Chronic hypoxia aggravates renal injury via suppression of Cu/Zn-SOD: a proteomic analysis

Daisuke Son,1 Ichiro Kojima,1 Reiko Inagi,1 Makiko Matsumoto,2 Toshiro Fujita,1 and Masaomi Nangaku1

1Division of Nephrology and Endocrinology, University of Tokyo School of Medicine, Tokyo; 2Discovery Research Laboratories, Kirin Pharma Company, Limited, Gunma, Japan

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Son D, Kojima I, Inagi R, Matsumoto M, Fujita T, Nangaku M. Chronic hypoxia aggravates renal injury via suppression of Cu/Zn-SOD: a proteomic analysis. Am J Physiol Renal Physiol 294: F62–F72, 2008.—Accumulating evidence suggests a pathogenic role of chronic hypoxia in various kidney diseases. Chronic hypoxia in the kidney was induced by unilateral renal artery stenosis, followed 7 days later by observation of tubulointerstitial injury. Proteomic analysis of the hypoxic kidney found various altered proteins. Increased proteins included lipocortin-5, calgizzarin, ezrin, and transferrin, whereas the decreased proteins were α2-microglobulin PGCL1, eukaryotic translation elongation factor 1α, and Cu/Zn superoxide dismutase (SOD1). Among these proteins, we focused on Cu/Zn-SOD, a crucial antioxidant. Western blot analysis and real-time quantitative PCR analysis confirmed the downregulation of Cu/Zn-SOD in the chronic hypoxic kidney. Furthermore, our laser capture microdissection system showed that the expression of Cu/Zn-SOD was predominant in the tubulointerstitium and was decreased by chronic hypoxia. The tubulointerstitial injury estimated by histology and immunohistochemical markers was ameliorated by tempol, a SOD mimetic. This amelioration was associated with a decrease in levels of the oxidative stress markers 4-hydroxy-2-nonenal and nitrotyrosine. Our in vitro studies utilizing cultured tubular cells revealed a role of TNF-α in downregulation of Cu/Zn-SOD. Since the administration of anti-TNF-α antibody ameliorated Cu/Zn-SOD suppression, TNF-α seems to be one of the suppressants of Cu/Zn-SOD. In conclusion, our proteomic analysis revealed a decrease in Cu/Zn-SOD, at least partly by TNF-α, in the chronic hypoxic kidney. This study, for the first time, uncovered maladaptive suppression of Cu/Zn-SOD as a mediator of a vicious cycle of oxidative stress and subsequent renal injury induced by chronic hypoxia.

chronic kidney failure; oxidative stress

ONCE RENAL DAMAGE REACHES a certain threshold, the progression of renal disease is consistent, irreversible and largely independent of the initial insult. The final common pathway in this process has been closely studied. The chronic hypoxia hypothesis, proposed by Fine et al. (11), emphasizes chronic ischemic damage in the tubulointerstitium as a final common pathway in end-stage kidney injury. Since its introduction, this fascinating hypothesis has been intensively investigated by many investigators (9, 20, 24). Despite intensive efforts to elucidate the pathomechanisms of chronic hypoxia on kidney damage, however, their complexity has hampered investigations and details remain scarce. Among others, mechanisms proposed to date include transdifferentiation (22), cell death (40–42), production of extracellular matrix (26, 27), and so on.

Advances in proteomics technology offer promise to substantially improve our understanding and treatment of the molecular basis of disease. In particular, deciphering the alterations that occur in proteins between health and disease enables pharmacologically relevant targets to be identified. Although molecular biological technologies such as microarray can identify large numbers of differentially expressed genes, a potential flaw is that they cannot take into account the multiple protein products of these genes or their functional significance. In contrast, proteome analysis can identify changes in protein expression, posttranslational modifications, protein–protein interactions, cellular and subcellular distribution, and temporal patterns of expression (17, 23, 28, 43, 52). In the present study we employed proteomics technology to study the pathophysiology of chronic hypoxia in the kidney.

MATERIALS AND METHODS

Animals and experimental design. All experiments were conducted in accordance with the Guide for Animal Experimentation, Faculty of Medicine, University of Tokyo, Japan. Six-week-old male Sprague-Dawley rats (Nisseizai, Saitama, Japan) weighing 160–200 g were housed in cages in a temperature- and light-controlled environment in an accredited animal care facility.

