Acatalasemia sensitizes renal tubular epithelial cells to apoptosis and exacerbrates renal fibrosis after unilateral ureteral obstruction

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Submitted 28 July 2003; accepted in final form 10 January 2004

Sunami, Reiko, Hitoshi Sugiyama, Da-Hong Wang, Mizuho Kobayashi, Yohei Maeshima, Yasushi Yamasaki, Noriyoshi Masuoka, Norio Ogawa, Shohei Kira, and Hirofumi Makino. Acatalasemia sensitizes renal tubular epithelial cells to apoptosis and exacerbrates renal fibrosis after unilateral ureteral obstruction. Am J Physiol Renal Physiol 286: F1030–F1038, 2004.—Tissue homeostasis is determined by the balance between oxidants and antioxidants. Catalase is an important antioxidant enzyme regulating the level of intracellular hydrogen peroxide and hydroxyl radicals. The effect of catalase deficiency on renal tubulointerstitial injury induced by unilateral ureteral obstruction (UUO) has been studied in homozygous acatalasemic mutant mice (C3H/AnLnCs-/-). Complete UUO caused interstitial cell infiltration, tubular dilation and atrophy, and interstitial fibrosis with accumulation of type IV collagen in obstructed kidneys (OBK) of both mouse groups. However, the degree of injury showed a significant increase in OBK of acatalasemic mice compared with that of wild-type mice until day 7. The deposition of lipid peroxidation products including 4-hydroxy-2-hexenal, malondialdehyde, and 4-hydroxy-2-nonenal was severer in dilated tubules of acatalasemic OBK. Apoptosis in tubular epithelial cells significantly increased in acatalasemic OBK at day 4. Expression of caspase-9, a marker of mitochondrial pathway-derived apoptosis, increased in dilated tubules of acatalasemic mice. The level of catalase activity remained low in acatalasemic OBK until day 7 without compensatory upregulation of glutathione peroxidase activity. The data indicate that acatalasemia exacerbated oxidative injury of renal tissue and sensitized tubular epithelial cells to apoptosis in OBK of UUO. This study demonstrates that catalase deficiency enhanced tubulointerstitial injury and fibrosis in a murine model of UUO and thus supports the protective role of catalase in this model.

catalase; acatalasemic mice; free radical; cell death; caspase

Catalase is an important anti-oxidant enzyme in cellular peroxisome and physiologically maintains tissue and cellular redox homeostasis. The enzyme regulates the level of intracellular hydrogen peroxide (H2O2) and hydroxyl radicals and thus plays a central role in defense against oxidative stress. In 1953, Rondini and Cudkowicz (26) reported for the first time that the kidney could generate H2O2 in normal and disease conditions in vivo by using inhibitors of catalase. They identified the kidney as a major site of the production of H2O2, a conclusion subsequently confirmed by several studies (8, 9). Takahara (34, 35) unexpectedly discovered Japanese patients with acatalasemia (also known as Takahara disease) by severe renal gangrene after exposure to H2O2. Changes in lipid and carbohydrate metabolism and the high incidence (12.7%) of diabetes mellitus in Hungarian acatalasemia suggest that this inherited catalase deficiency is a risk factor for atherosclerosis and diabetes mellitus (6, 7). A deficiency of catalase may predispose subjects to cumulative oxidant damage in pancreatic β cells (6, 7). However, the effect of acatalasemia on the progression of kidney disease is poorly understood.

An acatalasemic mouse strain, Csβ, was first described by Feinstein et al. (2–4). This catalase mutation was identified by screening blood catalase activity levels in a group of mice from irradiation studies and has thus been considered an X-ray-induced mutation. The mutation has been mapped to the catalase structural gene on chromosome 2 and exhibits additive inheritance of catalase activity in red blood cells from Csα (control) × Csβ F1 hybrid animals. The molecular basis of the Csβ catalase mutation has been reported, suggesting that the mutation does not act at the level of gene transcription or mRNA stability but rather at the level of mRNA translation and/or protein turnover (29). The mutation is characterized by modification of the enzyme active site but not of the antigenic site (4). The phenotypes of acatalasemic mice have been reported (2–4). Homozygous acatalasemic mutants are fertile and show no apparent developmental defects. Under normal conditions, no obvious abnormalities in histology and function of the kidneys are observed in these mice. However, after exposure to carbon tetrachloride, liver cells of acatalasemic mice died more rapidly than those of wild-type mice (40). Enhanced hepatocarcinogenesis was reported in acatalasemic mice treated with diethylnitrosamine (39). Because the supplementation of vitamin E, one of the antioxidants, lowered the incidence of mammary tumorigenesis induced by virus in acatalasemic mice (10), it is suggested that increased oxidative stress may be involved in virus-mediated cancer development in acatalasemia.

