Mitochondrial dysfunction is an early event in aldosterone-induced podocyte injury

Min Su,1* Asish-Roopchand Dhoopun,1,2* Yanggang Yuan,1,2 Songming Huang,1,2 Chunhua Zhu,1,2 Guixia Ding,1,2 Bicheng Liu,3 Tianxin Yang,4 and Aihu Zhang1,2

1Department of Nephrology, Nanjing Children’s Hospital, Nanjing Medical University, Nanjing, China; 2Institute of Pediatrics, Nanjing Medical University, Nanjing, China; 3Institute of Nephrology, Zhong Da Hospital, Southeast University, Nanjing, China; and 4Division of Nephrology, University of Utah and Veterans Affairs Medical Center, Salt Lake City, Utah

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Su M, Dhoopun AR, Yuan Y, Huang S, Zhu C, Ding G, Liu B, Yang T, Zhang A. Mitochondrial dysfunction is an early event in aldosterone-induced podocyte injury. Am J Physiol Renal Physiol 305: F520–F531, 2013. First published June 12, 2013; doi:10.1152/ajprenal.00570.2012.—We previously showed that mitochondrial dysfunction (MtD) is involved in an aldosterone (Aldo)-induced podocyte injury. Here, the potential role of MtD in the initiation of podocyte damage was investigated. We detected the dynamic changes of urinary protein, urinary F2-isoprostane and renal malondialdehyde levels, kidney ultrastructure morphology, mitochondrial DNA (mtDNA) copy number, mitochondrial membrane potential (ΔΨm), and nephrin and podocin expressions in Aldo-infused mice. Aldo infusion first induced renal oxidative stress, as evidenced by increased levels of urinary F2-isoprostane and renal malondialdehyde, and MtD, as demonstrated by reduced mtDNA, ΔΨm, and ATP production. Later, at 5 days after Aldo infusion, proteinuria and podocyte injury began to appear. In cultured podocytes, Aldo or hydrogen peroxide (H2O2) induced MtD after 2–8 h of treatment, whereas the podocyte damage, as shown by decreased nephrin and podocin expressions, occurred later after 12 h of treatment. Thus Aldo treatment both in vitro and in vivo indicated that MtD occurred before podocyte damage. Additionally, MtDNA depletion by ethidium bromide or mitochondrial transcription factor A (TFAM) shRNA induced MtD, further promoting podocyte damage. TFAM expression was found to be reduced in Aldo-infused mice and Aldo-treated podocytes. Adenoviral vector-mediated overexpression of TFAM prevented Aldo-induced MtD and protected against podocyte injury. Together, these findings support MtD as an early event in podocyte injury, and manipulation of TFAM may be a novel strategy for treatment of glomerular diseases such as podocytopathy.

Proteinuria is regarded not only as a clinical manifestation but also as an important prognostic factor in chronic kidney disease (2, 25). Recent clinical studies revealed that proteinuria is a strong independent risk factor for developing end-stage renal disease (ESRD; Ref. 15).Podocytes line the outer surface of the glomerular basement membrane and play an important role in the glomerular filtration barrier (2, 5, 26). Recent studies have demonstrated that podocyte injury is intimately related to proteinuria (2). Podocytes are reported to be injured in many types of proteinuric renal diseases, including nephrotic syndrome, diabetic nephropathy, and lupus nephritis (20, 41).

Once damaged, podocytopenia follows, ultimately culminating in glomerulosclerosis. Although studies on the molecular events of podocyte injury are accumulating, the early steps in the chain of events leading to podocyte injury remains unclear. Podocytes are terminally differentiated, high-energy-requiring cells that have lost mitotic activity and do not typically proliferate after injury (28). This characteristic is identical to that of neuronal cells (28). The cell body of a podocyte contains a prominent nucleus, a well-developed Golgi system, abundant rough and smooth endoplasmic reticulum, prominent lysosomes, and a substantial amount of mitochondria. Podocytes demand a high energy supply to maintain various cellular functions, including the organization of cytoskeletal and extracellular matrix proteins (6). It has been reported that mitochondrial dysfunction (MtD) is an early event in the pathology of Alzheimer’s disease (AD) that accumulates with age in AD transgenic mice (10). In addition to neurodegenerative diseases, MtD is also involved in several renal diseases and progressive disease processes, including glomerulosclerosis, in which podocyte injury is a crucial event in sclerosis formation (36).

