Protective role of cyclosporine A and minocycline on mitochondrial disequilibrium-related podocyte injury and proteinuria occurrence induced by adriamycin

Na Guan1,*, Ya-Li Ren2,*, Xiao-Ya Liu1, Ying Zhang3, Pei Pei3, Sai-Nan Zhu4 and Qingfeng Fan1,5,*

1Department of Pediatrics, Peking University First Hospital, Beijing 100034, China, 2Department of Electron Microscopy, Peking University First Hospital, Beijing 100034, China, 3Department of Central Laboratory, Peking University First Hospital, Beijing 100034, China, 4Department of Biostatistics, Peking University First Hospital, Beijing 100034, China and 5Present address: Renal-Electrolyte and Hypertension Division, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

Correspondence and offprint requests to: Na Guan; E-mail: guanna@163.com

*These authors contributed equally to this work.

ABSTRACT

Background. Dysfunction of mitochondria is involved in podocyte injury in some kidney diseases, but the relationship between abnormal mitochondrial morphology and podocyte injury as well as the underlying mechanism is still unclear. This study aims to investigate dynamic changes of mitochondrial morphology and the potential molecular events in an adriamycin (ADR)-induced podocyte injury model.

Methods. Podocyte apoptosis was evaluated by annexin V assay. Podocyte mitochondrial membrane potential (MMP) was measured with MitoCapture kit. Double staining was used to show the distribution changes of mitochondria and actin filament as well as mitofusin proteins and podocin. Mitochondrial shape descriptors were obtained using analySIS Image system. Effects of cyclosporine A (CsA) or minocycline (Mcy) on mitochondrial morphology were explored in ADR-induced nephropathy rats.

Results. ADR caused podocyte damage displaying as induction of cellular apoptosis and increase of activated caspase 3 and cytochrome c. The MMP level was decreased remarkably in ADR-treated podocytes. Mitochondrial morphological changes induced by ADR occurred rapidly from large and ellipsoid shape to the small, long and irregular. ADR significantly decreased surface area, perimeter and circularity, while increasing aspect ratio of mitochondria. In addition, mitochondria number transiently increased at 6 h following ADR application. Mitochondria intensity was increased along with punctate mitochondria formation, which co-localized with polymerized actin cytoskeleton in ADR podocytes. In ADR-induced nephropathy rats, 24-h proteinuria was decreased significantly by CsA or Mcy.
Mcy. ADR-induced abnormal changes of mitochondrial morphology were restored by CsA or Mcy. The induction of mitofusin proteins and the reduction of podocin in ADR rat glomeruli were rescued by CsA or Mcy.

Conclusions. Mitochondrial dysfunction may be an early event in ADR-induced podocyte damage, and the protective role of CsA or Mcy may be mediated partially by improving mitochondrial function through inhibiting the induction of mitofusin proteins.

Keywords: cyclosporine A, minocycline, mitochondria, mitofusin, podocyte

INTRODUCTION

The podocyte plays an important role in maintaining the integrity of the glomerular filtration barrier. Injury of the podocyte leads to proteinuria in a variety of glomerular diseases [1]. It has been demonstrated that the loss of glomerular podocytes due to cell death/apoptosis or detachment is a hallmark for the progression of kidney diseases to end stage renal failure [2], while the underlying mechanisms remain elusive.

The apoptotic events of cells can be initiated and/or propagated by diverse intracellular stress conditions, including oxidative stress, DNA damage, cytoplasmic Ca$^{2+}$ release, endoplasmic reticulum stress, mitochondrial dysregulation and many other stimulators [3]. The mitochondria are essential to multicellular life as a crucial organelle for ensuring the energy supply and cell homeostasis, as well as the production and clearance of reactive oxygen species (ROS) [4]. It has been revealed that the glomerular podocyte has abundant and complex mitochondrial networks [5]. Just recently, several studies indicated that mitochondria exert a key role for maintaining podocyte homeostasis, and their failure could lead to podocyte damage [5]. Morphological changes of podocyte mitochondria are definitively observed in patients with mitochondrial cytopathy or even focal segmental glomerular sclerosis (FSGS) [6]. In cultured podocytes, aldosterone application caused remarked dysfunction of mitochondria, including reduction of mitochondrial membrane potential (MMP) and ATP levels as well as mitochondrial DNA copy number, which may be responsible for decrease of the key podocyte slit diaphragm protein nephrin [7]. In addition, TGFβ1 could induce podocyte damage by stimulating mitochondrial oxidative phosphorylation and generation of ROS via the mTOR signaling pathway [8]. It was also reported that up-regulation of mitochondrial Nox4 may mediate TGFβ1-induced apoptosis in cultured podocytes. These findings emphasize again mitochondrial functions in podocytes. Nevertheless, quantitative morphological changes of mitochondria in damaged podocytes have not yet been reported. Furthermore, it is also worth investigating whether anti-proteinuric drugs commonly used in kidney diseases to decrease proteinuria or prevent rejection of kidney transplant [13]. It was also reported that CsA could affect mitochondrial function by preventing the mitochondrial permeability transition pore (MPTP) from opening, thus inhibiting cytochrome c release, a potent apoptotic stimulation factor [14]. However, it is unclear whether the anti-proteinuric role of CsA is mediated by improving mitochondrial function in kidney diseases. In addition, some studies suggested that minocycline (Mcy) has some favorable effects related to its ability to influence mitochondrial functioning [15]. There is no report about whether Mcy could be used as an anti proteinuric drug in glomerular diseases. Therefore, the role of anti proteinuric medication CsA and mitochondria targeted drug Mcy was investigated in ADR-induced rat nephropathy.

