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Original Article

Paeoniflorin inhibits TGF-β1-Smad2/3 and NF-κB signaling pathways in high fat diet-induced kidney injury and inflammatory responses

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Abstract: Due to the high morbidity and mortality rates, kidney diseases have been treated as a global health problem. However, the causes of their formation and development remains to be fully elucidated. High fat diet (HFD)-induced kidney injury has attracted increasing attention. Paeoniflorin, an anti-inflammatory chemical compound found in the herbal medicine derived from Paeonia lactiflora Pall. Previous investigations of paeoniflorin have found it possesses several pharmacological effects, including anti-inflammatory, neuroprotective effects, and suppression of insulin resistance. Therefore, in this study, we will examine the underlying molecular mechanisms of paeoniflorin in the inhibition of HFD-induced kidney fibrosis. Rats fed with HFD were treated with or without paeoniflorin to investigate whether it offers a protective effect, and to determine the underlying molecular mechanism that could remit renal fibrosis. Reverse transcription-quantitative polymerase chain reaction, western blot analyses, Masson's staining and immunohistochemical analyses were performed to analyze the mRNA and protein expression of associated indicators in the treatment groups. The oral glucose and insulin tolerance, urinary protein and serum creatinine analyses were also performed to indicate changes in the metabolic and kidney functions of the rats. ELISA analysis was utilized to measure the effects of the inflammatory cytokines in the serum. The results indicated that paeoniflorin treatment suppressed renal fibrosis and the production of inflammatory cytokines by altering expression of NF-κB pathways and TGF-β1/Smad-in the kidneys from the HFD-fed rats. These findings provide a potential approach for treating fat rich diet-stimulated kidney-related diseases using natural products.

Keywords: Paeoniflorin, high fat diet, kidney fibrosis, TGF-β1, inflammation

Introduction

Chronic kidney disease has been treated as life-threatening to individuals worldwide that can affect the function and structure of kidney [1]. Due to recurrent hospitalization, and accelerated mortality and morbidity rates, increasing investigations have been devoted to investigating the underlying molecular mechanisms. For the last stage of chronic kidney disease, renal fibrosis is the common pathway in the progression of chronic kidney disease towards kidney failure [2, 3]. The formation of fibrosis can be stimulated by chronic glomerulonephritis, chronic pyelonephritis, obstructive nephropathy, systemic lupus, hereditary kidney disease, diabetic nephropathy, hypertensive kidney disease, drug-induced kidney disease, hepatitis B or human immunodeficiency virus-induced kidney disease, and renal transplantation [4-8]. Among these factors, eating habits as a causal factor in inducing kidney disease have received wide attention. In previous reports, diet-induced increases in plasma oxidized low-density lipoprotein (LDL) have been found to promote early fibrosis in a porcine renal auto-transplantation model [9-11]. In addition, high fat diet (HFD)-induced obesity has been found to alter the expression of kidney-associated genes and lead to the formation of chronic kidney diseas-

