Inhibition of Sphingosine 1-Phosphate Receptor 2 Protects against Renal Ischemia-Reperfusion Injury

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ABSTRACT
Activation of the sphingosine 1-phosphate receptor 1 (S1P1R) protects against renal ischemia-reperfusion (IR) injury and inflammation, but the role of other members of this receptor family in modulating renal IR injury is unknown. We found that a selective S1P2R antagonist protected against renal IR injury in a dose-dependent manner. Consistent with this observation, both S1P2R-deficient mice and wild-type mice treated with S1P2R small interfering RNA had reduced renal injury after IR. In contrast, a selective S1P2R agonist exacerbated renal IR injury. The S1P2R antagonist increased sphingosine kinase-1 (SK1) expression via Rho kinase signaling in renal proximal tubules; the S1P2R agonist decreased SK1. S1P2R antagonism failed to protect the kidneys of SK1-deficient mice or wild-type mice pretreated with an SK1 inhibitor or an S1P1R antagonist, suggesting that the renoprotection conferred by S1P2R antagonism results from pathways involving activation of S1P1R by SK1. In cultured human proximal tubule (HK-2) cells, the S1P2R antagonist selectively upregulated SK1 and attenuated both H2O2-induced necrosis and TNF-α/cycloheximide-induced apoptosis; the S1P2R agonist had the opposite effects. In addition, increased nuclear hypoxia inducible factor-1α was critical in mediating the renoprotective effects of S1P2R inhibition. Finally, induction of SK1 and S1P2R in response to renal IR and S1P2R antagonism occurred selectively in renal proximal tubule cells but not in renal endothelial cells. Taken together, these data suggest that S1P2R may be a therapeutic target to attenuate the effects of renal IR injury.


AKI is a major clinical complication with high mortality, morbidity, and cost.1–2 Renal ischemia and reperfusion (IR) injury is a major cause of perioperative AKI for patients undergoing surgery involving the kidney, liver, or aorta.3,4 Unfortunately, the severity and incidence of AKI have been increasing, without any improvements in therapy or patient survival over the past 50 years.5 The incidence of renal dysfunction in high-risk patients after major cardiovascular, hepatobiliary, or aortic surgery approaches 70%–80%.3,4,6 Despite continued research searching for renal protective agents, there are no proven therapies to reduce AKI in the perioperative setting.1,7

Sphingolipids are pleiotropic regulators of kidney physiology that modulate diverse pathways of cell death, including necrosis, apoptosis, inflammation, and immunity.8,9 In particular, phosphorylation of sphingosine by sphingosine kinases (SK1 and SK2) leads to the formation of sphingosine 1-phosphate (S1P), a lysophospholipid targeting G-protein–coupled receptor that has diverse extracellular as well as intracellular effects.9 Of five G-protein–coupled receptors for S1P, activation of endothelial S1P1R receptor (S1P1R) reduces permeability and maintains the integrity of the vascular endothelial cell

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barrier. S1P2R activation also protects against cardiac, renal, and hepatic IR injury and inflammation. In contrast, S1P2R activation may have the opposite effects, with potentially adverse vascular signaling events. These previous studies suggest that a balance of S1P1R and S1P2R activation may modulate the tissue response to endogenous and exogenous S1P. However, unlike the better-characterized role of the S1P1R, the role of the S1P2R in tissue injury secondary to IR remains unclear. Moreover, the direct renal tubular effects of S1P2R activation have never been described. In this study, we aimed to test the role of S1P2R in modulating renal injury after IR.

RESULTS
Pharmacologic Blockade, Genetic Deletion, or In Vivo Knockdown of S1P2R Protects against Renal IR Injury in Mice
We initially tested the effects of selective S1P1R (W146), S1P2R (JTE-013), or S1P3R (CAY10444) blockade on renal IR injury in mice (Figure 1A); all drugs were given at a dose of 0.1 mg/kg body wt intraperitoneally 10 minutes before and 30 minutes after renal ischemia. Renal IR caused statistically significant increases in plasma creatinine in all groups. However, blockade of the S1P2R produced significant renal protection against renal IR injury after IR.

Figure 1. S1P2R activation modulates renal injury after IR. (A) Treatment with a selective S1P2R inhibitor (JTE-013; 0.1 mg/kg intraperitoneally) or S1P3R (CAY10444; 0.1 mg/kg intraperitoneally) antagonists did not decrease renal injury after IR (n=5–6 in each group). (B) JTE-013 protected the kidney against renal IR in a dose-dependent manner (0.01–0.1 mg/kg intraperitoneally given twice at 10 minutes before ischemia and at 30 minutes after reperfusion; n=6). (C) JTE-013 (0.1 mg/kg, single intraperitoneal injection) administered 10 minutes before ischemia or 30 minutes after reperfusion (Reperf) protected against renal IR injury. However, JTE-013 (0.1 mg/kg, single intraperitoneal injection) administered 60 minutes after reperfusion did not produce renal protection after IR (n=6 per group). (D) Mice deficient in the S1P2R (S1P2R−/−) or wild-type (WT) mice treated with S1P2R siRNA (50 μg intravenously 48 hours before IR) were protected against renal IR compared with wild-type mice or mice treated with scrambled control siRNA (n=4–6 per group). (E) Renal S1P1-5 mRNA expression after sham operation or 30 minutes of renal ischemia followed by 24 hours of reperfusion (IR). Representative reverse transcriptase PCR bands (top) and densitometric quantification of relative band intensities normalized to GAPDH (bottom, n=4) are shown. S1P2R induction was most robust after renal IR. (F) Evidence for in vivo gene knockdown of the S1P2R with siRNA treatment. Representative bands and densitometric quantification of relative S1P2R mRNA (normalized to GAPDH) in mouse kidney. Mice were injected with scrambled control siRNA or siRNA targeting the S1P2R 48 hours before renal IR (n=4). (G) Treatment with a selective S1P2R agonist (SID46371153, SID) exacerbated mild (20-minute) and severe (30-minute) renal injury after IR. Mice were treated with vehicle or SID46371153 (0.1 or 1 mg/kg, injected intraperitoneally 10 minutes before ischemia) and subjected to sham operation (n=4) or to 20 or 30 minutes of renal ischemia followed by 24 hours of reperfusion (IR, n=6). *P<0.05 versus vehicle-treated sham operated mice; #P<0.05 versus vehicle-treated mice subjected to renal IR. Error bars represent 1 SEM.
IR injury compared with vehicle-treated mice. Neither S1P1R nor S1P3R antagonist pretreatment affected renal IR injury. We subsequently showed dose-dependent renal protection with JTE-013, 0.05–0.1 mg/kg injected intraperitoneally 10 minutes before and 30 minutes after renal ischemia, which produced maximal renal protection in mice after IR injury (Figure 1B). We also tested whether blockade of S1P2R after renal ischemia protected against renal IR injury. Figure 1C shows that JTE-013, 0.1 mg/kg, injected intraperitoneally 10 minutes before ischemia or 30 minutes after reperfusion protected against renal IR injury. However, JTE-013 administered 60 minutes after reperfusion did not produce renal protection after IR.

