Oxidative stress mediated mitochondrial damage plays roles in pathogenesis of diabetic nephropathy rat

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Abstract. – OBJECTIVE: Diabetic nephropathy (DN) is considered as a complication of diabetes and accounts for about 40%. Reactive oxygen species (ROS) level continues to increase in DN. However, whether specific ROS levels can alleviate renal damage by improving mitochondrial function has not been investigated.

MATERIALS AND METHODS: DN model was established by intraperitoneal injection of streptozotocin (STZ). The rats were divided into normal group, STZ model group, and N-acetylcysteine (NAC) group. Fasting blood glucose was tested to assess the modeling. The renal injury was evaluated by using hematoxylin-eosin (HE) staining and periodate-Schiff staining. Serum creatinine and 24 h urinary protein levels were determined by renal function detection kit. The levels of ROS and malondialdehyde (MDA) were assessed by the kit to evaluate the effects of oxidative stress and NAC in rats. The mitochondrial damage marker Cyto C level was detected by Western blot.

RESULTS: Blood glucose, serum creatinine, and urinary protein levels were significantly increased in model group compared with the normal group (\(p<0.05\)). Blood glucose levels, serum creatinine, and urinary protein levels were markedly improved after the ROS and MDA levels reduced by NAC. Meanwhile, glomerular hypertrophy, mesangial matrix accumulation, and severe renal injury were observed in the model group. NAC administration markedly improved glomerular morphology and mesangial matrix aggregation. Cyto C expression in the model group increased significantly compared with normal control group (\(p<0.05\)), while NAC effectively inhibited Cyto C levels.

CONCLUSIONS: Oxidative stress-mediated mitochondrial damage is an important part of the pathogenesis of DN. Inhibition of ROS production can be a potential target for DN treatment.

Key Words: Oxidative stress, Mitochondrial damage, Diabetic nephropathy.

Introduction

Diabetic nephropathy (DN) is the most common microvascular complications of diabetes that affects 20%-40% of type 2 diabetes mellitus (T2DM) patients and may lead to end-stage renal disease. It seriously affects the morbidity and mortality of diabetes. The molecular pathological mechanisms leading to DN involve hyperglycemic-induced metabolism, hemodynamic and complex interactions between inflammation. These factors alter the function and morphology of the inner walls of blood vessels and interact with neighboring cells, leading to mitochondrial dysfunction and renal endothelial dysfunction, thus playing a crucial role in the development of DN.

Oxidative stress refers to the overproduction of high activity molecules such as reactive oxygen species (ROS) in the body by a variety of harmful stimuli, leading to the oxidation degree excesses oxide removal and the imbalance between oxidation and antioxidant system. The main characteristic of cellular oxidative stress is the continuous up-regulation of ROS levels. In recent years, it was found that oxidative stress is an important factor to promote the development of DN.

Mitochondria are the main place of cellular oxidative breath. In addition to supplying energy, participating in energy metabolism, and maintaining normal life activities of cells, mitochondria are also involved in processes such as cell differentiation, cell information transmission and apoptosis, and have the functions of regulating cell growth and cell cycle. Therefore, oxidative stress level is closely related to mitochondrial function. Cytochrome (Cyto) C locates in the space between outer membrane and endometrium of mitochondria. It is an important component of mitochondrial oxidative respiratory chain and plays a crucial role in apoptosis. Apoptosis inducer can trigger Cyto C release into the cytoplasm from...
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the mitochondria and bind to Apaf-1. The Cyto C/Apaf-1 complex activates caspase-9, which in turn activates caspase-3 and other downstream caspasers. The release of Cyto C occurs before the activation of caspasers and DNA breaks, thus is a hallmark of apoptosis. Therefore, we evaluated mitochondrial damage by detecting cytochrome C expression in this study.

Glutathione (GSH) is an important anti-oxidant in the body. N-acetylcysteine (NAC), a precursor of GSH, can directly and specifically inactivate ROS in vivo to inhibit oxidative stress. Up to now, there is still lack of report about whether specific inhibition of ROS levels can mitigate renal damage by improving mitochondrial function.

