Dysfunction of the PGC-1α/Mitochondria Axis Confers Adriamycin-Induced Podocyte Injury

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Running title: PGC-1α/mitochondria axis and ADR-induced podocytes injury
Abstract

Adriamycin (ADR)-induced nephropathy in animals is an experimental analogue of human focal segmental glomerulosclerosis (FSGS), which presents as severe podocyte injury and massive proteinuria and has a poorly understood mechanism. The current study was designed to test the hypothesis that the peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α)/mitochondria axis is involved in ADR-induced podocyte injury. Using MPC5 immortalized mouse podocytes, the ADR dose-dependently induced the downregulation of nephrin and podocin, cell apoptosis, and mitochondrial dysfunction based on the increase in mitochondrial reactive oxygen species (ROS) production, a decrease in mitochondrial DNA (mtDNA) copy number, and the reduction of mitochondrial membrane potential (MMP) and ATP content. Moreover, ADR treatment also remarkably reduced the expression of PGC-1α, an important regulator of mitochondrial biogenesis and function, in podocytes. Strikingly, PGC-1α overexpression markedly attenuated mitochondrial dysfunction, reduction of nephrin and podocin, and the apoptotic response in podocytes following ADR treatment. Moreover, the downregulation of PGC-1α and mitochondria disruption in podocytes were also observed in rat kidneys with ADR administration, suggesting that the PGC-1α/mitochondria axis is relevant to the in vivo ADR-induced podocyte damage. Taken together, these novel findings suggest that dysfunction of the PGC-1α/mitochondria axis is highly involved in ADR-induced podocyte injury. Targeting PGC-1α may be a novel strategy for treating ADR nephropathy and FSGS disease.

Keywords: podocyte injury; mitochondrial dysfunction; adriamycin nephropathy; PGC-1α
Introduction

Adriamycin is one of the most potent chemotherapeutic agents for treating 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 adriamycin 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 GFR, and tubulointerstitial fibrosis (22). Although a number of studies on adriamycin 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 the peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) plays a crucial role in oxidative metabolism and mitochondrial biogenesis, possibly by upregulating mitochondrial transcription factor A (TFAM), a direct
regulator of mtDNA 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 the 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, USA). Anti-
cytochrome c antibody and 2’,7’-dichlorofluorescein diacetate (DCFDA) were purchased from
Sigma (St. Louis, MO, USA). We used anti-nephrin, anti-podocin (Abcam, Cambridge, MA,
USA), anti-PGC-1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-β-actin
antibodies (Cell Signaling Technology, Beverly, MA, USA). SYBR Green master mix for real-
time PCR was purchased from Applied Biosystems (Foster City, CA, USA).

**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 Co., Ltd. (Shanghai, China). The animals were housed and
fed under standard conditions. The rats were randomly assigned to the following two groups (n=6 each): the ADR group received a single, slow tail-vein injection of 5 mg/kg ADR, and the control rats received pure saline injections. After 2 weeks, rats were anesthetized with 5 mg/kg of urethane. The kidneys were harvested for analysis of the protein levels and gene expression.

**Cell culture and treatments**

MPC5 conditionally immortalized mouse podocyte clonal cells (kindly provided by Peter Mundel at the Mount Sinai School of Medicine through Dr. Jie Ding at Peking University) were cultured and induced to differentiate as previously described (26). Briefly, podocytes were maintained at 33°C with interferon-γ for proliferation, and they were cultured at 37°C in the absence of interferon-γ to induce differentiation. All cells were between passages 3 and 5.

Before the experiments, the cells were subcultured to 80-90% confluency in various-sized culture vessels (six-well plates and 60 mm dishes), depending on the number of cells required for the protocols, and the cells were then incubated in 1% fetal bovine serum-supplemented medium for 24 h to encourage quiescence. To determine the proper stimulatory dose, we administered ADR concentrations of 50 nM to 200 nM and finally chose 200 nM for use in the main experiments. The duration of stimulation was based on the protocol requirements, which are illustrated for each experiment.

