Selective sphingosine 1-phosphate 1 receptor activation reduces ischemia-reperfusion injury in mouse kidney

Alaa S. Awad,1 Hong Ye,1 Liping Huang,1 Li Li,1 Frank W. Foss, Jr.,2 Timothy L. Macdonald,2 Kevin R. Lynch,3,4 and Mark D. Okusa1,4

Departments of 1Medicine, 2Chemistry, 3Pharmacology, and the 4Cardiovascular Research Center, University of Virginia, Charlottesville, Virginia

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Selective sphingosine 1-phosphate 1 receptor activation reduces ischemia-reperfusion injury in mouse kidney. Am J Physiol Renal Physiol 290: F1516–F1524, 2006. First published January 10, 2006; doi:10.1152/ajprenal.00311.2005.—The mechanisms involved in renal ischemia-reperfusion injury (IRI) are complex and appear to involve the early participation of bone marrow-derived cells. T lymphocytes participate in the pathogenesis of IRI. Sphingosine 1-phosphate (S1P) induces peripheral T cell depletion. Therefore, we hypothesized that S1P1 receptor activation protects kidney from IRI. FTY-720, a non-receptor-selective sphingosine analog, was given intraperitoneally to C57BL/6 mice, and animals were subjected to ischemia for 32 min followed by reperfusion for 24 h. Plasma creatinine, blood count, myeloperoxidase (MPO) activity, and renal histology were determined. IRI led to a marked increase in plasma creatinine, MPO activity, leukocyte infiltration, and vascular permeability. FTY-720 significantly decreased plasma creatinine in a dose-response manner with a maximal reduction of ~73% and ~69% with doses of 240 and 48 μg/kg, respectively. MPO, leukocyte infiltration, vascular permeability, and peripheral blood lymphocyte counts were markedly decreased with FTY-720 treatment. The protective effect of FTY-720 was reversed with VPC-44116, a selective S1P1 receptor antagonist. Furthermore, SEW-2871, a selective S1P1 agonist, significantly decreased plasma creatinine in a dose-response manner with a maximal reduction of ~70% with a dose of 10 mg/kg. Analysis of kidneys by light microscopy revealed minimal histological signs of ischemic injury with FTY-720 or SEW-2871 treatment compared with the vehicle group. Using RT-PCR, we found a time-dependent increase in the S1P1 mRNA expression following IRI that begins after 2 h with the maximum expression at ~4 h. We conclude that the protective effect of FTY-720 is due primarily to activation of S1P1 receptors. The mechanism of protection is not known but may be related to peripheral lymphocyte depletion or direct effects on kidney cells expressing S1P1 receptor.

FTY-720; inflammation; lymphocyte; acute renal failure

NOVEL THERAPEUTIC INTERVENTIONS for preventing or attenuating renal tissue injury following ischemia-reperfusion (IR) remain a focus of significant interest. Although the exact pathogenic mechanisms remain poorly defined, accumulating evidence supports the potential role of inflammation and involvement of the immune system. Previous studies have mainly focused on the participation of neutrophils as mediators of IR injury (IRI) (17, 32); however, recent reports show that lymphocytes contribute to injury (8, 40). The observation that injury is ameliorated by depletion of T cells and reconstituted with adoptive transfer of CD4+ cells is evidence that the tissue injury following IR is dependent on CD4+ cells (8), although the mechanism is not fully understood. Renal damage secondary to IRI is frequent in organ transplantation and adversely affects allograft survival. An important component of this injury is caused by initial adhesion of neutrophils and lymphocytes to endothelial cells.

