Attenuation of Ischemia/Reperfusion-Induced Renal Injury in Mice Deficient in Na\(^+\)/Ca\(^{2+}\) Exchanger

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ABSTRACT

Using Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1)-deficient mice, the pathophysiological role of Ca\(^{2+}\) overload via the reverse mode of NCX1 in ischemia/reperfusion-induced renal injury was investigated. Because NCX1\(^{-/-}\) homozygous mice die of heart failure before birth, we used NCX1\(^{+/+}\) heterozygous mice. NCX1 protein in the kidney of heterozygous mice decreased to about half of that of wild-type mice. Expression of NCX1 protein in the tubular epithelial cells and Ca\(^{2+}\) influx via NCX1 in renal tubules were markedly attenuated in the heterozygous mice. Ischemia/reperfusion-induced renal dysfunction in heterozygous mice was significantly attenuated compared with cases in wild-type mice. Histological renal damage such as tubular necrosis and proteinaceous casts in tubuli in heterozygous mice were much less than that in wild-type mice. Ca\(^{2+}\) deposition in necrotic tubular epithelium was observed more markedly in wild-type than in heterozygous mice. Increases in renal endothelin-1 content were greater in wild-type than in heterozygous mice, and this reflected the difference in immunohistochemical endothelin-1 localization in necrotic tubular epithelium. When the preischemic treatment with KB-R7943 was performed, the renal functional parameters of both NCX1\(^{+/+}\) and NCX1\(^{+/+}\) acute renal failure mice were improved to the same level. These findings strongly support the view that Ca\(^{2+}\) overload via the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange, followed by renal endothelin-1 overproduction, plays an important role in the pathogenesis of ischemia/reperfusion-induced renal injury.

Renal ischemia is characterized by the depletion of ATP and the development of intracellular acidosis, which alter cellular ionic homeostasis. In particular, elevated intracellular Ca\(^{2+}\) concentration causes cellular injury during ischemia and leads to irreversible renal damage during reperfusion (Schrier et al., 1987). An increase in the intracellular Na\(^+\) concentration has been shown to correlate with Ca\(^{2+}\) overload. The accumulation of intracellular Na\(^+\) concentration, which is caused by inhibition of the Na\(^+\)/K\(^+\) ATPase activity because of decreased ATP production (Cross et al., 1995) and activation of the Na\(^+\)/H\(^+\) exchange because of intracellular acidosis (Scholz et al., 1993), has been shown to activate the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1) and subsequently to cause Ca\(^{2+}\) overload. Therefore, the NCX1 plays a crucial role in cellular injury during ischemia and in cell death during reperfusion. In the last decade, the NCX1 has been cloned and the structure/function relationship intensively studied. In addition, many investigators have studied the pathophysiological significance of NCX1 in the abnormality of the circulatory system (Philipson and Nicoll, 2000).

The role of NCX1 in ischemia/reperfusion injury has been demonstrated using the selective NCX1 inhibitor KB-R7943. This compound has been reported to be a selective and potent inhibitor of the Ca\(^{2+}\) influx mode of Na\(^+\)/Ca\(^{2+}\) exchange in cardiomyocytes, smooth muscle cells, and NCX1-transfected fibroblasts (Iwamoto et al., 1996). Similar inhibitory effects of KB-R7943 on the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange were observed in cardiomyocytes, smooth muscle cells, and NCX1-transfected fibroblasts (Iwamoto et al., 1996). Simi...
Affinity for ET receptors are elevated in the postischemic period that renal ET-1 mRNA expression, ET-1 content, and its production and its ETA receptor-mediated actions are closely related to the pathogenesis of ischemic ARF (Yamashita et al., 2001).

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide (Yanagisawa et al., 1988) that has been implicated as a mediator of cardiac, vascular, and renal diseases associated with regional and systemic vasoconstriction (Rubanyi and Polokoff, 1994). This peptide is produced in various tissues, including endothelial cells, smooth muscle cells, and renal tubular epithelial cells and acts through activation of G protein-coupled ETA and ETB receptors (Rubanyi and Polokoff, 1994). A potential contribution of ET-1 to the pathology of ischemic ARF has been suggested based on findings indicating that renal ET-1 mRNA expression, ET-1 content, and its affinity for ET receptors are elevated in the postischemic period that renal ET-1 mRNA expression, ET-1 content, and its production and its ETA receptor-mediated actions are closely related to the pathogenesis of ischemic ARF (Gellai et al., 1995; Kuro et al., 2000; Matsumura et al., 1998). In the kidney, we first demonstrated the protective effects of KB-R7943 on ischemia/reperfusion-induced acute renal failure (ARF), and therefore suggested that Ca2+ overload via the reverse mode of NCX1 plays an important role in the pathogenesis of this renal disease (Yamashita et al., 2001).

