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Original Article

Functional mechanism of DPPA in diabetic nephritis via activating AKT signal pathway

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Abstract: Diabetic nephropathy (DN) is a common chronic disease. Diphenyl phosphoryl azide (DPPA) is a type of phosphatidic acid monomer that plays a critical role in regulating Raf-1 translocation and mitogen-activated protein (MAP) kinase cascade 2 and 3 activation. This study observed the effect of DPPA in DN and related mechanism. Rat was injected by STZ at 45-65 mg/kg to establish DN model. Venous blood was extracted after 48 h. Blood glucose ≥ 16.7 mmol/L and glycosuria at 3+~4+ were considered as modeling success. The rats were intraperitoneal injected with DPPA at 8-week old. Renal tissue was extracted and stained by HE to observe morphology. Fibronectin expression was evaluated by immunohistochemistry. AKT signaling pathway related molecules were tested by Western blot. A total of 48 rats were successfully modeled and divided into two groups, including DPPA group and normal saline group. Renal tissue section showed renal medulla disappeared and inflammatory cells infiltration in control group. Inflammatory cell infiltration significantly reduced in DPPA group compared with control. Immunohistochemistry demonstrated that fibronectin expression in renal tissue after DPPA treatment obviously reduced compared with control. Western blot revealed that p-AKT expression in DPPA group was markedly higher than that in control. DPPA alleviated DN by activating AKT signaling pathway.

Keywords: DPPA, DN, AKT

Introduction

There were 1.4 million people were diagnosed as diabetic nephropathy (DN) around the world every year, and more than 450,000 people died of DN [1]. According to the report published by the American College of Physicians, the incidence of DN was obviously higher than the other types of chronic disease between 1975 and 2005. Recent study showed that about 231,840 patients were diagnosed as DN, whereas more than 40,290 patients died in USA in 2015 [2]. Following the acceleration of rhythm of life in recent years, the incidence of diabetes increased year by year with the younger trend [3]. The latest statistics results demonstrated that there were 270,000 female were diagnosed as DN and nearly 700,000 died in our country in 2015 [4].

Dipalmitoyl phosphatidic acid (DPPA) is mainly produced by phospholipase D (PLD) hydrolysis of glycerol phospholipid. It is an important intermediate in the process of glycerol phospholipid metabolism, which has an obvious inhibitory effect on neovascularization. It plays a critical role in regulating Raf-1 translocation and mitogen-activated protein (MAP) kinase cascade 2 and 3 activation. It was reported that DPPA can promote the drug penetrating the cell membrane to enter the cell [5, 6]. Moreover, it could be metabolized to lyso-PA to increase Bcl-2 expression in Hela cells [7]. However, DPPA in diabetes showed no influence on Bcl-2 expression [8], suggesting it may play a different regulatory role in different disease process. The impact of DPPA on DN occurrence and development is still unclear. Fibronectin is a kind of macromolecule glycoprotein distributed on the cell surface with the function of promoting fiber connection between cells. It is an important indicator to evaluate damage degree in the process of DN.

Materials and methods

DN mode establishment

A total of 50 SD rats at 8 weeks old were purchased from Kunming Medical University labo-

ratory animal center. The rats were fasted for 8-12 h and intraperitoneal injected for STZ at 45-65 mg/kg. After 48-72 h, the venous blood was extracted from caudal vein. DN model success was evaluated by blood glucose ≥ 16.7 mmol/L and glycosuria at 3+~4+ continued for more than 1 week. The model was type I diabetes. Forty-eight rats were successfully modeling and equally divided into two groups. The rats in DPPA group received DPPA intraperitoneal injection at 0.1 mg/kg every 48 h for 4 weeks [9]. The rats in control received normal saline intraperitoneal injection at 0.1 mg/kg every 48 h for 4 weeks.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Kunming Medical University.

HE staining

The renal tissue was extracted and fixed in formalin. After dehydration, the tissue was embedded and cooled at 4°C. Then the tissue was cut into slice at 4 μm and roasted at 65°C for 1 h. Next, the slice was dewaxed and stained by hematoxylin for 1 h. After washed by water, the slice was further stained by eosin and observed under the microscope. The slice was diagnosed by three different pathologists.