2-Amino-2-[2-(octylphenyl)ethyl]-1,3-propanediol (FTY-720) is undergoing evaluation for use in reducing graft rejection (7, 16, 18). FTY-720 elicits lymphopenia resulting from a reversible redistribution of lymphocytes from the circulation to secondary lymphatic tissue (23, 31, 39). In tissues subjected to IRI, lymphocytes are known to contribute to early injury (8, 41). In this setting, FTY-720 has also been demonstrated to reduce IRI in kidney (38) and liver (1, 22). In vivo, FTY-720 is phosphorylated (FTY-720-P) by sphingosine kinase to form a potent sphingosine 1-phosphate (S1P) analog. S1P, found in high levels in blood (0.1–1 μM), is produced by phosphorylation of sphingosine and is a pleiotropic lysophospholipid mediator of a wide variety of biological processes through binding to a family of G protein-coupled receptors; S1P1, 5 (formerly Edg-1, -5, -3, -6, -8, respectively) (15). S1P is thought to regulate cell growth, suppress apoptosis, stress responses, calcium homeostasis, cell migration, angiogenesis, and vascular maturation (for a review, see Ref. 35). S1P is released by mononuclear phagocytes and platelets and binds to S1P1 in nanomolar affinities (6). FTY-720 interacts with S1P1, S1P3, S1P4, S1P5, but not S1P2 receptors (5, 23); thus the receptor subtype-mediating tissue protection following FTY-720 administration is not known. Therefore, we performed a series of experiments to determine the S1P receptor subtype that mediates renal tissue protection after IRI.

MATERIALS AND METHODS

Renal IR protocol. All animals were handled and procedures were performed in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6 mice (7–8 wk of age, Charles River Laboratories, Wilmington, MA) were allowed free access to food and water until the day of surgery. Mice were anesthetized with a regimen that consisted of ketamine (100 mg/kg ip), xylazine (10 mg/kg ip), and acepromazine (1 mg/kg ip) and were placed on a thermoregulated pad to maintain body temperature at 37°C. Both renal pedicles were identified through bilateral flank incisions and cross-clamped for 32 min and kidneys were observed for reperfusion on release of the clamps. Another group of sham-operated animals was subjected to a similar surgical procedure except that the
renal pedicles were not clamped. Surgical wounds were closed, and mice were returned to cages for up to 24 h. At the end of the experimental period, animals were reanesthetized, blood was obtained by cardiac puncture, and kidneys were removed for various analyses.

**Drug administration.** A 1 mM stock solution of FTY-720, a non-receptor-selective sphingosine analog (5, 23) (Novartis, Basel, Switzerland), or SEW-2871, a selective S1P1 agonist (10, 34), were prepared in a 3% fatty acid-free bovine serum albumin/PBS solution (Sigma, St. Louis, MO). Dose-response experiments were performed for FTY-720 (24, 48, 240, and 480 μg·kg⁻¹·dose⁻¹) or SEW-2871 (0.1, 1, and 10 μg·kg⁻¹·dose⁻¹). Compounds or vehicle (3% fatty acid-free bovine serum albumin/PBS solution) were administered by intraperitoneal injection 24 h before ischemia, 20 h before ischemia, and 32 min after ischemia. Additional experiments were performed with a selective S1P1 antagonist, VPC-44116 (10 μg·kg⁻¹·dose⁻¹) alone, or combined with FTY-720 (48 μg·kg⁻¹·dose⁻¹) (10).

**Blood pressure and heart rate measurement.** Systolic blood pressure and heart rate were measured by using a photoelectric sensor for pulse detection in mouse tail (IITC model 179, IITC/Life Science Instruments, Woodland Hills, CA). Mice were allowed to rest quietly for 10 min, and blood pressure was measured twice and averaged. Measurements were made at baseline before ischemia and 24 h after ischemia.

**Plasma creatinine and leukocyte counts.** Plasma creatinine was determined using a colorimetric assay according to the manufacturer’s protocol (Sigma). Anticoagulated blood was analyzed for leukocyte counts (HEMAVET 850, CDC Technologies, Oxford, CT).

**Myeloperoxidase activity.** Myeloperoxidase (MPO) activity to assess leukocyte infiltration was determined in kidney homogenates (28). Kidneys were harvested from mice subjected to the IR, and a portion of the kidney was snap-frozen in liquid N₂ until time of assay. Kidneys were homogenized in 10 Vol of ice-cold 50 mM potassium phosphate buffer, pH 7.4, using a Tekmar tissue grinder. The homogenate was centrifuged at 5,000 × g for 10 min, and the supernatant was discarded. The pellet was washed twice, resuspended in 10 Vol of ice-cold 50 mM potassium phosphate buffer with 0.5% hexadecyltrimethylammonium bromide, and sonicated. The suspension was subjected to three freeze-thaw cycles, sonicated for 10 s, and centrifuged at 15,000 × g for 15 min at 4°C. The resultant supernatant fluid was discarded. The pellet was washed twice, resuspended in 10 Vol of ice-cold 50 mM potassium phosphate buffer with 0.5% hexadecyltrimethylammonium bromide, and sonicated. The suspension was subjected to three freeze-thaw cycles, sonicated for 10 s, and centrifuged at 15,000 × g for 15 min at 4°C. The supernatant fluid was added to an equal volume of a solution consisting of 0.1% diiodoacetamide (10 mg/mL), 0.3% H₂O₂, and 45 mM potassium phosphate, pH 6.0. Absorbance was measured at 460 nm over a period of 5 min (3).

