European Review for Medical and Pharmacological Sciences 2019; 23: 4017-4026

Lixisenatide protects doxorubicin-induced renal fibrosis by activating wNF-κB/TNF-α and TGF-β/Smad pathways

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Abstract. – OBJECTIVE: The aim of this study was to investigate whether Lixisenatide, NF-κB/TNF-α, and TGF-β/Smad pathways exert clear regulatory roles in doxorubicin-induced renal fibrosis in rats, and to explore the possible underlying mechanism.

MATERIALS AND METHODS: 30 rats were randomly assigned into the sham group, the Doxorubicin (DOX) group and the Lixisenatide group, 10 in each group. Eight weeks after the specific animal procedure, serum and kidney samples of rats were collected. The serum levels of Cr and BUN were detected using relative commercial kits. The activities of malondialdehyde (MDA), total antioxidant capacity (T-AOC), catalase (CAT), reduced glutathione (GSH), and superoxide dismutase (SOD) in kidney homogenate were accessed using commercial kits. Meanwhile, pathological lesions in kidney tissues were evaluated by HE staining, immune-histochemical staining and TUNEL assay, respectively. Also, the protein levels of relative genes in NF-κB/TNF-α, and TGF-β/Smad pathways in rat kidneys were determined by immune-histochemical staining and Western blot, respectively.

RESULTS: Rats in the Lixisenatide group showed significantly lower levels of Cr and BUN. Activities of T-AOC, CAT, GSH, and SOD in the Lixisenatide group were significantly higher, whereas MDA activity was significantly lower than in the DOX group. Lixisenatide treatment remarkably alleviated DOX-induced pathological lesions and cell apoptosis in kidneys. Furthermore, the protein levels of relative genes in NF-κB/TNF-α and TGF-β/Smad pathways in rat kidneys were significantly downregulated in the Lixisenatide group when compared with the DOX group.

CONCLUSIONS: Lixisenatide protects doxorubicin-induced renal fibrosis in rats by inhibiting NF-κB/TNF-α and TGF-β/Smad pathways.

Key Words

Lixisenatide, Nrf2, NF-κB/TNF-α, TGF-β/Smad, Doxorubicin, Renal fibrosis.

Introduction

Recently, chronic kidney diseases (CKDs) have been concerned globally[1,2]. Due to the high expense of renal replacement therapy, CKD brings a heavy economic burden on affected patients and their families[2]. Therefore, it is of great significance to verify the mechanism of renal fibrosis progression, to prevent chronic renal failure and delay renal fibrosis progression as soon as possible[3]. Chronic renal failure is the final stage of the development of various CKDs. The most basic pathological change of chronic renal failure is renal fibrosis, including glomerular sclerosis and renal interstitial fibrosis. This may eventually lead to the loss of renal function[4]. Renal fibrosis is also an important process to determine chronic renal insufficiency. Meanwhile, the pathological changes can result in loss of normal nephron, proliferation of fibroblasts and myofibroblasts, as well as accumulation of excess extracellular matrix[5,6]. Previous studies[7,8] have also demonstrated that renal fibrosis has the characteristics of difficult treatment and progressive lesion, which is the key point in the treatment of chronic renal failure.

Doxorubicin (DOX) is the most widely used broad-spectrum and highly effective anti-tumor antibiotic. However, DOX causes severe kidney injury, which seriously limits its clinical application[9,10]. Reactive oxygen species (ROS) produced by excessive DOX metabolism in the body may be the main cause of oxidative damage in kidney[11,12]. Renal cells are highly energy-consuming, which require abundant ATP to maintain their vigorous metabolism and life activities[13]. Therefore, renal cells are very sensitive to oxidative stress and dysfunction caused by exogenous chemicals[13]. Furthermore, renal cells are major targets for DOX toxicity, whose damage is an important feature of DOX-induced nephrotoxicity[14].

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NF-κB/TNF-α exerts a multi-directional effect and unique regulatory mechanism that activates multiple pathways and genes[15]. The expression of NF-κB/TNF-α exists in almost all mammalian cells, which is activated by a number of stimulating conditions[16]. NF-κB/TNF-α pathway plays an important role in the normal physiological regulatory network of immune and inflammatory responses. Meanwhile, it also poses a crucial role in cancer and other diseases[17]. In addition, multiple growth factors have been confirmed to participate in the development and progression of renal fibrosis, including TGF-β1, TNF-α, PDGF, IGF-1, IL-4, ET-1, CTGF, and others[17]. Among them, TGF-β is a crucial inducing factor for ECM synthesis and accumulation. It has also been recognized as a basic fibrosis regulator. As is known to all, TGF-β1 is a fibroblast chemotactic compound, which serves as the strongest stimulator of fibroblast collagen production. Currently, it is considered the most critical and important factor in fibrosis[18].