Currently, we provide evidence that mitochondrial disequilibrium may be an early event in ADR-induced podocyte damage, and the protective role of CsA and Mcy may be mediated at least partially by improving mitochondrial function via inhibiting the induction of mitofusins 1 and 2.

MATERIALS AND METHODS

Podocyte culture and ADR induction

Mouse podocyte cell line MPC5 was a kind gift from Peter Mundel. Podocytes were cultured in RMPI 1640 medium supplemented with 10% fetal bovine serum (Gibco), 10 U/mL IFN-γ (Sigma) and 1% penicillin/streptomycin at 33°C in a 5% CO$_2$/95% air atmosphere. After 1 week, cells were transferred to the non-permissive conditions of 37°C and differentiated by removal of IFN-γ for 14 days [16]. The podocytes were treated with doxorubicin hydrochloride (ADR; Sigma) at 0.25, 0.5 and 1.0 µg/mL for 24 h, respectively. Or, the podocytes were treated with 0.5 µg/mL of ADR for 6, 12 and 24 h, respectively.
Apoptosis detection
Podocyte apoptosis was measured with annexin V-FITC Annexin V-FITC Kit (BD Pharmingen). Briefly, 1 x 10^6 cells were washed twice with cold phosphate-buffered saline (PBS), and re-suspended in 1 mL of 1x binding buffer. Then, 100 µL was transferred to a 5 mL culture tube, and 5 µL of FITC-annexin V and 5 µL of propidium iodide were added. The tube was incubated for 15 min at room temperature. Finally, 400 µL of 1x binding buffer was added and analyzed by flow cytometry (BD FACSAria).

MMP assay
Podocyte MMP was measured with MitoCapture according to the manufacturer’s instructions (BioVision). MitoCapture, a cationic dye, can accumulate and aggregate in mitochondria thus giving off a bright red fluorescence in healthy cells. In apoptotic cells, MitoCapture cannot aggregate in mitochondria due to the altered MMP, and thus it remains in the cytoplasm in its monomer form, fluorescing green color. Podocytes were collected, and incubated with MitoCapture dye at 37°C for 15 min. Green fluorescence signal was detected immediately by flow cytometry (BD FACSAria) [17].

Immunofluorescence staining
Podocytes were grown on collagen I-coated coverslips, and treated with 0.5 µg/mL of ADR for 3, 6, 12 and 24 h, respectively. Cells were fixed with 4% paraformaldehyde (PFA), and permeabilized with 0.25% TritonX-100. Mitochondria and actin cytoskeleton were revealed with MitoTracker (Cell Signaling) and Alexa 488-conjugated phalloidin (Invitrogen), respectively.

Tissue immunofluorescence staining was performed on frozen kidney sections from ADR-induced nephropathy rats. Tissue sections were fixed with 4% PFA, and permeabilized with 0.25% TritonX-100. To remove or decrease non-specific binding, sections were incubated with 10% goat serum. Then, mouse anti-mitofusin 1 or 2 antibody (Abcam) and rabbit anti-podocin antibody (Abcam) were applied for 2 h at room temperature. After three washes with PBS, Alexa 488-conjugated goat anti-mouse IgG and Alexa 594-conjugated goat anti-rabbit IgG (Invitrogen) were added and incubated for 1 h. After three washes, sections were mounted with ProLong gold antifade reagent (Invitrogen). Images were taken by using laser scanning confocal microscope (Olympus Fluoview FV 1000) equipped with a 100x oil immersion objective lens (NA 1.40).

Immunoblotting assay
Total protein was isolated with RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 50 mM Tris–HCl pH 7.4, 15% glycerol) supplemented with protease cocktail inhibitor (Roche). Protein concentration was measured using a BCA assay kit (Bio-Rad). Totally, 50 µg of protein was loaded on 12.5% SDS-PAGE, and then transferred to nitrocellulose membrane. Membranes were rinsed once with Tris-buffered saline containing 0.05% Tween-20 (TTBS), and then blocked for 30 min in 5% BSA/TTBS. The membranes were incubated overnight with the indicated primary antibodies at 4°C. After five washes with TTBS, membranes were incubated with HRP-conjugated secondary antibody for 1 h. The blots were developed with an ECL chemiluminescence detection kit (Pierce), and the specific band was scanned and quantified with Image J 1.49d (http://imagej.nih.gov/ij).