Aldosterone (Aldo), a key regulator of blood pressure and electrolytic balance, has recently attracted considerable attention for its involvement in the development and progression of cardiovascular disease and chronic kidney disease by reducing vascular compliance and promoting endothelial dysfunction and renal hypertrophy and fibrosis (38). It has been demonstrated that up to 85% of patients with primary hyperaldosteronism have evidence of proteinuria (31). In remnant kidney hypertensive rats (7) and stroke-prone, spontaneously hypertensive rats (30), the plasma Aldo concentration is markedly elevated, and an exogenous infusion of Aldo can reverse the renoprotective effects of angiotensin-converting enzyme (ACE) inhibitors. A growing body of evidence substantiates the pathologic role of Aldo in podocyte injury (35). We have previously shown that Aldo-induced podocyte injury is mediated by MtD, and preserving mitochondrial function is important for preventing podocyte injury (37, 40). However, whether the mitochondria link Aldo and the initiation and development of podocyte injury remains unclear. In this study, we extend the investigation to determine whether MtD occurs before podocyte damage and proteinuria and whether it initiates podocyte injury in Aldo-infused mice and Aldo-treated podocytes. Additionally, mitochondrial function in podocytes was evaluated after mtDNA depletion by ethidium bromide (EtBr) or mitochondrial transcription factor A (TFAM) short hairpin RNA (shRNA) or after adenovirus vector-mediated overexpression of TFAM. Our findings indicated that exposure to Aldo might critically affect mitochondrial function in podocytes. Thus the
resultant cell injury could be the consequence of mitochondrial dysfunction.

MATERIALS AND METHODS

Reagents, plasmids, and antibodies. Aldo, EtBr, and N-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, MO). Primary rabbit polyclonal antibodies were against nephrin (Abcam, Cambridge, MA), podocin, TFAM, and β-actin (Cell Signaling Technology, Beverly, MA). Peroxidase-conjugated goat anti-rabbit secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). MitoSOX was from Invitrogen (Carlsbad, CA). Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) was obtained from Calbiochem (San Diego, CA).

Mice. Eight-week-old male C57BL/6J mice (20–25 g body wt) were each implanted subcutaneously with an osmotic minipump (Duret, Cupertino, CA) by incision of the right flank region while

<table>
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TFAM, mitochondrial transcription factor A; COX I, cytochrome oxidase I; ND6, NADH dehydrogenase 6; mtDNA, mitochondrial DNA.

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Fig. 1. Urinary albumin excretion and podocyte damage in aldosterone (Aldo)-infused mice. A: albuminuria (d, day). Urinary albumin concentration was detected by ELISA and normalized to urine creatinine concentration, and results are expressed in μg/mg urine creatinine. B: kidney ultrastructure morphology changes of podocyte foot processes by electron microscopy (×12,000) at indicated periods of Aldo infusion. Arrow indicates podocyte foot process effacement. C: nephrin, podocin, and mitochondrial transcription factor A (TFAM) mRNA expression by real-time RT-PCR analysis in kidney cortex. D: nephrin, podocin, and TFAM protein expression by immunoblotting analysis in kidney cortex. E: nephrin and podocin expression by immunohistochemistry in kidney from sham or Aldo-infused mice at day 14. Shown are representatives of at least 3 independent experiments. Values represent means ± SE; n = 6. *P < 0.01 vs. sham by ANOVA.
animals were under light 3% isoflurane anesthesia. Aldo was infused at 300 µg·kg⁻¹·day⁻¹ for 2 wk, and all mice were on standard pelleted rodent chow and housed in an air-conditioned room with a 12:12-h light-dark cycle. The mice were killed on days 1, 3, 5, 7, and 14. Plasma samples and kidneys were immediately frozen in liquid nitrogen and stored at −80°C until use. All animal procedures were approved by the Nanjing Medical University Institutional Animal Care and Use Committee.

**Cell culture and virus infection.** MPC5 conditionally immortalized mouse podocyte clonal cells (27) (kindly provided by Peter Mundel at Mount Sinai School of Medicine through Jie Ding at Peking University, Beijing, China) were cultured and induced to differentiate as described previously (26). Podocytes were maintained without interferon-γ at 37°C for 14 days before experimentation to induce differentiation. In some instances, the cells were preincubated with EtBr (100 ng/ml), uridine (50 mg/ml), and sodium pyruvate (110 mg/ml) for 4 days to reduce mtDNA levels and cultivated further after the removal of EtBr for the indicated times. Adenoviral vectors encoding TFAM (Ad-TFAM) were obtained from Applied Biological Materials (Richmond, BC, Canada). Lentivirus expressing shRNA for TFAM were from Santa Cruz Biotechnology. Cells were infected with adenoviruses or lentiviruses for 24 – 48 h before the experiments according to the manufacturer’s instructions.