We also demonstrated that mice genetically deficient in S1P2R (S1P2R−/−) or wild-type mice treated with small interfering RNA (siRNA) targeting the S1P2R were also protected against renal IR injury compared with wild-type or scrambled control siRNA–treated mice (Figure 1D). In addition, we determined that renal cortical mRNA expression of S1P1R, S1P2R, and S1P3R were significantly increased after renal IR. Of these three S1P receptor subtypes, we observed the greatest induction in S1P2R (approximately sixfold) after renal IR (Figure 1E). In contrast, the expression of S1P1R and S1P3R did not change after renal IR. Figure 1F demonstrates significantly reduced S1P2R mRNA expression in the kidneys of mice treated with siRNA targeting S1P2R compared with scrambled control siRNA–treated mice. The S1P2R siRNA also prevented the increase in S1P2R mRNA expression after renal IR. Other S1P receptors (S1P1R, S1P3R, S1P4R, and S1P5R) were not affected by S1P2R siRNA treatment (not shown).

Selective Activation of the S1P2R Exacerbates Renal IR Injury in Mice

In contrast to the renal protective effects of S1P2R blockade, a selective S1P2R agonist, SID46371153, produced dose-dependent exacerbation of renal injury in mice subjected to mild (20-minute) or severe (30-minute) renal IR (Figure 1G). Plasma creatinine levels were similar in sham-operated mice treated with vehicle or those treated with SID46371153 alone.

**S1P2R Blockade or Deletion Reduces Renal Tubular Necrosis after Renal IR Injury**

Compared with vehicle-treated sham-operated mice (Figure 2A, representative of four experiments), significant renal injury was evident in vehicle-treated mice subjected to renal IR (Figure 2B), with severe tubular necrosis, corticomedullary congestion, and hemorrhage, and development of proteinaceous casts (30-minute renal ischemia and 24-hour reperfusion). However, mice treated with a selective S1P2R antagonist (JTE-013; 0.1 mg/kg) displayed markedly reduced renal tubular damage and necrosis after renal IR compared with vehicle-treated mice (Figure 2C). Consistent with this, wild-type mice treated with siRNA for S1P2R (Figure 2D) or S1P2R−/− mice (Figure 2E) had improved renal histologic findings after IR. In contrast, mice treated with a selective S1P2R agonist (SID46371153) showed increased renal injury after IR (Figure 2F).

The Jablonski scale Renal Injury Score for histology grading was used to grade renal tubular necrosis 24 hours after IR. Representative photomicrographs (magnification ×200) of hematoxylin and eosin–stained kidney sections. Mice were subjected to sham-operation (A) or to 30 minutes of renal ischemia followed by 24 hours of reperfusion (IR). Wild-type mice were treated with vehicle (B), JTE-013 (a selective S1P2R antagonist; 0.1 mg/kg intraperitoneally 10 minutes before ischemia and again 30 minutes after reperfusion) (C), S1P2R siRNA (50 μg intravenously 48 hours before ischemia) (D), or SID46371153 (a selective S1P2R agonist; 1 mg/kg intraperitoneally 10 minutes before ischemia) (F). We also subjected S1P2R−/− mice to renal IR (E). Photographs are representative of five independent experiments. (G) Summary of Jablonski scale Renal Injury Scores (scale, 0–4) for mice subjected to sham operation or renal IR. *P<0.05 versus vehicle-treated sham operation; #P<0.05 versus vehicle-treated mice subjected to renal IR. Error bars represent 1 SEM.
renal IR (Figure 2G). Thirty minutes of renal ischemia and 24 hours of reperfusion resulted in severe acute tubular necrosis in vehicle-treated mice (score, 2.6±0.4; n=5). In contrast, wild-type mice treated with JTE-013, S1P2R−/− mice, and wild-type mice treated with siRNA targeting the S1P2R had significantly lower Renal Injury Scores than vehicle-treated mice subjected to renal IR (Figure 2G). We also show that mice treated with SID46371153 showed even greater renal tubular necrosis after IR, consistent with significantly increased plasma creatinine.

S1P2R Blockade or Deletion Reduces Renal Apoptosis after Renal IR Injury

We failed to detect significant apoptotic cells positive for terminal deoxynucleotidyl transferase–mediated digoxigenin-dideoxyuridine nick-end labeling (TUNEL) in kidney sections from sham-operated mice (Figure 3A, representative of four experiments). Thirty minutes of renal ischemia and 24 hours of reperfusion resulted in increased apoptotic cells in the kidney (Figure 3B). In contrast, wild-type mice treated with JTE-013 (Figure 3C), wild-type mice treated with siRNA for S1P2R (Figure 3D), and S1P2R−/− mice (Figure 3E) had fewer apoptotic positive cells in the kidney scores compared with vehicle-treated mice subjected to renal IR. We also show that mice treated with SID46371153 showed even more apoptotic cells in the kidney after IR, consistent with significantly increased plasma creatinine (Figure 3F).