**Analysis of kidney leukocyte content by flow cytometry.** We used flow cytometry to analyze kidney leukocyte content. These studies assessed kidney content at 24 h of reperfusion of T cell (CD4⁺ and CD8⁺), B cells (B220), macrophage (F4/80), and neutrophil (Gr-1) in sham-, vehicle-, or FTY-720-treated mice. In brief, kidneys were harvested from mice subjected to the IR, and a portion of the kidney was snap-frozen in liquid N₂ until time of assay. Kidneys were homogenized in 10 Vol of ice-cold 50 mM potassium phosphate buffer, pH 7.4, using a Tekmar tissue grinder. The homogenate was centrifuged at 5,000 × g for 10 min, and the supernatant was discarded. The pellet was washed twice, resuspended in 10 Vol of ice-cold 50 mM potassium phosphate buffer with 0.5% hexadecyltrimethylammonium bromide, and sonicated. The suspension was subjected to three freeze-thaw cycles, sonicated for 10 s, and centrifuged at 15,000 × g for 15 min at 4°C. The resultant supernatant fluid was added to an equal volume of a solution consisting of 0.1% diiodoacetamide (10 mg/mL), 0.3% H₂O₂, and 45 mM potassium phosphate, pH 6.0. Absorbance was measured at 460 nm over a period of 5 min (3).

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**Analysis of kidney vascular permeability.** Kidneys were fixed in periodate-lysine-paraformaldehyde (4% paraformaldehyde) and embedded in paraffin, and 4-μm sections were cut. Sections were subjected to routine staining with hematoxylin and eosin (H and E), viewed by light microscopy (Zeiss AxiosSkop), and digital images were taken using SPOT RT Camera (software version 3.3; Diagnostic Instruments, Sterling Heights, MI) under ×200 and ×400 magnifications, Semiquantitative analysis of tubule injury was performed as previously described (29). Sections were viewed in a masked fashion (ASA) under ×400 magnification and the percentage of tubules showing epithelial necrosis was assigned the following scoring system: 0 = normal; 1 = <10%; 2 = 10–25%; 3 = 26–75%; 4 = >75%. Five to 10 fields from each of cortex, outer medulla, and inner medulla were evaluated scored and averaged.

**Reverse transcription-polymerase chain reaction.** The presence of S1P1, S1P2, S1P3, S1P4, and S1P5 receptor mRNAs in the kidney was confirmed via RT-PCR. RT-PCR was performed on the cDNA using the ThermoScript RT-PCR System with Platinum Taq DNA Polymerase according to the manufacturer’s protocol (Invitrogen, Life Technologies). The following PCR protocol was used: initial denaturation (95°C for 3 min), denaturation, annealing, and elongation program repeated 35 times (95°C for 45 s, 59.5°C for 60 s, 72°C for 60 s), final elongation (72°C for 7 min), and finally a holding step at 4°C. PCR oligonucleotide primers were designed as previously described (29) (Table 1).

**Quantitative real-time PCR.** Total RNA was extracted from kidneys using a NReasy Mini Kit (Qiagen, Hilden, Germany). Quality of RNA was confirmed by ethidium bromide staining in 2% agarose gel. Single-strand cDNA was synthesized using iScript cDNA Synthesis Kits (Bio-Rad, Hercules, CA) for two-step real-time RT-PCR. Gene-specific primers were designed using Beacon Designer Probe/Primer Design Software (Premier Biosoft International, Palo Alto, CA) (Table 1). Primers were obtained from Integrated DNA Technologies (Coralville, IA). Amplification products were verified by melting curves and agarose gel electrophoresis. Quantitative real-time PCR was performed using a MyIQ Single Color Real-Time PCR Detection System iCycler (Bio-Rad). Reactions were performed in duplicate, and threshold cycle numbers were averaged. Samples were calculated with normalization to GAPDH. Fold-overexpression was calculated according to the formula: OD₆₂₀ (EBD) = OD₆₂₀ – (1.426 × OD₇₄₀ + 0.030) (36). Data were expressed as micrograms of EBD per gram of dry kidney weight.
individual pairs of effect means were conducted by using the least squares methods of pooled variance. Unpaired Student’s *t*-tests were also used in some analysis. Data are expressed as means ± SE. Statistical significance was identified at *P* < 0.05.