Therefore, we used NCX1-selective or nonselective ETA/ETB-receptor antagonists and ET-converting enzyme inhibitors are known to attenuate the ischemia/reperfusion-induced impairment of renal function (Gellai et al., 1995; Kuro et al., 2000; Matsumura et al., 2000). Taken together, it seems likely that renal ET-1 overproduction and its ETA-receptor-mediated actions are closely related to the pathogenesis of ischemic ARF.

The purpose of this study was to determine the pathological role of Na+/Ca2+ exchange in the ischemia/reperfusion-induced ARF, using recently produced NCX1-deficient mice (Wakimoto et al., 2000). Homozygous NCX1-deficient mice (NCX1−/−) died between embryonic days 9 and 10 (Wakimoto et al., 2000). Their hearts did not beat and cardiac myocytes showed apoptosis. Therefore, we used NCX1−/− heterozygous mice, which were subjected to the renal ischemia followed by reperfusion, and impairment of renal function, histological damage, and changes in renal ET-1 content were compared with those in NCX1−/− wild-type mice. We report here that NCX1−/− heterozygous mice exhibit an attenuated ischemia/reperfusion-induced renal dysfunction and cell injury, and a lowered ET-1 overproduction in the postischemic kidney, indicating that the Na+/Ca2+ exchange mechanism and renal ET-1 system play an important role in the pathogenesis of postischemic ARF.

Materials and Methods

Animals. The generation of the NCX1-knockout mice has been described in detail previously (Wakimoto et al., 2000). Briefly, we cloned the NCX1 gene from a 129/SV mouse genomic library. The targeting vector was constructed by insertion of the neo cassette into the 3-kilobase pair XhoI/Xhol fragment containing exon 2 of NCX1 gene. The diphtheria toxin-A fragment gene was ligated to the 3′ position of the targeting vector for negative selection. The A3-1 embryonic stem cell line was transfected with the linearized targeting vector by electroporation. After G418 selection, homologous recombinants were identified by polymerase chain reaction and confirmed by Southern blot hybridization. Targeted embryonic stem cells were aggregated with eight cells from C57BL/6J (B6) mice, and chimeric blastocysts were implanted into the uterus of pseudopregnant ICR mice. Chimeric male mice were then mated to female B6 mice to confirm the germline transmission.

Surgery and Experimental Design. Male B6 mice (NCX1−/− and NCX1+/− mice; 15–20 g) 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, the right kidney was removed through a small flank incision made after pentobarbital anesthesia (50 mg/kg i.p.). After a 2-week recovery period, to induce ischemic ARF, these mice 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 for 45 min with a nontraumatic clamp. At the end of the ischemic period, the clamp was removed and blood reperfused. In some animals, KB-R7943 (10 mg/kg) or its vehicle (a mixture of 15% ethanol, 15% polyethylene glycol 400, and 70% saline) was administered as a slow bolus injection at 1 ml/kg into the external jugular vein, 5 min before the occlusion.

In sham-operated control animals, the left kidney was treated identically, except for clamping. Animals exposed to 45-min ischemia were housed in metabolic cages at 24 h after reperfusion; 24-h urine samples were taken and blood samples were drawn from the aorta at the end of 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.

In separate experiments, left kidneys were obtained 24 h after reperfusion to determine NCX1 protein expression and ET-1 content. Western Blotting. Tissue homogenate preparation, SDS-polyacrylamide gel electrophoresis, and immunoblotting were performed as described previously (Yamashita et al., 2001). Immunoblot analysis was performed with anti-NCX1 polyclonal antibody at 1:300 dilution with PBS (Iwamoto et al., 1998). Protein was measured with the bicinchoninic acid assay reagent (Pierce Chemical, Rockford, IL). The immunoblots were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences, Inc., Piscataway, NJ).

Blood and Urine Measurements. Blood urea nitrogen (BUN) and creatinine levels in plasma and urine were determined using commercial kits, the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemicals, Osaka, Japan), respectively. Urinary osmolality (Uosm) was measured by freezing point depression (Fiske, MA). Urine and plasma sodium concentrations were determined by flame photometer (205D; Hitachi, Hitachinaka, Japan). Fractional excretion of sodium (FENa, %) was calculated from the formula FENa = UNaV/Ucr × 100, where UNaV is urinary excretion of sodium, PNa is the plasma sodium concentration, and Ucr is creatinine clearance.

Histological Studies. Histological studies were done as described previously (Yamashita et al., 2001). Histopathological changes were analyzed for tubular necrosis and proteinaceous casts, as suggested by Solez et al. (1974). Tubular necrosis and proteinaceous casts were graded as follows: no damage (0 or 0), mild (≥ 1, unicellular, patchy isolated damage), moderate (+ or 2, damage less than 25%), severe (++) or 3, damage between 25 and 50%), and very severe (+++) or 4, more than 50% damage). Evaluations were made in a blind manner.