A chronic renal hypoxia model was used in which hypoxia was induced by unilateral renal artery stenosis (RAS) according to Goldblatt’s 2-kidney, 1-clip hypertension technique (50). Briefly, a U-shaped silver clip (0.23-mm internal diameter) was placed around the left renal artery through a midline abdominal incision under ketamine hydrochloride anesthesia (50 mg/kg). A sham operation, consisting of laparotomy and manipulation of the left renal pedicle without clipping, was performed as a control. Rats were killed 7 days after surgery, and the kidney cortex was harvested for analysis. This time point was chosen on the basis of our preliminary studies, which showed significant tubulointerstitial injury and minimal blood pressure change.

The first set of experiments involved proteomic analysis and real-time quantitative PCR analysis of the cortex in RAS and sham-operated rats. Kidney tissues were obtained by laser capture microdissection (LCM) for subsequent real-time quantitative PCR. Focal areas of necrotic tissue were observed in the clipped kidneys of some rats at death, and they were excluded from analyses. Three to six kidney samples without necrosis in each group were selected for further analysis.

In the second set of experiments, we evaluated the effects of SOD-mimetic tempol (4-hydroxy-TEMPO; Sigma-Aldrich, St. Louis, MO) in RAS rats. Rats were divided into three groups: 1) sham-operated rats receiving vehicle (sham; n = 6), 2) RAS rats receiving vehicle (RAS; n = 6), and 3) RAS rats receiving SOD-mimetic tempol (RAS + tempol; n = 6). Sham-operated rats receiving tempol

Address for reprint requests and other correspondence: M. Nangaku, Division of Nephrology and Endocrinology, Univ. of Tokyo School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan (e-mail: mnangaku-tky@umin.ac.jp).

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alone were also examined. Tempol at 1.5 mmol/kg or vehicle (0.9% saline) was given by single daily intraperitoneal administration beginning 2 days before clipping surgery until the day before death.

In the third set of experiments, we evaluated the effects of the anti-TNF-α monoclonal antibody infliximab (Centocor, Mallvern, PA) in RAS rats. Three groups of rats were compared: 1) sham-operated rats receiving vehicle (sham; n = 6), 2) RAS rats receiving vehicle (RAS; n = 6), and 3) RAS rats receiving infliximab (RAS + infliximab; n = 6). Infliximab at 16 mg/kg or vehicle (0.9% saline) was given intraperitoneally on alternate days from day 0 (day of clipping) to day 6 (total: 4 times).

To demonstrate hypoxia in the clipped kidney, we used male “hypoxia-sensing” transgenic rats of Wistar strain (39). These are transgenic rats harboring a transgene composed of hypoxia response element (HRE; enhancer) and the FLAG-tagged luciferase reporter gene, allowing us to detect hypoxic cells through hypoxia-inducible factor (HIF)-mediated cellular hypoxic response. Kidney tissues of rats of 3-day RAS were submitted to immunohistochemical analysis using anti-FLAG antibody.

Proteomic analysis. For proteomic analysis, kidneys were perfused with 0.9% saline to minimize blood contamination. The kidney samples were immediately frozen in liquid nitrogen and stored at –80°C until use. Three kidney samples from each group were homogenized together in lysis buffer [tissue protein extraction reagent (Pierce Biotechnology, Rockford, IL), protease inhibitor cocktail (Sigma-Aldrich), and 1 mM PMSF] and centrifuged at 20,000 g for 20 min at 4°C, and the resulting supernatant was used for protein analysis. Total protein concentration was determined using the Bradford method.

Immobiline DryStrip gels (Amersham Bioscience, Uppsala, Sweden; pH 3–10 non-linear, 13 cm) were rehydrated in a swelling solution [6 M urea, 2 M thiourea, 3% CHAPS, 1% Triton X-100, and DeStreak reagent (Amersham Bioscience)] that contained 250 μg of proteins for 12 h. Isoelectric focusing (IEF) was performed at 5,000 V for 15 h. After the IEF procedure, the gel strips were equilibrated with a buffer containing 100 mM Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, and 2% dithiothreitol for 45 min. For SDS gel electrophoresis, a 10–18% SDS gel gradient (14 × 14 cm) was prepared. Gel electrophoresis was carried out at 25 mA (constant current). The gel was stained in equilibration solution containing 0.02% Coomassie brilliant blue G-250 overnight.

