Tropical Journal of Pharmaceutical Research December 2021; 20 (12): 2539-2544
ISSN: 1596-5996 (print); 1596-9827 (electronic)
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Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v20i12.12

Original Research Article

Pachymic acid protects against kidney injury in mice with diabetic nephropathy by inhibiting the PI3K/AKT pathway

Weihao Li[1,2]*, Chong Wang[3], Mingming Zhang[4], Yingze Yuan[5], Zhiping Zhang[2], Xiaomei Liu[2], Feifei Zhang[2], Yuefeng Wu[1]*

[1]Key Laboratory of Animal Physiology, Biochemistry and Molecular Biology of Hebei Province, College of Life Sciences, Hebei Normal University, Shijiazhuang, Hebei Province 050024, China, [2]Special Clinical Laboratory, Second Hospital of Hebei Medical University, Hebei Province 050000, China, [3]Department of Laboratory Medicine, East Branch of Second Hospital of Hebei Medical University, Shijiazhuang, Hebei Province 050000, [4]Clinical Laboratory, Hebei General Hospital, Shijiazhuang, Hebei Province 050001, [5]Department of Laboratory Medicine, Jizhong Energy Xingtai MIG General Hospital, Xingtai, Hebei Province 054000, China

For correspondence: **Email: weihaoli001@163.com; wuyuefeng@hebtu.edu.cn; **Tel:** +86-0311-66636290; 86-0311-80787555*

Sent for review: 9 August 2021 *Revised accepted: 29 November 2021*

Abstract

Purpose: *To investigate the effect of pachymic acid (PA) on diabetic nephropathy (DN).*
Methods: *C57BL/6J mice were divided into three groups: control group, DN model group, and PA group. In the DN model and PA groups, the mice were injected intraperitoneally with streptozotocin (STZ) on five consecutive days to induce DN. In the control group, the mice were injected with saline. Then, mice in the PA group were treated with 5 mg/100 g PA for four consecutive weeks. Weight and fasting blood glucose were measured. The pathological condition of kidney tissue was examined by hematoxylin/eosin staining. Serum creatinine (Scr), urea nitrogen (BUN), urine protein (U-Pro), malondialdehyde (MDA), and superoxide dismutase (SOD) levels in kidney tissues were measured by enzyme immunosorbent assay (ELISA). Protein expression of AKT, PI3K, p-AKT, and p-PI3K in kidney tissues was evaluated by western blot.*
Results: *Compared with the control group, mice in the DN model group weighed less; had a higher degree of kidney tissue damage; higher fasting blood glucose, Scr, BUN, U-Pro, MDA, p-AKT, and p-PI3K levels; and lower SOD activity. Compared with the DN model group, the PA group showed improvements in weight and kidney damage; had lower fasting blood glucose, Scr, BUN, U-Pro, p-AK, and p-PI3K levels; and higher SOD activity.*
Conclusion: *PA treatment improves the renal function of DN mice and inhibits oxidative stress, probably by suppressing the PI3K/AKT pathway. These findings suggest that PA has potentials as a treatment for DN.*

Keywords: *Pachymic acid, Diabetic nephropathy, Oxidative stress, PI3K/AKT signaling pathway, Kidney injury*

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INTRODUCTION

Diabetic nephropathy (DN) is one of the most severe microvascular complications of diabetes mellitus and a key cause of end-stage renal disease [1]. Its pathological features include albuminuria, glomerulus damage, and relentless deterioration of kidney function [2]. DN results

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from the metabolic and hemodynamic changes caused by diabetes mellitus [3], for example, activation of glucose-dependent pathways within the diabetic kidney leads to accumulation of advanced glycation end products, renal polyol formation, and oxidative stress [3]. By understanding the pathogenic mechanisms of DN, effective treatments can be discovered, for example, insulin and renin-angiotensin system inhibitors treat DN by regulating the glycemic index and blood pressure, respectively [4]. However, the current treatments for DN have some side effects and toxicity and only delay the disease progression without curing it [4]. Therefore, it is essential to explore new and effective therapeutics for DN.