Lixisenatide is a human glucagon-like peptide-1 (GLP-1) receptor agonist. It not only has a hypoglycemic effect of glucose concentration-dependent insulin release, but also exerts the effect of body mass reduction[19]. Pharmacological studies have shown that Lixisenatide can stimulate the proliferation and differentiation of islet beta cells, increase insulin synthesis, delay gastric emptying and reduce food intake[20]. Lixisenatide has a high selectivity for GLP-1 receptors, which is four times than that of human GLP-1 receptor agonists. Authors[21] have indicated that 20 μg/d Lixisenatide is the best therapeutic and tolerated dose. In recent years, the effects of Lixisenatide on the occurrence and development of organ damage caused by toxin and ischemia have been well investigated[21]. In this study, we explored the role of Lixisenatide in alleviating DOX-induced renal fibrosis and its underlying mechanism. We aimed to provide an experimental basis for clinical application of Lixisenatide in patients with renal fibrosis.

Materials and Methods

Chemicals and Reagents

Lixisenatide was purchased from Sinopharm Chemical Reagent (Shanghai, China); Doxorubicin injection was obtained from Qilu Pharmaceutical (Jinan, China); Relative commercial kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China), including malondialdehyde (MDA), total antioxidant capacity (T-AOC), catalase (CAT), reduced glutathione (GSH), superoxide dismutase (SOD), Cr (creatinine), and blood urine nitrogen (BUN) determination kit.

Animals and Experimental Procedures

30 male Sprague Dawley (SD) rats weighing 180-220 g (Vital River Laboratory Animal Technology, Beijing, China) were housed in a standard environment. Rats were randomly assigned into three groups, including the sham group, the DOX group and the Lixisenatide group, 10 rats in each group. Rats in the sham group received nephrectomy and intraperitoneal injection with normal saline. Meanwhile, rats in the DOX group underwent nephrectomy and received postoperative administration of DOX twice for constructing the renal fibrosis model. After successful construction of the model, rats were intraperitoneally injected with saline. The animal procedure in the Lixisenatide group was the same as that of the DOX group, without intraperitoneal injection of Lixisenatide after model construction. This study was approved by the Animal Ethics Committee of Nantong University Animal Center.

Renal fibrosis model was established by right kidney extirpation and repeated administration of DOX in rats. After one week of habituation, rats were anesthetized with 10% chloral hydrate (5 mg/kg) and placed in the prone position on the surgical table. Skin preparation (2.5-3.0 cm) in the right waist of the rat was performed. A 2.0-3.0 cm incision was cut to 0.5-1.0 cm on the right side of the spine. Subsequently, the right kidney was exposed and the perirenal capsule was bluntly dissociated using curved forceps. Renal artery and vein were clamped and fixed with hemostats to avoid the damage of adrenal tissue. The right renal artery and vein, as well as the ureter, were ligated with 3-0 suture, followed by removal of the right kidney. Finally, the incision was sutured layer by layer.

After surgery, 0.8 million unit/kg penicillin was intramuscularly injected into the hind limb of rats for three consecutive days to prevent infection. On the 7th day after surgery, the surgical incision suture was removed after the verification of healing. On the 7th day after the extirpation of the right kidney, rats were injected with 5 mg/kg DOX through the tail vein. Subsequently, 3 mg/kg of doxorubicin was repeatedly injected on the 30th day after surgery. At the 8th week postoperatively, renal tissues were harvested for pathological examination. Extensive segmental or even glo-

merular sclerosis, balloon adhesion, renal tubular epithelial vacuolar degeneration, renal interstitial fibrosis and inflammatory cell infiltration indicated the successful construction of the renal fibrosis model in rats.

Assessment of Renal Function

The body weight of each rat was daily recorded before drug administration, and the changes in body mass in each group was calculated at the end of the animal procedure. After sacrifice, rat kidney was immediately harvested. The envelope and fat tissue were peeled off, followed by weighing the kidney mass. The renal index was calculated as kidney mass/body mass × 100%. Subsequently, 2 mL blood sample was collected. After 30 min of blood coagulation, blood samples were centrifuged at 3500 g/min for 10 min. Then the supernatant was harvested and the levels of Cr and BUN were detected using relative commercial kits.

Histological Examination

Kidney tissues were cut into coronal section, fixed with 10% paraformaldehyde and stained with hematoxylin and eosin. Histological lesions in kidney tissues were assessed by semi-quantitative detection of renal tubule injury and necrosis (0 grade = no injury, 1 grade = ≤ 10%, 2 grades = 11-25%, 3 grades = 26-45%, 4 grades = 46-75% and 5 grades = >76%). 5 fields were randomly selected for each sample, and the pathological lesions were evaluated (magnification 200×).

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay

TUNEL assay was conducted to detect renal cell apoptosis according to the instructions of ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Millipore, Billerica, MA). 5-μm paraffin section was counterstained with hematoxylin, and TUNEL-positive cells were counted in 10 randomly selected fields (magnification 200×). 5 slices of each kidney sample were chosen for cell counting.

