

AMPK α , hs-CRP and Fc γ R in diabetic nephropathy and drug intervention

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Abstract. The aim of this study was to investigate the roles of AMP-activated protein kinase α subunit (AMPK α), hypersensitive C-reactive protein (hs-CRP) and Fc γ receptor (Fc γ R) in diabetic nephropathy and drug intervention effects. Sixty Sprague Dawley male rats were randomly divided into the control (n=30) and observation (n=30) groups. The model of type 2 diabetic nephropathy was established by high-fat and high-glucose diet and streptozotocin injection. The rats in the observation group were treated with baicalein and the rats in control group did not receive any drug intervention. The pathological changes of kidneys were observed by hematoxylin and eosin (H&E) staining. The expression of AMPK α mRNA in renal tissue was detected by reverse transcription-polymerase chain reaction (RT-PCR). The levels of hs-CRP and Fc γ R were measured by enzyme-linked immunosorbent assay (ELISA) at 1, 4, 6 and 8 weeks after drug intervention and blood urea nitrogen (BUN) and the 24 h urinary micro-albumin (U-ALB) levels were compared at 1, 4, 6 and 8 weeks after intervention. After 8 weeks of drug intervention, the pathological changes of kidneys in the observation group were significantly lower than those in the control group ($p<0.05$), while the relative expression levels of AMPK α mRNA and protein in the control group were higher than those in the observation group ($p<0.05$). The levels of hs-CRP, BUN and 24 h U-ALB in the control group were significantly higher than those in the observation group at different time-points after drug intervention and the level of Fc γ R in the control group was significantly lower than that in the observation group ($p<0.05$). Baicalein may protect renal function by inhibiting the expression of AMPK α and

inflammatory reaction, and can also decrease BUN and 24 h U-ALB levels and improve the pathological changes of the kidney.

Introduction

Diabetes mellitus (DM) is a type of metabolic disease caused by insulin secretion dysfunction and/or abnormal insulin action, characterized by chronic hyperglycemia, carbohydrate, fat and protein metabolic disorders (1). DM causes a series of physiological and pathological changes in the body and chronic lesions in lung, heart, brain, kidney, nerve and other organs and even leads to functional defects and failure (2).

Diabetic nephropathy (DN) is one of the potentially destructive complications of DM, its incidence is high and ~20% of patients with type 2 diabetes are likely to be eventually complicated by DN (3). DN is the leading cause of end-stage renal disease, accounting for ~50% of the total of end-stage renal disease (4). High renal perfusion and high filtration, thickening of glomerular basement membrane and extracellular matrix accumulation dominated by mesangial area leads to diffuse and nodular glomerulosclerosis, which is clinically manifested as increased blood pressure, proteinuria, renal insufficiency and other symptoms with a great risk of cardiovascular death (5). AMP-activated protein kinase (AMPK) is the main sensor and modulator of a cellular energy state. In metabolic stress, AMPK inhibits anabolism and promotes the catabolic processes to restore the energy homeostasis (6), of which α subunits (AMPK α) are catalytically active and play important roles in liver glucose and lipid metabolism (7). At present, studies have shown that hypersensitive C-reactive protein (hs-CRP), as a risk factor of DN, can predict the risk of DN in patients with type 2 diabetes to a certain extent, pro-inflammatory immune Fc γ R is the Fc receptor in IgG constant region and involved in the inflammatory process of DN, which is closely related to DN development and progression (8).

This study clarified the effects of baicalein on the DN rats by detecting the levels of AMPK α , hs-CRP and Fc γ R.

Materials and methods

Experimental materials, apparatus and reagents. A total of 60 healthy adult Sprague-Dawley male rats

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(approximately 200 g) were provided by Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, China). Experimental apparatus and reagents included: Microtome produced by Leica Biosystems (Wetzlar, Germany), centrifugal machine manufactured by Beijing Guangan Medical Instrument Factory (Beijing, China), electronic balance manufactured by Changzhou Hongheng Electronic Equipment (Changzhou, China), UV-2000 UV analyzer produced by Shanghai Scientific Instrument Factory (Shanghai, China), microplate reader (Jiangsu Potebio Co., Ltd., Jiangsu, China), electrophoresis tank manufactured by Beijing Liuyi Instrument Factory (Beijing, China), streptozotocin (STZ; Sigma-Aldrich, Darmstadt, Germany), Astragalus injection manufactured by Gaoyou subsidiary of New Asia Pharmaceutical Co., Ltd. (Shanghai, China), rabbit anti-rat AMPK α (Cell Signaling Technology, Danvers, MA, USA), horseradish peroxidase-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX, USA), polyvinylidene fluoride membrane (PVDF; Roche, Indianapolis, IN, USA), western blotting luminescence reagent (Santa Cruz Biotechnology), agarose (Promega, Madison, WI, USA), TRIzol kit (Invitrogen Life Technologies, Carlsbad, CA, USA), hs-CRP and Fc γ R kits were provided by Shanghai Yueyan Biotechnology Co., Ltd. (Shanghai, China) and the primers were provided by Shanghai Yingjun Biotechnology Co., Ltd. (Shanghai, China).

