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

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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 lysosphospholipid 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 S1P1–5 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

Reanal 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 apecromazine (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 costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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 mL 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 (ITTC model 179, ITTC/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 15,000 g for 15 min at 4°C, and the resulting 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 o-diamisidine (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 flow cytometry to analyze kidney leukocyte content. These studies increase relative to sham. All the antibodies were purchased from eBioscience (San Diego, CA).

Analysis of kidney vascular permeability. Changes in vascular permeability were assessed by quantitating extravasation of Evans blue dye (EBD) into the kidney tissue. Briefly, 2% EBD (Sigma Biosciences, St. Louis, MO) was administered at a dose of 20 mg/kg via the jugular vein of sham, vehicle-treated, or FTY-720-treated (48 μg·kg⁻¹·dose⁻¹) mice 5 h after reperfusion. One hour later, mice were killed under anesthesia and perfused through the heart with PBS and EDTA for 5 min at a rate of 2.5 mL/min by a multisyringe pump (Harvard Apparatus, Holliston, MA). Kidneys were then removed, allowed to dry overnight at 60°C, and the dry kidney weight was determined. EBD was extracted in formamide (20 mL/g dry tissue; Sigma Biosciences), homogenized, and incubated at 60°C overnight. Homogenized kidneys were centrifuged at 5,000 g for 30 min. The extravasated EBD concentration in kidney was calculated against a standard curve (14), and absorbance was quantitated spectrophotometrically at 620 and 740 nm using the formula OD620 (EBD) = OD620 - (1.426 × OD740 + 0.030) (36). Data were expressed as micrograms of EBD per gram of dry kidney weight.

Kidney histology. 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 Axioskoph), 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 number 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 RNeasy 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 (Corvald, 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 2^−(ΔΔCt) = 2^−((En−Et)/2), where Rt is the threshold cycle number for the reference gene observed in the test sample, Et is the threshold cycle number for the experimental gene observed in the test sample, En is the threshold cycle number for the reference gene observed in the control sample, and En is the threshold cycle number for the experimental gene observed in the control sample. Samples that had expression levels ≥5-fold were considered overexpressed.

Statistical analysis. Comparisons between groups were examined by one-way ANOVA by using SPSS version 13.0 software for Windows (SPSS, Chicago, IL) program. Multiple comparisons of

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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 treatment (48 h; see MATERIALS AND METHODS). Blood was obtained following 24 h of reperfusion and plasma creatinine was assayed. Values are means ± SE; n = 4 for each group. *P < 0.005 compared with sham. +P < 0.005 compared with vehicle treatment.

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% and 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% and 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% and 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% and 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% and 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% and 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.
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 S1P\(_1\) 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 S1P\(_1\) 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\(^{-1}\)) and VPC-44116 (10 mg·kg\(^{-1}\)·dose\(^{-1}\)) alone or in combination. Values are means ± SE; \(n = 7\) for each group. *\(P < 0.05\) compared with vehicle.

Fig. 5. Effect of FTY-720 on plasma creatinine and leukocyte infiltration is reversed with VPC-44116, a selective S1P\(_1\) 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 (\(\Delta OD_{460} \cdot g^{-1} \cdot min^{-1}\)) 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.
was 1.5 not reverse this effect. Peripheral blood lymphocyte counts in treatment). Although as expected, FTY-720 reduced blood lymphocyte counts under normal control respectively). Similar to FTY-720 treatment, SEW-2871 re-

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/µl; control, 5.85 ± 0.14 K/µl, 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 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.

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 S1P1–3 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-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 S1P1 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 S1P5 receptors are expressed in spleen 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 mg/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, which is due to action on immune cells or kidney cells cannot be 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, barrier function (19), and reduction of monocyte adhesion (25). S1P activation of S1P1 is also important in reperfusion (4–6 h) (9, 28). Thus, our results are consistent with the findings of Sutton et al. Our results show that IRI led 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 results of Sutton et al. Our results show that IRI led to an increase in 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 IRI 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>
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<tr>
<th>S1P Receptors</th>
<th>Reperfusion Time, h</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>24</th>
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<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>
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<tr>
<td>S1P2</td>
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<td>6.5±4.3</td>
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<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>
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<tr>
<td>S1P4</td>
<td>3.5±1.8</td>
<td>6.0±2.4</td>
<td>4.6±1.5</td>
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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.
cular 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 and/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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