Biochemical Measurements

Rat abdominal aorta was dissociated after abdomen exposure. Abdominal aorta catheterization was performed below the branches of the renal artery. The proximal segment of the renal artery was clamped, the left renal vein was cut and the kidney was lavaged with cold saline. Until the color of kidney turned from red to pale, the left kidney was harvested and preserved in liquid nitrogen for subsequent use. Kidney homogenate was prepared and the levels of MDA, T-AOC, CAT, GSH, and SOD were determined using relative commercial kits.

Immuno-Histochemical Staining

Kidney slices were deparaffinized, hydrated in ethyl alcohol and blocked with blocking solution for 30 min. After incubation with primary antibodies (TGF-β1, Smad2/3, and α-SMA) at 4°C overnight and secondary antibody at room temperature for 1 h, immunohistochemistry results were captured using Nikon Eclipse 80i microscope.

Western Blot

Total protein was extracted using the radio-immunoprecipitation assay (RIPA) protein lysate (Beyotime, Shanghai, China). The concentration of extracted protein was quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The protein sample was separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). Then the membranes were incubated with primary and secondary antibodies based on the standard protocols of Western blot. Chemiluminescence was used to expose the protein bands on the membrane.

Statistical Analysis

Statistical Product and Service Solutions 22.0 (SPSS) Software package (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were expressed as $\bar{x}\pm s$. The *t*-test was used to compare the difference between the two groups. Categorical data were analyzed using χ^2-test or Fisher's exact test. $p<0.05$ was considered statistically significant.

Results

Lixisenatide Improved Renal Function in DOX-Induced Renal Fibrosis

Compared with the sham group, body weight and renal index of rats in the DOX group were remarkably reduced after animal procedures ($p<0.05$). This indicated that DOX-induced renal damage and successful construction of the rat model. Higher body weight and renal index were observed in the Lixisenatide group ($p<0.05$, Figure 1A, 1B).