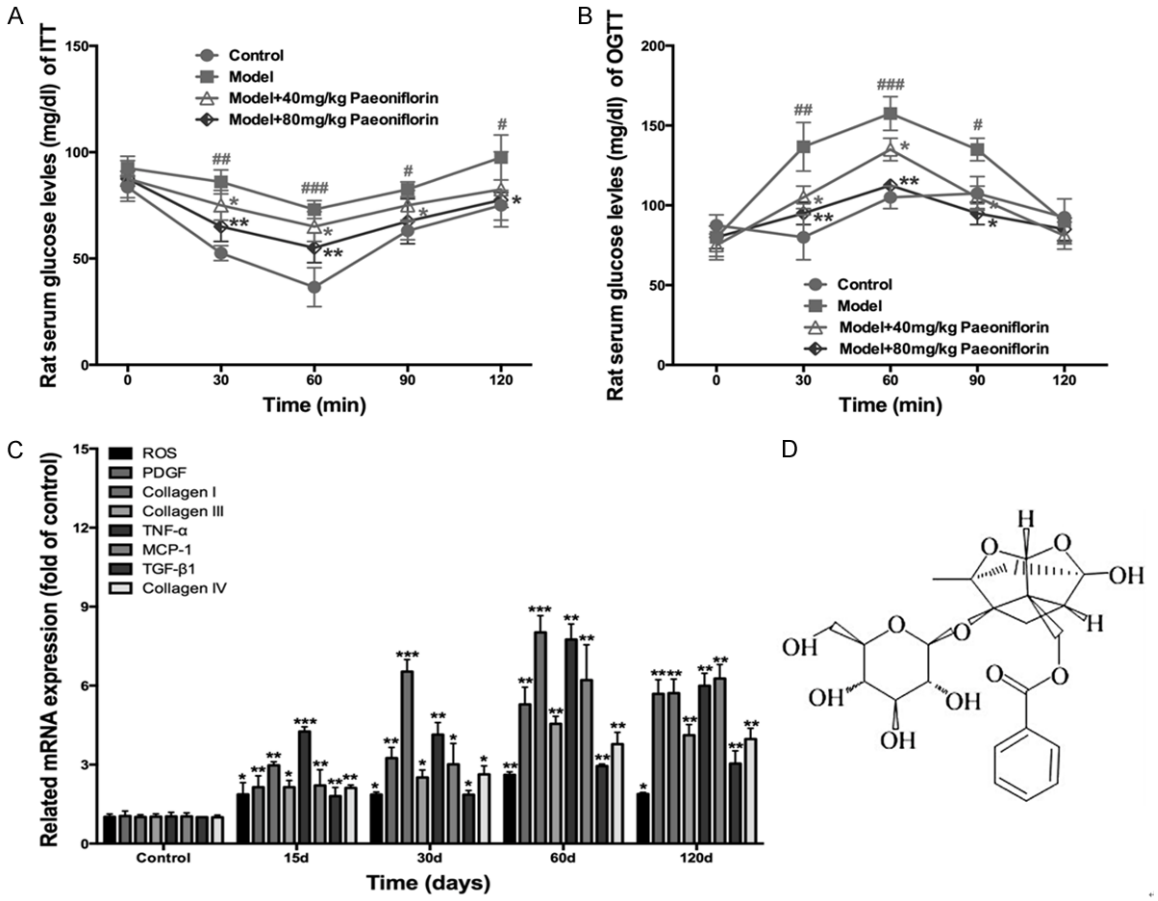

Figure 1. Paeoniflorin alters metabolism and kidney function in the high fat diet (HFD)-induced rats. A. Results of an insulin tolerance test (ITT) of the rats. B. Results of an oral glucose tolerance test (OGTT) of the rats. C. Reverse transcription-quantitative polymerase chain reaction analysis of the expression of renal fibrosis-associated genes at different experimental time points after HFD feeding. D. Structure of paeoniflor in. Data are expressed as the mean ± standard error of the mean (n=10-15). $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, vs. control group; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, vs. model group. TNF-α, tumor necrosis factor-α; TGF-β1, transforming growth factor-β1; PDGF, platelet-derived growth factor; MCP-1, monocyte chemoattractant protein-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

es [12-14]. These changes alter the normal physiological activity and promote type II diabetes, enhancing the development of kidney injury and formation of fibrosis [15]. However, although the substantial progress in medicine has expanded our knowledge of kidney-associated diseases, few novel therapies have been introduced. Therefore, additional intervention, including chemopreventive treatment from natural products, may be promising for the treatment of kidney injury.

Paeoniflorin (**Figure 1D**) is an anti-inflammatory chemical compound found in the herbal medicine, derived from Paeonia lactiflora Pall [16]. It also can be extracted from P. suffrsticosa Andrroot and P. delarayi Franch. Previous investigations of paeoniflorin have revealed several pharmacological effects, including anti-inflammatory effects, suppression of insulin resistance, and neuroprotective activities [17-20]. In the development of kidney fibrosis, the transforming growth factor-β1 (TGF-β1)/small mothers against decapentaplegic (Smads) pathway is considered to have the most marked effect in the promotion of fibrosis formation and kidney injury [21-23]. HFD-induced kidney damage has also been considered to be connected with the fibrosis development [24, 25]. However, the molecular mechanism of HFD-stimulated kidney fibrosis remains to be fully elucidated, and there are no associated reports on whether

Table 1. The list of primer sequences for reverse transcription-quantitative polymerase chain reactionRT-qPCR analysis

Gene	Forward primers (5'-3')	Reverse primers (5'-3')
TNF-α	TGTGCATTCGTCTCTCTTGG	GATGGTTGATGGAGGCACTT
TGF-β1	GCGGACTACTATGCTAAAGAGG	GTAGAGTTCCACATGTTGCTCC
ROS	GTGCCCACGTGAAGGAGTAT	ACTTGGGGACACCCTTTAGC
PDGF	CAGACAAGAAGAGGTTGCC	CGTCAGGCAGTTTGTATTGG
MCP-1	CTGTCACGCTTCTGGGCCTGT	GCAGCAGGTGAGTGGGGCAT
Collagen I	ACCTCCCGCCTGCCCATCAT	CACGAAGCAGGCAGGGCCAA
Collagen III	CAAGGTCCTTCTGGATCAAGTG	CCTTTATGCCTCTGTCACCTTG
Collagen IV	GCCCTACGTTAGCAGATGTACC	TATAAATGGACTGGCTCGGAAT
GAPDH	CAAGTTCAACGGCACAGTCAAGG	ACATACTCAGCACCAGCATCACC

TNF-α, tumor necrosis factor-α; TGF-β1, transforming growth factor-β1; PDGF, platelet-derived growth factor; MCP-1, monocyte chemoattractant protein-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

paeoniflorin has the ability to provide a positive treatment effect for fibrosis in rats. Therefore, in this study, the HFD was utilized to build the kidney fibrosis model to characterize the therapeutic effects of paeoniflorin.

