Amelioration of rhabdomyolysis-induced renal mitochondrial injury and apoptosis through suppression of Drp-1 translocation

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ABSTRACT
Introduction: Mitochondrial dysfunction plays an important role in acute kidney injury (AKI). Mitochondrial fission regulated by dynamin-related protein 1 (Drp-1) impairs the function of the mitochondria and the survival of cells. This study was conducted to explore the effects of suppression of Drp-1 accumulation in the mitochondria, on mitochondrial function and renal tubular cell apoptosis in rhabdomyolysis (RM)–induced AKI.

Methods: An RM model was induced by intramuscular injection of glycerol in Sprague Dawley rats. Twenty-four and 48 hours after intraperitoneal injections of mitochondrial division inhibitor 1 (Mdivi-1), we observed the functions of the kidney, changes in pathology, expressions of Drp-1 in tubular tissues (by immunohistochemistry and Western blot) and accumulation of Drp-1 and mitofusin 2 in tubular mitochondria (by Western blot). Mitochondrial function (ATP and ROS) and tubular epithelial cell apoptosis (by TUNEL) were also measured.

Results: RM induced Drp-1 accumulation, decreased ATP production and increased ROS in mitochondria. With increasing cytochrome c expression, cell apoptosis increased, whereas kidney function decreased. These changes were time-dependent. At different time points, despite not significantly influencing the overall expression of Drp-1, Mdivi-1 suppressed the accumulation of Drp-1, inhibited the insertion of proapoptotic Bax in mitochondria and inhibited the release of cytochrome c, thus ameliorating cell apoptosis.

Conclusions: To conclude, in RM-induced AKI, suppression of Drp-1 accumulation in mitochondria favors the maintenance of mitochondrial function and reduces the apoptosis of tubular cells. Regulation of the mitochondrial fusion–fission balance may offer a novel strategy for the prevention and treatment of RM-induced AKI.

Key words: Acute kidney injury, Apoptosis, Dynamin-related protein 1, Mitochondria, Rhabdomyolysis

INTRODUCTION

Rhabdomyolysis (RM) is defined as the massive breakdown of muscles, resulting in the release of myoglobin (Mb) into the bloodstream. RM is usually associated with trauma, natural disasters or drug overdose, among others (1). Mb can be freely filtered by the glomeruli; however, Mb filtration may cause acute kidney injury (AKI) (2). Previous studies have shown that AKI development is involved in renal vasoconstriction, Mb deposit obstructions in renal tubules, and the direct toxic effects of Mb taken in by proximal tubular cells. However, the mechanism of RM-induced AKI has yet to be completely elucidated. The direct toxicity of Mb on tubular cells has gained considerable attention (3). Mb is a prooxidant that can catalyze reactive oxygen species (ROS) generation and lead to lipid peroxidation (4). In addition, the Mb-induced oxidative stress pathway participates in AKI (5).

In addition to ATP, the mitochondria produce ROS, which plays a key role in the regulation of oxidative stress and apoptosis (6). Previous studies have reported that ferric ions in the Mb can directly impair the mitochondrial membrane and induce cell injury and apoptosis, indicating that...
mitochondrial injury participates in the development of RM-induced AKI (4, 7). The mitochondria, which are highly dynamic organelles, maintain their morphology and function through fusion and fission. This dynamic balance is regulated by mitofusin (Mfn1/Mfn2) and dynamin-related protein 1 (Drp-1) (8). Changes following mitochondrial dysfunction (usually presented in fission status), such as reduction in energy production and alteration of the membrane potential, stimulate cell apoptosis (9). However, whether or not the balance of fusion and fission in the mitochondria is influenced by RM remains unknown. According to a recent report by Funk and Schnellmann (10), expression of the mitochondrial fission protein Drp-1 in the kidney increases after AKI, which indicates persistent disruption of mitochondrial homeostasis.