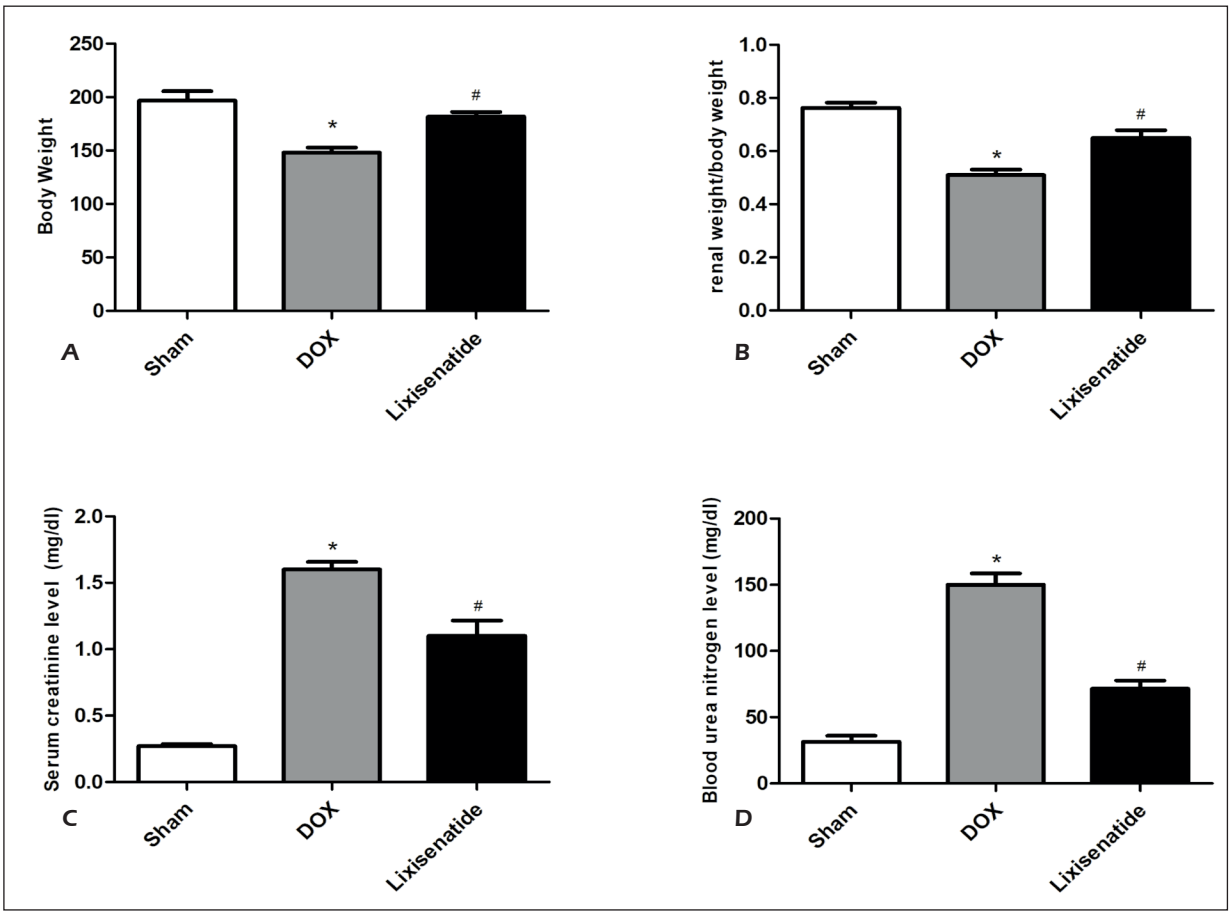

Figure 1. Lixisenatide alleviated renal function in DOX-induced renal fibrosis. **A**, Body weight of rats in the sham group (n=10), the DOX group (n=10) and the Lixisenatide group (n=10); **B**, The ratio of renal weight/body weight in the different treatment groups; **C**, Serum levels of creatinine in the different treatment groups; **D**, Blood levels of urea nitrogen in the different treatment groups. Data were presented as mean ± SD, *significant difference vs, sham group (p<0.05); #significant difference vs, DOX group (p<0.05).

Meanwhile, the quantitative analysis of renal function showed that the peripheral blood level of Cr in the DOX group and the Lixisenatide group was significantly higher than that of the sham group (p<0.05, Figure 1C). Also, the peripheral blood level of BUN in the DOX group and the Lixisenatide group was markedly higher than the sham group (p<0.05, Figure 1D). Although Lixisenatide treatment remarkably decreased BUN level, it still could not be reversed to baseline.

Lixisenatide Preserved Renal Histologic Architecture and Alleviated Neutrophil Infiltration

No significant pathological lesions were observed in kidneys harvested from the sham group. Pathological evaluation of kidney tissues in the DOX group showed significantly widening diffuse glomerular mesangial area, capillary lumen stenosis and focal segmental sclerosis of the glomerulus. Meanwhile, immune complex deposition was observed in the glomerular basement membrane, which was irregularly disordered. Most of the glomeruli were swollen and some glomeruli had a crescent formation. Mild to moderate hyperplasia of the mesangial matrix was found, and the number of sclerotic cells increased significantly. The renal sac was moderately dilated, with thickness of the cyst wall. Renal tubular epithelial cells showed granule degeneration, manifesting as cell disintegration, shedding and regeneration. Tubular lumen remarkably expanded with tubular shape in the lumen. Lymphatic-monocyte infiltration around the renal tubule was found, with immune complex deposition. Renal interstitium showed mild to moderate fibrosis and infiltration

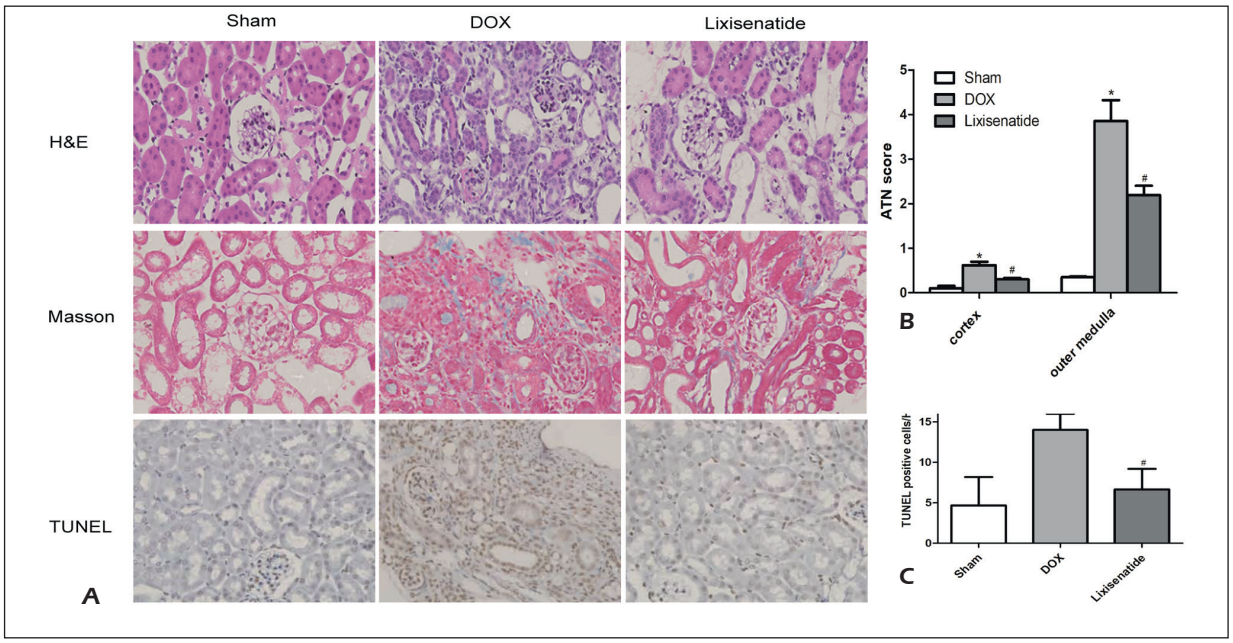

Figure 2. Lixisenatide prevented DOX-induced renal pathological lesions. Renal sections were stained with hematoxylin and eosin, and examined under a light microscope (magnification 200×). **A**, H&E staining, Masson staining and TUNEL assay of kidney tissues in the sham group, the DOX group, and the Lixisenatide group. **B**, Quantification of ATN score in cortex and outer medulla of the three groups. **C**, Quantification of TUNEL-positive cells by average number of 5 HPF in different groups. Data were expressed as mean ± SD.*significant difference vs, sham group (p<0.05); #significant difference *vs*, DOX group (p<0.05).

of inflammatory cells. The Lixisenatide group presented slighter pathological lesions than the DOX group (Figure 2A, 2B). The acute tubular necrosis (ATN) score in the DOX group was remarkably higher than the Lixisenatide group and the sham group (p<0.05, Figure 2B).

