Ultrastructure of mitochondria and the endoplasmic reticulum in renal tubules of Dahl salt-sensitive rats

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1Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin; 2Department of Nephrology, Tongji Hospital, Huazhong University of Science and Technology, Wuhan, Hubei, China; and 3The Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi’an Jiaotong University, Xi’an, Shaanxi, China

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He X, Liu Y, Usa K, Tian Z, Cowley AW Jr, Liang M. Ultrastructure of mitochondria and the endoplasmic reticulum in renal tubules of Dahl salt-sensitive rats. Am J Physiol Renal Physiol 306: F1190–F1197, 2014. First published April 2, 2014; doi:10.1152/ajprenal.00073.2014.—Metabolic and functional abnormalities in the kidney precede or coincide with the initiation of overt hypertension in the Dahl salt-sensitive (SS) rat. However, renal histological injury in SS rats is mild before the development of overt hypertension. We performed electron microscopy analysis in 7-wk-old SS rats and salt-insensitive consomic SS.13BN rats and Sprague-Dawley (SD) rats fed a 4% NaCl diet for 7 days. Long mitochondria (>2 μm) accounted for a significantly smaller fraction of mitochondria in medullary thick ascending limbs in SS rats (4% ± 1%) than in SS.13BN rats (8% ± 1%, P < 0.05 vs. SS rats) and SD rats (9% ± 1%, P < 0.01 vs. SS rats), consistent with previous findings of mitochondrial functional insufficiency in the medulla of SS rats. Long mitochondria in proximal tubules, however, were more abundant in SS rats than in SS.13BN and SD rats. The width of the endoplasmic reticulum, an index of endoplasmic reticulum stress, was significantly greater in medullary thick ascending limbs of SS rats (107 ± 1 nm) than in SS.13BN rats (95 ± 2 nm, P < 0.001 vs. SS rats) and SD rats (74 ± 3 nm, P < 0.01 vs. SS or SS.13BN rats). The tubules examined were indistinguishable between rat strains under light microscopy. These data indicate that ultrastructural abnormalities occur in the medullary thick ascending limbs of SS rats before the development of histological injury in renal tubules, providing a potential structural basis contributing to the subsequent development of overt hypertension.

METHODS

Animals

Male Dahl SS rats, consomic SS.13BN rats, and SD rats were used. Inbred SS and SS.13BN rats were produced at the Medical College of Wisconsin (15, 22, 23). SD rats were purchased from Harlan. Rats of each strain were divided into two groups. One group was maintained on an AIN-76A diet containing 0.4% NaCl from weaning to the time of tissue collection (n = 6 SS rats, 5 SS.13BN rats, and 4 SD rats, respectively). The other group was switched to a 4% NaCl diet at 6 wk of age for 7 days (n = 5 SS rats, 6 SS.13BN rats, and 4 SD rats, respectively). All groups were euthanized for tissue collection at 7 wk of age.
Electron Microscopy

The cortex and outer stripe of the outer medulla (OM) from the left kidney were separated and cut into pieces of ~1 mm³. The tissue pieces, obtained at a consistent depth from the kidney surface across all rats, were fixed in 2.5% gluteraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 60 min at 4°C, washed with 0.1 M cacodylate buffer, postfixed in 1% OsO₄ for 2 h on ice, dehydrated in ascending concentrations of acetone, and embedded in resin for routine electron microscopy. Fine slices of 60 nm thickness were stained with uranyl acetate and lead citrate and then examined under a Hitachi H-600 electron microscope at an accelerating voltage of 75 kV.

For each rat, five tubule cross-sections from at least three tissue blocks were selected randomly for morphological quantitative analysis. Metamorph Offline (version 7.7.0.0) software was used to analyze all images. The following parameters were examined.

Mitochondrial morphology. Under ×8,000 magnification, ~100 mitochondria were measured in each tubule segment to determine the percent distribution of mitochondria with the following various lengths: <1 µm (short), 1–2 µm (medium), and >2 µm (long). Mitochondrial shape was assessed by calculating the ratio of the short axis over the long axis.

Volume density of mitochondria as a percentage of cytoplasmic volume. The volume density of mitochondria was determined by a point-counting procedure using a grid (with each point spaced by 2.048 mm) with a final magnification of × 8,000. The equation applied was as follows: \( V_{mit} = \left( \frac{P_{mit}}{P_{cyt}} \right) \times 100 \), where \( V_{mit} \) is the volume density of mitochondria as a percentage of cytoplasmic volume, \( P_{mit} \) is the number of grid points that fell within mitochondria, and \( P_{cyt} \) is the number of points that fell within the cell but outside of the nucleus.

