Protective effects of osthole, a natural derivative of coumarin, against intestinal ischemia-reperfusion injury in mice

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Abstract. Intestinal ischemia/reperfusion (I/R) injury is considered to be associated with high morbidity and mortality rates. Osthole, a natural derivative of coumarin, has been shown to exert a variety of pharmacological and therapeutic effects under physiological and pathological conditions. In the present study, to investigate the protective effects of osthole against intestinal I/R injury, various doses of osthole (5, 10, 25 and 50 mg/kg) were pre-administered to mice subjected to intestinal I/R injury. A dose-dependent increase in the survival rate was observed in the osthole-treated mice. Pre-treatment with osthole (50 mg/kg) attenuated the destruction of epithelial cells within the villi induced by intestinal I/R injury, and suppressed oxidative stress, neutrophil infiltration and modulated nitric oxide (NO) levels. Moreover, the increased IkBα phosphorylation and nuclear factor (NF)-κB nuclear translocation induced by I/R injury were significantly decreased following pre-treatment with osthole. Taken together, our data demonstrate that osthole exerts protective effects against intestinal I/R injury in mice by suppressing oxidative stress, neutrophil infiltration and NO levels, partly through the inhibition of NF-κB nuclear translocation. Hence, the findings of the present study provide insight into the mechanisms through which osthole exerts its protective effects against intestinal I/R injury.

Introduction

Intestinal ischemia/reperfusion (I/R) injury represents a significant clinical problem in numerous situations, such as small bowel transplantation, cardiopulmonary bypass, acute mesenteric ischemia, abdominal aortic aneurysm surgery, intestinal obstruction, as well as hemorrhagic, traumatic and septic shock (1,2). Intestinal I/R injury triggers a highly complex cascade of events resulting in decreased contractile activity, increased microvascular permeability and the dysfunction of the mucosal barrier (3-7). Although the definitive pathogenesis regarding intestinal I/R injury remains obscure, the hallmarks of this condition have been well defined, including reactive oxygen species (ROS), recruitment of activated leukocytes, cytokine release, cellular apoptosis and mitochondrial dysfunction (8). ROS, the most critical effector of intestinal I/R injury, can react with all biological macromolecules (nucleic acids, proteins, carbohydrates and lipids) in a destructive manner and initiate a wide range of toxic oxidative reactions, which include the initiation of lipid peroxidation, the direct inhibition of mitochondrial respiratory chain enzymes and membrane sodium/potassium adenosine 5’-triphosphate-ase activity, inactivation of membrane sodium channels and other oxidative modifications of proteins (9,10). In addition, the overproduction of nitric oxide (NO) generated by inducible NO synthase (iNOS) is also characterized in intestinal I/R, which aggravates intestinal oxidative stress (11). The nuclear factor (NF)-κB pathway has been reported to play an important role in intestinal I/R which is responsible for ROS accumulation and cell apoptosis (12). Moreover, oxidative stress promotes inflammatory cell infiltration and mitochondrial dysfunction. Thus, the therapeutic strategy of removing free radicals and reducing NO overproduction may be potentially effective for protection against intestinal I/R injury.

However, to date, there is still lack of effective therapeutic treatments for intestinal I/R injury. Osthole, a natural derivative of coumarin, is an active constituent extracted from some medicinal plants (13) which has been shown to exert a variety of pharmacological and therapeutic effects. Accumulating evidence has demonstrated that osthole possesses a variety of pharmacological properties, including antitumor (14), anti-osteoporotic (15), anti-diabetic (16), anti-inflammatory (17) and anti-allergic properties (18). Recently, osthole has been demonstrated to exert therapeutic effects against cerebral ischemic stroke (19,20) and cardiac hypertrophy in rats (21).

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Thus, the effects of osthole on intestinal I/R injury need to be further investigated.

The aim of this study was to investigate the effect of osthole in a mouse model of intestinal I/R and delineate the underlying mechanisms. Using a mouse model, we first observed that pre-treatment with osthole exerted protective effects on mice with intestinal I/R injury in a dose-dependent manner. Pre-treatment with osthole markedly ameliorated villous height and attenuated the destruction of epithelial cells within the villi. The effect of osthole against intestinal I/R injury was evaluated by measuring the levels of myeloperoxidase (MPO), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH). Pre-treatment with osthole exerted protective effects, including the suppression of oxidative stress, neutrophil infiltration and NO levels. Increased iNOS phosphorylation and NF-κB nuclear translocation induced by I/R injury were also significantly decreased following pre-treatment with osthole. In this study, the effects of osthole in the early phase following intestinal I/R injury in mice were investigated to provide a mechanistic basis for the protective effects of osthole against intestinal I/R injury.

Materials and methods

Animals and experimental groups. Adult male BALB/c mice were obtained from the Laboratory Animal Center of the Fourth Military Medical University, Xi’an, China and housed under standard conditions. They were kept in cages at a fairly constant room temperature and were exposed to a 12/12 h light/dark cycle. Each animal was fasted overnight prior to surgery, but had access to water ad libitum. All animal experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986, under the guidelines of the National Health and Medical Research Council for the Care and Use of Animals for Experimental Purposes in China.