Methods

Model preparation and grouping. All the rats had access to food and water *ad libitum* for one week and were then divided into the observation (n=30) and control (n=30) groups using the random number table method. The control group was fed normally, while the observation group was fed with high-fat and high-sugar diet and a single injection of STZ (25 mg/kg). After 4 weeks, fasting blood glucose was detected and the level ≥ 7.8 mmol/l was regarded as the DM rat model. After 8 weeks, the DM rat model with 24 h urine microalbumin (24 h U-ALB) value of 30 and 300 mg was regarded as the DN rat model. After the establishment of DN model, 1 ml Astragalus injection was mixed into 5 ml normal saline for the gavage administration (400 mg/kg) of observation group for 8 consecutive weeks, and the control group was treated with the gavage administration with 3 ml distilled water.

Morphological observation of kidney. After 8 weeks of administration, the rats were laparotomized and the kidney tissues were taken as the samples, followed by fixation and dehydration and 70, 80, 90 and 95% ethanol was added in turn for treatment during dehydration, followed by soaking via xylene and embedding via paraffin. Microtome was used to cut the sample into 5 μ m sections, which were stained with H&E and sealed by neutral balsam, followed by observation of renal pathological changes under the microscope (Olympus Corporation, Tokyo, Japan). Ethics approval was obtained from Nanjing University of Chinese Medicine (Nanjing, China).

Detection of AMPK α in renal tissues. The mRNA expression in AMPK α in renal tissues was detected via RT-PCR: i) After 8 weeks of drug administration, 100 mg renal tissues were taken from the rats in each group and stored at -80°C; ii) the total RNA was extracted strictly according to the

Table I. AMPK α and β -actin primer sequences.

Item	Sequence
AMPK α	F: 5'-GTTCTACCTCGCCTCCAGTC-3' R: 5'-TGCTCCACCACCTCATCATC-3'
β -actin	F: 5'-ACTGGCATTGTGATGGACTC-3' R: 5'-AGGAAGGAAGGCTGGAAGAG-3'

F, forward; R, reverse.

instructions of TRIzol kit; the concentration and purity of RNA were detected and the concentration ratio was required to be between 1.8 and 2.0; iii) primer design: The experimental primers were designed and synthesized by Shanghai Yingjun Biotechnology (primer sequences are shown in Table I); iv) access RT-qPCR system (Promega) was used to amplify the total RNA into target DNA fragment; amplification conditions: Degeneration at 95°C for 2 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, a total of 35 cycles, extension at 72°C for 5 min; and v) after EB staining and agarose gel electrophoresis, PCR products were observed and analyzed quantitatively and the relative expression level of AMPK α mRNA was expressed by the gray level ratio of AMPK α mRNA to β -actin.

The protein expression in AMPK α in renal tissues was detected by western blot analysis: i) Iced cell lysis buffer (300 ml) was added into 100 ml renal tissues and then the solution received ultrasound examination 3 times (5 sec/time) after 30 min and was centrifuged at 8,600 x g for 60 min at 4°C and the supernatant was removed; ii) protein quantification was performed using Lorry method; total protein (40 μ g) was dissolved in the isopyknic buffer solution and boiled for 10 min, followed by polyacrylamide gel electrophoresis to separate the protein; iii) the protein was transferred onto the PVDF membrane; and iv) the PVDF membrane was placed into the blocking solution (dilution, 1:5,000), sealed for 30 min and placed overnight at 4°C; on the second day, the blocking solution was removed and rabbit anti-rat AMPK α monoclonal antibody (dilution, 1:5,000; cat. no. 5831; Cell Signaling Technology, Inc.) was added to incubate for 30 min at 37°C, followed by rinsing 3 times (5 min/time). Horseradish peroxidase-labeled goat anti-rabbit IgG polyclonal antibody (dilution 1:10,000; cat. no. 7074; Cell Signaling Technology, Inc.) was then added to incubate for 120 min at 37°C, followed by rinsing 3 times (5 min/time); and the PVDF membrane was placed into the cassette with X-ray film for exposure for 10 min, followed by conventional development and fixation. The relative expression level of AMPK α protein was expressed by the gray level ratio of AMPK α mRNA to β -actin protein.