## RESULTS

**FTY-720 attenuates renal IRI.** FTY-720, a nonselective sphingosine analog prodrug, was used to investigate the role of S1P receptors in IRI. FTY-720 was administered at four different doses (24, 48, 240, and 480 μg/kg). In mice whose kidneys were subjected to IR and treated with vehicle, we observed a fourfold increase in plasma creatinine compared with sham controls. Plasma creatinine for sham and vehicle was 0.23 ± 0.04 and 1.15 ± 0.13 mg/dl, respectively (*P* < 0.005; Fig. 1). In contrast, FTY-720 in all doses tested decreased plasma creatinine after IR compared with vehicle with a maximal effect achieved between 48 and 240 μg/kg (*P* < 0.005). The loss of significant protection from IRI with the highest doses of FTY-720 suggested the possibility that S1P receptor subtypes may have different functional and/or opposing effects. The effect of FTY-720 on renal tissue protection was independent of any change between vehicle and FTY-720 treatment (48 μg/kg) on the peripheral counts of neutrophils (1.04 ± 0.2 and 1.75 ± 0.3 K/μl), erythrocytes (6.8 ± 0.9 and 8.3 ± 0.5 M/μl), and platelets (557 ± 119 and 682 ± 38 K/μl), respectively. However, peripheral blood lymphocyte count with FTY-720 treatment (48 μg/kg) was decreased after IR (0.49 ± 0.05 K/μl) compared with the vehicle group (1.3 ± 0.3 K/μl; *P* < 0.05).

**FTY-720 reduced kidney leukocyte content after IRI.** IRI led to a significant increase in kidney leukocyte infiltration (948% of sham, *P* < 0.01) as assessed by MPO activity, a biochemical marker for the presence of neutrophils and macrophages. FTY-720 at doses of 48 and 240 μg/kg attenuated the increase in kidney MPO activity (250% of sham and 188% of sham, respectively, *P* < 0.05 to vehicle; Fig. 2). Using flow cytometry, we identified subpopulations of leukocytes that infiltrate into the kidney following IRI and after treatment with FTY-720. There was an increase in T cell (CD4: 11%; CD8: 14%), B cell (B220: 52%), macrophage (F4/80: 263%) and neutrophil (GR-1: 821%) infiltration in the kidney following IRI and after treatment with FTY-720. There was an increase in T cell (CD4: 11%; CD8: 14%), B cell (B220: 52%), macrophage (F4/80: 263%) and neutrophil (GR-1: 821%) infiltration in the kidney following IRI and after treatment with FTY-720. There was an increase in T cell (CD4: 11%; CD8: 14%), B cell (B220: 52%), macrophage (F4/80: 263%) and neutrophil (GR-1: 821%) infiltration in the kidney following IRI and after treatment with FTY-720.

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**Table 1. Primer pairs for PCR**

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Fig. 1. Effect of FTY-720 on plasma creatinine after renal ischemia–reperfusion injury (IRI). Mouse kidneys were subjected to 32-min ischemia and 24-h reperfusion and treated with vehicle or FTY-720 (24, 48, 240, and 480 μg/kg) on the peripheral counts of neutrophils (73% reduction from vehicle, *P* < 0.01; Fig. 3).
FTY-720 reverses the increase in kidney vascular permeability induced by IR. EBD binds to plasma proteins and its appearance in extravascular tissues reflects an increase in vascular permeability. Analysis of EBD extravasation in sham-, vehicle-, or FTY-720-treated mice 6 h after reperfusion is shown in Fig. 4. There was an increase in kidney tissue EBD content in the vehicle group (250.3 ± 32 μg EBD/g dry kidney wt) from sham control (85.7 ± 26 μg EBD/g dry kidney wt; P < 0.005). FTY-720 treatment (48 μg·kg⁻¹·dose⁻¹) significantly reduced EBD content (116.6 ± 21 μg EBD/g dry kidney wt; P < 0.05 from vehicle).

