Water restriction increases renal inner medullary manganese superoxide dismutase (MnSOD)

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Zhou X, Burg MB, Ferraris JD. Water restriction increases renal inner medullary manganese superoxide dismutase (MnSOD). Am J Physiol Renal Physiol 303: F674–F680, 2012. First published June 20, 2012; doi:10.1152/ajprenal.00076.2012.—Oxidative stress damages cells. NaCl and urea are high in renal medullary interstitial fluid, which is necessary to concentrate urine, but which causes oxidative stress by elevating reactive oxygen species (ROS). Here, we measured the antioxidant enzyme superoxide dismutases (SODs, MnSOD, and Cu/ZnSOD) and catalase in mouse kidney that might mitigate the oxidative stress. MnSOD protein increases progressively from the cortex to the inner medulla, following the gradient of increasing NaCl and urea. MnSOD activity increases proportionately, but MnSOD mRNA does not. Water restriction, which elevates renal medullary NaCl and urea, increases MnSOD protein, accompanied by a proportionate increase in MnSOD enzymatic activity in the inner medulla, but not in the cortex or the outer medulla. In contrast, Cu/ZnSOD and TNF-α (an important regulator of MnSOD) do not vary between the regions of the kidney, and expression of catalase protein actually decreases from the cortex to the inner medulla. Water restriction increases activity of mitochondrial enzymes that catalyze production of ROS in the inner medulla, but reduces NAPDH oxidase activity there. We also examined the effect of high NaCl and urea on MnSOD in Madin-Darby canine kidney (MDCK) cells. High NaCl and high urea both increase MnSOD in MDCK cells. This increase in MnSOD protein apparently depends on the elevation of ROS since it is eliminated by the antioxidant N-acetylcysteine, and it occurs without raising osmolality when ROS are elevated by antimycin A or xanthine oxidase plus xanthine. We conclude that ROS, induced by high NaCl and urea, increase MnSOD activity in the renal inner medulla, which moderates oxidative stress.

Antioxidant defenses include many antioxidants and enzymes. Which antioxidants or enzymes are upregulated depends on the nature of oxidative stress. For example, in oxidative stress associated with lead-induced hypertension, Cu/ZnSOD is increased in the brain and kidney, whereas MnSOD, catalase, or glutathione peroxidase remains unchanged (31). In celecoxib-induced oxidative stress, catalase activity is increased, while glutathione peroxidase activity is decreased, and SOD and glucose-6-phosphate dehydrogenase activities are unchanged (29). In general, superoxide is the ROS that is initially produced, and it is dismutated by SODs into hydrogen peroxide, which is less reactive. Hydrogen peroxide is then decomposed by catalase and peroxidases into water and oxygen. Therefore, SODs are a first line of defense against ROS. SODs can be divided into three categories according to the ion used as a cofactor and subcellular localization. The categories are mitochondrial MnSOD, cytosolic Cu/ZnSOD, and an extracellular form of Cu/ZnSOD (EC-SOD) (8). In the present study, we tested the hypothesis that hyperosmolality induces MnSOD, Cu/ZnSOD, or catalase by examining expression of these antioxidant enzymes in mouse kidney medullas before and after water restriction, which increases renal inner medullary interstitial osmolality. We find that hyperosmolality only significantly increases MnSOD protein abundance and activity in the inner medulla. Using cultured mouse inner medullary collecting duct (mIMCD3) and Madin-Darby canine kidney (MDCK) cells as models, we reveal that both high NaCl and urea increase MnSOD protein abundance.

REDOX STATE IS CRITICAL FOR cellular function, and redox balance is closely regulated by opposing pro- and antioxidant systems. Cells constantly produce reactive oxygen species (ROS) through aerobic metabolism. Under normal physiological conditions, ROS are rapidly detoxified by cellular antioxidants and antioxidant enzymes, thus minimizing oxidative stress. Disregulation by excessive production of ROS, suppression of antioxidants, or both, increases ROS, resulting in harmful oxidation of cellular constituents. Oxidative stress occurs in many illnesses, such as cardiovascular diseases (27), kidney diseases (11), and cancer (25).

