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Deterioration of ischemia/reperfusion-induced acute renal failure in SOD1-deficient mice

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Abstract

Reactive oxygen species (ROS) are likely candidates for involvement in ischemia/reperfusion-induced acute renal failure (ARF). In this study, the issue of whether superoxide dismutase (SOD1)-deficiency exacerbates the ischemia/reperfusion-induced ARF was examined. At two weeks after a right nephrectomy of mice, the left renal vessels were clipped to induce renal ischemia and were then released after 45 min. The severe renal damage observed at one day was partially recovered at seven days after the induction of ischemia. $SOD1^{-/-}$ mice suffer from severe ARF compared with $SOD1^{+/-}$ and $SOD1^{+/+}$ mice. The damage was more evident in aged animals (24–28 week old) than younger ones (10–12 week old). The expression of major antioxidative and redox enzymes, except for CuZnSOD, were substantially unchanged. Thus, the increased ARF in $SOD1^{-/-}$ mice appears to be mainly attributable to a deficiency in CuZnSOD. These data support the view that ROS are exacerbating factors in ischemia/reperfusion-induced ARF.

Keywords: *Superoxide dismutase, oxidative stress, ischemia reperfusion, acute renal failure*

Abbreviations: *ARF, acute renal failure; ROS, reactive oxygen species; SOD, superoxide dismutase; BUN, blood urea nitrogen; Cr, creatinine; 8-OHdG, 8-hydroxy guanine; AR, aldose reductase; AL, aldehyde reductase; GPX, glutathione peroxidase; GR, glutathione reductase; Prx, peroxiredoxin; TBS, 150 mM NaCl and 20 mM; Tris/HCl, pH 7.6; TBST, TBS containing 0.1% tween 20; iNOS, inducible nitric oxide synthase*

Introduction

Various pathogenic causes, such as endothelial cell injury, invasion of inflammatory cells and apoptotic cell death, are involved in acute renal failure (ARF) by ischemia. Since mitochondria are the primary organelles that are affected in renal ischemia, the depletion of ATP and the release of reactive oxygen species (ROS) from the electron transport system occur. ATP is serially degraded to hypoxanthine, xanthine and ultimately to uric acid. During the course of purine metabolism, xanthine oxidase converts molecular oxygen to superoxide [1]. The restoration of oxygen supply by reperfusion enhances the generation of superoxide, which initiates a radical chain reaction. The generated ROS leads to the oxidation of lipids, proteins and DNA [2].

An inflammatory response also occurs during ischemia/reperfusion-induced ARF [3]. Activated leukocytes adhere to endothelial cells and participate in this pathogenesis. Since activated neutrophils generate large amounts of ROS by NADPH oxidase and myeloperoxidase, neutrophils that invade the inflammatory lesion are another source for ROS.

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As a result of this, during ischemia/reperfusion, renal tissues are exposed to oxidative stress, which appears to be an exacerbating factor for ARF.

A number of proteins as well as low molecular weight antioxidants are protective against ROS. Superoxide dismutase (SOD) is generally thought to play a central role because it scavenges superoxide anions at the initial step of the radical chain reaction [4]. SOD1 and SOD2 encode CuZnSOD and MnSOD, respectively and represent a major intracellular superoxide-scavenging system in most mammalian cells. CuZn-SOD is constitutively present in the cytosol and intermembrane space of mitochondria [5,6] while MnSOD is exclusively located in the mitochondrial matrix and is expressed in an inducible manner. The elevation of MnSOD by inflammatory cytokines and preconditioning makes cells more resistant to harsh oxidative stress [7].

The finding that a mutation in SOD1 is a cause of familial amyotrophic lateral sclerosis has attracted the interest of many neurologists and other researchers [8]. While $SOD1^{-/-}$ mice grow normally under conventional breeding conditions [9], female $SOD1^{-/-}$ mice are infertile [10,11]. Hearing loss [12] and hepatocarcinogenesis [13] have been reported in aged $SOD1^{-/-}$ mice. Stress caused by intervention generally leads to a deterioration of the pathogenesis of $SOD1^{-/-}$ mice, which is more severe than for $SOD1^{+/+}$ mice [9,14–16]. In the case of an SOD2-deficiency, the homo $SOD2^{-/-}$ mice die by dilated cardiomyopathy during the neonatal stage [17].

