Dysfunction of the PGC-1α-mitochondria axis confers adriamycin-induced podocyte injury

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ADRIAMYCIN (ADR) is one of the most potent chemotherapeutic agents for the treatment of many cancer types in the clinic. However, the severe toxicity to noncancerous cells, particularly to cells in the kidney and heart, has greatly limited its application. ADR-induced kidney injury, which is called ADR nephropathy, is a severe clinical complication with high mortality and morbidity. In rodents, ADR can induce glomerular injury with a pathology similar to focal segmental glomerulosclerosis (FSGS), which is characterized by podocyte dysfunction, proteinuria, reduced glomerular filtration rate, and tubulointerstitial fibrosis (22). Although a number of studies on ADR nephropathy have been performed in recent decades, the therapeutic outcome of this disease remains unsatisfactory because of the uncertain pathogenesis and lack of specific therapeutic targets. The podocyte has recently become a focus in this field, and there is growing evidence that ADR is toxic for podocytes, which subsequently causes podocyte damage, FSGS development, and proteinuria (10, 39).

In addition to being useful for studying the kidney side effects of ADR, the ADR-induced nephropathy model mimics human FSGS. In the clinic, FSGS is a common pathological manifestation of nephrotic syndrome in children and an important cause of renal failure in adults (5, 12, 20). As a podocyte disease (28), FSGS has a poorly understood pathogenic mechanism and unsatisfactory response to traditional therapies such as steroids and immunosuppressants (12, 29). Assessing the potential mechanisms of podocyte injury in FSGS models will elucidate the pathogenesis of human FSGS.

Growing evidence has demonstrated that peroxisome proliferator-activated receptor (PPAR)-γ coactivator (PGC)-1α plays a crucial role in oxidative metabolism and mitochondrial biogenesis, possibly by upregulating mitochondrial transcription factor A (TFAM), a direct regulator of mitochondrial (mt)DNA replication (37). Importantly, recent studies have shown that mitochondrial dysfunction is involved in podocyte injury in pathological models such as excessive aldosterone administration (14, 32, 36). Moreover, our group found that endogenous PGC-1α has an important role in protecting podocyte integrity and function by maintaining normal mitochondrial function in response to aldosterone-induced podocyte injury (40). In the present study, we investigate the following: 1) whether PGC-1α and mitochondria function were altered in ADR-treated podocytes and animal kidneys, 2) whether PGC-1α regulates mitochondria function in ADR-induced podocyte injury, and 3) whether dysregulation of the PGC-1α-mitochondria axis mediates the ADR effect on podocytes.

MATERIALS AND METHODS

Reagents. Caelyx (liposomal ADR) was purchased from Merck (Whitehouse Station, NJ). Anti-cytochrome c antibody and 2’,7’-dichlorofluorescein (DCF) diacetate (DCFDA) were purchased from Sigma (St. Louis, MO). We used anti-nephrin (Abcam, Cambridge, MA), anti-podocin (Abcam), anti-PGC-1α (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin antibodies (Cell Signaling Technology, Beverly, MA). SYBR green master mix for real-time PCR was purchased from Applied Biosystems (Foster City, CA).

Animals. All experiments were performed with the approval of the experimental animal committee of Nanjing Medical University. Male Sprague-Dawley rats (170 ± 10 g) were purchased from Shanghai SLAC Laboratory Animals (Shanghai, China). Animals were housed and fed under standard conditions. Rats were randomly assigned to the following two groups (n = 6 rats/group): the ADR group received a single, slow tail vein injection of 5 mg/kg ADR, and the control group

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received pure saline injections. After 2 wk, rats were anesthetized with 5 mg/kg urethane. Kidneys were harvested for the analysis of protein levels and gene expression.

Cell culture and treatments. MPC5 conditionally immortalized mouse podocyte clonal cells (kindly provided by Peter Mundel, Mount Sinai School of Medicine, through J. Ding, Peking University) were cultured and induced to differentiate as previously described (26).

Before the experiments, cells were subcultured to 80–90% confluency in various-sized culture vessels (6-well plates and 60-mm dishes) depending on the number of cells required for the protocols, and cells were then incubated in 1% FBS-supplemented medium for 24 h to encourage quiescence. To determine the proper stimulatory dose, we administered ADR at concentrations from 50 nM to 200 nM and encouraged quiescence. To determine the proper stimulatory dose, we received pure saline injections. After 2 wk, rats were anesthetized with 5 mg/kg urethane. Kidneys were harvested for the analysis of protein levels and gene expression.

