Liraglutide protects from renal damage via Akt-mTOR pathway in rats with diabetic kidney disease

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Abstract. – OBJECTIVE: To explore the protective effect of liraglutide on renal damage in rats with diabetic kidney disease (DKD) through the protein kinase B-mammalian target of rapamycin (Akt-mTOR) pathway.

MATERIALS AND METHODS: A total of 45 specific pathogen-free male Sprague-Dawley rats were divided into healthy group (no diabetes, n=15), diabetes group (diabetes, n=15), and liraglutide group (diabetes + liraglutide intervention, n=15). The differences in the biochemical indexes, lesion degree, glomerular Nephrin expression level, and mRNA and protein expressions of Akt-mTOR in renal tissues were detected in three groups via hematoxylin-eosin (HE) staining, immunohistochemistry, Western blotting, and quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), respectively.

RESULTS: The albumin-to-creatinine ratio (ACR) and levels of serum creatinine (Scr), urine microalbumin (UmAlb), fasting blood glucose (FBG), glycated hemoglobin (HbA1c), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and total cholesterol (TC) in renal tissues were the lowest in healthy group and the highest in diabetes group, while they significantly declined in liraglutide group compared with those in diabetes group. Also, there were statistically significant differences (p<0.05). The level of high-density lipoprotein cholesterol (HDL-C) in renal tissues was the highest in healthy group and the lowest in diabetes group, while it was significantly increased in liraglutide group compared with that in diabetes group (p<0.05). The protein expression of Akt-mTOR in renal tissues was the lowest in healthy group and the highest in diabetes group, while it markedly declined in liraglutide group compared with that in diabetes group, displaying statistically significant differences (p<0.05). Similarly, the mRNA expression of Akt-mTOR in renal tissues was the lowest in healthy group and the highest in diabetes group, while it markedly declined in liraglutide group compared with that in diabetes group, displaying also statistically significant differences (p<0.05).

CONCLUSIONS: Liraglutide can significantly reduce the blood glucose and improve the renal function in rats by suppressing the protein expression of AKT-mTOR, thereby exerting a protective effect on renal damage in rats with DKD.

Key Words: Diabetic kidney disease, Liraglutide, Akt-mTOR.

Introduction

Currently, the morbidity rate of diabetic kidney disease (DKD) is still rising, making DKD the...
major cause of end-stage renal disease\textsuperscript{1}, whose important manifestations are systemic microvascular complications. Several studies have demonstrated that DKD is one of the most important complications of diabetic patients. DKD mostly develops from type 2 diabetes. The symptoms of DKD patients are not significant in the early stage, but proteinuria and renal function impairment will gradually occur with the progression of the disease. Therefore, DKD will ultimately develop into end-stage renal disease\textsuperscript{2,3}. Once it develops into the end stage, DKD is often more difficult to be treated than other kidney diseases\textsuperscript{4}.

Therefore, the timely prevention and treatment of DKD are of great significance in the protection of renal tissues\textsuperscript{5}. Liraglutide is a glucagon-like peptide-1 (GLP-1) analog with 97\% sequence homology to human GLP-1\textsuperscript{6}. The activity of liraglutide is mediated by the specific interaction between it and the GLP-1 receptor, leading to an increase in cyclic adenosine monophosphate\textsuperscript{7}. Moreover, liraglutide can enter the brain to promote neuronal and synaptic regeneration, cell repair, and improve cognitive impairment in different animal models. GLP-1, an endogenous incretin hormone, can promote pancreatic β-cells to secrete insulin in a glucose concentration-dependent manner\textsuperscript{8,9}. Reports\textsuperscript{10} have shown that liraglutide exerts a neuroprotective effect in degenerative diseases in many laboratory animals. Protein kinase B-mammalian target of rapamycin (Akt-mTOR) is a serine/threonine protein kinase, which, according to researches, can promote cellular anabolism and regulate cell growth and proliferation. Numerous studies have confirmed the abnormal activation of the Akt-mTOR signaling pathway in diabetic patients, which is also closely related to DKD, and reducing the expression of Akt-mTOR can prevent the occurrence and development of DKD\textsuperscript{11}. In this experiment, therefore, the differences in the biochemical indexes, lesion degree, glomerular Nephrin expression level, mRNA, and protein expressions of Akt-mTOR in renal tissues were detected in healthy group, diabetes group, and liraglutide group to explore the protective effect of liraglutide on renal damage in rats with DKD through the Akt-mTOR pathway.