The mitochondrial fusion–fission imbalance plays a crucial role in the development of RM-induced AKI. Therefore, interventions targeting this pathogenic mechanism could provide new insights into the protection of the kidney. However, related investigations on this topic are limited. We hypothesize that the suppression of mitochondrial fission (by mitochondrial division inhibitor 1 [Mdivi-1]) (11, 12) in tubular epithelial cells can improve kidney functions through the amelioration of mitochondrial dysfunction and reduction in cell apoptosis.

**Material and Methods**

**Glycerol-induced RM modeling in rats**

This study was performed according to the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health and was approved by the Institutional Animal Ethics Committee of West China Hospital, Sichuan University. Male Sprague Dawley rats weighing 200-250 g were used in this study. Animals were fed standard rat chow at 22°C to 24°C. Thirty rats were divided at random into 3 groups: the control group (group A, n = 6), the RM group (24-hour group, n = 6; 48-hour group, n = 6), and the Mdivi-1 treatment group (24-hour group, n = 6; 48-hour group, n = 6). Rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). RM was induced by the injection of 10 ml/kg of 50% glycerol water solution (Amresco, USA) into the leg muscles of rats, as described by Plotnikov et al (11). Age-matched control rats of the same strain were injected with only sterile saline. Afterward, the Mdivi-1 therapeutic protocol, which is used to treat AKI, was implemented: intraperitoneal injections of Mdivi-1 (50 mg/kg in dimethyl sulfoxide [DMSO]; Sigma, St. Louis, MO, USA) 1 hour before induction of RM, and 6 and 12 hours after induction of RM (13).

**Tissue sampling, histological examination and plasma creatinine detection**

At the end of the 24th and 48th hours, blood samples were collected, and rats from each group were sacrificed. The kidneys of the rats were dissected after ketamine anesthesia. The left kidney was divided into 2 parts. One part was preserved using a quick freeze method with liquid nitrogen, and the other was preserved with 0.9% saline. The contralateral kidney was fixed in 4% paraformaldehyde for 48 hours before being embedded in paraffin for histological examination and immunohistochemical staining. Formalin-fixed paraffin-embedded sections (4 µm) of the kidney tissues were stained with hematoxylin and eosin for light microscopy observation. The levels of plasma creatinine were measured by the Jaffé method (14).

**Immunohistochemistry**

Drp-1 protein was evaluated in the kidneys by immunohistochemical staining of 4-µm formalin-fixed paraffin-embedded sections. Drp-1 polyclonal antibodies (1:100 dilution; Abcam, UK) were used as primary antibodies. The sections were washed with phosphate-buffered saline (PBS) and incubated with biotinylated secondary antibody (1:100 dilution; Sigma). All slides were counterstained with hematoxylin. We investigated the stained tissues using the same optical microscope setup (Olympus BX15; Olympus, center valley, PA, USA) and measured the integral optical density using an image analysis system (Image-Pro Plus 6.0; Media Cybernetics, Silver Spring, MD, USA).

**Tubule isolation and mitochondrial extraction**

Tubule isolation from rat kidney tissues was performed by collagenase digestion, as described by Plotnikov et al (11). Rat kidney tissues were dissociated with 0.1% collagenase solution, large fragments were removed, and tubules were precipitated by gentle centrifugation. The mitochondria were extracted from tubule homogenates followed by multiple centrifugations in ice-cold isolation buffer, with pH 7.4, of 50 mM Tris/HCl, 250 mM sucrose, 5 mM EDTA and protease inhibitors. The mitochondria were collected, washed with PBS and then incubated in ice in 0.1 M Na2CO3 at pH 11.5 for 30 minutes. Finally, the pellet was collected for Western blot analysis to detect Mfn2, Drp-1 and inserted Bax.
Extraction of cytosolic and nuclear proteins

To isolate cytosolic components from nuclear fractions, we mechanically homogenized and treated tubular tissues using a Nuclear and Cytosolic Protein Extraction Kit (Signalway Biotechnology, Pearland, TX, USA) according to the manufacturer's instructions. The expressions of Drp-1 and Bax in the cytosol and that of proliferating cell nuclear antigen (PCNA) in the nucleus were then detected by Western blot analysis.

