Osthole protects against inflammation in a rat model of chronic kidney failure via suppression of nuclear factor-κB, transforming growth factor-β1 and activation of phosphoinositide 3-kinase/protein kinase B/nuclear factor (erythroid-derived 2)-like 2 signaling

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Abstract. Multiple pharmacological applications of osthole have been previously recognized, including antioxidant, anti-inflammatory, anti-platelet and estrogenic effects, and resistance to pain. The present study investigated the protective effects of osthole against inflammation in a rat model of chronic kidney failure (CRF) and the underlying mechanisms. Osthole treatment with significantly reversed CRF-induced changes in serum creatinine, calcium, phosphorus and blood urea nitrogen levels in CRF rats. Male Sprague-Dawley rats (age, 8 weeks) received 200 mg/kg 2% adenine suspension to induce CRF in the model group. In the osthole-treated group, rats received 200 mg/kg 2% adenine suspension + osthole (40 mg/kg, intravenously). The results revealed that treatment with osthole significantly inhibited CRF-induced tumor necrosis factor-α, interleukin (IL)-8 and IL-6 expression, and suppressed nuclear factor-κB (NF-κB) protein expression in CRF rats. Osthole treatment significantly attenuated the protein expression of transforming growth factor-β1 (TGF-β1), reduced monocyte chemoattractant protein-1 activity and increased the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) ratio in CRF rats. These results suggested that osthole protects against inflammation in a rat model of CRF via suppression of NF-κB and TGF-β1, and activation of PI3K/Akt/nuclear factor (erythroid-derived 2)-like 2 signaling. Therefore, osthole may represent a potential therapeutic agent for the treatment of CRF.

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Introduction

Chronic renal failure (CRF) is a clinical syndrome consisting of a series of symptoms and metabolic disturbances triggered by damages to renal structure and function (1). As the ageing population and morbidity of hypertension and diabetes mellitus increases, the morbidity of CRF is increasing. The incidence of chronic renal failure in China is ~1/10,000 (2). CRF is a common consequence of various advanced renal diseases and is associated with poor prognosis and high mortality, affecting the quality of life of patients, and imposes heavy pressure and economic burden to families and society (3).

Inflammation in patients with CRF is associated with renal failure, and may result from dialysis treatment and infectious diseases (4). Recurrent inflammation means that patients with CRF exist in a state of sustained light inflammation, which leads to complications including angiocardiopathy, malnutrition and anemia (5).

Monocyte chemoattractant protein-1 (MCP-1) is weakly expressed in healthy humans. In nephridial tissues it is secreted by renal mesangial, renal tubular epithelial, vascular endothelial and mononuclear cells at damaged interstitial sites (6). MCP-1 expression levels are significantly increased in injured renal tubular epithelial cells (7). Increased MCP-1 expression further activates T lymphocytes and basophilic leukocytes. MCP-1 expression maybe stimulated by the activity of nuclear factor-κB (NF-κB) (8).

As a constituent of *Fructuscnidii,* osthole [7-methoxyl-8-(3-methylbut-2-enyl)-2-chromenone] is a coumarin that possesses various pharmacological properties, including antioxidant, anti-inflammatory, anti-tumor, anti-platelet and estrogenic effects, and resistance to pain (9). It has been used clinically for numerous years for the treatment of skin and venereal diseases (10). Previous studies have demonstrated that osthole promotes learning and memory in a dementia mouse model via inhibition of acetylcholinesterase activities, enhancing activities of glutathione peroxidase and superoxide dismutase to scavenge oxygen free radicals (11,12). The present study confirmed that osthole protects against inflammation,

and demonstrated the underlying mechanisms in a rat model of CRF.

Materials and methods

Ethical statement and animals. Male Sprague-Dawley rats (n=40; age, 8 weeks; weight, 250-280 g) were obtained from the Animal Experiment Center, The Affiliated Hospital of Qingdao University (Qingdao, China) and were maintained in natural light conditions at a humidity of 50-60%, a constant temperature of 22±2˚C and with free access to food and water. The rats were randomly divided into control (n=10), osthole (n=10), model (n=10) and model + osthole (n=10) groups. In the control group, rats received normal food and water during the experiment. In the osthole group, rats were gavaged with 40 mg/kg osthole. In the model group, rats received 200 mg/kg 2% adenine suspension to induce CRF. In the model + osthole group, the rats received 200 mg/kg 2% adenine suspension + intravenously administered osthole (40 mg/kg).