Selective S1P₁ activation mediates tissue protection after IRI. To determine the specific receptor target of FTY-720, we used selective agonist and antagonist compounds. First, we coadministered FTY-720 (48 μg/kg) and VPC-44116 (10 mg/kg), a selective S1P₁ receptor antagonist (Ref. 10 and unpublished data, Lynch K). As demonstrated in Fig. 5A, the protective effect of FTY-720 was reversed with VPC-44116. Plasma creatinine for vehicle, FTY-720, VPC-44116, and FTY-720 + VPC-44116 was 0.83 ± 0.06, 0.29 ± 0.04 (P < 0.001, to vehicle), 1.01 ± 0.11 (P < 0.001, to FTY-720 treatment), and 0.62 ± 0.07 mg/dl (P < 0.05, to FTY-720 treatment), respectively. MPO assay was used to assess leukocyte infiltration as presented in Fig. 5B. The increase in kidney neutrophil infiltration was significantly reduced with FTY-720 treatment.

Fig. 3. Effect of FTY-720 on kidney leukocyte content after renal IRI. Mouse kidneys were subjected to 32-min ischemia and 24-h reperfusion and treated with vehicle or FTY-720 (48 μg·kg⁻¹·dose⁻¹). Kidney leukocyte subset content in mice was quantitated using flow cytometry by staining leukocytes with CD4-APC (L3T4), CD8a-PE (Ly2), B220-APC (CD45R), F4/80-APC (BM8), and GR-1-PE (Ly6G). Data were analyzed by Flowjo software 6.0. Flow cytometry data were conducted in 3 steps to eliminate the interference of the nonspecific staining by the dead cells. Filled bars, vehicle-; cross-hatched bars, FTY-720-treated group. Values are expressed as % change relative to vehicle, n = 6. Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.0001 compared with vehicle.

Fig. 4. Effect of FTY-720 on vascular permeability after renal IRI. Mouse kidneys were subjected to IRI and treated with FTY-720 (48 μg·kg⁻¹·dose⁻¹) alone or combination. Values are means ± SE; n = 7 for each group.

Fig. 5. Effect of FTY-720 on plasma creatinine and leukocyte infiltration is reversed with VPC-44116, a selective S1P₁ antagonist. A: mouse kidneys were subjected to IRI and treated with FTY-720 (48 μg·kg⁻¹·dose⁻¹) and VPC-44116 (10 mg·kg⁻¹·dose⁻¹) alone or combination. Values are means ± SE; n = 7 for each group. B: effect of FTY-720 on MPO is reversed with VPC-44116. MPO activity (ΔOD₄₆₀·g⁻¹·min⁻¹) was measured from mouse kidneys subjected to IRI and treated with FTY-720 (48 μg·kg⁻¹·dose⁻¹) and VPC-44116 (10 mg·kg⁻¹·dose⁻¹) alone or in combination. Values are means ± SE; n = 7 for each group.
(65% of vehicle, \( P < 0.05 \)) but this was reversed by the addition of VPC-44116 (105% vehicle, \( P < 0.05 \) to FTY-720 treatment). Although as expected, FTY-720 reduced blood lymphocyte counts; however, VPC11446 administration did not reverse this effect. Peripheral blood lymphocyte counts in vehicle, FTY-720, VPC-44116, and FTY-720 + VPC-44116 was 1.5 ± 0.4, 0.56 ± 0.07 (\( P < 0.05 \), to vehicle), 1.9 ± 0.27 (\( P < 0.005 \), to FTY-720 treatment), and 0.59 ± 0.1 K/\( \mu \)l (\( P = \) NS to FTY-720 treatment), respectively.