Interstitial fluid in the kidney medulla contains high levels of NaCl and urea, which provide an osmotic gradient to concentrate urine. High NaCl and urea increase ROS in cell culture (36) and in renal medullas in vivo (36). High NaCl also increases ROS in isolated hypotalamai (12) and in renal thick ascending limbs (28). High NaCl-induced ROS come mainly from mitochondria (35, 37). The ROS induced by high NaCl and urea oxidize (carboxylate) proteins, both in cell culture and in the kidney medulla in vivo (36). Nevertheless, despite elevated ROS, renal medullary cells normally are healthy, which suggests that hyperosmolality also induces antioxidant defense mechanisms. The purpose of the present studies was to identify those mechanisms in mouse renal medullas.

MATERIALS AND METHODS

Animal studies. C57BL/6 mice (3–5 mo old), purchased from Jackson Laboratory, were handled according to the procedures approved by Uniformed Services University Institutional Animal Care and Use Committee. The numbers of females and males were roughly equal. Mice were treated as outlined below. Mice were fed with gelled food (10) composed of mouse food powder (OpenSource Diet), water, and agar. Mice kept in metabolic cages were initially acclimated to gelled food containing 4 g food powder, 0.09 g agar, and 5 ml water/20 g body wt−1·24 h−1 (10). Then, the mice were randomly divided into two groups. One group (control) continued the same diet. The other group was subjected to water restriction by reducing the amount of water to 20% of the control. The water-restricted food was administered in 4 g food powder, 0.05 g agar, and 1 ml water/20 g body wt−1·24 h−1. Urine was collected every 24 h under mineral oil to prevent evaporation. Mice were weighed daily. After 72 h, the control and water-restricted mice
were euthanized. Both kidneys of each mouse were quickly excised and immediately sectioned on ice into the cortex, outer medulla, and inner medulla, including the papilla. Tissues were homogenized in 10 mM triethanolamine, pH 7.4, and 250 mM sucrose. The homogenates were used to measure the amounts of proteins (Western blot analysis), the activity of MnSOD and NADPH oxidase, and the ability of mitochondria to generate ROS. The protein concentrations in homogenates were measured with a bicinchoninic acid assay (Pierce).

Cells and chemicals. Subconfluent mIMCD3 cells (26) between passages 14 and 20 were cultured in low-glucose Dulbecco’s modified Eagle’s medium/Ham’s F-12 (1:1), containing 10% fetal bovine serum and 2 mM t-glutamine in 5% CO₂-95% air at 37°C (21). Subconfluent MDCK cells (ATCC) were incubated in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum. Cells remained subconfluent at the end of treatment at both tonicities. All chemicals were purchased from Sigma-Aldrich.

Western blot analysis. Protein samples were fractionated in NuPage 4–12% Bis-Tris gels and then transferred to nitrocellulose membranes (Invitrogen) according to the manufacturer’s protocol. The membranes were first blocked with Odyssey Blocking Buffer for 60 min at room temperature (Li-Cor) and then incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: mouse anti-MnSOD for mouse kidney (catalog no. MAB4081, Chemicon), rabbit anti-MnSOD for cultured cells (catalog no. 06-984, Upstate Biotechnology), rabbit anti-CuZnSOD (catalog no. 07–403, Upstate Biotechnology), rabbit anti-catalase (catalog no. ab15834, Abcam), rabbit anti-TNF-α and GAPDH (catalog nos. 3707 and 2118, respectively, Cell Signaling), rabbit anti-p47phox (catalog no. sc-14015, Santa Cruz Biotechnology), and mouse anti-grp94 (catalog no. 611414, BD Transduction Laboratories). After incubation with primary antibodies and a brief wash, the membranes were incubated with Alexa Fluor-conjugated secondary antibodies at room temperature for 60 min. Protein expression was analyzed by an Odyssey Infrared Imager (Li-Cor).

