Abstract. Previous studies have demonstrated that pterostilbene (Pter) prevents oxidative stress, suppresses cell growth and exhibits anti-fungal and anti-inflammatory effects. Pter is used to treat a number of clinical diseases, including Alzheimer's disease, various malignancies and hypercholesteremia. The aim of the present study was to investigate whether Pter protects against acute renal ischemia reperfusion injury (IRI) and inhibits oxidative stress, inducible nitric oxide synthase (iNOS) expression and inflammation in rats. A total of 40 adult male Sprague Dawley rats were divided into the following 5 groups at random: Control group, where rats were not subjected to renal IRI; IRI group, where rats were subjected to renal IRI; Pter 10 group, where rats underwent renal IRI and were treated with 10 mg/kg Pter; Pter 20 group, where rats underwent renal IRI and were treated with 20 mg/kg Pter; Pter 30 group, where rats underwent renal IRI and were treated with 30 mg/kg Pter. The results demonstrated that Pter treatment improved renal function following acute renal IRI. Compared with the untreated renal IRI group, myeloperoxidase, iNOS, interleukin (IL)-1β, IL-6 and tumor necrosis factor-α expression levels were significantly decreased (P<0.01), whereas IL-10 expression levels were significantly increased (P<0.01) following treatment with Pter in acute renal IRI rats. In addition, Pter significantly attenuated caspase-3 activity and the Toll-like receptor 4 (TLR4)/nuclear factor (NF)-κB signaling pathway induced by acute renal IRI (P<0.01). These results provide evidence to suggest that administration of Pter may protect against acute renal IRI and inhibit oxidative stress, iNOS expression and inflammation in rats via the TLR4/NF-κB signaling pathway.

Introduction

Acute renal ischemia reperfusion injury (IRI) refers to a pathological and physiological phenomenon whereby cells in ischemic tissues are damaged following the re-establishment of a blood supply (1). Renal injury following IRI does not improve and typically worsens over time. As an organ with high perfusion rates, the kidneys are sensitive to reperfusion following ischemia (2). Therefore, renal IRI is likely to occur following kidney transplant, kidney vascular surgery, extracorporeal shock wave lithotripsy or resuscitation (3). Renal IRI is associated with acute ischemic renal failure. In addition, renal IRI may occur as a result of delayed renal graft function or chronic renal allograft dysfunction (3).

Nuclear factor (NF)-κB, a key downstream factor of the Toll-like receptor (TLR/NF-κB signaling pathway, mediates essential functions by regulating inflammatory factors, cell proliferation and differentiation (4). As a result, systemic inhibition of NF-κB may reduce inflammation and have damaging consequences (5). The results of a previous study indicated that NF-κB serves important roles in inflammation of IRI (6).

The number of studies investigating resveratrol metabolins have increased in recent years. It has been demonstrated that resveratrol exhibits neuroprotective effects in cerebral ischemia (7). In addition, as the contents of these metabolins in plants are low and their structures are unstable, they are easily degraded when heated. Pterostilbene (Pter) is a derivative of resveratrol (8). Resveratrol is rapidly metabolized to produce Pter and piceid (9). The selectivity and stability of Pter is superior when compared with that of resveratrol (9). A previous study demonstrated that, similar to resveratrol, Pter inhibits oxidative stress, possesses anti-fungal properties and suppresses cell proliferation (8). In addition, Pter has been demonstrated to exhibit favorable effects in Alzheimer's disease (10). The aim of the present study was to investigate the protective effects of Pter in acute renal IRI, and to explore the potential underlying mechanisms involved.

Materials and methods

Animals. A total of 50 adult male Sprague Dawley (SD) rats (weight, 250-300 g; age, 8-10 weeks old) were purchased from
the Center of Experimental Animal Research at the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). All experiments in the present study were approved by the Institutional Animal Experimentation Committee of the Academic Medical Center of the First Affiliated Hospital of Zhengzhou University. SD rats (10 rats/cage) were maintained in a temperature-controlled room at 22-23 °C with a 12-h light/dark cycles, and provided with access to food and water ad libitum.