Materials and methods

Animals and drugs administration

The animals used in the present study comprised 6-8 week-old male Sprague-Dawley rats, weighing 200-220 g, which were purchased from the Animal Experimental Center of Nanjing Medical University (Nanjing, China). The Institutional Animal Care and Use Committee at Chinese Medicine Hospital of Jining approved all the animal experimental protocols. Paeoniflorin (CAS: 23180-57-6; ≥ 98% high-performance liquid chromatography) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and prepared in phosphate-buffered saline (PBS). The diet provided (D12492i; 60 kcal% fat) was obtained from Open Source Diets, Inc. (New Brunswick, NJ, USA). All rats were divided into four groups: i) Control; ii) HFD group as a model; iii) HFD+40 mg/kg paeoniflorin; iv) HFD+80 mg/kg paeoniflorin. Paeoniflorin was fed every two days. Following a 120-day experimental period, blood and liver tissues were collected for further investigation. In addition, the kidney tissue was obtained at 15, 30, 60 and 120 days for reveres transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.

Cells culture

HK2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and plated at a density of 1×10^4 cells/well in 6-well plates. The cells were treated with 20 mg/ml TGF-β1 for 12 h and then incubated with or without 1 mg/ml paeoniflorin for 24 h at 37°C and 5% CO_2.

Biochemical analysis

The rats were sacrificed after the final treatment by decapitation at 9:30-10:30 a.m. following a 16-h fast in order to avoid fluctuations in hormone levels due to circadian rhythms. Prior to this, the oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were operated, in accordance with the methods described in a previous report [26]. Extraction of the eyeball blood was performed, and kidney tissues were dissected and rapidly placed on ice, certain sections of which were immediately fixed for histological analysis. The left samples were stored in liquid nitrogen for RT-qPCR and western blot analyses. The serum concentrations of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 were measured using ELISA kits, according to the protocols (R&D Systems, Inc., Minneapolis, MN, USA). In addition, serum creatinine and blood urea nitrogen were characterized by a test kit from Jiancheng Biotech Co., Ltd. (Nanjing, China).

Histology analysis

The collected liver samples were treated for Masson's staining for determining the levels of fibrosis, as described previously [27]. Immunohistochemical (IHC) test was operated to analyze the expression of TGF-β1 in the kidney, and IHC-streptavidin-peroxidase (IHC-SP) analysis was used for determining the levels of IL-6 and TNF-α in the renal tissues. In addition, the expression levels of α-smooth muscle actin (SMA) and E-cadherin in the TGF-β1-induced

Table 2. Effects of paeoniflorin on physiological indexes and biochemical parameters in high fat-induced rats on day 120

Parameter	Control	HFD	HFD+Paeoniflorin 40 mg/kg	HFD+Paeoniflorin 80 mg/kg
Body weight gain (g)	398.60±6.00	324.4±10.23[c]	378.8±11.65[e]	393.00±9.24[f]
Liver weight (g)	4.23±0.34	2.63±0.57[b]	3.53±0.41	4.02±0.60[e]
Kidney weight (g)	1.27±0.06	1.51±0.09[a]	1.28±0.13[d]	1.26±0.07[d]
Urinary protein (mg/24 h)	5.12±0.63	11.29±1.31[b]	7.24±0.50[d]	6.01±1.01[e]
Serum creatinine (μmol/L)	43.22±2.11	92.45±2.41[b]	66.90±1.47[d]	61.37±2.77[e]
Blood urea nitrogen (mmol/L)	4.41±0.91	8.12±1.05[b]	7.52±1.11	5.07±0.56[d]
Serum IL-1β (ng/L)	6.27±0.73	11.84±0.57[a]	7.51±0.36	6.39±0.32[d]
Serum IL-6 (ng/L)	28.78±1.09	32.97±0.96[c]	30.51±1.26	29.03±1.12[f]
Serum TNF-α (ng/L)	24.86±2.38	32.64±1.72[b]	32.60±2.05	23.93 ±2.17[f]

Data are expressed as the mean ± standard error of the mean (n=15). [a]P<0.05, [b]P<0.01 and [c]P<0.001, vs. control group; [d]P<0.05, [e]P<0.01 and [f]P<0.001, vs. HFD group. TNF-α, tumor necrosis factor-α; IL, interleukin; HFD, high fat diet.

