Research Article

Inhibition of Mitochondrial Complex I Aggravates Folic Acid-Induced Acute Kidney Injury

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Keywords

Rotenone · Folic acid · Mitochondrial dysfunction · Acute kidney injury · Oxidative stress

Abstract

Background: Some researches revealed that mitochondrial dysfunction is associated with various kidney injury. However, the role of mitochondrial dysfunction in the pathogenesis of acute kidney injury (AKI) still needs evidence. **Methods:** We evaluated the effect of mitochondrial complex I inhibitor rotenone on folic acid (FA)-induced AKI in mice. **Results:** Strikingly, the mice pretreated with rotenone at a dose of 200 ppm in food showed exacerbated kidney injury as shown by higher levels of blood urea nitrogen and creatinine compared with FA alone group. Meanwhile, both renal tubular injury score and the expression of renal tubular injury marker neutrophil gelatinase-associated lipocalin were further elevated in rotenone-pretreated mice, suggesting the deteriorated renal tubular injury. Moreover, the decrements of mitochondrial DNA copy number and the expressions of mitochondrial Cytochrome c oxidase subunit 1, mitochondrial NADH dehydrogenase subunit 1, and mitochondria-specific superoxide dismutase (SOD2) in the kidneys of FA-treated mice were further reduced in rotenone-pretreated mice, indicating the aggravated mitochondrial damage. In parallel with the SOD2 reduction, the oxidative stress markers of malondialdehyde and HO-1 displayed greater increment in AKI mice with rotenone pretreatment in line with the deteriorated apoptotic response

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and inflammation. **Conclusion:** Our results suggested that the inhibition of mitochondrial complex I activity aggravated renal tubular injury, mitochondrial damage, oxidative stress, cell apoptosis, and inflammation in FA-induced AKI.

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Introduction

Acute kidney injury (AKI) has been estimated with a mortality rate of ~50% [1, 2]. It can arise in a variety of pathological conditions such as exposure to nephrotoxins and ischemia [3, 4]. After injury, inflammation, oxidative stress, and apoptosis are common pathological features of AKI, which may eventually lead to tubulointerstitial fibrosis and chronic kidney disease (CKD) [5]. Patients with AKI have a higher risk in the development of CKD and end-stage renal disease [6]. Currently, there are no satisfactory therapies for AKI.

Recently, some researches revealed an abnormal change of mitochondria in CKD models of UUO [7–9] and 5/6 nephrectomy [10]. Mitochondrial damage enhanced the production of reactive oxygen species (ROS) and the release of proapoptotic factors, resulting in the oxidative stress, cellular apoptosis, and inflammation [11, 12].

Rotenone could inhibit the activity of mitochondrial respiratory chain complex I via affecting the transfer of the iron-sulfur clusters and coenzyme Q to reduce the synthesis of ATP [13]. By reviewing the literatures, there exist inconsistent results about the effect of rotenone on kidney diseases. Sun and Zhang et al. [14, 15] reported that rotenone attenuates oxidative stress, inflammation, and fibrosis in CKD models of UUO and ischemia-reperfusion-induced CKD. Oppositely, Jiang et al. [16] demonstrated that rotenone induces nephrotoxicity in rats in line with the enhanced oxidative stress and apoptosis. So far, the role of mitochondrial inhibitors in AKI has not been well studied, and the role of mitochondrial inhibitor in different AKI models still needs to be defined. Folic acid (FA) overdose-induced AKI has been described in humans [17] and recapitulates the major processes of human AKI. Thus, FA-induced AKI animal model was widely used to investigate the pathogenesis and therapies of AKI. In the present study, we tested the role of mitochondrial complex I inhibitor rotenone in FA-induced AKI and the underlying mechanisms in mice.

Materials and Methods

In some experiments, we followed the methods from Zhang et al. [15].

Reagents and Antibodies

Rotenone (R8875) and FA (F7876) were purchased from Sigma (St. Louis, MO, USA). Antibodies against neutrophil gelatinase-associated lipocalin (NGAL; ab63929) were purchased from Abcam (Shanghai, China). BAX (50599-2-Ig, 1:1,000), HO-1 (10701-1-AP, 1:1,000), superoxide dismutase (SOD2; 24127-1-AP, 1:1,000), GAPDH (10494-1-AP, 1:1,000), and β -actin (20536-1-AP, 1:1,000) were purchased from Proteintech (Wuhan, China). Horseradish peroxidase-conjugated goat antirabbit secondary antibody was purchased from Beyotime (Haimen, China, A0208, 1:1,000). SYBR Green master mix for real-time polymerase chain reaction (RT-PCR) was provided by Applied Biosystems (4367660, Foster City, CA, USA).