Western blot analysis for Mfn2, Drp-1, Bax, cytochrome c, caspase-3 and PCNA

Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins from each sample were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (NuPAGE, Invitrogen, Carlsbad, CA, USA). The membranes were probed overnight using the corresponding antibodies. The membranes were then incubated with a secondary antibody (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hours. Bands were visualized by enhanced chemiluminescence (Santa Cruz Biotechnology), and sample loadings were normalized by immunoblotting with an anti-β-actin monoclonal antibody (1:2,000; Chemicon, Billerica, MA, USA).

TdT-mediated dUTP nick end labeling (TUNEL) assay

Paraffin-embedded specimens were cut into 5-µm sections. Staining was performed using an in situ cell-death detection kit (AP; Roche, USA), according to the manufacturer’s instructions. In brief, tissue sections were treated with 20 µg/mL proteinase K for 30 minutes, washed with PBS solution and incubated with fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase for 60 minutes. The sections were then incubated with converter alkaline phosphatase for 30 minutes. The reaction was developed with Fast Red Tablets in naphthol phosphate substrate (Laboratory Vision, USA). Ten villi and crypts in each section were observed under a fluorescence microscope. Afterward, the average number of apoptotic cells per 100 counted cells was defined as the apoptotic index (AI).

ATP measurement

Renal cortical tissue ATP was measured as described by Vives-Bauza et al (15). ATP was extracted from flash frozen kidney cortex with 0.4 M HClO4. ATP levels were determined using an ATP bioluminescence assay kit (Roche) and normalized to protein concentration.

Determination of ROS production in kidney tissue

Rat kidney homogenate was analyzed fluorometrically by measuring the oxidation of the nonfluorescent probe 20,70-dichloro-fluorescein diacetate (DCFDA) into the fluorescent metabolite DCF as described by Topo et al (16). In brief, 30 mL of kidney homogenate in PBS was mixed with 5 µm DCFDA and incubated for 30 minutes at room temperature. The mean fluorescence intensity was directly measured at excitation and emission wavelengths of 485 and 535 nm, respectively.

Statistical analysis

All values are expressed as means ± standard error of the mean. Multiple group means were compared by 1-way analysis of variance. A p value of <0.05 was considered statistically significant. SPSS 17.0 statistical software was used for analysis.

Results

Kidney function and histological changes

Twenty-four hours after injection with glycerol, the urine of rats in the RM group changed into a red-dark color (figure not shown). Meanwhile, the level of creatinine in the RM (24 h), Mdivi-1 (24 h), RM (48 h) and Mdivi-1 (48 h) groups increased significantly compared with the normal control group. The level of creatinine in the Mdivi-1 (24 h) group was significantly lower than that in the RM (24 h) group. In addition, the level of creatinine in the Mdivi-1 (48 h) group was lower compared with that in the RM (48 h) group (Fig. 1A). This result suggests that Mdivi-1 may improve kidney function. Histological changes in kidney tissues are described in Figure 1B. Swelling, granular degeneration and vacuolar degeneration of tubular epithelial cells appeared 24 hours after model induction. Some tubules were obstructed by Mb casts. More significant changes were observed after 48 hours, with exacerbating cell degeneration and detachment of the tubular brush border. Histological changes were relatively mild in the Mdivi-1 group at the same time points.

Expression of Drp-1 in the kidney

The results of immunohistochemistry showed that Drp-1 expression significantly increased after 24 hours; no difference
between the Mdivi-1 treated group and the RM group was found. The expression of Drp-1 was even higher after 48 hours; still no statistically significant difference was detected between the RM group and Mdivi-1 treated group (Fig. 2A, B). These findings were further confirmed by Western blot analysis, which showed that Drp-1 expression increased with time and was not inhibited by Mdivi-1 at both time points (Fig. 2C, D). These results suggest that Drp-1 expression in RM is up-regulated in the kidney and cannot be suppressed by Mdivi-1.

