–E, G, H, J, K, and M–O). This tubular damage was associated with a substantial neutrophil infiltrate in the outer medulla and inner cortex (Figure 5, A and B).

In contrast, *Jnk1*^{-/-} mice showed significant protection from acute kidney injury in the renal IRI model based on lower levels of serum creatinine and blood urea nitrogen (Figure 4, A and B). *Jnk1*^{-/-} mice also showed less severe histologic tubular damage as well as a reduction in tubular cell death identified by TUNEL and cleaved caspase 3 staining (Figure 4, F, I, and L–O). Furthermore, *Jnk1*^{-/-} mice exhibited less neutrophil infiltration (Figure 5, C and D).

Jnk2^{-/-} Mice Are Not Protected from IRI-Induced AKI

Wild-type and *Jnk2*^{-/-} mice were examined in a 24-hour model of bilateral IRI. Compared with the marked increase in JUN phosphorylation seen in the WT IRI group by Western blot analysis, *Jnk2*^{-/-} mice showed an approximately 50% reduction in JUN phosphorylation (Figure 6, C and D). However, *Jnk2*^{-/-} mice were not protected from the development of severe kidney injury following IRI based on serum creatinine and blood urea nitrogen levels (Figure 7, A and B), tubular damage, and *Kim1/Havcr1* levels (Figure 7, C, E, and F). Similarly, *Jnk2*^{-/-} mice were not protected from neutrophil or macrophage infiltration (Supplemental Figure S3, A–C and E–G, and Figure 8, A and B) or the up-regulation of *Ccl2*, *Nos2*, *Tnf*, or *Il6* mRNA levels (Figure 8, C–F).

Jnk1^{PT}*Jnk2*^{-/-} Mice Are Protected from IRI-Induced AKI

The protection of *Jnk1*^{-/-} mice in the renal IRI model could be due to JNK1 facilitating tubular cell necrosis and/or JNK1 in neutrophils and macrophages facilitating their recruitment and activation in the injured kidney. To address the first possibility, mice lacking *Jnk1* in proximal tubules

Figure 6 JNK signaling in ischemic/reperfusion injury (IRI). Mice of different genotypes were sacrificed 30 minutes after reperfusion. **A:** Serial sections from a *Jnk1*^{ff}*Jnk2*^{-/-} mouse, showing brush border staining for γ -glutamyltransferase (γ GT) and nuclear staining for phosphorylated JUN (p-JUN) Ser63. Many tubules show p-JUN staining, including many γ GT⁺ tubules. **B:** Serial sections from a *Jnk1*^{PT}*Jnk2*^{-/-} mouse, showing staining for γ GT and p-JUN Ser63. There is a lack of p-JUN staining in γ GT⁺ tubules, although some other tubules show p-JUN staining. **C:** Western blot analysis of p-JUN with TUBA1A/tubulin control for sham and 30-minute IRI groups. **D:** Quantification of p-JUN Western blot analysis. One-way analysis of variance with the Tukey multiple-comparisons test was performed. ****P* < 0.001. Scale bars = 100 μ m (**A** and **B**). WT, wild type.

(*Jnk1*^{ff} γ GT^{Cre} mice, termed *Jnk1*^{PT}) were generated on the *Jnk2*^{-/-} background (note, the *Jnk2*^{-/-} background was employed before it was known that JNK2 plays no role in the IRI model).

First, a pilot study was performed to assess JNK signaling at 30 minutes after reperfusion, a time of maximal JNK activation and before the loss of tubular morphology.⁸ *Jnk1*^{ff}*Jnk2*^{-/-} mice showed a strong induction of JUN Ser63 staining at 30 minutes in most tubular segments, including γ GT stained proximal tubules, in the inner cortex and outer medulla (Figure 6A). By contrast, *Jnk1*^{PT}*Jnk2*^{-/-} mice showed a major reduction in p-JUN Ser63 staining in γ GT⁺ proximal tubules at 30 minutes after reperfusion,

although staining in other tubular segments was still apparent (Figure 6B). Western blot analysis of kidneys showed an almost complete inhibition of JUN phosphorylation in *Jnk1*^{PT}*Jnk2*^{-/-} mice (Figure 7, Figure 6 C and D).

Compared with the severe renal dysfunction seen in WT and *Jnk2*^{-/-} mice at 24 hours after IRI, *Jnk1*^{PT}*Jnk2*^{-/-} mice showed a marked reduction in serum creatinine and blood urea levels (Figure 7, A and B). In addition, *Jnk1*^{PT}*Jnk2*^{-/-} mice showed less histologic tubular damage and a reduction in *Kim1/Havcr1* mRNA levels (Figure 7, D–F). Furthermore, *Jnk1*^{PT}*Jnk2*^{-/-} mice showed a reduction in neutrophil and macrophage infiltration (Supplemental Figure S3, D and H, and Figure 8, A and B) and a reduction in *Ccl2*, *Nos2*,