In this study, the role of SOD1 in ARF caused by ischemia/reperfusion was examined using SOD1 knock-out mice. This would lead to an understanding of the role of SOD1, which constitutively functions to scavenge superoxides, under these pathological conditions.

Materials and methods

Animals

Three pairs of B6 $SOD1^{+/-}$ mice, established by Matzuk et al. [11], were purchased from the Jackson Laboratories (Bar Harbor, ME) and bred in our institute, as described previously [16]. The animal room climate was maintained under specific pathogen free conditions at a constant temperature of 21–23°C with a 12 h alternating light–dark cycle. Animal experiments were performed in accordance with the declaration of Helsinki and the protocol was approved by the Animal Research Committee of this institution.

Surgery and experimental design

Two weeks prior to the start of the study, the right kidney was removed through a small dorsal incision made after pentobarbital anesthesia (40 mg/kg i.p.). Mice were anesthetized with pentobarbital (40 mg/kg i.p) and the left kidney was exposed through a small dorsal incision. To induce ischemia, the left renal artery and vein were clipped for 45 min. At the end of the ischemic period, the clip was released and blood was reperfused.

Blood measurements

Blood urea nitrogen (BUN) and creatinine (Cr) levels in plasma were determined using a commercial test system, the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemicals, Osaka, Japan), respectively.

Immunohistochemistry

Mice were sacrificed under ether anesthesia for evaluation by immunoblot and immunohistochemistry. Kidney tissue was excised and bisected. One half was frozen in liquid nitrogen, and stored at −80°C for a protein assay. The other half was fixed in Bouin's solution overnight, immersed sequentially in 50, 75 and 99% ethanol for 24 h each and then embedded in paraffin and sectioned at 4 μm for 8-hydroxy guanine (8-OHdG) immunostaining and hematoxylin and eosin (HE) staining. The tissue sections were incubated with 1% hydrogen peroxide in methanol and blocked for 30 min with 5% skim milk. They were then incubated with a mouse anti-8-OHdG monoclonal antibody (MOG-100 at a concentration of 5.0 μg/ml; Nikken Foods Co. Inc., Shizuoka, Japan) or a rabbit anti-nitrotyrosine antibody [18] (a generous gift from Prof. Joseph S. Beckman, The University of Alabama at Birmingham) overnight in a humidified chamber at 4°C. After rinsing, the sections were further incubated for 10 min at room temperature with blocking solution B. Blocking solutions A and B were intended to inhibit non-specific binding of the antibody to endogenous mouse immunoglobulin. A MAX-PO complex, in which the Fab' portion of the secondary antibody was conjugated with an amino acid polymer and peroxidase (Histofine mouse stain kit, 414,321; Nichirei, Tokyo, Japan) was placed on the tissue for 10 min. After a final rinse, specific immunolabeling was examined using 3,3′-diaminobenzidine (Nichirei, 415,171, Tokyo, Japan) as the chromogen, which was placed on the tissues for a few minutes. 3,3′-Diaminobenzidine development was stopped by washing the tissues with distilled water. The sections were then dehydrated, mounted and photographed.

SDS-PAGE and immunoblot analysis

SDS-PAGE and immunoblot analyses of kidney proteins were performed. Protein samples were subjected to 10–15% SDS-PAGE and then transferred to a Hybond® nitrocellulose membrane (Amersham Pharmacia) under semi-dry conditions by means of a Transfer-blot SD semi-dry transfer cell (Bio-Rad). The membrane was then blocked by incubation with 5% skimmed milk in

TBS (150 mM NaCl and 20 mM Tris/HCl, pH 7.6) for 1 h at room temperature. The membranes were then incubated with antibodies to the gene products of SOD1 [16], SOD2 [7], aldose reductase (AR) [19], aldehyde reductase (AL) [20], cytosolic glutathione peroxidase (GPX1) [21], glutathione reductase (GR) [22], peroxiredoxin (Prx) 1 [23], or Prx 4 [24] overnight at 4°C or 2 h at room temperature. After washing with TBS containing 0.1% Tween-20 (TBST), the membrane was incubated with 1:1,000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnogy) for 1 h at room temperature. After washing with TBST, the peroxidase activity on the membranes was detected by a chemiluminescence method using an ECL Plus® kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to X-ray films (Kodak, Rochester, USA).