Podocyte apoptosis. After treatment, annexin V-FITC and propidium iodide double staining (annexin V:FITC Apoptosis Detection Kit, BD Biosciences, San Diego, CA) were used to stain podocytes treated for each experiment.

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Virus infection. Adenoviral vectors encoding FLAG-tagged mouse PGC-1α (Ad-PGC-1α) were obtained from Addgene (Cambridge, MA). Cells were infected with adenoviruses for 24 h before ADR treatment as previously described (40).

Podocyte apoptosis. After treatment, annexin V-FITC and propidium iodide double staining (annexin V:FITC Apoptosis Detection Kit, BD Biosciences, San Diego, CA) were used to stain podocytes according to the manufacturer’s protocol. We quantified apoptosis with flow cytometry (Becton Dickinson).

PCR. Total RNA from cultured podocytes and the renal cortex was isolated using the TRIzol Total RNA Isolation kit (Invitrogen, Carlsbad, CA). Single-stranded cDNA was obtained by reverse transcription according to the manufacturer’s protocol. Total DNA from cultured podocytes and the renal cortex was isolated using a DNeasy Tissue kit (Invitrogen). The primer pair sequences are shown in Table 1.

Table 1. Primer sequences for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Nephrin (mouse)</td>
<td>5'-CCGAGGTACACAGGACACAA-3'</td>
<td>5'-CTCAAGCTCAACACCTTCA-3'</td>
</tr>
<tr>
<td>Podocin (mouse)</td>
<td>5'-CCGAAATCATTCTGCAAGC-3'</td>
<td>5'-AGGAGCGGAGGAGCAAGA-3'</td>
</tr>
<tr>
<td>PGC-1α (mouse)</td>
<td>5'-CTACTGCTTACCGAAGCT-3'</td>
<td>5'-TGAGGAGCTTACGAAGGGT-3'</td>
</tr>
<tr>
<td>mtDNA (mouse)</td>
<td>5'-TTTTTCTCATTCTGAGTTC-3'</td>
<td>5'-CCACCTTCATTTACGATTATATG-3'</td>
</tr>
<tr>
<td>18S rRNA (mouse)</td>
<td>5'-GGACCTGGAACTGGGACCAT-3'</td>
<td>5'-GCCGCTGAACTTTCTTTT-3'</td>
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Fig. 2. ADR induced mitochondrial dysfunction in a dose-dependent manner. A: mitochondrial (mt)DNA copy numbers. After 24 h of exposure to different concentrations of ADR (0–200 nM), total podocyte DNA was extracted. mtDNA copy numbers were detected by real-time PCR. B: representative images of podocytes stained with JC-1. Cells were treated with ADR or vehicle for 24 h and incubated with JC-1 dye for 30 min. C: quantitative analysis of JC-1 fluorescence by flow cytometry. D: Quantitative analysis of tetramethylrhodamine ethyl ester (TMRE) fluorescence by flow cytometry. Cells were treated with ADR or vehicle for 24 h and incubated with TMRE for 30 min. E: ATP content. Podocytes were incubated with ADR or vehicle for 24 h, and ATP content was measured and normalized to protein concentration. Values are means ± SD; n = 6 per group. *P < 0.05 vs. control according to ANOVA.

Fig. 3. ADR increased ROS production and cell apoptosis in a dose-dependent manner. A: ROS production in podocytes. Podocytes were incubated with different ADR concentrations (0–200 nM) for 24 h, and 2′,7′-dichlorofluorescein (DCF) fluorescence was measured by flow cytometry. B: mitochondrial superoxide was measured with MitoSOX red reagent by flow cytometry. C: mitochondrial morphology was imaged by transmission electron microscopy after 24-h incubation with different concentrations of ADR. D: ADR-induced podocyte apoptosis. Podocytes were incubated with ADR at the indicated concentrations (0–200 nM) for 48 h, and cell apoptosis was determined by flow cytometry. Values are means ± SD; n = 6 per group. *P < 0.05 vs. control according to ANOVA.
plates at 37°C in the dark at a final concentration of 5 μM for 10 min. Fluorescence was then measured as described above.