**Virus infection**

Adenoviral vectors encoding FLAG-tagged mouse PGC-1α (Ad-PGC-1α) were obtained from Addgene (Cambridge, MA, USA). Cells were infected with adenoviruses for 24 h prior to the ADR treatment as previously described (40).
**Podocyte apoptosis**

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

**PCR**

Total RNA from the cultured podocytes and renal cortex was isolated using the TRIzol Total RNA Isolation kit (Invitrogen, Carlsbad, CA, USA). Single-stranded cDNAs were obtained by reverse transcription according to the manufacturer’s protocol. Total DNA from the cultured podocytes and renal cortex was isolated using a DNeasy Tissue Kit (Invitrogen, Carlsbad, CA, USA). The primer pair sequences are given in tables 1. The mRNA and mtDNA copy numbers were detected with real-time PCR. Amplification was performed using the ABI 7300 Real-time PCR Detection System (Foster City, CA, USA) with SYBR Green master mix (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S rRNA served as internal controls for the mRNA and mtDNA, respectively. The thermal cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

**Reactive oxygen species (ROS) and mitochondrial superoxide measurement**

DCFDA is a cell-permeable fluorogenic dye that can be oxidized by H$_2$O$_2$ to produce fluorescence; therefore, DCFDA was used to monitor the intracellular generation of ROS as
previously described (16). To measure ROS, podocytes were cultured in six-well plates until they reached confluence. Plates were incubated with 10 μM DCFDA in the dark at 37°C for 30 min. Then, the podocytes were harvested and washed twice with Hanks’ balanced salt solution (Sigma, St. Louis, MO, USA), and the fluorescence was measured with flow cytometry.

The ROS generated by mitochondria were also measured with the MitoSOX Red reagent (Invitrogen, Carlsbad, CA, USA), an indicator of mitochondrial superoxide. Briefly, 1 ml of MitoSOX Red reagent working solution was incubated with the podocytes; the podocytes adhered to the six-well plates at 37°C in the dark at a final concentration of 5 μM for 10 min. The fluorescence was then measured as described above.

Mitochondrial membrane potential (MMP)

The mitochondrial membrane potential (MMP) was assessed by measuring the potential-dependent accumulation of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1; Molecular Probes) or tetramethylrhodamine ethyl ester (TMRE, Invitrogen-Molecular Probes, Eugene, OR, USA)(27). As previously described (1), the isolated mitochondrial pellet and dissociated podocytes were washed twice with Hank's balanced salt solution (Sigma); they were then incubated in the dark with JC-1 (7.5 μM) for 30 min at 37°C. Then, the mitochondria and cells were washed with JC-1 washing buffer. The fluorescence in the podocytes and isolated mitochondria was detected with fluorescence assisted cell sorting (FACS) and a FLUOstar Optima reader, respectively.

ATP measurement

ATP in the podocytes and renal cortex was detected with a luciferase-based bioluminescence
assay kit (Sigma-Aldrich, St. Louis, MO, USA) 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
Podocytes or renal cortex homogenate (100 mg) was lysed in protein lysis buffer with a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) for 20 min on ice. After centrifugation, the supernatant was harvested, and the lysate protein concentrations were measured with a BCA Protein Assay Kit (Pierce). Immunoblotting was performed with anti-nephrin (1:200), anti-podocin (1:500), anti-PGC-1α (1:200), or anti-β-actin antibody (1:1000); then, the samples were treated with horseradish peroxidase-labeled secondary antibodies and ECL visualization. Bands were visualized with a GS-800 Calibrated Densitometer (Bio-Rad, Philadelphia, PA, USA), and densitometry was performed with Quantity One Software (Bio-Rad, Philadelphia, PA, USA).

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

Statistical analysis
All results are presented as the mean ± SD (standard deviation). One-way or two-way analysis of
variance was used for the statistical analysis (SPSS 16.0). A p-value of less than 0.05 was considered statistically significant.

**Results**

**ADR induced podocyte injury and mitochondrial dysfunction**

First, we examined the effect of different ADR concentrations on the nephrin and podocin levels in cultured podocytes. When podocytes were exposed to 0-200 nM of ADR, both the mRNA and protein expressions of nephrin and podocin were downregulated in a dose-dependent manner (Figure 1A-D).

Given the recent evidence that mitochondrial dysfunction is involved in podocyte injury, we evaluated the cell mitochondrial function by measuring the superoxide production, mitochondrial membrane potential (MMP), 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, the mtDNA copy number (Figure 2A), MMP (Figure 2B-D), and podocyte ATP content (Figure 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 (Figure 3A-B). In the meantime, the mitochondrial morphology in the ADR-treated podocytes was remarkably altered, wherein there was swelling and fusion of the cristae (Figure 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 (Figure 3D).
ADR decreased PGC-1α expression

PGC-1α is a transcriptional coactivator of the peroxisome proliferator-activated receptor (PPAR)-γ and other nuclear hormones (13, 30, 42). The 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 (Figure 4A-C).