Oxygen consumption is high in the kidney because of the active solute transport and reabsorption; therefore, the renal tubulointerstitium is always at risk for oxidative injury. There are several reports of the increased oxidative stress in tubulointerstitial injury such as ischemia-reperfusion (22) and cisplatin toxicity (15). However, little is known about its involvement in the setting of unilateral ureteral obstruction (UUO) (25), which is a well-established model of renal tubulointerstitial fibrosis (13). We hypothesized that a defect in the antioxidant system by catalase deficiency may enhance renal...
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Mice that underwent surgery were killed under pentobarbital sodium anesthesia. Then, 0.2 ml of 3.5 mM H2O2 was added and after 0, 30, 60, and 120 min, the supernatant was removed and diluted with 0.1 M PPB to make a 0.2% solution. The samples including renal cortex were homogenized with a tissue homogenizer (Tokyo, Japan). The following primary antibodies were used for immunohistochemistry. Mouse monoclonal antibodies to 4-hydroxy-2-nonenal (4-HNE) (36), 4-hydroxy-2-hexenal (4-HHE), and malondialdehyde (MDA) were obtained from Nof Life Science (Tokyo, Japan). Mouse monoclonal anti-caspase 9/8 (Ab-2 clone 9CS10A2) was from Lab Vision (Fremont, CA); and rabbit polyclonal antibodies against type IV collagen (LSL, Tokyo, Japan) and biotinylated Galanthus nivalis (snowdrop) lectin were from Vector Laboratories (Burlingame, CA).

Renal catalese, glutathione peroxidase, and xanthine oxidase activity. When kidneys were harvested, each kidney was decapsulated, washed with saline, bisected coronally, blotted dry on gauze, and weighed as described previously (28). Whole kidney weight was expressed as a percentage of body weight determined at the time the mouse was killed. Dry kidney weight was not determined. After the harvesting of obstructed (OBK) or contralateral (CUK) kidneys from either wild-type or acatalasemic mice, samples were stored in a −80°C freezer until assay.

Catalase activity was determined by measuring the removal rate of 70 μM H2O2 based on the method of Masuoka et al. (18–20). Samples including renal cortex were homogenized with a teflon homogenizer in homogenization buffer [0.1 M potassium phosphate buffer (PPB), pH 7.2, containing 1 mM EDTA and 1% Triton X-100]. The samples were centrifuged for 30 min (11,000 g) at 4°C. The supernatant was removed and diluted with 0.1 M PPB to make a 0.2% homogenate. Then, 0.2 ml of 3.5 mM H2O2 was added and after 0, 30, and 60 s, 2 ml of the reaction mixture (containing <140 nmol of H2O2) were removed and placed in test tubes containing 2 ml of the reagent solution (consisting of 10 ml of 0.2 mM meso-tetraakis(4-methylpyridyl)-porphyrin (IV) pentachloride solution, 10 ml of 41.2 μM N,N-dimethylamine in 0.2 M hydrochloric acid, 10 ml of 8.56 mM 3-methyl-2-benzothiazolinone hydrazone solution in 0.2 M hydrochloric acid, and 1 ml of 20 mM EDTA solution). The mixture was incubated at 25°C for 1 h, and then the absorbance at 590 nm was measured. The hydrogen peroxide removal reaction was carried out at 0.0175–0.14 mM H2O2 at 37°C. The kinetic parameters were obtained from Lineweaver-Burk plot analysis of the removal rates.

Glutathione peroxidase (GPX) activity was determined by the method of Wakimoto et al. (38) with some modification. The assay is an indirect measure of the activity of cellular GPX. Oxidized glutathione is recycled to its reduced form by glutathione reductase. The oxidation of NADPH resulting in NADP+ is accompanied by a decrease in absorbance at 340 nm so that GPX activity can be monitored. To assay GPX, the tissue homogenate was added to a solution containing glutathione, glutathione reductase, and NADPH. The enzyme reaction was started by adding tert-butyl hydroperoxide as a substrate, and the 340-nm absorbance was recorded every 5 s for 1 min. The rate of decrease in the 340-nm absorbance was directly proportionate to GPX activity in the sample. Xanthine oxidase (XO) activity was measured by adding 40 mM sodium carbonate buffer containing 10 mM xanthine (pH 10.2) to the tissue homogenate. The absorbance at 293 nm was measured every 5 s for 90 s. The rate of increase in 293-nm absorbance was proportional to XO activity. Total protein concentration was determined by the biuret method using bovine serum albumin as a standard.