Assessment of renal function. After administration of osthole, blood samples were obtained from the inferior vena cava. Creatinine, calcium, phosphorus and blood urea nitrogen (BUN) serum levels were assayed in the core laboratory of The Affiliated Hospital of Qingdao University.

Hematoxylin and eosin (H&E) staining. After administration of osthole, rats were sacrificed with decollation under pentobarbital anesthesia (30 mg/kg; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), kidney tissues were fixed in 10% buffered formalin solution and subsequently embedded in paraffin wax following dehydration with alcohol and xylene. Kidney tissues were cut into 5 μm sections, stained with H&E and observed under a light microscope (Nikon Corporation, Tokyo, Japan).

Assessment of inflammation and MCP-1. Following osthole administration, blood samples were obtained from the inferior vena cava. ELISA kits specific for tumor necrosis factor-α (TNF-α; R019), interleukin (IL)-6 (R016) and IL-8 (R017) were used to quantify serum levels of the aforementioned proteins (all from Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. MCP-1 serum levels were quantified using an ELISA kit (E-EL-R0633c) from Elabscience Biotechnology Co., Ltd. (Bethesda, MD, USA).

Western blot analysis. The rat kidney tissue samples were obtained and proteins were extracted by homogenization using ice-cold radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Protein content was measured using a Bicinchoninic Acid assay kit (Beyotime Institute of Biotechnology). Total protein (~50 μg) was separated by 8-12% SDS-PAGE and subsequently transferred onto polyvinyldene difluoride membranes. Following this, membranes were blocked in 5% skimmed milk for 2 h at room temperature, and washed twice with TBS with Tween-20. The membrane blots were initially probed with primary antibodies as follows:

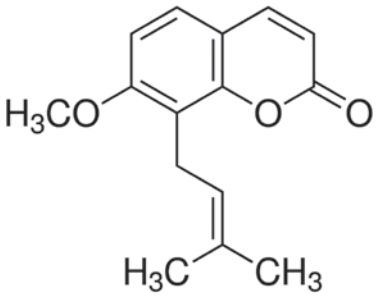

Figure 1. The chemical structure of osthole.

NF-κB/p65 (sc-109; 1:3,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), transforming growth factor-β1 (sc-57447; TGF-β1; 1:2,000; Santa Cruz Biotechnology, Inc.), protein kinase B (Akt; sc-8312; 1:2,000, Santa Cruz Biotechnology, Inc.), phosphorylated (p)-Akt (sc-7985-R; 1:2,000; Santa Cruz Biotechnology, Inc.), nuclear factor (erythroid-derived 2)-like 2 (Nrf2; 12,721; 1:2,000; Cell Signaling Technology, Inc.) and β-actin (AA128; 1:5,000; Beyotime Institute of Biotechnology) overnight at 4˚C. A horseradish peroxidase-conjugated secondary antibody (14,708; Cell Signaling Technology, Inc.) was subsequently incubated with the membrane at 37˚C for 1 h, and the membrane was visualized using an Enhanced Chemiluminescence system (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and quantified with Image Lab version 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All data were processed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Data analysis involved one-way analysis of variance followed by Scheffé's method, to correct for multiple comparisons. All results were expressed as the mean ± standard error. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Osthole protects renal function in the CRF rat. The chemical structure of osthole is presented in Fig. 1. Initially, the effect of osthole on renal function in the CRF rat was analyzed and serum creatinine, calcium, phosphorus and BUN levels were detected. No significant differences were observed between the control and control osthole groups in terms of creatinine ($P>0.05$; Fig. 2A), calcium ($P>0.05$; Fig. 2B), phosphorus ($P>0.05$; Fig. 2C) or BUN ($P>0.05$; Fig. 2D) serum levels. However, serum levels of creatinine, calcium and phosphorus in the CRF model group were significantly reduced compared with the control or control osthole groups. Meanwhile, BUN serum levels were increased in the CRF model group compared with the control and control osthole groups ($P<0.05$; Fig. 2D). Treatment with osthole reversed these alterations in the CRF rat, with creatine ($P<0.05$; Fig. 2A) calcium ($P<0.05$; Fig. 2B) and phosphorus ($P<0.05$; Fig. 2C) serum levels significantly increasing compared with the CRF rat model group, and BUN serum levels significantly decreasing compared with the CRF rat model group (Fig. 2D).