Mitochondrial membrane potential. Mitochondrial membrane potential (MMP) was assessed by measuring the potential-dependent accumulation of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carboxycyanine iodide (JC-1; Molecular Probes) or tetramethylrhodamine ethyl ester (Invitrogen-Molecular Probes) (27). As previously described (1), the isolated mitochondrial pellet and dissociated podocytes were washed twice with HBSS (Sigma) and then incubated in the dark with JC-1 (7.5 μM) for 30 min at 37°C. Mitochondria and cells were then washed with JC-1 washing buffer. Fluorescence in podocytes and isolated mitochondria was detected with FACS and a FLUOstar Optima reader, respectively.

ATP measurements. ATP in podocytes and the renal cortex was detected with a luciferase-based bioluminescence assay kit (Sigma) in a FLUOstar Optima reader according to the manufacturer’s instructions. Each total ATP level was calculated as the luminescence normalized to the protein concentration.

Western blot analysis. Podocytes or renal cortex homogenate (100 mg) was lysed in protein lysis buffer with a protease inhibitor cocktail (Sigma) for 20 min on ice. After centrifugation, the supernatant was harvested, and lystate protein concentrations were measured with a BCA Protein Assay kit (Pierce). Immunoblot analysis was performed with anti-nephrin (1:200), anti-podocin (1:500), anti-PGC-1α (1:200), or anti-β-actin antibody (1:1,000); samples were then treated with horseradish peroxidase-labeled secondary antibodies and ECL visualization. Bands were visualized with a GS-800 Calibrated Densitometer (Bio-Rad, Philadelphia, PA), and densitometry was performed with Quantity One Software (Bio-Rad).

Transmission electron microscopy. To evaluate mitochondrial morphology, live podocytes were collected, fixed in 1.25% glutaraldehyde and 0.1 M phosphate buffer, and postfixed in 1% OsO4 and 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 with an electron microscope (JEOL JEM-1010, Tokyo, Japan).

Fig. 4. ADR decreased peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α expression in a dose-dependent manner. Podocytes were incubated for 24 h with different concentrations of ADR (0–200 nM), and PGC-1α protein and mRNA expressions were detected by Western blot analysis and real-time RT-PCR. A: Western blots of PGC-1α and β-actin. B: densitometric analysis of the PGC-1α Western blot. C: real-time RT-PCR of PGC-1α. Values are means ± SD; n = 6 per group. *P < 0.05 vs. control according to ANOVA.

Fig. 5. Overexpression of PGC-1α in podocytes. Podocytes were infected with Ad-PGC-1α at a multiplicity of infection of 50 or empty vector [vehicle (Vehi)]. After infection with Ad-PGC-1α for 24 h, podocytes were treated with or without ADR (200 nM) for another 24 h, and PGC-1α protein and mRNA expressions were detected. A: Western blots of PGC-1α and β-actin. B: densitometric analysis of the PGC-1α Western blot. C: real-time RT-PCR of PGC-1α. Values are means ± SE; n = 6 per group. *P < 0.05 vs. control (Cntl); **P < 0.05 vs. the ADR-treated group according to ANOVA.

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Statistical analysis. All results are presented as means ± SD. One-way or two-way ANOVA was used for the statistical analysis (SPSS 16.0). P values of <0.05 were considered statistically significant.

RESULTS

ADR induced podocyte injury and mitochondrial dysfunction. First, we examined the effect of different ADR concentrations on nephrin and podocin levels in cultured podocytes. When podocytes were exposed to 0–200 nM of ADR, both mRNA and protein expressions of nephrin and podocin were down-regulated in a dose-dependent manner (Fig. 1, A–D).

Given the recent evidence that mitochondrial dysfunction is involved in podocyte injury, we evaluated cell mitochondrial function by measuring superoxide production, mitochondrial membrane potential, intracellular ATP content, and mtDNA copy number. mtDNA damage is a reliable, sensitive indication of mitochondrial dysfunction (38). In cells treated with different doses of ADR, mtDNA copy number (Fig. 2A), mitochondrial membrane potential (Fig. 2, B–D), and podocyte ATP content (Fig. 2D) were reduced in a dose-dependent manner.