Light microscopic studies. Obstructed or contralateral unobstructed kidneys were removed, fixed in 10% buffered formalin, and embedded in paraffin. Paraffin sections (3-μm thick) were stained with hematoxylin and eosin (HE) and Masson trichrome (28, 29). Two independent observers with no prior knowledge of the experimental design evaluated each tissue section using an Olympus light microscope (Olympus, Tokyo, Japan). The observers scored with a semi-quantitative scale designed to evaluate the degree of tubulointerstitial injury including tubular atrophy, dilation, simplification of the tubular lumen, and interstitial fibrosis. The severity of tubulointerstitial injury was scored from 0 to 4 as follows: 0, normal kidney; 1, mild change; 2, moderate change; 3, severe change; and 4, injury to the whole tissue. The degree of interstitial cell infiltration was scored in a range from 0 to 4 in the same manner as for the HE stain. The scores were determined in each section selected at random, and >20 fields were examined under ×100 magnification.

Electron microscopic studies. Electron microscopy was performed in the mouse kidney specimens as described previously (17, 31, 32). In brief, tissue blocks of kidney sections were immersed in 2.5% glutaraldehyde for 2 h at 4°C and postfixed with 1% osmium tetroxide. The blocks were then processed for routine dehydration, epon embedding, and thin sectioning and examined with an electron microscope (Hitachi, Tokyo, Japan). A cell was considered morphologically apoptotic if it displayed loss of cell volume, chromatid condensation along the nuclear membrane, or nuclear fragmentation into spherical structures containing condensed chromatin but was still surrounded by the cell membrane.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. DNA fragmentation associated with apoptosis was detected in situ by the addition of nucleotides to free 3′-hydroxyl groups in DNA as described previously (17, 31–33) using MEBSTAIN Apoptosis Kit Direct (Medical and Biological Laboratories, Nagoya, Japan). The formalin-fixed, paraffin-embedded sections were deparaffinized, stripped of proteins by incubation with proteinase K (20 μg/ml) for 30 min at 37°C, and then washed in distilled water. The sections were immersed in terminal deoxynucleotidyl transferase (TdT) buffer and then incubated with TdT and FITC-labeled dUTP at 37°C for 60 min. The reaction was terminated by transferring the slides to TB buffer (30 mM sodium citrate, 300 mM sodium chloride). The sections were washed and mounted in glycerol medium (Immunon, Pittsburgh, PA). Tissue sections for the positive control were treated with proteinase K, pretreated with DN buffer (30 mM Tris-HCl, pH 7.2, 140 mM potassium cacodylate, 4 mM MgCl2), followed by DNase I (0.7 μg/ml, Stratagene, La Jolla, CA), and then nick end-labeled as described above. The slides for the negative control were treated similarly except for the use of buffer lacking TdT. In kidney specimens, >20 of ×200 microscopic fields were examined in each animal at each time point with the use of an Olympus immunofluorescence microscope. The number of fluorescent-positive tubular or interstitial cells per field was determined to be the tubular or interstitial TdT-mediated dUTP nick end labeling (TUNEL) score, respectively.

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After the sections were washed three times with PBS, they were incubated with FITC-conjugated goat antirabbit IgG (dilution 1:1000; Nichirei, Tokyo, Japan) was used as the second antibody. Antibody for 30 min. Biotinylated rabbit anti-mouse IgG (dilution 1:1,000; Nichirei, Tokyo, Japan) used as the second antibody. Control sections using wild-type OBK sections were treated similarly but without addition of the primary antibody. Each section was washed three times in PBS and then incubated with the second antibody labeled with peroxidase (Nichirei). They were then placed in a diaminobenzidine-H2O2 solution, counterstained with hematoxylin, dehydrated, and enclosed in synthetic resin. Peroxidase products were determined on the basis of the intensity and distribution of deposition in the tubulointerstitium: 0, none or trace staining; 1, <10% positive; 2, 10–30% positive; 3, 30–70% positive; and 4, >70% positive. The score was determined in 20 randomly selected nonoverlapping ×200 fields in each section of the individual mouse renal cortex. The average number of scores from seven separate animals was calculated.