Protein patterns in the gel were recorded as digitalized images using a high-resolution scanner (GS-800 calibrated imaging densitometer; Bio-Rad, Hercules, CA). The scanned gel image was analyzed using a standard protocol under the PDQuest software (Bio-Rad). For protein identification, gel pieces containing the desired protein spots were excised, washed, and digested in gel with trypsin. The resulting peptides were then processed for analysis with an AXIMA-CFR MALDI-TOF mass spectrometer (Shimadzu Biotech, Kyoto, Japan). The search for protein identity based on the peptide mass fingerprint was performed with Mascot search software (Matrix Science, Boston, MA).

Laser capture microdissection. Frozen sections (8 μm) of kidney were prepared and mounted on glass slides for the LCM system. A Histogene LCM frozen section staining kit (Arcturus Engineering, Mountain View, CA) was used for fixation. First, the sections were rehydrated in 75% ethanol and distilled water for 30 s. After staining with Histogene staining solution for 20 s, they were washed with distilled water for 30 s and dehydrated in 75, 95, and 100% ethanol for 30 s each, followed by xylene for 5 min, and air-dried. The regions of glomeruli and the tubulointerstitium were then dissected by LCM with the use of an AutoPix LCM system (Arcturus Engineering) and dropped immediately onto the CapSure HS LCM caps (Arcturus Engineering). Thirty glomeruli or 5–10 tubulointerstitial areas per section (n = 3 in each group) were collected into a 0.5-ml tube, and total RNA was extracted using a Picopure RNA isolation kit (Arcturus Engineering). To synthesize cDNA from total RNA, we used SuperScript II reverse transcriptase (Life Technologies BRL, Rockville, MD).

Real-time quantitative PCR analyses. Total RNA was extracted from kidney cortex homogenates or cultured cells with ISOGEN (Nippon Gene, Tokyo, Japan). mRNA levels were assessed by real-time quantitative PCR using SYBR green PCR reagent (Qiagen, Hilden, Germany) and an iCycler PCR system (Bio-Rad) according to the manufacturer’s instructions. All reactions were performed in duplicate. The reproducibility was confirmed in three independent experiments, with representative data presented in RESULTS. Ct, or threshold cycle, was used for relative quantification of the input target number. The value of threshold cycle for the control sample was considered 1, i.e., 20. The number of threshold cycles for other samples was derived from cycles of the control sample and recorded as ΔCt. The amount of amplified molecules at the threshold cycle was calculated by 2ΔCt. The mRNA levels of genes were normalized to the levels of β-actin. Each gene and PCR primer were as follows: rat Cu/Zn-SOD (5′-CGGACATGGTTCCATGTTC-3′, 5′-CTGGAGCCCATGTITTTCCAT-3′), rat TNF-α (5′-CTTCTGCTTACGT-GAATCTCGGGGT-3′, 5′-TGGAACTGATGGAGGGAAGCC-3′), β-actin (5′-CTTCTAACATGCTGCTG-3′, 5′-TGGACTGATGCTAGTCAGGATGCG-3′), HIF-1α (5′-CATTCTTTGCTGAGTACACTA-3′), and human β-actin (5′-TCCCCCAACCTTGAAGATGTAGAAG-3′, 5′-AATTGCTCAGAATCGTACATT-3′).

Western blot analyses. Isolation of whole protein from the kidney cortex was performed by tissue homogenization in Tris-glycine buffer. Protein (10–30 μg) from each animal was resolved on a 12–15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Polyclonal rabbit anti-Cu/Zn-SOD antibody (1:1,000; StressGen, Victoria, Canada), polyclonal rabbit anti-annexin-5 (lipocortin-5) antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti-ezrin antibody (1:1,000; Zymed, San Francisco, CA), polyclonal goat anti-transferrin antibody (1:200; Santa Cruz), monoclonal mouse anti-α2u-globulin antibody (1:200; R&D Systems, Minneapolis, MN), polyclonal rabbit anti-actin antibody (1:300; Sigma-Aldrich), and horseradish peroxidase-conjugated secondary antibody (1:3,000; Jackson ImmunoResearch, West Grove, PA) were used. The ECL Western blotting system (Amersham Bioscience) was used for detection. The reproducibility was confirmed in three independent experiments, with representative data presented in RESULTS. The intensity of the band was quantified utilizing the NIH ImageJ software (National Institutes of Health, Bethesda, MD) and normalized by the band intensity of actin.