Surface density of the inner mitochondrial membrane. The surface density of the inner mitochondrial membrane (IMM) was calculated using the following equation: \( S_{IMM} = \frac{4}{\pi} \times \frac{l}{I} \times (P \times d) \), where \( S_{IMM} \) is the surface density of the IMM, \( I \) is the number of intersections of

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Fig. 1. The medullary thick ascending limb (mTAL) of Dahl salt-sensitive (SS) rats contained a smaller proportion of long mitochondria than SS.13BN or Sprague-Dawley (SD) rats. A: mitochondrial volume, surface density of the inner mitochondrial membrane (\( S_{IMM} \)), mitochondrial shape, and length distribution of mitochondria. B: representative electron microscopic images. \( n = 4–6 \). * \( P < 0.05 \) vs. SS rats.
the IMM with the test line. \( P \) is the number of points that fell within the mitochondrial reference area, and \( d \) is the scale dimension between points. The final magnification used was \( \times 60,000 \).

**Width of the ER.** The area and length of \( > 50 \) rough ER cisternae were measured to calculate the average width. The width did not include the ER membrane or attached ribosomes.

**Light Microscopy**

The right kidney was removed without flushing, fixed in 10% phosphate-buffered formalin for 24 h at room temperature, and then embedded in paraffin. Tissue sections of 4 \( \mu \)m thickness were prepared, deparaffined, and stained with Masson’s trichrome staining. Sections were observed under a Nikon Eclipse E400. In each section, 5 fields in the superficial cortex and 5 fields in the juxtamedullary cortex were randomly selected to examine proximal convoluted tubules (PCTs), 10 fields in each region were randomly selected to examine distal tubules, and the entire section was used to examine glomeruli. Five fields in the outer stripe and five fields in the inner stripe of the OM were randomly selected to examine nTALs. Tubular diameter and cell height were measured under \( \times 200 \) and \( \times 400 \) magnifications, respectively. Images were taken using a SPOT Insight camera program and SPOT Advanced (version 3.5.9) for Windows software. Metamorph Offline (version 7.7.0.0) software was used to analyze all images. The parameters evaluated were as follows.

**Glomerular density.** Glomerular density was calculated as the total glomerular number divided by the total renal cortex area.

**Tubular diameter.** Tubular diameter was defined as the length of the shortest possible straight line that passed through the center of a symmetrically sectioned tubule. Asymmetrically sectioned tubules were excluded from measurements. The diameter did not include the basement membrane.

**Tubular cell height.** Tubular cell height was measured along the diameter axis from the base to the apical surface of a tubular cell crossing the nucleus, not including the brush border.

![Graphs](image1)

**Fig. 2.** The proximal convoluted tubule (PCT) of SS rats contained a larger proportion of long mitochondria than in SS.13\(^{BN} \) or SD rats. A: mitochondrial volume, \( S_{\text{IMM}} \), mitochondrial shape, and length distribution of mitochondria. B: representative electron microscopic images. \( n \) = 4–6. *\( P < 0.05 \) vs. SS rats.
Tubular cell number. Tubular cell number was calculated as the number of cell nuclei in a tubular cross-section.

Renal interlobular artery thickness. Symmetrically sectioned round renal interlobular arteries (10–15 arteries/rat) were selected. Artery, artery wall, and artery lumen areas were measured separately.

Cast area. Cast area was expressed as the total cast area in the cortex divided by the total renal cortex area and the total cast area in the OM divided by the total renal OM area.

OM interstitial collagen. The OM interstitial collagen area was graded according to the following semiquantitative scoring from 0 to 5:

- 0: Normal
- 1: Mild increase
- 2: Moderate increase
- 3: Severe increase
- 4: Very severe increase
- 5: Extreme increase

Mitochondrial volume, mitochondrial shape, and length distribution of mitochondria are shown.

Fig. 3. Mitochondria in the distal tubule (DT) appeared similar in SS, SS.13BN, and SD rats. Mitochondrial volume, $S_{\text{IMM}}$, mitochondrial shape, and length distribution of mitochondria are shown. $n = 4–6$.