Animals

C57BL/6J mice were originally obtained from Jackson lab. This mouse colony was propagated at the Nanjing Medical University. Male mice with the age at 8–12 weeks old were used

for experiments. All mice were maintained on a 12 h light-dark cycle in a temperature-controlled ($22 \pm 2^\circ\text{C}$) room, were fed a standard rodent diet, and were allowed to free access to drinking water. The procedures were approved by the Nanjing Medical University Institutional Animal Care and Use Committees.

FA-Induced AKI Model and Rotenone Treatment

Mice were injected (i.p.) with FA at 240 mg/kg body weight. Sodium bicarbonate (0.3 M NaHCO_3 , the vehicle used for FA administration) alone was used as controls. To examine the effect of the inhibition of the mitochondrial complex I on FA-induced AKI, mice were pretreated with rotenone at 200 ppm in jelly diet for 24 h before FA i.p. injection. The dose of rotenone at 200 ppm in jelly diet used in this study was according to our previous studies [14, 15, 18–20]. The sham control mice were treated with jelly diet without rotenone. After FA administration for 72 h, the animals were killed and the kidney tissues were harvested for the evaluation of gene and protein expressions and histology. Six mice were used in each group.

Measurements of Blood Urea Nitrogen and Creatinine

Blood urea nitrogen (BUN) and creatinine levels were determined to assess the renal function. After the FA treatment for 72 h, the mice were sacrificed, and the blood samples were obtained from the inferior vena cava and anticoagulated with heparin (20 unit/mL blood). The plasma was isolated by centrifugation. Automatic biochemical analyzer was used to detect the concentration of BUN and creatinine.

Renal Histology and Tubular Injury Score

Kidney tissues from mice were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned transversely. Paraffin sections (3 μm) of renal tissue were stained with periodic acid-Schiff and examined by light microscopy. Ten nonoverlapping tubulointerstitial fields were randomly selected from each slide. The swelling of renal tubular epithelial cells, renal tubular atrophy, lumen dilatation, and brush edge shedding were observed under 200 $\times$ magnification by microscopy. According to the percentage of pathological injury area under a single field of vision, semi-quantitative scoring was performed by 4 points. The scoring criteria are as follows as described previously [21, 22]: the injury area is 0%, 0 points, normal; injury area <25%, 1 point, mild; injury area is 25–50%, 2 points, moderate; injury area is 50–75%, 3 points, severe; injury area >75%, 4 points, extremely severe.

Western Blotting

Kidney tissues (20 mg) were lysed used RIPA lysis (200 μL), which contained protease inhibitors. Immunoblotting was then performed using primary antibodies against NGAL (1:1,000), HO-1 (1:500), SOD2 (1:1,000), BAX (1:500), β -actin (1:1,000), and GAPDH (1:1,000), followed by addition of HRP-labeled secondary antibodies. The blots were visualized using Amersham ECL Detection Systems (Amersham, Buckinghamshire, UK). The intensity of the band was quantitatively analyzed using the gel imaging system scan (Bio-rad, USA).

Quantitative RT-PCR

Total DNA and RNA from kidney cortex tissues were isolated using the DNeasy Tissue Kit (Tiangen, China) and Trizol reagent (Takara), respectively. Primers were designed using Primer5 software (available at <http://frodo.wi.mit.edu/>) and synthesized by Invitrogen. The sequences of primers for quantitative RT-PCR were shown in Table 1. Reverse transcription was done by using Promega Reverse Transcription System according to the manufacturer's instruction (Madison, WI, USA). RT-PCR amplification was performed using the ABI 7500 RT-PCR Detection System (Foster City, CA, USA) and SYBR Premix Ex Taq (TaKaRa). The

Table 1. Sequences of primers for qRT-PCR

Gene	Primer sequence
GAPDH	5'-GTCTTCACTACCATGGAGAAGG-3' 5'-TCATGGATGACCTTGGCCAG-3'
NGAL	5'-GCAGGTGGTACGTTGTGGG-3' 5'-CTCTGTAGCTCATAGATGGTGC-3'
IL-6	5'-GCTTAGGCATAACGCACT-3' 5'-GGAAATCGTGGAATGAG-3'
ICAM-1	5'-CGCTTCCGCTACCATCAC-3' 5'-GGCGGCTCAGTATCTCCTC-3'
mt-COX1	5'-CCCAATCTCTACCAGCATC-3' 5'-GGCTCATAGTATAGCTGGAG-3'
mt-ND1	5'-AATCGCCATAGCCTTCCTAACAT-3' 5'-GGCGTCTGCAAATGGTTGTAA-3'
18s rRNA	5'-TTCGGAAGTGAAGCCATGATT-3' 5'-TTTCGCTCTGGTCCGTCTTG-3'

qRT-PCR, quantitative real-time-PCR; IL-6, interleukin-6; mt-COX1, mitochondrial Cytochrome c oxidase subunit 1; mt-ND1, mitochondrial NADH dehydrogenase subunit 1; NGAL, neutrophil gelatinase-associated lipocalin.

