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A novel and selective Na^+/Ca^{2+} exchange inhibitor, SEA0400, improves ischemia/reperfusion-induced renal injury

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Abstract

We evaluated the effects of SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline), a novel and selective Na^+/Ca^{2+} exchange inhibitor, on ischemic acute renal failure. Ischemic acute renal failure in rats was induced by clamping the left renal artery and vein for 45 min followed by reperfusion, 2 weeks after the contralateral nephrectomy. SEA0400 administration (0.3, 1 and 3 mg/kg, i.v.) before ischemia dose-dependently attenuated the ischemia/reperfusion-induced renal dysfunction and histological damage such as tubular necrosis. SEA0400 pretreatment at the higher dose suppressed the increment of renal endothelin-1 content after reperfusion. The ischemia/reperfusion-induced renal dysfunction was also overcome by post-ischemia treatment with SEA0400 at 3 mg/kg, i.v. In in vitro study, SEA0400 (0.2 and 1 μM) protected cultured porcine tubular cells ($LLC\text{-}PK_1$) from hypoxia/reoxygenation-induced cell injury. These findings support the view that Ca^{2+} overload via the reverse mode of Na^+/Ca^{2+} exchange, followed by endothelin-1 overproduction, plays an important role in the pathogenesis of ischemia/reperfusion-induced renal injury. The possibility exists that a selective Na^+/Ca^{2+} exchange inhibitor such as SEA0400 is useful as effective therapeutic agent against ischemic acute renal failure in humans.
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Keywords: Ischemia; Reperfusion; Acute renal failure; Na^+/Ca^{2+} exchanger; Endothelin-1

1. Introduction

Ischemic acute renal failure is a frequent clinical syndrome with high morbidity and mortality (Thadani et al., 1996). Reperfusion of previously ischemic renal tissue initiates a complex cellular events that results in injury and the eventual death of renal cells due to a combination of apoptosis and necrosis (Lieberthal and Levine, 1996). The molecular mechanisms underlying the ischemia/reperfusion-induced renal injury are poorly understood, but it has been reported that several causal factors (ATP depletion, reactive oxygen species, phospholipase activation, neutrophil infiltration, vasoactive peptides, etc.) are contributive to the pathogenesis of this renal damage (Edelstein et al., 1997). Intracellular Ca^{2+} accumulation has also been considered as one of the factors which cause the ischemia/reperfusion-induced renal injury (Schrier et al., 1987). Although physiological and pathophysiological mechanisms of Ca^{2+} overload in ischemic kidney have not been fully elucidated, there is evidence indicating that increased cytosolic Ca^{2+} may be an important mediator of epithelial cell necrosis, which is characteristic of ischemic acute renal failure (Schrier et al., 1984; Wilson et al., 1984). Moreover, Ca^{2+} entry blockers exert a protective effect against ischemic acute renal failure (Schrier et al., 1987).

It is becoming clear that intracellular Ca^{2+} overload plays an important role in the ischemia/reperfusion injury in several organs. In cardiac ischemia, there is accumulating evidence that Ca^{2+} entry via the reverse mode of Na^+/Ca^{2+} exchanger after the reperfusion leads to heart dysfunction (Tani and Neely, 1989). In addition, it has been recently demonstrated that a Na^+/Ca^{2+} exchanger inhibitor (KB-R7943, 2-[2-[4-(4-nitorobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate) improves the ischemia/reperfusion injury, both in isolated cardiomyocytes and in the whole heart (Nakamura et al., 1998; Ladilov et al., 1999; Elias et al., 2001). In the kidney, we first demonstrated the protective effects of KB-R7943 on ischemia/reperfusion-induced acute renal failure, and therefore suggested that Ca^{2+} overload via

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the reverse mode of Na^+/Ca^{2+} exchanger plays an important role in the pathogenesis of this renal disease (Kuro et al., 1999). KB-R7943 has been reported to selectively inhibit the reverse mode of Na^+/Ca^{2+} exchanger in cardiomyocytes, smooth muscle cells, and Na^+/Ca^{2+} exchanger-transfected fibroblasts (Iwamoto et al., 1996; Watano et al., 1996). On the other hand, some workers have questioned as to whether KB-R7943 is a selective inhibitor of Na^+/Ca^{2+} exchanger based on findings that KB-R7943 affects various proteins, including several ion channels and receptors, at concentration used to inhibit Na^+/Ca^{2+} exchanger (Sobolevsky and Khodorov, 1999; Pintado et al., 2000; Matsuda et al., 2001).

2-[4-[(2,5-Difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400) is a newly developed Na^+/Ca^{2+} exchanger inhibitor (Matsuda et al., 2001). This compound was extremely more potent than KB-R7943 in inhibiting extracellular Na^+-dependent Ca^{2+} uptake in cultured neurons, astrocytes, and microglia. In addition, SEA0400 at the concentration range that inhibited Na^+/Ca^{2+} exchanger did not significantly affect ion transporters, ion channels, receptors and enzymes, thereby indicating that SEA0400 is a highly selective inhibitor of Na^+/Ca^{2+} exchanger (Matsuda et al., 2001). A similar Na^+/Ca^{2+} exchanger-selective characteristic of SEA0400 was observed in isolated guinea-pig ventricular myocytes (Tanaka et al., 2002). Thus, SEA0400 would be a valuable pharmacological tool to investigate the pathophysiological role of Na^+/Ca^{2+} exchanger in ischemia/reperfusion-induced tissue injury. In fact, Matsuda et al. (2001) and Takahashi et al. (2003) clearly indicated that SEA0400 had a protective effect against ischemia/reperfusion injuries in brain and heart, respectively.

In the present study, we first examined whether pre- or post-ischemic treatment with SEA0400 would be have a protective effect on the ischemia/reperfusion-induced renal dysfunction and tissue injury. In addition, we asked if SEA0400 could suppress the ischemia/reperfusion-induced enhancement of endothelin-1 production, which is closely related to the pathogenesis of renal ischemia/reperfusion injury (Kuro et al., 2000). In the previous study, we originally found a decreasing effect of KB-R7943 on the endothelin-1 overproduction in the post-ischemic kidney, and suggested a close relationship between ischemia/reperfusion-induced enhancement of endothelin-1 production and Ca^{2+} overload via the reverse mode of Na^+/Ca^{2+} exchanger (Yamashita et al., 2001). Finally, we evaluated the effect of SEA0400 on the hypoxia/reoxygenation-induced cell injury in LLC-PK_1 cells (cultured porcine tubular cells), which have the characteristics of proximal tubules.