Indirect immunofluorescence was performed as described previously (28, 33). Briefly, surgically removed kidney specimens were immediately snap-frozen, and unfixed cryostat sections (4-μm thick) were prepared. The sections were washed in PBS three times, 5 min each, and then incubated with rabbit polyclonal antibodies against type IV collagen (dilution 1:30; LSL, Tokyo, Japan) as the primary antibody in PBS for 1 h at room temperature. Each section was washed three times in PBS and incubated with FITC-conjugated goat anti-rabbit IgG (dilution 1:80; Zymed Laboratories, San Francisco, CA) as the secondary antibody in PBS for 30 min. After the section was washed with PBS three times, it was mounted with fluoromount-G. Deposition of type IV collagen in the tubulointerstitium of renal cortex was assessed semiquantitatively by fluorescence microscopy as described previously (28, 33). The type IV collagen deposition score was determined on the basis of the intensity and distribution of type IV collagen in the tubulointerstitium: 0, none; 1, trace; 2, mild; 3, moderate; and 4, severe staining. The score was determined in each section selected at random, and >20 fields were examined under ×100 magnification.

Determination of serum malondialdehyde levels by measuring thiobarbituric acid-reactive substance. Blood samples were withdrawn from orbital veins of wild-type and acatalasemic mice under pentobarbital sodium anesthesia. Blood was collected into microcentrifuge tubes and was then immediately centrifuged to isolate the serum. The concentration of serum creatinine and blood urea nitrogen was measured by a standard assay. Levels of serum MDA were determined as an indicator of lipid peroxidation after a protocol described previously (24). Briefly, 0.083 N H2SO4 and 10% acetic acid were added to serum and centrifuged at 3,000 g for 10 min. Samples were mixed with 0.8% thiobarbituric acid and boiled for 1 h at 95°C. N-butanol was added, mixed for 2 min, and then centrifuged at 3,000 g for 10 min. The absorbance of the supernatant was measured at 515 nm by a spectrophotometer (F-2500, Hitachi, Tokyo, Japan). Serum MDA levels were expressed as nanomoles per milliliter.

Statistical analyses. Data are shown as means ± SE and were analyzed by the Mann-Whitney U-test or one-way ANOVA using the StatView program (Hulinks, Tokyo, Japan). P < 0.05 denoted the presence of a statistically significant difference.

RESULTS

Acatalasemia accelerates atrophy of OBK. Body weight was similar between both groups, and there were no significant changes in each group throughout the experiment (Table 1). As shown in Table 2, OBK weight of wild-type mice showed no significant changes through day 7. However, OBK weight of acatalasemic mice significantly decreased at day 7 compared with that at day 4. Moreover, it was significantly less than that of wild-type mice at day 7. CUK weight of acatalasemic mice significantly increased at day 7 and was significantly heavier than that of wild-type mice at day 7. Because the volume fraction of the tubulointerstitium is much larger than that of glomeruli, we further examined microscopic changes in renal tubulointerstitium in experimental animals.

Acatalasemia enhances tubular dilatation, atrophy, interstitial fibrosis, and deposition of type IV collagen in OBK. OBK uniformly developed obstructive nephropathy characterized by progressive tubulointerstitial changes, whereas the glomeruli and blood vessels remained normal throughout the experimental period. Acatalasemic OBK demonstrated significant tubulointerstitial injuries including tubular dilatation, atrophy, and simplification of the tubular epithelium compared with wild-type OBK (Fig. 1, A and B, E and F; Fig. 2A). It also showed more severe interstitial changes, including fibrosis, inflammatory cell infiltration (Fig. 2B), increased number of fibroblasts, and interstitial expansion, than wild-type OBK. Type IV collagen was clearly stained in interstitial spaces in wild-type OBK (Fig. 1G). In acatalasemic mice, there was significantly more deposition of interstitial type IV collagen in OBK at day 4 compared with that in wild-type mice (Fig. 1H). No significant changes were noted in the tubulointerstitium of sham-operated kidneys (Fig. 1, C and D) or CUK (data not shown) by light microscopy. There was no particular change in renal function (serum creatinine and blood urea nitrogen) as reported previously (Table 3) (28).

Excess lipid peroxidation in acatalasemic mice. Modification of proteins and lipids by oxidative stress is believed to play a central role in a variety of biological activities caused by increased oxidative stress, such as apoptosis and extracellular

<table>
<thead>
<tr>
<th>Table 1. Body weight of mice with unilateral ureteral obstruction</th>
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<tr>
<td>Wild-type, g</td>
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<td>Acatalasemic, g</td>
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Values are means ± SE of 7 animals/group. Sham, sham-operated.