cycling program includes a preliminary denaturation (95 °C for 10 min) followed by 40 cycles (95 °C for 15 s and 60 °C for 1 min). The relative mitochondrial DNA copy number was normalized to the 18S rRNA level encoded by the nuclear DNA, and mRNA levels were normalized to GAPDH and calculated using the comparative cycle threshold ($\Delta\Delta Ct$) method.

Malondialdehyde Determination

We used a commercial kit to quantify the generation of malondialdehyde (MDA) according to the manufacturer's protocol. In brief, kidney tissue samples were homogenized and sonicated in RIPA buffer on ice. Tissue lysates were then centrifuged at 12,000 *g* for 15 min at 4 °C to collect the supernatant. Renal MDA was measured using a Lipid Peroxidation MDA assay kit (Beyotime). MDA levels were detected using multimode microplate readers (Spectramax M5) at 532 nm and normalized by the protein concentration.

In situ Transferase dUTP Nick End Labeling Assays

For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, kidney sections were stained as per the manufacturer's instructions (TUNEL Detection Kit, 11684817910, Roche). The number of TUNEL-positive nuclei per field was evaluated in 5 fields per section and 5 sections per kidney.

Enzyme-Linked Immunosorbent Assay

The kidney tissue was homogenized in phosphate-buffered saline and then centrifuged for 5 min at 10,000 rpm. Supernatant was diluted at 1:50 with enzyme immunoassay buffer. The plasma tumor necrosis factor- α (TNF- α) level was determined by an enzyme-linked immunosorbent assay kit (DKW12-2720, Dakewe Bioengineering Co., Ltd., China) according to the manufacturer's instructions.

Fig. 1. Rotenone exacerbated FA-induced renal injury. **A** BUN. **B** Blood serum creatinine. **C** Representative images of renal PAS staining. **D** Tubular injury score. Data were presented as means $\pm$ SE, $n = 6$ in each group. BUN, blood urine nitrogen; FA, folic acid; Scr, serum creatinine.

Statistical Analysis

The quantitative data of the experiment were expressed by means $\pm$ SEM. Statistical analysis was performed using SPSS 16.0 software. Statistical significance was determined by Student *t* test for comparisons of 2 groups. Multigroup comparisons were performed using ANOVA multiple comparisons. $p < 0.05$ was considered statistically significant.

Results

Rotenone Exacerbated FA-Induced Renal Injury

To evaluate the effect of rotenone on renal function in FA-treated mice, we measured BUN and creatinine levels. Compared with the FA alone group, rotenone treatment further increased BUN (from 18.08 ± 0.76 to 121.02 ± 1.08 mm) and serum creatinine (from 67.40 ± 3.45 to 368.13 ± 7.91 μ M) levels in AKI mice (Fig. 1A, B). In line with the renal dysfunction, periodic acid-Schiff staining of kidney tissues showed that FA-induced renal tubular injury was further aggravated by rotenone pretreatment as shown by the more severe tubular dilation, protein cast formation, and brush border loss (Fig. 1C, D). Furthermore, the tubular injury marker NGAL was measured to better evaluate the renal tubular injury. As shown by the data, both protein and mRNA levels were significantly upregulated by FA treatment, which was further elevated by rotenone pretreatment (Fig. 2A–C). These data suggested that inhibition of mitochondrial complex I activity by rotenone aggravated acute renal tubular injury induced by FA.

Fig. 2. Effect of rotenone pretreatment on the regulation of tubular injury marker NGAL induced by FA. **A** Western blotting analysis of NGAL. GAPDH was used as loading control. **B** Densitometry of Western blots in A. **C** qRT-PCR analysis of NGAL. Data were presented as means $\pm$ SE, $n = 6$ in each group. FA, folic acid; NGAL, neutrophil gelatinase-associated lipocalin.