Establishment of ADR rat nephropathy
All animal protocols were approved by the Animal Experimentation Committee of Peking University First Hospital. The rats were fed with standard diet, and water was given at ad libitum. Alternating 12-h cycles of light and dark were maintained for rats. Male Sprague-Dawley rats weighing 120–160 g received one tail vein injection of ADR (Sigma) at 0.7 mg/100 g body wt diluted in 0.9% saline [18]. An equal volume of saline was injected by tail vein in rats as control (Ctl). Four weeks after injections, the ADR-injected rats were further divided into three groups: (i) ADR alone, (ii) ADR plus CsA and (iii) ADR plus Mcy. CsA (1 mg/100 g body wt) was intraperitoneally injected daily, and Mcy (5 mg/100 g body wt) was fed daily for a total of 2 weeks. After initial injections, the rats were killed at 2 weeks (Ctl group, n = 3; ADR group, n = 3), 4 weeks (Ctl group, n = 3; ADR group, n = 6) and 6 weeks (Ctl group, n = 8; ADR group, n = 7; ADR plus CsA group, n = 8; ADR plus Mcy group, n = 7).

At each time point, 24-h urine was collected from each group and proteinuria was assessed by using a Pyrogallol red-molybdate dye-binding method. Kidneys were harvested immediately and fixed with ice-cold 1% glutaraldehyde in 0.1 M PBS for transmission electron microscopy (JEOL-1230, Japan).

Electron microscopy in cultured podocytes
A total of 1 x 10^6 cells were fixed with ice-cold 1% glutaraldehyde in 0.1 M PBS for 24 h at 4°C followed by washing three times with 0.2 M sucrose. Cells were then post-fixed in 1% osmium tetroxide at room temperature for 1 h, dehydrated in graded ethanol, embedded with epon 812, sectioned using an ultramicrotome (Leica, Germany), and stained with 2% uranyl acetate and lead citrate. The sections were photographed with a transmission electron microscopy (JEOL-1230, Japan).

Stereology analysis of mitochondrial morphology
Forty photographs in each case were taken according to the rule of equidistant zigzag movement under the magnification of 15 000 in cell specimen and 25 000 in the rat glomerular area. The grid size with 2000 × 2000 nm^2 was superimposed on each cell photograph. Because of the lower number of mitochondria in rats, equidistant grid lines of 1000 nm spacing on each picture were set in order to get satisfied number of ~50. One grid was a test frame.

The intersections of horizontal and vertical lines were test points that were used in the following. Mitochondria that intersect with the grids, whether horizontal or vertical lines, were defined as effective measure targets. The target number of mitochondria is about 200 in each case. Mitochondrial shape descriptors and size measurements were obtained using analySIS Image Processing software, including (i) surface area (mitochondrial size) that is reported in squared nanometers; (ii) circumference (mitochondrial perimeter) in nanometers, (iii) aspect ratio that is computed as (major axis/Minor axis) reflecting the length-to-width ratio of mitochondria, (iv)
circularity that is calculated as $4\pi (\text{surface area}/\text{perimeter}^2)$, a two-dimensional index of sphericity with values of 1 indicating perfect spheroids and (v) mitochondrial density calculated as the number of mitochondria cross-sections per square nanometer in the whole cell area or only in the plasma area excluding nuclei. The density value is obtained by dividing the area of all test points in the case by the number of effective mitochondria in the same case. According to the rule of stereology, the associated area of one test point is equivalent to the area of one test frame [19, 20]. Computed values were imported into Microsoft Excel and Prism 6 (GraphPad software) for data analysis.

**Statistical analyses**

All data are expressed as means ± SEM. ANOVA was used to compare differences of multiple groups. A P-value of <0.05 was considered as significant differences.

**RESULTS**

**Apoptosis induction and mitochondrial dysfunction in ADR-treated podocytes**

Podocyte damage was firstly analyzed by assessing the cellular apoptosis with FITC-annexin V assay in cultured podocytes treated with different concentrations of ADR for 24 h. Compared with non-treated cell (5.5 ± 0.9), the percentage of apoptotic cells was significantly increased (P < 0.01) in podocytes treated with 0.25 (219.3 ± 2.5), 0.5 (377.7 ± 6.4) and 1.0 µg/mL (497.5 ± 8.7) of ADR, respectively. In addition, 0.5 and 1.0 µg/mL of ADR decreased (P < 0.05) in podocytes treated with 1.0 µg/mL (241.3 ± 14.15) of ADR (Figure 1D), indicating reduction of cell area and only in plasma area was transiently increased (P < 0.01) at 6 h following ADR application (Figure 1B and C).