Qualitative PCR. Mouse tissues were sonicated with an ice-cold RNAzol RT kit (Molecular Research Center). Total RNA from mIMCD3 cells was extracted with an RNA Extraction minikit (Qiagen). cDNAs were synthesized with a High Capacity cDNA Reverse Transcription Kit using 100 ng total RNA/μl reaction buffer as recommended (Applied Biosystems). Expression of MnSOD mRNA was measured by Taqman-based qualitative PCR (qPCR) in an Applied Biosystems 7900HT with the forward primer 5'-TGGGTGTGTTGTCTTCCA-3', the reverse primer 5'-GACCTGAATACCCACTTCA-3', and the probe 5'- CCGTCTCTCGAGACATGAAGCC-3'. This set was designed using a GenScip online program. The amount of 200 ng total inner medulla RNA/reaction or 8 ng total cell RNA/reaction was used for qPCR. 18S rRNA was used to control for the amount of cDNA used in each analysis. Hyperosmolality does not affect expression of 18S rRNA (4). The 18S rRNA primers and probe were purchased from Applied Biosystems.

MnSOD activity. MnSOD activity was measured fluorometrically (39). This method measures reduction of superoxide generated by a xanthine/xanthine oxidase system. Briefly, the tissue homogenate (20 μg/assay) was preincubated in a black viewplate (Packard) with 5 mM NaCN at room temperature for 10 min to inhibit CuZnSOD activity (30), then incubated in an assay buffer containing 50 mM K₃PO₄ pH 7.8, 1 mM DETAPAC, 1 unit catalase, 0.5 mg/ml salmon sperm DNA, 0.1 mM xanthine, 50 μM xanthine oxidase, and 10 μM dihydroethidium (freshly prepared) at room temperature for 5 min. Fluorescence was excited at 485 nm and recorded at 610 nm (39). The difference in readings without and with tissue homogenates represents MnSOD activity.

Mitochondrial enzyme and NADPH oxidase activities. Mitochondrial enzyme activity to produce ROS was measured in tissue homogenates, as previously described (39). Briefly, the homogenate (20 μg/assay) was incubated in 50 mM K₃PO₄ pH 7.8, 1 mM DETAPAC, 1 unit catalase, 0.5 mg/ml salmon sperm DNA, 80 μM amytacycin A, 5 mM succinate, and 10 μM dihydroethidium in a black viewplate at 37°C for 30 min.

To measure NADPH oxidase activity, amytacycin A and succinate were replaced by 0.1 mM NADPH. Fluorescence was measured with excitation at 485 nm and emission at 610 nm.

Statistical analyses. Results are expressed as means ± SE. For unpaired comparisons of mouse kidneys, the mean result for the first mouse in the control group was set to one, and all other results were normalized to that value. Statistical analyses were performed by a two-tail paired or unpaired t-test or repeated measures ANOVA. Post hoc comparison was made by a Tukey analysis. P ≤ 0.05 is considered significant.

RESULTS

Expression of MnSOD protein and MnSOD activity increase progressively from the mouse renal cortex to the inner medulla, but MnSOD mRNA does not differ between the regions. In kidneys from mice given water ad libitum, the abundance of MnSOD protein (Fig. 1, A and B) and MnSOD activity (Fig. 1C) increase progressively from the cortex to the outer medulla to the inner medulla. MnSOD protein abundance is 2.34-fold higher in the inner medulla, including the papilla. MnSOD protein abundance increases progressively from the cortex (Cor) to inner medulla to outer medulla. MnSOD activity increases progressively from the Cor to IM. D: MnSOD mRNA does not differ between the regions. *P < 0.05 vs. Cor. #P < 0.05 vs. the OM (repeated measures ANOVA, n = 3).