Expression of Mfn2, Drp-1 and inserted Bax in mitochondria

To investigate changes in mitochondrial fusion and fission, we investigated the level of Mfn2, Drp-1 and Bax in the mitochondria through Western blot. We found that RM stimulated not only the overall expression of Drp-1 in the kidney but also the accumulation of Drp-1 in the mitochondria in a time-dependent manner. At different time points, Drp-1 expression in the mitochondria and in the RM.
group significantly differed from that in the Mdivi-1 group (Fig. 3A, B). This result suggests that Mdivi-1 decreases Drp-1 accumulation in the mitochondria despite not being able to inhibit the general expression of Drp-1 in the kidney. Nonetheless, according to our study, Mfn2 expression in the mitochondria only increased slightly 24 and 48 hours after model induction. The expression levels were also similar in the RM and Mdivi-1 treated groups, indicating that RM can induce a mild increase in Mfn2 expression in the mitochondria whereas Mdivi-1 poses little effect (Fig. 3A, C). These findings suggest that RM is likely to influence the balance of mitochondrial dynamics through its stimulative impact on mitochondrial fission, whereas Mdivi-1 may only influence Drp-1 translocation from cytosol to mitochondria, as well as Drp-1 accumulation in the mitochondria.

In normal mitochondria, the insertion of Bax is not common. This study showed that 24 hours after induction, the amount of Bax in the mitochondria of renal tubules was markedly elevated with increasing time and was even higher another 24 hours later. Treatment using Mdivi-1 obviously reduced the insertion of Bax (Fig. 3A, D). Exactly 24 and 48 hours after model induction, Bax expression in the Mdivi-1 and RM groups revealed statistical differences (p<0.05). This result indicates that RM can induce the insertion of Bax in the mitochondria and that this effect can be mitigated by Mdivi-1.

Expressions of Drp-1 and Bax in cytosol

We determined the expressions of Drp-1 and Bax in the cytosol through Western blot analysis and found that the expression of Drp-1 in the cytosol was lower than that in the mitochondria under RM conditions. At the same time point, the cytosolic Drp-1 expression in the RM group was significantly higher than that in the Mdivi-1 group (Fig. 3E, F). The variation in cytosolic Bax was consistent with Drp-1 (Fig. 3E, G). These results further confirm that Mdivi-1 reduces RM-induced Drp-1 accumulation in the mitochondria and inhibits Bax insertion.

Function of mitochondria

RM increased the production of ROS; the change was more obvious at 48 hours than at 24 hours after induction. After Mdivi-1 intervention, ROS production significantly decreased. This result is indicative of the inhibitory effect of Mdivi-1 on RM-induced production of ROS and the protective effect of Mdivi-1 on mitochondrial functions (Fig. 4A). About 24 hours after model induction, we also found that ATP, the marker of respiratory function in the mitochondria, markedly decreased. This change was more significant 48 hours after induction, indicating that RM suppresses mitochondrial respiratory function. At both time points, ATP production in the Mdivi-1 treated group was higher than that in the RM group, revealing the beneficial effects of Mdivi-1 in alleviating RM-induced impairment of mitochondrial respiratory function (Fig. 4B).
Expression of apoptosis-related proteins of cytochrome c and caspase-3

Similar to the changes in mitochondrial function, marked elevation of cytochrome c was observed in the RM group after 24 hours. Cytochrome c is a protein released after mitochondrial damage and is involved in the initiation of apoptosis. The increase in cytochrome c peaked 48 hours after induction. This result suggests that RM induces cytochrome c release in a time-dependent manner. Compared with the RM group, cytochrome c was expressed at significantly lower levels in the Mdivi-1 treated group (Fig. 5A, B), indicating the protective role of Mdivi-1 on mitochondrial function via maintenance of the mitochondrial membrane. Changes in caspase-3, a protein playing a central role in the pathway of mitochondrial apoptosis, were consistent with those in cytochrome c (Fig. 5A, C). Thus, mitochondrial apoptosis, following the activation of cytochrome c, occurs through the caspase-3 pathway.