2. Materials and methods

2.1. Animals and experimental design

2.1.1. Experiment 1

Male Sprague–Dawley rats (280–320 g, 10 weeks old, Japan SLC, Shizuoka) were housed in a light-controlled room with a 12-h light/dark cycle and access to food and water was ad libitum. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences. Two weeks before the study (8 weeks of age), the right kidney was removed through a small flank incision made following pentobarbital anesthesia (50 mg/kg, i.p.). After a 2-week recovery period, these rats were randomly separated as follows: (1) sham-operated control ($n=6$); (2) vehicle-treated acute renal failure ($n=6$); (3) pre-ischemic treatment with SEA0400 (0.3, 1 and 3 mg/kg, i.v.) in acute renal failure ($n=7$); (4) post-ischemic treatment with SEA0400 (3 mg/kg, i.v.) in acute renal failure ($n=7$). To induce ischemic acute renal failure, the rats were anesthetized with pentobarbital (50 mg/kg, i.p.), and the left kidney was exposed through a small flank incision. The left renal artery and vein were occluded with a nontraumatic clamp for 30 or 45 min. At the end of the ischemic period, the clamp was released for blood reperfusion. SEA0400 or its vehicle (a lipid emulsion containing 20% soybean oil) was administered (pre-ischemic treatment, 30-min before the ischemia; post-ischemic treatment, immediately after reperfusion) as a slow bolus injection at a volume of 1 ml/kg into the external jugular vein. In sham-operated control animals, the left kidney was treated identically, except for clamping. Animals exposed to 30- or 45-min ischemia were housed in metabolic cages at 24 h after reperfusion; 5-h urine samples were taken, and blood samples were drawn from the aorta at the end of the urine collection period. The plasma was separated by centrifugation. These samples were used for measurements of renal functional parameters. The kidneys were excised and examined using a light microscope.

2.1.2. Experiment 2

Some rats from each group, separated as in Experiment 1, were killed at 2, 6 and 24 h after the 45-min ischemia/reperfusion, and their left kidneys were obtained to determine endothelin-1 content.

2.1.3. Experiment 3

In separate experiments, we examined the effect of SEA0400 (3 mg/kg, i.v.) on the acute damage of renal function after ischemia/reperfusion. Animals were uninephrectomized, as described above. After a 2-week recovery period, the rats were anesthetized with sodium thiobutabarbital (Inactin, 100 mg/kg, i.p.) and placed on a heated surgical tray that maintained rectal temperature between 37 and 38 °C. After tracheotomy, the right femoral artery and vein were cannulated to monitor arterial blood pressure and for infusion of 0.9% saline containing 1.0% inulin and 0.3% *p*-aminohippuric acid (0.02 ml/min), respectively. The right carotid artery and vein were also cannulated for blood sampling and for infusion of 2.5% mannitol/0.45% saline (0.08 ml/min), which ensures urine production after the ischemia, respectively. After making an abdominal midline

incision, a polyethylene cannula was inserted into the left ureter for urine collection. A 60- to 90-min period was allowed for stabilization of mean arterial pressure and urine flow. After the equilibration period, urine samples were collected during two 20-min control clearance periods. Results for the second control period served as basal values for renal function. Following the control periods, SEA0400 or its vehicle was administered intravenously by the slow bolus injection at a volume of 1 ml/kg. Thirty minutes after injection, left renal artery and vein were occluded with a nontraumatic clamp for 30 min. At the end of the ischemic period, the clamp was released for blood reperfusion. Urine samples were then collected during five consecutive 20-min periods (E1–E5). Blood samples (0.2 ml each) were obtained at 20 min before drug injection and at 80 and 120 min after the injection, respectively. The blood loss was replaced by injecting an equal volume of blood from donor rats. Plasma was immediately separated by centrifugation. In preliminary experiments, no urine production was usually observed after 45-min ischemia and reperfusion.

2.2. Hypoxia and reoxygenation in LLC-PK1

LLC-PK_1 (American Type Culture Collection, Manassas, VA), a porcine kidney cell line, was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 50 μg/ml streptomycin, and 50 U/ml penicillin at 37 °C in a CO_2 incubator (95% air, 5% CO_2). When the cells cultured in 24-well plates became confluent, the culture medium was changed to DMEM without glucose and serum and the cells were exposed to the hypoxic condition using an Anaero Pack Pouch (Mitsubishi Gas Chemical, Tokyo, Japan), in which the oxygen concentration was less than 1% within 1 h after the exposure. After 6 h of hypoxia, the cells were put in a CO_2 incubator for 1 h in the DMEM to which glucose was added at the beginning of reoxygenation. After the exposure of the cells to hypoxia and reoxygenation, lactate dehydrogenase (LDH) activity in the culture supernatant for 7 h was measured with a commercial kit. SEA0400 (0.2, 1 μM) added to the medium at the beginning of hypoxia and/or reoxygenation. The drug concentrations were determined based on the previous studies, in which these concentrations of SEA0400 specifically inhibited Na^+-dependent Ca^{2+} uptake and Ca^{2+} paradox injury in cultured cells (Matsuda et al., 2001; Takahashi et al., 2003). LDH release was expressed as a percentage of total cellular LDH activity.

2.3. Blood and urine measurements

Blood urea nitrogen and creatinine levels in plasma and urine were determined using a commercial assay kit, BUN-test-Wako and Creatinine test-Wako (Wako). Urinary osmolality was measured by freezing point depression (Fiske Associates, Norwood, MA, USA). Urine and plasma sodium