**Isolation of glomeruli and glomerular mitochondria.** Isolation of glomeruli was performed as previously reported (40). Mitochondria from glomeruli and cultured podocytes were isolated using a kit (MITO-ISO1) purchased from Sigma using the manufacturer’s protocol. Isolated mitochondria were resuspended in storage buffer (0.25 mol/l sucrose and 25 mmol/l Tris base, pH 7.0) provided by the kit, and the protein concentration was determined by the Bradford method.

**Quantitative real-time PCR and RT-PCR.** Total DNA and RNA from cultured podocytes and isolated glomeruli were extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA) and Trizol reagent (Invitrogen, respectively). Oligonucleotides were designed using the Primer3 software (available at http://frodo.wi.mit.edu/) and synthesized at Invitrogen. The sequences of primer pairs are shown in Table 1. Real-time PCR was used to detect mtDNA copy number as previously described (37), and real-time RT-PCR was used to determine the target gene expression. Reverse transcription was performed using a reaction kit (Reverse Transcriptase System; Promega, Madison, WI) according to the manufacturer’s protocol. Real-time PCR amplification was performed as previously described (40). Relative amounts of mtDNA copy number were normalized to 18S rRNA levels encoded by the nuclear DNA, and mRNA values were normalized to the GAPDH gene control values and calculated using the comparative cycle threshold (2−ΔΔCt) method.

**Western blotting.** Podocytes or kidney cortex was lysed in protein lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 200 mM sodium fluoride, and 4 mM sodium orthovanadate as protease inhibitors, pH 7.5) for 15 min on ice. Protein concentration was measured as above. Immunoblotting was performed with primary antibodies against TFAM (1:1,000), nephrin (1:200), podocin (1:500), α-actin (1:1,000), followed by the addition of horseradish peroxidase-labeled secondary antibodies. The blots were visualized with the Amersham ECL Detection System (GE Healthcare, Piscataway, NJ).

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**Fig. 2. Renal oxidative stress and mitochondrial function in Aldo-infused mice.**

**A:** urinary F₂-isoprostane (F₂-IsoP) and kidney malondialdehyde (MDA) levels.

**B:** mitochondrial membrane potential (ΔΨₘ), mitochondrial DNA (mtDNA) copy number, and ATP production. Urinary F₂-isoprostane excretion, kidney MDA in the cortex, ΔΨₘ in isolated mitochondria, mtDNA copy number, and ATP content were detected as described in MATERIALS AND METHODS. Values represent means ± SE; n = 6. *P < 0.01 vs. sham by ANOVA. #P < 0.01 vs. Aldo infusion for 1 day by ANOVA.
Densitometric analysis was performed using Quantity One Software (Bio-Rad, Hercules, CA).

Mitochondrial superoxide anion production, mitochondrial membrane potential, and ATP production analysis. Podocytes mitochondrial superoxide anion production was measured by MitoSOX as previously described (13). Mitochondrial membrane potential (ΔΨm) in podocytes and isolated mitochondria was determined using the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1; Molecular Probes) as described previously (40). For the quantitation of mitochondrial superoxide anion and ΔΨm, MitoSOX, and JC-1 fluorescence levels were analyzed by flow cytometry. ATP levels were determined with a luciferase-based bioluminescence assay kit (Sigma) in a FLUOstar Optima reader according to the manufacturer’s instructions.

Electron microscopy. Fresh kidney tissues were fixed in 1.25% glutaraldehyde/0.1M phosphate buffer and postfixed in 1% OsO4/0.1 M phosphate buffer. Ultrathin sections (60 nm) were cut on a microtome, placed on copper grids, stained with uranyl acetate and lead citrate, and examined under an electron microscope (JEOL JEM-1010, Tokyo, Japan).

Immunostaining. The kidneys were fixed in 4% paraformaldehyde and imbedded in paraffin. Paraffin sections of each specimen were cut at 4 μm (Cryostat 2800 Frigocut-E; Leica Instruments), and a standard protocol using xylene and graded ethanol was employed to deparaffinize and rehydrate the tissue. The sections were washed with PBS and treated with blocking buffer containing 50 mM NH4Cl, 2% BSA, and 0.05% saponin in PBS for 20 min at room temperature. The sections were then incubated overnight at 4°C with rabbit polyclonal anti-nephrin or anti-podocin antibodies at 2–5 g/ml. After being washed with PBS, the secondary antibody was applied, and the signals were visualized using an ABC kit (Santa Cruz Biotechnology).