Pachymic acid (PA) is a lanostane-type triterpenoid from *Poria cocos*, which is used in traditional Chinese medicine [5]. It has been reported that PA exerts sedative, hypnotic, anti-inflammatory, anticancer, antioxidative, and antihyperglycemic effects [5]. For instance, PA was shown to induce apoptosis and suppress growth of pancreatic cancer by targeting endoplasmic reticulum stress [6]. PA has also been shown to prevent oral inflammation and to promote odontoblastic differentiation [7]. Moreover, PA protects against renal injury induced by sepsis by inhibiting oxidative stress and inflammatory responses [8]. However, the effect of PA on DN has not been reported.

In this study, a mouse model of DN was developed, the DN mice were treated with or without PA, and then weight, fasting blood glucose, pathological condition, renal function, and oxidative stress were evaluated. The effect of PA on PI3K/AKT signaling in the mouse model of DN was also evaluated.

EXPERIMENTAL

Reagents

Pachymic acid (PA, cat no. IP0010, white crystalline powder with purity ≥ 99.9 %), streptozotocin (STZ, cat. no. S8050，HPLC ≥ 98 %), and the hematoxylin and eosin (HE, cat. no. G1120) staining kit were purchased from Solarbio Life Sciences (Beijing, China). Serum creatinine (Scr, cat. no. JL20488), superoxide dismutase (SOD, cat. no. JL12237), and urea nitrogen (BUN, cat. no. JL20491) ELISA reagent kits were obtained from Jianglaibio (Shanghai, China). The urine protein (U-Pro, cat. no. RJ17462) ELISA reagent kit was purchased from Shanghai Renjie Biotechnology Co. Ltd (Shanghai, China). The malondialdehyde (MDA, cat. no. ym-r00398) ELISA reagent kit was obtained from Shanghai Yuanmu Biotechnology Co., Ltd (Shanghai, China). Anti-AKT (cat. no. ab18785), anti-PI3K (cat. no. ab191606), anti-p-AKT (cat. no. ab38449), anti-β-actin (cat. no. ab8226), goat anti-rabbit IgG H&L (HRP) (cat. no. ab205718), and rabbit anti-mouse IgG H&L (HRP) (cat. no. ab205719) antibodies were purchased from ABCAM (Cambridge, United Kingdom). Anti-p-PI3K antibodies were obtained from Shanghai Youning Biotechnology Co., Ltd (Shanghai, China).

Animals and the DN model

Forty C57BL/6J male mice (weight: 18–22 g, age: 6–8 weeks) were obtained from Zhejiang Experimental Animal Center (Hangzhou, Zhejiang, China). The mice were housed in specific pathogen-free rooms with 50-70 % humidity at 20-26℃ under a 12 h light/12 h dark cycle and were allowed free access to water and feed. All animal experiments were approved by the Research Ethics Committee of the Second Hospital of Hebei Medical University (approval no. 2017-P042) and were conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

To construct the DN mouse model, 30 C57BL/6J mice were selected randomly and injected intraperitoneally with 50 mg/kg STZ, which was diluted to 10 mg/ml with cold citrate-citric acid buffer (pH 4.5, Sigma-Aldrich), for five consecutive days. Ten C57BL/6J mice were used as the control group, and they were injected with an equal amount of citrate-citric acid buffer for five consecutive days under similar conditions. At 7 and 14 d after injection, blood glucose levels (6 h fasting) were measured using blood from the tail vein and a blood glucose monitor (Accu-Chek, Roche Applied Science, Penzberg, Germany). Mice with fasting blood glucose levels > 13.9 mmol/L on both days were designated diabetic indicating successful construction of the DN model. A total of 26 DN mice were obtained and divided randomly into two groups: the DN model group and the PA group. For the PA group, PA was diluted in saline containing 0.5 % DMSO to 1 mg/mL, and DN mice were treated with the diluted PA at a dose of 5 mg/100 g by intragastric administration for four consecutive weeks. For the DN model and control groups, mice were treated with an equal amount of saline by intragastric administration for four consecutive weeks. During the administration period, two mice in the DN model group died and one mouse in the PA group died, thus 10 mice from each group were chosen randomly for further analysis.