Using von Kossa method, the amount of black reaction products deposition in necrotic tubular epithelium was also determined by microscopic observation.

Primary Culture of Proximal and Distal Tubular Cells. Proximal and distal tubular cells were prepared from NCX1−/− and NCX1−/− mice with a modification of methods described previously (Gesek et al., 1987). Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and the left kidney was perfused with ice-cold modified Krebs-Henseleit buffer (KHB) through the thoracic aorta after ligation of the aorta and vena cava above the renal
vessels. Modified KHB contains the following: 118 mM NaCl, 4.0 mM KCl, 1.0 mM KH₂PO₄, 27.2 mM NaHCO₃, 1.25 mM CaCl₂, 1.20 mM MgCl₂, 5.0 mM glucose, and 10 mM HEPES. The kidney was removed and the cortex was cut into 1-mm-thick slices, bagged in a Pack Pouch (Mitsubishi Bas Chemical Co., Inc., Tokyo, Japan), in and the cells were exposed to the hypoxic condition using an Anaero culture medium was changed to DMEM without glucose and serum.

When the cells cultured in 24-well plates became confluent, the treated for 10 min before the repletion of [Ca²⁺/H9262]. The complex was visualized with 3,3-diaminobenzidine secondary antibody (Nichirei, Tokyo, Japan) at 37°C in atmosphere of 95% O₂/5% CO₂. The slices were then washed with KHB and transferred to an ice-cold solution. Nephron segments were isolated from the cortex region under microscope. Proximal tubules (segments 1–3) just after the glomerulus and distal convoluted tubules just after the thick ascending limb were excised. These isolated tubules were then explanted for 4 to 5 days on 35-mm dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Measurement of [Ca²⁺/H9262] in Proximal and Distal Tubular Cells. [Ca²⁺/H9262] was monitored using Fluo-3-acetoxymethyl ester as a fluorescent Ca²⁺ indicator. Cells in 35-mm dishes were loaded with 4 μM Fluo-3-acetoxymethyl ester for 40 min at 37°C in 1 ml of balanced salt solution (BSS; 10 mM HEPES-Tris (pH 7.4), 146 mM NaCl, 4 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, and 10 mM glucose). Loaded cells were then washed twice with BSS. Cells were exposed to Ca²⁺/H9262-free BSS containing 0.2 mM EGTA for 10 min and then to BSS containing 2 mM Ca²⁺/H9262 KB-R7943 (10 μM) was pretreated for 10 min before the repletion of [Ca²⁺/H9262]. Fluorescence signals from single tubular cells with excitation at 488 nm were monitored by confocal laser scanning microscope system (MRC1024; Bio-Rad, Hercules, CA). The fluorescence intensity of individual cells (F) was normalized to that (F₀) before adding 2 mM Ca²⁺/H9262.

Renal ET-1 Assay. ET-1 was extracted from the kidney, as described elsewhere (Fujita et al., 1995). Briefly, kidneys were weighed and homogenized for 60 s in 8 ml of ice-cold organic solution (chlo-roform/methanol, 2:1, including 1 mM N-ethylmaleimide). The homogenates were left overnight at 4°C and then 0.4 ml of distilled water was added after which the homogenates were centrifuged at 1500g for 30 min and the resultant supernatant was stored. Aliquots of the supernatant were diluted 1/10 with a 0.09% trifluoroacetic acid solution and applied to Sep-Pak C18 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 (RIA). The clear solution was subjected to RIA. The recovery of ET-1 was 80%. RIA for tissue ET-1 was done, as described previously (Matsumura et al., 1990b).

Immunohistochemistry. Excised left kidneys were preserved in phosphate-buffered 10% formalin, after which the kidneys were chopped into small pieces, embedded in paraffin wax, and cut at 3 μm. Tissue sections were incubated for 30 min at 37°C with anti-ET-1 polyclonal antibody (Peptide Institute, Inc., Osaka, Japan) or with anti-NCX1 polyclonal antibody (Iwamoto et al., 1998) at 1:2000 and 1:300 dilution with PBS, respectively. After washing with PBS, the sections were further incubated with goat anti-rabbit biotinylated secondary antibody (Nichirei, Tokyo, Japan) at 37°C for 10 min and then the streptavidin-horseradish peroxidase (Nichirei) was applied for 5 min. The complex was visualized with 3,3-diaminobenzidine.