DCF fluorescence, an index of ROS production, and MitoSOX, an indicator of mitochondrial superoxide, were used to assess the mitochondrial oxidative stress. After 60 min of ADR treatment, both were strikingly enhanced (Fig. 3, A and B). In the meantime, the mitochondrial morphology in the ADR-treated podocytes was remarkably altered, with swelling and fusion of the cristae (Fig. 3C). In agreement with mitochondrial dysfunction, ADR-induced podocyte apoptosis was significantly increased in a dose-dependent manner, and there was a noticeable effect at 100 nM and a maximal effect at 200 nM (Fig. 3D).

ADR decreased PGC-1α expression. PGC-1α is a transcriptional coactivator of PPAR-γ and other nuclear hormones (13, 30, 42). Repression of PGC-1α caused abnormal mitochondrial morphology and downregulation of mitochondria-related enzymes (6). In agreement with mitochondrial dysfunction, ADR significantly downregulated podocyte PGC-1α expression in a dose-dependent manner (Fig. 4, A–C).

PGC-1α overexpression inhibited ADR-induced mitochondrial dysfunction and podocyte injury. Considering the reduction of PGC-1α in ADR-treated podocytes, we next studied whether PGC-1α overexpression can ameliorate ADR-induced podocyte injury and protect against the mitochondrial dysfunction observed in other injury models (21, 23, 40). After Ad-PGC-1α infection of podocytes, mRNA and protein expression of PGC-1α were significantly increased in both control and ADR-treated cells compared with empty vector-treated groups (Fig. 5, A–C). Next, we evaluated podocyte injury by measuring nephrin and podocin expression levels and apoptosis. As shown in Fig. 6, PGC-1α overexpression significantly restored nephrin and podocin downregulation and inhibited apoptosis in ADR-treated cells. Furthermore, mitochondrial dysfunction parameters (ROS production, mtDNA, mitochondrial membrane potential, and ATP content) were evaluated. As shown in Fig. 7, ROS production was remarkably increased, which was in contrast with the significant reduction of mitochondrial membrane potential, mtDNA, and ATP content after ADR treatment. After PGC-1α overexpression, all of these impaired parameters were strikingly improved (Fig. 7, A–F).

ADR reduced PGC-1α and TFAM expression levels in rat kidneys. To further determine whether a ADR-induced PGC-1α reduction occurs in vivo, we examined mRNA and protein expression levels of PGC-1α in ADR-treated rats. As
Fig. 7. PGC-1α overexpression blocked ADR-induced mitochondrial dysfunction. Podocytes were infected with Ad-PGC-1α at a multiplicity of infection of 50 or empty vector (vehicle). After 24-h infection with Ad-PGC-1α, podocytes were treated with or without ADR (200 nM) for another 24 h. A: quantitative analysis of ROS production with DCF fluorescence. B: quantitative analysis of mitochondrial superoxide with MitoSOX fluorescence. C: mitochondrial membrane potential measurement by JC-1. D: mitochondrial membrane potential measurement by TMRE. E: real-time PCR of mtDNA copy number. F: ATP content. Values are means ± SD; n = 6 per group. *P < 0.05 vs. control; **P < 0.05 vs. the ADR-treated group according to ANOVA.

Fig. 8. ADR reduced expressions of PGC-1α and mitochondrial transcription factor A (TFAM) in kidneys of ADR-treated rats. Rats were randomly assigned to the following two groups (n = 6 rats/group): the ADR group received a single tail vein injection of 5 mg/kg ADR and the control group received a saline injection. A: Western blots of PGC-1α and β-actin. B: densitometric analysis of the PGC-1α Western blot. C: real-time RT-PCR analysis of PGC-1α. D: real-time RT-PCR analysis of TFAM. Values are means ± SD. *P < 0.05 vs. control.
shown in Fig. 8, A–C, both mRNA and protein expression levels of PGC-1α were significantly decreased by ADR treatment. At the same time, TFAM, a key activator of mitochondrial transcription and a participant in mitochondrial genome replication, was robustly decreased by ADR, suggesting a severe disruption of mitochondria in ADR-treated kidneys (Fig. 8D).