Detection of other indexes. At 1, 4, 6 and 8 weeks after drug intervention, blood (4 ml) was collected from the abdominal aorta of rats, placed in ethylene diamine tetraacetic acid (EDTA) anticoagulant tube and centrifuged at 1,400 x g for 10 min. Subsequently, the supernatant was removed and stored at -20°C, and 24 h urine was collected to be tested. The levels

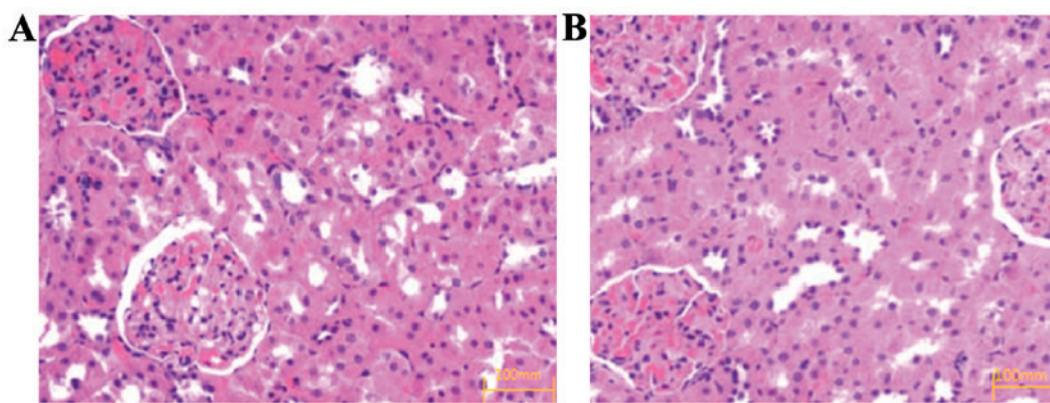


Figure 1. H&E staining results. Renal pathological changes of rats in the (A) control and (B) observation groups (magnification, x400). The degree of renal pathological change in the observation group is significantly relieved compared with that in the control group. H&E, hematoxylin and eosin.

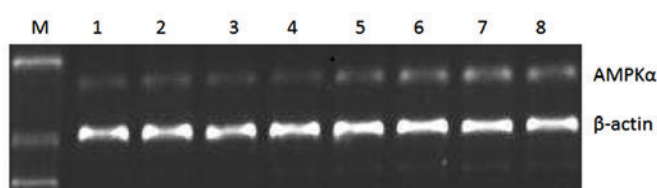


Figure 2. Gel electrophoresis results of RT-PCR amplification of AMPKα mRNA in rats in both groups. M, DNA marker; 1-4, control group; 5-8, observation group; AMPKα, AMP-activated protein kinase α.

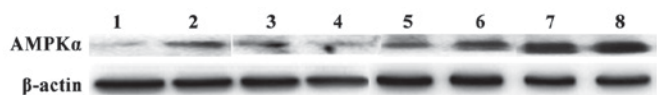


Figure 3. Results of AMPKα protein expression in both groups. 1-4, control group; 5-8, observation group. AMPKα, AMP-activated protein kinase α.

of hs-CRP, FcγR, BUN and 24 h U-ALB in rats in each group were detected. The levels of hs-CRP and FcγR in rats were detected via ELISA according to the instructions of the kit. A microplate reader was used to read the OD value at the wavelength of 450 nm to calculate the concentrations of hs-CRP and FcγR. The serum BUN level was detected by continuous monitoring assay with urease-glutamate dehydrogenase and 24 h U-ALB was detected via immunological transmission turbidimetry.

Statistical analysis. Data were processed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA) software. Measurement data were presented as mean ± standard deviation (SD) and Students' t-test was used for intergroup comparison. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Pathological changes in renal tissues. H&E staining showed that after 8 weeks of drug intervention, the glomerular sclerosis, mesangial cell and mesangial matrix hyperplasia occurred in the control group (Fig. 1A) and the degree of renal pathological change in the observation group was significantly relieved compared with that in the control group (Fig. 1B).

Table II. Comparison of AMPKα expression in rats between the two groups.

Group	Relative expression level of AMPKα mRNA	Relative expression level of AMPKα protein
Observation group	0.493 ± 0.074	0.978 ± 0.124
Control group	0.278 ± 0.062	0.456 ± 0.083
t-test	12.198	19.161
P-value	< 0.001	< 0.001

AMPKα, AMP-activated protein kinase α.

