

# Expression of sphingosine-1 phosphate receptor in rat renal ischemia-reperfusion injury

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**Abstract.** Sphingosine-1 phosphate receptor (S1PR) has come to the fore as a mediator of extracellular signaling through its interaction with G-protein-coupled receptors, which results in the induction of peripheral T-cell depletion. The mechanisms involved in renal ischemia-reperfusion (I/R) injury are complex, but appear to involve the early participation of bone marrow-derived cells, such as T lymphocytes. In this study, we investigated the expression of S1PR in a rat model of renal I/R injury. By means of a laparotomy, the right kidney was harvested and the left renal artery and vein were clamped. The kidney was reperfused after 90 min of ischemia, and rats were sacrificed at 0, 3, 6, 12 and 24 h after reperfusion. S1PR expression was analyzed by immunohistochemistry, and was observed only in endothelial cells of the normal kidneys. From 0 to 3 h after reperfusion, S1PR expression gradually became stronger in endothelial cells, reaching its peak intensity at 3 h after reperfusion. Twelve hours after reperfusion, necrosis had extended throughout the ischemic kidney, and nearly all the tubular epithelial cells had been destroyed. From 3 to 12 h after reperfusion, S1PR expression gradually weakened. At 24 h after reperfusion, levels of S1PR expression had almost reached those of the normal kidneys. In conclusion, S1PR was found to be expressed in a rat model of renal I/R injury. Several hours after achieving the maximum level of S1PR expression, the maximum level of renal I/R injury was observed. These results suggest a relationship between S1PR and renal I/R injury.

## Introduction

Renal ischemia-reperfusion (I/R) injury, an invariable consequence of renal transplantation, is a clinically significant problem involving the onset of acute tubular necrosis (ATN) when the transplantation includes a long ischemic interval or when using a kidney from a cardiac arrest donor. The longer the ischemic interval, the higher the incidence rate of ATN. It is therefore clinically important to identify methods of reducing renal I/R injury (1).

The identification of mediators of the I/R injury cascade (2) has generated new target sites. Through these, it is possible to abrogate the effect of I/R injury using anti-neutrophil preparations (3), free radical scavengers (4,5) and monoclonal antibodies to block adhesion molecules and the inflammatory cytokine cascade (6-8). The mechanisms involved in renal I/R injury are complex, but appear to involve the early participation of bone marrow-derived cells, as T lymphocytes have been shown to participate in its pathogenesis. Sphingosine-1 phosphate receptor (S1PR) has come to the fore as a mediator of extracellular signaling through its interaction with G-protein-coupled receptors, which results in the induction of peripheral T-cell depletion (9).

Several studies have found that the administration of a non-selective S1PR agonist reduces injury resulting from renal I/R (10-13). However, there have been no studies addressing the relationship between S1PR expression and renal I/R injury. In the present study, we investigated this relationship in a rat model of renal I/R injury.

## Materials and methods

**Ischemia-reperfusion model.** Male Lewis rats (270-320 g) were subjected to laparotomy under pentobarbital sodium anesthesia. The right kidney was harvested and the left renal artery and vein were clamped with a hemostasis clip for 90 min. The clip was subsequently removed to permit reperfusion, and the abdomen was closed during I/R. The left kidneys

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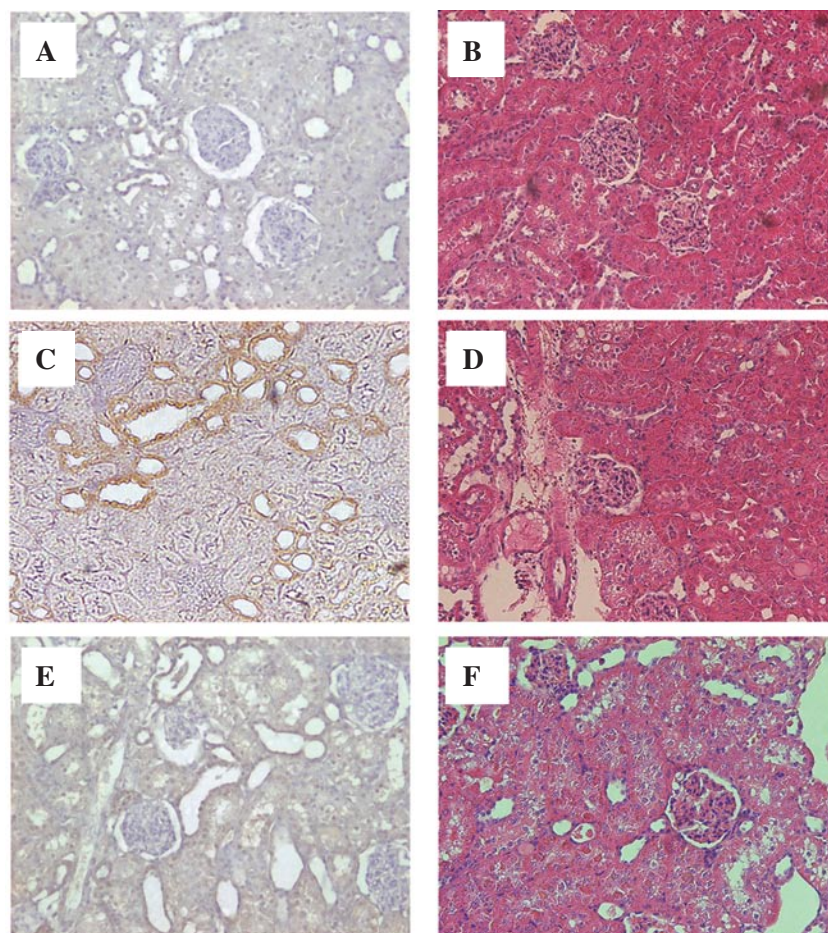