Disruption of MMP is one of the earliest intracellular events that occur following induction of apoptosis [3, 7]. Here, MMP level was assessed with a cationic dye MitoCapture. As compared with Ctl, the cleaved caspase 3 and cytochrome c were detected using immunoblot assay. In comparison with Ctl cell (0.82 ± 0.01), aspect ratio that reflects the length-to-width ratio of mitochondria, remarkably increased (P < 0.01) at 6 (1.91 ± 0.05), 12 (1.81 ± 0.05) and 24 h (1.72 ± 0.06) following ADR administration against Ctl cell (1.39 ± 0.04), providing the evidence that irregular and fragmented mitochondria were present. Consistently, compared with Ctl (1.56 ± 0.04), aspect ratio that reflects the length-to-width ratio of mitochondria, remarkably increased (P < 0.01) at 6 (1.91 ± 0.05), 12 (1.81 ± 0.05) and 24 h (1.72 ± 0.06) following ADR administration against Ctl cell (1.39 ± 0.04), providing the evidence that irregular and fragmented mitochondria were present. Consistently, compared with Ctl (1.56 ± 0.04), aspect ratio that reflects the length-to-width ratio of mitochondria, remarkably increased (P < 0.01) at 6 (1.91 ± 0.05), 12 (1.81 ± 0.05) and 24 h (1.72 ± 0.06) following ADR administration against Ctl cell (1.39 ± 0.04), providing the evidence that irregular and fragmented mitochondria were present.

**Dynamic changes of mitochondria and actin cytoskeleton in ADR-treated podocytes**

Distribution of mitochondria and actin cytoskeleton was analyzed in podocytes treated with 0.5 µg/mL of ADR at different time periods. Normal podocytes displayed large and complex mitochondrial networks as well as filamentous actin cytoskeleton. In ADR-treated cells, mitochondria fluorescence intensity increased obviously along with remarked morphological alterations. Against Ctl, mitochondria became short and fragmented at 3 h after ADR application. Since 6 h, linear mitochondrial network disappeared, and aggregated mitochondria were present in normal or non-treated cells, whereas mitochondrial fragmentation was observed in ADR-treated podocytes as evidenced by a significant increase of small, long and irregular mitochondria (Figure 2A).

**Dynamic alteration of mitochondrial morphology in ADR-treated podocytes**

Timing effects of 0.5 µg/mL of ADR on mitochondrial morphology were investigated in cultured podocytes. Electron microscopy images were used to evaluate morphological changes of mitochondria following ADR application. Large and round mitochondria were present in normal or non-treated cells, whereas mitochondrial fragmentation was observed in ADR-treated podocytes as evidenced by a significant increase of small, long and irregular mitochondria (Figure 2A). Quantification of mitochondrial shape is required to determine if changes in mitochondrial morphology are linked to the specific pathophysiological functions of podocytes [5]. The stereology parameters of mitochondria were obtained using analySIS Image Processing software. Quantitative analyses showed that ADR-treated podocytes exhibited 42.4, 32.1 and 30.2% reduction (P < 0.01) of mitochondrial surface area at 6, 12 and 24 h, respectively. In comparison with Ctl (2191 ± 60.80 nm), mitochondrial perimeter decreased significantly (P < 0.01) at 6 (1735 ± 68.14 nm), 12 (1866 ± 55.72 nm) and 24 h (1840 ± 75.56 nm) following ADR application. These data indicate that ADR-induced small mitochondrial formation in podocytes. In addition, circularity, a two-dimensional index of sphericity with values of 1 indicating perfect spheroids, obviously decreased (P < 0.01) at 6 (0.76 ± 0.01), 12 (0.78 ± 0.01) and 24 h (0.79 ± 0.01) after ADR administration against Ctl cell (0.82 ± 0.01), providing the evidence that irregular and fragmented mitochondria were present. Consistently, compared with Ctl (1.56 ± 0.04), aspect ratio that reflects the length-to-width ratio of mitochondria, remarkably increased (P < 0.01) at 6 (1.91 ± 0.05), 12 (1.81 ± 0.05) and 24 h (1.72 ± 0.06) following ADR administration against Ctl cell (1.39 ± 0.04), providing the evidence that irregular and fragmented mitochondria were present.
observed persisting to 24 h. Rearrangement of actin cytoskeleton also occurred in ADR-treated cells. At 3 and 6 h following ADR application, actin polymerization was revealed displaying as the increased and aggregated staining of actin cytoskeleton at cell periphery. In comparison with the untreated cells, intracellular actin filaments disappeared in ADR-treated podocytes. Moreover, co-localization of punctate mitochondria and polymerized actin cytoskeleton was observed in ADR-treated cells, especially at 3 and 6 h (Figure 3).

**Protective role of CsA and Mcy in ADR-induced rat nephropathy**

Rat nephropathy was induced by a single tail vein injection of ADR. Twenty-four hour proteinuria was assessed at 2, 4 and 6 weeks. As compared with the corresponding Ctl, induction of proteinuria was significantly detected (P < 0.01) at 4 weeks (167.92 ± 23.56 mg versus 12.50 ± 1.82 mg) and 6 weeks (226.86 ± 40.42 mg versus 12.0 ± 1.57 mg) in ADR rats (Figure 4A). Ultrastructural changes of podocyte foot processes (FPs) were revealed using transmission electron microscopy. In Ctl kidneys, thin and long FPs were well conserved, whereas FPs diffusion occurred at 2 weeks, and effacement was widely observed at 4 and 6 weeks after ADR injections (Figure 4B).