S1P2R Blockade Reduces Renal Pro-Inflammatory Gene Expression after Renal IR Injury

We found significantly increased mRNA expression of proinflammatory genes (TNF-α, intracellular adhesion molecule-1, ICAM-1, MCP-1, MIP-2) in vehicle-treated mice subjected to renal IR (Figure 4). Mice were injected with vehicle or JTE-013 (a selective S1P2R antagonist; 0.1 mg/kg intraperitoneal) 10 minutes before sham surgery or 10 minutes before ischemia and 30 minutes after reperfusion. Treatment with JTE-013 significantly inhibited the increases in pro-inflammatory TNF-α, ICAM-1, MCP-1 and MIP-2 expression after renal IR. *P<0.05 versus vehicle-treated sham operation; #P<0.05 versus vehicle-treated mice subjected to renal IR. Error bars represent 1 SEM.

Figure 3. S1P2R blockade reduces renal apoptosis after renal IR injury. Representative fluorescence photomicrographs (four experiments) of kidney sections illustrating apoptotic nuclei TUNEL fluorescence staining; original magnification ×100) in the outer medulla of the kidneys of mice subjected to sham operation (A) or to 30 minutes of renal ischemia followed by 24 hours of reperfusion (IR). Wild-type mice were treated with vehicle (B), JTE-013 (a selective S1P2R antagonist; 0.1 mg/kg intraperitoneally 10 minutes before ischemia and again 30 minutes after reperfusion) (C), S1P2R siRNA (50 μg intravenously 48 hours before ischemia) (D) or SID46371153 (a selective S1P2R agonist; 1 mg/kg intraperitoneally 10 minutes before ischemia) (F). We also subjected S1P2R−/− mice to renal IR (E).

Figure 4. S1P2R blockade reduces renal pro-inflammatory gene expression after renal IR injury. Renal pro-inflammatory mRNA expression after sham operation or 30 minutes of renal ischemia followed by 5 hours of reperfusion (IR; n=4 per group). Representative reverse transcription PCR bands and densitometric quantifications of relative mRNA band intensities normalized to GAPDH are shown. Mice were injected with vehicle or JTE-013 (a selective S1P2R antagonist; 0.1 mg/kg intraperitoneal) 10 minutes before sham surgery or 10 minutes before ischemia and 30 minutes after reperfusion. Treatment with JTE-013 significantly inhibited the increases in pro-inflammatory TNF-α, ICAM-1, MCP-1 and MIP-2 expression after renal IR. *P<0.05 versus vehicle-treated sham operation; #P<0.05 versus vehicle-treated mice subjected to renal IR. Error bars represent 1 SEM.
monocyte chemotactic protein-1, or macrophage inflammatory protein-2) in vehicle-treated mice subjected to renal IR 5 hours earlier (collected 5 hours after surgery; Figure 4), and JTE-013 treatment significantly reduced expression of these pro-inflammatory mRNAs.

**Inhibition of Rho or Rho-Associated Protein Kinase Prevents S1P2R-Mediated Exacerbation of Renal IR Injury**

Because S1P2R can activate Rho guanosine triphosphatase as well as Rho-associated protein kinase (ROCK),16 we tested whether a selective S1P2R agonist, SID46371153, targets Rho, as well as ROCK to exacerbate renal dysfunction after IR injury. We pretreated mice with a specific Rho inhibitor C3-exotoxin20 (0.2 mg/kg administered intraperitoneally 1 hour before renal ischemia) or with a selective inhibitor of ROCK21 (Y27632; 10 mg/kg given intraperitoneally 1 hour before renal ischemia). We determined that both C3-exotoxin (creatinine, 1.8±0.2 mg/dl; n=4) and Y27632 (creatinine, 1.9±0.2 mg/dl; n=4) attenuated the renal dysfunction induced with 1 mg/kg SID46371153 plus renal IR (creatinine, 3.0±0.2 mg/dl; n=6). Moreover, treating mice with C3-exotoxin (creatinine, 1.6±0.2 mg/dl; n=4) or Y27632 (creatinine, 1.9±0.2 mg/dl; n=4) alone protected against renal IR injury.

Figure 5. S1P2R blockade upregulates renal SK1 expression in mice. Renal sphingosine kinase mRNA (A, C, E, and G) and protein (B, D, F, and H) expression in wild-type mice treated with JTE-013 (0.1 mg/kg intraperitoneally 6 hours before [A and B]; n=4), wild-type mice treated with S1P2R siRNA (50 μg intravenously 48 hours before [C and D]; n=4), S1P2R−/− mice (E and F; n=4) and wild-type mice treated with SID46371153 (a selective S1P2R agonist; 1 mg/kg, intraperitoneally 6 hours before sham operation or renal IR) (G and H; n=4). I. Renal S1P lyase protein expression in wild-type mice treated with JTE-013 (0.1 mg/kg intraperitoneally 6 hours before sham operation or renal IR) or with vehicle (n=3–4). S1P2R deletion or blockade significantly and selectively increased SK1 expression compared with vehicle-treated mice (n=4–6 per group) without affecting S1P lyase expression. Conversely, S1P2R agonist significantly decreased SK1 expression compared with vehicle-treated mice. *P<0.05 versus respective control mice (vehicle, scramble, or wild-type). Error bars represent 1 SEM.
Regulation of SK1 mRNA and Protein Expression with S1P2R Modulation In Vivo

Because a prior study suggested that deletion or downregulation of S1P2R led to an induction of SK1 in mouse embryonic fibroblasts,22 and because induction of SK1 has been shown to protect against cardiac and renal IR injury,23–25 we tested whether SK1 mRNA and protein expression are induced with selective S1P2R blockade. Figure 5, A and B shows that kidneys from mice treated with JTE-103 (0.1 mg/kg) had significantly increased SK1 mRNA and protein expression in 6 hours with no changes in SK2 expression. Consistent with these findings, kidneys from S1P2R−/− mice or wild-type mice treated with S1P2R siRNA show significantly and selectively increased SK1 mRNA and protein expression (Figure 5, C–F). In contrast, activation of S1P2R with SID46371153 downregulated SK1 mRNA and protein expression in the kidney (Figure 5, G and H).