Osthole treatment protects against kidney tissue damage in the CRF rat. To observe the effect of osthole on kidney

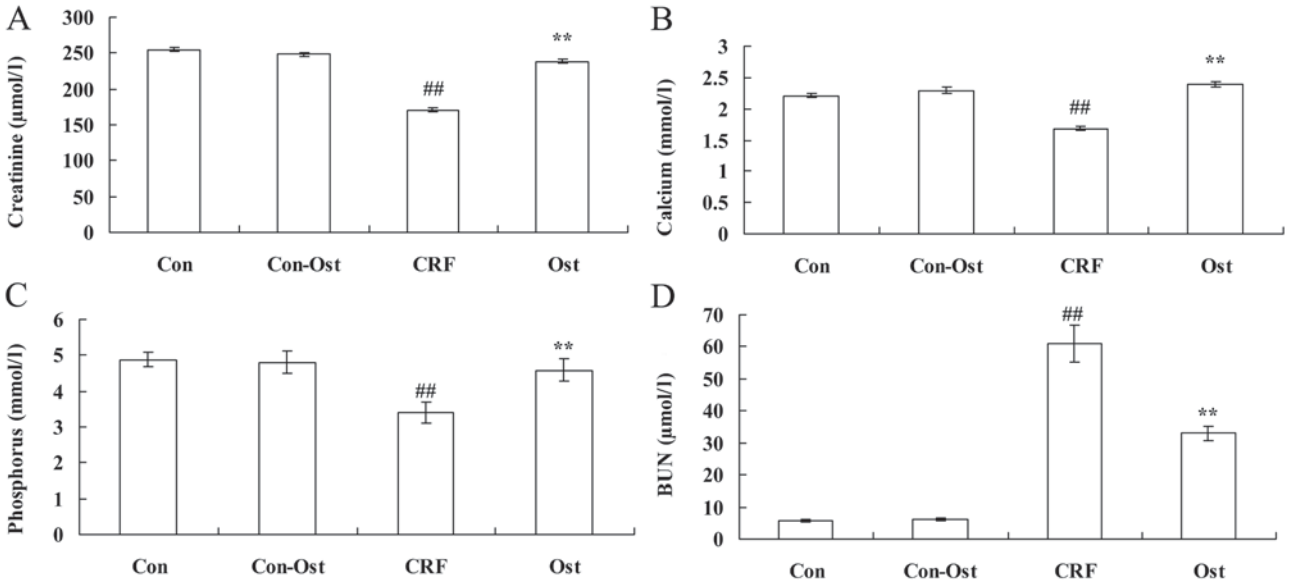

Figure 2. Osthole preserves renal function in the CRF rat. Osthole protects against CRF-induced changes in serum (A) creatinine, (B) calcium, (C) phosphorus and (D) BUN. Data are presented as the mean ± standard error. **P<0.01 vs. Con; $^{\#\#}$P<0.01 vs. CRF. CRF, chronic renal failure; BUN, blood urea nitrogen; Con, control group; Ost, control + osthole group; Ost, CRF + osthole group.

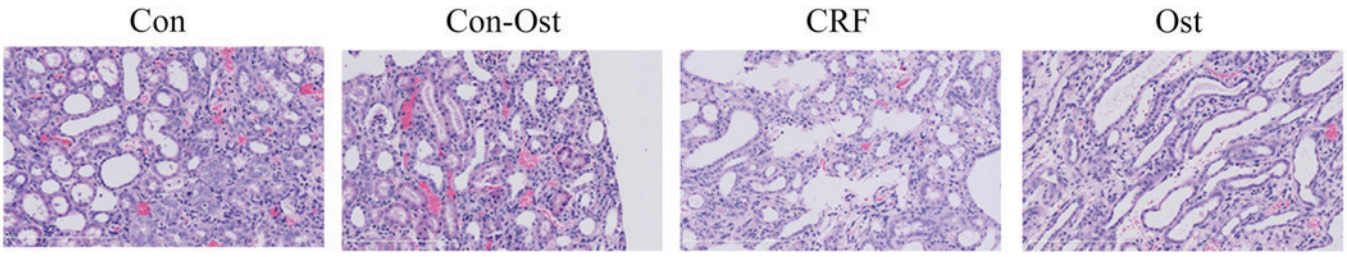

Figure 3. Osthole protects against kidney tissue damage in the CRF rat. Representative photomicrographs of H&E-stained rat kidney tissues (magnification, x40). CRF, chronic renal failure; Con, control group; Con-Ost, control + osthole group; Ost, CRF + osthole group.