Immunohistochemistry

The tissue was fixed in formalin and dehydrated on the second day. Then the tissue was embedded and cooled at 4°C. Next, the tissue was cut into slice at 4 μm and roasted at 65°C for 1 h. After dewaxing, the tissue was repaired in citric acid buffer with pH=6.0. After that, the tissue was further steeped in 0.3% hydrogen peroxide. After blocked by 10% FBS for half an hour, the slice was incubated in primary antibody overnight. Next, the slice was washed by PBS and incubated in secondary antibody at 37°C for 1 h. At last, the slice was developed by DAB and washed by PBS. After counter staining by hematoxylin for 3 min, the slice was observed under the microscope.

Western blot

Total protein was extracted from tissue using Beyotime protein extraction kit and quantified by BCA. The protein was separated by SDS-PAGE and transferred to PVDF membrane at 250 mA for 90 min. After blocked by 5% skim milk for 1 h, the membrane was incubated in primary antibody overnight. Then the membrane was incubated in secondary antibody at room temperature for 1 h. At last, the membrane was added with ECL reagent and developed in X-ray.

Statistical analysis

All data were presented as $\bar{x}\pm s$ and compared by t test and one-way ANOVA with post-hoc Tukey HSD. All data analysis was performed using Image-Pro-Plus 6.0 and GraphPad Prism 5 software. $P<0.05$ was depicted as statistical significance.

Results

HE staining detection of renal tissue changes in rat DN model

HE staining showed that compared with the control group, renal cytoplasmic red staining became lighter in DPPA treatment after 1 week, 2 weeks, and 12 weeks. On the first week, the renal medulla disappeared and inflammatory cells infiltrated in DPPA group. On the contrary, DPPA group exhibited fewer number of renal tubule with expanded lumen, better integrity of the glomerulus, and less inflammation infiltration. On the second week, the above mentioned changes aggravated in both of experimental group and control. The rats in control presented renal interstitial fibroblasts hyperplasia, renal tubular dissolution and necrosis, and glomerular number decreased. Compared with control, the rats in experimental group exhibited slighter lesions. On the 12^{th} week, the glomerular structure was damaged in control and the integrity was worse than that in DPPA group (**Figure 1**).

Fibronectin changes in rat DN model

Fibronectin plays an adhesive role between cells. As is known to all, fibronectin is closely associated with tissue fibrosis. Thus, we used immunohistochemistry to test fibronectin expression in renal tissue. Fibronectin expression in DPPA group was obviously lower than that in glomerulus from control. In addition, glomerulus structure was seriously damaged in control compared with DPPA group. The expres-