Hypoxia and Reoxygenation in LLC-PK₁. LLC-PK₁ (American Type Culture Collection, Manassas, VA), a porcine kidney cell line, was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 50 μg/ml streptomycin, and 50 U/ml penicillin at 37°C in a CO₂ incubator (95% air, 5% CO₂). 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 Bas Chemical Co., Inc., 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 CO₂ 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 (Wako Pure Chemicals). KB-R7943 (10 μM) was added to the medium at the beginning of hypoxia and/or reoxygenation. LDH release was expressed as a percentage of total cellular LDH activity.

Statistical Analysis. Values are mean ± S.E.M. For statistical analysis, we used one-way analysis of variance followed by Bonferroni’s or Dunnett’s multiple comparison tests. Histological data 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.

Results

Expression and Localization of NCX1 Protein. To justify the use of NCX1<sup>+/−</sup> heterozygous and NCX1<sup>+/−</sup> wild-type mice, NCX1 protein expression in the kidney of these animals was examined. As shown in Fig. 1, NCX1 protein level in renal tissues of NCX1<sup>+/−</sup> mice was about half of that seen in NCX1<sup>+/−</sup> mice. On the other hand, protein levels of Na⁺/K⁺-ATPase, sarcoplasmic reticulum Ca²⁺-ATPase (type 2) and L-type voltage-dependent Ca²⁺ channel did not differ between NCX1<sup>+/−</sup> and NCX1<sup>+/−</sup> mice (data not shown). In addition, an immunohistochemical study clearly indicated that a staining for NCX1 protein expression was much more intense in tubular epithelial cells of renal cortex of NCX1<sup>+/−</sup> wild-type mice than in those of NCX1<sup>+/−</sup> mice (Fig. 2).

[Ca²⁺], Rise Evoked by the Reverse Mode of Na⁺/Ca²⁺ Exchange in Cultured Renal Tubular Cells. [Ca²⁺]<sub>120 KDa</sub> reple-

![Fig. 1. NCX1 protein expression in renal tissues of NCX1<sup>+/−</sup> wild-type and NCX1<sup>+/−</sup> heterozygous mice. Each column and bar represents the mean ± S.E.M. (n = 6). *, P < 0.01, compared with wild-type mice.](image-url)
tion after a period of [Ca\(^{2+}\)]\(_i\) depletion is known to cause Ca\(^{2+}\) overloading via Na\(^+\)/Ca\(^{2+}\) exchange in cardiomyocytes or neuronal cells, a process called the Ca\(^{2+}\) paradox (Chapman and Tunstall, 1987). To assess the functional difference of Na\(^+\)/H\(^+\) exchange in renal tubules between NCX1\(^{-/-}\) and NCX1\(^{+/+}\) mice, we examined [Ca\(^{2+}\)]\(_i\) rise evoked by the Ca\(^{2+}\) paradox in cultured renal tubular cells using Ca\(^{2+}\) indicator Fluo-3. When distal tubular cells of NCX1\(^{-/-}\) mice were exposed with a Ca\(^{2+}\), Mg\(^{2+}\)-free buffer for 10 min and then placed in a buffer containing 2 mM Ca\(^{2+}\), [Ca\(^{2+}\)]\(_i\) markedly increased. In the proximal tubular cells, [Ca\(^{2+}\)]\(_i\) rise was also observed, although being significantly smaller than that in distal tubular cells (Fig. 3). These [Ca\(^{2+}\)]\(_i\) responses were blocked over 90% by pretreatment with KB-R7943 (10 \(\mu\)M), an inhibitor for the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange, but not significantly affected by verapamil (10 \(\mu\)M), L-type Ca\(^{2+}\) channel blocker, or SK&F96365 (50 \(\mu\)M), a blocker of store-operated Ca\(^{2+}\) channels (data not shown). In NCX1\(^{-/-}\) mice, on the other hand, the [Ca\(^{2+}\)]\(_i\) elevations in both tubular cells induced by 2 mM Ca\(^{2+}\) were markedly down-regulated compared with the case of NCX1\(^{+/+}\) mice.