We also measured plasma and kidney S1P levels with HPLC.15,23 We determined that both plasma (13.5 ± 2 pmol/μl; n = 4) and kidney (10.2 ± 2.8 pmol/mg protein; n = 4) S1P increased significantly after renal IR compared with values in sham-operated animals (plasma S1P, 0.6 ± 0.3 pmol/μl; n = 4; P < 0.05; kidney S1P, 1.4 ± 0.2 pmol/mg protein; n = 4; P < 0.05). Furthermore, consistent with increased SK1 mRNA and protein expression, selective S1P2R blockade with JTE-013 treatment also increased plasma (1.7 ± 0.3 pmol/μl; P < 0.05) and renal (3.7 ± 0.8 pmol/mg protein; n = 4; P < 0.05) S1P levels compared with values in vehicle-treated sham-operated mice.

Renal S1P levels can also be modulated by changes in S1P lyase (an enzyme that degrades S1P to phosphoethanolamine and palmitaldehyde).26 Therefore, we also performed immunoblotting for S1P lyase in mouse kidney tissues subjected to sham-operation or to renal IR after vehicle or JTE-013 injection to test whether S1P degrading capacity changed after IR injury or with selective S1P2R blockade. We determined that mouse kidney S1P lyase protein expression did not change 5 hours after renal IR or with S1P2R blockade with JTE-013 (Figure 5I).

Inhibition of SK1 or S1P1R Signaling Prevents Renal Protection with S1P2R Blockade

Plasma creatinine increased significantly in wild-type, SK1−/−, and SK2−/− mice subjected to renal IR injury compared with sham-operated mice. JTE-013 protected wild-type mice and SK2−/− mice against renal IR injury (Figure 6A). In contrast, SK1−/− mice were not protected against renal IR injury after JTE-013 treatment. We also treated some wild-type mice with SK1-II (a selective but nonspecific SK inhibitor) or with CAY10621 (a SK1-specific inhibitor) before induction of renal IR injury. Vehicle, SKI-II, or CAY10621 administration had no
detrimental effects on renal function in sham-operated mice (data not shown). SKI-II or CAY10621 treatment blocked the renal protective effects of JTE-013, providing additional evidence that renal protection with S1P,R blockade is mediated by the induction of SK1 activity (Figure 6B). We subsequently determined that JTE-013 (0.1 mg/kg) failed to protect against renal IR injury when mice were also treated with a selective S1P,R antagonist (W146, 0.1 mg/kg given intraperitoneally 20 minutes before ischemia and 20 minutes after reperfusion; Figure 6C). Therefore, it appears that additional S1P synthesized with JTE-013–mediated induction of SK1 protects against renal IR via pathways involving activation of S1P,R.

Regulation of SK1 Expression in Human Proximal Tubule-2 Cells In Vitro by S1P2R Modulation and Rho Kinase

Figure 7, A–D shows that human proximal tubule-2 (HK-2) cells treated with JTE-013 (1 μM) or with SID46371153 (1 μM) for 6 hours significantly increased and decreased, respectively, SK1 mRNA and protein expression, with no changes in SK2 expression. We also show that a specific ROCK inhibitor, Y27632, significantly increased SK1 mRNA and protein expression in HK-2 cells (Figure 7, E and F) and in mouse kidney (Figure 7, G and H), mimicking the effects of a selective S1P,R antagonist (JTE-013).

S1P2R Modulation Regulates HK-2 Cell Necrosis and Apoptosis In Vitro

HK-2 cells pretreated with the selective S1P2R antagonist JTE-013 were protected against H2O2-induced necrosis (5 mM H2O2 for 2 hours; Figure 8) and apoptosis (20 ng/ml TNF-α and 10 μg/ml cycloheximide treatment for 16 hours; Figure 9). In contrast, a selective S1P,R agonist, SID46371153, potentiated H2O2-induced HK-2 cell necrosis (Figure 8). Moreover, SID46371153 potentiated apoptotic cell death in HK-2 cells (data not shown).

Critical Role of HIF-1α in S1P2R Antagonist–Induced Upregulation of SK1 and In Vivo Renal Protection

Because HIF-1α is known to be involved in SK1 induction in human endothelial cells,27 we tested the hypothesis that JTE-013 induces SK1 via an HIF-1α–dependent mechanism in HK-2 cells. When HK-2 cells were pretreated with 10 μM of 2-methoxyestradiol (a potent HIF-1α inhibitor) 30 minutes before exposure to JTE-013 (1 μM for 6 hours), induction of

Figure 7. Inhibition of S1P2R or Rho kinase upregulates SK1 expression in human proximal tubule cells. SK mRNA (A and C) and protein (B and D) expression in HK-2 cells treated with vehicle, JTE-013, or SID46371153 for 6 hours (n=4 for each group). JTE-013 (a selective S1P2R antagonist; 1 μM) significantly and selectively induced SK1 mRNA (A) and protein (B) expression compared with vehicle treatment (0.001% DMSO). In contrast, SID46371153 (a selective S1P2R agonist; 1 μM) significantly and selectively reduced SK1 mRNA (C) and protein (D) expression compared with vehicle treatment (0.2% DMSO). (E–H) A specific Rho kinase (ROCK) inhibitor Y27632 increased SK1 mRNA and protein expression in HK-2 cells (E and F) and in mouse kidney (G and H) mimicking the effects of a selective S1P2R antagonist (JTE-013). *P<0.05 versus vehicle treatment. Error bars represent 1 SEM.
S1P2R modulates necrosis of HK-2 cells. Lactate dehydrogenase (LDH) released after H2O2-induced necrosis in HK-2 cells (n=5–6 for each group, expressed as a percentage of total LDH released). Vehicle (DMSO in saline, 0.001% for JTE-013 and 0.2% for SID46371153; JTE-013 (0.1 or 1 μM), or SID46371153 (1 or 5 μM) was applied 30 minutes before H2O2 treatment. JTE-013 attenuated whereas SID46371153 significantly exacerbated LDH release in a dose-dependent manner. *P<0.05 versus respective vehicle treatment. Error bars represent 1 SEM.