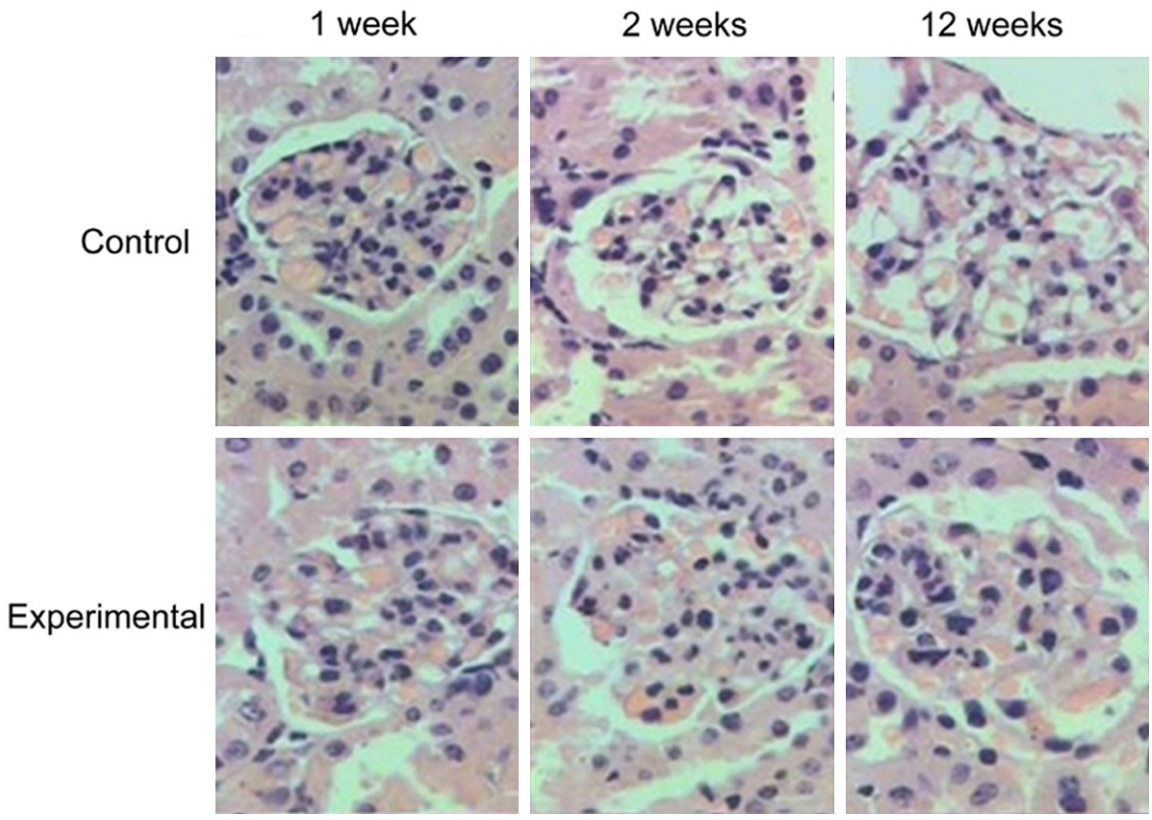

Figure 1. HE staining detection of renal tissue changes in rat DN model.

sion of fibronectin further confirmed our successful modeling and elaborated pathological changes in renal tissue (**Figure 2**).

DPPA activated AKT signaling pathway to retard DN

Fibronectin played a key role in extracellular matrix, while the latter is mainly regulated by AKT signaling pathway. Renal tissue was extracted to detect fibronectin, AKT, and p-AKT protein expression. It was found that fibronectin expression in renal tissue was in accordance with immunohistochemistry on the 12th week. Meanwhile, p-AKT level in experimental group was significantly higher than that in control, suggesting DPPA activated AKT signaling pathway to retard DN process (**Figure 3**).

Discussion

Following the improvement of quality of life, an increasing number of diabetes patients need better treatment. These patients may also concomitant with DN that affects normal life activities. Chronic renal disease can evolve into renal interstitial fibrosis and even kidney tumor [10-12]. Drug intervention plays a decisive role in this process, such as the small molecule drugs affecting the intermediate of diabetes. DPPA is an important intermediate in glycerol phospholipid metabolism process. Recent studies reported that DPPA can influence diabetes in addition to renal cancer upon gene model rat, which changed the understanding of hypoglycemic drugs. It was showed that DPPA can suppress UspA2 gene expression, whereas the latter was an important gene for fibronectin production. Therefore, it affected fibronectin gene expression and reduced protein level [13]. We suspected that whether small molecule drugs such as DPPA can influence DN occurrence. Thus, we established rat DN model and found that DPPA can obviously improve the pathological process of renal fibrosis, proving that DPPA had a therapeutic effect in the renal interstitial fibrosis stage.

AKT signaling pathway is involved in cell proliferation and differentiation. It was revealed that the activation of AKT signaling pathway may