Histological and immunohistochemical analysis. Tissues were fixed in methyl Carnoy’s solution and paraffin-embedded. Sections (3 μm) were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. Indirect immunoperoxidase methods were used to identify the following antigens: vimentin with mouse monoclonal antibody V9 (Dako, Carpinteria, CA); monocytes/macrophages with mouse monoclonal antibody ED-1 (Chemicon, Temecula, CA); FLAG with mouse monoclonal antibody M2 (Sigma-Aldrich); SOD1 antibody V9 (Dako, Carpinteria, CA); and nitrotyrosine, and HNE antibodies were used for computer-assisted morphometry of renal histology. Semiquantitative analysis of tubulointerstitial injury was performed in a blinded manner using 10 randomly selected fields of cortex per cross section of PAS staining (n = 6 in each group). Injury was graded on a scale of 0 to 5+ on the basis of the percentage of tubular cellularity, basement membrane thickening, cell infiltration, dilation, atrophy, sloughing, or interstitial widening as follows: 0, no change; 1, <10% tubulointerstitial injury; 2, 10–25% injury; 3, 25–50% injury; 4, 50–75% injury; and 5, 75–100% injury (25).

Sections stained immunohistochemically with anti-vimentin, ED1, nitrotyrosine, and HNE antibodies were used for computer-assisted
morphometry. Positive areas for respective antibody expression in the tubulointerstitium were quantified at a \( \times 200 \) magnification in a blinded manner using NIH ImageJ. Ten tubulointerstitial areas per section were randomly selected from each group for quantification (\( n = 6 \) in each group).

**Cell culture.** Human renal proximal tubular epithelial cells (RPTEC) were purchased from Clonetics (Walkersville, MD) and cultured in complete epithelial medium (REGM BulletKit; Clonetics), as supplied, according to recommended instructions. The cells were subcultured and used at passages 3–5. At 80–90% confluence, the medium was changed to serum-free REGM, and cells were subjected to TNF-\( \alpha \) treatment (R&D Systems).

**Serum TNF-\( \alpha \) estimation by ELISA.** Serum TNF-\( \alpha \) levels were determined using an ELISA. The ELISA was performed by adding 100 \( \mu l \) of each sample to wells in a 96-well plate of a commercially available rat ELISA kit (BD Biosciences, San Diego, CA). The samples were tested in duplicate. The TNF-\( \alpha \) ELISA was performed according to the manufacturer’s instructions, and final results are expressed as picograms of TNF-\( \alpha \) per milliliter.

**Statistical analysis.** All data are means \( \pm SD \). Statistical analyses were performed using the \( t \)-test. Nonparametric data were analyzed with the Mann-Whitney test when appropriate. Differences with \( P \) values \( < 0.05 \) were considered significant.

**RESULTS**

**Induction of chronic renal hypoxia model.** Chronic renal hypoxia model was induced by the unilateral RAS. Seven days after the surgery, histology of the RAS group showed significant tubulointerstitial injury including tubular atrophy, tubular dilatation, basement membrane thickening, and cell infiltration with little change in glomeruli (Fig. 1, A and B). Immunohistochemical analysis revealed vimentin- and ED1-positive cells

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**Fig. 1.** Histological and immunohistochemical analysis of markers of tubulointerstitial injury. In contrast to sham rats (A), marked tubulointerstitial injury was observed in chronic hypoxic kidneys of 7-day renal artery stenosis (RAS; B) as shown by periodic acid-Schiff (PAS) staining. RAS kidneys also showed enhanced vimentin staining (E) and massive macrophage infiltration (H) in the tubulointerstitial area compared with sham kidneys (D and G). Contralateral kidneys showed no histological (C) or immunohistochemical changes (F and I) compared with sham kidneys. Magnification, \( \times 200 \) for PAS and vimentin and \( \times 400 \) for ED-1.