**Surgical preparation and experimental protocol.** SD rats were injected with 75 mg/kg ketamine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 0.5 mg/kg dexmedetomidine (Sigma-Aldrich; Merck KGaA) and 0.05 mg/kg atropine-sulfate (Sigma-Aldrich; Merck KGaA). For the duration of the procedure, body temperatures were maintained at 37±0.5 °C using an external thermal heating pad. The left kidney was exposed and immobilized using a Lucite kidney cup in the left flank. The renal vessels were carefully separated, and the nerves and adrenal gland were preserved. A polyethylene catheter was used to isolate, ligate and cannulate the left ureter for urine collection. Following stabilization for 30 min, the left kidney was treated with a polyethylene catheter and blood flow was recovered. Following a stabilization period of 30 min, rats were randomly divided into the following 4 groups (n=10): IRI group, consisting of rats that underwent the IRI procedure alone; Pter 10 group, consisting of rats that underwent the IRI procedure and gavaged with 10 mg/kg/day Pter at 24 h after IRI surgery; Pter 20 group, where rats underwent the IRI procedure and gavaged with 20 mg/kg/day Pter at 24 h after IRI surgery; Pter 30 group, where rats underwent the IRI procedure and were gavaged with 30 mg/kg/day Pter at 24 h after IRI surgery. A additional control group, consisting of SD rats (n=10) that had not undergone the renal IRI procedure was included. Pter was administered over the course of 2 weeks as described previously (11,12).

**Renal function.** For the analysis of blood urea nitrogen (BUN) levels and creatinine concentration, urine was collected after rats had received 2 weeks of Pter treatment from the left ureter for 10 min. BUN levels were determined using a Urea Nitrogen (BUN) diacetyl monoxime test kit from Stanbio Laboratory L.P. (Boerne, TX, USA) according to the manufacturer’s instructions. The creatinine concentration in urine samples was analyzed using a creatinine kit from Tiangen Biotech Co., Ltd. (Beijing, China) according to the manufacturer’s instructions. Subsequently, total protein was quantified using a BCA kit (Beyotime Institute of Biotechnology, Nanjing, China).

**Histological analysis.** Following reperfusion and 2 weeks of Pter treatment, renal tissue samples were harvested under anesthesia and fixed using 4% paraformaldehyde for 24 h at room temperature. Tissue samples were dehydrated, made transparent, waxed and embedded, before being cut into 5-µM thick sections and stained with hematoxylin-eosin at room temperature for 15-20 min. The sample slices were examined using a BX51 microscope (Olympus Corporation, Tokyo, Japan).

**Myeloperoxidase (MPO) level.** Following reperfusion and 2 weeks of Pter treatment, renal tissue samples were harvested and stored at -80 °C. MPO levels were detected using an MPO test kit (Beyotime Institute of Biotechnology). Renal tissue samples were homogenized in cold 5 mM sodium phosphate buffer and centrifuged at 12,000 x g for 5 min at 4 °C. The concentration of MPO was determined using the Bradford assay. MPO was expressed as U/g protein.