Analysis of renal lipid peroxides, urinary albumin, and F2-isoprostane. Levels of lipid peroxide [malondialdehyde (MDA) content] were determined in kidney cortex using MDA assay kit (Beyotime Institute of Biotechnology, Shanghai, China) and spectrophotometric measurement of thiobarbituric acid-reactive substances, according to the manufacturer’s instructions. Results are expressed as nanomoles per milligram of protein. Enzyme immunoassay kits were used to determine urinary concentrations of albumin and creatinine (Exocell, Philadelphia, PA) and F2-isoprostane (Cayman, Ann Arbor, MI), according to the product instructions. For all assays, samples were run in duplicate and results averaged. Urinary albumin and F2-isoprostane concentration was normalized to urine creatinine concentration to adjust for the specific hydration status.

Statistical analysis. All data are presented as means ± SE. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s test with SPSS 11.5 statistical software. P < 0.05 was considered significant.

RESULTS

MtD occurs before proteinuria and podocyte injury in Aldo-infused mice. We first examined the dynamic changes of MtD, proteinuria, and podocyte injury in Aldo-infused mice. Urinary protein excretion increased after 5 days of Aldo infusion in mice and continued to increase until day 14 (Fig. 1A). Examination of kidney ultrastructure morphology showed foot-process effacement after 5 days of Aldo treatment, which appeared as sheets covering the glomerular basement membrane, with disappearance of the slit diaphragm gap (Fig. 1B). Consistent with the proteinuria and podocyte damage, the expressions of

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**Fig. 3. Aldo-induced mitochondrial dysfunction (MtD) in podocytes.** A: mitochondrial superoxide anion production. *Left:* representative images of podocytes stained with MitoSOX. Cells in chamber slides were exposed to vehicle or Aldo (100 nM) for 2 h in the presence of MitoSOX. *Right:* quantitation of MitoSOX by fluorescence flow cytometry. Podocytes were treated with 100 nM Aldo for the indicated periods of time, the fluorescence was detected by flow cytometry. *B:* effect of Aldo on mitochondrial function in podocytes. Confluent podocytes were treated with 100 nM Aldo for the indicated periods of time, and ΔΨm, mtDNA copy number, and ATP production were detected. C: effect of MnTBAP and N-acetyl-l-cysteine (NAC) on Aldo-induced MtD in podocytes. Confluent podocytes were pretreated with MnTBAP (100 μM) or NAC (5 mM) for 30 min and then stimulated by 100 nM Aldo for further 24 h. ΔΨm and mtDNA copy number were detected. Values represent means ± SE; n = 6. ∗P < 0.01 vs. control (Cntl) by ANOVA. **P < 0.01 vs. Aldo treatment by ANOVA.
nephrin and podocin decreased after 5 days in Aldo-infused mice (Fig. 1, C–E). The serum blood urea nitrogen and creatinine levels did not significantly differ among the sham and Aldo-infused mice at different time points (data not shown).

We used several independent parameters to evaluate MtD: reactive oxygen species (ROS) production, \( \Delta \Psi_m \), mtDNA copy number and ATP production. MtD can enhance ROS production/accumulation and may contribute further to mitochondrial damage (10). Both urinary F2-isoprostane, a specific marker of renal oxidative stress, and renal MDA, a measurement of renal lipid oxidation, showed a progressive rise that was noticeable after 3 days of Aldo infusion (Fig. 2A). \( \Delta \Psi_m \) was decreased in isolated mitochondria (Fig. 2B), which was noticeable after 3 days and sharply decreased after 14 days of Aldo infusion. The mtDNA copy number in the kidney cortex at 1 day after Aldo infusion was increased (139%), but it significantly decreased after day 3 and reached a deep reduction at day 14 (58%; Fig. 2B). We next measured ATP production in the kidney cortex. As shown in Fig. 2B, ATP production was downregulated at day 3 compared with sham controls and reached a deep reduction at day 14. These results indicated that MtD occurred before proteinuria and podocyte damage.

TFAM is an essential protein factor for the initiation of mtDNA transcription (17). We then analyzed TFAM mRNA and protein levels in the kidney cortex in Aldo-infused mice. In parallel to the changes in mtDNA, TFAM expression was upregulated on day 1 after Aldo infusion, compared with sham controls, but was then downregulated until day 14 (Fig. 1, C and D).