Assessment of weight and fasting blood glucose

After treatment, the mice were weighed using an electronic scale (Beijing Langke Xingye Weighing Equipment CO., LTD, Beijing, China) and blood glucose levels (6 h fasting) were measured using blood from the tail vein and a blood glucose monitor (Accu-Chek, Roche Applied Science).

Biochemical analysis

Blood was collected from the tail vein and centrifuged to obtain serum for the further analysis. Serum Scr, BUN, and U-Pro levels were measured using the corresponding commercial ELISA kits.

Examination of renal morphology

The mice were anesthetized with 10 % chloral hydrate, and then the kidneys were removed and divided into four equal parts. Three of the four parts were frozen in liquid nitrogen. The remaining part was fixed with 4 % paraformaldehyde for 24 h, dehydrated gradually using a graded ethanol series, rinsed with xylene, embedded in paraffin, and sliced into 4 μm thick sections. The sections were mounted onto glass slides and stained with hematoxylin for 10 min and eosin for 3 min at room temperature. Finally, the morphology was evaluated using a light microscope (Olympus Corporation, Tokyo, Japan).

Oxidative stress measurement

Frozen kidney was weighed, homogenized in 0.1 M ice-chilled phosphate buffer pH 7.4, centrifuged at 3000 rpm for 20 min, and supernatant was collected. The levels of MDA, an oxidative stress biomarker, and SOD, an antioxidant enzyme, in the supernatant were measured using commercial ELISA kits.

Western blotting

Frozen kidney was lysed with RIPA lysis buffer containing a protease inhibitor cocktail (Beyotime), centrifuged at 4°C for 15 min at 12,000 rpm, and the supernatant was collected. The protein concentration of the supernatant was quantified by the BCA method. After boiling for 10 min, proteins were resolved by 10 % SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% skim milk in TBST for 2 h and then incubated with AKT, PI3K, p-AKT, p-PI3K, or β-actin primary antibodies overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. Detection was performed using an enhanced chemiluminescence kit (ECL; Thermo Fisher Scientific, Waltham, MA, USA). Semi-quantification was performed using ImageJ software.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis among groups was carried out using Student's t-test or one-way ANOVA with Sidak's multiple comparison test in GraphPad Prism 8.0 software. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Effect of PA on weight and fasting blood glucose of DN mice

In this study, a mouse model of DN was established by STZ injection. DN mice were treated with PA to determine the effect of PA on DN. The DN model group showed a decrease in weight ($t = 5.763$, $p < 0.001$) but an increase in fasting blood glucose ($t = 11.291$, $p < 0.001$) when compared with the control group (Table 1). Compared with the DN model group, the PA group showed an increase in weight ($t = 3.284$, $p = 0.004$) and a decrease in fasting blood glucose ($t = 4.992$, $p < 0.001$). Thus, PA treatment improved the weight and fasting blood glucose level of DN mice.

Effect of PA on renal morphology of DN mice

HE staining was used to examine the effect of PA on kidney morphology.

Table 1: The weight and fasting blood glucose of DN and non-DN mice treated with or without PA

Group	Weight (g)		Fasting blood glucose (mmol/L)	
	Before intervention	***After intervention***	***Before intervention***	***After intervention***
Control	21.63 ± 1.04	24.92 ± 1.03	7.26 ± 0.16	7.59 ± 0.09
DN	19.41 ± 1.01[a]	21.75 ± 1.40[a]	17.18 ± 1.51[a]	18.57 ± 2.91[a]
PA	19.66 ± 1.38[a]	23.48 ± 0.89[b]	17.15 ± 2.58[a]	12.65 ± 2.37[b]

Renal tissues from the control group had normal structure and no obvious pathological changes, whereas renal tissues from the DN model group showed atrophy and necrosis of renal tubular epithelial cells, inflammatory infiltration of renal interstitial cells, and glomerulus damage (Figure 1). Compared with the DN model group, PA attenuated the glomerulus damage, the atrophy and necrosis of renal tubular epithelial cells, and decreased inflammatory infiltration of renal interstitial cells in DN mice (Figure 1). Therefore, PA attenuated renal tissue injury morphology in DN mice.