HK2 cells were analyzed using the IHC method with the following antibodies: Anti-E-cadherin (cat. no. 24E10) Rabbit mAb (cat. no. CST-3195; Cell Signaling Technology, Danvers, MA, USA), anti-α-SMA antibody (cat. no. ab125044; Abcam, Cambridge, MA, USA) and anti-TGF-β1 antibody (ab92486; Abcam). The tissues were then analyzed using an Olympus-CX31-LV320 microscope (Olympus, Tokyo, Japan).

RT-qPCR

The RNA was purified from kidney tissues by total RNA isolation system (Roboklon, Berlin, Germany) kits. In detail, 2 μg of total RNA was reverse transcribed through the M-MLV-RT system (Promega Corporation, Madison, WI, USA). The procedure was performed at 42°C for 1 h and terminated by deactivation of the enzyme at 70°C for 10 min. qPCR analysis was performed using an SYBR Green kit in an ABI PRISM 7900HT detection system. Invitrogen (Thermo Fisher Scientific, Inc.) produced all primers for qPCR, the sequences of which are listed in **Table 1**. The thermal cycling conditions were shown below: Pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 sec, annealing at 58°C for 15 sec and extension at 72°C for 20 sec. The gene expression levels were according to the housekeeping gene (GAPDH) and expressed as a fold of the control. The amount of the target gene was normalized to the endogenous reference (HPRT) and then relative to a calibrator (control animal), using the $2^{-\Delta\Delta Cq}$ method [28-30]. Steady-state mRNA levels are expressed as an n-fold difference relative to the calibrator.

Western blot analysis

Cells or tissues were washed in PBS and destroyed in lysis buffer [PBS containing 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM orthovanadate and Roche protease cocktail inhibitor (Roche, Basel, Switzerland)]. The final supernatants were got by centrifugation at 12,000 x g for 20 min. Protein concentrations were measured by a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.) with bovine serum albumin (BSA) as a standard. Equal quantities of total protein were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were then blocked in 5% BSA followed by immunoblotting using the following antibodies at dilutions of 1:1,000: Rabbit anti-nuclear factor-κB (NF-κB; ab8856), anti-TGF-β1 (ab92486), rabbit anti-GAPDH (ab-9485), anti-inhibitor of NF-κB (IκBα; ab32-518), anti-IκB kinase β (IKKβ; ab7217), anti-Smad2 (ab40855) and anti-Smad3 (ab40854) (Abcam, Cambridge, MA, USA). After that, the TSA buffer was used to wash the membranes and incubated them with horseradish peroxidase-labeled goat anti-rabbit. The western blot bands were visualized using a GE Healthcare ECL Western Blotting Analysis System (GE Healthcare Piscataway, NJ, USA) and exposed to Kodak X-ray film (Kodak, Rochester, NY, USA).