Rotenone Treatment Deteriorated Renal Mitochondrial Damage Induced by FA

Mitochondrial dysfunction has been considered as one of the pathogenic factors in kidney injury [23]. Thus, we measured mtDNA copy number and mitochondrial genes of mitochondrial Cytochrome c oxidase subunit 1 (mt-COX1) and mitochondrial NADH dehydrogenase subunit 1 (mt-ND1) to evaluate the mitochondrial abnormality. As shown by Figure 3A–C, in FA-treated mice, the mtDNA copy number and the expressions of mt-COX1 and mt-ND1 were obviously reduced, which was further worsened in rotenone-treated AKI mice, suggesting deterioration of mitochondrial abnormality. Furthermore, we also measured mitochondria-specific SOD2 and found that rotenone treatment led to more severe reduction of SOD2 levels in the kidney of mice challenged with FA (Fig. 3D, E), suggesting the impairment of the antioxidant system in mitochondria.

Rotenone Aggravated Renal Oxidative Stress Induced by FA

Dysfunctional mitochondria serve as the important source of ROS production. Contrasting to the reduced SOD2 expression in rotenone-treated AKI mice, the levels of kidney MDA were further enhanced compared to FA alone group (Fig. 4A). Similarly, another oxidative stress marker HO-1 was markedly higher in the kidneys of mice with rotenone pretreatment than

Fig. 3. Rotenone pretreatment worsened renal mitochondrial damage induced by FA. **A** mtDNA copy number was determined by qRT-PCR. **B** qRT-PCR analysis of mt-COX1. **C** qRT-PCR analysis of mt-ND1. **D** Western blotting analysis of SOD2, GAPDH was used as loading control. **E** Densitometry of Western blots in **D**. Data were presented as means $\pm$ SE, $n = 6$ in each group. FA, folic acid; mt-COX1, mitochondrial Cytochrome c oxidase subunit 1; SOD2, superoxide dismutase; mt-ND1, mitochondrial NADH dehydrogenase subunit 1.

that in FA alone group (Fig. 3C). These findings indicated that rotenone treatment promoted renal oxidative stress in FA-induced AKI mice possibly through the impairment of mitochondrial function.

Rotenone Aggravated Renal Cellular Apoptosis Induced by FA

Cellular apoptosis is a common feature of many forms of AKI. By TUNEL staining, we found that the TUNEL-positive nuclei were almost undetectable in the control kidneys, while the number of TUNEL positive nuclei was significantly increased after FA insult. In contrast, rotenone pretreatment evidently increased the number of TUNEL-positive nuclei compared to the FA alone group (Fig. 5A, B). To better evaluate the apoptotic response, we further measured BAX expression in the kidneys and found that FA-induced upregulation of BAX protein was more robustly increased in rotenone-pretreated animals (Fig. 5C, D). These data suggested that rotenone could aggravate apoptotic response in FA-induced AKI.

Rotenone Promoted the Renal Inflammation Induced by FA

It is well known that the inflammation is involved in the pathogenesis of AKI [24]. By quantitative RT-PCR, we found that the inflammatory markers interleukin-6 (IL-6) were markedly elevated in FA-treated mice, which was further upregulated in rotenone-pretreated mice (Fig. 6A). Furthermore, we measured circulating levels of TNF- α by enzyme-linked immunosorbent assay and observed a trend increment in rotenone-pretreated mice compared to FA alone group (Fig. 6C). However, the upregulation of ICAM-1 mRNA in kidneys induced by FA was unaltered by rotenone (Fig. 6B). These data suggested that rotenone promoted the inflammatory response in FA-induced AKI.

Fig. 4. Rotenone aggravated renal oxidative stress induced by FA. **A** Determination of renal tissue MDA. **B** Western blotting analysis of HO-1, β -actin was used as loading control. **C** Densitometry of Western blots in **B**. Data were presented as means $\pm$ SE, $n = 6$ in each group. FA, folic acid; MDA, malondialdehyde.

Evaluation of the Rotenone Toxicity in Mice

Finally, we assessed the toxicity of rotenone in mice. As shown by the data, 4-day rotenone administration did not significantly affect the body weight, and the circulation levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), BUN, and Cr (Fig. 7A–F). These data suggested rotenone at a dose of 200 ppm in food did not cause the obvious organ injury under the normal condition.