Figure 9. S1P2R blockade attenuates HK-2 cell apoptosis induced by TNF-α (20 ng/ml) and cycloheximide (CHX; 10 μg/ml). Representative poly(adenosine diphosphate-ribose) polymerase (PARP) and caspase-3 fragmentation (n=4 for each group) as indices of HK-2 cell apoptosis. JTE-013 (0.1 or 1 μM) was applied 30 minutes before induction of apoptosis, and cells were harvested 16 hours later. S1P2R receptor blockade reduced PARP and caspase fragmentation in HK-2 cells.

mRNA encoding SK1 was significantly attenuated (Figure 10A) without affecting SK2 expression.

We next tested whether inhibition of S1P2R directly increases nuclear HIF-1α and subsequent DNA binding in HK-2 cells and in mouse kidneys (Figure 10, B and C). In HK-2 cells, a selective S1P2R antagonist, JTE-013, dose-dependently increased nuclear HIF-1α and DNA binding, which was completely inhibited by 10 μM of 2-methoxyestradiol (given 30 minutes before JTE-013 treatment; Figure 10B). We also showed that JTE-013 increases nuclear HIF-1α in sham-operated mouse kidneys (Figure 10C). Renal IR (30-minute renal ischemia and 24-hour reperfusion) increased nuclear HIF-1α, which was further increased with JTE-013 (Figure 10C). Treating mice with a selective HIF-1α inhibitor, YC-1 (50 mg/kg given intraperitoneally), and SC221724 (20 mg/kg given intraperitoneally) completely prevented the increase in mouse kidney HIF-1α DNA binding. Finally, we showed that mice pretreated with a selective HIF-1α inhibitor, YC-1 (50 mg/kg given intraperitoneally), or SC221724 (20 mg/kg given intraperitoneally) were not protected against renal IR injury with JTE-013 treatment (Figure 10D). Taken together, these data demonstrate a critical role of increased nuclear HIF-1α in mediating the renal protective effects of S1P2R inhibition.

The next series of experiments were performed to determine whether S1P2R modulates nuclear translocation of HIF-1α by affecting existing HIF-1α protein or by synthesizing new HIF-1α. HK-2 cells were subjected to 24 hours of normoxia or to hypoxia (2% O2). A selective S1P2R antagonist (JTE-013; 1 μM) directly increased renal tubular HIF-1α mRNA and protein expression under both normoxic and hypoxic conditions (Figure 11A). In addition, a selective S1P2R agonist (SID46371153; 1 μM) decreased HIF-1α mRNA expression in normoxic HK-2 cells. SID46371153 also reduced HIF-1α mRNA and protein expression in hypoxic HK-2 cells (Figure 11B). JTE-013 increased and SID46371153 attenuated hypoxia-driven increases in SK1 mRNA and protein expression (Figure 11, A and B). A prolyl hydroxylase inhibitor dimethyloxaloylglycine (DMOG) (1 mM) reversed the SID46371153-mediated decreases in SK1 and HIF-1α protein expression in HK-2 cells. Finally, SID46371153 reduced the induction of HIF-1α in mouse kidneys subjected to renal IR (Figure 11C). Preventing this reduction of HIF-1α expression with DMOG injection (5 mg per mouse intraperitoneally; creatinine, 2.36±0.2 mg/dl; n=4) blocked the exacerbation of renal injury with SID46371153 (creatinine, 3.0±0.2 mg/dl; n=6). Therefore, these data suggest that S1P2R directly modulates new synthesis of HIF-1α that directly contributes to increased SK1 synthesis and renal protection.

Selective Regulation of S1P2R and SK1 in Proximal Tubule Cells after Renal IR or JTE-013 Treatment