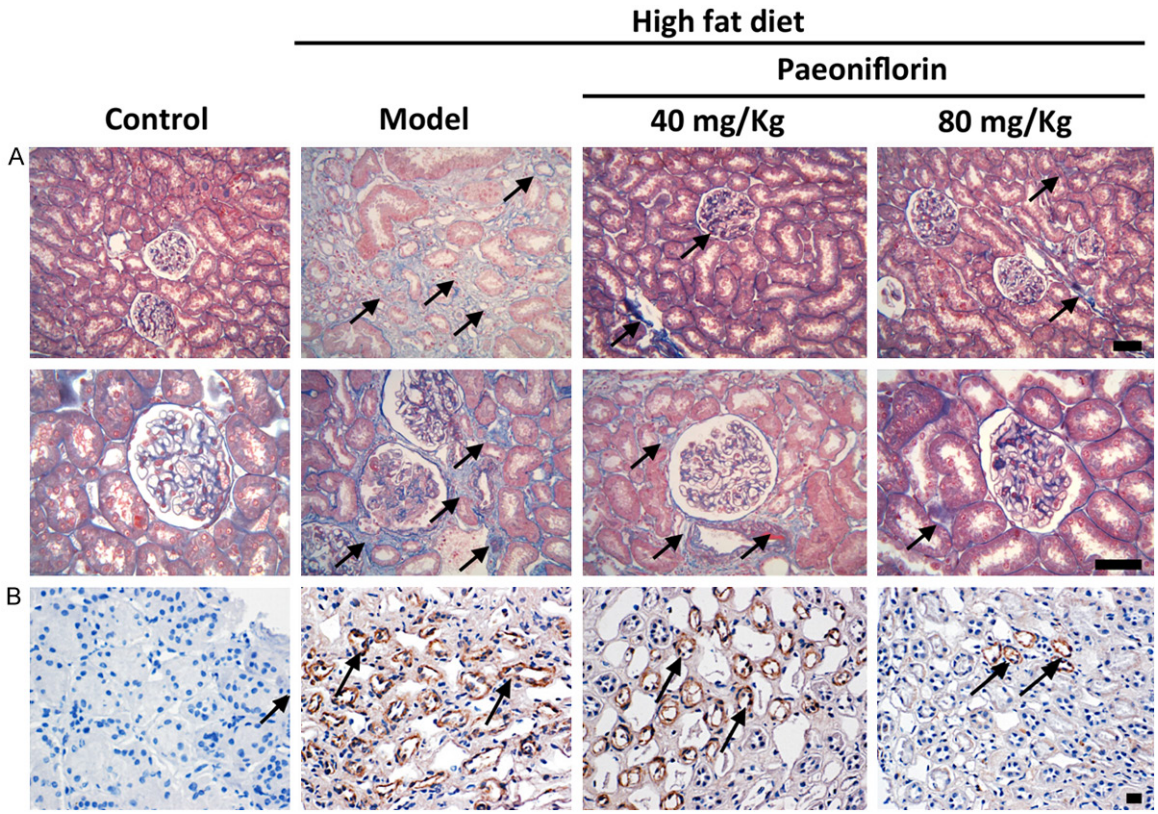

Figure 2. Paeoniflorin suppresses kidney fibrosis formation and the expression of collagen and TGF-β1. A. Masson's staining analysis for collagen in high fat diet-induced renal fibrosis in rats. Black arrows indicate collagen expression. B. Immunohistochemical analysis of the protein expression of TGF-β1 in the kidney of the high fat diet-induced rats. Black arrows indicate TGF-β1-positive cells. TGF-β1, transforming growth factor-β1.

All the bands were characterized by ImageJ version 1.48.

Statistical analysis

In this study, the mean ± standard error of the mean was used to analyze the data. The targetted tissues and control groups were compared using Graph Pad PRISM by one-way analysis of variance with Dunn's least significant difference test. $P<0.05$ was recognized to state the significant difference statistically.

Results

Effect of paeoniflorin on metabolism and kidney function

In this study, the metabolic changes of HFD-fed rats with and without paeoniflorin were characterized. It is easy to see that the paeoniflorin could alter metabolism and kidney function in HFD-induced rats. As shown in **Table 2**, the rats in HFD+Paeoniflorin group on day 120 exhibited increased body and liver tissues' weight compared with the HFD-fed only groups, but close to the values in control groups. However, although the kidney tissues' weight is similar to the control group from the rats in HFD+Paeoniflorin group, this value is much less than the weight from HFD-fed only group on day 120. Results of insulin tolerance test (ITT) and oral glucose tolerance test (OGTT) of the rats are shown in **Figure 1A** and **1B**. The results showed that the insulin tolerance and oral glucose tolerance was changed far away from the normal control groups in the HFD model groups. However, both of them were changed to close to the normal control groups when adding paeoniflorin in HFD model groups. In addition, as shown in **Table 2**, the use of ELISA for serum analysis showed that HFD enhanced the release of inflammatory cytokines, including IL-6, IL-1β, and TNF-α. The serum levels of urinary protein and serum creatinine were also elevated in the HFD group and were downregulated by paeoniflorin treat-

ment. Taken together, the HFD was capable of altering metabolic activities and weakening renal function. However, these responses may be reversed by paeoniflorin administration in a dose-dependent manner.

Paeoniflorin suppresses the formation of kidney fibrosis and the expression of associated genes

Following the 120-day experimental period, in this study, RT-qPCR was utilized to study the expression levels of fibrosis-associated genes in the kidney, collected at different time points. As the experimental period progressed (**Figure 1C**), the expression levels of collagen I, III and IV, the major markers for the determination of fibrosis, increased in a time-dependent manner; Moreover, upregulated levels of ROS, TGF-β1 pathway activation, platelet-derived growth factor (PDGF) and monocyte chemoattractant protein-1 (MCP-1) were observed as the duration of exposure to the HFD increased. The results of the Masson's staining in **Figure 2A** demonstrated that the HFD significantly enhanced the expression of collagen and TGF-β1. These data are consistent with the data from the RT-qPCR analysis. Compared with the model group, the formation of kidney fibrosis and expression of associated genes may be decreased by different concentrations of paeoniflorin.