Aldo-induced MtD occurs before cell damage in cultured podocytes. Given the observation that MtD occurred before the podocyte damage and proteinuria in Aldo-infused mice, we next detected whether these in vivo findings extended to an in vitro model of Aldo-treated podocytes. We found that Aldo induced mitochondrial superoxide anion production in a time-dependent manner, which became noticeable at 2 h and was sustained until 24 h (Fig. 3A). Consistently, Aldo induced \( \Delta \Psi_m \) collapse and decreases in mtDNA copy number and ATP production after 4–8 h of treatment in cultured podocytes (Fig. 3B). As mtDNA has been reported to be a major target for mitochondrial superoxide anion (12), we characterized the role

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**Fig. 4. Aldo-induced podocyte injury.** A: nephrin, podocin, and MnSOD mRNA expressions. B: nephrin, podocin, and MnSOD protein expressions. Left: representative immunoblots. Right: densitometric analysis. Confluent podocytes were treated with 100 nM Aldo for the indicated periods of time, and nephrin, podocin, and MnSOD mRNA and protein expressions were assessed by real time RT-PCR analysis and immunoblotting analysis, respectively. C: effect of Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) and NAC on Aldo-induced podocytes injury. Confluent podocytes were pretreated with MnTBAP (100 \( \mu \)M) or NAC (5 mM) for 30 min, and then stimulated by 100 nM Aldo for further 24 h. Nephrin, podocin, and MnSOD mRNA expressions were assessed by real time RT-PCR analysis. Values represent means ± SE; \( n = 6. \) *\( P < 0.01 \) vs. control by ANOVA. #\( P < 0.01 \) vs. Aldo treatment by ANOVA.
of ROS using the manganese superoxide dismutase (MnSOD) mimic MnTBAP and a widely used antioxidant, NAC, as a ROS scavenger. As shown in Fig. 3C, both MnTBAP and NAC abrogated the Aldo-induced collapse of $\Delta\Psi_m$ and the Aldo-induced decrease in the mtDNA copy number.

To evaluate podocyte injury, we used the expression of the glomerular slit diaphragm proteins nephrin and podocin as two independent parameters. As shown in Fig. 4, A and B, nephrin and podocin expressions were downregulated at 12 h in Aldo-treated podocytes. Both MnTBAP and NAC notably inhibited the decreases in nephrin and podocin induced by Aldo (Fig. 4C). Therefore, these data also indicated that MtD occurred before Aldo-treated podocyte injury.

MnSOD is a critical antioxidant enzyme that removes mitochondrial superoxide free radicals (22). We next examined the effect of Aldo treatment on MnSOD expression. As shown in Fig. 4, A and B, MnSOD expression was induced in a time-dependent manner. Notably, after the depletion of ROS by the cell-permeable ROS scavenger MnTBAP or NAC, the elevation of MnSOD expression was significantly abrogated (Fig. 4C), indicating a key role of ROS in MnSOD upregulation.

**Hydrogen peroxide induced MtD and podocyte injury.** We next examined the effects of $\text{H}_2\text{O}_2$ on MtD and podocyte injury to test the hypothesis that the induction of oxidative stress by means other than Aldo would also elicit the same detrimental response to mitochondrial dysfunction. As shown in Fig. 5, mitochondrial superoxide anion production was significantly increased, $\Delta\Psi_m$ and the mtDNA copy number were significantly decreased after 2–4 h of treatment of podocytes with $\text{H}_2\text{O}_2$, and the inhibition of nephrin and podocin expression occurred 8–12 h after $\text{H}_2\text{O}_2$ treatment. These data indicated that $\text{H}_2\text{O}_2$ elicited the same detrimental response to mitochondrial dysfunction as Aldo.