This study established a rat DN model and adopted ROS inhibitor NAC to explore the role of oxidative stress and its induced mitochondrial damage in DN.

Materials and Methods

Main Materials and Reagents

β-actin antibody was purchased from Kangcheng Biological Company (Shanghai, China). Col IV and fibronectin (FN) antibodies were purchased from Abcam Biotechnology (Cambridge, MA, USA). Rabbit Anti-Mouse IgG (H + L) and Rabbit Anti-Mouse IgG (H + L) were purchased from Proteintech Co., Ltd. (Wuhan, China). Rat reactive oxygen species (ROS) enzyme-linked immunosorbent assay (ELISA) kit and MDA detection kit were purchased from AMAX (Wuhan, China). HE staining kit and periodate-Schiff kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Main Instruments

Gel imaging system UVP Multispectral Imaging System (Sacramento, CA, USA). PS-9 semi-dry transfer electrophoresis instrument was purchased from Jinnix Ltd. (Dalian, China). Thermo-354 microplate reader was purchased from Thermo Fisher Scientific Company (New York, NY, USA).

Experimental Animals

Female Wistar rats aged 6-8 weeks old and weighed 180-220 g were purchased from Zhejiang University Experimental Animal Center (Zhejiang, China). The rats were raised in a clean animal room with temperature at 24°C and the relative humidity at 60%. The day/night cycle was 12 h. The rats were free to eat and drink, and the bedding was changed daily to avoid infection.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Yiwu Central Hospital (Zhejiang, China).

Modeling and Administration

The rats were divided into normal group, model group, and NAC group. After adaptive breeding, the rat was intraperitoneally injected with STZ (45 mg/kg) dissolved in freshly prepared citrate buffer. In the NAC group, NAC (5 mg/kg) was injected through the tail vein at 2 h before STZ injection to inhibit the increase of ROS. Two weeks later, the rats were sacrificed and the model was confirmed by HE staining and blood glucose level detection.

Hematoxylin-Eosin (HE) Staining

After the rats were sacrificed, the kidneys were fixed with 4% paraformaldehyde for 24 h and then dehydrated to in 30% sucrose concentration at 4°C. After optimal cutting temperature compound (OCT) embedding, they were frozen in a microtome for continuous freezing. The frozen sections were dried and subjected to conventional HE staining. After gradient ethanol dehydration, the sections were hyalinized in xylene. At last, the sections were observed under an optical microscope and photographed.

Periodate-Schiff Staining

After conventional frozen, the section was treated with 0.5% periodic acid oxidation for 10 min and washed by distilled water. Then, the section was incubated in Schiff reagent at 37°C for 30 min and redyed in Mayer hematoxylin for 1 min. After gradient ethanol dehydration, the section was sealed by neutral balsam.

Fasting Blood Glucose and Serum Creatinine Detection

The rat was fasted for one day at 36 h after modeling. The blood sample was taken from the tail vein and blood glucose was measured by glucometer. The whole blood was centrifuged to obtain serum. Serum creatinine was detected on automatic biochemical analyzer by basic picric acid colormetric method according to the manual.
24 h Urine Protein Detection

Fung’s metabolic cage was used to collect 24 h urine on the day before scarification. The 24 h urine protein was measured on biochemical automatic biochemical analyzer.

Total Protein Extraction

A total of 100 mg rat ovary samples was stored at -80°C. The sample was lysed by 1 ml radioimmunoprecipitation assay (RIPA) potent lysate (Beyotime Biotechnology, Shanghai, China) for 15 min and centrifuged at 12000 × g and 4°C for 10 min. The supernatant was tested by bicinechonic acid (BCA) method to determine protein concentration. At last, the sample was boiled for 5 min to denature the protein.

Western Blot

Total protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane at 300 mA for 1 h. Next, the membrane was incubated in primary antibody (ColIV, FN, cyto C, 1:1000) at 4°C overnight. After washed by Tween/Tris-buffered salt solution (TTBS) solution, the membrane was incubated in secondary antibody (1:1000) at 37°C and tested by enhanced chemiluminescence (ECL).