To further demonstrate that S1P1 receptor activation mediates renal tissue protection, we used SEW-2871, a selective S1P1 agonist (34). As shown in Fig. 6, the increase in plasma creatinine after IR was reduced in a dose-dependent manner, with the highest reduction at a dose of 10 mg/kg. This dose was not associated with any hemodynamic changes as blood pressure and heart rate were not different between vehicle (132.8 ± 1 mmHg and 384.2 ± 18 beats/min, respectively) and SEW-2871 (10 mg/kg, 132.8 ± 2 mmHg, and 366 ± 4 beats/min, respectively). Similar to FTY-720 treatment, SEW-2871 reduced peripheral lymphocyte counts under normal control conditions (SEW-2871, 1.52 ± 0.1 K/\( \mu \)l; control, 5.85 ± 0.14 K/\( \mu \)l, \( P < 0.05 \)). Light microscopic analysis of H and E staining of the renal medulla following IRI revealed a loss of brush-border villi, tubular necrosis, and obstruction of proximal tubule cells in vehicle-treated mice (Fig. 7). The severity of the injury was reduced markedly in kidneys from mice treated with FTY-720 or SEW-2871. Outer medulla are shown for sham (A and E), vehicle (B and F), FTY-720 (48 mg/kg, C and G), or SEW-2871 (10 mg/kg, D and H) under ×200 and ×400 magnifications, respectively. A semiquantitative score was assigned based on the masked reading according to the severity of injury. Both FTY-720 and SEW-2871 treatment reduced tubular injury and ischemic necrosis following IRI in mice (FTY-720: 1.8 ± 0.3, \( P < 0.05 \) and SEW-2871: 0.8 ± 0.1, \( P < 0.01 \)) from vehicle-treated mice (3 ± 0.4).

**SIP receptor mRNA expression in mouse kidney.** We sought to determine the expression of S1P receptors mRNA in the kidney. Total RNA from normal mouse kidneys was extracted and subjected to PCR. S1P1, S1P2, S1P3, and S1P4 mRNA were detected in whole mouse kidney (Fig. 8A). No bands were demonstrated in controls for water with S1P1–5 primers, water for GAPDH and cDNA with no primers (data not shown). Conversely, S1P5 mRNA was not found in mouse kidney extracts. By using quantitative real-time PCR, we assessed the S1P1–4 receptor expression in whole kidney extracts. The rank order for S1P mRNA expression in kidney was found to be S1P1 > S1P3 > S1P2 > S1P4 (Fig. 8B).

**Renal IRI induces an early increase in S1P1 mRNA expression.** We next measured the relative expression of S1P1,2,3,4 receptor mRNA expression following IRI. Using real-time PCR, we found a time-dependent increase in the S1P1 mRNA expression after IR that began after 2 h with maximum expression after 4 h (10-fold increase from control; \( P < 0.05 \); Fig. 9 and Table 2). S1P1 mRNA expression showed a threefold increase after 4 h (\( P < 0.05 \) from control). In contrast, expression of S1P2 and S1P4 mRNA was not significantly different from control at any time point (Table 2).

**DISCUSSION**

The present study demonstrates that S1P1 receptor activation has a protective effect on renal function and histology following IR. The nonselective S1P receptor agonist FTY-720 demonstrated significant protection, an effect that was reversed with a selective S1P1 antagonist, VPC-44116. Furthermore, the protective effect of FTY-720 is mimicked by a selective S1P1 agonist, SEW-2871. Although previous studies have demonstrated that FTY-720 is protective following kidney IRI (38), our studies extend these preliminary observations and provide data on the optimal dose of FTY-720 that confers renal tissue protection and evidence that the specific activation of S1P1 receptors expressed on leukocytes and/or kidney tissue mediates tissue protection.

The naturally occurring ligand, S1P, binds to five related G protein-coupled receptors, termed S1P1–5 (15). S1P receptors regulate a wide variety of important cellular functions, including cell survival, cell motility, adherens junction assembly, cytoskeletal rearrangements, and apoptosis (for a review, see Refs. 15, 33, 35). S1P1 receptors are expressed in many tissues including but not limited to lymphocytes, endothelial cells and mesangial cells; S1P2 receptors are expressed in lymphoid tissue, and S1P3 receptors are expressed in splanchnic and white matter tracts of the central nervous system (15, 21, 24).

FTY-720 is rapidly phosphorylated in vivo by sphingosine kinase to form FTY-720-phosphate (FTY-720-P) and that FTY-720-P is the biologically active principle (5). A phosphorylated form of FTY-720 subsequently was found to be an agonist for S1P1,3,5 but not S1P2 receptors (5, 23) and was postulated to be the mediator of the immunosuppressive effect of FTY-720 (5, 23). The relative contributions of different mechanisms of action of FTY-720 may depend on the receptor subtypes.