Table 1. Effect of water restriction on urine and plasma osmolality and body weight

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Water Restriction</th>
<th>P Value</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine osmolality, mosmol/kgH₂O</td>
<td>2.201 ± 0.206</td>
<td>4.780 ± 4.3</td>
<td>&lt;0.0005</td>
<td>6</td>
</tr>
<tr>
<td>Plasma osmolality, mosmol/kgH₂O</td>
<td>304 ± 4.3</td>
<td>338 ± 11.0</td>
<td>&lt;0.05</td>
<td>3</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>23.1 ± 3.9</td>
<td>20.4 ± 4.1</td>
<td>0.65</td>
<td>3</td>
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the inner medulla than in the cortex (Fig. 1B), and MnSOD activity is 3.25-fold higher (Fig. 1C). Importantly, the progressive increases in MnSOD between these regions correlate with increasing osmolality (mainly NaCl and urea) between the regions (9). In contrast, MnSOD mRNA does not differ between the kidney regions (Fig. 1D). Also, the abundance of catalase protein decreases from the cortex to the inner medulla (Fig. 1, A and B), but the abundance of Cu/ZnSOD protein and of TNF-α protein does not differ between the kidney regions (Fig. 1, A and B).

Water restriction increases MnSOD protein abundance and enzymatic activity in the mouse renal inner medulla. As noted above, the corticomedullary gradient of MnSOD expression parallels a corresponding increase in osmolality within the kidney. Water restriction elevates urinary and medullary os-
molality. We restricted water to test further the relationship between hyperosmolality and MnSOD. Water restriction significantly increases urine and plasma osmolality but has no significant effect on body weight (Table 1). After the 3 days of water restriction, the abundance of MnSOD protein increases by 2.49-fold in the inner medulla (Fig. 2, A and B) and MnSOD activity increases by 2.62-fold (Fig. 2C). The essentially proportionate increase in MnSOD protein and enzyme activity indicates that the activity increases because more enzyme protein is present. In contrast, water restriction does not significantly change MnSOD protein in the outer medulla or the cortex (Fig. 3). Water restriction also does not significantly affect expression of Cu/ZnSOD, catalase, or TNF-α protein in the cortex or the outer medulla (Fig. 3). We conclude that water restriction increases MnSOD activity in the kidney inner medulla, most likely because the resulting increased inner medullary interstitial osmolality elevates the amount of MnSOD protein.

High NaCl increases abundance of MnSOD protein in both mIMCD3 and MDCK cells, and high urea only increases MnSOD protein in MDCK cells. NaCl and urea are the main determinants of renal inner medullary interstitial hyperosmolality. We used mIMCD3 and MDCK cells to assess the importance of NaCl and urea for elevating MnSOD. In mIMCD3 cells, high NaCl increases the abundance of MnSOD protein, but high urea does not (Fig. 4A). The high NaCl-induced increase in MnSOD protein occurs within 8 h after NaCl is added (Fig. 4B). As with water restriction in the inner medulla, high NaCl does not affect MnSOD mRNA expression (Fig. 4C). In MDCK cells, both high urea and high NaCl increase the abundance of MnSOD protein (Fig. 4D). We propose that the water restriction-induced increase in MnSOD protein in the mouse renal inner medulla most likely is caused by the increase in interstitial NaCl and, possibly, also by the increase in urea.

**High NaCl-induced ROS mediate the high NaCl-induced increase in MnSOD protein.** Knowing that the antioxidant N-acetylcysteine prevents high NaCl-induced increase of ROS (35, 38), we applied it to determine whether ROS are necessary for the high NaCl-induced increase in MnSOD protein. Addition of N-acetylcysteine (10 mM) completely eliminates the effect of high NaCl on MnSOD protein expression in mIMCD3 cells (Fig. 5A), showing that ROS are necessary. To determine whether ROS are sufficient to elevate MnSOD protein, we increased ROS without increasing NaCl. Antimycin A increases mitochondrial production of ROS (22), and xanthine oxidase plus xanthine produces superoxide. When antimycin A (1 μM) or xanthine oxidase (32 mM) plus xanthine (100 μM) is added to mIMCD3 cells, MnSOD protein abundance increases, even in the absence of added NaCl (Fig. 5B). We conclude that high NaCl-induced elevation of ROS is necessary and sufficient to produce the high NaCl-induced increase in MnSOD protein.