Materials and Methods

Instruments and Reagents

Liraglutide was purchased from Novo Nordisk A/S (Copenhagen, Denmark), reverse transcription kit from Fermentas (Thermo Fisher Scientific, Waltham, MA, USA), real-time fluorescence quantitative kit from TaKaRa (Otsu, Shiga, Japan), and real-time fluorescence quantitative polymerase chain reaction (qPCR) instrument from Bio-Rad (Hercules, CA, USA).

A total of 45 healthy adult male Sprague-Dawley rats weighing 180-220 g and aged 6-8 weeks old were provided by the Chengdu University of Traditional Chinese Medicine Animal Center. This research was approved by the Animal Ethics Committee of Chengdu University of Traditional Chinese Medicine.

Laboratory Animals

The above rats were fed in the specific pathogen-free animal room in separate cages under the room temperature of (22±2)\degree C, the humidity of 50-60\%, and 12/12 h light/dark cycle. Also, they had free access to food and water.

Routine Biochemical Detection

After drug treatment for 5 weeks, the rats were placed in metabolic cages, and the urine was collected and used for the detection of renal function. The detection indexes included urine microalbumin (UmAlb), albumin-to-creatinine ratio (ACR), and serum creatinine (Sr). Then, the blood was drawn from the abdominal aorta in each group and centrifuged at 1000 rpm using the high-speed refrigerated centrifuge. The serum separated was taken to detect the blood glucose and blood lipid, and the detection indexes included fasting blood glucose (FBG), glycated hemoglobin (HbA1c), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C).

Detection of Lesion Degree of Renal Tissues Via Hematoxylin-Eosin (HE) Staining

After drug treatment for 5 weeks, the rats were sacrificed via dislocation, the renal tissues were isolated, treated with 4\% paraformaldehyde/phosphate-buffered-saline (PBS; pH 7.4) at 4\degree C for 48 h, washed with running water, dehydrated with 70\%, 80\%, and 95\% ethanol, and treated with 100\% ethanol. Then, reagents were removed with xylene and tissues were embedded in paraffin and sliced into 4 μm-thick sections. After deparaffinization, the sections were washed with water and air-dried, and the nucleus was stained with reagent A (HE staining kit, Shanghai Bogoo Biological Technology Co., Ltd.,
Liraglutide in diabetic kidney

Shanghai, China) for 10 min and microscopically examined, showing the blue color. Next, the sections were washed with water, air-dried, and treated with reagent B at room temperature for 15 min. After the reagent B was washed away, the sections were transparentized with 95% alcohol, anhydrous alcohol, and xylene, and sealed with neutral balsam. After staining, 6 sections in each group was observed in the field of renal cortex randomly selected under a microscope (200×), and semi-quantitatively scored.

Detection of Glomerular Nephrin Protein Expression Using Immunohistochemical Method

The paraffin sections of renal tissues in three groups were deparaffinized and washed with water, followed by antigen retrieval using the citrate buffer (pH 6.0) kit. Subsequently, the sections were washed with PBS (pH 7.4) for 3 times, incubated with the 3% hydrogen peroxide solution (H₂O₂: pure water = 1:9) in a dark place at room temperature for 25 min, and incubated again with the primary antibodies of Nephrin (1:100) and Z0-1 (1:100) in a wet box containing a small amount of water at 4°C overnight. After the sections were washed with PBS for 3 times (5 min/time), they were incubated with the secondary antibodies at room temperature for 2 h, followed by color development using diaminobenzidine (DAB) for a certain time and controlled under the microscope (Solarbio, Beijing, China). Then, the sections were washed to terminate the color development, counterstained with Harris hematoxylin for 3 min, dehydrated, and sealed with neutral balsam. Finally, the glomerular Nephrin expression was analyzed using the image analysis system (Image-Pro Plus 6.0, Silver Springs, MD, USA) and observed in the field randomly selected under the microscope (×200).