**MtDNA depletion induced podocyte injury.** We next determined whether MtD could induce podocyte damage. Depletion of mtDNA is confirmed as an important cause of MtD (23). Exposure to a low concentration of EtBr selectively reduces the mtDNA content in numerous cell types, and this effect is reversible after EtBr removal from the culture medium (4, 24). Using this technique, we generated mtDNA-depleted podocytes that were auxotrophic for uridine and pyruvate, as previously reported with other cell types (4, 24). To confirm mtDNA depletion in EtBr-treated cells, we performed real-time PCR with specific primers targeting different mtDNA regions. As shown in Fig. 6A, exposure to EtBr for 24 h resulted in 15–20% reduction of PCR products for cytochrome oxidase I (COX I), D-loop, and NDH dehydrogenase 6 (ND6). After 4 days of treatment, mtDNA was depleted to levels <20% of control. Removal of EtBr gradually restored the mtDNA content to 70–80% of normal within 7 days (reverted cells). Meanwhile, mitochondrial superoxide anion generation significantly increased, while $\Delta\Psi_m$ was significantly inhibited in mtDNA-depleted cells (Fig. 6B), which recovered upon withdrawal of the chemical. Furthermore, nephrin and podocin expressions were significantly downregulated in mtDNA-depleted cells and restored in reverted cells (Fig. 6, C and D). These data all indicated that MtD could induce podocyte damage.

**Suppression of endogenous TFAM expression induces MtD and podocyte injury.** It has been demonstrated that downregulation of TFAM induces mtDNA depletion (17). To confirm EtBr-mediated MtD and podocyte injury, we transfected podocytes with TFAM shRNA and detected the expressions of nephrin and podocin. TFAM expression was inhibited by TFAM shRNA 48 h after transfection (~80%; Fig. 7A and B). As shown in Fig. 7C, downregulation of TFAM by shRNA reduced $\Delta\Psi_m$ (by 55%) and mtDNA copy number (by 64%),

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Fig. 5. Hydrogen peroxide ($\text{H}_2\text{O}_2$)-induced MtD and podocyte injury in podocytes. A: mitochondrial function in podocytes. Confluent podocytes were treated with 100 µM $\text{H}_2\text{O}_2$ for the indicated periods of time, and mitochondrial superoxide anion production, $\Delta\Psi_m$, and mtDNA copy number were detected. B: nephrin and podocin protein expressions. Left: representative immunoblots. Right: densitometric analysis. Podocytes were treated as in A, and nephrin and podocin protein expressions were assessed by immunoblotting analysis. Values represent means ± SE; $n = 6$. *$P < 0.01$ vs. control by ANOVA.
TFAM overexpression blocks Aldo-induced MtD and podocyte injury. We next tested whether TFAM overexpression prevents podocyte injury by improving mitochondrial function using TFAM expressing adenoviral vectors. We first detected the effect of Aldo on TFAM expression in podocytes. As shown in Fig. 8, podocytes were exposed to 0–100 nM of Aldo, which dose dependently reduced TFAM expression with a significant effect at 100 nM (Fig. 8, A and B). A time-course experiment after 100 nM Aldo treatment showed that its effects were time dependent, with a significant reduction in TFAM expression observed after 12 h of treatment (Fig. 8, C and D). Both MnTBAP and NAC notably inhibited the decrease in TFAM induced by Aldo (Fig. 8, E and F).

Podocytes were infected with increasing multiplicities of infection of Ad-TFAM. As shown in Fig. 9A, a dose-dependent induction of TFAM protein levels was observed in podocytes infected with Ad-TFAM. Ad-TFAM at multiplicities of infection of 50 induced a >3.5-fold increase in TFAM expression. TFAM overexpression inhibited Aldo-induced mitochondrial superoxide anion production and restored $\Delta \Psi_m$ and mtDNA copy number (Fig. 9B).

We next determined whether TFAM overexpression conferred protection against Aldo-induced podocyte injury. As shown in Fig. 9, C and D, TFAM overexpression inhibited Aldo-induced reduction of nephrin and podocin. Thus TFAM overexpression inhibited Aldo-induced podocyte injury by inhibiting MtD.

DISCUSSION

In the present study, we observed that the levels of kidney MDA and urine F2-isoprostane, markers of renal oxidative stress, increased significantly after 3 days of Aldo infusion and remained substantially increased until day 14. ROS play a key role in the progression of renal injury, and increased generation of ROS is a common risk factor for podocyte injury (9). In recent years, it has become clear that one of the most important pathways by which Aldo exerts its negative effects on the kidney...
is through production of ROS (29, 38). Patni et al. (29) demonstrated that diphenylene iodonium chloride (DPI), an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, partially attenuates Aldo-induced tubule cell apoptosis. Our previous study showed that after 60 min of Aldo treatment, a significant amount of ROS is released in podocytes (40). Consistent with this finding, renal ROS production was significantly induced as early as 3 days after Aldo infusion in mice in this study. The mitochondrial respiratory chain is the major source of ROS, and excessive oxidative stress may result from damaged mitochondria (21). In cultured podocytes, using a mitochondrial specific superoxide indicator, MitoSOX, we found that ROS production originated from the mitochondria after 2 h of Aldo stimulation.

