Paeoniflorin ameliorates acute necrotizing pancreatitis and pancreatitis-induced acute renal injury

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Abstract. Acute renal injury caused by acute necrotizing pancreatitis (ANP) is a common complication that is associated with a high rate of mortality. Paeoniflorin is the active ingredient of paeonia radix and exhibits a number of pharmacological effects, such as anti-inflammatory, anticancer, analgesic and immunomodulatory effects. The present study detected the potential treatment effects of paeoniflorin on acute renal injury induced by ANP in a rat model. The optimal dose of paeoniflorin for preventing acute renal injury induced by ANP was determined. Then, the possible protective mechanism of paeoniflorin was investigated. The serum levels of tumor necrosis factor (TNF) -α, interleukin (IL) -1β and IL -6 were measured with enzyme-linked immunosorbent assay kits. Renal inflammation and apoptosis were measured by immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. The expression of nitric oxide in kidney tissues was also evaluated. The p38 mitogen-activated protein kinases (MAPKs) were measured by western blotting. The results shown that paeoniflorin may ameliorate acute renal injury following ANP in rats by inhibiting inflammatory responses and renal cell apoptosis. These effects may be associated with the p38MAPK and nuclear factor-κB signal pathway.

Introduction

Acute necrotizing pancreatitis (ANP) is a sudden and serious inflammatory disease of the human digestive system (1). Clinical data has indicated that ANP is a serious inflammatory disease with a systemic inflammatory response and multiple organ dysfunction (2). Although the pathogenesis of ANP is not completely clear, the activation of inflammatory cytokines is key in the etiology (3-5). During ANP, a number of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 are released into the blood, which can result in sepsis (6), and mortality (7). Thus, controlling the initial inflammatory response is important for controlling ANP and renal injury induced by ANP. ANP is not only localized to the pancreas but can also activate inflammatory responses throughout the body, it is a systemic disease and can result in systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction (8). ANP-induced renal injury is a frequent and early complication of ANP, and the mortality rate of patients with ANP-induced renal failure is ~71.2% (9). Thus, it is essential to protect renal function in patients with ANP.

Paeoniflorin (PF) is the active ingredient of Radix Paeoniae (10), a traditional herbal medicine and the dried root of Paeonia lactiflora Pallas. PF exhibits numerous pharmacological effects, such as anti-inflammatory, anticancer, analgesic (11) and immunomodulatory effects (12-14). PF can inhibit the systemic inflammatory response and improve the survival of rats with lipopolysaccharide (LPS)-induced sepsis (15). However, the pharmacological effect of PF on ANP and renal injury induced by ANP remains unclear. Evidence demonstrates that the p38 mitogen-activated protein kinase (MAPK) signaling pathway is closely associated with the nuclear factor (NF)-κB signaling pathway, which is a critical cell signaling pathway in the inflammatory response. Thus the p38 MAPKs signaling pathway is a focus of research into the inflammatory response. p38MAPKs are members of the MAPKs signaling pathway, which includes p38MAPKs, c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase kinase. Its function is associated with the growth and apoptosis of cells (16). The p38 and JNK signaling molecules are key in the inflammatory signaling pathway. p38MAPKs can be triggered by proinflammatory cytokines, such as TNF-α and IL-6 (17,18), and can mediate the inflammatory response induced by LPS (19). The p38MAPK signaling pathway has been demonstrated to be important in the progress of ANP and the organ dysfunction following ANP (20-22).

Thus, the present study used sodium taurocholate to generate an ANP model in rats and investigate the pharmacological
effect of PF in renal injury induced by ANP, and whether the p38 MAPK pathway is important in this process. The aim of the present study was to obtain the optimal dose of PF for preventing acute renal injury induced by ANP, and to investigate the effects and underlying mechanisms of PF on acute renal injury following ANP.