ROS and MDA Levels Detection

ROS level was determined by ELISA according to the manual. Specially, the serum was added to the 96-well plate and incubated in HRP conjugated secondary antibody at 37°C for 1 h. After washed for 5 times, the plate was reacted with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate avoid of light for 20 min. At last, the plate was read on microplate reader at 490 nm to obtain the absorbance value.

MDA was detected by the kit upon the principle of its reaction with thiobarbituric acid to produce red color. The sample was measured at 530 nm to calculate the MDA concentration.

Statistical Analysis

All data were analyzed on SPSS 18.0 software (SPSS Inc., Released 2009. PASW Statistics for Windows, Chicago, IL, USA). The Student’s t-test was used to compare the differences between two groups. Tukey’s post-hoc test was used to validate the ANOVA for comparing measurement data between groups. p < 0.05 was considered as statistical significance.

Results

HE Staining

HE staining is the basic staining method to observe cell morphology changes [11]. Compared with the normal group, glomerular volume significantly increased in diabetic rat. NAC treatment led the renal enlargement unobvious. As shown in Figure 1, glomerular hypertrophy was evident in the diabetic model group, and NAC treatment effectively prevented such histological change.

Periodate-Schiff Staining

Periodate-Schiff staining is a method for detecting polysaccharides, glycolipids, and mucins in glycogen and tissues [11]. Kidney tissue showed blue cell nucleus and bright purple cytoplasm after periodic acid-Schiff staining. In the diabetic model group, mesangial matrix aggregation was more evident, whereas NAC treatment markedly reduced the phenomenon (Figure 2).

Fasting Blood Glucose, Serum Creatinine, and 24 h Urine Protein Content Changes

Fasting blood glucose is an important indicator to assess the severity of diabetes [12]. As shown in Figure 3A, the fasting blood glucose
level in the diabetic group was significantly increased compared with the normal control group ($p<0.05$). NAC intervention declined the blood glucose level.

24 h urine protein is the earliest and most common use of clinical indicator of DN, which is an independent factor of the cardiovascular risk in T2DM patients13,14. The 24 h urinary protein results were shown in Figure 3B. Compared with the normal control group, the 24 h urinary protein level was significantly elevated in the diabetic model group ($p<0.05$). NAC treatment apparently decreased the level of 24 h urine protein ($p<0.05$).

Creatinine is a small molecule substance that can pass through the glomerular filtration and is rarely absorbed in the renal tubules. The creatinine produced daily is almost always excreted from the urine and generally not affected by urine volume. Clinical serum creatinine is one of the main ways to evaluate renal function. Serum creatinine elevation refers to impaired renal function15. It was found that serum creatinine level was markedly up-regulated ($p<0.05$) in diabetic model group and significantly decreased after NAC treatment ($p<0.05$) (Figure 3C).

**ColIV and Fibronectin Protein Expressions in Renal Tissue**

ColIV and FN in renal tissues were also used to detect the degree of renal injury. As shown in Figure 4, and the expressions of ColIV and FN in diabetic model group were significantly increased. NAC treatment markedly inhibited the expressions of ColIV and FN.

**ROS and MDA Levels in Renal Tissue**

Oxidative stress is a key factor that leads to mitochondrial damage. Oxidative stress presents as a significant increase of ROS and MDA levels. ROS and MDA levels were significantly increased in the diabetic model group ($p<0.05$), while NAC markedly reduced ROS and MDA levels ($p<0.05$) (Figure 5).

**Cytochrome C Expression in Renal Tissue**

Cyto C is a crucial electron mediator during biooxidation. Cyto C is released from the mitochondria into the cytoplasm during mitochondria damage, which can induce apoptosis through Caspase and other pathways. Cytoplasmic cyto C expression was markedly enhanced in the diabetic model renal tissue compared with the normal

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**Figure 2.** Periodate-Schiff staining of diabetic rat kidney.