Next, we wanted to determine the specific kidney cell type that induces S1P2R and SK1 in response to renal IR or selective S1P2R antagonism. Mice were injected with vehicle or with 0.1 mg/kg JTE-013, then subjected to sham operation or to renal IR (Figure 12). We isolated proximal tubules (with Percoll density gradient separation) and renal endothelial cells (with Dynabeads coupled to CD144 antibody). We determined that renal IR induced SK1 in both renal proximal tubule cells (Figure 12A) and endothelial cells (Figure 12B). However, JTE-013 increased SK1 mRNA and protein expression only in mouse proximal tubules (Figure 12A) and not in renal endothelial cells (Figure 12B). Renal IR also did not induce S1P2R mRNA in mouse kidney endothelial cells (Figure 12B). In addition, we
Figure 10. HIF-1α mediates S1P2R antagonist-induced upregulation of sphingosine kinase-1 and in vivo renal protection. (A) Induction of SK1 by S1P2R blockade is mediated by HIF-1α. Representative reverse transcriptase PCR bands and densitometric quantification of SK mRNA (band intensities normalized to glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) in HK-2 cells treated with vehicle (0.001% DMSO; n=4) or JTE-013 (1 μM; n=4) for 6 hours. We blocked HIF-1α function with a post-transcriptional inhibitor 2-methoxyestradiol (2ME; 10 μM) given 30 minutes before vehicle or JTE-013 treatment. Inhibition of HIF-1α by 2-methoxyestradiol significantly attenuated the increase in SK1 mRNA with JTE-013 treatment. (B) HIF-1α DNA binding activity in nuclear extracts from HK-2 cells treated with vehicle or JTE-013 (n=4) for 6 hours. JTE-013 increases nuclear translocation of HIF-1α, which is inhibited by 2-methoxyestradiol (10 μM, given 30 minutes before JTE-013 treatment). (C) JTE-013 increases HIF-1α nuclear translocation in kidneys of mice subjected to sham operation (n=4) or 30 minutes of renal ischemia followed by 24 hours of reperfusion (IR) (n=4). Pretreatment with a selective HIF-1α inhibitor, YC-1 (50 mg/kg intraperitoneally), or SC221724 (20 mg/kg intraperitoneally), blocked the increase in kidney HIF-1α DNA binding. (D) YC-1 (50 mg/kg intraperitoneally) or SC221724 (20 mg/kg intraperitoneally) prevented renal protection with JTE-013 (n=4–6 in each group). Plasma creatinine was measured from 24 hours after reperfusion. *P<0.05 versus vehicle treatment (A and B) or vehicle sham (C and D); #P<0.05 versus JTE-013 treatment (A and B) or vehicle IR (C and D); $P<0.05 versus JTE-013 IR (C). Error bars represent 1 SEM. COS-7, African Green Monkey kidney fibroblast cell line.

Also observed a robust induction of SK1 mRNA and protein in freshly isolated mouse proximal tubules from naïve mice treated with 1 μM JTE-103 for 6 hours (Figure 12C). Finally, JTE-013 failed to induce SK1 and S1P2R in immortalized human endothelial cells (EA.hy926 cells) (Figure 12D).

**Discussion**

S1P is an endogenous lysophospholipid ligand that regulates cellular necrosis, apoptosis, and inflammation in many cell types by binding to cell surface G-protein-coupled high-affinity S1P receptors. Five identified S1PRs produce distinct as well as overlapping intracellular signaling events. S1P1R, S1P2R, and S1P3R in particular are widely expressed in many cell types, including the brain, liver, heart, kidney, and immune system. S1P1R couples exclusively through G12/13 pathway, whereas S1P2R and S1P3R can couple with Gi, Gq, or G12/13 pathways. Of five S1P receptor subtypes, the S1P1R has been the most extensively studied. Activation of the S1P1R protects against cardiac and hepatic IR injury and reduces systemic and tissue inflammation by directly affecting lymphocyte egress. In the kidney, activation of the S1P1R with SEW2871 or with FTY720 protects against renal IR injury by producing anti-inflammatory effects on infiltrating pro-inflammatory T lymphocytes, as well as by eliciting direct cytoprotective effects on renal proximal tubule cells. However, other S1P receptor subtypes are also expressed in the kidney in addition to the S1P1R. Renal proximal tubule cells, endothelial cells, and immune cells present in the kidney also express S1P2R, S1P3R, and S1P4R subtypes. In particular, unlike the well characterized renal protective effects of S1P1R, to our knowledge the physiologic and pathophysiologic roles of S1P2R modulation of renal IR injury have never been studied previously.

In this study, we provide direct evidence for a detrimental role of renal S1P2R activation in IR injury. We complemented pharmacologic S1P2R blockade with genetic (SIP2R−/−), as well as gene deletion (with siRNA knockdown of S1P2R) studies. All three approaches of S1P2R inhibition produced a similar degree of renal protection after IR, as well as selective induction of SK1, implicating it as a mechanism of renal protection. Previous studies of S1P2R physiology and pharmacology were limited by lack of a selective S1P2R agonist. In this study, we used a novel and highly selective agonist for the S1P2R (SID46371153). SID46371153 was developed from high-throughput screening of nearly 100,000 compounds and was found to be selective for the S1P2R. In addition, the activity of SID46371153 was inhibited by the S1P2R selective antagonist JTE-013.

Global gene deletion studies have shown that unlike S1P1R, S1P2R is not necessary for normal vascular development. However, S1P2R deficiency results in deafness and balance.
abnormality due to defects in hair cells and stria vascularis. Several prior studies in other organs and cell types suggested a pro-inflammatory and pro-apoptotic role for S1P2R activation.17,35,36 Blockade of S1P2R significantly inhibited H2O2-induced permeability in the rat lung perfused model,37 and HEK-293 cells overexpressing the S1P2R were prone to cell rounding and apoptosis.38 Moreover, S1P2R signaling modulates mast cell function, including degranulation and cytokine/chemokine release.39 The S1P2R also stimulates pro-inflammatory macrophage activities, including macrophage retention and inflammatory cytokine secretion.40

Activation of the S1P2R in endothelial cells resulted in ROCK- and phosphatase and tensin homolog–dependent disruption of adherens junctions and increased paracellular permeability.16 Our studies also support an important role for proximal tubular Rho and ROCK activation in S1P2R-mediated exacerbation of renal injury after IR. In addition, specific inhibitor of Rho or ROCK mimicked the renal protective effects of S1P2R antagonist JTE-013. Finally, we show in this study that inhibition of ROCK mimics S1P2R antagonist–mediated induction of SK1. Therefore, we propose that S1P2R-mediated activation of Rho and ROCK may lead to increased renal injury after IR by downregulation of cytoprotective SK1. Because several studies also suggested a protective role for S1P2R activation by reducing cardiac and hepatic IR injury,41,42 it is likely that S1P2R produces tissue and cell type–specific modulation of injury and inflammation.