The level of apoptosis in the renal tubules

Figure 6A presents the results of TUNEL analysis, in which the nucleus of apoptotic cells were stained red and normal cells were stained blue. About 24 hours after model
induction, the apoptotic cells in the RM group were slightly
greater in number than in the control group. After 24 hours
of Mdivi-1 treatment, the apoptosis rate decreased in the
Mdivi-1 treated group and was lower than the untreated
group. After 48 hours, apoptosis was more significant in
the RM group. Mdivi-1 also decreased apoptosis at the
latter time point. These findings suggest that apoptosis of
tubular epithelial cells in RM occurs in a time-dependent
manner and can be suppressed by Mdivi-1. The trends
in changes in apoptosis are consistent with those for the
signaling proteins described above (Fig. 6B).

Level of proliferation in the renal tubules

PCNA is a kind of DNA polymerase δ auxiliary protein pres-
ent in the nucleus that can reflect the state of cell prolifera-
tion. Our results showed that normal tubules did not express
PCNA. Nonetheless, RM can stimulate tubular expression
of PCNA in a time-dependent manner. At the 24- or 48-hour
time point, the level of PCNA expression was relatively low.
Moreover, Mdivi-1 treatment did not affect PCNA expres-
sion in renal tubules (Fig. 7).

**Discussion**

In AKI caused by different etiologies, the reduction in
the number of tubular epithelial cells due to excessive apo-
ptosis or necrosis is a major intrinsic change; impairment of
the mitochondria has been identified as the primary cause
of excessive apoptosis and necrosis (17, 18). After the mi-
tochondria are damaged, the respiratory complex breaks
down, the membrane potential is altered and permeability
increases, followed by the release of proapoptotic factors,
such as cytochrome c (19). Nath et al confirmed that renal
mitochondrial respiration is disrupted early and further di-
minishes after glycerol injection (20). Our results are con-
sistent with previous reports. We found that 24 hours after
glycerol injection, energy production in the mitochondria
decreased, accompanied by an increase in the produc-
tion of ROS and the release of proapoptotic factors; these
changes led to the increased apoptosis of tubular epithelial
cells. These changes were time-dependent, indicating that
cell apoptosis following the dysfunction of the mitochondria
in RM induced the development and progression of AKI.
However, the mechanism of mitochondrial damage, a key
contributor to renal tubular cell death during RM-induced
AKI, remains largely unknown.

Mitochondrial function largely depends on the balance of
fusion and fission. Fusion favors the stability of normal cel-
lar function and membrane permeability. Mfn2 is required
for placentation, which plays a crucial role in mitochondrial
fusion. Mitochondrial fission is associated with a reduction
in energy production, a change of membrane potential, and
the release of proapoptotic factors, all of which threaten
cell survival (21). Drp-1, an important regulatory protein for
mitochondrial fission (gene locus: 12p11.21), is located in
the cytosol with an N-terminal guanosine triphosphatase
(GTPase) domain. The principal pathway for Drp-1 induced
mitochondrial fission is as follows: Drp-1 assembles from
the cytosol onto the mitochondria at focal sites of division
and forms spiral chains around membrane constriction sites;
this process facilitates guanosine triphosphate hydrolysis
and organelle fission. After the completion of fission, Drp-1 translocates to the cytosol (22). Previous studies have confirmed the involvement of mitochondrial fission induced by Drp-1 in disorders of other systems, such as neurological and cardiovascular diseases (23, 24). However, investigations into Drp-1 in the kidney are limited. Brooks et al (25) reported that Drp-1 silencing by siRNA or expression of Drp-1 dominant-negative gene ameliorates mitochondrial segmentation, cytochrome c release, and cell apoptosis. According to our data, RM stimulated the overall expression of Drp-1 in the kidney, which is consistent with previous studies. Gene chip analysis of other diseases shows that some molecules (i.e., p53, androgen) can bind to specific loci of Drp-1 and regulate the expression of Drp-1 (26, 27). However, the signaling pathway, signal factor, and influencing level (transcription or translation) involved in Drp-1 regulation in RM require further investigation. Translocation of Drp-1 is usually induced by stimulatory factors (i.e., signals for apoptosis), and Drp-1 is recruited by the mitochondrial outer membrane protein Fis for accumulation in the mitochondria (22). We also observed the increasing accumulation of Drp-1 in the mitochondria, which indicates that mitochondrial impairment may be a signal itself that stimulates the translocation of Drp-1 and mitochondrial fission, resulting in cell apoptosis. Another interesting finding in our research is that RM slightly increases Mfn2 expression in the mitochondria. We speculate, under the condition of mitochondrial damage and fission, that the increase in Mfn2 may be a feedback response to compensate for the loss of mitochondria, which is a self-antagonistic regulation. However, further studies are necessary to test this hypothesis. Our study also found that PCNA expression in tubules began to increase at the early stage of RM-induced AKI. This indicated that the regeneration program of injured tubules had started. However, we did not observe any intrinsic relationship between PCNA expression and Drp-1 translocation.