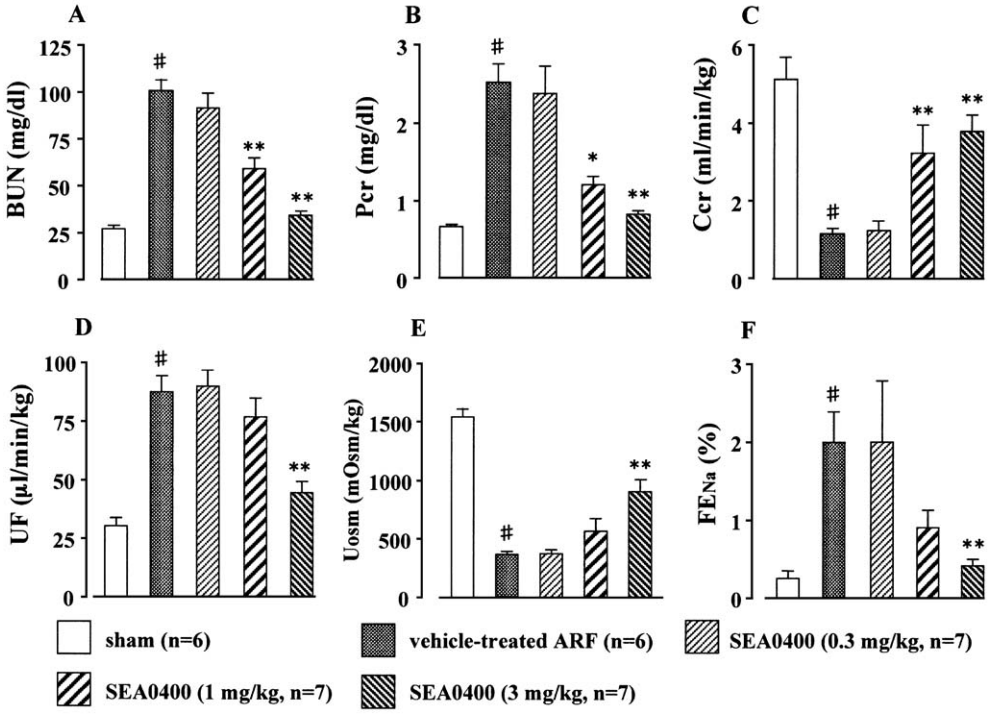

Fig. 1. Effects of SEA0400 administered before ischemia on blood urea nitrogen (BUN, A), plasma creatinine concentration (Pcr, B), creatinine clearance (Ccr, C), urine flow (UF, D), urinary osmolality (Uosm, E), and fractional excretion of sodium (FE_{Na}, F) at 24 h after ischemia/reperfusion. At 24 h after reperfusion 5-h urine was collected. Each value represents the mean ± S.E.M. $^{\#}P<0.01$, compared with sham-operated rats; $^{*}P<0.05$, $^{**}P<0.01$, compared with vehicle-treated ARF rats. ARF, acute renal failure.

concentrations were determined using a flame photometer (Hitachi, 205D, Hitachinaka, Japan). Fractional excretion of sodium (FE_{Na}, %) was calculated from the following formula: $FE_{Na} = U_{Na}V/(P_{Na} \times Ccr) \times 100$, where $U_{Na}V$ is urinary excretion of sodium, P_{Na} is the plasma sodium concentration, and Ccr is creatinine clearance. Glomerular filtration rate was estimated from the inulin clearance. Urine and plasma inulin levels were measured spectrofluorometrically. Renal plasma flow was estimated from the *p*-aminohippuric acid clearance. Urine and plasma *p*-aminohippuric acid levels were measured by colorimetry according to the Bratton–Marshall method.

2.4. Histological studies

Excised left kidneys were processed for light microscopic observation according to standard procedures. The kidneys were then preserved in phosphate-buffered 10% formalin; after which the kidneys were chopped into small pieces, embedded in paraffin wax, cut at 3 μm and stained with hematoxylin and eosin. Histopathological changes were analyzed for tubular necrosis, proteinaceous casts, and medullary congestion. These were graded as no change (− or 0), mild (± or 1), moderate (+ or 2), severe (++ or 3), and very severe

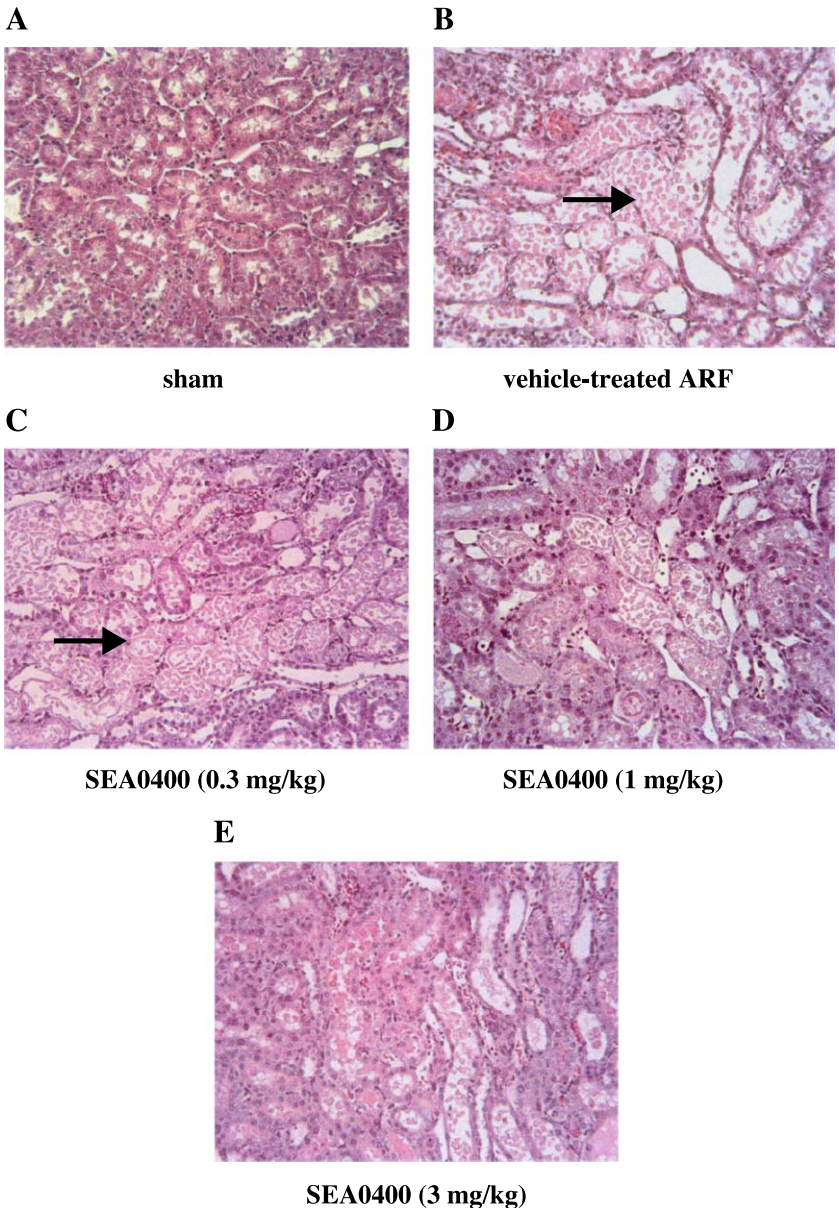

Fig. 2. Light microscopy of the outer zone outer stripe of medulla of the kidney of ARF rats treated with vehicle (B), SEA0400 (C, 0.3 mg/kg), SEA0400 (D, 1 mg/kg), and SEA0400 (E, 3 mg/kg) at 24 h after ischemia/reperfusion, and sham rats (A). Drugs were given intravenously 30 min before the ischemia. Arrows indicate tubular necrosis (hematoxylin–eosin staining, magnification ×200). ARF, acute renal failure.

(+++ or 4) based on the microscopical observations of each section.