Measurement of SOD activity

SOD activity was determined using WST-1 [2-(4-iodophenyl)-3-(4-nitrrophenyl)-5-(2,4-disulphophenyl)-2*H*-tetrazolium] (Dojindo) for detection of superoxide anion as described previously [24]. The reaction mixture contained an appropriate amount of diluted xanthine oxidase (Roche), 0.1 mM xanthine (Wako), 0.025 mM WST-1, 0.1 mM EDTA, 50 mM $NaHCO_3$, pH 10.2, in a total volume of 1 ml. The increase in the absorbance at 438 nm was monitored at 25°C for 1 min. One unit is defined as the amount of enzyme required to inhibit 50% of the absorbance change of 0.06/min and is equivalent to 0.8 units determined by the standard procedure using cytochrome *c* assay according to the manufacture's protocol. The activity was recalculated and the presented data were corresponding to that by the standard assay. MnSOD activities were defined as 1 mM NaCN-resistant activity.

Statistical analysis

Statistical analyses of the data were carried out using the Mann–Whitney *U*-test. The number of samples are shown in parenthesis in each figure. A value of $P < 0.05$ was considered to be significant. ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$.

Results

Comparison of renal function of $SOD1^{-/-}$ mice with $SOD1^{+/+}$ mice

We first examined how an SOD1 deficiency affects kidney function under normal conditions. Kidney weight and serum levels of BUN and Cr did not differ significantly between $SOD1^{+/+}$ and $SOD1^{-/-}$ mice at 25 weeks of age (Figure 1). A histological examination show that kidneys in $SOD1^{-/-}$ mice were normal. Thus, in spite of pivotal role of SOD1 in suppressing ROS levels, its deficiency did not affect kidney function under our breeding conditions.

Kidney function of $SOD1^{+/+}$ and $SOD1^{-/-}$ mice after ischemia/reperfusion injury

We then examined the effects of ischemia/reperfusion on the kidney function of $SOD1^{+/+}$ and $SOD1^{-/-}$ mice. Mice were right-nephrectomized two weeks prior to the start of the study. Ischemic ARF was initiated by clipping the left renal blood vessels for 45 min followed by release. We first examined the effects on young animals (12–14 week old) 1 week after the induction of ischemia (Figure 2). Although dysfunction of kidneys was observed in mice as the result of ischemia/reperfusion, there was only small change in the levels of BUN and Cr between groups of mice with ischemic-reperfusion and sham-operation. This would be due to recovering from damage after

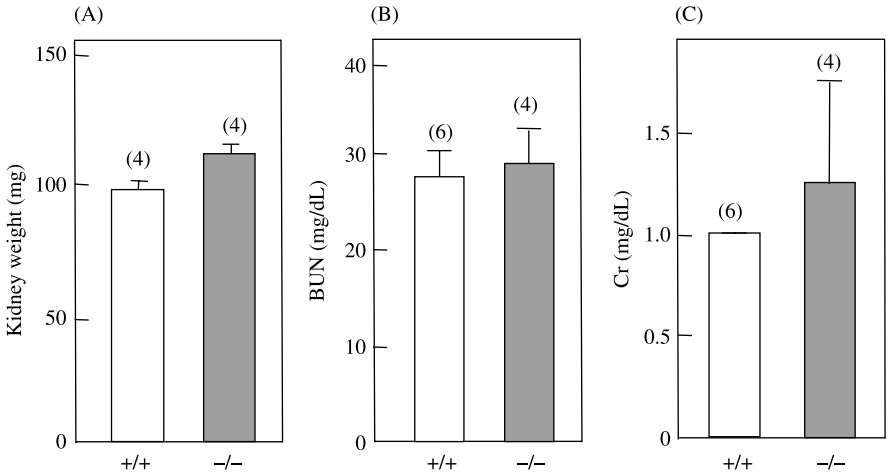

Figure 1. Kidney weight and BUN and creatinine levels. About 25-week-age mice were sacrificed and their kidney weight measured (A). Plasma levels of BUN (B) and Cr (C) were assayed under normal conditions.