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**Fig. 2.** Expression of the “hypoxia-responsive” transgene. Hypoxia in the tubular cells was demonstrated by an increase in the hypoxia-responsive transgene expression in kidneys of hypoxia-sensing transgenic rats. After 3 days of RAS, expression of the hypoxia-responsive transgene was upregulated ubiquitously (B) compared with sham-operated kidneys (A). Magnification, \( \times 400 \).
in the tubulointerstitium in the RAS group (Fig. 1, D, E, G, and H). The contralateral kidney showed no histological or immunohistochemical changes (Fig. 1, C, F, and I). These pathological findings show that 7-day RAS caused significant tubulointerstitial damage and suggest that tubular cells are more susceptible to hypoxia than glomeruli. Systolic blood pressure of rats in the RAS group showed a mild increase compared with that of the sham group at day 7 (113 ± 11.1 vs. 97.2 ± 6.7 mmHg, P < 0.05).

Demonstration of hypoxia of the kidney after renal artery stenosis. To obtain the evidence of hypoxia in the kidney after renal artery stenosis, we utilized the hypoxia-sensing transgenic rats and investigated the hypoxia-responsive transgene expression. In the kidneys with 3-day RAS, expression of hypoxia-responsive transgene was significantly upregulated ubiquitously compared with that of sham-operated kidneys (Fig. 2).

Proteomic analysis of chronic hypoxic kidneys. We performed proteomic analysis to obtain protein expression profiles in the chronic hypoxic kidney. Kidney samples were homogenized together in either the RAS or sham group, and two-dimensional electrophoresis was performed (Fig. 3). Differentially expressed protein spots between two gels of each sample group were analyzed. The numbers of increased and decreased spots with a greater than threefold change were 86 and 57, respectively. We selected five increased and three decreased spots that had shown remarkable changes. These spots were analyzed using MALDI-TOF mass spectrometry. Identified proteins and their data are shown in Table 1; number labeling corresponds to spot number in Table 1.

Table 1. Proteomic analysis data

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Proteins</th>
<th>NCBI I.D.</th>
<th>Ratio (RAS/Sham)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased spots</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Lipocortin-5 (annexin-5)</td>
<td>gi 2981437</td>
<td>3.47</td>
</tr>
<tr>
<td>2</td>
<td>S100 calcium binding protein</td>
<td>gi 51854249</td>
<td>4.78</td>
</tr>
<tr>
<td>3</td>
<td>Ezrin</td>
<td>gi 52138521</td>
<td>5.94</td>
</tr>
<tr>
<td>4</td>
<td>Transferrin</td>
<td>gi 33187764</td>
<td>6.74</td>
</tr>
<tr>
<td>5</td>
<td>Mixture</td>
<td>---</td>
<td>4.82</td>
</tr>
<tr>
<td>Decreased spots</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>α2-globulin PGCL1</td>
<td>gi 56971469</td>
<td>0.237</td>
</tr>
<tr>
<td>7</td>
<td>SOD1 (Cu/Zn-SOD)</td>
<td>gi 8394328</td>
<td>0.229</td>
</tr>
<tr>
<td>8</td>
<td>Eukaryotic translation elongation factor 1ox2</td>
<td>gi 15805031</td>
<td>0.059</td>
</tr>
</tbody>
</table>

Spot numbers correspond to those in Figure 3. RAS, renal artery stenosis. "Mixture" proteins consisted of 3-oxoacid CoA transferase (gi 34854196), methylmalonate semialdehyde dehydrogenase gene (gi 13591997), dihydropyrimidinase (gi 13928984), and adenylyl cyclase-associated protein homolog MCH1 (gi 539940).

key antioxidative enzyme with a potentially important role in progression of chronic kidney disease.

Decreased protein and mRNA levels of Cu/Zn-SOD in chronic hypoxic kidneys. To rule out artifacts in proteomic analysis, we performed Western blot analysis of Cu/Zn-SOD. Results showed that the expression of Cu/Zn-SOD protein was significantly decreased in the RAS group compared with the sham group (P < 0.05; Fig. 4A), confirming the reduction of Cu/Zn-SOD protein level seen in proteomic analysis. To clarify whether this decrease in Cu/Zn-SOD protein was due to downregulation of mRNA, we performed real-time quantita-
tive PCR analysis of Cu/Zn-SOD, utilizing the cortex of the experimental animals. In RAS kidneys, the mRNA level of Cu/Zn-SOD was significantly decreased compared with sham kidneys \((P < 0.05; \text{Fig. 4B})\). These findings suggest that chronic renal hypoxia suppresses the expression of Cu/Zn-SOD mRNA and depletes Cu/Zn-SOD protein in association with the development of tubulointerstitial injury.