2.5. Renal endothelin-1 assay

Endothelin-1 was extracted from the kidney, as described elsewhere (Fujita et al., 1995). Briefly, kidneys were weighed and homogenized for 60 s in 8 volumes of ice-cold organic solution (chloroform/methanol, 2:1, including 1 mM *N*-ethylmaleimide). The homogenates were left overnight at 4 °C, then 0.4 volume of distilled water was added; after which the homogenates were centrifuged at 1500 × *g* for 30 min and the resultant was stored. Aliquots of the supernatant were diluted 1:10 with a 0.09% trifluoroacetic acid solution and applied to Sep-Pak C_{18} cartridges. The sample was eluted with 3 ml of 63.3% acetonitrile and 0.1% trifluoroacetic acid in water. Eluates were dried in a centrifugal concentrator, and the dried residue was reconstituted in assay buffer for radioimmunoassay. The clear solution was subjected to radioimmunoassay. The recovery of endothelin-1 was approximately 80%. Radioimmunoassay for tissue endothelin-1 was done, as described elsewhere (Matsumura et al., 1990), using endothelin-1 antiserum (a generous gift from Dr. Marvin R. Brown, Department of Medicine, University of California, San Diego, CA). This serum dose not cross-react with big endothelin-1.

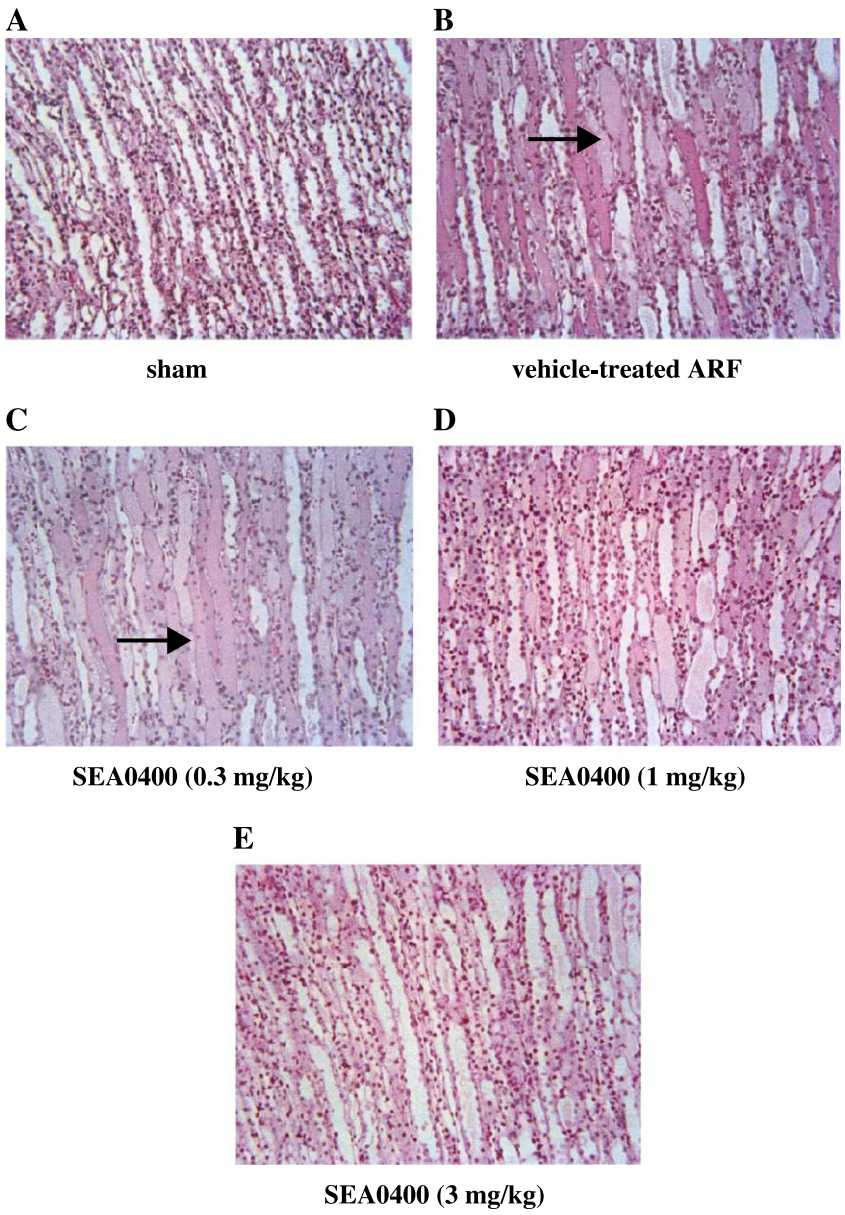

Fig. 3. Light microscopy of the inner zone of medulla of the kidney of ARF rats treated with vehicle (B), SEA0400 (C, 0.3 mg/kg), SEA0400 (D, 1 mg/kg), and SEA0400 (E, 3 mg/kg) at 24 h after ischemia/reperfusion, and sham rats (A). Drugs were given intravenously 30 min before ischemia. Arrows indicate proteinaceous casts in tubuli (hematoxylin–eosin staining, magnification × 200). ARF, acute renal failure.

2.6. Drugs

SEA0400 (Taisho Pharmaceutical, Saitama, Japan) was dissolved in a lipid emulsion containing 20% soybean oil. Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and Wako.

2.7. Statistical analysis

Values were expressed as mean ± S.E.M. For statistical analysis, we used one-way analysis of variance followed by a Bonferroni's multiple comparison test. Histological date were analyzed using the Kruskal–Wallis nonparametric test combined with the Steel-type multiple comparison test. For all comparisons, differences were considered significant at $P < 0.05$.

3. Results

3.1. Renal function after ischemia/reperfusion and effects of pre-ischemic treatment with SEA0400

As shown in Fig. 1, renal function of rats subjected to 45-min ischemia showed a marked deterioration when measured 1 day after the reperfusion. As compared with sham-