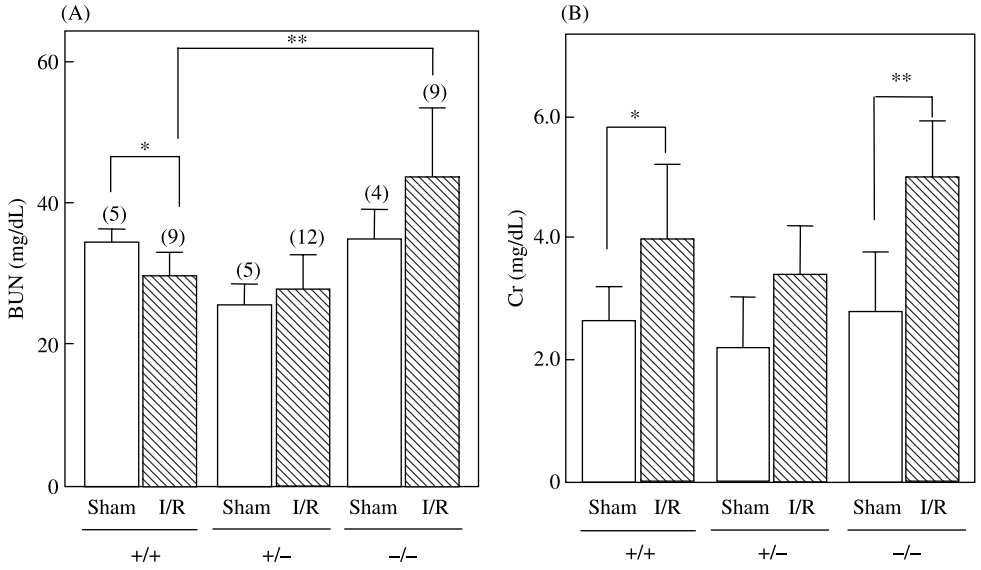

Figure 2. Renal function parameters of young mice at one week after ischemia-reperfusion. Serum BUN (A) and Cr (B) were measured for $SOD1^{+/+}$, $SOD1^{+/-}$, or $SOD1^{-/-}$ mice (12–14 week old) one week after ischemia/reperfusion with the number indicated in parenthesis.

reperfusion. Thereafter, ischemic ARF was examined at the acute stage at 24 h after ischemia/reperfusion (Figure 3). The serum levels of BUN and Cr indicated that ARF was more severe at 24 h and was significantly enhanced in $SOD1^{-/-}$ mice compared with $SOD1^{+/+}$ mice. When the same study was performed using older mice at 24–28 weeks of age, ARF was enhanced to a greater extent in the $SOD1^{-/-}$ mice (Figure 4).

Histological examination of kidneys after ischemia-reperfusion

An immunohistochemical examination was then performed on the kidneys of 24–28 week old mice with or without ischemia/reperfusion. Renal tissue injury characterized by renal tubular expansion, vacuolation of renal tubular epithelial cells and defluvium of the renal tubule epithelium was more severe in $SOD1^{-/-}$ mice than $SOD1^{+/+}$ mice (Figure 5). The levels of 8-OHdG, a marker of oxidative damage in DNA, were elevated in $SOD1^{-/-}$ mice compared to $SOD1^{+/+}$ mice. In the sham-operated groups, 8-OHdG expression was detected not only in the glomerulus but also in the renal tubules of the kidney in $SOD1^{-/-}$ mice. This expression was dramatically enhanced following renal ischemia/reperfusion. An immunohistochemical examination using an anti-nitrotyrosine antibody indicated that nitrotyrosine was present in normal renal tubules in $SOD1^{+/+}$ mice and was elevated after ischemia/reperfusion. The intensity of the staining was enhanced