We demonstrate a selective induction of SK1 mRNA and protein with S1P2R blockade in renal proximal tubule cells in vivo and in HK-2 cells in vitro without affecting S1P degrading enzyme S1P lyase. Our findings agree with findings in mouse embryonic fibroblasts, where S1P2R deletion resulted in a marked upregulation of SK1.22 We also demonstrate that induction of renal tubular SK1 plays a critical role in renal protection induced with S1P2R blockade because JTE-013 failed to protect against renal IR in mice treated with selective SK1 inhibitors (SK1-II or CAY10621) or mice deficient in SK1. Furthermore, we showed that activation of the S1P2R led to downregulation of renal tubular SK1, providing a potential mechanism of exacerbated renal IR injury with S1P2R activation. In addition, our findings imply that tonic S1P2R activation by endogenous S1P may reduce additional S1P generation by decreasing SK1 synthesis. Finally, our data suggest that although both endothelial and renal proximal tubule cells may make more S1P in response to renal IR, only the proximal tubule cells upregulate S1P1R to respond to increased renal S1P.

SK1 is a well-known mediator of tissue protection, growth, and survival.22,23,43 In acute lung injury, overexpression of SK1 was protective.44 Furthermore, in cardiac IR injury, SK1 activation plays a potent cytoprotective role and SK1–deficient myocytes had increased injury after ischemia.25 Overexpression of SK1 would indeed increase the synthesis of endogenous S1P in the renal cortex and in proximal tubules. All five S1PR subtypes bind to S1P, with similar nanomolar affinities.45 For example, S1P1R, S1P2R, and S1P3R bind to S1P

Figure 11. S1P2R modulates renal proximal tubular HIF-1α synthesis. (A and B) HK-2 cells were subjected to normoxia (95% room air plus 5% CO2) or to hypoxia (2% O2, 93% N2, and 5% CO2) for 24 hours (representative of three experiments). A selective S1P2R antagonist (JTE-013; 1 μM) increased renal tubular HIF-1α mRNA and protein expression under both normoxic and hypoxic conditions (A). A selective S1P2R agonist (SID46371153; 1 μM) decreased HIF-1α mRNA expression in normoxic renal proximal tubule cells (B). SID46371153 also reduced HIF-1α mRNA and protein expression in hypoxic renal proximal tubule cells. Hypoxia increased SK1 mRNA and protein expression, and this increase was potentiated by JTE-013 and attenuated by SID46371153 (A and B). A prolyl hydroxylase inhibitor DMOG (1 mM) reversed the SID46371153-mediated decreases in SK1 and HIF-1α expression in HK-2 cells. (C) Mice were subjected to 30 minutes of renal ischemia and 5 hours of reperfusion. SID46371153 reduced the induction of HIF-1α in mouse kidneys subjected to renal IR (representative of four experiments). *P<0.05 versus sham operation group; #P<0.05 versus vehicle-treated renal IR group. Error bars represent 1 SEM.
translocation and renal protection afforded by JTE-13. HIF-1 is a heterodimeric transcription factor composed of an α and a β subunit. Under normoxic conditions, prolyl-hydroxylation and ubiquitination of the oxygen-dependent degradation domain of HIF-1α results in rapid HIF-1α degradation. With hypoxia or ischemia, HIF-1α stabilizes and interacts with HIF-1β, forming the HIF-1 heterodimer. Nuclear translocation of HIF-1 allows binding to the hypoxia-responsive element with subsequent induction of several cytoprotective genes, including vascular endothelial growth factor and erythropoietin. Consistent with this, previous studies have demonstrated that HIF-1α activation protects against renal IR injury.

Mechanistically, our results suggest that S1P2R can directly modulate new HIF-1α mRNA and protein synthesis. We therefore propose that increased nuclear HIF-1α binding with JTE-103 is due to increased synthesis of HIF-1α rather than by affecting degradation or nuclear translocation of existing HIF-1α protein. Increased synthesis and enhanced nuclear translocation of HIF-1α with S1P2R inhibition may lead to a novel therapeutic approach in attenuating AKI due to renal IR or hypoperfusion. Our data suggest that blockade or deletion of S1P2R removes the tonic inhibition of HIF-1α with subsequent SK1 induction (Figure 13).

In summary, we demonstrate in this study that S1P2R in the kidney modulates renal IR injury by regulating renal proximal tubular SK1 synthesis via Rho kinase and HIF-1α (Figure 13). Clinically, pretreatment (before renal ischemia or hypoperfusion) or post-treatment is an excellent option for the frequent clinical scenarios where patients are subjected to renal injury during surgical procedures (e.g., kidney transplantation, open heart surgery, aorto-vascular surgery, or liver transplantation). We showed here that a selective S1P2R blockade with JTE-013 provided significant renal protection when given immediately before renal ischemia or even 30 minutes after reperfusion. Collectively, these findings may have important clinical implications because they imply that S1P2R antagonism might be a viable therapeutic option in attenuating the effects of renal IR.

**CONCISE METHODS**

**Materials**

Full drug names used in this study are listed in Supplemental Material.
Renal proximal tubule cell

**Figure 13.** A schematic of proposed cellular mechanisms of renal protection with S1P2R inhibition in renal proximal tubules. Our data suggest that selective S1P2R blockade with JTE-013 results in increased HIF-α mRNA and protein synthesis and SKI induction leading to new S1P synthesis with subsequent S1P R activation. Consistent with this hypothesis, selective S1P2R blockade with W146 inhibits the renal protective effects of JTE-013. Our findings also imply that tonic S1P2R stimulation directly reduces HIF-α mRNA synthesis, leading to attenuated HIF-α transcription factor activity. Moreover, tonic S1P2R activation by endogenous S1P places brake on additional S1P generation by reducing SK1 synthesis and activity. Finally, we showed that inhibition of Rho and ROCK signaling mimics S1P2R antagonist–mediated induction of SK1 and also attenuates S1P2R agonist–mediated exacerbation of renal function. Therefore, we also propose that S1P2R–mediated activation of Rho and ROCK may lead to increased renal injury after IR. (−) represents inhibition.