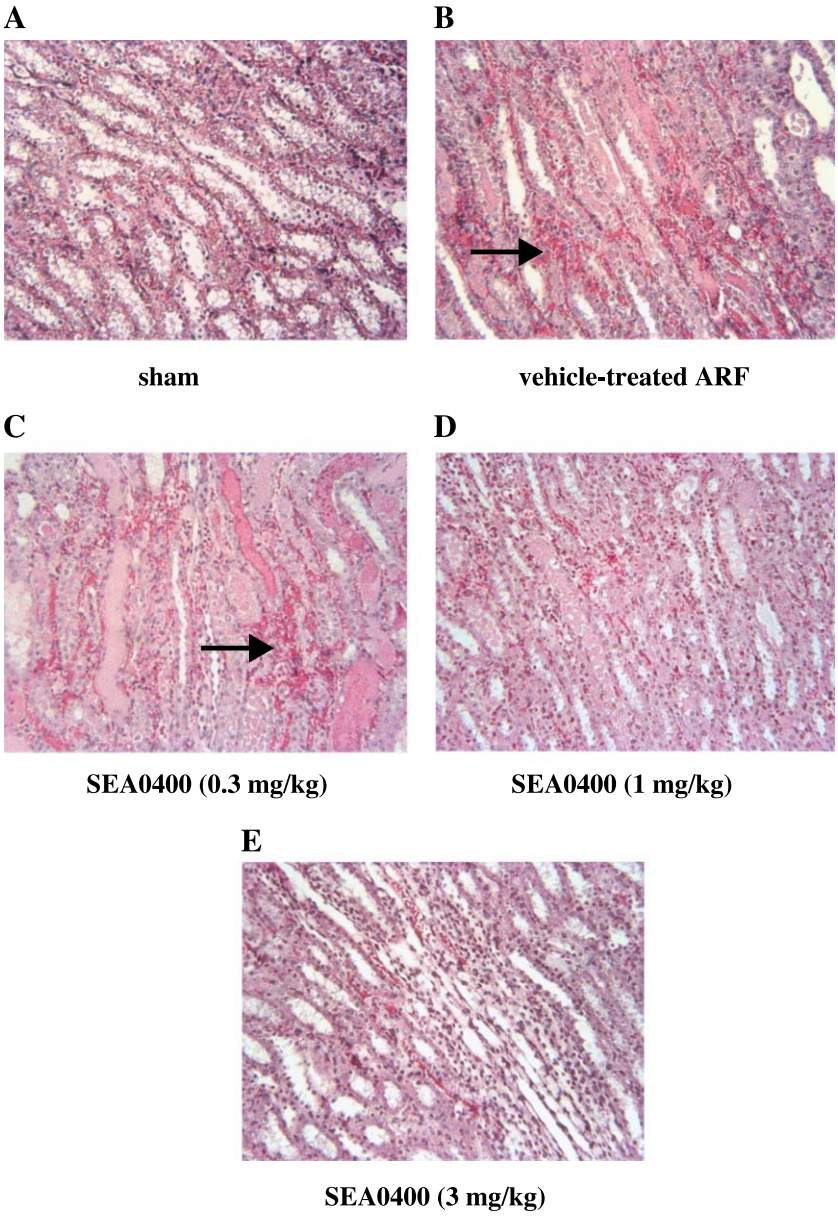

Fig. 4. Light microscopy of the outer zone inner stripe of medulla of the kidney of ARF rats treated with vehicle (B), SEA0400 (C, 0.3 mg/kg), SEA0400 (D, 1 mg/kg), and SEA0400 (E, 3 mg/kg) at 24 h after ischemia/reperfusion, and sham rats (A). Drugs were given intravenously 30 min before ischemia. Arrows indicate congestion and hemorrhage (hematoxylin–eosin staining, magnification ×200). ARF, acute renal failure.

Table 1
Histopathological changes of kidneys in ARF rats

Histopathological changes/grade		Tubular necrosis					Protein casts					Medullary congestion				
		–	±	+	++	+++	–	±	+	++	+++	–	±	+	++	+++
sham (n=6)		6	0	0	0	0	6	0	0	0	0	6	0	0	0	0
vehicle-treated ARF (n=6)		0	0	0	2	4	0	0	0	5	1	0	0	3	3	0
ARF + SEA0400	0.3 mg/kg (n=6)	0	0	0	2	4	0	0	1	2	3	0	0	1	4	1
	1 mg/kg (n=6)	0	1	5	0	0	0	3	3	0	0	1	2	3	0	0
	3 mg/kg (n=6)	1	2	2	1	0	2	2	2	0	0	3	2	1	0	0

Data are expressed as the number of animals with histopathological changes.
Grades: no changes (–), mild (±), moderate (+), severe (++), very severe (+++).
ARF, acute renal failure.

operated rats, vehicle-treated acute renal failure rats showed significant increases in blood urea nitrogen, plasma creatinine concentration, urine flow, and FENa, and significant decreases in creatinine clearance and urinary osmolality. Pretreatment with SEA0400 (0.3 mg/kg, 1 mg/kg, 3 mg/kg, i.v.) efficiently attenuated the acute renal failure-induced renal dysfunction in a dose-dependent manner. The administration of SEA0400 (3 mg/kg) to sham-operated rats produced no significant effects in their renal function (data not shown).

3.2. Histological renal damage after ischemia/reperfusion

Histopathological examinations revealed severe lesions in the kidney of vehicle-treated acute renal failure rats (1 day after the 45-min ischemia). These changes were characterized by tubular necrosis (Fig. 2, outer zone, outer stripe of medulla), proteinaceous casts in tubuli (Fig. 3, inner zone of medulla), and medullary congestion and hemorrhage (Fig. 4, outer zone, inner stripe of medulla). Pretreatment with SEA0400 dose-dependently attenuated the development of all these lesions (Tables 1 and 2).

3.3. Renal function after ischemia/reperfusion and effects of post-ischemic treatment with SEA0400

In a preliminary experiment, we examined the effect of SEA0400 (3 mg/kg) treatment immediately after the 45-min ischemia, but the agent failed to improve significantly the ischemia/reperfusion-induced renal dysfunction. We next evaluated the effect of the post-ischemic treatment under the condition of 30-min ischemia. As shown in Fig. 5, SEA0400, at a dose of 3 mg/kg, almost completely overcame the renal dysfunction induced by the 30-min ischemia and reperfusion.

3.4. Effects of SEA0400 on immediate renal function changes after the ischemia and reperfusion

Fig. 6 shows immediate changes in renal function after 30-min ischemia followed by reperfusion, in vehicle- or SEA0400 (3 mg/kg i.v.)-treated anesthetized rats. In the vehicle-treatment group, basal values of renal plasma flow and glomerular filtration rate averaged 2.69 ± 0.33 and 0.80 ± 0.11 ml/min/g kidney wt., respectively. For the SEA0400 pretreated group, similar results were obtained. During the first 20-min period after the reperfusion, the levels of renal plasma flow and glomerular filtration rate were extremely low in both groups (near zero). Thereafter, these renal hemodynamic parameters gradually increased over the 100-min observation period, in the same manner in both groups.

3.5. Effects of SEA0400 on the hypoxia/reoxygenation-induced injury in LLC-PK1

We assessed the effect of SEA0400 on hypoxia/reoxygenation-induced cell injury in LLC-PK_1 cells, which have the characteristics of proximal tubules, by measuring

Table 2
Effects of SEA0400 on histopathological changes of kidneys in ARf rats

		Tubular necrosis	Protein casts	Medullary congestion
vehicle-treated ARF (n=6)		3.67 ± 0.21	3.17 ± 0.45	2.50 ± 0.47
ARF + SEA0400	0.3 mg/kg (n=6)	3.67 ± 0.21	3.33 ± 0.44	3.00 ± 0.46
	1 mg/kg (n=6)	1.83 ± 0.41[b]	1.50 ± 0.46[b]	1.33 ± 0.46[a]
	3 mg/kg (n=6)	1.50 ± 0.49[b]	1.00 ± 0.50[b]	0.67 ± 0.49[b]

Each value represents the mean ± S.E.M. of histopathological change/grade.
Grades: no changes (0), mild (1), moderate (2), severe (3), very severe (4).
ARF, acute renal failure.
[a] $P < 0.05$, compared with vehicle-treated ARF rats.
[b] $P < 0.01$, compared with vehicle-treated ARF rats.