Detection of Protein Expression of Akt-mTOR in Renal Tissues Via Western Blotting

The cells were added with an appropriate amount of radioimmunoprecipitation assay (RIPA) lysis buffer and the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; RIPA: PMSF = 100:1; Beyotime, Shanghai, China). The renal tissues of rats were cut into pieces, added with lysis buffer (10:1), homogenized using the homogenizer, and transferred into an Eppendorf (EP) tube. Next, by centrifugation at 14000 rpm and 4°C for 30 min using the high-speed refrigerated centrifuge. Then, the protein supernatant was collected and subjected to a heating bath at 95°C for 10 min for protein denaturation. The protein sample prepared was placed in the refrigerator at −80°C for later use, while the protein was quantified using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). After that, the sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) gel was prepared, and the protein sample was loaded into the gel well for electrophoresis under the constant pressure of 80 V for 2.5 h. After, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) using the semi-dry transfer method. The PVDF membranes were immersed in Tris-Buffered Saline and Tween-20 (TBST) containing 5% skim milk powder and shaken slowly for 1 h on a shaking table to be sealed. Then, the protein was incubated with the primary antibody diluted with 5% skim milk powder, rinsed with TBST for 3 times (10 min/time), incubated again with the secondary antibody at room temperature for 2 h, and rinsed again with TBST twice and with TBS once (10 min/time). Finally, the protein was detected using the enhanced chemiluminescence (ECL) reagent, followed by exposure in a dark room. The relative expression of the protein was analyzed using Image-Pro Plus v6 (Media Cybernetics, Silver Spring, MD, USA), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference.

Detection of mRNA Expression of Akt-mTOR in Renal Tissues Via Real Time-qRT-PCR

The expressions of Akt and mTOR in renal tissues in the three groups were detected via RT and qPCR. First, the tissue samples were taken from the cryopreserved tube, drained off, ground in liquid nitrogen in the 5 mL tube, and thoroughly homogenized using the tissue homogenizer. Then, the liquid was transferred into the clean 1.5 mL EP tube, placed at room temperature for 5-10 min, fully lysed, and centrifuged at 1200 rpm for 5 min. After the precipitate was discarded, chloroform was added (200 μL/mL), shaken evenly, and placed at room temperature for 15 min, followed by centrifugation at 12000 rpm and 4°C for 15 min. The supernatant was aspirated into another centrifuge tube, added with isopropanol (0.7-1-fold volume of the supernatant), and placed at room temperature for 10-30 min, followed by centrifugation at 12000 rpm.
for 10 min. Once the supernatant was discarded, 75% ethanol (1 mL/mL) added into the centrifuge tube, gently shaken to suspend the RNA precipitate, followed by centrifugation at 12000 rpm and 4°C for 5 min. After the supernatant was discarded as far as possible, the precipitate was blown dry on a super clean bench for 10-20 min and dissolved with 10-50 μL of diethylpyrocarbonate (DEPC)-treated ddH₂O solution (Beyotime, Shanghai, China). Its concentration was determined using the NanoDrop One microspectrophotometer. Then, reverse transcription was performed (4.5 μL of RNase-free dH₂O, 2 μL of 5×RT reaction buffer, 0.5 μL of random primer, 0.5 μL of Oligo dT, and 2 μL of RNA). The cDNA samples were divided into three portions (diluted at 1:20), and 3 μL of complementary deoxyribose nucleic acid (cDNA) was taken for PCR amplification. The amplification level of target genes was verified using 5% agarose gel electrophoresis. The image acquisition and analysis software LabWorks 4.0 was adopted for quantitative and data processing. To obtain reliable data, the detection was repeated for 3 times in each group. The changes in relative expression levels of target genes were analyzed using the 2⁻ΔΔCt method, and the primer sequences are shown in Table I.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>Akt</td>
<td>F</td>
<td>5'-GAACGACGTAGCCATTGTGAAG-3'</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-TGAGAAGTTGTTAGTGGGGACT-3'</td>
</tr>
<tr>
<td>mTOR</td>
<td>F</td>
<td>5'-TGACAGCCATCATCAAAGAGA-3'</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-CAGGAAATCCCATAGCAATA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F</td>
<td>5'-CAGAGCCCCCTTGTGGGATC-3'</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-GGCCCTCGTCGCCACATAGG-3'</td>
</tr>
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</table>

Statistical Analysis
Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. The Chi-square test and t-test were performed for the differences in the biochemical indexes, lesion degree, glomerular Nephrin expression level, and mRNA and protein expressions of Akt-mTOR in renal tissues in healthy group, diabetes group, and liraglutide group. p<0.05 suggested significant differences.