<table>
<thead>
<tr>
<th>Table 2. Kidney/body weight ratio of mice with unilateral ureteral obstruction</th>
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<td></td>
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<tr>
<td>Wild-type</td>
</tr>
<tr>
<td>Left (ligated)</td>
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<tr>
<td>Right (contralateral)</td>
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<tr>
<td>Acatalasemic</td>
</tr>
<tr>
<td>Left (ligated)</td>
</tr>
<tr>
<td>Right (contralateral)</td>
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</table>

Values are means ± SE of 7 animals/group. *P < 0.05 vs. wild-type left (ligated) at day 7. †P < 0.05 vs. wild-type right (contralateral) at day 7. §P < 0.05 vs. acatalasemic left (ligated) at day 4. †§P < 0.05 vs. acatalasemic right (contralateral) at day 4.
matrix expansion (16, 30). The major content of cell membrane is lipid, and thus lipid peroxidation in tissue may cause renal injury. We next examined whether acatalasemia influenced lipid peroxidation products in the tubulointerstitial compart-

ment of OBK and in serum. There was an increase in HHE, MDA, or HNE antibody labeling of various patches in dilated tubules of wild-type OBK (Fig. 3, A and C). More intense antibody binding was observed in the cytoplasm of dilated

Fig. 1. Renal histology and type IV collagen deposition in wild-type or acatalasemic mice. Light micrographs of wild-type (A and higher magnification in E) or acatalasemic (B and higher magnification in F) obstructed kidneys and wild-type (C) or acatalasemic (D) sham-operated kidneys at day 7 are shown. Note significant tubular dilation, atrophy, and interstitial expansion in acatalasemic obstructed kidneys compared with wild-type. Sham-operated kidneys are microscopically normal. Immunofluorescent micrographs of wild-type (G) or acatalasemic (H) obstructed kidneys at day 4 stained with type IV collagen are also shown. Note increased deposition of type IV collagen in acatalasemic obstructed kidneys. A–F: Masson-trichrome stain. Scale bars: 500 (A and B) and 200 μm (C–H).
tubules in acatalasemic OBK (Fig. 3, B and D, and Table 4). HHE staining peaked at day 1, and MDA or HNE staining peaked at day 4 after UUO and decreased thereafter in acatalasemic OBK. Sham-treated kidneys or CUK gave a weak antibody reaction (data not shown). Serum MDA levels detected by the thiobarbituric acid-reactive substance (TBARS) method increased in acatalasemia 7 days after UUO, without a significant difference between the two groups (Table 3).

Table 3. Laboratory data of blood samples from mice with unilateral ureteral obstruction

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
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<tr>
<td>Serum creatinine, mg/dl</td>
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<tr>
<td>Wild-type</td>
<td>0.09±0.07</td>
<td>0.20±0.14</td>
<td>0.07±0.06</td>
<td>0.10±0.08</td>
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<tr>
<td>Acatalasemic</td>
<td>0.10±0.03</td>
<td>0.12±0.08</td>
<td>0.07±0.09</td>
<td>0.08±0.08</td>
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<tr>
<td>Blood urea nitrogen, mg/dl</td>
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<td></td>
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<tr>
<td>Wild-type</td>
<td>30.9±7.1</td>
<td>35.5±16.4</td>
<td>26.9±2.0</td>
<td>27.4±4.4</td>
</tr>
<tr>
<td>Acatalasemic</td>
<td>25.5±2.9</td>
<td>31.8±11.3</td>
<td>27.0±2.5</td>
<td>20.6±13.1</td>
</tr>
<tr>
<td>TBARS, nmol/ml</td>
<td></td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>9.2±0.8</td>
<td>6.4±0.1</td>
<td>8.5±3.0</td>
<td>13.9±12.7</td>
</tr>
<tr>
<td>Acatalasemic</td>
<td>8.7±1.3</td>
<td>9.0±4.7</td>
<td>6.6±1.2</td>
<td>22.6±19.0</td>
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</table>

Values are means ± SE of 7 animals/group. TBARS, thiobarbituric acid-reactive substance.