After 8 weeks of drug intervention, the relative expression levels of AMPKα mRNA and protein in the observation group were higher than those in the control group ($p < 0.05$) (Table II). The results of gel electrophoresis of RT-PCR products and western blot analysis are shown in Figs. 2 and 3.

Comparison of serum hs-CRP level in rats between the two groups after drug intervention. The hs-CRP levels in the observation group at 1, 4, 6 and 8 weeks after intervention were significantly lower than those in the control group ($p < 0.05$) (Table III).

Comparison of serum FcγR level in rats between the two groups after drug intervention. FcγR levels in the observation group at 1, 4, 6 and 8 weeks after intervention were significantly higher than those in the control group ($p < 0.05$) (Table IV).

Comparison of serum BUN level in rats between the two groups after drug intervention. BUN levels in the observation group at 1, 4, 6 and 8 weeks after intervention were significantly lower than those in the control group ($p < 0.05$) (Table V).

Comparison of 24 h U-ALB level in rats between the two groups after drug intervention. U-ALB levels in the observation group at 1, 4, 6 and 8 weeks after intervention were significantly lower than those in the control group ($p < 0.05$) (Table VI).

Table III. Comparison of hs-CRP level in rats between the two groups after drug intervention (mg/l).

Group	Case	1 week after intervention	4 weeks after intervention	6 weeks after intervention	8 weeks after intervention
Observation group	30	7.52 \pm 2.73	5.75 \pm 2.26	3.47 \pm 1.35	1.83 \pm 0.56
Control group	30	12.36 \pm 3.42	13.43 \pm 3.63	14.22 \pm 3.15	15.45 \pm 3.53
t-test		6.058	9.837	17.181	20.872
P-value		<0.001	<0.001	<0.001	<0.001

hs-CRP, hypersensitive C-reactive protein.

Table IV. Comparison of Fc γ R level in rats between the two groups after drug intervention (ng/ml).

Group	Case	1 week after intervention	4 weeks after intervention	6 weeks after intervention	8 weeks after intervention
Observation group	30	22.57 \pm 3.74	25.68 \pm 3.16	26.67 \pm 3.38	27.63 \pm 3.58
Control group	30	17.46 \pm 3.47	18.53 \pm 3.62	18.42 \pm 3.16	19.15 \pm 3.23
t-test		5.486	8.150	9.766	9.633
P-value		<0.001	<0.001	<0.001	<0.001

Fc γ R, Fc γ receptor.

Table V. Comparison of BUN level in rats between the two groups after drug intervention (mmol/l).

Group	Case	1 week after intervention	4 weeks after intervention	6 weeks after intervention	8 weeks after intervention
Observation group	30	9.08 \pm 2.53	8.23 \pm 2.16	6.87 \pm 1.75	4.43 \pm 1.35
Control group	30	18.35 \pm 2.46	18.73 \pm 2.13	19.82 \pm 2.14	24.45 \pm 2.43
t-test		14.388	18.958	25.658	39.446
P-value		<0.001	<0.001	<0.001	<0.001

BUN, blood urea nitrogen.

Table VI. Comparison of 24 h U-ALB level in rats between the two groups after drug intervention (mg).

Group	Case	1 week after intervention	4 weeks after intervention	6 weeks after intervention	8 weeks after intervention
Observation group	30	85.53 \pm 7.35	78.75 \pm 5.16	59.48 \pm 4.38	47.83 \pm 3.57
Control group	30	164.39 \pm 9.47	193.43 \pm 9.65	203.22 \pm 9.36	209.45 \pm 9.43
t-test		36.032	57.410	76.184	87.793
P-value		<0.001	<0.001	<0.001	<0.001

U-ALB, urinary micro-albumin.

Discussion

DN is one of the major complications of DM, and DM is the main cause of end-stage renal disease (9). The main cause of DN is the damage to glomerular microvascular structure and

function caused by the long-term high-glucose environment, which is generally regarded as the result of environmental and genetic factors. Its pathogenesis is very complex, including the mutual influences of insulin resistance, hemodynamic changes, cytokines, oxidative stress, glucose metabolism disorders

and genetic background (9). DN has the characteristics of nodular or diffuse glomerular sclerosis; thus, it is also known as diabetic glomerulopathy with non-specific manifestations, whose early manifestations include renal tubular hypertrophy and hyperplasia, renal tubular fibrosis and thickening of basilar membrane (10). Additionally, high glucose increases the glucose filtration rate of glomeruli and directly stimulates the basilar side of renal tubules, resulting in the increased glucose load in renal tubules and damage to the renal tubular epithelial cells (11). High glucose also induces platelet aggregation, forms microthrombus, promotes glomerular sclerosis, and increases glomerular permeability. Consequently, proteinuria is increased, thus causing tubulointerstitial damage, forming a vicious cycle and deteriorating the effects of the disease (12).