FTY-720 was demonstrated to prolong allograft survival in several transplant animal models (16, 18). The possible mechanism of its immunomodulation was related to sequestration of peripheral blood lymphocytes to secondary lymphoid organs and selective induction of infiltrated lymphocyte apoptosis (23, 31, 39). Recently, FTY-720 has been found to be able to prevent IRI in the liver (1, 22) and kidneys (38); however, these studies mainly focused on FTY-720-induced lymphope-
nia. Our study extends these findings and demonstrates that specific S1P1 receptor activation by FTY-720 induces tissue protection. This conclusion is drawn from studies using SEW-2871, a specific S1P1 agonist with no demonstrated activity against S1P2–5 (10, 34). Furthermore, we demonstrate that the administration of this compound protected kidneys from IRI in a dose-dependent manner. The protective effect of the nonselective compound FTY-720 was reversed with VPC-44116, a selective S1P1 antagonist (10). VPC-44116 is the phosphonate analog of VPC-23019, which is a phosphate and has the same profile at the S1P receptors as VPC-23019 (Ref. 10 and unpublished results, Lynch K). Interestingly, the effect of VPC-44116 in blocking FTY-720 on plasma creatinine and MPO activity was not associated with reversal of lymphopenia induced by FTY-720 treatment. These data suggest the possibility that FTY-720 may have additional effects independent of lymphocytes. Together, our results strongly suggest that S1P1 activation reduces IRI and this receptor type is the primary

Fig. 7. S1P1 agonists reduce morphological changes associated with IRI. Kidneys were fixed and paraffin embedded, and 4-μm sections were cut and stained with hematoxylin and eosin. Shown are histological sections of kidney outer medulla for sham under ×200 and ×400 magnifications (A and E), or following IRI treated with vehicle (B and F), FTY-720 (48 μg/kg; C and G), or SEW-2871 (10 mg/kg; D and H), respectively.
kidneys was extracted and subjected to real-time PCR. S1P1, 2, 3, 4 receptor relative to S1P1 receptor mRNA is shown. Total RNA from normal mouse mRNA is factored for kidney GAPDH mRNA. Whether the protective effect of FTY-720 barrier function (24). Whether the protective effect of FTY-720 on endothelial cells may lead to maintenance of endothelial (19, 25) and reduction of monocyte adhesion (2). These effects stimulation of endothelial cell nitric oxide synthase enzyme, for cell survival and proliferation of vascular endothelial cells, (MW) markers are shown to the mouse kidneys was extracted and subjected to RT-PCR. Molecular weight markers are shown to the left. Edg 1, 3, 4, 6, and 8 correspond to S1P 1, 3, 2, 4, and 5, respectively. B: expression of kidney S1P2, 3, 4 receptor mRNA relative to S1P1 receptor mRNA is shown. Total RNA from normal mouse kidneys was extracted and subjected to real-time PCR. S1P1, 2, 3, 4 receptor mRNA is factored for kidney GAPDH mRNA, n = 4 for each group. *P < 0.0001. #P < 0.005.

Fig. 8. S1P receptor mRNA expression in kidney. A: total RNA from normal mouse kidneys was extracted and subjected to real-time PCR. Molecular weight (MW) markers are shown to the left. Edg 1, 3, 4, 6, and 8 correspond to S1P 1, 3, 2, 4, and 5, respectively. B: expression of kidney S1P2, 3, 4 receptor mRNA relative to S1P1 receptor mRNA is shown. Total RNA from normal mouse kidneys was extracted and subjected to real-time PCR. S1P1, 2, 3, 4 receptor mRNA is factored for kidney GAPDH mRNA, n = 4 for each group. *P < 0.0001. #P < 0.005.

target of FTY-720 in mediating tissue protection. The tissue-protective effect of FTY-720 was associated with attenuation of lymphocyte, macrophage, and neutrophil infiltration, a result consistent with the prevailing view that inflammatory cell infiltration appears to play an important role in the pathogenesis of IRI (13, 27, 40).