Acatalasemia sensitizes tubular epithelial cells to apoptosis in OBK. A morphological study suggested that cell deletion by apoptosis may play an important role in the pathogenesis of renal tubular atrophy associated with obstructive nephropathy in rats (5). Because oxidative stress could induce apoptosis in tubulointerstitial cells, we next investigated whether oxidative stress enhanced by acatalasemia sensitized renal cells to apoptosis. The TUNEL technique quantitatively demonstrated a significant increase in the total number of positive cells in acatalasemic OBK compared with wild-type (Fig. 4, A and B, and Fig. 5A). Apoptosis in acatalasemic OBK peaked at day 4 and decreased thereafter, suggesting tissue fibrosis replaced the tubulointerstitial cell compartment at day 7. Tubular epithelial cell apoptosis consisted of >90% of total positive cells, indicating that the contribution of interstitial cell apoptosis was relatively low in the acatalasemic UUO model. The time course study demonstrated that tubular apoptosis in the cortex (Fig. 5B) and medulla (Fig. 5C) showed a similar pattern. To examine which parts of the tubular cells were undergoing apoptosis, an electron microscopic study was performed. Apoptosis was observed in both distal (Fig. 4C) and proximal (Fig. 4D).
4D) tubular epithelial cells in acatalasemic OBK. It was also observed in collecting duct cells (data not shown). Apoptosis is known to proceed along a pathway characterized by activation of a caspase cascade. Therefore, we examined caspase 9 expression, which recently was shown to play a role in the mitochondrial pathway as an effector molecule for apoptosis (14) and to be activated in a murine UUO model (37). Immunostaining for caspase 9 demonstrated that both precursor and active subunits were strongly expressed in atrophic tubules of acatalasemic OBK (Fig. 4F) compared with wild-type (Fig. 4E).

No significant compensation of GPX for catalase in acatalasemic OBK. Renal catalase activity from acatalasemic mice showed a 4.6-fold ($P < 0.01$) decrease compared with wild-type mice at the start of the experiment, and it remained low during the experiment (Fig. 6A). The renal catalase activity in wild-type mice continued to decrease until day 7 after UUO.

### Table 4. Accumulation of lipid peroxidation products in obstructed kidneys of mice with unilateral ureteral obstruction

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4-Hydroxy-2-hexenal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.60±1.49</td>
<td>2.50±1.51</td>
<td>3.00±1.73</td>
<td>2.83±2.22</td>
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<tr>
<td>Acatalasemic</td>
<td>0.80±1.66</td>
<td>4.71±1.97</td>
<td>3.75±1.66</td>
<td>2.57±1.81</td>
</tr>
<tr>
<td><strong>Malondialdehyde</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.00±1.00</td>
<td>2.50±0.50</td>
<td>1.00±1.00</td>
<td>5.00±1.00*</td>
</tr>
<tr>
<td>Acatalasemic</td>
<td>1.00±1.00</td>
<td>3.00±1.00</td>
<td>5.00±1.00†</td>
<td>4.50±0.50</td>
</tr>
<tr>
<td><strong>4-Hydroxy-2-nonenal</strong></td>
<td></td>
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<tr>
<td>Wild-type</td>
<td>0.90±1.81</td>
<td>3.00±1.41*</td>
<td>3.83±1.06*</td>
<td>3.42±1.76*</td>
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<tr>
<td>Acatalasemic</td>
<td>0.50±1.05</td>
<td>4.29±1.74†</td>
<td>5.14±1.64†</td>
<td>3.29±2.05</td>
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</table>

Values are means ± SE of 7 animals/group. *$P < 0.05$ vs. wild-type sham. †$P < 0.05$ vs. acatalasemic sham. ‡$P < 0.05$ vs. wild-type at the same time point.

Fig. 4. Tubular epithelial cell apoptosis and expression of caspase 9 in obstructed kidneys of wild-type or acatalasemic mice. Fluorescent micrographs of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive tubular epithelial cells (arrowheads) in wild-type (A) or acatalasemic (B) obstructed kidneys at day 7 are shown. Electron micrographs of tubular epithelial cell apoptosis (arrows) in dilated distal tubules (C) and higher magnification of apoptosis (white arrow) in proximal tubule (D) of acatalasemic obstructed kidneys at day 7 are demonstrated. Note that brush borders (white asterisks; D) are clearly visible in a proximal tubular epithelial cell. Immunoperoxidase staining of caspase 9, a marker of mitochondria-derived apoptosis, in wild-type (E) or acatalasemic (F) obstructed kidneys at day 7 are also shown. TL, tubular lumen. Scale bars: 100 (A, B, E, F), 10 (C), and 3 μm (D).
Because high catalase levels are found in erythrocytes (23), we removed residual blood by perfusing kidneys with saline and compared catalase activity between perfused and unperfused kidneys. The catalase activity of saline-perfused kidneys was 6.0 ± 0.2% lower than that of unperfused kidneys. To examine the effect of acatalasemia on other renal antioxidant enzymes, we investigated the activity of GPX (Fig. 6B) in OBK of wild-type or acatalasemic mice. However, there was no compensatory upregulation of GPX in acatalasemic OBK. Moreover, there were no significant changes in XO activity between the two mice groups (Fig. 6C). Because XO produces superoxide anion from oxygen during the conversion of hypoxanthine to xanthine, it is suggested that the level of reactive oxygen species production mediated by XO may be similar in both OBK.