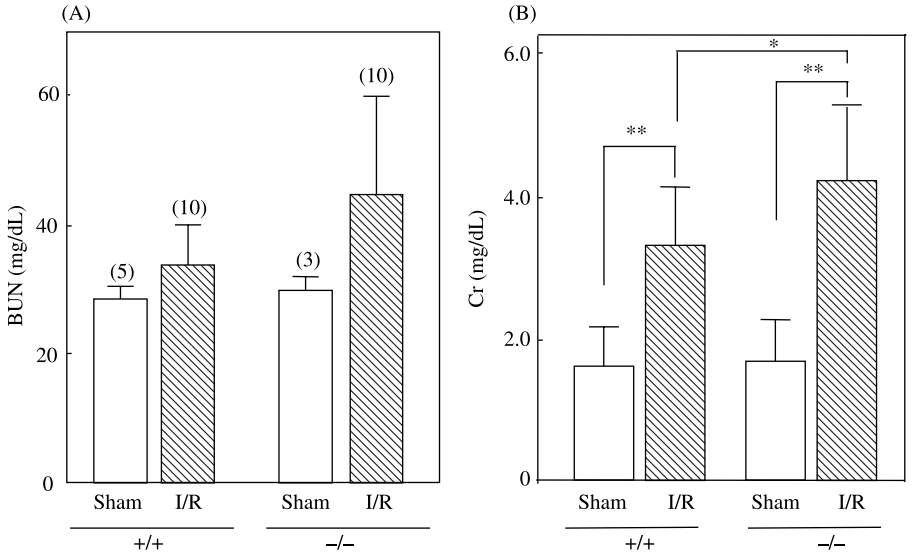

Figure 3. Renal function parameters of young $SOD1^{+/+}$ and $SOD1^{-/-}$ mice at 24 h after ischemia-reperfusion. Serum BUN (A) and Cr (B) were measured for $SOD1^{+/+}$ or $SOD1^{-/-}$ mice (12–14 week old) at 24 h after ischemia/reperfusion with the number indicated in parenthesis.

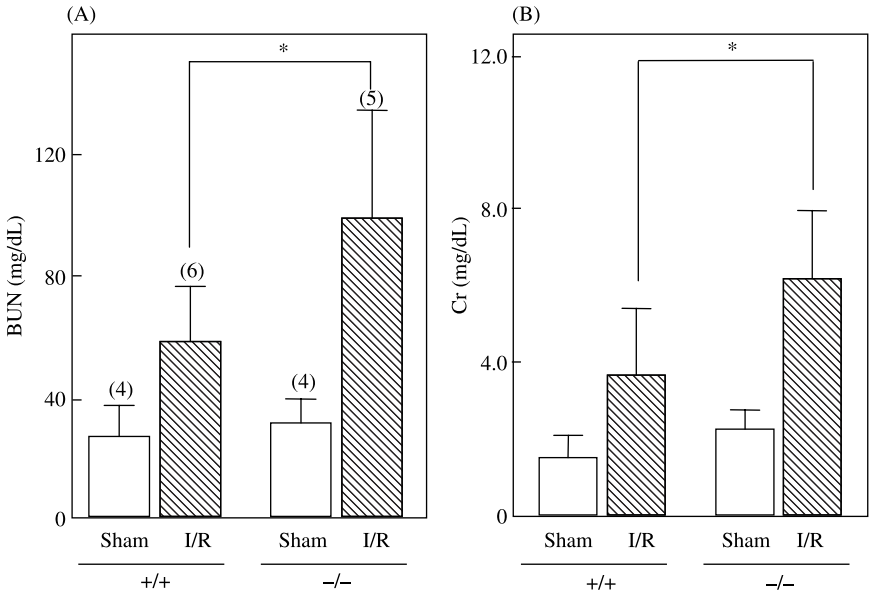

Figure 4. Renal function parameters of SOD1$^{+/+}$ and SOD1$^{-/-}$ 24–28 week older mice at 24 h after ischemia-reperfusion. Serum BUN (A) and creatinine (B) were measured for SOD1$^{+/+}$ or SOD1$^{-/-}$ mice (24–28 week old) 24 h after ischemia/reperfusion with the number indicated in parenthesis.

