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Original Research Article

Polydatin ameliorates renal fibrosis in a streptozotocin–induced rat model of diabetic nephropathy by inhibiting TLR4/NF-κB signaling

Huimin Niu[1]*, Gang Li[2], Yanhong Qiao[1], Feng Wang[1]
[1]Department of Nephrology, Heping Hospital affiliated to Changzhi Medical College.Changzhi, Shanxi 046000, China;
[2]Department of Urology, Heji Hospital affiliated to Changzhi Medical College, Changzhi, Shanxi 046011, China

For correspondence: **Email: NiuhuiminatHJH@163.com; **Tel:** +86-355-3128406*

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Abstract

Purpose: *To determine the effects of polydatin (PD) on a streptozotocin (STZ)-induced rat model of diabetic nephropathy (DN) and NRK-52E cells treated with high glucose (HG).*
Methods: *Sprague Dawley rats received 65 mM STZ to model DN in vivo. NRK-52E cells were treated with HG, to model DN in vitro. Both models were treated with PD. Fasting blood glucose, kidney/body weight, urinary protein, serum creatinine, and blood urea nitrogen levels, interstitial injury score, as well as protein expression levels of connective tissue growth factor (CTGF), fibronectin, and collagen I were determined in DN rats after PD treatment. Enzyme-linked immunosorbent assay was used to measure inflammatory factors. Protein expression was determined by Western blot analysis while apoptosis was assessed by flow cytometry.*
Results: *STZ successfully induced DN in rats. PD treatment significantly reduced kidney/body weight; decreased fasting blood glucose, urinary protein, serum creatinine, and blood urea nitrogen levels; lowered interstitial injury scores; and downregulated protein expression levels of CTGF, fibronectin, and collagen I. It also inhibited inflammatory reaction and suppressed Toll-like receptor (TLR4)/nuclear factor (NF)-κB signaling. Furthermore, PD suppressed apoptosis, reduced inflammatory factor levels, and suppressed TLR4/NF-κB signaling induced by HG in NRK-52E cells.*
Conclusion: *PD exerts a protective role in DN by decreasing interstitial injury, reducing renal fibrosis, inhibiting inflammatory responses, and suppressing cell apoptosis, at least, partly via inactivation of TLR4/ NF-κB pathway.*

Keywords: *Polydatin, Diabetic nephropathy, Streptozotocin, NRK-52E, TLR4/NF-κB pathway*

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INTRODUCTION

Diabetes is a chronic metabolic syndrome, and diabetic nephropathy (DN) is one of the most serious complications [1,2]. It is predicted that the global population of diabetes patients will exceed 370 million by the year 2025. Approximately 30% of people with diabetes will ultimately develop DN [3], and the incidence of DN has increased steadily worldwide over the past several decades [4]. Mesangial cell proliferation and extracellular matrix (ECM)

accumulation are key pathological features of DN that can lead to renal fibrosis, glomerulosclerosis, mesangial expansion, and renal hypertrophy [5]. Increasing evidence also indicates that metabolic and hemodynamic factors contribute to DN development [6]. Importantly, pro-inflammatory cytokines and chemokines have been shown to accelerate DN progression [7]. Despite substantial research progress on DN pathogenesis, better understanding of the mechanism is also needed to identify novel therapeutic targets for DN. The Toll-like receptor (TLR) family is composed of 10 members and can trigger downstream inflammatory signaling pathways [8]. TLRs can reportedly enhance nuclear factor kappa B (NF-κB) activity [9], suggesting they are very important in the development of inflammatory and immune diseases. Among the 10 members, TLR4 is an important component of inflammatory signaling pathway activation mediated by the innate immune response. A previous study showed that TLR4 was actively involved in DN pathogenesis [10]. Polydatin (PD) is a crystalline compound extracted from *Polygonum cuspidatum* and is a glycoside compound of resveratrol [11]. PD has anti-inflammatory and anti-oxidative effects and is commonly used in the treatment of chronic bronchitis, shock, hepatitis, and even DN. However, the mechanism underlying the efficacy of PD in DN remains unclear. PD prevented lipopolysaccharide-induced acute lung injury through the TLR4/MyD88/NF-κB pathway and reduced uric acid and nephropathy renal injury in fructose-treated mice by reducing oxidative stress and inflammatory responses [12]. These findings raised the possibility that PD may exert a protective role in DN via the TLR4/NF-κB pathway. To validate this hypothesis *in vivo* and *in vitro*, we used Sprague Dawley (SD) rats treated with streptozotocin (STZ) and NRK-52E cells were treated with high glucose (HG), respectively.