DISCUSSION

In the present study, we induced UUO-mediated renal tubulointerstitial injury in acatalasemic as well as wild-type mice and compared the progression of renal tubular injury and fibrosis associated with oxidative stress. The results showed that acatalasemia enhanced lipid peroxidation, sensitized tubular epithelial cells to apoptosis, and accelerated tubular atrophy and interstitial fibrosis. The data demonstrated a mechanistic insight into the oxidative stress-induced renal tubulointerstitial injury in acatalasemic disease conditions.

Catalase is a major enzyme that catalyzes the decomposition of H₂O₂ (1). The mammalian catalase has a molecular mass of ≈240,000 Da and forms tetrahomodimers. The enzyme is localized in the matrix of peroxisomes in mammalian cells. High catalase levels are found in the liver, kidney, and erythrocytes in mammals. Distribution of catalase in the kidney is restricted to the proximal tubules and not to the distal, Henle’s loops, collecting duct tubules, or glomeruli. The expression of catalase in mice is stronger in the proximal tubules in the juxtamedullary cortex rather than in the superficial cortex, as detected by immunohistochemistry (43). This may partly explain our findings that tubulointerstitial injury in acatalasemic OBK is prominent in the deep cortex and medulla (Fig. 1). It is speculated that UUO itself could injure various areas other than the proximal tubules.

Genetic defects of catalase were first described by Takahara (34, 35) in Japanese individuals who exhibited a deficiency of blood catalase enzyme activity (acatalasemia). Acatalasemia was first believed to be a molecular disease specific to some races, but patients with acatalasemia were also found in several different countries (7, 23). Short-term clinical manifestations of human acatalasemia appear predominantly in the mouth. In moderate cases, oral ulcerations develop, whereas more severe forms manifest as alveolar gangrene and atrophy, resulting in widespread loss of teeth. No long-term health effects of intracellular catalase deficiency in humans have been reported. So far, there are no precise reports of renal injury in patients with acatalasemia. Our findings should provide an important mechanistic insight into the treatment of these patients.

Two major antioxidant enzymes, catalase and GPX, are involved in degradation of H₂O₂ into nontoxic water and oxygen. These enzymes act in a complementary fashion to metabolize H₂O₂ generated by the course of metabolic and other processes in the kidney. GPX constitutes the first, and catalase the second, line of defense against the buildup of H₂O₂. GPX represents a high-affinity, low-capacity degradative system, whereas catalase represents a relatively low-affinity, high-capacity system. We first speculated that GPX could compensate for catalase deficiency in acatalasemic OBK; however, no compensatory upregulation of GPX was recognized in OBK (Fig. 6B) or CUK homogenate (data not shown).
It is speculated that protein synthesis including antioxidant enzymes may be strongly inhibited in UUO kidney (12).

We observed that UUO induced a gradual decrease in both catalase and GPX activities in wild-type mice (Fig. 6, A and B). This finding is consistent with the previous report that mRNA and proteins of catalase and Cu, Zn-SOD are downregulated in the UUO kidney (25). Moreover, we demonstrated that catalase activity in the kidney was persistently low regardless of UUO in acatalasemic mice (Fig. 6A). The transcriptional regulation of catalase gene and protein is reported. Catalase mRNA is expressed in acatalasemic animals, but point mutation in the intron causes abnormality of alternative splicing to produce abnormal proteins. The mutation is purely structural, being characterized by modification of the enzyme active site but not of the antigenic site (4). Our preliminary study suggested that catalase immunoreactivity is still present in acatalasemic mouse kidney (unpublished observations).