Discussion

In a number of experimental studies, rotenone is used to induce the Parkinson's disease in animals [25–27]. Studies in cancer research indicated that rotenone could be effective in treating cancers including liver and breast cancer [28, 29]. Recent studies from our group reported that rotenone treatment protected the kidneys from obstructive injury and ischemia-reperfusion-induced CKD, possibly via inhibiting the activity of the damaged mitochondria [14, 15]. In ischemia-reperfusion-induced CKD, we applied the rotenone to the animals after acute injury, which significantly attenuated the progression of AKI to CKD. In addition, another

Fig. 5. Rotenone aggravated renal cellular apoptosis induced by FA. **A** Representative images of renal TUNEL staining (green). **B** Quantification of TUNEL positive cell in **A**. **C** Western blotting analysis of BAX, GAPDH was used as loading control. **D** Densitometry of Western blots in **C**. Data were presented as means $\pm$ SE, $n = 6$ in each group. FA, folic acid; TUNEL, transferase dUTP nick end labeling.

Fig. 6. Rotenone promoted renal inflammation induced by FA. **A, B** The mRNA expressions of IL-6 and ICAM-1 in renal tissues were detected by qRT-PCR. **C** Enzyme-linked immunosorbent assay analysis of renal TNF- α protein level. Data were presented as means $\pm$ SE, $n = 6$ in each group. FA, folic acid; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α .

study found that rotenone could ameliorate aldosterone-induced renal injury [30]. However, the role of rotenone in AKI has not been studied. In the present study, our results showed that rotenone further exacerbated FA-induced renal injury possibly by aggravating mitochondrial dysfunction, oxidative stress, apoptosis, and inflammation in the kidney.

Mitochondrial dysfunction has been recognized as a pathogenic feature in FA-induced kidney injury [31]. Our results displayed FA treatment significantly decreased mtDNA copy number and the expressions of mitochondrial gene mt-COX1 and mt-ND1, while rotenone pretreatment makes the situation worse, suggesting the deterioration of mitochondrial abnormality. Mitochondrial damage not only results in the reduction of ATP level but also leads to the excessive production of ROS, which directly causes cellular injury and subsequently promotes

Fig. 7. Evaluation of rotenone toxicity in mice. **A** Body weight after 4-day rotenone treatment. **B–F** Blood levels of aspartate aminotransferase (**B**), alanine aminotransferase (**C**), LDH (**D**), BUN (**E**), and serum creatinine (**F**). Data were presented as means $\pm$ SE, $n = 6$ in each group. Scr, serum creatinine; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

mitochondrial dysfunction [32, 33]. It is well accepted that oxidative stress is involved in the pathogenesis of FA-induced kidney injury. Previous study has shown that rotenone could decrease the activity of antioxidant enzymes in the body [34], leading to the accumulation of a large number of free radicals, which causes oxidative damage [34]. Several groups have reported that rotenone administration may result in massive production of ROS [35, 36]. Verma et al. [37] reported that the MDA levels were significantly enhanced after rotenone treatment in animals. In agreement with previous studies mentioned above, in the present study, rotenone administration further decreased SOD2 level in renal tissue accompanied with increased kidney MDA content and HO-1 expression, indicating that rotenone might enhance the oxidative stress response via decreasing the activity antioxidant enzyme SOD2 in mitochondria.

Oxidative stress and other detrimental factors adversely affect mitochondrial function and leading to the cell apoptosis. Previous findings have shown that apoptosis acts as another important mechanism in FA-induced AKI [38]. As shown by the results, significant apoptosis was observed in kidneys after treatment with FA as illustrated by TUNEL staining and BAX measurement, while rotenone pretreatment aggravated FA-induced apoptotic response. Rotenone-induced oxidative stress has been shown to cause cell apoptosis [39, 40]. In turn, oxidative stress could lead to more severe mitochondrial damage and cell apoptosis [34, 41].

Inflammation plays an important role in the occurrence and development of AKI [24]. In the present study, FA treatment significantly upregulated the expressions of IL-6, TNF- α , and ICAM-1. While, rotenone pretreatment further upregulated the IL-6 expression. IL-6 is produced mainly by activated macrophages [42] and exerts its effects by recruiting neutrophils to the peritubular region [43]. Also, it has been demonstrated that oxidative stress could trigger the inflammatory response in many pathological processes [44]. Thus, this enhanced inflammatory response could be triggered by the mitochondrial oxidative stress caused by mitochondrial complex I inhibitor rotenone.

Conclusion

In summary, this study examined the role of mitochondrial complex I inhibitor rotenone in FA-induced AKI for the first time. The findings demonstrated that the inhibition of mitochondrial activity could be pathogenic in FA-induced AKI, which offered direct evidence showing the role of mitochondrial dysfunction in promoting AKI.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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Data Availability Statement

All the data used to support the findings of this study are included within the article.

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