CsA is an anti proteinuric medication commonly used in kidney diseases [13]. Several studies reported that Mcy could improve mitochondrial function [15]. Therefore, the effects of CsA and Mcy were explored in ADR-injected rats. At 4 weeks following ADR injections, CsA and Mcy were administrated once a day totally for 2 weeks. Quantitative results of 24-h proteinuria showed that compared with rats injected with ADR alone (226.86 ± 40.42 mg), proteinuria was decreased significantly (P < 0.05) in CsA-treated (113.10 ± 35.18 mg) or Mcy-treated (153.6 ± 26.71 mg) rats (Figure 4C). Electron microscope image showed that ADR led to widely diffusion and effacement of podocyte FPs, which was restored or recovered effectively by CsA or Mcy treatment (Figure 4D).

**Effects of CsA and Mcy on podocyte mitochondrial morphology in ADR-induced rat nephropathy**

In saline-injected Ctl rat, round or ellipsoid mitochondria were orderly arranged in podocytes with intact mitochondrial membrane and regularly arranged cristae in the form of...
concentrical ring or vertical line (Figure 5). At 2 weeks after ADR injections, mitochondrial membrane in podocyte cell body seemed disappear and intra-mitochondrial structure became unclear. At 4 weeks, podocyte mitochondria became swollen with vague or partly ruptured mitochondrial membrane, and disorganized cristae. At 6 weeks, podocyte

**Figure 2**: Time-dependent alteration of mitochondrial morphology in ADR-treated podocytes. Differentiated mouse podocytes were treated with 0.5 µg/mL of ADR for the indicated time periods. Electron microscopy images were used to evaluate mitochondria morphological changes. (A) Representative images are shown, and the typical mitochondrion was enlarged. Bar = 2 µm. (B) Mitochondrial shape descriptors and size measurements were obtained using analySIS Image Processing, including (i) surface area, (ii) circumference, (iii) aspect ratio (major axis/minor axis) reflecting the length-to-width ratio of mitochondria, (iv) circularity $[4\pi \cdot \text{surface area}/\text{perimeter}^2]$, a two-dimensional index of sphericity with values of 1 indicating perfect spheroids and (v) mitochondria density calculated as the number of mitochondria in whole cell area or only in the plasma area. Totally, 200 mitochondria each group were analyzed. Computed values were imported into Prism 6 for data analysis. ADR resulted in a reduction of mitochondrial area, circumference and circularity, while led to an increase of aspect ratio of mitochondria. In addition, mitochondria number both in whole cell area and in only plasma area increased remarkably only at 6 h following ADR application. *P < 0.01 versus 0 µg/mL; #P < 0.05 versus 0 µg/mL.
mitochondria were highly swollen with remarked loose and dissolved mitochondrial cristae (Figure 5). Treatment with CsA, especially Mcy, remarkably alleviated ADR-induced mitochondrial changes, presenting with regularly arranged mitochondria though some dilated crista spaces and vacuoles were observed (Figure 5).

**Effects of CsA and Mcy on podocyte mitofusin distribution in ADR-induced rat nephropathy**

Mitofusin protein is a key player in mediating mitochondrial fusion required for maintenance of mitochondrial morphology and thus functioning [22]. Distribution change of two isoforms of mitofusin protein, namely mitofusins 1 and 2 as well as a
podocyte marker podocin was investigated in ADR rat nephropathy. Normal rat kidneys showed a high expression level of podocin with a sharp linear distribution along glomerular capillary loops (Figure 6). In normal rat glomeruli, very low expression of mitofusin 1 was observed only in mesangial area (Figure 6A), whereas mitofusin 2 displayed a linear distribution and co-localized with podocin along glomerular capillary loops though its abundance was very low (Figure 6B).

In ADR rat glomeruli, fluorescence intensity of mitofusin 1 increased obviously at 2 weeks and continued to 6 weeks, whereas podocin displayed punctate or dot-linear distribution pattern and exhibited obvious reduction of intensity (Figure 6A). Moreover, mitofusin 1 was mainly localized in cell body of podocyte from 2 to 6 weeks, and also present in cellular junctions at 6 weeks where podocin expression disappeared after ADR injections (Figure 6A). Abundance of mitofusin 2 increased obviously at 2 weeks, especially at 4 weeks in ADR rat glomeruli. Increased mitofusin 2 was mainly localized in cell body of podocyte, and also present in cell junctions co-localizing with punctate podocin at 2 and 4 weeks. Interestingly, mitofusin 2 level decreased evidently in some area of glomeruli at 6 weeks (Figure 6B).

To explore the effects of CsA and Mcy on the distribution changes of mitofusin protein, at 4 weeks following ADR injections, CsA or Mcy was administrated to rats once a day totally for 2 weeks. Treatment with CsA or Mcy obviously down-regulated expression level of mitofusins 1 and 2, and effectively restored podocin expression (Figure 6C and D).