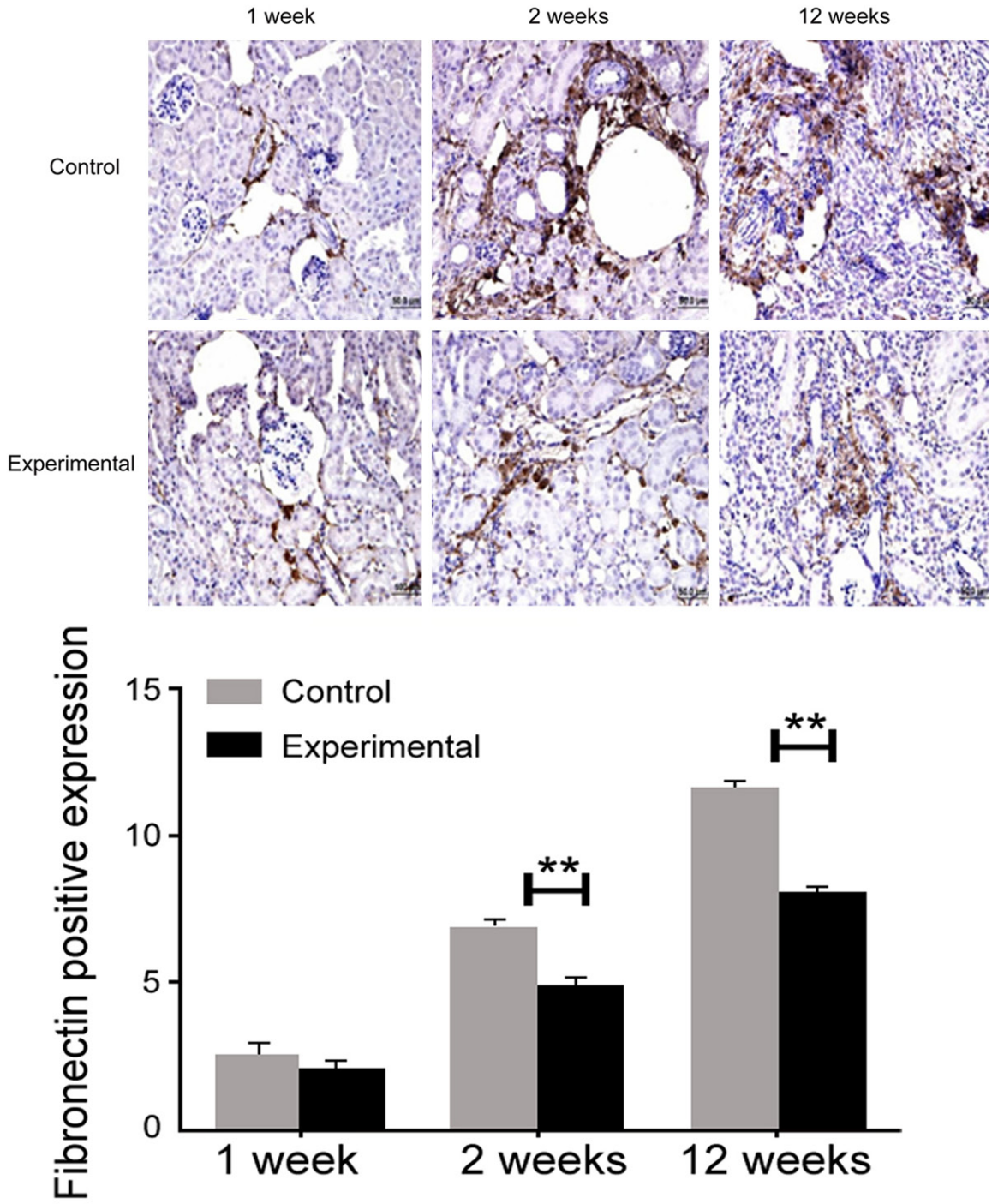

Figure 2. Fibronectin changes in rat DN model. **P<0.01.

decrease the incidence of diabetes [14]. Pathological diagnosis of early DN includes glomerular hypertrophy and glomerular extracellular matrix thickening in homogeneity. As one of the important adhesion molecules in the extracellular matrix, fibronectin may affect DN formation [15]. Therefore, the occurrence of DN has an inseparable relationship with fibronectin.

It was considered that high glucose is an important reason of kidney mesangial cells infinite proliferation [16]. Renal hypertrophy in early diabetes is often accompanied by the change

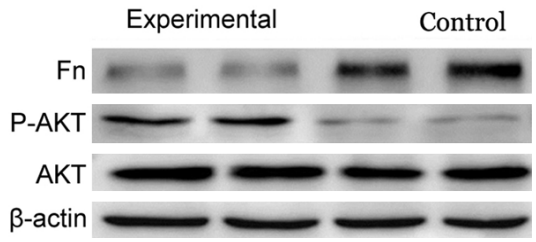

Figure 3. DPPA activated AKT signaling pathway to retard DN.

of signaling pathway [17, 18]. AKT plays an important role in cell cycle by silencing CDK expression to suppress cell proliferation. Fibronectin played a key role in extracellular matrix, while the latter is mainly regulated by AKT signaling pathway. It was revealed that fibronectin can change the direction of cell mitosis to affect AKT signaling pathway by altering cellular polarity [19]. DPPA treatment significantly changed fibronectin expression in renal tissue, thus we speculated that DPPA may influence the pathological process by affecting AKT signaling pathway. Immunohistochemistry staining showed the fibronectin expression in DPPA group was obviously reduced compared with control. It was demonstrated that the mesangial cells in AKT deletion mouse did not occur hyperplasia even induced by high glucose [20-22]. Therefore, elucidating the mechanism of DN is of great significance to reveal the occurrence and development of DN and retard renal function deterioration.

Conclusion

It was reported that fibronectin play a decisive effect on fibrosis. We found DPPA markedly declined fibronectin expression, while fibronectin downregulation activated AKT signaling pathway to retard DN progress. We discovered that DPPA can affect AKT signaling pathway in DN.

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Disclosure of conflict of interest

None.

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