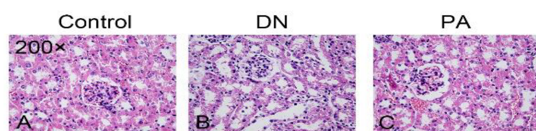

Figure 1: Effect of PA on the renal morphology of DN mice. Sections of renal tissue from DN mice were HE stained and visualized by light microscopy. Control group: normal mice without any treatment, DN group: DN model mice, PA group: DN model mice treated with PA

Effect of PA on kidney function in DN mice

The levels of kidney function indicators Scr, BUN, and U-Pro demonstrate how well the kidneys are working [9]. Scr, BUN, and U-Pro levels were higher in the DN model group than in the control group (t = 9.402, t = 11.293, t = 13.020, all p < 0.001; Table 2). Scr, BUN, and U-Pro levels were lower in the PA group than in the DN model group (t = 4.461, p = 0.001; t = 6.638, p < 0.001; t = 5.994, p < 0.001; Table 2). Thus, treatment with PA improved kidney function in DN mice.

Table 2: Levels of serum Scr, BUN, and U-Pro in control, DN model, and PA group mice

Group	Scr (mg/dL)	BUN (mg/dL)	U-Pro (mg/24h)
Control	0.44±0.07	14.77±2.29	2.91±0.31
DN	0.66±0.03[a]	30.49±3.76[a]	12.68±2.35[a]
PA	0.51±0.11[b]	20.59±2.85[b]	7.52±1.37[b]

Effect of PA on oxidative stress in DN mice

To study the effect of PA on oxidative stress, levels of the oxidative stress biomarker MDA and the antioxidant enzyme SOD were measured. The MDA level was higher (t = 9.913, p < 0.001) and SOD activity was lower (t = 10.016, p < 0.001) in the DN model group than in the control group (Table 3). The MDA level was lower (t = 6.196, p < 0.001) and SOD activity was higher (t = 5.152, p < 0.001) in the PA group than in the DN model group. Thus, PA attenuated oxidative stress in DN mice.

Table 3: The MDA level and SOD activity in renal tissues in control, DN model, and PA group mice

Group	MDA (nmol/mg)	SOD (U/mg)
Control	6.65 ± 0.63	321.32 ± 19.88
DN	17.34 ± 3.35[a]	225.44 ± 22.83[a]
PA	10.00 ± 1.67[b]	272.12 ± 17.32[b]

Effect of PA on the PI3K/AKT signaling pathway in DN mice

The effect of PA on the PI3K/AKT signaling pathway in DN mice was assessed by western blot. Expression of p-PI3K and p-AKT was upregulated in the DN model group when compared with the control group (t = 26.074, t = 24.429, all p < 0.05; Figure 2). However, expression of p-PI3K and p-AKT decreased upon PA treatment (t = 4.236, t = 4.631, all p < 0.05; Figure 2). Thus, PA may inhibit PI3K/AKT signaling in DN mice.

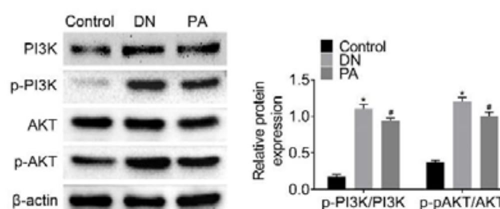

Figure 2: Effect of PA on PI3K/AKT signaling in DN mice. Protein expression of PI3K, AKT, p-PI3K, and p-AKT in DN mice was evaluated by western blot and quantified using Image J software. Control group: normal mice without any treatment, DN group: DN model mice, PA group: DN model mice treated with PA. DN group versus control group, $^{*}p$ < 0.05; PA group versus DN group, $^{\#}p$ < 0.05

DISCUSSION

DN is a severe complication of diabetes and its major clinical features include albuminuria, hyperglycemia, polydipsia, polyphagia, and polyuria [1,2].