Paeoniflorin inhibits TGF-β1 and NF-κB signaling pathway activation in HFD-induced rats

This study also evaluated the function of a TGF-β1 signaling pathway in the development of renal fibrosis. IHC analysis of the expression of TGF-β1 in the kidney (**Figure 2B**) proved that TGF-β1 activation was downregulated as the paeoniflorin dose increased. In addition, IHC-SP analysis was used to determine the levels of IL-6 (**Figure 3A**) and TNF-α (**Figure 3B**) in renal tissue. As expected, the HFD enhanced the levels of IL-6 and TNF-α, and these were inhibited by paeoniflorin administration. In addition, as shown in **Figure 3C**, the relative protein expression of the TGF-β1 pathway that was utilized to evaluate its activation in HFD-fed rats was characterized by western blot. The bands showed that the TGF-β1/Smad and NF-κB signaling pathways were related to the development of fibrosis and were significantly activated, compared with the control. These were suppressed by paeoniflorin treatment.

Effect of paeoniflorin on TGF-β1-induced HK2 cells

The present study also performed *in vitro* experiments to examine the effect of paeoniflorin on TGF-β1-induced HK2 cells. As expected (**Figure 4**), TGF-β1 incubation promoted morphological change and caused the expression of α-SMA in HK2 cells, as well as downregulated the expression of E-cadherin. This indicated that TGF-β1 was a key inducer, with the ability to promote collagen formation and activate kidney fibrosis-associated signaling pathways. However, paeoniflorin treatment was found to reverse the responses observed in the TGF-β1 group.

Discussion

Due to its high morbidity and mortality rates, chronic kidney diseases have become a public health concern. Recently, the renal fibrosis and damage have increased markedly. Fibrosis is the inappropriate repair of the kidney tissues from the chronic injury including alcohol-induced damage, chronic inflammation, autoimmune disease, metabolic and parasitic diseases [31]. It even could develop into renal failure and uremia if the fibrosis is going mildly. In contrast to the suggestion that fibrosis is an irreversible state, there is evidence that the fibrosis can be reversible. Therefore, hundreds of millions of individuals worldwide have suffered from kidney fibrosis [32]. In addition, the kidney diseases that was resulted from eating habits have received wide attention. HFD-induced obesity can alter the expression of kidney-associated genes and lead to the formation of chronic kidney diseases [12, 13]. Diabetes can also enhance the development of kidney injury and formation of fibrosis [33]. Therefore, the efficient way to cure chronic renal disease is the inhibition of fibrogenesis at the early stage.

Paeoniflorin, a natural product isolated from the dried root of Paeonia lactiflora Pall (Ranunculaceae), has shown neuroprotective, anti-inflammatory and antifibrotic effects in the liver [17-19]. However, to the best of our current understanding, the molecular mechanisms of how fibrosis forms in chronic renal diseases,