**Western blot analysis.** Following reperfusion and 2 weeks of Pter treatment, renal tissue samples were collected and homogenized in RIPA buffer (Beyotime Institute of Biotechnology) containing 1% protease inhibitor cocktail. Tissue samples were centrifuged at 12,000 x g for 5 min at 4 °C, and the protein concentration of the sample supernatant was determined using a BCA kit (Beyotime Institute of Biotechnology). A total of 50 µg protein per lane was loaded and separated by 10% SDS-PAGE, and transferred to nitrocellulose membranes (EMD Millipore; Billerica, MA, USA). The membranes were blocked in Tris-buffered saline with Tween-20 (TBST) plus 5% non-fat dry milk for 1 h at 37 °C, and incubated overnight at 4 °C with primary antibodies against inducible nitric oxide synthase (iNOS; dilution, 1:3,000, sc-649), TLR4 (dilution, 1:4,000; sc-10741), NF-κB (dilution, 1:4,000; sc-10741), IL-1β (dilution, 1:4,000; sc-10741) and β-actin (sc-10731, 1:4,000, all from Santa Cruz Biotechnology). The membranes were subsequently washed with TBST, and then probed with a goat anti-rabbit IgG secondary antibody (dilution, 1:5,000; 7074; Cell Signaling Technology, Inc., Danvers, MA, USA) at room temperature for 1 h. β-actin was used as a loading control. The blots were visualized with ECLplus (Beyotime Institute of Biotechnology) reagent and quantified using the Bio-Rad Laboratories Quantity One software 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Determination of interleukin (IL)-10, IL-1β, IL-6 and tumor necrosis factor (TNF)-α expression levels.** Following reperfusion and 2 weeks of Pter treatment, whole blood samples were collected and centrifuged at 1,000 x g for 10 min at 4 °C. Serum was used to measure IL-10 (EKO418), IL-1β (EKO393), IL-6 (EKO412) and TNF-α (EKO526) levels using ELISA kits (Boster Biological Technology Co., Ltd., Wuhan, China) according to manufacturer’s protocol. Samples were read using a spectrophotometer at an absorbance of 450 nm.

**Detection of caspase-3 activation.** Following reperfusion and 2 weeks of Pter treatment, renal tissue samples were harvested and homogenized in RIPA buffer (Beyotime Institute of Biotechnology) containing 1% protease inhibitor cocktail. Tissue samples were centrifuged at 12,000 x g for 5 min at 4 °C, and the protein concentrations of the sample supernatants were...
determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). An equal quantity of total protein (10 µg) from each sample was used to measure caspase-3 activity using caspase-3 activity kits (C1115; Beyotime Institute of Biotechnology) in the dark. Subsequently, total protein was quantified using a BCA kit (Beyotime Institute of Biotechnology). Samples were read with a spectrophotometer at an absorbance of 405 nm. Caspase-3 activity was normalized to β-actin expression.

Statistical analysis. The results are expressed as the mean ± standard deviation. SPSS software (version, 18.0; SPSS, Inc., Chicago, IL, USA) was used to perform statistical analyses. One-way analysis of variance was used for comparisons among multiple groups followed by Tukey’s test, and the independent samples t-test was used to compare the difference between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Pter protects against renal function. The chemical structure of Pter is indicated at Fig. 1. When compared with the control group, IRI induced a significant increase in the concentration of BUN and creatinine in rat urine samples, which indicated that IRI significantly impaired renal function (P<0.01; Fig. 2). By contrast, Pter treatment significantly attenuated the IRI-induced increase in BUN and creatinine levels in rats (P<0.01; Fig. 2), which suggests that Pter may protect against IRI-induced impairment of renal function.

IRI induces morphological changes. Compared with the control group, IRI-induced renal cell death was markedly observed in IRI-induced model rats (Fig. 3). By contrast, Pter treatment markedly reduced the IRI-induced increase in morphological scores (Fig. 3).

Pter protects against the IRI-induced elevation in MPO levels. In the IRI-induced model group, MPO levels were significantly increased when compared with the control group (P<0.01; Fig. 4). Following the administration of Pter, the IRI-induced increase in MPO levels was significantly suppressed when compared with the untreated IRI model group (P<0.01; Fig. 4).

Pter protects against the IRI-induced increase in iNOS protein expression levels. In order to investigate the effects of Pter on the iNOS-mediated signaling pathway, iNOS protein expression levels in the renal tissues of Pter-treated rats that had undergone renal IRI were determined. Western blot analysis demonstrated that IRI significantly increased iNOS protein expression levels in the IRI model group when compared with the control group (P<0.01; Fig. 5). By contrast, administration
of Pter significantly inhibited the IRI-induced elevation in iNOS protein expression levels (P<0.01; Fig. 5).