**Western blot analysis of other proteins identified by proteomics.** To verify the results of proteomic analysis, we performed Western blot analysis of four identified proteins other than Cu/Zn-SOD. Lipocortin-5, ezrin, and transferrin proteins were significantly increased, whereas α-2u-globulin protein was significantly decreased \((P < 0.05; \text{Fig. 5})\). The result of Western blot analysis was accordant with that of proteomic analysis.

**Dominant expression of Cu/Zn-SOD mRNA and protein in the tubulointerstitium.** Given that damage in our model was dominant in the tubulointerstitial area, we speculated that there might be a difference in the degree of Cu/Zn-SOD mRNA reduction according to histological area, i.e., glomeruli and tubulointerstitium. To examine this, we used a LCM system to separately dissect the intended tissue areas under direct visualization.

Quantitative real-time PCR analysis showed that Cu/Zn-SOD mRNA level in sham kidneys was 2.84-fold higher in the tubulointerstitial than in the glomerular area \((P < 0.05; \text{Fig. 6})\). To confirm this, we performed immunohistochemical analysis of Cu/Zn-SOD with tubular markers in sequential paraffin-embedded sections. The Cu/Zn-SOD staining pattern was almost identical to that of THP, a marker of the thick ascending
Cu/Zn-SOD staining (Fig. 7). In contrast, Cu/Zn-SOD staining could hardly be seen in the glomeruli, proximal tubules, or collecting ducts. Furthermore, quantitative real-time PCR analysis of the LCM showed that the Cu/Zn-SOD mRNA level was decreased in both the glomerular and tubulointerstitial areas of chronic hypoxic kidneys (Fig. 6), particularly in the tubulointerstitium. On the basis of these data, we conclude that Cu/Zn-SOD is expressed mainly in the distal tubules and that downregulation of Cu/Zn-SOD in the chronic hypoxic kidney can be attributed to its decrease in the corresponding region.

SOD mimetic ameliorates hypoxia-induced tubulointerstitial injury. Cu/Zn-SOD is a major antioxidant that protects a range of tissues from various oxidative stresses. Renal hypoxia is known to cause significant oxidative stress, and it is likely that a relative scarcity of Cu/Zn-SOD increases the susceptibility of renal tissue to ischemic injury. We speculated that the decrease in Cu/Zn-SOD caused by chronic renal hypoxia contributes to the further deterioration of tubulointerstitial damage, establishing a vicious cycle of hypoxia-induced renal damage. To verify this hypothesis, we investigated whether antioxidant supplementation could ameliorate this tubulointerstitial damage.

Tempol administration to RAS rats significantly ameliorated tubulointerstitial injury compared with vehicle-treated RAS rats (Fig. 8, Table 2). The number of vimentin-positive cells was significantly decreased in tempol-treated RAS rats compared with vehicle-treated RAS rats. Furthermore, tempol administration significantly reduced macrophage infiltration, as represented by ED1-positive cell count in the interstitium (Fig. 8, Table 2). Blood analysis revealed serum creatinine was not elevated in RAS rats, probably due to compensation of contralateral kidneys (Table 2). Systolic blood pressure of the rats in the RAS and RAS + tempol groups was significantly higher than that of rats in the sham group (Table 2). Tempol administration into sham-operated rats did not induce any changes of the kidney estimated by histological and immunohistochemical analysis (Fig. 8, D, H, and L).

This improvement in tubulointerstitial injury by tempol was associated with the amelioration of oxidative stress in chronic hypoxic kidneys. As shown by immunohistochemical analysis, HNE and nitrotyrosine staining were significantly greater in kidneys of the RAS group than in those of the sham group (Fig. 9, Table 2). This increase in HNE and nitrotyrosine staining was significantly ameliorated by tempol administration (Fig. 8, Table 2).

Suppression of Cu/Zn-SOD is caused by TNF-α in chronic hypoxic kidneys. To investigate the mechanism of Cu/Zn-SOD suppression, we decided to utilize RPTEC, and we stimulated the cells with various agents. Under anoxic conditions (0.2% O₂) or angiotensin II treatment, Cu/Zn-SOD mRNA of RPTEC showed no significant changes during 24 h of incubation with real-time quantitative PCR analysis (data not shown). We then speculated that some proinflammatory cytokines might be involved, and we tried TNF-α stimulation. Cu/Zn-SOD mRNA showed significant and dose-dependent suppression by TNF-α treatment at 24 h (Fig. 10).