Fig. 4. The width of the endoplasmic reticulum (ER) in the mTAL was largest in SS rats, medium in SS.13BN rats, and smallest in SD rats. A: width of the ER in mTALs. B: representative electron microscopic images of mTALs. Red arrows indicate the rough ER. C: width of the ER in PCTs and DTs. $n = 4–6$. *P < 0.05 vs. SS rats; #P < 0.05 vs. SS.13BN rats.
Long mitochondria (>2 μm), an indication of healthy mitochondria (>1 μm), accounted for 4 ± 1% of all mitochondria in mTALs in SS rats, 8 ± 1% in SS.13BN rats (P < 0.05 vs. SS rats), and 9 ± 1% in SD rats (P < 0.01 vs. SS rats). The fraction of short mitochondria (<1 μm) tended to be greater in SS rats than in the other two strains, although the difference did not reach statistical significance. Similarly, the ratio of the short axis to the long axis of mitochondria tended to be greater in SS rats and lower in SD rats. Mitochondrial volume density (as a percentage of cytoplasmic area) was ∼25% in mTALs and was not significantly different among the three rat strains. The surface density of the IMM was also not significantly different.

In contrary, PCTs of SS rats contained a significantly larger fraction of long mitochondria and a smaller fraction of short mitochondria than SS.13BN or SD rats (Fig. 3). The ratio of the short axis to the long axis of mitochondria tended to be lower in SS rats. Mitochondrial volume density was ∼35% in PCTs of SS rats, which tended to be higher than in SS.13BN and SD rats.

Mitochondria in the distal tubule appeared similar between SS, SS.13BN, and SD rats (Fig. 3).

As shown in Fig. 4, A and B, the width of the rough ER, an index of ER stress, was significantly larger in mTALs in SS rats (107 ± 1 nm) than in SS.13BN rats (95 ± 2 nm) by 13% and SD rats (74 ± 3 nm) by a substantial 45%. The width in SS.13BN rats was significantly larger than in SD rats by 28%. The width of the rough ER in the distal tubule tended to show the same trend as the mTAL, but the difference between rat strains did not reach statistical significance (Fig. 4C). The width of the ER in PCTs was not significantly different between rat strains.

**Light Microscopy**

No significant differences were observed for the diameter or tubular cell height of PCTs or mTALs, glomeruli density, or lumen or wall areas of interlobular arteries among the three rat strains.

Table 1. *Morphometric analysis of the kidney under light microscopy*

<table>
<thead>
<tr>
<th></th>
<th>SS Rats</th>
<th>SS.13BN Rats</th>
<th>SD Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4% NaCl diet</td>
<td>4% NaCl diet</td>
<td>0.4% NaCl diet</td>
</tr>
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<td>Glomeruli, number of glomeruli/mm² cortex</td>
<td>3.7 ± 0.2</td>
<td>3.5 ± 0.1</td>
<td>3.6 ± 0.3</td>
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<td>PCT diameter, μm</td>
<td></td>
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<tr>
<td>Superficial</td>
<td>38.0 ± 0.6</td>
<td>38.2 ± 0.1</td>
<td>38.0 ± 0.6</td>
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<tr>
<td>Juxtamedullary</td>
<td>38.7 ± 0.4</td>
<td>39.1 ± 0.6</td>
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<tr>
<td>PCT cell height, μm</td>
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</tr>
<tr>
<td>Superficial</td>
<td>11.7 ± 0.3</td>
<td>11.3 ± 0.2</td>
<td>11.3 ± 0.2</td>
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<tr>
<td>Juxtamedullary</td>
<td>11.8 ± 0.3</td>
<td>11.2 ± 0.2</td>
<td>11.5 ± 0.1</td>
</tr>
<tr>
<td>DT diameter, μm</td>
<td>26.2 ± 0.3</td>
<td>29.2 ± 0.1*</td>
<td>26.4 ± 0.2</td>
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<td>DT cell height, μm</td>
<td>8.9 ± 0.2</td>
<td>8.6 ± 0.1</td>
<td>8.8 ± 0.1</td>
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<td>DT cell number, number of cells/DCT</td>
<td>9.6 ± 0.2</td>
<td>10.0 ± 0.2</td>
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<td>mTAL diameter, μm</td>
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<tr>
<td>Outer stripe</td>
<td>26.1 ± 0.2</td>
<td>27.2 ± 0.5</td>
<td>26.7 ± 0.4</td>
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<tr>
<td>Inner stripe</td>
<td>26.2 ± 0.4</td>
<td>26.7 ± 0.8</td>
<td>25.5 ± 0.4</td>
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<tr>
<td>mTAL cell height, μm</td>
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<td></td>
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</tr>
<tr>
<td>Outer stripe</td>
<td>8.8 ± 0.2</td>
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<td>9.3 ± 0.2</td>
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<tr>
<td>Inner stripe</td>
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<td>8.1 ± 0.3</td>
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<td>Interlobular artery</td>
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<td>Lumen/vessel</td>
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<td>0.19 ± 0.02</td>
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<td>Wall/vessel</td>
<td>0.80 ± 0.02</td>
<td>0.81 ± 0.02</td>
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</table>