**Pter protects against IRI-induced alterations in IL-10, IL-1β, IL-6 and TNF-α expression levels.** To investigate the effects of Pter on inflammation, IL-10, IL-1β, IL-6 and TNF-α expression levels in the renal tissues of Pter-treated rats that had undergone renal IRI were determined. Compared with the control group, IRI significantly increased the expression levels of IL-1β, IL-6 and TNF-α, and significantly decreased IL-10 expression levels (P<0.01; Fig. 6). By contrast, Pter treatment significantly reversed the expression levels of these factors when compared with the IRI model group (P<0.01; Fig. 6).

**Pter protects against IRI-induced caspase-3 activation.** When compared with the control group, IRI significantly increased caspase-3 activity in rats from the IRI model group (P<0.01; Fig. 7). However, treatment with Pter significantly inhibited the IRI-induced increase in caspase-3 activity (P<0.01; Fig. 7).

**Pter protects against the IRI-induced increase in TLR4 protein expression levels.** In order to determine whether Pter influences TLR4 protein expression levels in rats following IRI, western blot analysis was performed. As indicated in Fig. 8, TLR4 protein expression levels were significantly increased in the IRI group when compared with the control group (P<0.01). By contrast, Pter treatment significantly reduced the IRI-induced increase in TLR4 protein expression levels (P<0.01; Fig. 8).
Pter protects against the IRI-induced increase in NF-κB protein expression levels. To investigate the effects of Pter on NF-κB protein expression levels in rats following IRI, the expression levels of this protein were determined by western blot analysis. The results indicated that NF-κB protein expression levels were significantly increased in IRI model rats when compared with those in the control group (P<0.01; Fig. 9). However, administration of Pter significantly decreased NF-κB protein expression levels in rats following IRI when compared with those in the untreated IRI group (P<0.01; Fig. 9).

Discussion

A previous study investigated strategies for the prevention of renal IRI (3). Furthermore, alternative studies have included investigating the protective mechanisms involved in renal endogenesis, including pre-treatment and pre-conditioning, and have gained particular attention (13). In addition, previous study examining the drug-induced activation or inhibition of various factors that protect renal tissues, have demonstrated clinical significance to the outcome of renal transplants and functional recovery, as well as patient survival following transplantation (14). The aim of the present study was to determine whether Pter significantly inhibited IRI-induced alterations to BUN and creatinine concentration levels, as well as histological scores in a rat model of renal IRI.

As a typical enzyme of neutrophil granulocytes, MPO is a dependable indicator of the level of neutrophil granulocyte infiltration in tissues (15). In addition, MPO activity is proportional to number of neutrophil granulocytes, which may be used to indicate the level of infiltration of these cells in the spinal cord (16). The quantitative results of previous studies suggest that, among the different renal IRI mechanisms, the participation of inflammatory cells, specifically neutrophil granulocytes, may be important during renal IRI (16). When IRI occurs, neutrophil granulocytes have been demonstrated to accumulate and migrate to ischemic regions. In addition, the accumulation of blood platelets impairs angiogenesis. The presence of swollen vascular endothelial cells leads to narrowing of the lumen and reduces blood flow. In addition, increased numbers of neutrophil granulocytes may stimulate the production of cytokines. iNOS, which is activated by these inflammatory mediators, may subsequently produce NO, which is involved in nerve injury and the production of oxygen radicals. Following the adherence of hemameba and vascular endothelial cells, the cytoskeleton may be reconstructed, which leads to an increase in the space between endothelial cells and damages the endothelium of the blood-brain barrier. MPO exists predominantly in the azurophilic granules of neutrophil granulocytes. It has been demonstrated that the expression of MPO in renal IRI tissues is increased, which suggests that inflammation may be an important mechanism underlying renal IRI. In the present study, administration of Pter significantly suppressed MPO levels and iNOS protein expression levels following renal IRI in rats. A previous study reported that Pter attenuated inflammation via suppression of MPO levels and the TLR4/NF-κB signaling pathway in rat hearts following ischemia-reperfusion (17). In addition, Pan et al (17) demonstrated that Pter inhibited lipopolysaccharide-induced iNOS expression in murine macrophages.
A previous study demonstrated that IL-10 exhibits protective effects against IRI (18). In T-cells co-cultured with an anti-CD3 monoclonal antibody and macrophages, the expression of TNF-α and IL-6 were upregulated (19). Following stimulation of NF-κB signaling pathways via death-1/B7-H1, IL-10 was upregulated (20). By employing the IL-10 monoclonal antibody for neutralization, the level of pro-inflammatory factors, such as TNF-α and IL-6 are increased (21). In the present study, Pter treatment significantly suppressed the expression levels of IL-1β, IL-6 and TNF-α, and significantly increased IL-10 expression levels in renal IRI rats. Notably, Tsai et al. (22) revealed that Pter inhibited mouse skin carcinogenesis via inhibition of iNOS production and downregulated the inflammatory response.