in SOD1$^{-/-}$ mice. Mesangium cells were positively stained both in sham-operated kidneys and in the case of renal ischemia/reperfusion.

Levels of antioxidative/redox enzymes in kidneys after ischemia/reperfusion

To examine possible involvement of MnSOD, a mitochondria-specific isozyme, in the ischemia/reperfusion-induced ARF in SOD1-deficinet mice, we measured SOD activities and also assessed protein levels of CuZnSOD and MnSOD (Figure 6). Contribution of the MnSOD activity was about 25% of total SOD activities in mouse kidneys. MnSOD activity was slightly lower, although not significant, in the kidneys after ischemia/reperfusion. Immunoblots confirmed that the CuZnSOD was detected in kidneys from the

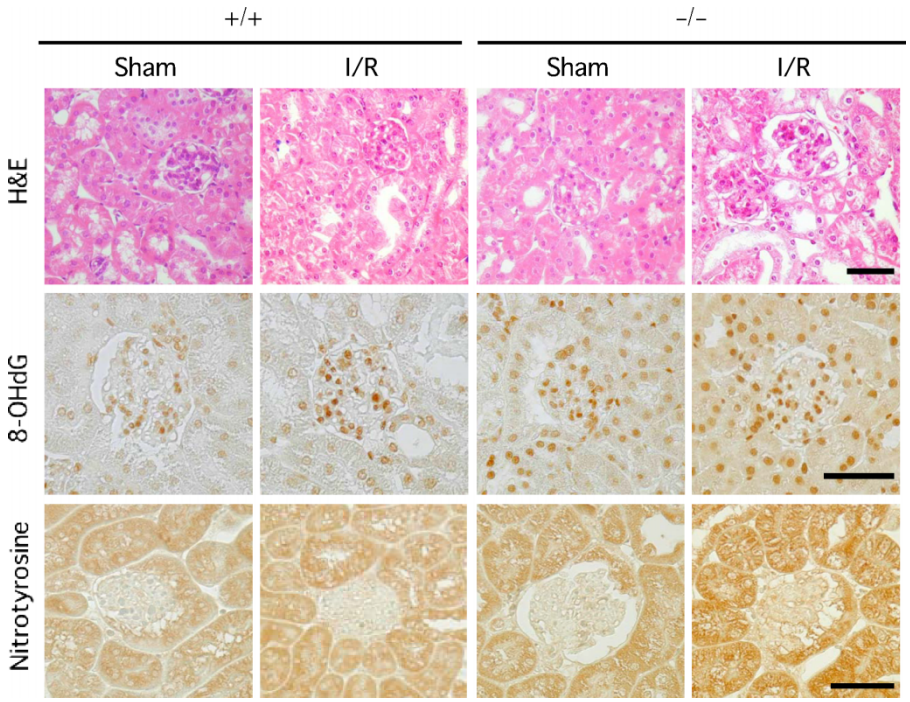

Figure 5. Immunohistochemical analysis of kidney after ischemia/reperfusion. Renal sections of 24–28 week old mice at 24 h after ischemia/reperfusion were reacted with an anti-8-OHdG and an anti-nitrotyrosine antibody as well as HE. Typical data from several experiments are shown.