Values are means ± SE; n = 4–6 rats/group. PCT, proximal convoluted tubule; DT, distal tubule; mTAL, medullary thick ascending limb. *P < 0.05 vs. Dahl salt-sensitive (SS) rats on a 0.4% NaCl diet and SS.13BN and Sprague-Dawley (SD) rats on a 4% NaCl diet.
strains on the 0.4% NaCl diet or the 4% NaCl diet for 7 days (Table 1). The diameter of the distal tubule was significantly larger in SS rats on the 4% NaCl diet than in the other groups by ~10%. The height or number of cells in the distal tubule was not significantly different among groups.

Fibrosis and inflammation scores in the OM and cast areas in the cortex and OM were significantly higher in SS rats fed the 4% NaCl diet for 7 days than in SS,13BN or SD rats on the 4% NaCl diet or SS rats maintained on the 0.4% NaCl diet (Fig. 5). The histological injury appeared to be higher in SS rats on the 0.4% NaCl diet than in the other two strains, but the difference did not reach statistical significance except for inflammation scores in SS and SD rats. Red casts accounted for ~90% of the casts in the cortex and 75% in the OM (Fig. 5, C and D). The remaining casts were blue casts. Both types of casts showed similar differences among rat groups.

Despite the significant differences among rat groups, renal histological injury was mild in all conditions. Even in SS rats fed the 4% NaCl diet for 7 days, fibrosis and inflammation affected not much more than 5% of the renal OM (average score: around 1). Fibrosis and inflammation only occurred sporadically in the cortex and were not sufficient for reliable quantification. Cast areas were <2% of the OM area and ~0.4% of the cortex area in SS rats on the 4% NaCl diet and lower in other groups.

**DISCUSSION**

The present study is the first systematic analysis of renal tubular ultrastructure in a model of hypertension. The results indicate that shortening of mitochondria and dilation of the rough ER occurred in the mTALs of 7-wk-old SS rats exposed to a high-salt diet for just 7 days. It provides a potential structural basis for mitochondrial functional deficiency and ER stress that might participate in driving the development of substantial, overt hypertension.

Fig. 5. Fibrosis, inflammation, and tubular casts affected <5% of the kidney area in SS, SS,13BN, and SD rats on 0.4% NaCl diets or 7 days (7d) of 4% NaCl diets. A: fibrosis scores. A score of 1 indicates that <5% of the area was affected. B: inflammation scores. A score of 1 indicates that <5% of the area was affected. C: cast area (expressed as a percentage of the total area) in the cortex. D: cast area (expressed as a percentage of the total area) in the outer medulla. n = 4–6. *P < 0.05 vs. SS rats on the 4% NaCl diet; #P < 0.05 vs. SS rats on the 0.4% NaCl diet.
Mitochondrial and metabolic deficiencies are emerging as a new mechanism contributing to the development of hypertension (13). Intravenous infusion of succinate, an intermediate in the tricarboxylic acid cycle, or inducible overexpression of uncoupling protein 1 in vascular smooth muscle cells, which uncouples respiration and oxidative phosphorylation, caused hypertension in mice (2, 10). Succinate activation of the GPR91 receptor is a potential link between high glucose and renin release (17). Fumarase, an enzyme in the tricarboxylic acid cycle, was genetically altered and functionally insufficient in the renal medulla of SS rats compared with SS.13BN rats before blood pressure substantially diverged between the two rat strains (22, 23). Experimental elevation of fumaric acid, the substrate of fumarase that mimics the condition in SS rats, exacerbates salt-induced hypertension in SS.13BN rats (23). Mitochondria isolated from mTALs of SS rats contained several proteins with altered abundance compared with SS.13BN rats and exhibit a lower capability of O2 utilization (27). The shortening of mitochondria found in the present study would be consistent with compromised mitochondrial function in the mTALs of SS rats, although it may not completely explain the functional differences between rat strains. In some cases, cellular failure to remove defective mitochondria may result in extensively extended mitochondria with poor function. However, short and fragmented mitochondria are associated with poor mitochondrial function in most cases (14).