Furthermore, we administrated anti-TNF-α monoclonal antibody (infliximab) to RAS rats and examined the causal relation between TNF-α expression and Cu/Zn-SOD suppression. As shown by histological analysis, infliximab administration significantly ameliorated the tubulointerstitial injury of chronic hypoxic kidneys (Fig. 11, A–D), and serum TNF-α level estimation by ELISA revealed a significant TNF-α increase in RAS rats and significant TNF-α inhibition by administration of infliximab (Fig. 11E). Western blot analysis revealed that the Cu/Zn-SOD protein level of infliximab-treated RAS kidneys was increased significantly compared with that of
vehicle-treated RAS kidneys (Fig. 12). These findings suggest that Cu/Zn-SOD suppression is partly caused by increased expression of the inflammatory cytokine TNF-α in chronic hypoxic kidneys.

**DISCUSSION**

Our proteomic analysis in chronic hypoxic kidneys revealed changes to various proteins, including a decrease in Cu/Zn-SOD. Cu/Zn-SOD was predominantly expressed in the distal tubules and was reduced in that area by chronic hypoxia.

Administration of SOD-mimetic tempol ameliorated oxidative stress and associated tubulointerstitial injury in chronic renal hypoxia. In vitro study of TNF-α stimulation and in vivo study of anti-TNF-α antibody administration suggested that the upregulated inflammatory cytokine TNF-α might be involved in the mechanism of suppression of Cu/Zn-SOD in chronic hypoxic kidneys.

Chronic renal hypoxia was induced using unilateral RAS. The induction of chronic hypoxia in this model has been confirmed by microelectrode (29) and in hypoxia-sensing
transgenic animals in this study. The morphological and immunologic changes in a 28-day chronic renal ischemia model have been well characterized by Truong et al. (44). In their study, tubular changes were seen as early as 3 days after RAS, with tubular atrophy, thickening of tubular basement membrane, and interstitial inflammation/fibrosis, followed by the gradual development of glomerulosclerosis occurring thereafter. Pathological changes observed in our 7-day renal hypoxia model were consistent with these previous observations. We employed a relatively short time point for analysis to minimize the effects of secondary changes. At this time point, blood pressure showed only a mild increase, and contralateral kidneys were histologically normal.

Our proteomic analysis revealed a decrease in Cu/Zn-SOD protein levels in the cortex in chronic hypoxic kidneys, while real-time quantitative PCR analysis confirmed a parallel decrease in Cu/Zn-SOD mRNA levels. LCM is a technique for isolating pure cell populations from a heterogeneous tissue section via direct visualization of the cells. Using LCM, we determined that the predominant expression of Cu/Zn-SOD mRNA occurred in the tubulointerstitial area in normal kidneys and confirmed its greater reduction in the tubulointerstitium than elsewhere in chronic hypoxic kidneys. To our knowledge, real-time quantitative PCR analysis confirmed a parallel decrease in Cu/Zn-SOD mRNA levels. LCM is a technique for isolating pure cell populations from a heterogeneous tissue section via direct visualization of the cells. Using LCM, we determined that the predominant expression of Cu/Zn-SOD mRNA occurred in the tubulointerstitial area in normal kidneys and confirmed its greater reduction in the tubulointerstitium than elsewhere in chronic hypoxic kidneys. To our knowledge,
this is the first study to demonstrate a reduction in Cu/Zn-SOD protein and mRNA levels in chronic hypoxic kidneys. The biological significance of this decrease in Cu/Zn-SOD was further emphasized by the demonstration that the SOD-mimetic tempol ameliorated hypoxia-induced tubulointerstitial injury.

SODs are important antioxidant enzymes that guard against superoxide toxicity. Various SOD enzymes employ either a copper, manganese, iron, or nickel cofactor to carry out the disproportionation of superoxide. Whereas SOD3 refers to extracellular Cu/Zn-SOD (EC-SOD), intracellular Cu/Zn-SOD is designated as SOD1 in mammals (Cu/Zn-SOD). SOD1 is a cytosolic enzyme that serves the critical function of limiting intracellular superoxide concentrations during various oxidant stresses such as ischemia and reperfusion injury (5, 13). Superoxide can react with and denature important cellular enzymes, including aconitase and fumarase (12, 14, 16, 21). It also rapidly reacts with nitric oxide to form the potent oxidant peroxynitrite, which in turn causes protein nitration and cell death (4, 51).