Materials and methods

Reagents. PF (C_{22}O_{29}H_{11}· FW, 480.45; purity ≥95%) was purchased from Nanjing Tcm Institute of Chinese Materia Medica (Nanjing, China). Rat TNF-α, IL-1β and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from the Huaeui Biology Technology Company (Wuhan, China). Nitric oxide (NO) detection kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit anti-NF-κBp65 (1:200; ab16502), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2,500; ab8245) and anti-caspase3 (1:100; ab2302) polyclonal antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-p-p38 (1:1,000; 8690) and anti-p-(1:1,000; 4511) polyclonal antibodies were purchased from Cell Signaling Technologies, Inc. (Hercules, CA, USA). The terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kits was purchased from Roche Diagnostics (Indianapolis, IN, USA).

Animals. A total of 128 Sprague Dawley (SD) rats (weight, 180-200 g), were purchased from the Center of Experimental Animals of Wuhan University (Wuhan, China). The rats were maintained in specific pathogen-free conditions at room temperature under a 12 h light-dark cycle with ad libitum access to water. The rats underwent fasting prior to operation. The rats were maintained in accordance with the principles of the 1983 Declaration of Helsinki. The present study was approved by the Ethics Committee of Wuhan University (Wuhan, China).

Experimental design. In the first part of the present study, 48 SD rats were randomly divided into six groups (8 rats/group), as follows: i) The sham-operated group; ii) the ANP group; iii) the ANP+P1 group (50 mg/kg PF); iv) the ANP+P2 group (100 mg/kg PF); v) the ANP+P3 group (150 mg/kg PF); and vi) the vehicle group (normal saline at the same volume). In order to induce severe ANP, the rats were fasted overnight and given fresh tap water ad libitum. Anesthesia was administered by intraperitoneal injection with 10% chloral hydrate (3 ml/kg; Wuhan Goodbio Technology Co., Ltd., Wuhan, China). The pancreatic bile duct was cannulated through the duodenum, and ANP was induced by a standardized retrograde infusion of a freshly prepared 5% sodium taurocholate solution (1 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) into the biliary-pancreatic duct. Isotonic saline solution (20 ml/kg) was injected into the back of the rats to compensate for fluid loss. A sham-operated group received isotonic saline solution instead of sodium taurocholate. For the PF-treated groups, PF was dissolved in vehicle (normal saline) and intravenously injected into the femoral vein 30 min following the generation of the ANP model. The rats were sacrificed 12 h following the induction of ANP by exsanguination following anesthesia, as the pancreatic damage reached a climax at that time point (23). The effect of PF was evaluated by determining the levels of serum amylase (AMY), serum lipase (LIPA), aspartate-transaminase (AST), alanine-aminotransferase (ALT), and the pathological changes of pancreas shown by hematoxylin & cosin (H&E) staining.

Following the initial experiment, the 100 mg/kg dose of PF was determined to be the optimum dose. Subsequently, 72 SD rats were divided into three groups: i) The 3 h group; ii) the 6 h group; and iii) the 12 h group. These groups were further divided into three subgroups (8 rats/subgroup), including the sham-operated group, the ANP group and the ANP+PF group (PF group in the figures). For each part of study the rats were sacrificed by exsanguination under anesthesia. Blood samples were collected from the postcava and plasma was collected following centrifugation at 12,000 x g for 10 min at 4°C. The pancreas and kidneys were harvested and immediately frozen in liquid nitrogen, after which they were stored at -80°C for subsequent experiments. Sections of the pancreas and kidneys were fixed in 4% paraformaldehyde (Wuhan Goodbio Technology Co., Ltd.).

Enzyme assay. The serum levels of AMY, LIPA, AST, ALT, BUN and Cr were measured using the Automatic Biochemistry Analyzer (Olympus Corporation, Tokyo, Japan), according to a standard technique (24).

Histopathological examination. The paraffin-embedded tissues were cut into 3-µm sections for pathological examination by H&E staining. The H&E results were observed under a light microscope (Olympus BX55; Olympus Corporation) by two experienced pathologists who were blinded to the groups. The pancreatic pathological scores were measured according to the degree of edema, inflammation, vacuolization and necrosis as described by Schmidt et al (25). The kidney pathology score was used for evaluating the severity of the renal injury. For each kidney, 100 cortical tubules from at least 10 different views were analyzed. Higher scores represented more severe damage (maximum score per tubule was 10), with points given for the following: The presence and extent of tubular epithelial cell flattening (1 point), brush border loss (1 point), cell membrane bleb formation (1 or 2 points), cytoplasmic vacuolization (1 point), cell necrosis (1 or 2 points), interstitial edema (1 point) and tubular lumen obstruction (1 or 2 points) (26).