**Renal Function after Ischemia/Reperfusion.** As shown in Fig. 4, renal functional parameters of mice subjected to 45-min ischemia showed a marked deterioration, as measured 48 h after reperfusion. Compared with each sham-operated control animals, both NCX1\(^{-/-}\) and NCX1\(^{+/+}\) mice exhibited increases in BUN, plasma creatinine concentration (Per), urine flow (UF) and FENa, and decreases in Ccr, and Uosm. However, ischemia/reperfusion-induced changes in renal functional parameters of NCX1\(^{-/-}\) mice were considerably small, compared with cases in NCX1\(^{-/-}\) mice (BUN: NCX1\(^{-/-}\), 78.0 \(\pm\) 8.8 versus NCX1\(^{+/+}\), 142.6 \(\pm\) 7.4 mg/dl; Per, NCX1\(^{-/-}\), 0.80 \(\pm\) 0.10 versus NCX1\(^{+/+}\), 1.34 \(\pm\) 0.05 mg/dl; Ccr, NCX1\(^{-/-}\), 2.15 \(\pm\) 0.23 versus NCX1\(^{+/+}\), 0.95 \(\pm\) 0.13 ml min\(^{-1}\) kg\(^{-1}\); UF, NCX1\(^{-/-}\), 88.1 \(\pm\) 10.4 versus NCX1\(^{+/+}\), 102.6 \(\pm\) 10.7 \(\mu\)l min\(^{-1}\) kg\(^{-1}\); Uosm, NCX1\(^{-/-}\), 663 \(\pm\) 73 versus NCX1\(^{+/+}\), 501 \(\pm\) 18 mOsM/kg; FENa, NCX1\(^{-/-}\), 1.05 \(\pm\) 0.16 versus NCX1\(^{+/+}\), 1.93 \(\pm\) 0.12%). On the other hand, there were no significant differences in renal functional parameters between NCX1\(^{-/-}\) and NCX1\(^{+/+}\) sham-operated control mice.

**Histological Renal Damage after Ischemia/Reperfusion.** Histological examination revealed severe lesions in the kidney of NCX1\(^{-/-}\) mice (48 h after the ischemia/reperfusion). These changes were characterized by tubular necrosis (Fig. 5b, outer zone outer stripe of medulla) and proteinaceous casts in tubuli (Fig. 5f, inner zone of medulla). In NCX1\(^{+/+}\) mice, histologically evident damage was significantly less than that seen in NCX1\(^{-/-}\) mice (Figs. 5, d and h; Table 1).

**Ca\(^{2+}\) Deposition after Ischemia/Reperfusion.** Figure 6 shows light micrographs of Ca\(^{2+}\) deposition demonstrated by von Kossa method in the kidney subjected to 45-min ischemia followed by reperfusion. Ca\(^{2+}\) deposition in medullary tubular epithelium of kidney of NCX1\(^{-/-}\) mice was more evident compared with the case of NCX1\(^{+/+}\) mice.

**Effects of KB-R7943 on the Ischemia/Reperfusion-Induced Renal Dysfunction.** To further evaluate the possible involvement of NCX1 in the ischemia/reperfusion-induced renal injury, the effect of pharmacological blockade of NCX1 was examined. As shown in Fig. 7, preischemic treatment with KB-R7943 improved the renal functional parameters of both NCX1\(^{-/-}\) and NCX1\(^{+/+}\) ARF mice to the same level.

**Effects of KB-R7943 on the Hypoxia/Reoxygenation-Induced Injury in LLC-PK1.** LLC-PK1 cells, derived from pig kidney, have characteristics of proximal tubules. We evaluated the effect of KB-R7943 on the hypoxia/reoxygenation-induced cell injury in LLC-PK1. Hypoxia/reoxygenation technique is known as in vitro model system of ischemia/reperfusion-induced renal injury. As shown in Fig. 8, an enhanced LDH release from the cells exposed to hypoxia followed by reoxygenation was markedly suppressed by the treatment with KB-R7943 during the hypoxia. Similar suppressive effect of KB-R7943 was also observed by the addition at the beginning of reoxygenation.

**Renal ET-1 Content after Ischemia/Reperfusion.** To confirm the contribution of ET-1 to ischemia/reperfusion-induced renal injury both in NCX1\(^{-/-}\) and NCX1\(^{+/+}\) mice, we measured renal ET-1 content at 24 h after reperfusion. As shown in Fig. 9, renal ET-1 content was significantly increased by the ischemia/reperfusion, both in NCX1\(^{-/-}\) and NCX1\(^{+/+}\) mice, compared with that seen in each sham mice. However, ischemia/reperfusion-induced changes in renal ET-1 content of NCX1\(^{-/-}\) mice were considerably small, compared with cases in NCX1\(^{+/+}\) mice (NCX1\(^{-/-}\), 0.71 \(\pm\) 0.06 versus NCX1\(^{+/+}\), 1.26 \(\pm\) 0.23 ng/g tissue).

**Immunohistochemical Analysis.** To determine the localization of renal ET-1 peptide expression after the ischemia/reperfusion, an immunohistochemical study was done. As clearly indicated in Fig. 10, a staining for ET-1 peptide expression was intense in tubular lumen containing necrotic cells, and it was more prominent in NCX1\(^{-/-}\) than in NCX1\(^{+/+}\) mice.
Discussion

We investigated the pathological role of NCX1 in ischemia/reperfusion-induced renal injury using NCX1-knockout mice. Because NCX1<sup>−/−</sup> homozygous mice die of heart failure before birth (Wakimoto et al., 2000), we used NCX1<sup>−/+</sup> heterozygous mice, in which NCX1 protein expression in renal tissues was decreased to about half of those of NCX1<sup>+/+</sup> wild-type mice. Furthermore, expression of NCX1 protein in the tubular epithelial cells and Na<sup>+</sup>/Ca<sup>2+</sup> exchange activity of renal tubules were markedly attenuated in the heterozygous mice, thereby indicating the usefulness of these mice in examining the renal pathophysiological role of NCX1.