PGC-1α overexpression inhibited ADR-induced mitochondrial dysfunction and podocyte injury

Considering the reduction of PGC-1α in the 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). Following the Ad-PGC-1α infection of podocytes, the mRNA and protein expressions of PGC-1α were significantly increased in both the control and ADR-treated cells compared with the empty vector groups (Figure 5A-C). Next, we evaluated podocyte injury by measuring the nephrin and podocin expression levels and apoptosis. As shown in Figure 6, PGC-1α overexpression significantly restored nephrin and podocin downregulation and inhibited apoptosis in ADR-treated cells. Furthermore, the mitochondrial dysfunction parameters (ROS production, mtDNA, MMP, and ATP content) were evaluated. As shown in Figure 7, ROS production was remarkably increased, which was in contrast with the significant reduction of MMP, mtDNA and ATP content after ADR treatment. Following PGC-1α overexpression, all of these impaired parameters were strikingly improved (Figure 7A-F).
ADR reduced the PGC-1α and TFAM expression levels in rat kidneys.

To further determine whether ADR-induced PGC-1α reduction occurs in vivo, we examined the protein and mRNA expression levels of PGC-1α in ADR-treated rats. As shown in Figure 8(A-C), both the protein and mRNA 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 the mitochondria in ADR-treated kidneys (Figure 8D).

Discussion

In ADR-induced nephropathy model, loss of podocyte foot processes, podocyte depletion, and the consequent progression to glomerusclerosis account for the major pathological lesions (4). Although a number of studies have assessed the effect of ADR on the podocytes, the mechanism of ADR-induced podocyte injury is still uncertain. Mitochondria are important energy production organelles, and oxidative phosphorylation (OXPHOS) is associated with the generation of the by-product superoxide anion (O₂⁻) (18). Our recently published study suggests that the PGC-1α/mitochondria axis has an important role in mediating aldosterone-induced podocyte damage (40). 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 co-activate nuclear respiratory factor 1, which is thought to interact with transcription factor A (TFAM). TFAM plays an important role in mitochondrial DNA 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 treating patients with neutral lipid storage disease. Lin et al.(24) reported that adiponectin exerts a cytoprotective effect through the PPARα/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. Restoring 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 the 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 mitochondria-derived ROS production, which could be an important cause of the cellular ATP content reduction.

Programmed cell death is initiated when over-activated 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 pro-inflammatory substances, such as cytochrome c and mtDNA, thereby initiating the pro-apoptotic 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 overwhelms the removal ability of mitophagy, apoptosis dominates. In agreement with this finding, we found that a 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 have investigated mitochondria-targeted therapeutic strategies for podocytopathy via targeting the sirtun1-PGC-1α axis (35, 40), silencing Rho-associated coiled coil-containing protein kinase 1(36), inhibiting mTOR, or controlling mitophagy (17). Thomas et al.(34) found that injecting recombinant human mitochondrial transcription factor A, 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 treating podocytopathy.

Conflict of Interests

All the authors declared no competing interests, including relevant financial interests, activities,
relationships, and affiliations.

Acknowledgments

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References

31. Strand JM, Scheffler K, Bjoras M, and Eide L. The distribution of DNA damage is defined by region-specific susceptibility to DNA damage formation rather than repair differences. DNA repair 2014.


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Figure Legends

Figure 1. ADR downregulated nephrin and podocin expression in a dose-dependent manner.

(A) Podocytes were treated with different ADR concentrations for 24 h, nephrin and podocin protein expression were detected with Western blotting. (B) Densitometric analysis of Western blots of nephrin and podocin. (C) Real-time RT-PCR analysis of nephrin. (E) Real-time RT-PCR analysis of podocin. N=6 in each group. The values represent the mean \pm SD. *, P<0.05 versus control according to the ANOVA test.

Figure 2. ADR induced mitochondrial dysfunction in a dose-dependent manner.

(A) The mtDNA copy numbers. After 24 h exposure to different concentrations of ADR (0-200 nM), the total podocyte DNA was extracted. The 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 TMRE fluorescence by flow cytometry. Cells were treated with ADR or vehicle for 24 h and incubated with TMRE for 30 min. (E) The ATP content. Podocytes were incubated with ADR or vehicle for 24 h, and the ATP content was measured and normalized to protein concentration. N=6 in each group. Values represent the mean \pm SD. *, P<0.05 versus control according to the ANOVA test.