Results
Differences in Biochemical Indexes in Renal Tissues
The levels of ACR, Scr, UmAlb, FBG, HbA1c, TG, LDL-C, and TC in renal tissues were the lowest in healthy group and the highest in diabetes group, while they significantly declined in liraglutide group compared with those in diabetes group. Also, there were statistically significant differences (p<0.05). The level of HDL-C in renal tissues was the highest in healthy group and the lowest in diabetes group, while it was significantly increased in liraglutide group compared with that in diabetes group. There were also statistically significant differences (p<0.05) (Tables II, III, and IV).

Lesion Degree of Renal Tissues Detected Via HE Staining
According to the results of HE staining, compared with diabetes group, the mesangial cell proliferation and vacuolar degeneration were alleviated, and the tubular dilatation or atrophy, fibrous

Table II. Comparison of renal function indexes in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>ACR [mg/g]</th>
<th>Scr [μmol/L]</th>
<th>UmAlb [mg/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy group</td>
<td>102.4 ± 23.1</td>
<td>117.5 ± 20.5</td>
<td>47.4 ± 25.9</td>
</tr>
<tr>
<td>Diabetes group</td>
<td>134.5 ± 23.5*</td>
<td>153.4 ± 24.2*</td>
<td>67.4 ± 21.4*</td>
</tr>
<tr>
<td>Liraglutide group</td>
<td>119.1 ± 25.6**</td>
<td>127.7 ± 21.4**</td>
<td>53.8 ± 23.5**</td>
</tr>
</tbody>
</table>

*p < 0.05 vs. healthy group, *p < 0.05 vs. diabetes group.
tissues, and inflammatory cells were reduced in liraglutide group. In healthy group, the mesangial structure and renal tubules were normal, the tubular basement membrane was smooth and intact, and there were no interstitial widening and inflammatory cell infiltration (Figure 1).

Glomerular Nephrin Protein Expression Detected Via Immunohistochemistry

It was observed in immunohistochemical staining that the glomerular Nephrin protein was arranged uniformly and showed the blue-black color in healthy group. The glomerular Nephrin protein expressed was significantly decreased and arranged disorderly in diabetes group compared with that in healthy group, while it was increased in liraglutide group compared with that in diabetes group ($p<0.05$) (Figure 2).

Akt-mTOR Protein Expression Detected Via Western Blotting

The results of Western blotting manifested that the protein expression of Akt-mTOR in renal tissues was the lowest in healthy group and the highest in diabetes group, while it significantly declined in liraglutide group compared with that in diabetes group, displaying statistically significant differences ($p<0.05$) (Figure 3).

Akt-mTOR mRNA Expression Detected Via qRT-PCR

Similarly, the mRNA expression of Akt-mTOR in renal tissues was the lowest in healthy group and the highest in diabetes group, while it markedly declined in liraglutide group compared with that in diabetes group, also displaying statistically significant differences ($p<0.05$) (Figure 4).

<table>
<thead>
<tr>
<th>Group</th>
<th>FBG (mmol/L)</th>
<th>HbA1c (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy group</td>
<td>6.9 ± 0.7</td>
<td>7.3 ± 0.8</td>
</tr>
<tr>
<td>Diabetes group</td>
<td>9.8 ± 0.9*</td>
<td>8.7 ± 0.7*</td>
</tr>
<tr>
<td>Liraglutide group</td>
<td>7.3 ± 1.1**</td>
<td>7.9 ± 0.5***</td>
</tr>
</tbody>
</table>

* $p<0.05$ vs. healthy group, *$p<0.05$ vs. diabetes group.

<table>
<thead>
<tr>
<th>Group</th>
<th>HDL-C (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>LDL-C (mmol/L)</th>
<th>TC (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy group</td>
<td>3.4 ± 0.2</td>
<td>2.1 ± 0.3</td>
<td>1.9 ± 0.3</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Diabetes group</td>
<td>1.7 ± 0.1*</td>
<td>3.8 ± 0.2*</td>
<td>3.6 ± 0.1*</td>
<td>4.0 ± 0.3*</td>
</tr>
<tr>
<td>Liraglutide group</td>
<td>2.6 ± 0.2**</td>
<td>2.5 ± 0.2*#</td>
<td>2.3 ± 0.2*#</td>
<td>2.9 ± 0.2**#</td>
</tr>
</tbody>
</table>

* $p<0.05$ vs. healthy group, *$p<0.05$ vs. diabetes group.