Oxidative stress can occur as a result of either excess reactive oxygen species production, an impaired antioxidant system, or a combination. The acceptance by oxygen of one electron, as occurs during the respiratory burst of mitochondria and that is effected by the enzymes XO, NADH/NADPH oxidase, lipooxygenase, cyclooxygenase and so on, yields superoxide anion (O$_2^-$). This anion, in turn, is converted to H$_2$O$_2$ by the action of SOD. We found no differences in XO activity between OBK of acatalasemic and wild-type mice (Fig. 6C), suggesting that the production of reactive oxygen species, at least in part mediated by XO, may be similar in both OBK, although we have not examined the activity of SOD yet.

Gobe and Alxelsen (5) were the first to report the role of apoptosis in progressive renal tubular atrophy of hydronephrosis in rats. Apoptosis is generally known to proceed along distinct pathways, which later converge into a common pathway characterized by activation of a caspase cascade. Caspases (cysteinyl aspartate-specific proteinase) are cytosolic enzymes that belong to a family with 14 members, 12 of which are found in mammals. We showed increased caspase 9 expression in diluted tubules of acatalasemic mice compared with wild-type mice. Caspase 9 may play a role in the mitochondrial pathway as an effector molecule for apoptosis (14). Mitochondria can generate O$_2^-$ by converting oxygen, and thus they are also an important source of free radicals. It is reported that several caspases were activated in a murine UUO model (37).

Immunostaining for caspase 9 demonstrated that both precursor and active subunits were strongly expressed in atrophic tubules of acatalasemic OBK (Fig. 4), suggesting the involvement of mitochondria in the development of apoptosis in acatalasemic tubular cells. Excess oxidative stress can induce necrotic cell death in tubular epithelial cells in vitro (15); however, we did not observe cell necrosis in acatalasemic animals by light or electron microscopy in this study.

We found a significant accumulation of lipid peroxidation products in diluted tubules of acatalasemic UO kidney (Fig. 3). This was associated with increased tubular cell apoptosis in acatalasemia (Fig. 4). Kawada et al. (11) reported increased oxidative stress in the interstitium of UUO kidneys. The formation of reactive oxidative products detected by immunoreactivity with Nε-carboxymethyl-lysine and heme oxygenase-1 in the interstitium is supposed to play important roles in the UUO kidney. Ricardo et al. (25) reported increased formation of O$_2^-$ and H$_2$O$_2$ in slice cultures from OBK at 96 h. Our finding indicates the presence of increased oxidative stress, in particular in tubules of acatalasemic animals, and it may be involved in tubular cell loss by apoptosis and the subsequent progression of tubular atrophy. There is a discrepancy between early increase in the deposition of lipid peroxidation products in the kidney from day 1 (Table 4) and relatively late increase in serum TBARS at day 7 with acatalasemia (Table 4), suggesting that peroxidation of cell and tissue is an early molecular event. We investigated peroxidation products in OBK by immunohistochemistry at day 14, but no significant changes between wild-type and acatalasemic mice were observed (data not shown). It is speculated that tissue fibrosis was so severe after 14 days of obstruction that there were few tubulointerstitial cells to be peroxidized.

Currently, there are few reports regarding factors worsening tubulointerstitial injury in the UUO model (21, 42). Our data that catalase deficiency accelerates renal tubulointerstitial injury will give new insight into the treatment of tubulointerstitial disease. Consequently, future work on the development of novel therapeutic strategies, including catalase supplementation (41) or detoxification of hydroxyl radicals (27) by utilizing acatalasemic mice, should be sought. To conclude, acatalasemia exacerbates tubulointerstitial injury by excess oxidative stress and tubular cell apoptosis. Acatalasemic mice become a good model for investigating the mechanism of oxidative stress-induced renal injury, because there have been no reports of catalase gene knockout mice until now.

ACKNOWLEDGMENTS

We thank T. Hashimoto and Y. Saito for assistance in electron microscopy and S. Kameshima and S. Ariyoshi for technical assistance. We also are grateful to Prof. K. Takei in the Department of Biochemistry and Dr. H. Yamamoto in the Department of Public Health, Okayama University Graduate School of Medicine and Dentistry, and Prof. N. Kashihara in the Division of Nephrology, Department of Internal Medicine, Kawasaki Medical School, Kurashiki, Japan, for support.

GRANTS

This work was supported by Research Grant C-15590851 (to H. Sugiyama) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

REFERENCES

ACATALASEMIA SENSITIZES RENAL TUBULAR CELLS TO APOPTOSIS