AMPK is a heterotrimer comprising three subunits: α , β and γ , with α playing a catalytic role and the other two subunits playing roles of maintaining stability (13). AMPK widely exists in a number of systems, including liver, skeletal muscle, adipose tissue, kidney and pancreas. AMPK is a cellular energy metabolic regulator that realizes the complex activity regulation via sensitization of changes in cellular energy state to maintain the energy supply-demand balance in various links of cellular material metabolism (14). AMPK blocks glucogenesis-related enzymes, leading to reduction of glucogenesis, which plays a key role in fatty acid and sugar metabolism and is closely related to insulin resistance. After activation of AMPK, the blood lipids and blood sugar can be decreased, thereby alleviating the symptoms of DM (15). At the same time, AMPK can promote the glucose uptake and utilization for peripheral tissues, which is realized in two ways. Firstly, AMPK can induce the transfer of glucose transporter 4 to serosa, thereby increasing the rate of glucose transfer; secondly, AMPK can promote the activity of phosphofructokinase, thereby regulating and enhancing the glycolysis to enhance the glucose uptake capacity of peripheral tissues and ensure normal sugar metabolism. The results of the present study showed that the expression of AMPK α mRNA and protein in the observation group at 8 weeks after drug intervention was higher than those in the control group ($p < 0.05$), suggesting that AMPK α expression is upregulated after drug intervention and AMPK activation can decrease the fat and cholesterol synthesis, enhance the mechanization of fatty acids, and regulate lipid and glucose metabolism, thereby alleviating the symptoms of DN.

Hs-CRP is an acute-phase index of micro-inflammatory response that can activate the complement system in the body and enhance the leukocyte phagocytosis by binding to the chromatin and can play a regulatory role by stimulating cell activation (16). Hs-CRP can cause inflammatory response of the body, which is an important pathological process of DN (17). Previous findings have shown that hs-CRP is an independent risk factor of obesity and type 2 diabetes and hs-CRP is closely related to DN (18). An increasing number of studies have shown that the immune inflammatory mechanism is important in the occurrence and development of DN. Fc γ R belongs to the Ig superfamily, which is widely expressed in the hematopoietic system and can regulate the inflammatory and immune response and ensure the dynamic balance of DN (19).

Clinical treatment of DN usually includes correcting lipid metabolism disorders, controlling blood pressure and blood

sugar, reducing proteinuria and protecting renal function (20). A large number of studies have shown that traditional Chinese medicine has a unique effect on DN prevention and treatment). Astragalus injection is the medicine refined by Astragaloside extracted from Astragalus, which can invigorate qi strengthening superficies, arrest sweat and detoxify, promote granulation, eliminate the swelling and promote urination (21). The results of the present study showed that after the intervention with Astragalus injection at different time-points, the hc-CRP level in the control group was significantly higher than that in the observation group, whereas the Fc γ R level was significantly lower than that in the observation group ($p < 0.05$), which may be because Astragalus has an anti-inflammatory effect and can downregulate the expression of chemokines and adhesion molecules and inhibit the release of inflammatory factors, thereby reducing the infiltration of inflammatory cells. At the same time, Astragalus can adjust the immune dysfunction and activate Fc γ R, thereby delaying the progression of DN.

BUN and U-ALB are the main indexes of evaluating the renal function and the long-term hyperglycemia state may cause damage to the glomerular filtration membrane, filtering out U-ALB, can reflect the degree of glomerular injury (22). Findings have confirmed that one of the key factors of forming proteinuria in DN is the podocyte injury (23). The results of the present study showed that the concentrations of BUN and U-ALB in observation group at different time-points after drug intervention were significantly lower than those in control group ($p < 0.05$). H&E staining showed that the degree of renal pathological changes in the observation group was significantly relieved compared with that in the control group, which may be because the anti-oxidative stress effect of baicalein may downregulate the expression of podocyte integrin-linked protease, thus delaying the progression of disease. Baicalein effectively inhibited the accumulation of tubulointerstitial extracellular matrix, eliminated the free radicals, improved the microcirculation, increased the renal blood flow and reduced the urinary protein, thus protecting the kidney.

In conclusion, AMPK α , hs-CRP and Fc γ R play important roles in the development and progression of DN. The interference in AMPK α , hs-CRP and Fc γ R expression via baicalein can delay the progression of DN, thus increasing the survival time and life quality of patients.

Competing interests

The authors declare that they have no competing interests.

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