**Murine Model of Renal IR Injury**

After Columbia University Institutional Animal Care and Use Committee approval, we subjected male C57BL/6 (Harlan, Indianapolis, IN) as well as S1P2R−/−, SK1−/−, and SK2−/− mice34,49,50 (congenically derived on a C57BL/6 background for approximately 10 generations and kindly provided by Dr. Richard L. Proia, National Institutes of Health, Bethesda, MD) to 20 or 30 minutes of renal IR, as described elsewhere.15,56 Table 1 lists the primer sequences utilized.

**Detection of Renal Tubular Apoptosis**

We detected apoptosis after renal IR with TUNEL staining as described elsewhere15 using a commercially available in situ cell death detection kit (Roche, Indianapolis, IN) according to the instructions provided by the manufacturer.

**Reverse Transcription PCR**

Semi quantitative reverse transcriptase PCR assays for pro-inflammatory mRNAs, as well as SK1, SK2 and S1P1R, mRNA, were performed as described elsewhere.15,56 Table 1 lists the primer sequences utilized in this study.

**siRNA Preparation and Delivery to Mice In Vivo**

A chemically synthesized 21-nucleotide siSTABLE (stability-enhanced siRNA) sequence specific for S1P2R (5′-UAACUCGCGGCUUGG-UUUU-3′) were custom made and purchased from Dharmacon Research (Lafayette, CO) in 2′-hydroxyl, annealed, desalted, and dialyzed duplex form for in vivo use. The double-stranded sequence for S1P2R siRNA was injected as 50 μg of siRNA (100 μL) intravenously 48 hours before renal ischemia.

**Induction of HK-2 Cell Necrosis, Apoptosis, and Hypoxia**

Necrotic injury in HK-2 cells was induced with exposure to 5 mM H2O2 for 2–4 hours and lactate dehydrogenase released into cell culture media
was measured as described elsewhere.\(^{57,58}\) To induce apoptosis, HK-2 cells were exposed to TNF-\(\alpha\) (10 ng/ml) plus cycloheximide (5 \(\mu\)g/ml) for 16 hours as described elsewhere.\(^{59}\) Some cells were pretreated with vehicle (diluted DMSO), JTE-013 (0.1 \(\mu\)M), or SID46371153 (0.1–1 \(\mu\)M) 30 minutes before induction of cell death. HK-2 cells treated with vehicle (0.2% DMSO), DMOG (1 mM), or SID46371153 plus DMOG and subjected to hypoxia (2% O\(_2\), 93% N\(_2\), 5% CO\(_2\)) or normoxia (95% room air in 5% CO\(_2\)) for 24 hours.

**Immunoblotting Analyses**

Mouse kidney cortical tissues (including corticomedullary junction), HK-2 cell lysates, or freshly isolated mouse kidney proximal tubules (Percoll gradient separation) were collected for immunoblotting analyses of HIF-1\(\alpha\), SK1, SK2, and \(\beta\)-actin (internal protein loading control) as described elsewhere.\(^{15}\) We also probed for these proteins in immortalized human umbilical vein endothelial cells (EA.hy926). Finally, we performed immunoblotting for S1P lyase in mouse kidney tissues subjected to sham operation or to renal IR after vehicle or JTE-013 injection.

**HIF-1\(\alpha\) DNA-Binding Assay**

Nuclear extracts from mouse kidney tissues and HK-2 cells were prepared using the Transfactor Extraction Kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer’s instructions. HIF-1\(\alpha\) DNA-binding activity in nuclear extracts was determined using a TransFactor Family Colorimetric kit specific for HIF-1\(\alpha\) (BD Biosciences Clontech) according to the manufacturer’s instructions. Nuclear extracts from COS-7 cells treated with CoCl\(_2\) (Active Motif, Carlsbad, CA) and nuclear extracts incubated with a HIF-1\(\alpha\)-specific competitor oligonucleotide served as positive and negative controls, respectively.

**Isolation of Mouse Kidney Proximal Tubules and Endothelial Cells**

Mouse kidney proximal tubules were isolated using the methods described by Vinay and colleagues (collagenase digestion followed by Percoll density gradient separation).\(^{60}\) Renal endothelial cells were isolated by magnetic beads separation according to the manufacturer’s instruction (Invitrogen, Carlsbad, CA).

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Table 1. Reverse transcriptase PCR primers used in this study

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<th>Primers</th>
<th>Species</th>
<th>Sequences (Sense/Antisense)</th>
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<th>Cycle Number</th>
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Respective anticipated reverse transcriptase PCR product size, PCR cycle number for linear amplification, and annealing temperatures used for each primer.
Determination of Kidney and Plasma S1P

Five hours after renal IR or sham operation, mouse plasma and kidneys (lysed in 150 mM Tris-HCl, 150 mM NaCl, 1% Triton-X, 10 mM EDTA, 1 mM Na3VO4, 100 mM NaF plus protease and phosphatase inhibitors) were collected for S1P measurement as described elsewhere.23,36

Statistical Analyses

The data were analyzed with a t test for comparing means between two groups. One-way ANOVA plus a Tukey post hoc multiple comparison test was used for comparing multiple groups. Two-way ANOVA plus a 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 statistical significance. All data are expressed throughout the text as mean ±SEM.

ACKNOWLEDGMENTS

We thank R. L. Proia for providing the S1P2R knockout mice. This work was supported by National Institutes of Health Grant R01 GM-067081.

DISCLOSURES

None.

REFERENCES


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CORRECTION


The above-mentioned manuscript stated that all experiments were approved by the Columbia University Medical Center Institutional Animal Care and Use Committee (IACUC). Although the majority of experiments were approved, some of the signaling inhibitors (siRNA, CAY10444, CAY10621, SKI-2, SID46371153, and Rho kinase inhibitors) were administered without itemized IACUC approval. All drugs were administered to anesthetized animals in experiments that otherwise conformed to the IACUC-approved protocol. These omissions do not invalidate the scientific findings from the study. The authors sincerely apologize for this event.