In the present study, the ischemia/reperfusion-induced renal dysfunction and histological damage was moderate in NCX1<sup>−/+</sup> mice compared with cases in NCX1<sup>+/+</sup> mice. Hist-

![Fig. 3. [Ca<sup>2+</sup>], elevations evoked by the Ca<sup>2+</sup> paradox in cultured proximal and distal tubular cells from NCX1<sup>−/−</sup> wild-type and NCX1<sup>−/+</sup> heterozygous mice. Fluo-3-loaded cells were exposed to a Ca<sup>2+</sup>, Mg<sup>2+</sup>-free BSS containing 0.2 mM EGTA for 10 min and then to BSS containing 2 mM Ca<sup>2+</sup>, with or without 10 μM KB-R7943. KB-R7943 was pretreated for 10 min before the repletion of Ca<sup>2+</sup>. The fluorescence intensity of individual cells was normalized to that (F<sub>0</sub>) before adding 2 mM Ca<sup>2+</sup>. Each point and bar represents the mean ± S.E.M.](image-url)
Fig. 4. Renal functional parameters of NCX1<sup>−/−</sup> wild-type and NCX1<sup>−/−</sup> heterozygous mice, with or without ARF. BUN (a), Pcr (b), Ccr (c), UF (d); Uosm (e), and FENa (f). Each column and bar represents the mean ± S.E.M. #, P < 0.01, compared with wild-type sham mice; †, P < 0.01, compared with heterozygous sham mice; *, P < 0.01, compared with wild-type ARF mice.

Fig. 5. Light microscopy of outer zone outer stripe of medulla (a–d) and inner zone of medulla (e–h) of the kidney of NCX1<sup>−/−</sup> wild-type and NCX1<sup>−/−</sup> heterozygous mice, with or without ARF. Arrows indicate tubular necrosis (b and d) and proteinaceous casts in tubuli (f and h) (hematoxyline and eosin staining).
TABLE 1
Histopathological changes in kidneys of wild-type and heterozygous mice, with or without ARF

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<th>Wild-Type</th>
<th>Heterozygous</th>
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<tr>
<td></td>
<td>Sham Mice (n = 5)</td>
<td>ARF Mice (n = 6)</td>
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<tr>
<td>Histopathological changes/grade</td>
<td>(0 1 2 3 4)</td>
<td>(0 1 2 3 4)</td>
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<tr>
<td>Tubular necrosis</td>
<td>5 0 0 0 2</td>
<td>5 0 0 0 2</td>
</tr>
<tr>
<td>Protein casts</td>
<td>5 0 0 0 2</td>
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Data are expressed as the number of animals with histopathological changes. Values in parentheses represent the mean ± S.E.M. of histopathological change/grade. Grades: no changes (< 0), mild (1 ≤ 2), moderate (3 ≤ 4), severe (5 ≤ 6), very severe (7 ≤ 8).

ARF, acute renal failure.
* P < 0.01, compared with wild-type sham mice.
+ P < 0.01, compared with heterozygous sham mice.
- P < 0.01, compared with wild-type ARF mice.

Fig. 6. Light microscopy of Ca" transfer into medulla of the kidney of NCX1+/- wild-type and NCX1+/- heterozygous mice, with or without ARF. Ca" deposition in medullary tubular epithelium of kidney of NCX1+/- ARF mice (b) was more evident compared with the case of NCX1+/- ARF mice (d) (von Kossa staining).

![sham: wild-type](a) ARF: wild-type](b)

![sham: heterozygous](c) ARF: heterozygous](d)

chemically visualized Ca" deposition in medullary tubular epithelium of postischemic kidney of NCX1+/- mice was less evident than that seen in NCX1+/- mice. On the other hand, pharmacological blockade of NCX1 with KB-R7943 improved the renal dysfunction observed in both NCX1+/- and NCX1+/- ARF mice to the same level. An increment of ET-1 content in postischemic kidney of NCX1+/- mice was also less than that observed in NCX1+/- mice, and this difference reflected an immunohistochemical localization of ET-1 in tubular lumen-containing necrotic cells. These findings suggest that Ca" overload via the reverse mode of NCX1, followed by renal ET-1 overproduction, plays an important role in the pathogenesis of ischemia/reperfusion-induced renal injury.