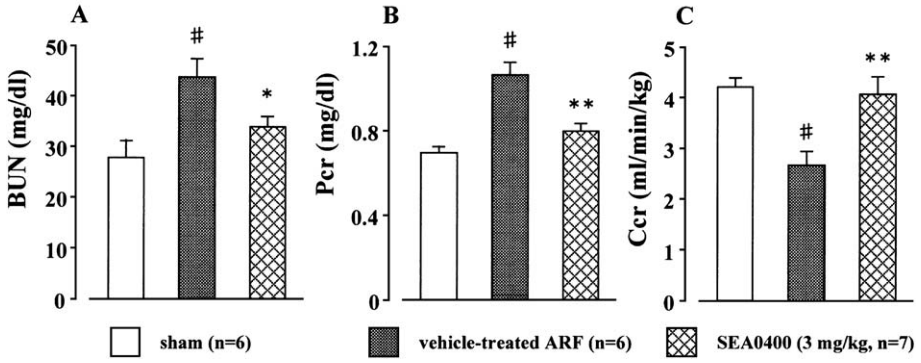

Fig. 5. Effects of SEA0400 administered after reperfusion on blood urea nitrogen (BUN, A), plasma creatinine concentration (Pcr, B), and creatinine clearance (Ccr, C) at 24 h after ischemia/reperfusion. At 24 h after reperfusion, 5-h urine was collected. Each value represents the mean ± S.E.M. $^{\#}P<0.01$, compared with sham rats; $^{*}P<0.05$, $^{**}P<0.01$, compared with vehicle-treated ARF rats. ARF, acute renal failure.

LDH release from the cells. Hypoxia/reoxygenation technique is known as in vitro model system of ischemia/reperfusion-induced renal injury (Yonehana and Gemba, 1999). Fig. 7 shows that LDH release from the cells exposed to hypoxia followed by reoxygenation notably increased compared with the normoxic control. The cell injury under the hypoxia/reoxygenation condition was significantly improved by the treatment with SEA0400 during hypoxia and reoxygenation, in a concentration-dependent manner. A similar protective effect of SEA0400 was also observed with addition at the beginning of reoxygenation.

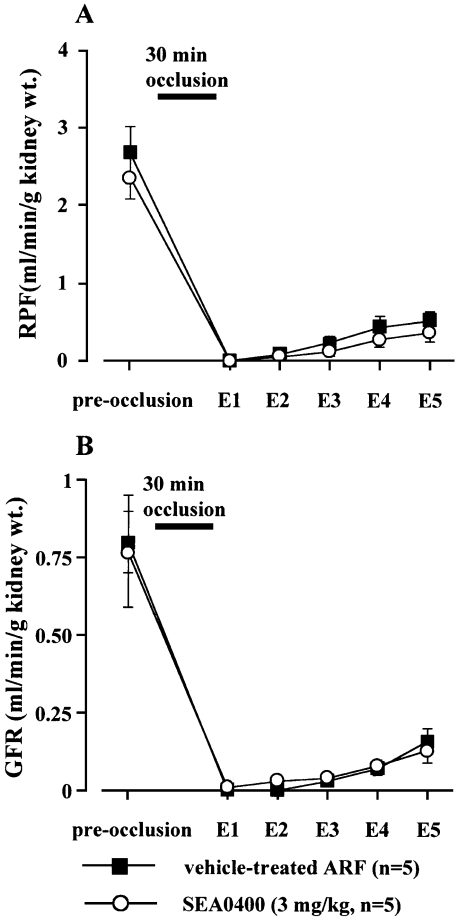

Fig. 6. Effects of SEA0400 on renal plasma flow (RPF, A) and glomerular filtration rate (GFR, B) immediately after the ischemia/reperfusion. SEA0400 was given intravenously 30 min before the ischemia. Each point and bar represents the mean ± S.E.M. ARF, acute renal failure.

3.6. Endothelin-1 levels in the kidney

To confirm the contribution of endothelin-1 to ischemic acute renal failure, we measured renal endothelin-1 levels at 2, 6 and 24 h after ischemia/reperfusion. As shown in Fig. 8, renal endothelin-1 content in vehicle-treated acute renal failure rats increased significantly at 2 h after the reperfusion. This increment was more marked at 6 h after reperfusion, and, thereafter, the increased level appeared to decrease gradually but remained higher even at 24 h after the reperfusion, compared with those in sham-operated

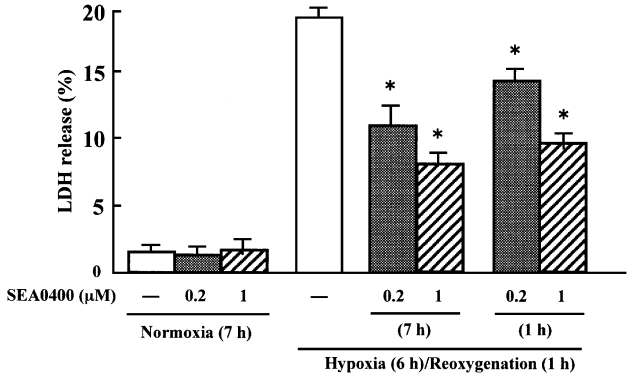

Fig. 7. Effects of SEA0400 on the LDH release induced by hypoxia/reoxygenation in LLC-PK1 cells. The cells were exposed to 7 h of normoxia or 6 h of hypoxia followed by 1 h of reoxygenation. SEA0400 was added to the culture medium at the beginning of hypoxia and/or reoxygenation. Each column and bar represents the mean ± S.E.M. from four separate experiments. $^{*}P<0.01$, compared with hypoxia and/or reoxygenation without SEA0400.