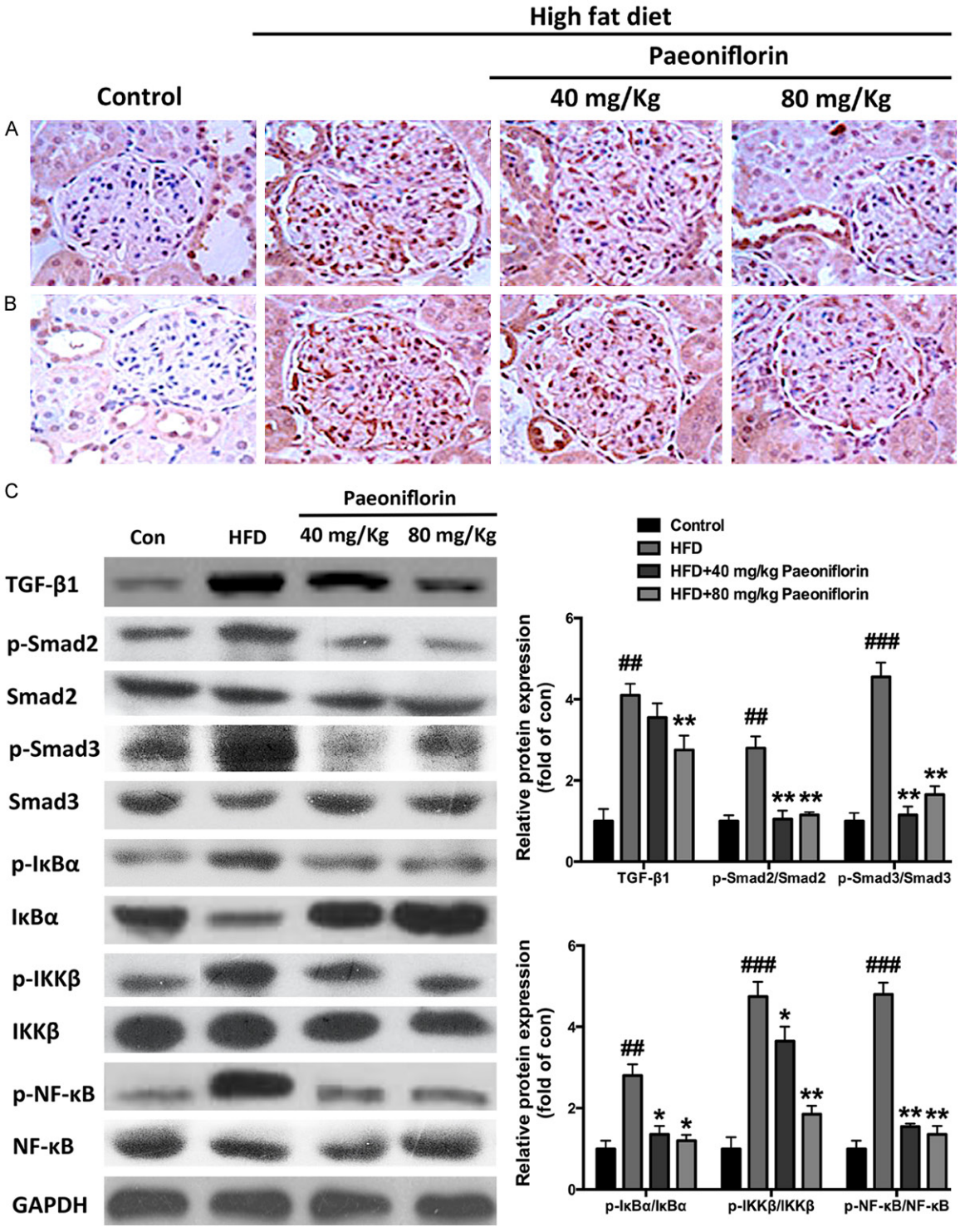

Figure 3. Effects of paeoniflorin on TGF-β1 and NF-κB signaling pathway activation. Immunohistochemical-streptavidin-peroxidase analysis of the expression levels of (A) IL-6 and (B) TNF-α in the kidney of high fat diet-fed rats. (C) Western blot analysis for the expression of TGF-β1 and NF-κB signaling pathway-associated proteins in the kidney of high fat diet-fed rats. Data are expressed as the mean ± standard error of the mean (n=10-15). $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, vs. control group; $^{*}P<0.05$, $^{**}P<0.01$ vs. model group. TGF-β1, transforming growth factor-β1; Smad, small mothers against decapentaplegic; p-, phosphorylated; NF-κB, nuclear factor-κB; IκBα, an inhibitor of NF-κB α; TNF-α, tumor necrosis factor-α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Con, control; HFD, high fat diet.