**DISCUSSION**

In the ADR-induced nephropathy model, loss of podocyte foot processes, podocyte depletion, and the consequent progression to glomerulosclerosis account for the major pathological lesions (4). Although a number of studies have assessed the effect of ADR on podocytes, the mechanism of ADR-induced podocyte injury is still uncertain. Mitochondria are important energy production organelles, and oxidative phosphorylation is associated with generation of the byproduct superoxide anion (18). Our recently published study (40) suggests that the PGC-1α-mitochondria axis has an important role in mediating aldosterone-induced podocyte damage. However, it is still unknown whether this axis also contributes to other pathological events in podocytes. In present study, we found that ADR-induced podocyte injury is accompanied by downregulation of PGC-1α and severe mitochondria dysfunction.

PGC-1α can interact with and coactivate nuclear respiratory factor 1, which is thought to interact with TFAM. TFAM plays an important role in mtDNA synthesis (8). Therefore, PGC-1α can regulate mitochondrial biogenesis. PGC-1α loss impairs mitochondrial respiratory function and enhances mitochondrial oxidative stress and apoptotic susceptibility (2, 25, 40). Haemmerle et al. (15) reported that the PGC-1α signaling pathway is a potential therapeutic target for the treatment of patients with neutral lipid storage disease. Lin et al. (24) reported that adiponectin exerts a cytoprotective effect through the PPAR-α-PGC-1 signaling pathway. In agreement with these hypotheses, we found that PGC-1α is a sensitive regulator of mitochondrial function in ADR-treated podocytes. ADR treatment remarkably downregulated PGC-1α expression in both in vitro podocytes and animal kidneys. Restoration of PGC-1α in podocytes protected against ADR-induced injury, possibly via promoting mitochondria biogenesis and inhibiting oxidative stress.

In adverse conditions, the mitochondrial respiratory chain is one of the most prominent sources of intracellular ROS production (7). In response to excess ROS, mitochondrial thiols are rapidly oxidized, and ADP and ATP exchange is impaired to the point that ATP synthase is partially blocked (3, 33). In the present study, we detected a robust increase in mitochondrial-derived ROS production, which could be an important cause of the cellular ATP content reduction.

Programmed cell death is initiated when overactivated stress causes mtDNA mutations and protein misfolding. ROS cause mtDNA damage and protein misfolding (31). The damaged mitochondria often become highly permeabilized, which can subsequently cause the release of cytotoxic and proinflammatory substances, such as cytochrome c and mtDNA, thereby initiating the proapoptotic cascade (11, 41). To eliminate the dangerous consequences of depolarized mitochondria, intracellular mitophagy is initiated (19). Mitochondrial dysfunction is responsible for the induction of mitophagy (9). When mitochondrial damage overwhels the removal ability of mitophagy, apoptosis dominates. In agreement with this finding, we found that the striking apoptotic response in ADR-treated cells is in parallel with increased mitochondria damage and ROS production. The attenuation of mitochondrial dysfunction by PGC-1α overexpression significantly blocked ROS production and cell apoptosis. Given that mitochondrial dysfunction may be an early event and important pathogenic factor for podocyte injury, an increasing number of studies investigated mitochondria-targeted therapeutic strategies for podocytopathy via targeting the sir-tuin1-PGC-1α axis (35, 40), silencing Rho-associated coiled-coil-containing protein kinase 1 (36), inhibiting mammalian target of rapamycin, or controlling mitophagy (17). Thomas et al. (34) found that injecting recombinant human mitochondrial TFAM, a downstream target of PGC-1α, improves mitochondrial respiration in the brain and peripheral tissues of aged mice. In the present study, we found that TFAM, a downstream target of PGC-1α and key regulator of mitochondrial transcription and mitochondrial genome replication, was significantly reduced by ADR treatment in rat kidneys. This result also indicates that the PGC-1α effect on mitochondrial function in the ADR model is possibly due to TFAM action. However, more in vivo studies are needed to elucidate the in vivo function of PGC-1α and TFAM in both ADR-induced podopathy and mitochondrial dysfunction.

In summary, we present new evidence that PGC-1α and mitochondrial dysfunction contribute to ADR-induced podocyte injury and that PGC-1α overexpression prevents ADR-induced podocyte damage by restoring mitochondrial function. These findings provide new insights into the pathogenic process of ADR-induced podocyte injury. Furthermore, this study suggests that targeting PGC-1α and/or mitochondria might be a promising strategy for the treatment of podocytopathy.

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**AUTHOR CONTRIBUTIONS**


**DISCLOSURES**

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

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