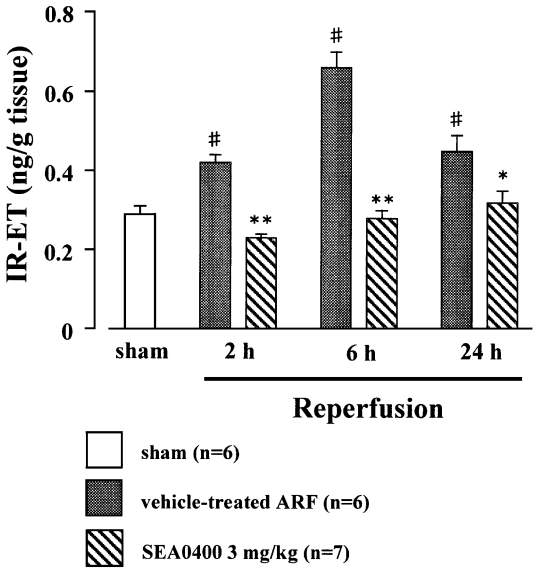

Fig. 8. Effects of SEA0400 administered before ischemia/reperfusion on immunoreactive endothelin-1 (IR-ET) content in the kidney of ARF rats at 2, 6 and 24 h after reperfusion. Each column and bar represents the mean ± S.E.M. $^{\#}P<0.01$, compared with sham rats; $^{*}P<0.05$, $^{**}P<0.01$, compared with vehicle-treated ARF rats. ARF, acute renal failure.

control rats. SEA0400 (3 mg/kg) almost completely suppressed the ischemia/reperfusion-induced increases in renal endothelin-1 contents.

4. Discussion

SEA0400 was demonstrated to have a protective effect on post-ischemic brain damage in a middle cerebral artery occlusion model (Matsuda et al., 2001). Most recently, it has been reported that SEA0400 efficiently improved myocardial ischemia/reperfusion injury, both in vivo and in vitro (Takahashi et al., 2003; Magee et al., 2003). This evidence strongly supports the view that Ca^{2+} influx via the reverse mode of Na^{+}/Ca^{2+} exchanger plays a critical role in brain and myocardial ischemic injury. On the other hand, there is little pharmacological information as to whether the Ca^{2+} influx mode of Na^{+}/Ca^{2+} exchanger is involved in the pathogenesis of ischemia/reperfusion-induced renal injury. The only available evidence is that KB-R7943 exhibits a protective effect on the ischemia/reperfusion-induced renal dysfunction and tissue injury, by pre- or post-ischemic treatment (Kuro et al., 1999; Yamashita et al., 2001). However, the specificity of KB-R7943 for Na^{+}/Ca^{2+} exchanger is now questionable. Sobolevsky and Khodorov (1999) noted that KB-R7943 blocked *N*-methyl-D-aspartate channels in isolated hippocampal neurons. Arakawa et al. (2000) found that KB-R7943 significantly inhibited the store-operated Ca^{2+} entry in cultured neurons and astrocytes. Moreover, it has become evident that KB-R7943 possesses nonspecific effects on various proteins including nicotinic acetylcholine receptor (Pintado et al., 2000). In contrast, SEA0400, at the concentration range that inhibits Na^{+}/Ca^{2+} exchanger activity, does not significantly affect ion transporters, ion channels, receptors and enzymes (Matsuda et al., 2001), thereby indicating that SEA0400 is a highly selective inhibitor of Na^{+}/Ca^{2+} exchanger. These findings led us to examine the effect of SEA0400 on the ischemia/reperfusion-induced renal injury.

In the present study, we demonstrated that the pre-ischemic treatment with SEA0400 overcame the ischemia/reperfusion-induced renal dysfunction and tissue injury. In addition, the post-ischemic treatment also showed a protective effect against the ischemia/reperfusion-induced renal dysfunction. Thus, this selective Na^{+}/Ca^{2+} exchanger inhibitor may be useful in the treatment for ischemia/reperfusion-induced acute renal failure. In a previous study, we originally found that pre- or post-ischemic treatment with KB-R7943 (10 mg/kg, i.v.) efficiently improved the post-ischemic renal injury (Yamashita et al., 2001). SEA0400 exhibited a qualitatively similar effect at relatively lower doses (1–3 mg/kg, i.v.), compared with KB-R7943. It has been reported that the potencies of SEA0400 in inhibiting the Na^{+}/Ca^{2+} exchanger activity were about 100 and 10 times of those of KR-R7943 in cultured rat cardiomyocytes and in isolated rat perfused hearts, respectively (Matsuda et al., 2001; Takahashi et al., 2003). In the 5-min ischemia/reperfusion arrhythmia model of anesthetized rats, 0.3–1 mg/kg (i.v.) of SEA0400 markedly reduced the incidence of ventricular fibrillation (Takahashi et al., 2003). In the same experimental model, KB-R7943 exerted a similar cardioprotective action by i.v. injection of 10 mg/kg, but not of 1 mg/kg (Nakamura et al., 1998). Thus, although the efficacies of SEA0400 in inhibiting Na^{+}/Ca^{2+} exchanger are different among preparations and organs, this compound seems to be a selective and potent Na^{+}/Ca^{2+} exchanger inhibitor, and may be an effective therapeutic agent for cases of the post-ischemic organ damage.

In a pilot study, SEA0400 treatment after the 45-min ischemia failed to improve significantly the ischemia/reperfusion-induced renal dysfunction. On the other hand, as previously reported (Yamashita et al., 2001), a post-ischemic treatment with KB-R7943 overcame the 45-min ischemia/reperfusion-induced renal injury. The reason for the discrepancy is unclear. However, based on the fact that SEA0400 is a highly selective and potent inhibitor of Na^{+}/Ca^{2+} exchanger, the above effect of KB-R7943 might be related to its nonspecific action other than the Na^{+}/Ca^{2+} exchanger inhibition. Alternatively, SEA0400 administered after the reperfusion may be unable to overcome the severe renal injury, although the reason is unknown. Further studies are required to clarify this problem.

There is accumulating evidence showing that the influx mode of Na^{+}/Ca^{2+} exchanger plays a crucial role in the myocardial ischemia/reperfusion injury. Overexpression of Na^{+}/Ca^{2+} exchanger gene in the heart therefore increases the susceptibility to ischemia/reperfusion-induced cardiac dysfunction (Cross et al., 1998). The above view is also

supported strongly by a recent pharmacological study by Magee et al. (2003), who found that the cardioprotective action of SEA0400 was more efficient than that of KB-R7943, in isolated rabbit heart subjected to regional ischemia and reperfusion. In the kidney, Na^+/Ca^{2+} exchanger plays an important role in transcellular Ca^{2+} reabsorption in distal and connecting nephron tubules (Reilly et al., 1993). One of the physiological roles of Na^+/Ca^{2+} exchanger in these epithelial cells is to regulate the intracellular Ca^{2+} level (Dai and Quamme, 1994). In contrast to the cases in cardiac tissues, there has been little available information on the pathological role of Na^+/Ca^{2+} exchanger in renal tissues. Most recently, we investigated the pathological role of Na^+/Ca^{2+} exchanger in ischemia/reperfusion-induced renal injury, using NCX1$^{\pm}$ heterozygous mice (Wakimoto et al., 2000), and found that ischemia/reperfusion-induced renal dysfunction, tissue damage and Ca^{2+} accumulation in necrotic tubular epithelium were observed more markedly in NCX1$^{+/+}$ wild type than in NCX1$^{\pm}$ heterozygous mice (Yamashita et al., 2003). NCX1 is the predominant isoform of Na^+/Ca^{2+} exchangers present in the heart, brain and kidney (Shigekawa and Iwamoto, 2001). In addition, when the pre-ischemic treatment with KB-R7943 was performed, the diminished renal functional parameters of both animals were improved to the same level. Taken together with findings in the present study using SEA0400, a selective and potent Na^+/Ca^{2+} exchanger inhibitor, Ca^{2+} overload via the reverse mode of Na^+/Ca^{2+} exchanger plays an important role in the pathogenesis of ischemia/reperfusion-induced renal injury.