EXPERIMENTAL

Reagents

PD (purity >99 % by high-performance liquid chromatography) was provided by PureOne Biotechnology Co. Ltd. STZ was supplied by Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

Animals and treatment

SD rats (225 – 250 g) were provided by Changzhou Cavens Experimental Animal Company, China. All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committees of the United States [13] and approved by the Ethics Committee of Peace Hospital Affiliated with Changzhi Medical College (approval no. 2017CMC035). Rats were housed in individual metabolic cages with *ad libitum* access to standard laboratory chow and water for 7 days. The use of animals in the laboratory was in accordance with international principles.

The rats were divided into five groups: normal control (NC), DN, DN + 20 PD, DN + 40 PD, and DN + 80 PD. Rats in the DN, DN + 20 PD, DN + 40 PD, and DN + 80 PD groups were injected intraperitoneally with 65 μM STZ (pH 4.5, dissolved in citrate-phosphate buffer) to induce diabetes [14]. Rats in the NC and NC + PD groups were injected intraperitoneally with 0.1 mM/L citrate-phosphate buffer. After administration of STZ, experiments were performed to validate successful construction of the model.

After 1 week, the diabetic rats were treated with different concentrations of PD (20, 40, or 80 μM) dissolved in carboxymethyl cellulose (0.5 %). At 0, 2, 4, 6, and 8 weeks after PD treatment, fasting blood glucose and body weight were measured. Finally, rats were sacrificed after 8 weeks for further analysis. Urine was collected on the day before the end of this experiment. Blood samples were obtained, and serum was collected by centrifugation (3,000 × g, 15 min). Kidney tissues were collected and weighed. Both blood and kidney samples were immediately stored at -80 °C.

Assessment of interstitial injury using Masson staining

Kidney tissue was fixed with 4% polyformaldehyde buffer before it was cut into 3-μm paraffin sections. Masson staining was performed to observe renal interstitial matrix quality under optical microscopy, and a semi-quantitative score was tabulated for each section. The scoring criteria were as follows: 0 for no interstitial injury, 0.5 for injury <5%, 1.0 for injury 5–20%, 1.5 for injury 21–35%, 2.0 for injury 36–50%, 2.5 for injury 51–65%, and 3.0 for injury >65% [15].

Enzyme-linked immunosorbent assay (ELISA)

Levels of interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein-1 (MCP-1) were

determined using ELISA kits (R & D Systems, Minneapolis, MN, USA).

Cell culture

Rat renal tubule epithelial NRK-52E cells were provided by Yubo Bio-Technique and cultured in Dulbecco's modified eagle medium in a 5% CO_2 humidified incubator (33°C), supplemented with 0.055 mM glucose, penicillin (100 U/mL), and streptomycin (100 U/mL). The cells were divided into five groups: NC, HG, HG + 10 PD, HG + 20 PD, and HG + 40 PD. Cells in the NC group were treated with 0.055 mM glucose. Cells in the HG, HG + 10 PD, HG + 20 PD, and HG + 40 PD groups were treated with 0.3 mM glucose. Cells in HG + 10 PD, HG + 20 PD, and HG + 40 PD groups were treated with PD (0.1, 0.2, or 0.4 mM, respectively).

Western blot analysis

Cells in 60-mm dishes were incubated at 37°C under 5% CO_2 for 24 h and then collected and washed. Before precooling, cell lysate was added to the cells. Then the solution was vortexed, put on ice for 20 min, and centrifuged (12,000 × g, 20 min). Protein concentrations were measured with a micro bicinchoninic acid protein assay kit. Aliquots of cell lysate (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were then blocked with anti-connective tissue growth factor (CTGF), anti-fibronectin, anti-collagen I, anti-TLR4, anti-p-IκBα, anti-IκBα, anti-p-p65, anti-p65, anti-BCL-2, and anti-cleaved-caspase-3 antibodies. After washing, protein bands were visualized with enhanced chemiluminescent (ECL) reagents and imaged using a ChemiDoc Imaging system. Western blot examination was repeated at least three times.

Apoptosis analysis by flow cytometry

Cells were inoculated on plates at a density of 4 × 10^5 cells per well and then treated with 0.25% trypsin. The cell suspension was stained with Annexin V-fluorescein isothiocyanate (BD Biosciences, San Jose, CA, USA) and propidium iodide in binding buffer for 30 min at 37°C, using a FACSCalibur flow cytometer. Flow cytometry analysis for apoptosis was repeated at least three times.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and are expressed as mean ± standard deviation (SD). SPSS version 17 (SPSS Inc., Chicago, IL, USA) was used, and $p < 0.05$ was considered statistically significant.