Currently, there remains a lack of effective treatments for DN. PA has been reported to ameliorate renal injury. For example, Jiang *et al* showed that PA affects ferroptosis and attenuates acute kidney injury caused by ischemia-reperfusion [5]. Cai *et al* suggested that PA inhibits inflammation and activates the Nrf2/HO-1 pathway to ameliorate acute kidney injury induced by sepsis [8]. Based on these findings, we investigated the effect of PA on DN.

In this study, a mouse model of DN was constructed and the effect of PA on the weight, renal morphology, and kidney function of DN mice was evaluated. DN mice presented with lower body weights than normal mice, and PA treatment increased the weight of DN mice. In addition, the control group had no obvious pathological changes, but the DN model group showed atrophy and necrosis of renal tubular epithelial cells, inflammatory infiltration of renal interstitial cells, and glomerulus damage. The renal injury morphology in DN mice was attenuated by PA treatment. Scr, BUN, and U-Pro levels were upregulated in DN mice, and this upregulation was attenuated by PA treatment. Scr and BUN are the metabolic waste products of creatine and proteins, respectively, and are excreted upon glomerular filtration; the amount of Scr and BUN excreted indicates the glomerulus filtering capability [9]. Albuminuria is also used as a biomarker of kidney injury [10]. Taken together, PA treatment improved the weight and renal function and attenuated the renal injury morphology in DN mice.

PA also has antioxidant and antihyperglycemic effects [5]. Hyperglycemia induces excessive production of oxygen free radicals and enhances lipid peroxidation to form lipid peroxides, such as MDA [11]. MDA is a known biomarker of cell membrane damage and oxidative stress [12]. In addition, formation of reactive oxygen species (ROS), an important part of cellular metabolism, results in oxidative damage to biomolecules, including lipids, proteins, and nucleic acids [13]. SOD, a powerful antioxidant enzyme, participates in the first line of defense against ROS [14]. PA was shown to activate the Nrf2/HO-1 signaling pathway and inhibit oxidative stress in acute kidney injury caused by sepsis [8]. PA treatment can decrease the MDA level and increase SOD activity, thereby limiting oxidative stress-induced damage in acute lung injury [15].

In this study, PA treatment decreased the fasting blood glucose level in DN mice. Additionally, DN mice had a higher MDA level and lower SOD activity than control mice, and PA treatment significantly decreased the MDA level and increased SOD activity in DN mice compared with the DN model group. Thus, PA decreased fasting blood glucose levels and suppressed oxidative stress in kidney tissues in DN mice.

Activation of the PI3K/AKT pathway was found to associate closely with DN progression [16]. Zang *et al* showed that high glucose activates PI3K/AKT signaling in DN and that inhibition of PI3K/AKT by TUG1 suppresses mesangial cell proliferation and fibrosis [16]. Lu *et al* demonstrated that high glucose stimulates ROS production, which leads to an increase in TGF-β1 expression and activation of the PI3K/AKT pathway in DN [17]. Pang *et al* showed that PA treatment attenuates brain ischemia/reperfusion injury via PI3K/AKT signaling [18]. In this study, the DN model group expressed higher levels of p-PI3K and p-AKT than the control group, and this increased expression of p-PI3K and p-AKT was suppressed by PA treatment in DN mice. Thus, PA inhibits the PI3K/AKT signaling pathway in DN.

CONCLUSION

PA improves kidney function and inhibits oxidative stress in DN mice, probably by inhibiting the PI3K/AKT pathway. These findings expand understanding of the pathologic mechanism of DN and indicate that PA has potential for the treatment and management of DN. This is a preliminary study on the protective role of PA in DN, and further basic biological, animal, and clinical experiments are required to validate these findings.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Weihao Li and Chong Wang designed the study and supervised the data collection. Mingming Zhang, Yingze Yuan, and Zhiping Zhang analyzed and interpreted the data. Xiaomei Liu, Feifei Zhang, and Yuefeng Wu prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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