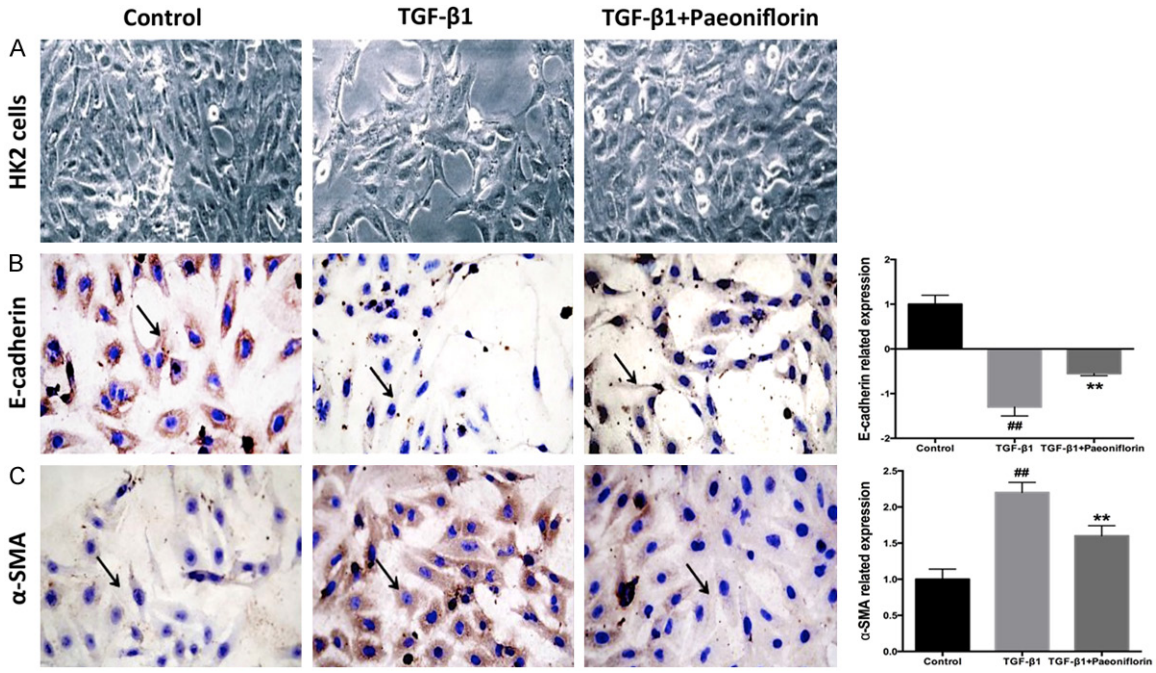

Figure 4. Effect of paeoniflorin on TGF-β1-induced HK2 cells. HK2 cells were plated at 1×10^4 cells/well in 6-well plates and treated with 20 mg/ml TGF-β1 for 12 h, followed by incubation with or without 1 mg/ml paeoniflorin for 24 h. A. Effects of TGF-β1 incubation on morphological changes in HK2 cells. B. Effects of TGF-β1 administration on the expression of E-cadherin in HK2 cells. C. Effects of TGF-β1 administration on the expression of α-SMA in HK2 cells. Data are expressed as the mean ± standard error of the mean (n=10-15). Black arrows indicate TGF-β1-positive cells. ##P<0.01 vs. control group, **P<0.01 vs. model group. TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin.

and whether there is an effective treatment strategy to prevent this, remain to be fully elucidated. Therefore, this study showed that paeoniflorin led to recovery from a renal function via suppressing serum creatinine and urine protein excretion. The results also demonstrated that paeoniflorin attenuated the progression of fibrosis by downregulating the expression of collagen I, collagen III, collagen IV, IL-6, IL-1β, TNF-α and MCP-1 in the kidneys. In addition, paeoniflorin suppressed renal fibrosis and inflammation via altering the expression of the TGF-β1/Smads and NF-κB pathways in the kidneys of the HFD-fed rats. An HFD has been regarded as a causal factor in a number of diseases, including non-alcoholic fatty liver disease, nerve injury, renal injury and diabetes [34]. In previous reports, unilateral ureteral obstruction and drugs, including cyclosporin A, adrenomedullin and adenine have been widely used to induce renal fibrosis in models, which have been widely used in the investigation of kidney-associated diseases [35]. However, whether an HFD causes kidney fibrosis, and whether the TGF-β1 pathways are involved in the developmental process remain to be fully elucidated. As previously reported, the TGF-β1/Smads signaling pathway is critical for the development of liver and kidney fibrosis and has been the significant focus in the investigation of embryonic development, organ formation, cell proliferation, differentiation and apoptosis [36]. Therefore, this study could demonstrate that an HFD could significantly enhance the expression of TGF-β1, the activation, and phosphorylation of Smads, and promoted the formation of kidney fibrosis. Paeoniflorin treatment downregulated these responses in a typical dose-dependent manner.

In addition, it was hypothesized that paeoniflorin suppresses kidney fibrosis by suppressing HFD-induced inflammatory responses. TLRs, the signal transduction molecules, is very important for the induction of immunity in host defense. Moreover, the TLR4 activation could utilize myeloid differentiation factor-88 (MyD88). MyD88, the adaptor protein, could utilize

IL-1 receptor to lead to the nuclear translocation of NF-κB, subsequently initiating the transcription of genes linked to inflammation. Despite the close link between inflammation and fibrosis, the crosslink among these key events and the intracellular signal transduction pathways remains to be fully elucidated. TLR4 is activated by LPS in HK2 cells that can lead to NF-κB activation, and the subsequent transcriptional activation of pro-inflammatory mediators, including TNF-α. TNF-α also activates the NF-κB signaling pathway in other cells, which is crucial for their survival. The upregulation of pro-inflammatory mediators is involved in chronic kidney inflammation, including IL-6, TNF-α, and IL-1β, which are important for the pathogenesis of renal fibrosis. The present study demonstrated the association between the inflammation-associated signaling pathways of TLR4/NF-κB and fibrosis. The present study also found that paeoniflorin significantly reduced the expression levels of TNF-α, p-NF-κB, IL-6, and IL-1β that have been induced by HFD in the kidneys of the rats, which indicated the protective effect of paeoniflorin on inhibiting inflammation in rats with fibrosis.

In conclusion, the results from this study could be utilized to prove that paeoniflorin may be a potential therapeutic candidate for HFD-stimulated kidney-related diseases.

Disclosure of conflict of interest

None.

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