In normal cardiac cells, NCX1 extrudes Ca" from sarcoplasm to maintain the intracellular Ca" concentration at the diastolic level. In contrast, in ischemic cardiac cells where intracellular pH decreases, the intracellular Na" concentration rises through the Na"/H" exchange system, which in turn increases the intracellular Ca" concentration through the Na"/Ca" exchange system (Dennis et al., 1990). The Ca" overload via this system seems to contribute to the ischemia/reperfusion injury in the heart (Tani and Neely, 1989; Cross et al., 1998). This view may be applicable to the case of the postischemic ARF. Although the pathological mechanisms of Ca" overload in ischemic kidney have not been fully elucidated, there is substantial evidence indicating that increased cytosolic Ca" may be an important mediator of epithelial cell necrosis, which is a characteristic of ischemic ARF and that Ca" overload is a primary factor in certain types of cell injury (Wilson et al., 1984). In addition, a preischemic treatment with Ca" channel blockers has been known to exert a protective effect against the ischemia/reperfusion-induced renal injury (Goldfarb et al., 1983; Yamashita et al., 2001). Most recently, we found that KB-R7943, a selective and potent inhibitor of the Ca" influx mode of Na"/Ca" exchange (Iwamoto et al., 1996; Watano et al., 1996), efficiently attenuated the ischemia/reperfusion-induced renal injury in both cases of pre- and postischemic treatments, thereby suggesting that Ca" overload via the reverse mode of the Na"/Ca" exchange is a crucial factor in the pathology of postischemic renal insufficiency, and that an inhibitor of NCX1 may be an beneficial therapeutic agent for the postischemic ARF (Yamashita et al., 2001).

In the present study, using NCX1+/- heterozygous mice we confirmed the pathophysiological importance of Ca" handling via NCX1 in the ischemia/reperfusion-induced renal injury. The level of NCX1 protein expression in renal tissues of the NCX1+/- mice was about half of that seen in NCX1+/- wild-type mice. Ca" influx via Na"/Ca" exchange, which is abolished by a selective NCX1 inhibitor KB-R7943, in proximal and distal tubular cells were much less potent in NCX1+/- mice than in NCX1+/- mice. In addition, we obtained the evidences that protein levels of Na"/K"-ATPase, sarcoplasmic reticulum Ca"-ATPase, and L-type voltage-dependent Ca" channel did not differ between NCX1+/- and NCX1+/- mice (T. Iwamoto, unpublished data). These findings seem to justify the usefulness of these animals in renal pathophysiological study. However, an attenuation of ischemia/reperfusion-induced renal injury observed in NCX1+/- mice was only partial. Thus, to elucidate more precisely the pathophysiological role of NCX1 in the postischemic ARF, further studies using NCX1+/- homozygous mice are needed. To attain this, adult NCX1+/- mice should be produced by the tissue (heart)-specific transgenic rescue. Furthermore, the rescued adult NCX1+/- mice may provide new information on the physiological role of NCX1 in regulatory mechanisms of renal function, although we observed no significant
differences in renal function between NCX1+/− and NCX1+/− sham-operated control mice (Fig. 4). Alternatively, transgene technique using kidney-specific promotor to overexpress NCX1 protein and/or a conditional knockout technique would be also useful.

There is growing evidence that ET-1 is closely related to the development of the ischemic ARF. It has been demonstrated that ET-1 content (Shibouta et al., 1990; Matsumura et al., 2000) and ET-1 mRNA expression (Firth and Ratcliffe, 1992; Wilhelm et al., 1999) are elevated in renal tissues after ischemia/reperfusion. Our previous study has shown that daily oral administration of the ETA-selective antagonist ABT-627, but not the ET B-selective antagonist A-192621, had a beneficial effect on ischemia/reperfusion-induced renal...
under the hypoxic condition, both in tubular cells and endo-
genous expression and the peptide production are up-regulated.
Overproduction of ET-1 in the ischemic kidney occurs in vas-
tubular necrosis. However, it remains obscure whether an
which are then sloughed off during the development of acute
the onset of renal ischemia and then transported across the
quantities in the peritubular capillary network shortly after
they hypothesized that ET-1 is first expressed in increased
expressed at this site causes ongoing vasoconstriction in the
ing tubules, and leads to tubular necrosis. Taken together,
markedly in NCX1
mice, compared with the case in NCX1
mice. When the
heterozygous mice, with or without ARF. Arrows indicate endothelin-1 peptide
expression in lumens of necrotic tubular cells. The peptide expression was
more intense in NCX1
(b) than in NCX1
mice (d).