In addition to peripheral lymphocyte cell depletion, activation of S1P1 receptors has other potential actions that could be important in renal tissue protection. Activation of S1P1 receptors on dendritic cells reduces IL-12 and IL-10 production (26). IL-12 activates natural killer T cells (4), which contribute importantly to the innate immune system through direct cytotoxicity. IL-10 is a potent anti-inflammatory cytokine and has an important role in the innate immune system through direct cytotoxicity. IL-10 is a potent anti-inflammatory cytokine and has been shown to protect kidneys from acute injury (11). S1P1 receptor activation in vascular endothelial cells is implicated in angiogenesis and the maturation of the vascular system in mammals (19, 20, 30). S1P activation of S1P1 is also important for cell survival and proliferation of vascular endothelial cells, stimulation of endothelial cell nitric oxide synthase enzyme (19, 25) and reduction of monocyte adhesion (2). These effects on endothelial cells may lead to maintenance of endothelial barrier function (24). Whether the protective effect of FTY-720 is due to action on immune cells or kidney cells cannot be determined by our study. However, our studies do demonstrate a pronounced increase in S1P1 mRNA expression well before the peak of kidney leukocyte infiltration, which suggests that S1P1 receptors along with S1P2–4 are expressed in kidney resident cells. In this regard, we performed experiments in which we measured the vascular permeability of EBD in kidney tissue with FTY-720 treatment. EBD has been used routinely to measure vascular permeability in various tissues including kidney (12). Independent confirmation that IRI leads to an increase in vascular permeability was demonstrated by Sutton et al. (37) using fluorescent dextran molecules between 3,000 and 500,000 Da. The molecular weight of albumin is 67,000 Da, and thus the leakage of EBD conjugated to albumin following IRI in the current study is consistent with the findings of Sutton et al. Our results show that IRI led to an increase in kidney EBD content, an effect that was reduced with FTY-720. We believe that our results are consistent with extravasation of EBD into tissue induced by IR and reduced following treatment with FTY-720. It is possible that a small contribution of EBD measured in ischemic kidney tissue may be due to that retained in the vasculature due to capillary plugging by leukocytes. However, it should be emphasized that leukocyte plugging of peritubular capillaries is minimal early in reperfusion (4–6 h) (9, 28). Thus our results are consistent with the early effect of FTY-720 treatment to reduce vascular permeability. Whether the effect of FTY-720 to preserve vas-

Table 2. Kidney S1P mRNA expression following IRI

<table>
<thead>
<tr>
<th>S1P Receptors</th>
<th>Reperfusion Time, h</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1P1</td>
<td>3.3 ± 0.3</td>
<td>11.3 ± 2.5*</td>
<td>5.0 ± 1.1*</td>
<td>2.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>S1P2</td>
<td>1.1 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>2.1 ± 0.8</td>
<td>6.5 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>S1P3</td>
<td>1.9 ± 0.4</td>
<td>3.6 ± 0.8*</td>
<td>2.1 ± 0.6</td>
<td>1.7 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>S1P4</td>
<td>3.5 ± 1.8</td>
<td>6.0 ± 2.4</td>
<td>4.6 ± 1.5</td>
<td>0.9 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

Values reported as means ± SE. IRI, ischemia-reperfusion injury. Mouse kidneys were subjected to 32 min of ischemia followed by 2, 4, 6, or 24 h of reperfusion. Kidneys were harvested at the appropriate time, total RNA was extracted, and real-time PCR was performed. S1P mRNA expression relative to sham is shown. *P < 0.05 compared with sham.
cicular permeability is due to a direct effect on endothelial cells or to an indirect effect through immune cells is not certain. Whether S1P3 is involved in IRI and plays a role in endothelial or epithelial function of the kidney is not clear at this time. In our study, S1P3 expression is also increased after IRI. S1P3 activation is thought to lead to a loss of epithelial integrity in the lung (14) and may have a similar function in the kidney. Thus selective compounds that activate S1P1 but not S1P3 might be advantageous. Additional studies are needed to explore this mechanism.

In summary, our results extend previous studies by defining the dose range of tissue protection observed with FTY-720. These preclinical studies have important implications before its use for the prevention of human kidney injury. Second, the use of additional selective pharmacological agents strongly implicates the role of S1P1 receptors in the action of FTY-720 in IRI. Whether FTY-720 mediates tissue protection through action on immune or nonimmune kidney cells will need to be addressed in future studies.

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