**DISCUSSION**

Mutations of mitochondria DNA were detected by in situ PCR in podocytes in primary FSGS. Refs. [23, 24] provided direct evidence that dysfunction of mitochondria may participate in the pathogenesis of podocyte damage. However, morphological change of mitochondria has not been fully investigated in glomerular podocytopathies, and quantitative analysis is required to determine if changes in mitochondrial morphology are linked to the specific pathophysiological functions of podocytes. In this study, stereology analyses of mitochondria morphological changes based on electron microscope images have been performed in an ADR-induced podocyte injury model in vivo and in vitro. Although CsA is widely used as an immunosuppressant drug in kidney diseases to decrease proteinuria or prevent rejection of kidney transplant, the underlying mechanism is still unclear. Some studies reported that Mcy has some favorable effects related to its ability to influence mitochondrial functioning [15], while whether Mcy has anti-proteinuric effects by protecting podocyte from mitochondria damage is worth studied. Therefore, effects of CsA and Mcy on mitochondrial...
morphology were investigated in ADR-induced rat nephropathy model.

ADR-induced podocyte damage was first evidenced by induction of cellular apoptosis in a dose-dependent manner (Figure 1A). Caspase 3, an effector caspase related to the initiation of the death cascade, has been implicated as an important marker of the cell’s entry point into the apoptotic signaling pathway [21]. Immunoblot assay showed that the cleaved caspase 3 as an active form was increased significantly at 12 and 24 h following ADR application in cultured podocytes (Figure 1B). We also found that the cytosolic level of cytochrome c was induced remarkably since 6 h and continued increase to 24 h in ADR-treated podocytes (Figure 1C). Cytochrome c, a component of the electron transport chain in mitochondria, is also an intermediate in apoptosis. Upon release from mitochondria, cytochrome c could activate caspase 3 via caspase 9, a cysteine protease [21]. These data implied that activation of caspase 3 during ADR-induced podocyte apoptosis may be mediated by cytochrome c since there was a close correlation between the time courses of cytochrome c release from the mitochondria and caspase 3 activation. The induction of cytochrome c prompted us to investigate whether mitochondria are involved in this process of ADR-induced podocyte damage. Disruption of MMP is one of the earliest intracellular events that occur following induction of apoptosis [3, 7]. Mitochondrial morphology transition is also linked with susceptibility to mitochondrial permeability transition [25]. Here, MMP level was assessed with a cationic dye MitoCapture. In apoptotic cells, MitoCapture remains in cytoplasm in its monomer form and emits green fluorescence indicative of reduction of MMP level [17]. As compared with non-treated cells, flow cytometry detected a significant increase of green fluorescent signal in podocytes treated with 0.5 or 1.0 µg/mL of ADR (Figure 1D), providing evidence that mitochondria dysfunction may be involved in ADR-induced podocyte injury. It should be noted that ADR induced a moderate increase of MMP level while a

FIGURE 5: Effects of CsA and Mcy on podocyte mitochondrial morphology in ADR-induced rat nephropathy. Rat nephropathy was induced by a single tail vein injection of ADR (0.7 mg/100 g body wt) or saline as Ctl. At 4 weeks after ADR injections, CsA or Mcy was given for total 2 weeks. Electron microscopy was used to evaluate mitochondrial morphology changes. In Ctl rats, podocyte exhibited round or ellipsoid mitochondria with intact mitochondrial membrane and regularly arranged cristae. In ADR-injected rats, podocyte mitochondria were swollen obviously with vague mitochondrial membrane and dissolved cristae, which was rescued by CsA, especially by Mcy treatment. Representative pictures are shown, and the typical mitochondrion was enlarged. Bar = 0.5 µm.
remarkable induction of caspase 3 and cytochrome c in cultured podocytes, suggesting that mitochondria are not the only upstream pathway responsible for ADR-induced podocyte apoptosis. In numerous studies, the mechanism underlying ADR-induced podocyte injury has been investigated, and shown to be modulated by both non-immune and immune factors [26]. In cultured mouse podocytes, it was also reported that ADR dose dependently impaired mitochondrial function displaying as the increase in ROS production, decrease in mitochondrial DNA copy number and reduction of MMP level [27].