Lixisenatide Decreased the Apoptosis of Renal Tubular Cells after DOX-Induced Renal Fibrosis

As shown in Figure 2A and 2C, the number of TUNEL-positive cells in kidney tissues of the DOX group was remarkably higher than the sham group. The Lixisenatide group showed significantly less TUNEL-positive cells than that of the DOX group (p<0.05).

Lixisenatide Decreased ROS Production and Tissue Impairment by Enhancing Antioxidant Capacity

Previous studies have found that DOX can significantly impair the antioxidant capacity of kidney tissue and increase the production of ROS. In our study, Lixisenatide administration remarkably restored the antioxidant capacity in renal fibrotic tissues. Compared with the DOX group, the level of MDA was significantly decreased after Lixisenatide administration (Figure 3A). Additionally, we also found a higher level of ROS in kidney tissues of the DOX group than that of the Lixisenatide group (Figure 3B). Moreover, results also indicated that the activities of T-AOC, CAT, GSH and SOD were remarkably higher in the Lixisenatide group compared with those of the DOX group (Figure 3C-3F).

Lixisenatide Activated NF-κB/TNF-α Signal Pathway in DOX-Induced Renal Fibrosis

To investigate whether Lixisenatide protected DOX-induced renal damage through activating NF-κB/TNF-α pathway, serum samples of rats were collected. Subsequently, the levels of cytokines were detected. Results showed that the levels of TNF-α, IL-1β, and IL-6 in the Lixisenatide group were markedly lower than the DOX group (Figure 4A-4C).

Kidney tissues of rats in the Lixisenatide group and the DOX group were collected as well. Western blot results showed that the protein levels of NF-κB, TNF-α, VCAM-1, ICAM-1, and MCP-1 in the Lixisenatide group were significantly lower than the DOX group (Figure 4D).