In addition to being a source of ROS, mitochondria themselves can be the target of ROS, causing mtDNA damage (12). Mitochondrial encoded gene expression is largely regulated by the mtDNA copy number (11, 12). The loss or mutation of mtDNA is associated with some human glomerular diseases. For example, a reduction in mtDNA copy number in the kidney cortex accounts for MtD in Finnish type congenital nephrotic syndrome (32). MtDNA is prone to oxidative stress, since it lacks histone-like coverage and is localized to the inner mitochondrial membrane, a major site of ROS production (12). Our present study demonstrated that mtDNA was extremely sensitive to ROS. While Aldo treatment induced a 44% increase of mtDNA copy number after 1 day of Aldo infusion, a 16% decrease was observed after 3 days, and it further reduced to ~45% by day 14. In cultured podocytes Aldo decreased the mtDNA copy number after 8 h of stimulation, which was sustained until 24 h. In line with the mtDNA damage, Aldo decreased \( \Delta \Psi_m \). Moreover, our data demonstrated that exogenous \( H_2O_2 \) significantly induced the generation of mitochondrial ROS and decreases \( \Delta \Psi_m \) and the mtDNA copy number, indicating that \( H_2O_2 \) elicits the same detrimental response to mitochondrial dysfunction as Aldo.

MnSOD is well known as one of the major antioxidant enzymes targeting mitochondrial superoxide anion, and it catalyzes the dismutation of superoxide anion into hydrogen peroxide, protecting cells from damage by oxidative stress (22). In the present study, we found that MnSOD was remarkably increased following Aldo exposure. Both the MnSOD mimic MnTBAP and NAC completely reversed MnSOD up-regulation, indicating that ROS are also involved in the augmentation of MnSOD. Consistent with our results, Abid et al. (1) has shown that vascular endothelial growth factor induces ROS release in coronary artery endothelial cells and further increases MnSOD expression. Moreover, the decrease in
mitochondrial copy number and the collapse of ΔΨm induced by Aldo were also abrogated by pretreatment with MnTBAP or NAC. Therefore, we postulated that MnSOD upregulation may serve as a protective mechanism against the formidable threat of Aldo-induced ROS in podocytes and that it may reflect an insufficient compensatory effect of the antioxidative system.

We found that ROS production increased and the ΔΨm, ATP production, and mtDNA copy number had decreased by day 3, whereas the proteinuria and podocyte damage occurred after 5 days of Aldo infusion, indicating that MtD in the kidney occurred before the renal damage. These data were verified in vitro, where MtD induced by Aldo or H2O2 treatment in podocytes occurred before the renal damage. These data were verified in days of Aldo infusion, indicating that MtD in the kidney whereas the proteinuria and podocyte damage occurred after 5 days.

To explore the direct effect of MtD on podocyte injury, we treated podocytes with 100 mg/ml EtBr based on the high sensitivity of mtDNA to low concentrations of EtBr, which specifically inhibits mtDNA replication and transcription, thus inducing mtDNA depletion without affecting nuclear DNA (4, 24). MtDNA has four main regions: D-loop, rRNA, tRNA, and genes that encode proteins. The noncoding D-loop region contains one of the origins of replication and the promoters for mtDNA transcription. MtDNA encodes 13 polypeptides of the respiratory chain including COX I and ND. D-loop, COX I, and ND6 have been previously proven to be useful markers for mtDNA depletion (24). Using these techniques, Moro et al. (24) demonstrated that mtDNA depletion alters fundamental biological processes involved in the pathophysiology of prostate carcinogenesis such as cell migration and apoptosis. In this study, we demonstrated that mtDNA depletion induced MtD, as evidenced by the generation of mitochondrial superoxide anion; reduction of ΔΨm, mtDNA copy number, and ATP production; and podocyte injury, which were reversible after EtBr removal. These data indicated that MtD could directly induce podocyte damage.