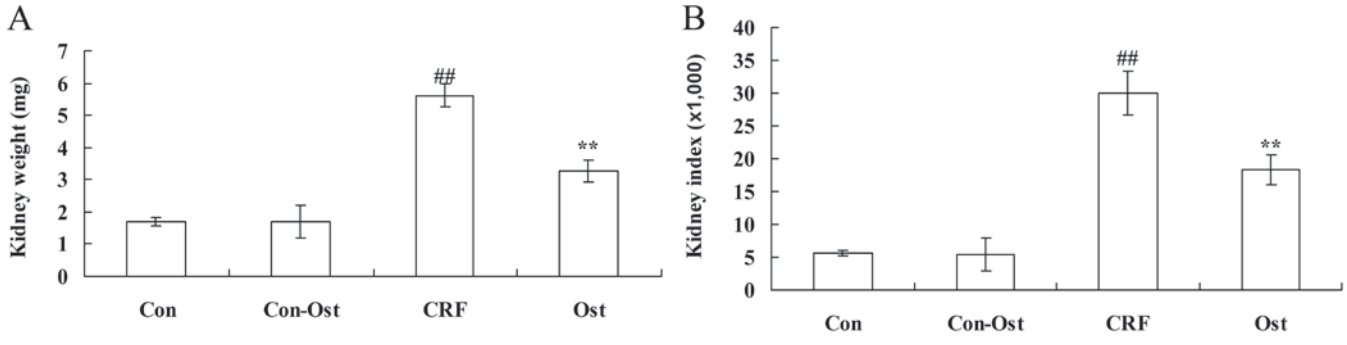

Figure 4. Osthole preserves kidney mass and renal index in the CRF rat. (A) Kidney mass and (B) kidney index in CRF rats. Data are presented as the mean ± standard error. **P<0.01 vs. Con, $^{\#\#}$P<0.01 vs. CRF. CRF, chronic renal failure; Con, control group; Con-Ost, control + osthole group; Ost, CRF +osthole group.

tissue damage in the CRF rat, kidney tissues were stained with H&E following the administration of osthole. There was no visible kidney tissue damage in the control or control osthole groups (Fig. 3). However, kidney tissue damage was observed in the CRF rat model group, and treatment with osthole visibly suppressed CRF-induced kidney tissue damage (Fig. 3).

Osthole treatment preserves the renal index in the CRF rat. CRF-induced kidney weight and index were assessed. No significant differences were observed in kidney weight (P>0.05; Fig. 4A) or kidney index (P>0.05; Fig. 4B) between the control and control osthole groups. Kidney weight and index in the CRF rat model group were significantly increased compared with the control (P<0.01; Fig. 4A) and control osthole (P<0.01;