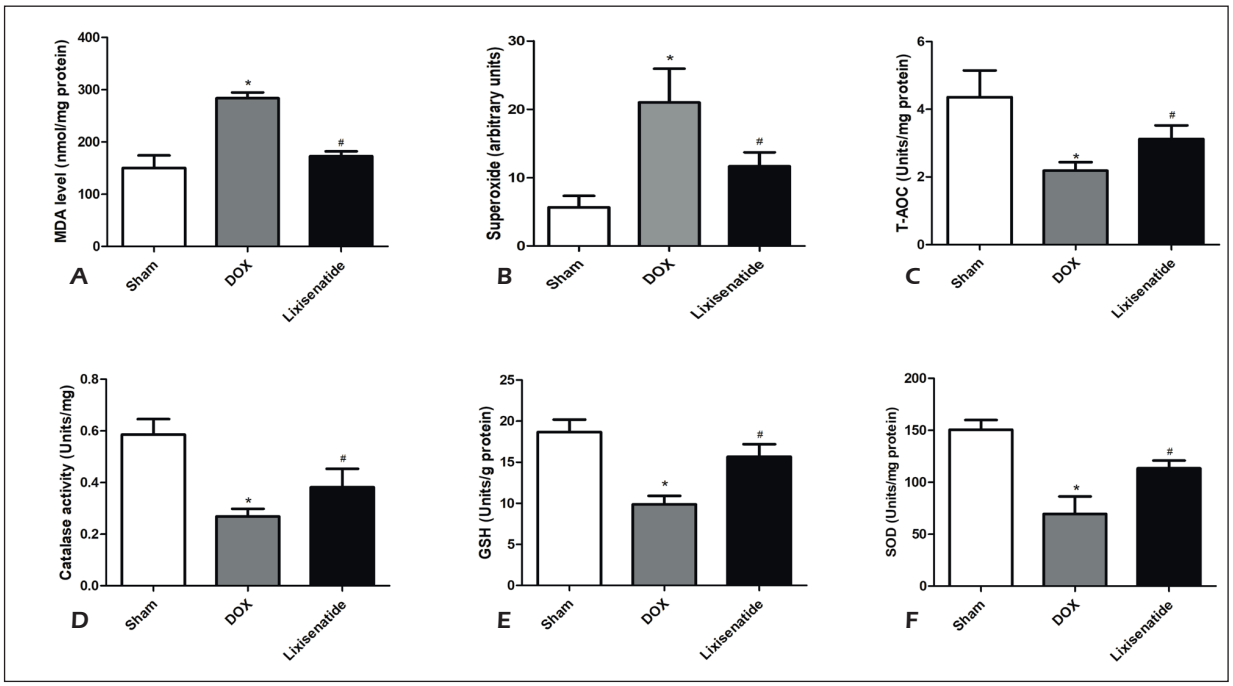

Figure 3. Lixisenatide attenuated oxidative stress. **A**, Content of malondialdehyde (MDA) in kidney tissues. **B**, Content of ROS was expressed as arbitrary units per millimetre squarefield. **C**, Content of total antioxidant capacity (T-AOC) in kidney tissues. **D**, Content of catalase (CAT) activity in kidney tissues. **E**, Content of reduced glutathione (GSH) in kidney tissues. F, Content of superoxide dismutase (SOD) in kidney tissues. Data were expressed as mean ± SD. *significant difference vs, sham group (p<0.05); #significant difference vs, DOX group (p<0.05).

Lixisenatide Activated TGF-β/Smad Pathway in DOX-Induced Renal Fibrosis

Furthermore, immune-histochemical staining showed that positive expressions of TGF-β1, Smad2/3 and α-SMA in kidney tissues of the DOX group were significantly higher than the sham group. By contrast, the Lixisenatide group presented lower levels of these genes (Figure 5A). Moreover, Western blot results also found that the protein expression levels of TGF-β1, Smad2/3, Smad4, collagen I, collagen IV, and α-SMA were remarkably downregulated in the Lixisenatide group when compared with the DOX group (Figure 5B).

Discussion

Many advances have been made in the study of chronic renal fibrosis, such as theories of intact nephron, trade-off hypothesis, glomerular hyperfiltration, dyslipidemia, uremic toxins and nutritional deficiencies. Renal fibrosis is a common pathway for various kidney diseases to develop into chronic renal failure[1,3,4]. Therefore, renal fibrosis and other organ fibrosis are hotspots in medical researches[5]. Organ fibrosis is a pathological process in which abnormal growth and excessive deposition of extracellular matrix can be found in tissues due to inflammation-induced necrosis of organ parenchymal cells[21]. Organ fibrosis and sclerosis are different stages of development in the same pathological process. Meanwhile, organ fibrosis is an important intermediate link and a necessary pathway for further development of organ sclerosis[21,22]. Scholars[6-8] have indicated that renal fibrosis includes tubulointerstitial fibrosis and glomerular sclerosis. Meanwhile, the two lesions are closely related. It is generally believed that renal fibrosis is an inevitable step in the progression of chronic renal failure.

Injury stimuli first alter the local immune microenvironment, leading to renal intrinsic cell activation, pro-inflammatory cytokine production and release, mononuclear-macrophages, recruitment of T lymphocytes to lesioned renal tissue. Subsequently, mesangial cells, myofibroblasts and tubular epithelial cells can be activated or transdifferentiated by relevant cytokines. A large amount of ECM is produced and deposited,