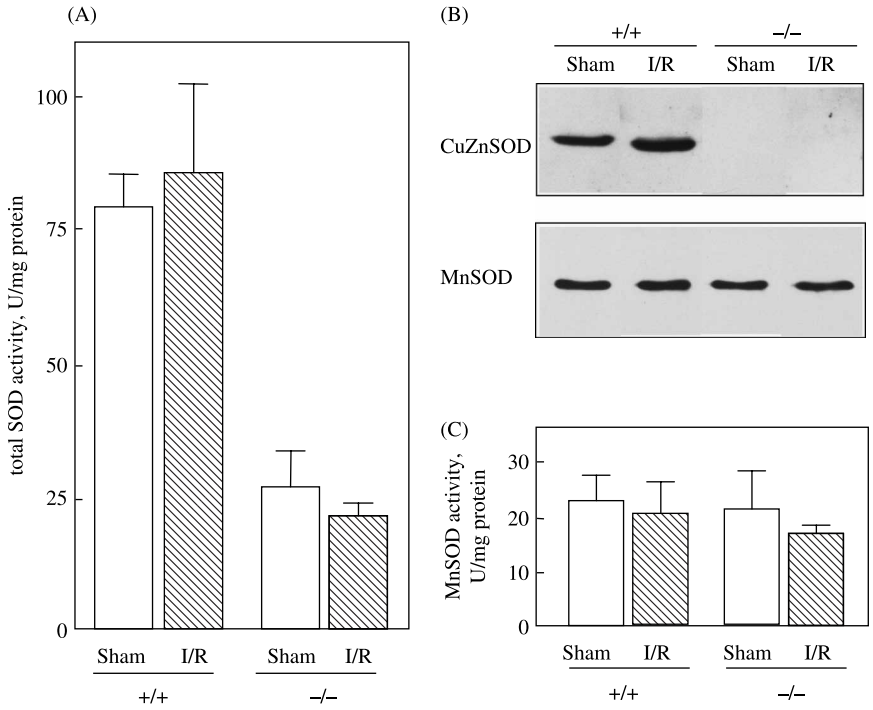

Figure 6. SOD activities and protein levels in kidney tissues. SOD activity was determined in the absence (A) or presence of 1 mM NaCN (C). Immunoblot analyses were performed using the anti-CuZnSOD and anti-MnSOD antibodies, respectively (B). Typical data from several experiments are shown. $n = 3$ for each group except for sham-operated $SOD1^{-/-}$ mice ($n = 2$).

$SOD1^{+/+}$ mice only. Levels of the MnSOD protein showed no difference between the sham operation and ischemia/reperfusion group both in $SOD1^{+/+}$ and $SOD1^{-/-}$ mice.

We also examined levels of other antioxidative/redox systems by immunoblot analyses (Figure 7). GPX1 and GR are glutathione-related antioxidative/redox enzymes that are present abundantly in kidney [21]. Aldo-keto reductase family, including AR and AL as major enzymes [19], is also protective to oxidative stress by reducing toxic carbonyl compounds generated by the oxidation reaction. In addition, thioredoxin-dependent peroxidase, referred to as Prx, also functions to reduce peroxides [24]. The levels of these antioxidative/redox enzymes, GPX1, GR, AR, AL. Prx1 and Prx4 appeared to be not significantly different between the sham operation and ischemia/reperfusion group both in $SOD1^{+/+}$ and $SOD1^{-/-}$ mice. Thus, an SOD1-deficiency appears to have little or no effect on the expression of these genes in kidneys under ischemia/reperfusion conditions.

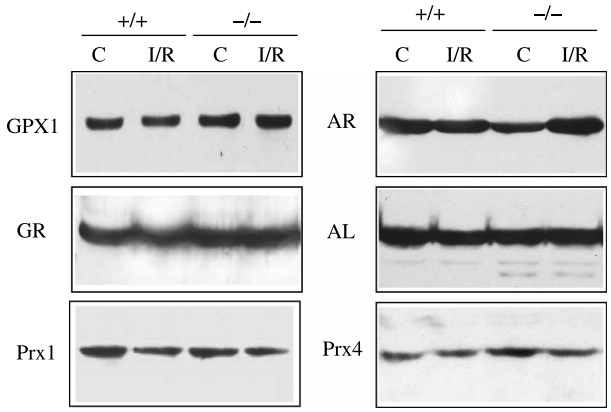

Figure 7. Immunoblot analysis of some antioxidative/redox proteins. Soluble proteins were prepared from kidneys of 24–28 week old mice 24 h after ischemia/reperfusion and subjected to immunoblot analysis using antibodies against GPX1, GR, AR, AL, Prx1, and Prx4. Typical data from several experiments are shown.