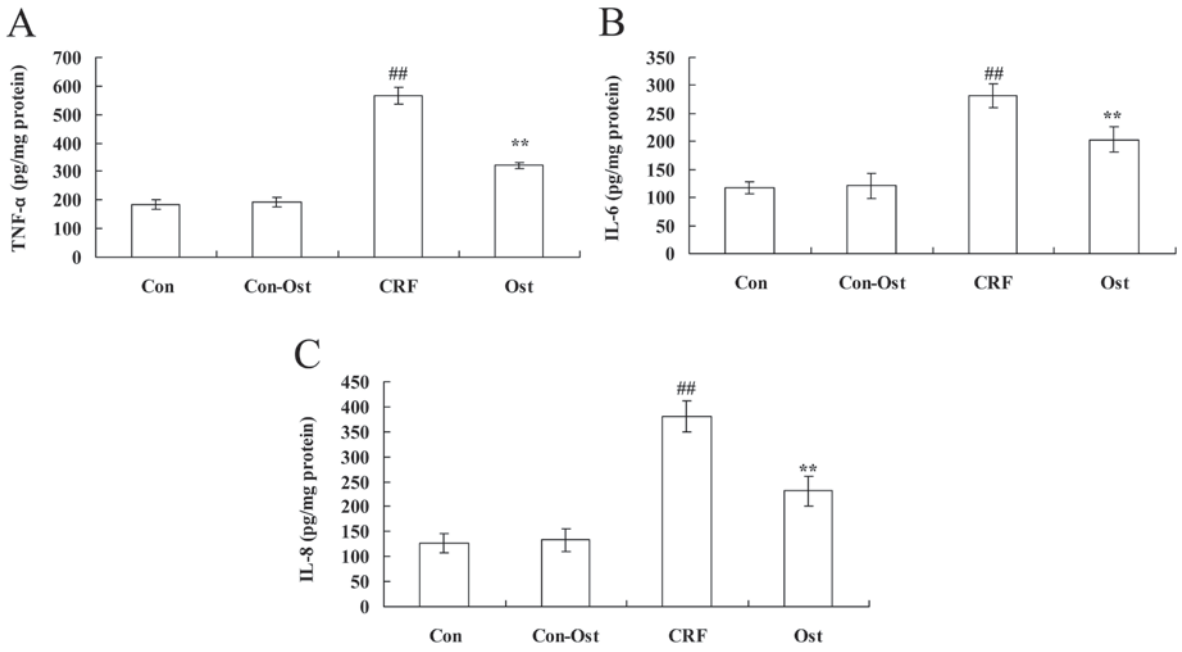

Figure 5. Osthole preserves serum levels of cytokines in CRF rats. Serum levels of (A) TNF-α, (B) IL-6 and (C) IL-8, as assessed by ELISA. Data are presented as the mean ± standard error. **P<0.01 vs. Con, $^{\#\#}$P<0.01 vs. CRF. TNF-α, tumor necrosis factor-α; IL, interleukin; CRF, chronic renal failure; Con, control group; Con-Ost, control + osthole group; Ost, CRF + osthole group.

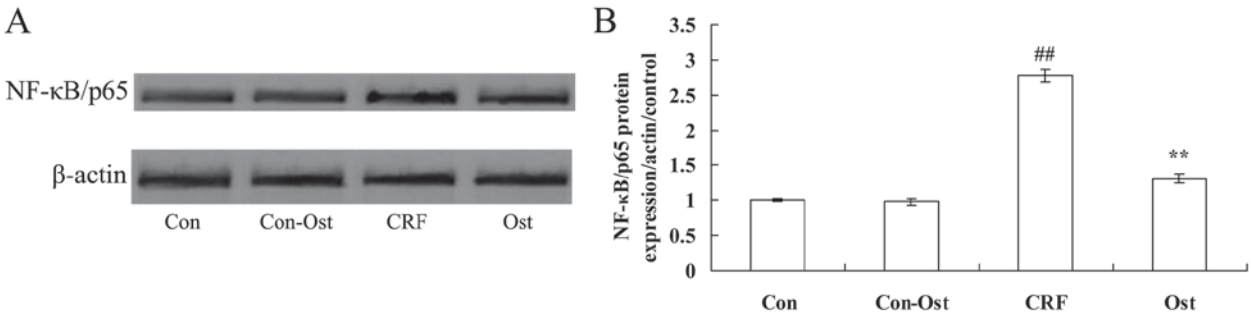

Figure 6. Osthole preserves NF-κB/p65 protein expression levels in the CRF rat. (A and B) Representative western blot images and quantification of protein expression levels of NF-κB/p65. Data are expressed as the mean ± standard error. **P<0.01 vs. Con; $^{\#\#}$P<0.01 vs. CRF. NF-κB; nuclear factor-κB; CRF, chronic renal failure; Con, control group; Con-Ost, control + osthole group; Ost, CRF + osthole group.

Fig. 4B) groups; however, osthole treatment significantly decreased kidney weight and index compared with the CRF rat model group (P<0.01 and P<0.01, respectively; Fig. 4A and B, respectively).

Osthole protects against inflammation in the CRF rat. To elucidate whether the CRF rat model exhibited inflammation, ELISA was used to analyze the serum levels of TNF-α, IL-6 and IL-8. No significant differences were observed in TNF-α (Fig. 5A), IL-6 (Fig. 5B) and IL-8 (Fig. 5C) serum levels between the control and control osthole groups (P>0.05). However, TNF-α, IL-6 and IL-8 serum levels were significantly increased in the CRF rat model group compared with the control groups (P<0.01). Osthole treatment significantly decreased TNF-α, IL-6 and IL-8 serum levels compared with the CRF rat model group (P<0.01).

Osthole prevents NF-κB/p65 increase in the CRF rat. Based on the above data, to further investigate the mechanisms underlying the effect of osthole on CRF, NF-κB/p65 protein expression levels were measured in CRF rats. No significant differences were observed between the control and osthole groups. NF-κB/p65 protein expression was significantly increased in the CRF rat model group compared with the control and control osthole groups (P<0.01). However, osthole treatment significantly suppressed the increase of NF-κB/p65 protein expression compared with the CRF rat model group (P<0.01; Fig. 6).