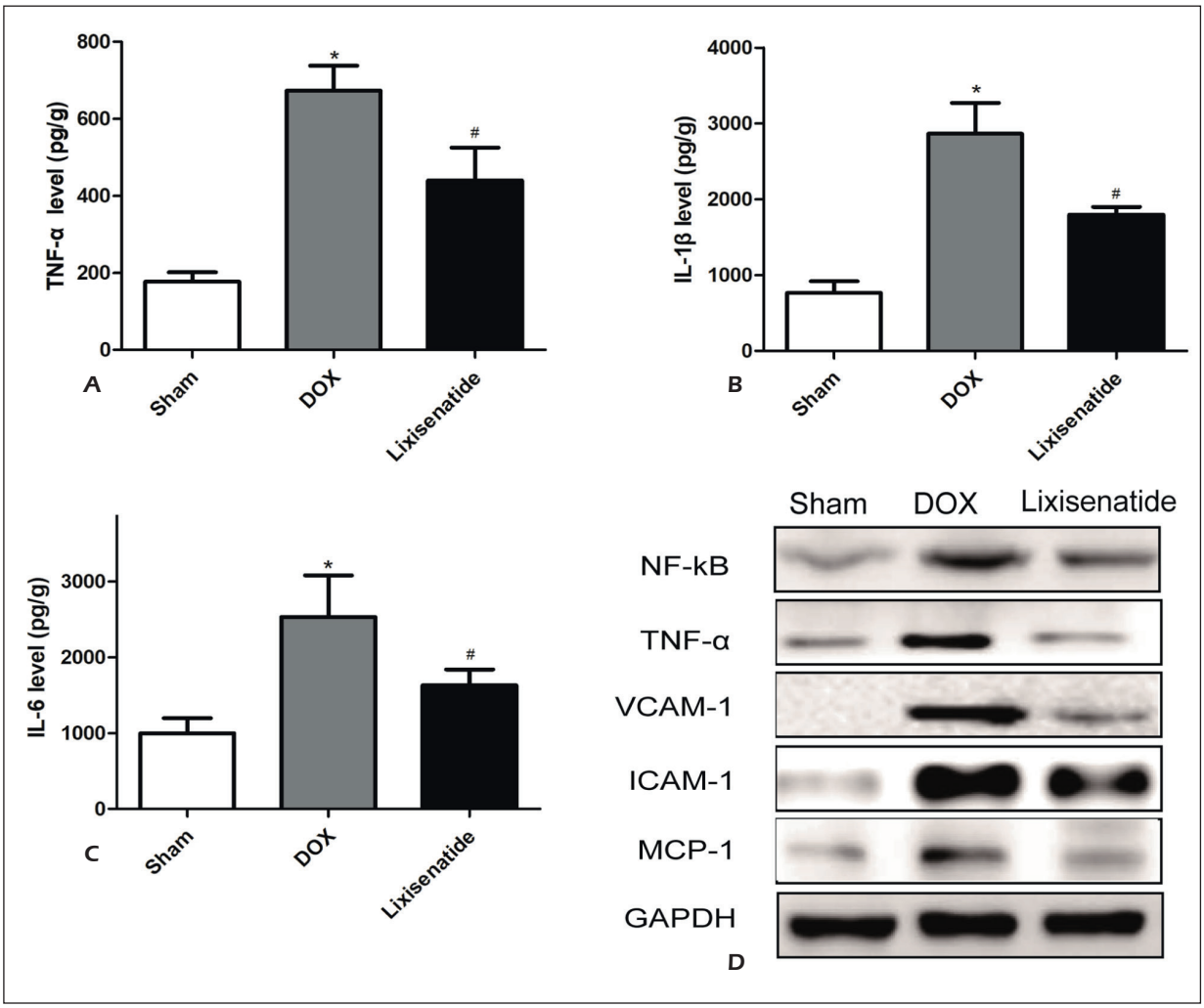

Figure 4. Lixisenatide decreased the levels of NF-κB relevant cytokines, as well as the expressions of NF-κB/TNF-α relevant proteins. **A**, Content of TNF-α in myocardial tissues. **B**, Content of IL-1β in myocardial tissues. **C**, Content of IL-6 in myocardial tissues. **D**, Protein expressions of NF-κB, TNF-α, VCAM-1, ICAM-1, and MCP-1 in different groups. GAPDH was used as an internal control to normalize the volume of protein expression. *significant difference vs. sham group (p<0.05); #significant difference vs. DOX group (p<0.05).

which further causes fibrous scar formation, microvascular network reduction, renal tissue remodeling, renal parenchymal damage and even the loss of renal function[3-5]. Inflammation is the basic pathological change of CKD and is also the triggering factor of renal fibrosis, mainly characterized by immune cell infiltration and inflammatory mediator secretion[21,22]. During renal fibrosis, the tubulointerstitial injury is caused by the activation of multiple cytokines and chemokines. It is observed that many chemokine receptors, such as CXCL13, CX3CR1, CCR1, CCR2, monocyte chemotactic factor 1 and osteopontin are upregulated in lesioned kidneys[23,24]. Current researches have demonstrated that the pharmacological effects of Lixisenatide on treating fibrotic lesions are closely related to the inhibition of the inflammation and collagen proliferation, as well as the enhancement of collagenase activity and collagenolysis[25].

Lipid metabolism often occurs in patients with CKD. Foam cells formed by macrophage phagocytic lipoproteins usually exist in areas of glomerular sclerosis and interstitial fibrosis[4,8]. Some researches have shown that macrophages, mesangial cells and tubular cells can produce reactive oxygen free radicals and oxidized lipoproteins. Oxidize low-density lipoproteins stimulate the expressions of inflammatory and fibrotic cytokines, and induce cell apoptosis. Meanwhile, they also produce reactive oxygen free radicals, ulti-