TFAM, a transcriptional factor for mtDNA, enhances mtDNA transcription in a promoter-specific fashion, indicating that it plays an important role in regulating mtDNA copy number (17,
Fig. 9. TFAM overexpression restored Aldo-induced MtD and prevented podocyte damage. A: TFAM expression in infected podocytes. Podocytes were infected with increasing multiplicities of infection (10, 20, and 50) of adenovirus (Ad)-TFAM, and the expression of TFAM was examined by immunoblotting 24 h after infection. Left: representative immunoblots. Right: densitometric analysis. MOI, multiplicities of infection. B: effect of TFAM overexpression on mitochondrial function in Aldo-treated podocytes. After infection with Ad-TFAM or empty vector (Vehi) for 24 h, podocytes were treated with Aldo (100 nM) for another 24 h. Mitochondrial superoxide anion production, \( \Delta F_m \) and mtDNA copy number were determined and there are no significant differences between control and Aldo treatment of the Ad-TFAM group. C: nephrin and podocin mRNA expression. D: nephrin and podocin protein expression. Left: representative immunoblots; Right: densitometric analysis. Podocytes were treated as in A, and nephrin and podocin mRNA and protein expression was determined by real-time RT-PCR and immunoblotting analysis, respectively. Values represent means ± SE, \( n = 6. *P < 0.01 \) vs. control group by ANOVA. **P < 0.01 vs. Aldo-treated vehicle group by ANOVA.

Fig. 10. Schematic representation of the relationships among risk factors, mitochondrial dysfunction (mitochondrial oxidative stress, mtDNA damage, and mitochondrial enzymatic activity decrease), and podocyte injury.
19). Disruption of the Tfam gene causes depletion of mtDNA, loss of mitochondrial transcripts, loss of mtDNA-encoded polypeptides, and severe respiratory chain deficiency both in vivo and in vitro (16, 18). Deficiency of TFAM results in increased ROS via hydrogen peroxide production (3). Moreover, targeted disruption of Tfam in cardiac myocytes was shown to induce deletion of mtDNA and dilated cardiomyopathy (34). Therefore, overexpression of TFAM may prevent mitochondrial function and protect cells from injury by up-regulating mtDNA, inhibiting oxidative stress, and restoring the mitochondrial respiratory chain (14, 33). To confirm the direct induction of MtD on podocyte damage and to prevent the effect of Aldo on podocyte injury, we suppressed TFAM expression by shRNA and measured mitochondrial function and podocyte injury. Similar to the effects of EtBr and Aldo on podocytes, inhibition of TFAM expression by shRNA significantly induced MtD, as evidenced by overproduction of mitochondrial superoxide anion, deletion of mtDNA, and collapse of ΔΨm. In line with the depletion of TFAM and mtDNA, podocyte damage occurred with the downregulation of nephrin and podocin, the markers of podocyte injury. Conversely, overexpression of TFAM prevented MtD, as demonstrated by amelioration in decline of mtDNA copy number, prevention of ROS overproduction, and restoration of mitochondrial enzymic activity, as well as protective effects from cell injury in Aldo-treated podocytes. Thus MtD is proposed as an important link between risk factors (such as Aldo and oxidative damage) and the initiation and development of podocyte damage (Fig. 10).

Our present study clearly demonstrated that Aldo inhibited TFAM expression. In Aldo-infused mice, renal TFAM expression was decreased from day 3 until day 14. Consistent with these findings, Hagiwara et al. (8) have demonstrated that TFAM expression is decreased in rats with puromycin amionic-nucleoside-induced focal segmental glomerulosclerosis. In our study, Aldo decreased TFAM expression in a time- and dose-dependent fashion. Moreover, the Aldo-induced increase in mitochondrial ROS preceded the TFAM decrease. Both MnT-BAP and NAC notably inhibited the decrease in TFAM induced by Aldo. TFAM overexpression prevented Aldo-induced oxidative stress and the Aldo-induced decreases in mtDNA copy number and mitochondrial electron transport function, which contributed to podocyte protection. These results suggested that Aldo induced mitochondrial ROS production, decreasing TFAM expression and inducing podocyte injury. TFAM deficiency further increased ROS and decreased the mtDNA copy number, creating a vicious circle of podocyte injury. Our earlier study also demonstrated that overexpression of proliferator-activated receptor-γ (PPARY) coactivator-1α (PGC-1α), PPARγ, or PPARγ agonist rosiglitazone protected against podocyte damage via prevention of MtD (37, 40). Therefore, these findings indicated that preventing MtD is a key step to protecting podocytes.

In summary, we present here new evidence that MtD contributes to Aldo-induced podocyte injury. More importantly, overexpression of TFAM protects against Aldo-induced podocyte injury via restoration of mitochondrial function. These findings support MtD as an initiating event in podocyte injury induced by Aldo and suggest that novel strategies for treatment of glomerular diseases such as podocytopathy may be developed based on manipulation of TFAM.