Apoptotic signaling pathways are dependent on caspase enzymes, which serve important roles in the renal tubular epithelium during renal IRI (20). The caspase family of enzymes consist of key proteases involved in cell apoptosis (20). Caspase-3 is the predominant protease for the activation of cell apoptosis (20). Caspase inhibitors inhibit cell apoptosis and the inflammatory response by downregulating the proteinase activities of caspase-1 and caspase-3 (23). In the present study, treatment with Pter significantly inhibited the IRI-induced activation of caspase-3 following renal IRI. Consistent with these observations, Wang et al. (24) demonstrated that Pter attenuated inflammation via the TLR4/NF-κB signaling pathway in ischaemia-reperfusion rats.

NF-κB serves a key role in inflammation during renal IRI in mice and NF-κB inhibition in specific T-cells may reduce IRI when compared with NF-κB inhibition in non-specific T cells (25). However, NF-κB exhibits a wide range of functions, therefore, inhibition of NF-κB may not be sufficient to prevent the damaging effects of renal IRI (26). In addition, the effects of NF-κB may exhibit different effects in different organs. Although inhibition of NF-κB may reduce systemic inflammation, it may exacerbate renal IRI (26). The results of the present study demonstrated that Pter significantly decreased NF-κB protein expression levels in the renal tissues of rats that had undergone renal IRI. Cichocki et al. (27) reported that Pter inhibited 12-O-tetradecanoylphorbol-13-acetate-activated NF-κB and iNOS expression in the mouse epidermis.

The TLR family consists of several members, including TLR4, TLR2 and TLR9 (28). The extent of renal injury in mice with TLR2, myeloid differentiation primary response gene 88 and TLR4 deficiencies was reduced when compared with that of wild-type mice (29). A previous study involving mice with a deficiency in TLR4 demonstrated that the stimulation of TLR4 by endogenous ligands serves an important role in mediating IRI (30). When IRI occurs, TLR4 expression in renal tubular epithelial cells is upregulated, which suggests that TLR4 may be involved in a positive feedback loop (31). The results of the present study suggested that Pter may significantly reduce the renal IRI-induced increase in TLR4 protein expression levels in rats. Similarly, Wang et al. (24) revealed that Pter attenuated inflammation in a rat model of ischemia-reperfusion via the TLR4/NF-κB signaling pathway.

In conclusion, the results of the current study demonstrated that Pter may protect against renal IRI in rats potentially via inhibition of oxidative stress, iNOS expression and inflammation. In addition, the protective effects of Pter may be associated with inhibition of the TLR4/NF-κB signaling pathway. Further studies are required to investigate the specific mechanisms underlying the effects of Pter treatment during renal IRI.

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