Osthole prevents TGF-β1 protein expression increase in the CRF rat. In the present study, TGF-β1 protein expression was identified following treatment with osthole in the CRF rat. No significant changes in TGF-β1 protein expression were observed between the control group and the control osthole group (P>0.05; Fig. 7). However, TGF-β1 protein expression was significantly higher in the CRF rat model group compared with the control group (P<0.01; Fig. 7). However, treatment with osthole significantly inhibited the CRF-induced increase

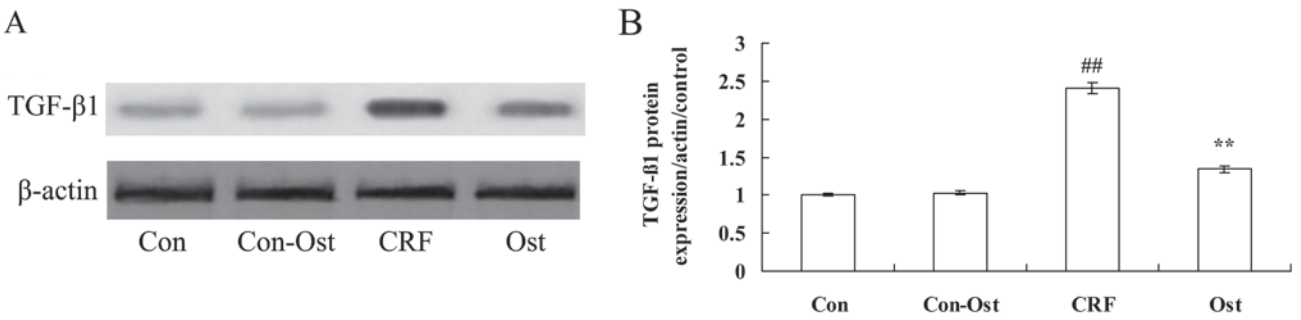

Figure 7. Osthole preserves TGF-β1 protein expression levels in the CRF rat. (A and B) Representative western blot images and quantification of protein expression levels of TGF-β1. Data are expressed as the mean ± standard error. **P<0.01 vs. Con; ##P<0.01 vs. CRF. TGF-β1, transforming growth factor-β1; CRF, chronic renal failure; Con, control group; Con-Ost, control + osthole group; Ost, CRF + osthole group.

of TGF-β1 protein expression, compared with the CRF rat model group (P<0.01; Fig. 7).

Osthole protects against MCP-1 activity increase in the CRF rat. To understand the biological involvement of osthole in the CRF rat, MCP-1 activity was examined to analyze the molecular functions of osthole. There was no significant difference in MCP-1 activity between the control group and the control osthole group (P>0.05; Fig. 8). There was a significant increase in MCP-1 activity in the CRF rat model group compared with the control group (P<0.01; Fig. 8), but treatment with osthole significantly reduced the CRF-induced increase of MCP-1 activity compared with the CRF rat model group (P<0.01; Fig. 8).

Osthole promotes Akt activation in the CRF rat. p-Akt and Akt protein expression levels were subsequently examined. The p-Akt/Akt ratio did not differ significantly between the control and the control osthole groups (P>0.05) and the p-Akt/Akt ratio of the CRF rat model group was significantly decreased compared with the control group (P<0.01). Treatment with osthole significantly increased the p-Akt/Akt ratio compared with the CRF rat model group (P<0.01; Fig. 9).

Osthole treatment increases Nrf2 protein expression levels in the CRF rat. Western blot analysis revealed no significant differences in Nrf2 protein expression levels between the control and control osthole groups (P>0.05), and Nrf2 protein expression was significantly decreased in the CRF rat model group compared with the control group (P<0.01). However, Nrf2 protein expression levels were significantly increased following osthole treatment, compared with the CRF rat model group (P<0.01; Fig. 10).

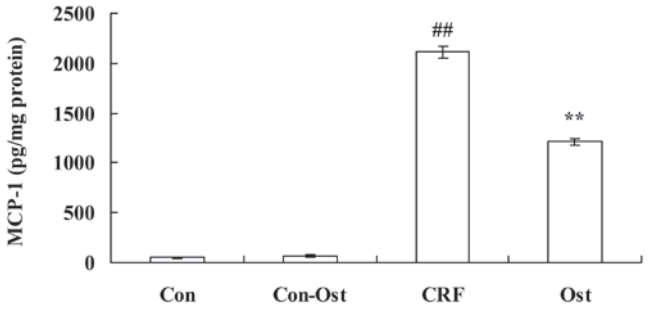

Figure 8. Osthole protects against MCP-1 activation in the CRF rat. Representative western blot images and quantification of protein expression levels of MCP-1. Data are expressed as the mean ± standard error. **P<0.01 vs. Con; ##P<0.01 vs. CRF. MCP-1, monocyte chemoattractant protein-1; CRF, chronic renal failure; Con, control group; Con-Ost, control + osthole group; CRF, Ost, CRF + osthole group.