Figure 3. ADR increased the 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 DCF fluorescence was measured by flow cytometry. (B) Mitochondrial superoxide was measured with the 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. N=6 in each group. The values represent the mean ± SD. *, P<0.05 versus control according to the ANOVA test.

Figure 4. ADR decreased PGC-1α expression in a dose-dependent manner.

Podocytes were incubated for 24 h with different concentrations of ADR (0-200 nM), the PGC-1α protein and mRNA expressions were detected by Western blot and real-time RT-PCR. (A) Western blot of PGC-1α. (B) Densitometric analysis of PGC-1α Western blot. (C) Real-time RT-PCR of PGC-1α. N=6 in each group. The values represent the mean ± SD. *, P<0.05 versus control according to the ANOVA test.

Figure 5. Overexpression of PGC-1α in podocytes.

Podocytes were infected with Ad-PGC-1α at multiplicities of infection (MOI) 50 or empty vector (vehicle). After infection with the Ad-PGC-1α for 24 h, podocytes were treated with or without ADR (200 nM) for another 24 h, and the PGC-1α protein and mRNA expressions were detected. (A) Western blot of PGC-1α. (B) Densitometric analysis of PGC-1α Western blot. (C) Real-time RT-PCR of PGC-1α. N=6 in each group. The values represent the mean ± SEM. *, P<0.05 versus control; **, P<0.05 versus ADR-treated group according to the ANOVA.
Figure 6. PGC-1α overexpression inhibited ADR-induced podocyte injury.

After 24 h Ad-PGC-1α transfection, podocytes were treated with or without ADR (200 nM) for another 48 h. (A) Western blots of nephrin and podocin. (B) Densitometric analysis of nephrin and podocin proteins. (C) Real-time RT-PCR analysis of nephrin and podocin. (D) Analysis of cell apoptosis. N=6 in each group. The values represent the mean ± SD. *, P<0.05 versus control; **, P<0.05 versus ADR-treated group according to the ANOVA.

Figure 7. PGC-1α overexpression blocked ADR-induced mitochondrial dysfunction.

Podocytes were infected with Ad-PGC-1α at multiplicities of infection (MOI) 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) MMP measurement by JC-1. (D) MMP measurement by TMRE. (E) Real-time PCR of mtDNA copy number. (F) ATP content. N=6 in each group. The values represent the mean ± SD. *, P<0.05 versus control; **, P<0.05 versus ADR-treated group according to the ANOVA.

Figure 8. ADR reduced expressions of PGC-1α and TFAM in the kidneys of ADR-treated Rats. The rats were randomly assigned to two groups (n=6 in each group); the ADR group received a single tail-vein injection of 5 mg/kg ADR and the control rats received saline injection. (A) Western blot analysis of PGC-1α. (B) Densitometric analysis of PGC-1α Western blot. (C) Real-time RT-PCR analysis of PGC-1α. (D) Real-time RT-PCR analysis of TFAM. The values represent the mean ± SD. *, P<0.05 versus control; **, P<0.05 versus ADR-treated group according to the ANOVA.
Figure 1

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C

Nephrin/GAPDH (fold over control)

D

Podocin/GAPDH (fold over control)
Figure 4

A

B

C

![Graph A: Bar chart showing PGC-1α/β-actin expression levels at 50, 100, and 200 nM compared to control.]

![Graph B: Bar chart showing PGC-1α/GAPDH expression levels at 50, 100, and 200 nM compared to control.]

![Graph C: Bar chart showing PGC-1α/GAPDH expression levels at 50, 100, and 200 nM compared to control.]

The graphs indicate a decrease in PGC-1α expression with increasing concentrations of the substance.
Figure 5

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B

![Bar graph showing PGC-1α/β-actin fold change in control and ADR groups.]

C

![Bar graph showing PGC-1α/GAPDH fold change in control and ADR groups.]

* denotes significant difference at p<0.05, ** denotes significant difference at p<0.01.
Figure 7

A. ROS production (fold over control)

B. MitosOX fluorescence (fold over control)

C. JC-1 590/520nm (fold over control)

D. TMRM fluorescence (fold over control)

E. mtDNA/18s rRNA (fold over control)

F. ATP content (fold over control)
Figure 8

A

Control          ADR

PGC-1α

B-actin

B

Relative protein expression (fold over control)

Control          ADR

C

PGC-1α/GAPDH (fold over control)

Control          ADR

D

TFAM/GAPDH (fold over control)

Control          ADR