In the present study, ADR-induced distribution change of mitochondria was analyzed in cultured podocytes. As previously described [5], our immunofluorescence staining revealed that normal podocytes present with large and complex mitochondrial networks (Figure 3). ADR treatment obviously increased fluorescence intensity of mitochondria along with rapid morphological alterations evidenced by the occurrence of short and fragmented mitochondria at 3 h. Since 6 h, linear mitochondrial network disappeared, and aggregated mitochondria were observed until upto 24 h (Figure 3). In addition, reorganization of actin cytoskeleton was revealed in ADR-treated cells, displaying as polymerization or aggregation of actin cytoskeleton at the cell periphery and decrease or disappearance of intracellular actin filaments. Moreover, co-localization of punctate mitochondria and polymerized actin cytoskeleton was observed in ADR-treated cells, especially at 3 and 6 h (Figure 3). These findings suggest that actin cytoskeleton may be related to the immobilization of mitochondria at the cell periphery in ADR-treated podocytes, which may be important for retaining mitochondria at the sites where high ATP utilization is required.
Based on electron microscopy, morphological changes of mitochondria were further analyzed. Large and round mitochondria were present in normal cells, whereas mitochondrial fragmentation was observed in ADR-treated podocytes as evidenced by a significant increase of small, long and irregular mitochondria (Figure 2A). It was reported that increased fission of mitochondria may be associated with fragmentation of mitochondria, displaying as small and punctate shape [28]. In addition, some studies have shown that fragmentation of mitochondria is not only a phenomenon, but also a trigger required for cellular apoptosis by release of proapoptotic factors such as cytochrome c and ROS production [10, 29]. To determine whether changes in mitochondrial morphology are linked to the specific pathophysiological functions of podocytes, the stereology parameters of mitochondria were obtained and quantitative analyses were then performed for the first time. In comparison with Ctl, ADR-treated podocytes exhibited reduction of mitochondrial surface area and circumference that occurred at 6 h and persisted to 24 h, indicating that ADR induced a rapid formation of small mitochondria. In addition, circularity, a two-dimensional index of sphericity with values of 1 indicating perfect spheroids, was also rapidly decreased after ADR administration, providing the evidence that irregular and fragmented mitochondria were present. Consistently, aspect ratio that reflects the length-to-width ratio of mitochondria was remarkably increased at 6, 12 and 24 h in ADR-treated podocytes, indicative of the formation of long mitochondria. We also evaluated the mitochondria number and found that mitochondria density both in the whole cell area and only in plasma area was transiently increased only at 6 h following ADR application (Figure 2B). Increased number of abnormal mitochondria was previously reported in injured podocytes in patients with mitochondria cytopathy [23]. In diabetic rats, it was also observed that mitochondria number increased before glomerular changes [30]. Moreover, mitochondrial morphological changes were studied in cultured podocytes treated with different concentrations of ADR. Electron microscopy images disclosed that ADR-induced dynamic changes of mitochondrial shapes from large and round morphology to the small, thin and irregular (Supplementary Figure S1A). Stereological quantitative analyses showed that ADR dose dependently reduced mitochondrial circumference, surface area and circularity, while increased aspect ratio compared with Ctl (Supplementary Figure S1B). It should be noted that mitochondria morphological change accompanied by decrease of MMP level and induction of podocyte apoptosis. Although the underlying mechanism is still unclear, our results at least suggested that dynamic changes of mitochondria morphology may be an early event that occurs in ADR-induced podocyte damage, and may be used as a potential marker to identify podocyte injury in patients with proteinuria.

To confirm and explore new potential targets that can be used to treat proteinuric podocytopathies, the role of mitochondria was investigated in ADR-induced rat nephropathy model. Induction of proteinuria was significantly detected at 4 and 6 weeks after ADR injections (Figure 4A). Ultrastructural changes of podocyte FPs were also revealed using electron microscopy in ADR rat kidneys, presenting with FPs diffusion at 2 weeks, and widely effacement at 4 and 6 weeks (Figure 4B). Abnormal podocyte mitochondria morphology was also observed in ADR rat glomeruli. In the normal rat kidney, round or ellipsoid mitochondria were orderly arranged in podocytes with intact mitochondrial membrane and regularly arranged cristae, whereas in the ADR nephropathy rat, podocyte mitochondria were swollen obviously with vague or partly ruptured mitochondrial membrane, and remarked loose and dissolved mitochondrial cristae (Figure 5). Mutations to mitochondria DNA and reductions in mitochondria DNA copy number have been identified as contributors to ADR-induced injury [31]. It was found that CsA can prevent opening of MPTP by binding to cyclophilin D and inhibit cell apoptosis in myocardial cells [32], neuron [33] and skeletal muscle cells [34]. In addition, some studies suggested that Mcy can inhibit cell injury or apoptosis by protection of mitochondria in liver cells [35], neurons [36, 37] and germ cells [38]. In the present study, the effects of CsA and Mcy on mitochondrial function and podocyte injury were explored in ADR rat nephropathy. At 4 weeks following ADR injections, CsA or Mcy was administered once a day for total 2 weeks. Induction of proteinuria and ultrastructural changes of FPs in ADR rats was prevented significantly by CsA or Mcy treatment (Figure 4C and D). Moreover, treatment with CsA, especially Mcy, remarkably alleviated ADR-induced mitochondrial shape changes, presenting with regularly arranged mitochondria although some dilated crista spaces and vacuoluses were present (Figure 5).