Discussion

CRF refers to chronic progressive renal parenchymal damage and occurs for various reasons (13). Its primary manifestations include retention of metabolites and poisonous substances, water imbalance, electrolyte and acid-base disturbance, and abnormity of endocrine function (14). It has been predicted that 10% people suffer from chronic renal disease, and the morbidity of CRF in China is ~1/10,000 (15). CRF is a common consequence of primary and secondary renal diseases, and as it presents with a poor prognosis and high mortality, it affects the quality of life of patients (16). In the present study, treatment with osthole was demonstrated to increase serum creatinine, calcium and phosphorus levels, and decrease BUN serum levels, kidney weight and kidney index in a CRF rat model.

The inflammatory reaction, additionally known as the acute phase response, is a systemic reaction resulting from tissue damage (17). When an organism is damaged or infected, pro-inflammatory substances stimulate monocytes to produce pro-inflammatory cytokines, including ILs and TNF-α. These cytokines result in aberrant synthesis of acute reactive proteins (18). Acute reactive proteins contain positive and negative acute reactive proteins. Positive acute reactive proteins increase during the inflammatory reaction, and include C-reactive protein, serum amyloid A, fibrinogen, ferritin, binding beads, C3, α1-Acid glycoprotein and ceruloplasmin (19). Negative acute reactive proteins decrease during the inflammatory response, including albumin, prealbumin, retinol binding protein and siderophilin. In the present study, treatment with osthole was confirmed to significantly decrease CRF-induced alterations to TNF-α, IL-6 and IL-8 serum levels in rats via suppression of NF-κB/p65 protein expression. In addition, Tsai *et al* (11) demonstrated that osthole attenuates neutrophilic inflammation and oxidative stress in lung injury.

Previous studies have demonstrated that renal tubular epithelial cell-epithelial mesenchymal transition is one of the mechanisms underlying renal interstitial fibrosis, and

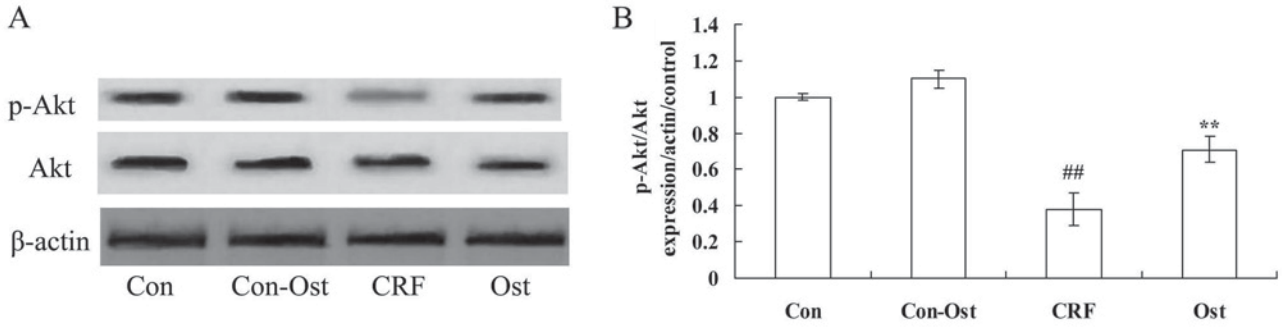

Figure 9. Osthole promotes Akt phosphorylation in the CRF rat. (A and B) Representative western blot images and quantification of protein expression levels of Akt. Data are expressed as the mean ± standard error. **P<0.01 vs. Con, ##P<0.01 vs. CRF model group. CRF, chronic renal failure; p-, phosphorylated; Akt, protein kinase B; Con, control group; Con-Ost, control + osthole group; Ost, CRF + osthole group.