Mammalian Cu/Zn-SOD is highly expressed in the liver, kidney, and motor neurons (2, 32). Knockout studies indicate that elimination of the Cu/Zn-SOD gene in rodents is associated with a variety of pathological conditions, including a decrease in life span (10) and vulnerability to motor neuron loss after axonal injury (33). Various mutations in the human gene Cu/Zn-SOD are known to lead to inherited forms of amyotrophic lateral sclerosis (6, 35). Recent studies have also emphasized a role for Cu/Zn-SOD in a model of acute renal failure induced by lipopolysaccharide (LPS) (3). In response to a moderate dose of LPS, Cu/Zn-SOD-deficient mice sustained significantly greater acute renal failure and tubular injury compared with wild-type mice. In contrast, LPS-induced acute renal failure was equivalent between wild-type and Mn-SOD transgenic mice. These studies support the biological significance of Cu/Zn-SOD in vivo.

Cu/Zn-SOD has been shown to play a role in a variety of other disease models. Several studies using an acute ischemia-reperfusion injury model in rats found that Cu/Zn-SOD decreased after ischemia-reperfusion injury, whereas Mn-SOD remained stable (36). Overexpression or exogenous administration of Cu/Zn-SOD ameliorated ischemia-reperfusion injury of the kidneys, heart, and brain, suggesting a pathogenic role for the suppression of Cu/Zn-SOD (7, 8, 38, 48, 53). Downregulation of Cu/Zn-SOD in association with increased oxidative stress in the kidney was seen in several other experimental diseases models, including a chronic kidney failure model of the remnant kidney (45), carboplatin nephrotoxicity (18), and the metabolic syndrome (34). Furthermore, recent proteomic
analyses using isobaric tagging detected a decrease in the abundance of Cu/Zn-SOD in the remnant kidney of the FVB/N mouse associated with the development of renal lesions (46). These findings suggest a pathogenic role of the suppression of Cu/Zn-SOD.

It is likely that the suppression of Cu/Zn-SOD exacerbates oxidative stress in chronic hypoxic kidneys. Although oxidative stress requires the presence of oxygen to produce reactive oxygen species, it is well known that oxidative stress also plays a crucial role in hypoxic tissue injury. This apparently paradoxical characteristic of oxidative stress in hypoxic conditions can be explained, at least in part, by relative reoxygenation of the tissues. Other mechanisms include the activation of xanthine oxidase induced by hypoxia (31, 37). Oxidative stress, in turn, damages the kidney via multiple mechanisms. Of note, oxidative stress per se aggravates hypoxia in the kidney. Oxidative stress reduces the efficiency of oxygen use by disturbing mitochondrial respiration, thereby contributing to the resulting hypoxia in the kidney (30, 49). The consumption of vasodilatory nitric oxide by oxidative stress can also result in a decrease in kidney blood flow, further aggravating renal hypoxia. In our present study, administration of SOD-mimetic tempol clearly improved both tubulointerstitial injury as well as the accumulation of oxidative stress products in chronic hypoxic kidneys. Because compensation by the contralateral nonstenotic kidney masked any deterioration in baseline renal function in our rats, we were unable to demonstrate an improvement in renal function with tempol.

Although predominant injury to Cu/Zn-SOD-rich tubular cells may explain the marked reduction in Cu/Zn-SOD in chronic renal hypoxia, we observed a significant increase in Cu/Zn-SOD gene and protein expression even after normalization by actin level. We speculated that one or more inhibitory factors produced by chronic hypoxia may suppress the expression of Cu/Zn-SOD. Oxidative stresses such as reactive oxygen species upregulates Cu/Zn-SOD as the defensive mechanism (54), but little is known about the inhibitory factors. Recently, Afonso et al. (1) showed that TNF-α downregulates Cu/Zn-SOD promoter via JNK/AP-1 signaling pathway. Human Cu/Zn superoxide dismutase 1 promoter via JNK/AP-1 signaling pathway. Free Radic Biol Med 41: 709–721, 2006.