RESULTS

PD decreased interstitial injury and renal fibrosis in DN rats

As shown in Figure 1, fasting blood glucose (Figure 1A), body weight (Figure 1B), kidney/body weight ratio (Figure 1C), 24-h urinary protein (Figure 1D), serum creatinine (Figure 1E), blood urea nitrogen (Figure 1F), interstitial injury score (Figure 1G), and relative protein expression levels (CTGF, fibronectin, and collagen I) (Figure 1H) were significantly increased in the DN group compared to normal rats. However, PD treatment (20, 40, or 80 mg/kg) significantly decreased these indexes in a dose-dependent manner. CTGF can trigger cellular processes underlying fibrosis, such as ECM synthesis and cell adhesion, proliferation, and migration. CTGF is also positively related to fibronectin and collagen I [16]. Collectively, these results indicate that STZ successfully induced DN in the rat model, and PD decreased interstitial injury and renal fibrosis in DN rats.

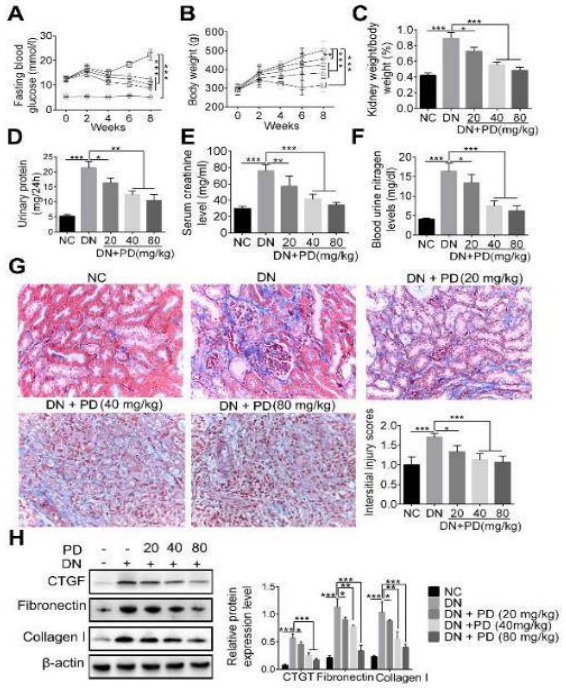

Figure 1. PD decreased biochemical indexes and reduced renal fibrosis in DN rats. (A) Fasting blood glucose; (B) body weight; (C) kidney weight/body weight ratio; (D) urinary protein; (E) serum creatinine; (F) blood urea nitrogen; (G) interstitial injury scores; and (H) relative protein expression levels of CTGF, fibronectin, and collagen I; *$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$, n = 10/group

PD decreased inflammatory factor levels in DN rats

The effects of PD on inflammatory response in DN rats were analyzed by determining the levels of IL-1β, IL-6, and MCP-1. The levels of all three proteins were significantly increased in serum and renal cortex from DN rats as compared to normal rats. However, PD treatment (20, 40, or 80 mg/kg) significantly and dose-dependently decreased the production of these inflammatory factors (Figure 2A and B). These results indicated that PD decreased the levels of inflammatory factors in the serum and renal cortex of STZ-induced DN rats.

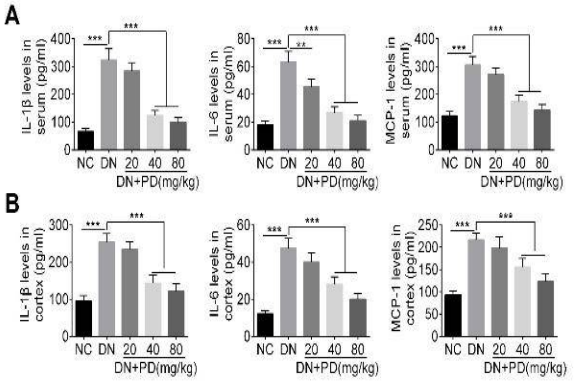

Figure 2: PD decreased inflammatory factor levels in DN rats. Concentrations of IL-1β, IL-6, and MCP-1 in the serum (A) and renal cortex (B) of DN rats. ** $p < 0.01$ and *** $p < 0.001$, n = 10/group

PD suppressed TLR4/NF-κB pathway in DN rats

The effects of PD on the TLR4/NF-κB pathway were examined by determining protein levels in DN rats. Compared to the NC group, the ratios of phosphorylated-IκBα (p-IκBα)/IκBα and phosphorylated-p65(p-p65)/p65, and the protein levels of TLR4 were significantly increased in DN rats. However, compared with DN group, PD treatment (20, 40, or 80 mg/kg) significantly decreased the ratios of p-IκBα/IκBα and p-p65/p65, and the protein level of TLR4 in a dose-dependent manner.