Mitochondria in PCTs, however, are longer in SS rats than in salt-insensitive rat strains. Mitochondrial fission was prominent in the cerebral cortex of SS rats after 9 wk of an 8% NaCl diet (18). Cardiac mitochondria from SS rats consume more O2 and produce more H2O2 than BN rats (1). Substitution of mitochondrial genomes from spontaneously hypertensive rats into SS rats improved aerobic treadmill running capacity and survival, whereas mitochondrial genomes from SS rats did not appear to have an effect on spontaneously hypertensive rats (12). Furosemide-sensitive O2 consumption in isolated mTALs did not appear to differ between SS rats and Dahl salt-resistant rats maintained on a Purina diet containing 0.22% Na+ (9). Together, these findings suggest mitochondrial dysfunction in SS rats might be cell type dependent and influenced by genomic context.

ER stress is a cellular response to the accumulation of unfolded or misfolded proteins, which could result from several conditions including aberrant redox states or Ca2+ levels in the cell (24). ER stress leads to cell cycle arrest, alterations of protein production, and, eventually, cell death. The role of ER stress in the development of hypertension has been recently recognized. Young et al. (26) demonstrated that ER stress in the brain subfornical organ contributed to ANG II-induced hypertension in mice. The results of the present study suggest that ER stress in the mTAL could contribute to the development of hypertension in the SS rat. It remains to be determined what initiates ER stress in the mTAL of the SS rat and what might mediate its effect on blood pressure. Oxidative stress, which could initiate ER stress, occurs in the renal medulla, including the mTAL, of SS rats and is known to contribute to the development of hypertension in SS rats (7, 21). The oxidative stress in the medulla of SS rats could, in part, be due to mitochondrial insufficiencies (23). It would be interesting to examine whether mitochondrial insufficiencies contribute to the dilation of the ER in the mTAL of the SS rat. mTALs of SS rats on a high-salt diet contain an increased number of proliferative cells that might be arrested in the G1/S phase of the cell cycle (25), which could result, in part, from ER stress.

It would not be practical to ascertain the cause-effect relationship between an ultrastructural alteration and hypertension with any single experiment. That is because it is difficult to specifically change any ultrastructural feature with an experimental intervention, especially in vivo. Evidence for a pathophysiological role of ultrastructural alterations would have to come from multiple lines of studies that correlate ultrastructural alterations with biochemical and functional changes whose contribution to hypertension can be experimentally tested. Such evidence in mTALs of SS rats is currently preliminary for the ER but strong for mitochondria, as discussed above.

It is a crucial challenge in salt-sensitive hypertension research to understand the mechanisms underlying the development of hypertension, not just the consequences of later-phase, overt hypertension. However, it is difficult, probably impossible, to find a time point where the kidney structure has stabilized and blood pressure is still completely normal in SS rats on the AIN-76A diet. Nephrogenesis in rats is not complete until 2–3 wk postnatal, and kidneys continue to mature for another 2–3 wk after that. However, small increases in blood pressure and renal injury likely already occur in 6-wk-old SS rats even if they have been maintained on a low-salt diet (8, 11, 15, 25). What we could reasonably look for was structural changes that precede or coincide with the initiation of substantial, overt hypertension. Exposure of 6- to 7-wk-old SS rats to the 4% salt diet for 7 days represents a critical time point where substantial, overt hypertension is just beginning to develop (22). We cannot rule out the possibility that some of the ultrastructural changes we observed were the consequence of small increases in blood pressure in SS rats. However, the time point we chose to study was a reasonable compromise and was informative for identifying structural changes that might contribute to the subsequent development of overt hypertension.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: X.H. and M.L. conception and design of research; X.H., Y.L., and K.U. performed experiments; X.H. and M.L. analyzed data; X.H., Y.L., and K.U. interpreted results of experiments; X.H. and M.L. drafted manuscript; X.H. and M.L. edited and revised manuscript; X.H., Y.L., K.U., Z.T., A.W.C.J., and M.L. approved final version of manuscript.

REFERENCES