Although the role of the mitochondria in AKI has gained increased attention, direct evidence that addresses mitochondrial intervention in RM-induced AKI remains lacking. Plotnikov et al reported that the antioxidant SKQR1 has renal protective effects and can improve mitochondrial function and reduce cell death in RM and ischemia-reperfusion–induced AKI (11). Similar findings were observed in our research; however, the underlying mechanism here appeared to be different. We performed an intervention that involved the use of Mdivi-1 (a specific inhibitor for Drp-1) to directly target mitochondrial fission induced by Drp-1 because of the special effects of Drp-1 in RM-induced AKI. Cassidy-Stone et al showed that Mdivi-1 can prevent configuration changes, influence the activity of GTPase and inhibit the accumulation of Drp-1 by binding to its specific loci (28). Our study also confirmed that Mdivi-1 can significantly suppress the RM-induced accumulation of Drp-1 on the mitochondrial membrane, which indicates that the specific effect of Mdivi-1 occurs during Drp-1 translocation but not during gene expression. After Mdivi-1 treatment, insertion of Bax onto the mitochondrial membrane was markedly reduced. Only fission promotes the insertion of the proapoptotic factor Bax (29). Therefore, Mdivi-1 can be speculated to reduce mitochondrial fission, suppress the release of proapoptotic factors and decrease cell apoptosis by playing a protective role in the kidney. These phenomena can be attributed to improvements in the balance between the fusion and fission of mitochondria. Hypothetically, inhibition of fission and stimulation of fusion are both beneficial for mitochondrial function; however, the current research only obtained evidence on the impact of fission and not fusion.

To conclude, the imbalance between mitochondrial fusion and fission plays a crucial role in the dysfunction of mitochondria and apoptosis of cells in RM-induced AKI. Mitochondrial dysfunction and cell apoptosis are closely
associated with increased Drp-1 expression and RM-stimulated translocation. Suppressing the accumulation of Drp-1 in the mitochondria maintains the stability of mitochondrial function, improves energy production, reduces the release of proapoptotic factors and inhibits the apoptosis of tubular epithelial cells. We speculate that the regulation of the mitochondrial fusion–fission balance may offer a novel strategy for the prevention and treatment of RM-induced AKI.

Several limitations, however, were present in the study: (a) only short-time outcomes (limited to the early phase of RM-induced AKI) were assessed; (b) the effects of Mdivi-1 could be assumed to attenuate with time; and (c) the mechanism of the influence of RM on Drp-1 and Mfn2 has yet to be confirmed. Further studies are necessary to address these issues.

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IC-IRB Approval: This study was approved by the Institutional Animal Ethics Committee of West China Hospital, Sichuan University, and was performed according to the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

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