Figure 1. Representative S1PR expression and H&E staining. A and B show the normal kidney before ischemia. S1PR expression was most intense in endothelial cells at 3 h after reperfusion (C). Six hours after reperfusion, slight destruction of the tubular epithelial cells was noted (D). Twelve hours after reperfusion, necrosis extended throughout the ischemic kidney (F), while 12 h after reperfusion, S1PR expression gradually became weaker in endothelial cells (E). A, C and E, immunohistochemistry; B, D and F, H&E staining.

were reperfed. The rats were sacrificed at 0, 3, 6, 12 and 24 h after reperfusion, then the kidneys were harvested for immuno-histochemistry. All procedures involving animals were conducted according to the ethical guidelines for animal experimentation of Osaka City University.

**Immunohistochemical staining.** Samples of ischemic and non-ischemic kidney tissue were fixed in 10% buffered formalin for 24 h for immunohistochemical staining. Staining was performed using the Vectastatin avidin-biotin peroxidase complex kit (Vector Laboratories, Burlingame, CA, USA). Kidney tissues sectioned onto microscope slides were deparaffinized, then the slides were immersed for 45 min in 0.3% peroxide in methanol to deplete endogenous peroxidase activity. Non-specific binding sites were saturated with 0.2% bovine serum albumin and normal rabbit serum diluted 1:66.7 in PBS for 20 min. Primary antibodies against rabbit polyclonal to EDG1 (Abcam, Cambridge, UK), a lysophospholipid receptor which binds sphingosine-1 phosphate, and control normal serum (Vector Laboratories) were used at a dilution of 1:50 and applied to the tissue sections. Sections were then incubated in a humidified chamber at room temperature for 30 min, and subsequently washed with PBS for 10 min. Biotinylated goat anti-rabbit IgG (Vector Laboratories)

was applied to the sections, followed by incubation at room temperature for 30 min. After washing with PBS for 10 min, the slides were incubated with avidin DH-biotinylated peroxidase (Vector Laboratories) for 45 min. Finally, color was developed by immersion in a peroxidase substrate solution containing 0.02% peroxide, 3,3' diaminobenzidine tetrahydrochloride, 0.04% nickel chloride and 0.01% hydrogen peroxide in 0.05 M Tris-HCl, pH 7.2, for 2-7 min.

**Analysis of acute tubular necrosis and S1PR expression.** For the quantification of ATN, the degree of ATN was graded on a scale of 0-3 by two blinded observers, defined as no necrosis, mild, moderate or severe necrosis. Necrosis, capillary congestion, interstitial edema, casts, destruction and flat and extended areas of tubular epithelial cells were also assessed. Similarly, S1PR immunostainings were graded on a scale of 0-4 according to the intensity of immunostaining, with grade 4 being the maximum. Quantification of ATN and S1PR expression was conducted by the same two pathologists throughout the study.

**Statistical analysis.** Results are presented as the mean  $\pm$  SD. Analyses of data were carried out using analysis of variance (ANOVA) (14).

## Results

**H&E staining and immunohistochemistry of S1PR.** S1PR expression was only observed in endothelial cells of the normal kidneys (Fig. 1A). H&E staining showed normal architecture of the kidney before ischemia (Fig. 1B). From 0 to 3 h after reperfusion, S1PR expression gradually became stronger in endothelial cells. S1PR expression was most intense in endothelial cells at 3 h after reperfusion (Fig. 1C). Six hours after reperfusion, the internal spaces of the tubular epithelial cells were expanded, and slight destruction of the cells was noted (Fig. 1D). Twelve hours after reperfusion, necrosis extended throughout the ischemic kidney and nearly all the tubular epithelial cells had been destroyed (Fig. 1F). However, from 3 to 12 h after reperfusion, S1PR expression gradually became weaker in endothelial cells (Fig. 1E, 12 h after reperfusion). At 24 h after reperfusion, levels of S1PR expression almost reached those of the normal kidneys.