dysfunction and tissue injury (Kuro et al., 2000). In addition,
an ET-converting enzyme inhibitor, phosphoramidon (Mat-
sumura et al., 1990a), was found to overcome ischemia/reper-
fusion-induced renal injury (Vemulapalli et al., 1993; Mat-
sumura et al., 2000). Taken together, it seems likely that the
up-regulation of renal ET-1 production and ET
receptor-
mediated actions are responsible for the pathogenesis of isch-
emic ARF. In the present study, there was an only moderate
increment of ET-1 content in the postischemic kidney of
NCX1
mice, compared with the case in NCX1
mice. In immuno-
histochemical study to determine the localization of ET-1 peptide in the postischemic kidney, an enhanced staining
pattern was observed in necrotic tubular cells more
markedly in NCX1
mice than in NCX1
mice. When the
present study was in progress, similar localization pattern of
ET-1 peptide in the kidney after the ischemia and 24 h of
reperfusion was demonstrated by Wilhelm et al. (2001). Pre-
viously, Wilhelm et al. (1999) demonstrated increased ET-1
expression in the peritubular capillary network of the kidney
after ischemia. Because this capillary bed is an extension of the
efferent arteriole of the glomerulus and represents the
primary blood supply of the tubules, it seems likely that ET-1
expressed at this site causes ongoing vasoconstriction in the
peritubular capillary network and hypoxia in the neighbor-
tubules, and leads to tubular necrosis. Taken together,
they hypothesized that ET-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 cells,
which are then sloughed off during the development of acute
tubular necrosis. However, it remains obscure whether an
overproduction of ET-1 in the ischemic kidney occurs in vas-
cular endothelium, in tubular cells or in both, because ET-1
gene expression and the peptide production are up-regulated
under the hypoxic condition, both in tubular cells and endo-
thelial cells (Kourembanas et al., 1991; Ong et al., 1995).

One possible candidate for factors causing ET-1 overpro-
duction in the kidney exposed to the ischemia/reperfusion
may be an intracellular Ca
accumulation, which is an
important mediator for the pathogenesis of ischemia/reper-
fusion injury of the kidney (Schrier et al., 1987). An increase
of calcium entry has been known to induce the expression of
ET-1 gene in endothelial cells (Rubanyi and Polokoff, 1994).
We demonstrated that KB-R7943 efficiently improves the
renal dysfunction and tissue injury induced by the ischemia/
reperfusion, accompanying the suppression of increase in
ET-1 content in the kidney after the ischemia/reperfusion
(Yamashita et al., 2001). Taken together, ET-1 overproduc-
tion seems to be positioned down-stream to the Ca
over-
load, in the cascade of ischemia/reperfusion-induced renal
injury, although ET-1 overproduction may result in the fur-
ther increase of intracellular Ca
level.

The medullary thick ascending limb of the loop of Henle
and the proximal tubule (pars recta), both situated in the
outer medulla of the kidney, are the nephron segments that
are most susceptible to ischemic injury (Brady et al., 2000).
Also in our study, an ischemia/reperfusion produced a
marked medullary tubular necrosis, in which ET-1 peptide
was abundantly observed. On the other hand, NCX1
is
known to be abundant in distal tubular portion, compared
with proximal portion (Yu et al., 1992; Bourdeau et al., 1993).
We also observed a marked Na
/Ca
exchange activity in
the distal tubules, compared with the case using proximal
tubes. However, based on that proximal tubules are more
sensitive to ischemic injury, NCX1 expressed in proximal
portions may be more important in the ischemia/reperfusion-
induced renal injury. It remains to be determined whether
NCX1 protein is localized and functions in necrotic site of
the nephron.

In separate experiments, we noted the ameliorative effect
of KB-R7943 on hypoxia/reoxygenation-induced injury in
LLC-PK
, cells, which are derived from pig kidney and have
characteristics of proximal tubules. Hypoxia/reoxygenation
technique using LLC-PK
is known as in vitro model system
of ischemia/reperfusion-induced renal tubular injury (Yone-
hana and Gemba, 1999). KB-R7943 was effective by the
treatment not only during the hypoxia but also after the
hypoxia, suggesting that Ca
influx via NCX1 during reoxy-
genation is more important in the hypoxia/reoxygenation-
induced cell injury. This observation is in agreement with our
previous report indicating that both pre- and postischemic
treatments with KB-R7943 overcame the ischemia/reperfu-
sion-induced renal injury (Yamashita et al., 2001).

In conclusion, NCX1
mice exhibited an attenuated de-
velopment of the ischemia/reperfusion-induced renal injury.
It seems most likely that Ca
overload via the reverse mode
of Na
/Ca
exchange, followed by renal ET-1 overproduc-
tion, plays an important role in the pathogenesis of ischemia/
reperfusion-induced ARF. Taken together with the pharma-
cological evidence that an inhibitor of NCX1 could overcome
the ischemia/reperfusion-induced renal injury in both cases of
pre- and postischemic treatments, selective and potent
inhibitors of NCX1 may be beneficial in the treatment of
ischemic ARF in humans.

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