Enzyme-linked immunosorbent assay (ELISA). The serum levels of TNF-α, IL-1β and IL-6 were measured using ELISA kits, according to the manufacturer’s instructions.

Immunohistochemistry assay. The paraffin-embedded kidney tissues were cut into 3-µm sections, and the levels of NF-kB and caspase-3 were determined by immunohistochemical analyses. Briefly, sections were deparaffinized and dehydrated using a graded series of ethanol solutions. The slides were immersed in 10 mM citrate buffer (pH 6.0) and boiled for 15 min in a microwave for antigen retrieval. Then the slides were allowed to cool at room temperature. Hydrogen peroxidase (0.3%) was used to halt the endogenous peroxidase activity for 15 min at room temperature. Non-specific binding was blocked by incubation with goat serum (5%) for 10 min, after which the sections were
Table I. Effect of paeoniflorin on enzyme levels.

| Group               | AMY (U/l)       | LIPA (U/l)     | ALT (U/l)     | AST (U/l)     |
|---------------------|-----------------|----------------|---------------|---------------|
| Sham-operated       | 1755.7±214.0    | 4417.9±553.1   | 40.1±4.8      | 127.9±37.2    |
| ANP                 | 8287.5±1394.5   | 14599.5±1741.5 | 183.5±54.6    | 618.8±165.7   |
| ANP+Paeoniflorin (mg/kg) |                |                |               |               |
| 50                  | 7244.1±793.2    | 14190.3±803.1  | 158.6±24.8    | 480.3±70.4    |
| 100                 | 6333.1±683.9    | 12675.0±735.2  | 114.1±9.4     | 357.6±51.7    |
| 150                 | 5244.3±445.8    | 11362.8±905.0  | 275.2±84.5    | 514.0±65.6    |
| NS                  | 1865.3±205.9    | 4489.7±649.3   | 50±17.9       | 142.6±29.3    |

Data are expressed as the mean ± standard deviation; n=8 in each group. *P<0.05 vs. the sham-operated group; †P<0.05 vs. the ANP group; ‡P<0.05 vs. the paeoniflorin 50 mg/kg group. AMY, amylase; LIPA, lipase; ALT, alanine-aminotransferase; AST, aspartate-transaminase; ANP, acute necrotizing pancreatitis; NS, normal saline.

incubated overnight at 4°C with rabbit anti-NF-κBp65 and anti-caspase3 primary antibodies, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:5,000; 1081; Pierce Biotechnology, Inc., Rockford, IL, USA). The staining results were visualized using 3,5-diaminobenzidine (DAB; Wuhan Goodbio Technology Co., Ltd.) and images of the slides were captured under the BX53 microscope. In each immunohistochemistry staining analysis, phosphate-buffered saline was used instead of the primary antibody as a negative control.

TUNEL. The TUNEL assay was performed for evaluating renal cell apoptosis. The renal paraffin-embedded tissues were cut into 3-µm sections as reported in the H&E and immunohistochemistry assay. The sections were deparaffinized, hydrated and incubated with proteinase-K (Wuhan Goodbio Technology Co., Ltd.) for 15 min at room temperature. The residual steps were conducted according to the protocol provided by the recommendations in situ cell death detection kit, POD (Roche Diagnostics). Hematoxylin was used for staining the nucleus after DAB staining. The results of TUNEL were observed with the Olympus BX53 microscope. The positive results of TUNEL were brown staining in the nucleus.

NO assay. Kidney tissues were thawed and homogenized in phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (Nanjing Jiancheng Bioengineering Institute). NO production was measured using an NO detection kit, according to the manufacturer’s instructions.