The proximal tubule (pars recta) is one of the nephron segments that are susceptible to ischemic renal injury (Brady et al., 2000). In addition, we have recently observed that the KB-R7943-sensitive Ca^{2+} paradox-like component in cultured proximal tubular cells was greater in wild type than in NCX1$^{\pm}$ heterozygous mice (Yamashita et al., 2003). In the current study, we noted the ameliorative effect of SEA0400 on hypoxia/reoxygenation-induced injury in LLC-PK$_1$ cells, which are derived from pig kidney and have the characteristics of proximal tubules. Hypoxia/reoxygenation technique using LLC-PK$_1$ is known as an in vitro model system of ischemia/reperfusion-induced renal tubular injury (Yonehana and Gemba, 1999). SEA0400 (0.2 and 1 μM) was effective with treatment not only during hypoxia plus reoxygenation but also after hypoxia (only during reoxygenation), suggesting that Ca^{2+} influx via Na^+/Ca^{2+} exchanger during reoxygenation is more important in the hypoxia/reoxygenation-induced cell injury. This result is in accord with the present findings that both pre- and post-ischemic treatments with SEA0400 overcame the ischemia/reperfusion-induced renal injury. Taken together, Ca^{2+} influx via the reverse mode of Na^+/Ca^{2+} exchanger might occur in the proximal tubules, mainly after the reperfusion, and this event plays a crucial role in the ischemia/reperfusion-induced tubular necrosis. Based on that many clinical cases of ischemic acute renal failure cannot be predicted, a selective Na^+/Ca^{2+} exchanger inhibitor such as SEA0400 may be useful in the treatment of human ischemic acute renal failure.

In contrast to the protective effect on tubular injury, the treatment with SEA0400 failed to improve the renal hemodynamics such as total renal plasma flow and glomerular filtration rate, which were markedly decreased after the ischemia/reperfusion. This suggests that the SEA0400-induced improvement of impaired renal function and tissue injury, observed at 1 day after the ischemia/reperfusion, is not due to acute renal hemodynamic changes after the reperfusion. However, since renal hemodynamics were measured at the whole-organ level, a possibility that decreases in glomerular microcirculation and/or peritubular capillary blood flow after the ischemia/reperfusion might be attenuated by SEA0400 treatment, cannot be ruled out.

It is acknowledged that endothelin-1 is closely related to the development of the ischemia/reperfusion-induced acute renal failure. Renal endothelin-1 mRNA expression (Firth and Ratcliffe, 1992; Wilhelm et al., 1999) and tissue content of endothelin-1 peptide (Shibouta et al., 1990) are elevated in the post-ischemic kidney. Both selective endothelin ET_A and nonselective endothelin ET_A/ET_B receptor antagonists have been indicated to improve the ischemia/reperfusion-induced renal dysfunction (Gellai et al., 1995; Birck et al., 1998; Kuro et al., 2000). The inhibition of endothelin-1 biosynthesis by the treatment with endothelin-converting enzyme inhibitor also markedly ameliorates the renal injury induced by ischemia/reperfusion (Vemulapalli et al., 1993; Matsumura et al., 2000). These findings suggest that the up-regulation of renal endothelin-1 production and its ET_A receptor-mediated action contribute to the pathogenesis of ischemic acute renal failure. In our recent study, there was an only moderate increment of endothelin-1 content in the post-ischemic kidney of NCX1$^{\pm}$ heterozygous mice, compared with the case in NCX1$^{+/+}$ wild-type mice, thereby suggesting the possible involvement of NCX1-mediated Ca^{2+} overload for endothelin-1 overproduction in the kidney subjected to ischemia/reperfusion (Yamashita et al., 2003). In immunohistochemical study, an enhanced staining was observed in necrotic tubular cells more markedly in the wild-type mice than in the heterozygous mice (Yamashita et al., 2003). In this study, SEA0400 abolished completely the increases in renal endothelin-1 content at 2, 6 and 24 h after reperfusion. Taken together, it is reasonable to consider that Ca^{2+} overload via the reverse mode of Na^+/Ca^{2+} exchanger, followed by renal endothelin-1 overproduction, is involved in the pathogenesis of ischemia/reperfusion-induced renal injury. In vascular endothelial cells, an increase of calcium entry has been known to induce the expression of endothelin-1 gene (Rubanyi and Polokoff, 1994). Furthermore, the increase in intracellular Ca^{2+} concentration via the reverse mode of Na^+/Ca^{2+} exchanger is functional in vascular endothelial cells (Schneider et al., 2002). Since endothelin-1 gene expression and the peptide production are up-

regulated under the hypoxic condition, both in tubular cells and endothelial cells (Kourembanas et al., 1991; Ong et al., 1995), it remains to be determined where endothelin-1 overproduction following the ischemia/reperfusion and Ca^{2+} overload via the reverse mode of Na^+/Ca^{2+} exchanger occurs (in vascular endothelium, tubular cells or possibly in both). One available evidence is that endothelin-1 is first expressed in increased quantities in the peritubular capillary network shortly after the onset of renal ischemia and then transported across the basement membrane of the adjacent tubular epithelial cell, which are then sloughed off during the development of acute tubular necrosis (Wilhelm et al., 2001).

In summary, we found that both pre- and post-ischemic treatments with SEA0400, a selective and potent Na^+/Ca^{2+} exchanger inhibitor, exerted an efficient protective effect against the ischemia/reperfusion-induced renal dysfunction and tissue injury, in vivo. We also noted that SEA0400 markedly suppressed the hypoxia/reoxygenation-induced cell injury in LLC-PK_1 cells. SEA0400-induced improvement of impaired renal function and tissue injury after the ischemia/reperfusion does not seem to be due to acute renal hemodynamic changes after the reperfusion. In addition, SEA0400 suppressed the renal endothelin-1 overproduction observed after the reperfusion. We conclude that Ca^{2+} overload via the reverse mode of Na^+/Ca^{2+} exchanger, following by endothelin-1 overproduction, plays an important role in the pathogenesis of ischemia/reperfusion-induced renal injury. The possibility exists that a selective Na^+/Ca^{2+} exchange inhibitor such as SEA0400 is useful as effective therapeutic agent against ischemic acute renal failure in humans.

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