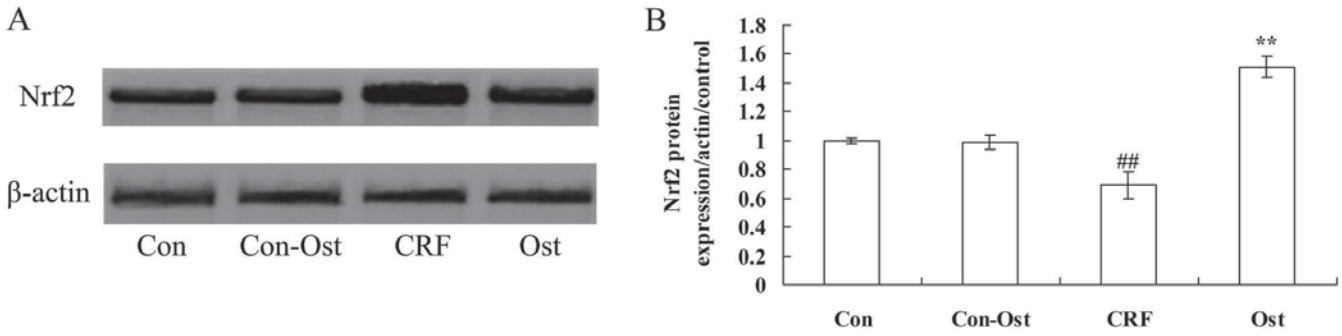

Figure 10. Osthole preserves Nrf2 protein expression levels in the CRF rat. (A and B) Representative western blot images and quantification of protein expression levels of Nrf2. Data are expressed as the mean ± standard error. **P<0.01 vs. Con, ##P<0.01 vs. CRF. CRF, chronic renal failure; Nrf2, nuclear factor (erythroid-derived 2)-like 2; Con, control group; Con-Ost, control + osthole group; Ost, CRF + osthole group.

may involve TGF-β1 (20,21). Cytokines may be involved in promoting or resisting renal interstitial fibrosis (22). The increase of TGF-β1 expression is an important factor for promoting renal interstitial fibrosis (23). As a transduction molecule, mothers against decapentaplegic (Smad) protein transduces TGF-β1 family signals to the nucleus. The TGF-β1/Smad pathway is a final and common pathway of renal fibrosis (24). In the TGF-β1/Smad signaling pathway, an active transcription complex produced by Smad2/3 enters the nucleus while Smad6 inhibits the transduction of TGF-β1 signals (25). Liu *et al* (26) reported that osthole inhibits hepatic fibrosis and hepatic stellate cells via TGF-β1 or endothelin-1. In the present study, treatment with osthole significantly inhibited CRF-induced TGF-β1 protein expression in rats.

Renal tubular epithelial cells are involved in the progression of renal interstitial fibrosis. For tubular interstitial lesions, renal tubular epithelial cells are affected by CRF of the luminal surface and basement membrane, including stimulation by excessive proteins in tubular fluid, growth factors, glucose peptides and oxygen free radicals (27). On the other hand, renal tubular epithelial cells are additionally stimulated by TGF-β1 and NF-κB, produced by mononuclear cells. Under the influence of these factors, renal tubular epithelial cells are activated. Increased secretion of MCP-1 and chemokine (C-C) motif ligand 5 results in the accumulation of inflammation cells, early inflammation and initiation of fibrosis. Consequently, inflammatory cell infiltration is an initiation factor for interstitial fibrosis and runs via the whole process of fibrosis (28). In the present study, treatment with osthole was demonstrated to significantly inhibit CRF-induced MCP-1 activity in rats. Hua *et al* (29) additionally confirmed that osthole mitigates progressive immunoglobulin A nephropathy by decreasing renal MCP-1 expression (30).

Akt is a downstream signaling molecule of phosphatidyl Inositol 3-kinase and may be activated upstream of NF-κB (31). Akt promotes the transcriptional activity of NF-κB in multiple ways. It activates IκB kinase and accelerates its degradation, which further promotes the nuclear translocation of NF-κB (32). In addition, it phosphorylates the Ser529 and -536 sites of NF-κB/p65 and strengthens the transcriptional activity of NF-κB (33). This signal cascade reaction finally results in cell multiplication and migration. Yang *et al* (34) demonstrated that osthole improves and accelerates focal segmental glomerulosclerosis via promotion of Nrf2 and suppression of NF-κB. Yao *et al* (35) reported that osthole induces vasodilation via the Akt-eNOS-NO signaling pathway in rats. In the present study, osthole treatment was demonstrated to significantly promote Akt and Nrf2 protein expression levels in CRF rats.

In conclusion, osthole treatment increased serum creatinine, calcium and phosphorus levels, and decreased BUN serum levels, kidney weight and kidney index scores of a CRF rat model. Therefore, osthole may protect against inflammation and MCP-1 activity, implicating its role as a potential therapeutic agent for the treatment of CRF via suppression of NF-κB and TGF-β1, and activation of Akt and Nrf2 in later clinical and pathological stages.

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