**Statistical analysis of S1PR expression and acute tubular necrosis.** As shown in Table I, S1PR expression scores were significantly higher at 3 and 6 h than at 0, 12 and 24 h after reperfusion. ATN scores gradually increased with time after reperfusion (Table I).

## Discussion

Renal transplantation is a viable therapeutic approach for patients with end-stage renal disease. However, renal I/R injury, which is an invariable consequence of renal transplantation resulting from aortic cross-clamping and resuscitation after systemic hypotension, is a clinically significant problem. Recent studies of I/R injury have focused on the function of neutrophils, the action mechanisms of inflammatory cytokines, tissue factors, intercellular adhesion molecule-1, oxygen-free radicals, vascular plugging, edema and other complications (15).

It has been accepted that organ dysfunction and multi-organ failure following I/R injury is due not to one specific pathway, but is rather mediated by various pathophysiologic processes. The role of neutrophils, lymphocytes, cytokine secretion and endothelial cell adhesion molecules in the pathogenesis of I/R injury has been well described (10). While early studies mainly focused on the participation of neutrophils as mediators of I/R injury, recent studies have also demonstrated the importance of T lymphocyte involvement.

Several studies have found that the non-selective S1PR agonist FTY-720 has a protective effect in rat renal I/R injury (10). FTY-720 is an analogue of sphingosine and acts on the sphingosine receptors, of which there are five. S1PR is required for thymocytes to leave the thymus and for T and B lymphocytes to leave the lymph nodes (11). FTY-720 binds to the group of G-protein-coupled S1PRs. Awad *et al* applied FTY-720 and a selective S1PR agonist (SEW2871) in a mouse renal I/R injury model (32 min ischemia and 24 h reperfusion). Treatment with FTY-720 and SEW2871 resulted in improved histological appearance and a decrease in serum creatinine, vascular permeability and neutrophil infiltration (9). Lien *et al* reported that SEW2871 improved renal function with a reduction in serum creatinine levels and a significant

Table I. Statistical analysis of S1PR expression and ATN scores.

Time after reperfusion (h)	S1PR expression score (n=30)	ATN score (n=30)
Before ischemia	0.6±0.5	None
0	0.7±0.5	1.6±0.7
3	2.6±0.8 <sup>a</sup>	2.4±0.4
6	2.0±0.7 <sup>a</sup>	3.1±0.7
12	1.2±0.7	3.2±0.5
24	0.9±0.7	3.4±0.6

To quantify S1PR expression and ATN, the degree of S1PR expression and ATN was graded on a scale of 0-4 by two blinded observers. Data are presented as the mean±SD. Analyses of data were carried out using the analysis of variance (ANOVA). S1PR expression scores were significantly higher at 3 and 6 h than at 0, 12 and 24 h after reperfusion. ATN scores gradually increased with time after reperfusion. <sup>a</sup>P<0.01.

reduction in ATN scores 24 h after ischemia in a mouse renal I/R injury model, and that SEW2871 ameliorated renal I/R injury by inhibiting lymphocyte regression and reducing pro-inflammatory molecules (13).

During the process of I/R injury, inflammatory reactions are activated and inflammatory cytokines, such as tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$ , as well as arachidonic acid metabolites, such as thromboxanes and prostaglandins, are released (16). The conversion of arachidonic acid to thromboxanes and prostaglandins is catalyzed by cyclooxygenase (COX)-2 (17).

Takeyoshi *et al* found that peroral administration of FK3311 (1 mg/kg), a COX-2 inhibitor, reduced the degree of neutrophilic leukocyte invasion, tissue destruction and thromboxane B<sub>2</sub> at 6 h after reperfusion in a canine 60-min renal warm I/R injury model (18). They also reported that the 2-day survival rate was significantly higher upon peroral administration of FK3311 (1 mg/kg) in the COX-2 inhibitor group than in the control inhibitor group in a canine 15-min lung warm I/R injury model (19). In a previous study, we too found COX-2 to be induced in rat renal I/R injury using immunohistochemistry (20).

The adipose-derived plasma protein adiponectin (APN) has various protective effects on cardiovascular disease. Ikeda *et al* reported that APN is required for full COX-2 induction by I/R injury in the heart *in vivo*. In rat neonatal cardiac myocytes, APN-induced COX-2 expression was reduced by treatment with a sphingosine kinase-1 inhibitor or siRNA-targeting sphingosine kinase-1. Treatment with a S1PR antagonist also diminished COX-2 expression in response to APN stimulation (21). These reports support the relationship between S1PR and COX-2 in I/R injury.

In this study, S1PR was expressed in endothelial cells, reaching maximum intensity 3 h after I/R injury. From 12 to 24 h after I/R injury, maximum tissue damage was observed. These results suggest that S1PR plays an important role in renal I/R injury, particularly in I/R-induced ATN.



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