**Figure 3.** Fasting blood glucose, serum creatinine, and 24 h urine protein content changes. *$p<0.05$, compared with NC; $^{#}p<0.05$, compared with DM.
control group. Cytoplasmic Cyto C levels were apparently decreased after NAC administration (Figure 6).

**Discussion**

DN is the most important complication of diabetes with upward trend in China. It has become the second cause of end-stage renal disease, only after glomerulonephritis. Because of its complex metabolic disorders, end-stage renal disease is often more difficult than the other kidney diseases in the treatment. Therefore, timely prevention and treatment is of great significance for the delay of diabetic nephropathy16. In this study, the role of oxidative stress-mediated mitochondrial damage in diabetic nephropathy was explored.

Oxidative stress refers to the degree of oxidation beyond removal when the body subjected to a variety of harmful stimuli, leading to oxidation and anti-oxidant system imbalance and tissue damage. In recent years, some theories connected high ROS production with various metabolic-related chronic diseases, such as atherosclerosis and diabetes17. Previous studies suggested that oxidative stress is an important cause of diabetic nephropathy, and some of the alleviating effects of traditional Chinese medicine on diabetes are related to the inhibition of oxidative stress18. However, the role of oxidative stress-mediated mitochondrial damage in diabetic rats has not been reported.

ROS is an important product of oxidative stress19. MDA is one of the key products of membrane lipid peroxidation, and its production can exacerbate membrane damage. Therefore, the content of MDA is a common indicator in physiological and physiological research of plant senescence, and can indirectly assess the degree of membrane damage20. In the present work, we found ROS and MDA elevation in the kidneys of diabetic rats, confirming the existence of hyperoxidative stress in the model. NAC, a precursor of GSH, is a thiol-containing antioxidant that exerts tissue protection in vivo through converting to GSH21. In early researches, NAC inhibited the expression of adhesion molecules on neutrophils and vascular endothelial cells, thus alleviating lung injury22. It also entered leukocytes and converted to physiological antioxidants to increase intracellular levels of reduction, leading to inactivation of ROS in cells and media. In our work, ROS and MDA levels were significantly inhibited by the oxidative stress inhibitor NAC, indicating that NAC markedly suppressed oxidative stress.

It is well-known that the increase of ROS source and the decrease of antioxidant capacity are the important factors leading to the enhancement of oxi-
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It has been reported that an increasing production of ROS in DN patients is accompanied by diminished antioxidant capacity. The main sources of ROS production are oxidative respiratory chain and NADPH oxidase pathway, accompanied by the activation of multiple oxidative signaling pathways and looping between multiple signaling pathways. The oxidative signaling cascade results in oxidation damage continue to expand. Increased urinary protein level and decreased serum creatinine clearance are important features of diabetic renal injury. Diabetic nephrotic syndrome without treatment can gradually developed to chronic renal failure within four years. In the present investigation, the administration of NAC inhibited ROS level, urinary protein level, and serum creatinine level, and improved glomerular morphology and function upon HE staining and periodate-Schiff staining.

Cyto C is an important protein of mitochondrial damage induced by oxidative stress. Elevated cyto C levels in cytoplasm are crucial markers of mitochondrial damage. Maintaining mitochondrial integrity is necessary to maintain normal renal cell function. It was showed that mitochondria play a key role in acute kidney injury caused by surgery, chemotherapy, and shock. In this report, we used NAC to inhibit ROS levels, which significantly decreased the level of cyto C and improved mitochondrial function.

The kidneys are one of the organs that is sensitive to oxidative stress. In this study, the use of ROS inhibitor NAC confirmed that inhibition of oxidative stress in diabetes can reduce renal mitochondrial damage and thereby improve renal function. The inhibition of ROS production is an important target for the treatment of diabetic nephropathy.

Conclusions

Oxidative stress-mediated mitochondrial damage is a key part of the pathogenesis of DN. The suppression of oxidative stress alleviated mitochondrial damage and improved renal injury. The inhibition of the oxidative stress processes or suppression of the ROS production can be a potential target for the treatment of DN in clinical. Therefore, we recommend targeting oxidative stress pathway as an optimal anti-diabetic therapy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References