Discussion

Parller et al. [2] first reported on the protection of kidneys from ischemic ARF by infusing of SOD. The involvement of oxidative stress and nitrosative stress in ischemic ARF has been implicated [25,26] and the production of ROS during ischemic injury has been demonstrated [27]. Its involvement in ARF is supported by the finding that the radical scavenger, edaravone [28] and an antioxidant, *N*-acetyl cysteine [29], effectively ameliorate the injury. Regarding the antioxidative system, an attenuated expression of SOD1, GPX and catalase has been reported in ischemia/reperfusion-induced ARF [30,31]. The findings herein indicate that an SOD1-deficiency exacerbated ischemia/reperfusion-induced ARF.

Ischemic ARF includes various injuries in renal tissues. Mitochondria appear to be pivotal target organelles in cells during renal ischemia/reperfusion [32]. MnSOD activity was slightly decreased without

any change in the MnSOD protein after I/R in rat kidneys (Figure 6), which is consistent with the observation using similar model in rats [32]. The overexpression of SOD2 protects against ischemia/reperfusion-induced ATP depletion [33]. Since a portion of the SOD1 product is localized in the mitochondrial intermembrane space as well as the cytosol [5,6], the detrimental effects of an SOD1-deficiency may partly be caused by its absence in this particular compartment.

Regarding reactive nitrogen species, the harmful molecule that directly damages cells appears to be peroxynitrite [34], formed from the reaction of NO and superoxide. Hence, SOD not only scavenges superoxide, but also suppresses peroxynitrite formation by eliminating superoxide. An immunohistochemical examination in fact indicated that the levels of nitrotyrosine, produced from tyrosine reacted with peroxynitrite [18], were elevated in the kidneys of $SOD1^{-/-}$ mice (Figure 6). Inhibition of inducible nitric oxide synthase (iNOS) reduces ARF, probably via the suppression of peroxynitrite formation [35,36]. Contrary to the detrimental effects of NO, iNOS-derived NO may be involved in the prolonged protective effects of ischemic preconditioning in the mouse kidney [37]. NO, which is originated from induced iNOS during the ischemic preconditioning, would play a role in vasodilation under severe ischemia. Since iNOS is a cytosolic enzyme, elimination of superoxide in cytosol by the SOD1 protein would have advantage to avoid the conversion of NO to toxic peroxynitrite and accentuate the beneficial function. In addition, a part of CuZnSOD localizes mitochondria and hence, overexpression of SOD1 would also exert protective effects at some extent, as observed in SOD2 overexpressed mice [33].

Other than mitochondria, ROS are generated by the action of xanthine oxidase during purine metabolism [1] and by neutrophils attached to endothelial cells, as seen in most inflammatory process [3]. Activated neutrophils then produce ROS via two major enzymatic systems. One is NADPH-oxidase, which produces superoxide by transferring electrons from NADPH to molecular oxygen. Neutrophils also release myeloperoxidase that produces HOCl, which, by reaction with nitrite, leads to nitrotyrosine formation [38]. The resulting ROS functions against infection at an inflammatory lesion, but, in the meantime, damages nearby cells. Thus, endothelial cells would suffer from damage by ROS from neutrophiles. In addition, SOD, by scavenging superoxide, would ameliorate neutrophil-mediated endothelial cell damage. A recent study has shown that the implantation of functional endothelial cells into the kidney exhibits a renoprotective effect [34]. Thus, the combined utilization of antioxidant and anti-inflammatory agents may be effective in the treatment of ischemic ARF.

In conclusion, while the endogenous SOD1 product may not be essential for renal function under normal conditions, its antioxidative function is prerequisite for pathological conditions when large amounts of ROS are generated, such as in ischemia/reperfusion injury.

Acknowledgements

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