Figure 7 JNK1 in proximal tubules promotes acute kidney injury. Groups of wild-type (WT), *Jnk2*^{-/-}, and *Jnk1*^{PT}*Jnk2*^{-/-} mice underwent bilateral ischemic/reperfusion injury (IRI), with WT sham mice as controls. **A**: Serum creatinine. **B**: Blood urea nitrogen (BUN). **C** and **D**: Periodic acid–Schiff staining of kidney sections. **C**: *Jnk2*^{-/-} IRI, showing extensive tubular damage with loss of brush border, nuclear loss, sloughing of cells into the lumen, and cast formation. **D**: *Jnk1*^{PT}*Jnk2*^{-/-} IRI, showing significant, but reduced, tubular damage. **E**: Percentage of tubules showing injury. **F**: Real-time PCR for *Kim1/Havcr1* mRNA levels. One-way analysis of variance with the Tukey multiple-comparisons test was performed. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale bars = 100 μm (**C** and **D**).

and *Il6* mRNA levels; however, the reduction in *Tnf* mRNA levels seen compared with WT IRI mice did not reach statistical significance compared with the *Jnk2*^{-/-} IRI group (Figure 8, C–F).

JNK1 Activates the RIP3/MLKL Pathway of Necroptosis in Tubular Epithelial Cells

The role of JNK1 and JNK2 in oxidant-induced cell death was investigated in primary cultures of tubular epithelial cells isolated from *Jnk1*^{-/-}, *Jnk2*^{-/-}, and WT mice. Tubular cells from WT and *Jnk2*^{-/-} mice showed an equivalent dose-dependent

increase in cell death in response to hydrogen peroxide stimulation, whereas *Jnk1*^{-/-} tubular cells were significantly protected against cell death (Figure 9A). CC-930 treatment significantly reduced hydrogen peroxide–induced cell death in both WT and *Jnk2*^{-/-} cells (Figure 9, B and D), whereas no further protection against cell death was seen in *Jnk1*^{-/-} cells (Figure 9C). Components of the necroptosis pathway were examined at 4 hours after hydrogen peroxide stimulation in cultured tubular epithelial cells. Both WT and *Jnk2*^{-/-} cells showed hydrogen peroxide–induced phosphorylation of RIP3 and MLKL, but this was absent in *Jnk1*^{-/-} cells (Figure 10).

Figure 8 JNK1 in proximal tubules promotes renal inflammation. Groups of wild-type (WT), *Jnk2*^{-/-}, and *Jnk1*^{PT}*Jnk2*^{-/-} mice underwent bilateral ischemic/reperfusion injury (IRI), with WT sham mice as controls. **A**: Quantification of immunostaining for neutrophils. **B–F**: Real-time PCR analysis of kidney mRNA levels of *Cd68* (**B**), *Ccl2* (**C**), *Nos2* (**D**), *Tnf* (**E**), and *Il6* (**F**). One-way analysis of variance with the Tukey multiple-comparisons test was performed. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Discussion

This study has demonstrated that pharmacologic inhibition of JNK signaling provides substantial protection against IRI-induced acute renal failure. Genetic studies identified that it is the JNK1 enzyme within the proximal tubule that plays a key role in the development of acute renal failure following IRI.

The demonstration that CC-930 provides substantial protection against acute renal failure following IRI provides significant new information. Wang et al⁹ showed that treatment with the JNK inhibitor SP600125 provides significant protection against acute renal failure in a rat bilateral IRI model. However, SP600125 is not selective for JNK as it inhibits 22 other kinases with equal or greater potency than JNK isoforms.^{20,21} Previous studies with CC-401 used a mild model of rat bilateral IRI in which serum creatinine was increased only 2.5-fold.⁸ Therefore, this finding was replicated in the more challenging situation of severe IRI and CC-930 JNK inhibitor was used as it has greater potency and a considerably longer half-life compared with CC-401.^{11,22} In the current study, the rat IRI model induced

a 5.4-fold increase in serum creatinine at 24 hours after IRI, and CC-930 treatment reduced serum creatinine levels by 60%. Furthermore, CC-930 treatment was effective in inhibiting JNK signaling, reducing tubular damage and necrosis, and reducing leukocyte infiltration and inflammation. These findings support the potential for a pharmacologic approach to preventing anticipated renal IRI-induced acute renal failure.

Previous studies have shown a considerable redundancy between JNK1 and JNK2 signaling in terms of JUN phosphorylation in tubular cells in the obstructed mouse kidney.⁴ In the current study, both JNK1 and JNK2 made a substantial contribution to JNK signaling in the kidney following IRI. Genetic deletion of *Jnk2* reduced JUN phosphorylation by approximately 50%, and then combined *Jnk2* deletion and *Jnk1* deletion in the proximal tubule reduced JUN phosphorylation by approximately 90%. However, global *Jnk1*, but not *Jnk2*, deletion provided substantial protection against IRI-induced acute renal failure. This finding was supported by *in vitro* studies showing protection of *Jnk1*^{-/-}, but not *Jnk2*^{-/-}, tubular cells from reactive oxygen species-induced cell death.