PD suppressed HG-induced apoptosis of NRK-52E cells

Apoptosis of NRK-52E cells was significantly induced by HG treatment compared to the NC group, but this was significantly inhibited by PD (Figure 4A). As shown in Figure 4B, HG treatment markedly increased CFGF, fibronectin, collagen I, and pro-apoptotic protein cleaved caspase-3 levels but decreased expression of the anti-apoptotic protein BCL-2. Again, PD treatment significantly reversed these effects. The results indicated that PD inhibited HG-induced apoptosis of NRK-52E cells.

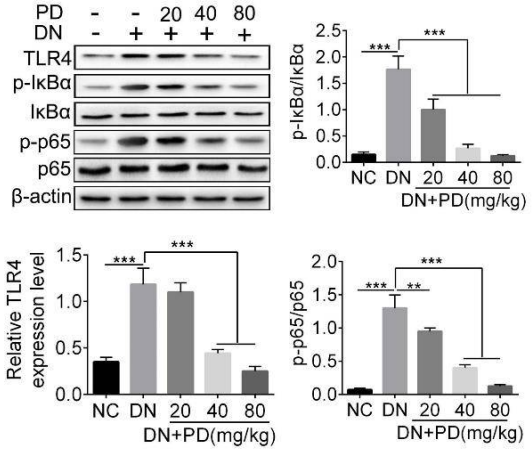

Figure 3: PD suppressed the TLR4/NF-κB pathway in DN rats. The protein levels of p-IκBα, IκBα, TLR4, p-p65, and p65 were analyzed by western blot; ** $p < 0.01$ and *** $p < 0.01$, n = 10/group

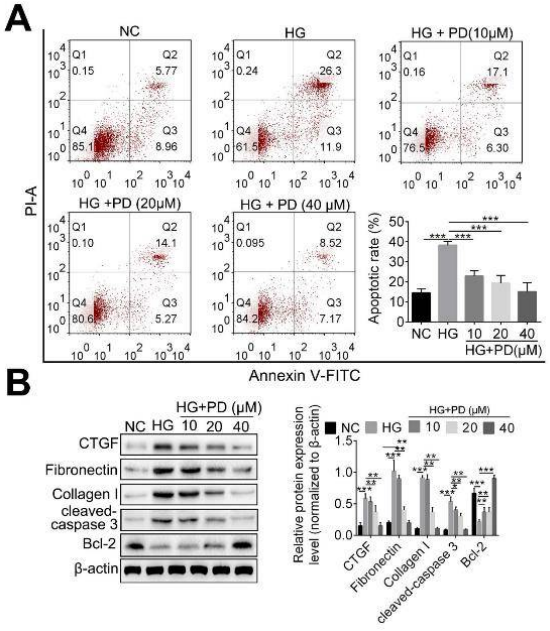

Figure 4: PD suppressed HG-induced apoptosis of NRK-52E cells. (A) The apoptotic rates of NRK-52E cells in the NC, HG and HG+PD groups measured with flow cytometry. (B) Protein levels of CFGF, fibronectin, collagen I, cleaved caspase-3, and BCL-2 in NRK-52E cells treated with HG; ** $p < 0.01$ and *** $p < 0.001$

PD reduced inflammatory factor levels in HG-treated NRK-52E cells

The effects of PD on inflammatory factors in NRK-52E cells were analyzed. Compared with the NC group, levels of IL-1β, IL-6, and MCP-1 in

NRK-52E cells were increased significantly after HG treatment (Figure 5 A–C). However, PD treatment markedly and dose-dependently decreased the levels of all three proteins in HG-treated NRK-52E cells, suggesting that PD suppressed the inflammatory response induced by HG.

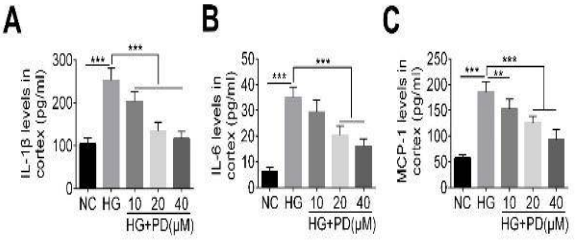

Figure 5. PD reduced inflammatory factor levels in HG-treated NRK-52E cells. IL-1β (A), IL-6 (B), and MCP-1 (C) levels in NRK-52E cells in the NC, HG and HG+PD groups. ** $p < 0.01$ and *** $p < 0.001$

PD suppressed TLR4/NF-κB signaling in HG-treated NRK-52E cells

The effects of PD on the TLR4/NF-κB pathway were detected by determining the protein levels of p-IκBα, IκBα, TLR4, p-p65, and p65 in HG-treated NRK-52E cells. Compared to the NC group, the ratios of p-IκBα/IκBα and p-p65/p65 and the protein level of TLR4 were markedly enhanced in HG-treated NRK-52E cells. However, these levels were dramatically decreased following PD treatment. These results indicate that PD suppressed TLR4/NF-κB signaling in HG-treated NRK-52E cells.