Water restriction increases activity of the mitochondrial enzymes that produce ROS in the renal inner medulla, but decreases NADPH oxidase activity. To further determine what enzymatic activity might be responsible for the increase in

![Fig. 4. A: high NaCl, but not high urea, elevates MnSOD protein in mIMCD3 cells. Additions of 250 mosmol/kg H2O of NaCl (N550) or urea (U550) were made to the 300 mosmol/kg H2O control medium for 24 h. *P < 0.05 vs. control, n = 3, paired t-test. B: time course of high NaCl-induced increase in MnSOD protein in mIMCD3 cells. MnSOD protein abundance was measured at the indicated times after NaCl was increased. *P < 0.05 vs. control at time 0, repeated measures ANOVA, n = 3. C: high NaCl does not increase MnSOD mRNA in mIMCD3 cells. Procedure was as in B, except MnSOD mRNA was measured. D: high NaCl and high urea both elevate MnSOD protein in Madin-Darby canine kidney (MDCK) cells. MDCK cells were treated as in A.]
inner medullary ROS caused by water restriction, we measured the activity of two possible sources of the ROS, namely, mitochondrial enzymes and NADPH oxidase. Water restriction causes a 71% increase in the generation of ROS by mitochondrial enzymes and NADPH oxidase. Water restriction not only increases it by 41% (Fig. 6B), but reduces NADPH oxidase activity there. High NaCl and high urea both increase MnSOD in MDCK cells. The antioxidant N-acetylcysteine eliminates the effect of high NaCl on MnSOD. ROS elevated by antimycin A or xanthine oxidase plus xanthine without raising osmolality simulates the effect of high NaCl on MnSOD. We conclude that ROS, induced by high NaCl and urea, increase MnSOD activity in the renal inner medulla, which moderates the oxidative stress.

MnSOD is an 88.6-kDa homotetramer that is synthesized in the cytosol and imported into the mitochondrial matrix (1–3, 14, 32). Each subunit contains one Mn atom that cycles from Mn(III) to Mn(II), then back to Mn(III), during two-step dismutation of superoxide (3). MnSOD is critical for protecting cells from oxidative stress. Mice whose MnSOD is knocked out die of oxidative injury within 3 wk of age (16, 18). MnSOD activity is low during the injurious oxidative stress associated with diabetic nephropathy (15), transplant rejection (20), and ischemia-reperfusion injury (7). On the other hand, MnSOD can be a prooxidant, as well as an antioxidant. In the presence of nitric oxide, MnSOD utilizes hydrogen peroxide to produce superoxide and the even more toxic oxidant peroxynitrite (19).

Our present study shows that water restriction increases the amount of MnSOD protein in mouse renal inner medullary cells in vivo (Fig. 2, A and B), accompanied by a proportionate increase in MnSOD activity (Fig. 2C). Since MnSOD mRNA does not change (Fig. 2D), the increased protein expression of MnSOD apparently results from faster translation of the existing mRNA or slower degradation of the protein. We do not know which mechanism is responsible. A redox-sensitive protein was found to bind to the 3'-untranslated region of MnSOD RNA and enhance MnSOD translation, but the protein was not identified (5). Also, various microRNAs, including miR-17* (34), regulate MnSOD at the translational level.

High NaCl increases MnSOD protein in mIMCD3 cells in culture (Fig. 4, A and B), and both high NaCl and high urea increase MnSOD protein in MDCK cells (Fig. 4D). Based on these results, we suggest that elevation of inner medullary interstitial NaCl and urea during water restriction raises MnSOD protein, which ameliorates the harmful effects of the accompanying increase in ROS by dismutating the additional superoxide that is produced. Oxidative stress in the kidney medulla is greater in mice with kidney-specific knockout of MnSOD (24), which supports this idea. To investigate it further, we plan to compare renal medullary function and cell death in response to water restriction in knockout vs. wild-type mice.

High NaCl-induced ROS are a byproduct of mitochondrial oxidative metabolism, as shown by experiments in cell culture (35, 37). Po2 decreases progressively from the cortex to inner medulla (6). Nevertheless, oxidative metabolism is the principal source of energy in the renal inner medulla despite a low Po2 of ~25 mmHg. The oxygen, although low, supports a rate of mitochondrial aerobic metabolism in the inner medulla.