Figure 9 JNK1 promotes tubular cell death. Primary tubular cells were cultured from wild-type (WT), $Jnk1^{-/-}$, and $Jnk2^{-/-}$ mice. **A**: Tubular cell death induced by varying doses of hydrogen peroxide. **B–D**: Tubular cell death induced by 1 mmol/L H_2O_2 with or without CC-930 in WT cells (**B**), $Jnk1^{-/-}$ cells (**C**), and $Jnk2^{-/-}$ cells (**D**). One-way analysis of variance with the Tukey multiple-comparisons test was performed. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. a.u., arbitrary unit.

The finding that JNK1, and not JNK2, mediates IRI-induced acute renal failure is consistent with some, but not other, studies of nonrenal IRI. $Jnk2^{-/-}$ mice show protection in a model of liver IRI.²³ In addition, in a liver

transplant model, donor $Jnk2^{-/-}$ organs showed reduced damage and survived longer compared with wild-type livers.²⁴ By contrast, in a cardiac model of IRI, both $Jnk1^{-/-}$ and $Jnk2^{-/-}$ mice showed significantly reduced

Figure 10 JNK1 induces receptor interacting serine/threonine kinase 3 (RIP3)/mixed lineage kinase domain-like pseudokinase (MLKL) activation in tubular cells. Primary tubular cells were cultured from wild-type (WT), $Jnk1^{-/-}$, and $Jnk2^{-/-}$ mice. **A**: Western blot analysis of RIP3 phosphorylation and MLKL phosphorylation in tubular cells 4 hours after stimulation with 1 mmol/L H_2O_2 with TUBA1A/tubulin control. **B**: Quantification of phosphorylated RIP3 (p-RIP3) blots. **C**: Quantification of phosphorylated MLKL (p-MLKL) blots. One-way analysis of variance with the Tukey multiple-comparisons test was performed. *** $P < 0.001$.

myocardial damage.²⁵ However, the current findings are consistent with a previous study in the obstructed kidney in which tubular cell apoptosis was significantly reduced in *Jnk1*^{-/-} mice, but not in *Jnk2*^{-/-} mice.⁴

Studies using CC-930 treatment and global *Jnk1*^{-/-} mice indicate that systemic blockade of JNK not only reduces tubular cell necrosis, but also reduces neutrophil and macrophage infiltration, macrophage activation, and general markers of inflammation. The question is whether JNK signaling is important in multiple cell types in renal IRI. Although JNK signaling, as assessed by JUN phosphorylation, occurred predominantly in tubular epithelial cells in the IRI kidney, it does not preclude the importance of JNK signaling in infiltrating leukocytes because activation of this pathway may be transient and therefore difficult to detect by immunostaining. For example, JNK signaling is important for inducing a proinflammatory macrophage phenotype and renal injury in experimental glomerulonephritis,^{18,26} whereas JNK inhibition in adoptively transferred macrophages is sufficient to prevent acute macrophage-mediated glomerular injury.²⁷ However, a pathogenic role for macrophages in the induction of acute renal injury following IRI is uncertain.²⁸ *Jnk1* deletion in the proximal tubule was effective in reducing tubular cell damage and death as well as reducing neutrophil and macrophage infiltration, macrophage activation, and inflammatory markers. Thus, preventing the release of danger-associated molecular patterns from injured and dying tubular cells may be of greater importance in driving the leukocyte infiltration and activation than JNK activation per se in these cells, although studies of JNK deletion in myeloid cells to confirm this have yet to be performed.

Given that *Jnk1*^{-/-}, but not *Jnk2*^{-/-}, mice were protected in the renal IRI model, it follows that there is a specific JNK1 target involved in the induction of tubular cell death. A central role for the necroptosis pathway in mediating IRI-induced acute kidney injury has been shown by using mice lacking the key enzymes RIP3 or MLKL and the use of the RIP1 inhibitor Necrostatin-1.^{29,30} In the *in vitro* studies, deletion of *Jnk1*, but not *Jnk2*, in tubular cells prevented activation (phosphorylation) of RIP3 and MLKL at 4 hours after exposure of cells to reactive oxygen species. This indicates that JNK1 signaling is an early and essential component of the necroptosis pathway in tubular cells exposed to oxidative stress. Studies in other cell types and other animal models have shown that JNK and RIP3/MLKL act in an amplification loop to promote necroptotic cell death.^{31,32} However, this is the first study to demonstrate that JNK1 is required for RIP3 and MLKL activation in a necroptotic pathway. Further studies are required to identify the intermediate steps between JNK1 activation and RIP3 activation. One limitation of these *in vitro* studies is that they focus on oxidative stress-induced cell death without examining ischemia, an important cause of cell death in the IRI model.

In conclusion, the current study demonstrates that JNK1, but not JNK2, plays a critical role in acute renal failure following IRI. In particular, JNK1 activation in the proximal tubule promotes tubular cell damage and necrosis, with cell culture studies identifying a role for JNK1 in activation of the RIP3/MLKL pathway of necroptosis. Finally, pharmacologic blockade of JNK signaling has therapeutic potential for prevention of anticipated renal ischemia/reperfusion injury.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2021.02.004>.

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