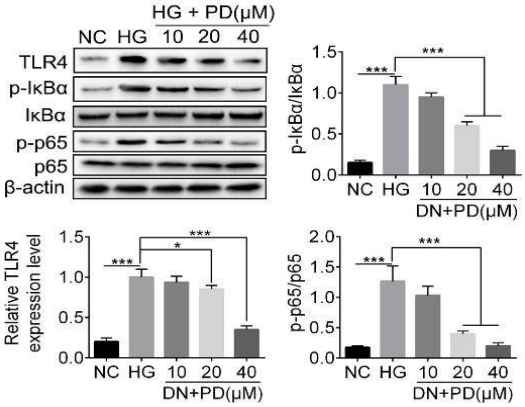

Figure 6: PD suppressed TLR4/NF-κB signaling in HG-treated NRK-52E cells; ** $p < 0.01$ and *** $p < 0.001$

DISCUSSION

DN is one of the major causes of end-stage renal disease [17]. Renal fibrosis typically occurs in all kidney diseases at the terminal stage [18]. In our study, PD significantly decreased interstitial injury scores and reduced relative protein levels of CTGF, fibronectin, and collagen I in a dose-dependent manner. CTGF can trigger cellular processes related to fibrosis, such as cell adhesion, proliferation, migration, and ECM accumulation. CTGF is also positively related to fibronectin and collagen I [16]. PD also reduced the relative protein expression levels of CTGF, fibronectin, and collagen I in a dose-dependent manner in HG-treated NRK-52E cells. Collectively, these results indicate that PD exerted a protective role in DN by decreasing interstitial injury and reducing renal fibrosis.

Moreover, PD reduced the production of inflammatory factors IL-1β, IL-6, and MCP-1 both *in vitro* and *in vivo*. Tang *et al* [19] reported that PD could inhibit the IL-1β-induced inflammatory response in human osteoarthritic chondrocytes. Importantly, pro-inflammatory cytokines and chemokines have been shown to accelerate DN progression [7]. Flow cytometry analysis showed that PD inhibited HG-induced apoptosis of NRK-52E cells by increasing cleaved caspase-3 levels but decreasing BCL-2 expression. Consistently, Zhang *et al* [20] found that PD attenuated ischemia/reperfusion-induced apoptosis in the rat myocardium. PD may exert a protective role in DN by reducing inflammatory factor production and suppressing cell apoptosis.

One study demonstrated that PD ameliorated the development of DN and improved various pathological changes [21]. PD has been shown to reverse renal dysfunction, ameliorate renal inflammation, and reduce fasting glucose in STZ-induced DN rats [12]. In addition, PD suppressed HG-induced proliferation of glomerular mesangial cells and inhibited ECM accumulation, which suggests that PD can be used for DN treatment [22]. TLR4 can activate the NF-κB signaling pathway, which is involved in regulating inflammation [23]. This leads to the production of chemokines and inflammatory cytokines, which contribute to DN progression. Ma *et al* showed that HG can promote TLR4 activation, NF-κB activation, and interstitial fibrosis in podocytes and tubular epithelial cells [24]. Lin *et al* reported that TLR4 silencing inhibited HG-induced expression of inflammatory cytokines such as IκB/NF-κB, IL-6, C-C motifs, and CCL-2. Some studies reported that PD depressed the NF-κB pathway in glomerular mesangial cells [21,25]. These investigations implicated the involvement of TLR4/NF-κB signaling in DN progression. The results of the present study suggest that PD significantly suppressed the TLR4/NF-κB pathway *in vitro* and *in vivo*. There, the protective role of PD in DN may be at least partially

mediated through regulation of the TLR4/NF-κB pathway.

CONCLUSION

The results demonstrate that PD exerts a protective role in DN by decreasing interstitial injury, inhibiting renal fibrosis, reducing the inflammatory response, and suppressing cell apoptosis, at least, partly by downregulation of TLR4/NF-κB signaling.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was completed by the researchers listed in this article. All liabilities related to the content of this article will be borne by the authors. Huimin Niu and Gang Li designed all the experiments and revised the paper. Yanhong Qiao performed the experiments, and Feng Wang wrote the manuscript.

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