**DISCUSSION**

We find that MnSOD protein and activity increase progressively from the cortex to inner medulla along with the increase in NaCl and urea, but MnSOD mRNA does not. Cu/ZnSOD and TNF-α (an important regulator of MnSOD) do not vary between the regions of the kidney, and expression of catalase protein actually decreases from the cortex to inner medulla. Water restriction increases MnSOD protein in the inner medulla, but not in the cortex or outer medulla, and has no significant effect on protein abundance of Cu/ZnSOD, catalase, and TNF-α in the cortex, outer medulla, or inner medulla. Water restriction increases the activity of mitochondrial enzymes that catalyze production of ROS in the inner medulla, but reduces NADPH oxidase activity there. High NaCl and high urea both increase MnSOD in MDCK cells. The antioxidant N-acetylcysteine eliminates the effect of high NaCl on MnSOD. ROS elevated by antimycin A or xanthine oxidase plus xanthine without raising osmolality simulates the effect of high NaCl on MnSOD. We conclude that ROS, induced by high NaCl and urea, increase MnSOD activity in the renal inner medulla, which moderates the oxidative stress.
that is similar to that in the liver and higher than that in skin or muscle (6). Most of the ATP in the inner medulla is produced by mitochondrial oxidative phosphorylation, rather than anaerobic glycolysis (6). Mitochondria and NADPH oxidase are the major source of ROS in the renal medulla, and in the papilla mitochondria are essentially the only source of ROS (39). The present study shows that water restriction increases activity of mitochondrial enzymes that catalyze production of ROS, but inhibits NADPH oxidase activity (Fig. 6). These data together with our own and other previous studies in cell culture show that high NaCl-induced ROS derive mainly from mitochondria and support the argument that water restriction increases mitochondrial production of ROS in the inner medulla. Mitochondria produce ROS through premature leakage of electrons to oxygen molecules from respiratory chains. High NaCl could increase leakage of electrons by reorganizing the cytoskeleton or by compression of lipid voids (37). Increased MnSOD in mitochondria may be essential, since, otherwise, mitochondria have limited protection from oxidative stress (13).

Catalase catalyzes decomposition of hydrogen peroxide to water and oxygen. Expression of catalase protein decreases from the cortex to the inner medulla (Fig. 1, A and B) and does not increase in response to water restriction (Fig. 2, A and B). However, being unaware of measurements of hydrogen peroxide in the renal medulla in response to water restriction, we cannot evaluate whether it changes. Furthermore, catalase is not the only enzyme that decomposes hydrogen peroxide. Thioredoxins, peroxiredoxins, peroxidases, and reductases, some of which are located in kidney mitochondria, have the same function (23). Whether water restriction affects these enzymes is unknown.

TNF-α increases MnSOD mRNA (33), and high NaCl stimulates synthesis of TNF-α in human limbal epithelial cells (17), which suggested that increased TNF-α might be involved in the water restriction-induced increase in MnSOD protein in the renal inner medulla. However, our results do not support that possibility since TNF-α protein is not higher in the renal inner medulla than in other regions of the kidney (Fig. 1, A and B), and water-restriction does not affect the amount of TNF-α in the inner medulla (Fig. 2, A and B) or increase MnSOD mRNA there (Fig. 1D).

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

Author contributions: X.Z. provided conception and design of research; X.Z. performed experiments; X.Z., M.B.B., and J.D.F. analyzed data; X.Z., M.B.B., and J.D.F. interpreted results of experiments; X.Z. prepared figures; X.Z. drafted manuscript; X.Z., M.B.B., and J.D.F. edited and revised manuscript; X.Z., M.B.B., and J.D.F. approved final version of manuscript.

**Fig. 6.** Mice were treated as in Fig. 2. A: WR increases activity of mitochondrial enzymes that catalyze production of ROS, but decreases NADPH oxidase activity in the mouse renal IM. *P < 0.05 vs. control, nonpaired t-test, n = 6. B–D: WR does not significantly affect protein abundance of components of NADPH oxidase in the renal IM, Cor, or OM (the large mean decrease in p47phox is not statistically significant because of large variation in the control in the IM and in both control and WR groups in the OM).
REFERENCES