The mitochondrion is a dynamic organelle in cells. Dynamic fusion and fission of the mitochondrion affected the shape of itself. Mitofusin protein as a key player in mediating mitochondrial fusion is necessary for maintaining mitochondrial morphology and thus functioning [22]. Distribution change of mitofusins 1 and 2 was further investigated in ADR rat nephropathy. In normal rat glomeruli, very low expression of mitofusin 1 was observed only in mesangial area (Figure 6A), whereas mitofusin 2 displayed a linear distribution and co-localized with the podocyte slit diaphragm marker podocin along capillary loops though its abundance was very low (Figure 6B). These data show that mitofusin 2 is a predominant mitofusin protein that may be responsible for mitochondrial fusion in normal podocytes. In ADR rat glomeruli, podocin displayed a punctate or dot-linear distribution pattern and exhibited obvious reduction of intensity (Figure 6). Nevertheless, our results showed that ADR injections caused distinct changes of mitofusins 1 and 2. Intensity of mitofusin 1 increased obviously at 2 weeks and continued to 6 weeks. Moreover, mitofusin 1 was mainly localized in podocyte cell body from 2 to 6 weeks, and also present at cellular junctions at 6 weeks where podocin expression disappeared (Figure 6). Abundance of mitofusin 2 also increased obviously at 2 weeks, especially at 4 weeks. Interestingly, mitofusin 2 level decreased evidently in some area of glomeruli at 6 weeks. Moreover, increased mitofusin 2 was mainly localized in podocyte cell body, and also present at cellular junctions where co-localized with punctate podocin at 2 and 4 weeks (Figure 6B). These findings proposed a distinct role of mitofusin proteins during podocyte injury evidenced by the fact that mitofusin 2 may be responsible for podocin redistribution at cellular junctions while mitofusin 1 may be related to podocin loss from cell.
junctons. Our data first manifested that the function of mitofusin proteins may depend on its location both in normal and injured podocytes. In addition, our results suggested that induction of mitofusins 1 and 2 plays a distinct role in mediating podocyte mitochondrial dysfunction via affecting the balance of fusion and fission of mitochondria. In podocytes, it was reported that the hyperglycemic condition can induce mitochondria fission via ROCK-mediated Drp1 phosphorylation at Ser600 [39]. Treatment of CsA or Mcy dramatically downregulated mitofusins 1 and 2 expression levels, and effectively restored podocin expression (Figure 6C and D).

Taken together, our findings demonstrated that abnormalities of mitochondrial morphology, density and function may be early events in ADR-induced podocyte damage, and the protective role of CsA and Mcy may be mediated partially by improving mitochondrial function via inhibiting Akt activation. Nevertheless, the proapoptotic effect of mitofusin 2 is independent of its function in mitochondrial fusion but mainly mediated by inhibition of Akt signaling and the resultant activation of mitochondrion apoptotic pathway [40]. Alternatively, activation of Akt modulated mitochondrial morphology displaying as elongated mitochondria, delayed the opening of MPTP and reduced cell death following simulated ischemia-reperfusion injury in HL-1 cardiac cell line. Interestingly, the effects of Akt on inducing mitochondrial elongation seemed to be dependent on mitofusin 1, not mitofusin 2 [41]. Therefore, the exact function and proximal signaling mediated by mitofusin proteins should be further investigated in mitochondria disequilibrium related podocytopathies.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No. 81100502 to N.G. and No. 30801250 to Q.F.) and the Program for New Century Excellent Talents in University (NCET-12-0006 to N.G.).

CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES


2. Tharaux PL, Huber TB. How many ways can a podocyte die? Semin Nephrol 2012; 32: 394–404


9. Sak MN. Mitofusin function is dependent on the distinct tissue and organ specific roles of mitochondria. J Mol Cell Cardiol 2011; 511: 881–887


The efficacy of recombinant human soluble thrombomodulin for the treatment of shiga toxin-associated hemolytic uremic syndrome model mice

Kazuhide Suyama, Yukihiro Kawasaki, Kyohei Miyazaki, Syuto Kanno, Atsushi Ono, Shinichiro Ohara, Masatoki Sato and Mitsuaki Hosoya

Department of Pediatrics, Fukushima Medical University School of Medicine, Fukushima City, Fukushima, Japan

Correspondence and offprint requests to: Yukihiro Kawasaki; E-mail: kyuki@fmu.ac.jp

ABSTRACT

Background. Recombinant human soluble thrombomodulin (rhTM) is a promising therapeutic natural anticoagulant that is comparable to antithrombin, tissue factor pathway inhibitor and activated protein C. In order to clarify the efficacy of rhTM for the treatment of typical hemolytic uremic syndrome (t-HUS), we examined changes in renal damage in t-HUS mice treated with rhTM or vehicle alone.

Methods. We used severe and moderate t-HUS mice injected with shiga toxin (Stx) and lipopolysaccharide (LPS). The severe t-HUS mice were divided into two subgroups [an rhTM subgroup (Group A) and a saline subgroup (Group B)] along with the moderate t-HUS mice [an rhTM subgroup (Group C) and a saline subgroup (Group D)]. Groups E and F were healthy mice treated with rhTM or saline, respectively.

Results. All mice in Group B died at 80–90 h post-administration of Stx2 and LPS whereas all mice in Group A remained alive. Loss of body weight, serum creatinine level, endothelial injury and mesangiolysis scores at 24 h after administration in the t-HUS mice treated with rhTM were lower than those in t-HUS mice treated with saline. The levels of hemoglobin at 6 h and platelet counts at 24 h after administration in Group A were higher than those in Group B. Serum interleukin (IL)-6, IL-1β and chemical hypoxia and ischemia/reperfusion injury by inhibition of the mitochondrial calcium uniporter. Toxicol Appl Pharmacol 2013; 273: 172–179

Received for publication: 25.9.2014; Accepted in revised form: 11.12.2014

The Author 2015. Published by Oxford University Press on behalf of ERA-EDTA. All rights reserved.