Western blot analysis. The fresh kidney tissues were homogenized in ice-cold lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) in the presence of a protease inhibitor cocktail (Wuhan Goodbio Technology Co., Ltd.). Proteins were measured using the Bradford Protein Assay with bovine serum albumin (Beyotime Institute of Biotechnology) as a standard. In brief, equal quantities of protein samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% skimmed milk in TBST buffer (Tris-buffered saline containing 0.1% Tween-20) at room temperature for 2 h and then incubated with a rabbit polyclonal anti-GAPDH antibody (1:2,500, Abcam), rabbit polyclonal antibody anti-P38 (1:1,000, Cell Signaling Technologies, Inc.) and rabbit polyclonal anti-p-P38 antibody (1:1,000, Cell Signaling Technologies, Inc.) at 4°C overnight. After extensive rinsing with TBST, the blots were incubated with secondary antibody HRP-conjugated goat anti-rabbit (1:5,000, Pierce Biotechnology, Inc.) at room temperature for 1.5 h, and then detected the expression of GAPDH, P38 and p-P38 by enhanced chemiluminescent reagent (Immobilon Western HRP Substrate; EMD Millipore). The intensities of the bands on the western blots were quantified using Quantity One software, version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. The data are presented as the mean ± standard deviation. Data analyses were conducted using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA). Data between all groups was compared by one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Optimum dose of PF. The present study analyzed the serum AMY, LIPA, ALT and AST levels in ANP rats treated with various doses of PF (50, 100 and 150 mg/kg) in order to determine the optimum dose of PF. All doses of PF were able to significantly reduce the levels of AMY and LIPA, as compared with the ANP group (P<0.05). However, the 100 mg/kg PF group was the only PF-treated group in which both the levels of AST and ALT were significantly decreased, as compared with the ANP group. Therefore, 100 mg/kg PF was selected as the optimum dose for treating ANP (Table I).

Histopathological assay. Representative pathological changes in pancreatic tissue are shown in the Fig. 1. Normal histological features of the pancreas were observed in the sham-operated group (Fig. 1A). In the ANP group, pancreatic edema, interstitial leukocyte infiltration, hemorrhage and necrosis were observed (Fig. 1B). In the ANP+100 mg/kg PF group, the severity of pancreatic histological injuries was significantly
reduced compared with the ANP group (Fig. 1C). As shown in Fig. 1D the histopathological score in the pancreas was lower in the PF group compared with that in the ANP group at the corresponding time points (P<0.05).

To examine the effect of PF on acute renal injury induced by ANP, the pathological changes in the kidney were analyzed 3, 6 and 12 h after rat ANP models were successfully generated. The renal damage was shown to be the most severe at the 12 h time point. The loss of brush border, cell necrosis, sloughing of tubular epithelial cell, cast formation and dilation of tubules in the cortex were observed clearly in the ANP group. The ANP+PF group had less severe features of renal
injury and the renal histological scores were significantly decreased compared with the ANP group at the corresponding time points as shown in Fig. 2 (P<0.05).

Analysis of serum AMY, LIPA, BUN and Cr. Compared with the levels of AMY, LIPA, BUN and Cr in the sham-operated group, the levels in the ANP group were significantly increased at each time point (Fig. 3). Peak serum AMY level was observed at the 6 h time point in the ANP group and the peak serum LIPA, BUN and Cr levels were observed at the 12 h time point. In the ANP+PF groups the serum enzyme levels were significantly reduced at the corresponding time points compared with the ANP group (P<0.05).

ELISA assay. PF reduced the serum levels of TNF-α, IL-1β and IL-6 in the ANP rats. The serum levels of TNF-α, IL-1β and IL-6 were determined using ELISA. It was found that the serum levels of TNF-α, IL-1β and IL-6 were progressively upregulated from 3 to 12 h. The ANP group showed significantly higher levels of TNF-α, IL-1β and IL-6 compared with the sham-operated group. The PF treatment resulted in a marked decrease of the enzyme expression in the ANP+PF group compared with the ANP group (P<0.05; Fig. 4D). Furthermore, the levels of TNF-α, IL-1β and IL-6 were significantly decreased in the ANP+PF group, as compared with the ANP group (P<0.05; Fig. 4D).