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Figure 1. Lesion degree of renal tissues detected via HE staining (200×).
Figure 2. Glomerular Nephrin protein expression detected via immunohistochemistry (200×).

Figure 3. Akt-mTOR protein expression in renal tissues in each group. A, Akt-mTOR protein expression in renal tissues. B, Relative Akt protein expression in renal tissues in each group. C, Relative mTOR protein expression in renal tissues in each group. *p<0.05 vs. healthy group, #p<0.05 vs. diabetes group.
Discussion

Currently, the cause of DKD has not been clarified, but about 30% of patients with type 2 diabetes will suffer from DKD according to epidemiological studies. The main manifestations of DKD decline in renal function, disorders of blood glucose, and blood lipid. Many researches have demonstrated that insulin resistance and hyperglycemia are the key influencing factors for DKD. Therefore, the strict control of blood glucose plays an important role in the prevention of DKD and improvement of renal function. The early treatment in the early stage of diabetes can delay the development of diabetes and reduce the damage to human body.

In the present study, the levels of ACR, Scr, UmAlb, FBG, HbAlc, TG, LDL-C, and TC in renal tissues were the lowest in healthy group and the highest in diabetes group, while they significantly declined in liraglutide group compared with those in diabetes group. Also, there were statistically significant differences. The level of HDL-C in renal tissues was the highest in healthy group and the lowest in diabetes group, while it significantly increased in liraglutide group compared with that in diabetes group. Besides, there were statistically significant differences. In healthy group, the mesangial structure and renal tubules were normal, the tubular basement membrane was smooth and intact, and there was no interstitial widening and inflammatory cell infiltration. Compared with diabetes group, the mesangial cell proliferation and vacuolar degeneration were alleviated, the tubular dilatation or atrophy, fibrous tissues, and inflammatory cells were reduced in liraglutide group. Moreover, the results of immunohistochemical staining revealed that the glomerular Nephrin protein was arranged uniformly and showed the blue-black grains in healthy group. The glomerular Nephrin protein expressed was significantly decreased and arranged disorderly in diabetes group compared with that in healthy group, while it was increased in liraglutide group compared with that in diabetes group. Above results indicate that liraglutide can improve renal function in rats by altering blood glucose and blood lipid in renal tissues. Consistent with the research results of Liou et al., obtained in the treatment of diabetes with GLP-1 agonist liraglutide in subjects receiving kidney transplant, liraglutide has a definite efficacy in the treatment of early DKD. This can effectively control the blood glucose level, increase the insulin sensitivity, significantly improve the renal function, and regulate the secretion of adipocytokines. The abnormalities of Akt-mTOR signaling pathway are also closely related to DKD.

The protein expression of Akt-mTOR in renal tissues was the lowest in healthy group and the highest in diabetes group, while it markedly declined in liraglutide group compared with that in diabetes group, displaying statistically significant differences. Similarly, the mRNA expression of Akt-mTOR in renal tissues was the lowest in healthy group and the highest in diabetes group, while it remarkably declined in
liraglutide group compared with that in diabetes group, also displaying statistically significant differences (p<0.05). The above results demonstrate that liraglutide can reduce the expression of AKT-mTOR in rats, thereby alleviating renal tissue lesion and renal damage in rats. Such findings are consistent with Li et al. In the work on the ability of lncRNA Rik to reduce cell proliferation and fibrosis in DKD through AKT-mTOR signal transduction they show that liraglutide effectively protects the kidney structure and suppresses cell proliferation and fibrosis in diabetes rats by inhibiting the activation of AKT-mTOR. In addition, liraglutide can significantly reduce the blood glucose level and improve the renal function in DKD rats by regulating the PI3K-AKT-mTOR pathway, thereby improving the condition of disease in rats.

Conclusions

We demonstrated that liraglutide could significantly reduce the blood glucose and improve the renal function in rats by suppressing the protein expression of AKT-mTOR, thereby exerting a protective effect on renal damage in rats with DKD.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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