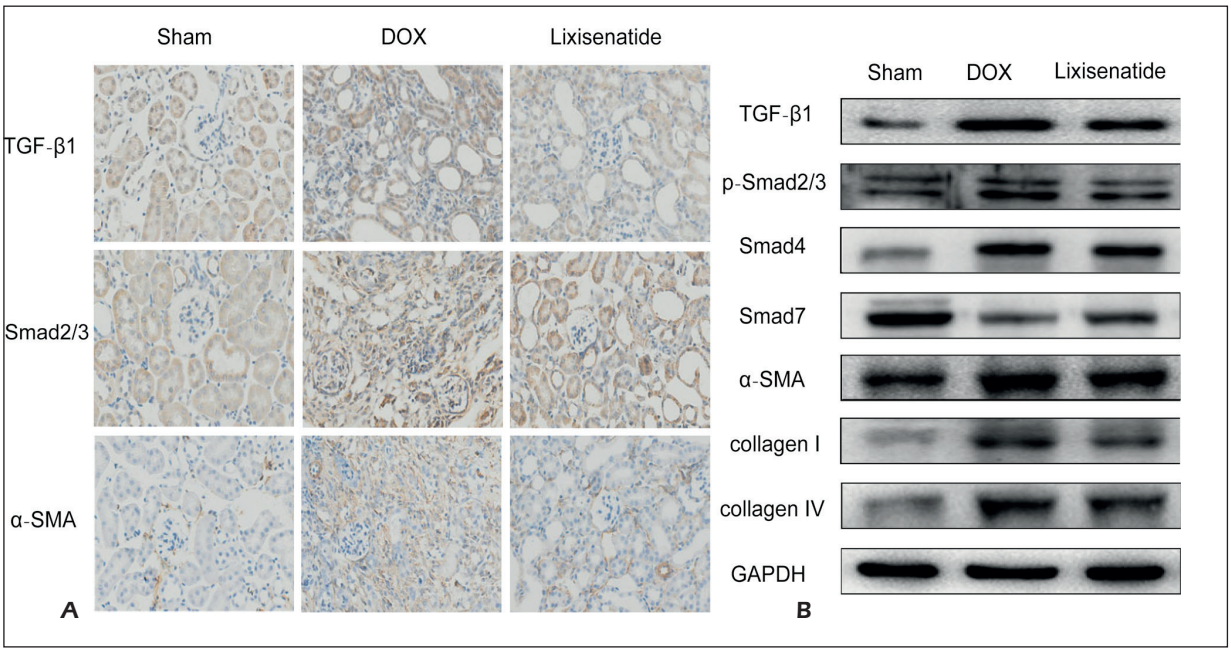

Figure 5. Lixisenatide supplementation enhanced the expression levels of TGF-β/Smad relevant proteins. **A**, Immuno-histochemical staining of TGF-β1, Smad2/3, and α-SMA. **B**, Protein expressions of TGF-β1, Smad2/3, Smad4, Smad7, collagen I, collagen IV, and α-SMA in different groups. GAPDH was used as an internal control to normalize the volume of protein expression. Data were expressed as mean ± SD. *significant difference vs. sham group (p<0.05); #significant difference vs. DOX group (p<0.05).

mately leading to massive invasion of macrophages, apoptosis and accumulation of extracellular matrix and tissue damage[5-7].

Nuclear factor-kappa B (NF-κB) is a fast-response transcription factor commonly found in the form of heterodimers composed of p50/p65[15,16]. Due to its binding to IκB, NF-κB exists in the cytoplasm in an inactive state[18]. NF-κB can be stimulated by multiple stimulators, which is subsequently dissociated from IκB, translocated into the nucleus in an active state, and bond to the κB site. The expression levels of downstream target genes are regulated by NF-κB[15,18]. It is known that the target genes of NF-κB are widely involved in pathological processes, such as hepatic inflammation, fibrosis and apoptosis[16]. A large number of studies have shown that the activity of NF-κB is significantly increased in patients with steatohepatitis, indicating its role in the injury of non-alcoholic steatohepatitis (NASH). Lipid peroxidation during NASH activates NF-κB. Subsequently, activated nuclear NF-κB promotes the production of other inflammatory factors, aggravates inflammatory response and in turn activates NF-κB, eventually forming a positive feedback regulation. The gradual expansion of inflammatory response remarkably damages hepatocytes[16,17].

Recent studies have shown that certain factors and cytokines (such as IL-1) in DOX-induced renal fibrosis model are involved in the process of tubule-interstitial injury, including TGF-β, CTGF, IL-1, MCP-1, and osteopontin. They also promote the accumulation of extracellular matrix[26]. Phenotypic transformation of mesangial cells, glomerular or tubular epithelial cells have been proved to be essential in renal tissue sclerosis or fibrosis. Meanwhile, it has been reported that under the induction of certain growth factors, renal interstitial fibroblasts can be transformed into myofibroblasts, renal tubular epithelial cells or glomerular epithelial cells (podocytes or Bowman's capsule epithelial cells). Previous works[27] have indicated that MCP-1 is capable of inducing trans-differentiation of renal tubular epithelial cells to myofibroblasts as well. The increased amount of renal interstitial fibroblasts is a crucial marker of interstitial fibrosis, serving as an evaluable marker of renal dysfunction and the prognosis of affected patients[27]. In addition, renal tubule-interstitial extracellular matrix, such as collagen I, II, IV, laminin, and fibronectin are significantly upregulated in DOX-induced renal fibrosis model.

In the present study, NF-κB expression in the DOX group was significantly higher than that of

the Lixisenatide group, indicating that ROS accumulation during the progression of renal fibrosis could activate NF-κB. Moreover, Nrf2 was highly expressed in the Lixisenatide group than that of the DOX group, suggesting that Lixisenatide activated Nrf2 and alleviated inflammatory response induced by renal fibrosis.

Conclusions

We found that lixisenatide protects pathological kidney lesions, oxidative stress and inflammatory response after doxorubicin-induced renal fibrosis in rats by inhibiting NF-κB/TNF-α and TGF-β/Smad pathways. Therefore, it might be a potential treatment target for renal fibrosis.

Conflict of Interests

The authors declare that they have no conflict of interest.

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