PF decreases renal NO production. The levels of NO in renal tissues from the ANP group were markedly increased compared with those in the sham-operated group. Treatment with PF significantly reduced the NO production (P<0.05; Fig. 5).
Discussion

This study is the first to observe the therapeutic effect of PF on ANP and the acute renal injury induced by ANP in rat models. PF is one of the active ingredients of Radix Paeoniae, and it is an important traditional herbal medicine with a number of pharmacological effects, including antihyperlipidemic and anti-inflammatory effects. The results of this study showed that PF can ameliorate ANP and protect the kidneys in ANP. This effect was demonstrated by the serum levels of enzymes and the histological changes in the pancreas and kidneys. Acute renal injury is a common complication of ANP and it is associated with increased risk of mortality (27). The mechanism of the acute renal injury following ANP is complicated. The inflammatory cascades that are initiated by endothelial dysfunction can be augmented by the generation of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6. Using ELISA, the serum levels of the TNF-α, IL-1β and IL-6 were shown to be lower in the PF+ANP group compared with the ANP group at each time point. In a previous study by Jiang et al (15), PF was shown to inhibit systemic inflammation and improve survival in the experimental rat models of sepsis.

Another important mechanism underlying the development of acute renal injury following ANP is acute ischemia (28). ANP results in a decreased circulating blood volume, which causes renal ischemia due to decreased blood flow to the kidneys. Ischemia results in apoptosis of renal cells which reduces kidney function. Therefore, in order to investigate the effect of PF on kidney cell apoptosis, a TUNEL assay was conducted and the expression of caspase-3 was analyzed. The ratio of the TUNEL-positive cells was shown to increase in the ANP groups and the majority of TUNEL-positive cells were located at the cortex of the pancreas.
kidney. The mechanisms underlying the apoptosis of renal tissues are associated with cellular cytotoxicity and DNA damage. In addition, NO was shown to have an important role in the initiation of apoptosis in experimental renal IR (29). A previous study demonstrated that NO production resulted in cytotoxicity and DNA damage (29). In the present study, PF was shown to inhibit NO-mediated apoptosis in acute renal injury induced by ANP. The results showed that PF was able to ameliorate NO production and inhibit tubular cell apoptosis. PF is known to promote the dilatation of blood vessels, which may increase blood perfusion to the renal cortex and inhibit cell apoptosis. Therefore, these results suggested that PF may attenuate renal damage by reducing NO-induced apoptosis of renal cells.

Caspase-3, which is a member of the caspase family that includes caspase-1 to caspase-12, has a key function in apoptosis (30). In a previous study, the role of caspase-3 in apoptosis was closely associated with the Bax/Bcl-2 signaling pathway. Activation of the initiator caspase-8 is triggered by the extrinsic death signaling pathway (31). The other initiator caspase, caspase-9, is predominantly activated by the release of pro-apoptotic proteins in the intrinsic pathway (32).

Table II. Serum levels of TNF-α, IL-1β and IL-6.

| Group             | TNF-α (pg/ml) | IL-1β (pg/ml) | IL-6 (pg/ml) |
|-------------------|---------------|---------------|--------------|
| Sham-operated     |               |               |              |
| 3 h               | 154.78±11.94  | 106.21±6.52   | 113.36±9.77  |
| 6 h               | 155.84±14.59  | 103.98±6.28   | 116.56±11.17 |
| 12 h              | 152.45±12.83  | 105.89±9.02   | 118.78±11.43 |
| ANP               |               |               |              |
| 3 h               | 251.39±18.06* | 257.50±16.31* | 271.44±17.00*|
| 6 h               | 317.35±20.76* | 351.41±14.94*| 355.00±27.80*|
| 12 h              | 366.90±13.13* | 430.39±26.26*| 441.64±31.19*|
| ANP+Paeoniflorin  |               |               |              |
| 3 h               | 189.44±11.84* | 178.54±13.23* | 173.33±15.03*|
| 6 h               | 261.88±16.47* | 275.76±20.28*| 244.51±15.37*|
| 12 h              | 286.23±11.23* | 315.60±11.04*| 288.29±12.71*|

Serum levels of TNF-α, IL-1β and IL-6 measured by enzyme-linked immunosorbent assay kits. Data are expressed as the mean ± standard deviation; n=8 in each group. *P<0.05 vs. the sham-operated group; †P<0.05 vs. the ANP group. ANP, acute necrotizing pancreatitis; TNF-α, tumor necrosis factor-α; IL, interleukin.
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