The mice were randomly divided into 3 groups (n=13 per group): i) Sham group: all the surgical steps were performed; however, intestinal I/R was not induced. The animals were kept under anesthesia for the duration of the intestinal I/R procedure. ii) I/R group: intestinal I/R was induced and this group served as the control for the osthole-treated group. iii) Osthole group: a single dose of osthole (50 mg/kg daily for 3 days) was injected via the tail vein. Osthole (>98% purity) was a gift from Dr W. Liu (Department of Neurology, Xijing Hospital, Fourth Military Medical University, Xi’an, China). The mice in each group were subdivided into 3 subgroups. The first subgroup was used for histological examination and immunofluorescence staining (n=4 per group); the second subgroup was used for detecting MPO, GSH, SOD, MDA and NO levels (n=6 per group); and the third subgroup was used for western blot analysis (n=3 per group).

Model of intestinal I/R injury. Following an acclimation period of at least 3 days, the mice were prepared for surgery under deep sodium pentobarbital anesthesia. All procedures were performed with the animals breathing spontaneously and body temperature was maintained at 37°C using a water-circulating heating pad. The animals were subjected to I/R as previously described (22). Briefly, a midline laparotomy was performed. The superior mesenteric artery was identified and isolated, and the blood supply to the intestine was interrupted for 1 h by occlusion of the superior mesenteric artery using a microvascular clamp. Ischemia was confirmed by the loss of pulsation of the mesenteric artery and its branches or a pale color of the intestine. Following the ischemic phase, the clamp was removed to allow the restoration of the blood flow (reperfusion) for 6 h, which was confirmed by the return of the pulses, re-establishment of the pink color and enhanced intestinal peristalsis. Once reperfusion was secured, the wound was sutured and the animal was allowed to awaken from the anesthesia. The mice were administered an overdose of pentobarbital prior to euthanasia.

Histology and tissue injury scoring. For isolation of the intestinal segments, tissue from the entire intestine was cut into 6 equal segments, of which a section of 1-1.5 cm at the middle of each segment was collected for histological analysis. Formalin-fixed and paraffin-embedded tissue sections were cut (4-μm-thick) and stained with hematoxylin and eosin for histological examination. The above procedure was performed by a physician. Intestinal sections from each animal were scored for mucosal damage as previously described (23). Briefly, a score of 0 was assigned to a normal villus; villi with tip distortion were scored as 1; villi lacking goblet cells and containing Guggenheim’s spaces were scored as 2; villi with patchy disruption of the epithelial cells were scored as 3; villi with exposed but intact lamina propria and epithelial cell sloughing were assigned a score of 4; villi in which the lamina propria was exuding were scored as 5; and finally, villi displaying hemorrhage or denudation were scored as 6. All histological analyses were performed by a pathologist in a blinded manner. Five random high-power fields (×200) were examined per sample.

MPO assay. The intestinal samples were weighed and homogenized in a solution prepared from the assay kit (Jiancheng Institute of Biotechnology, Nanjing, China), and homogenates were obtained and used for MPO assay. The reaction was initiated by adding hydrogen peroxide in the medium and 3,3,5,5-tetramethylbenzidine was used as an oxidizable dye to produce yellow color compounds. The optical density was recorded at 460 nm and the results were expressed as U/g wet tissue.

Detection of MDA, GSH and SOD in intestinal tissue. The intestinal tissues were homogenized in 0.05 mol/l phosphate buffer. The homogenates were centrifuged at 4,000 rpm for 20 min at 4°C and the MDA, GSH and SOD content in the supernatant was measured using the corresponding kits (Jiancheng Institute of Biotechnology). SOD activity and MDA levels were measured using the xanthine oxidase and thiobarbituric acid method, respectively. SOD activity was measured at 500 nm. One SOD activity unit was defined as the enzyme amount causing 50% inhibition in 1 ml reaction solution/mg tissue protein and the result was expressed as U/mg protein. MDA was determined at 532 nm and the results were expressed as nmol/mg protein. GSH levels were measured through a reaction using dithiobisnitrobenzoic acid. GSH activity was determined at 420 nm and the results were expressed as mg/g protein.

NO synthesis can contribute to nitrite accumulation. The concentration of nitrite in the intestinal samples was measured...
following a reaction with Griess reagent (sulfanilamide 1%, naphthyl ethylene diamine 0.01%, H$_3$PO$_4$ 5%) to reflect the production of NO at 550 nm. The results were expressed as µmol/g protein. Detailed procedures for the above measurements were performed according to the instructions of kit. The protein content was determined using a kit based on the Bradford assay and bovine serum albumin was used as a standard.

**Western blot analysis.** Total protein lysates from frozen tissues were prepared in ice-cold RIPA buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS and protease inhibitor cocktails). Nuclear extracts were prepared using a Nuclear Protein Extraction kit (Beyotime Biotechnology, Shanghai, China). Protein samples were immunoblotted with antibodies to IκB, p-IκB and NF-κB-p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and the protein-antibody immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA). For detection, an ECL chemiluminescence system (GE Healthcare, Piscataway, NJ, USA) was used. Signals were detected and quantified using the LAS-3000 image analyzer (FujiFilm, Tokyo, Japan).

**Immunofluorescence staining.** Staining was performed on slides of 4-µm-thick sections from intestinal tissue. Following deparaffinization and dehydration, the slides were immersed in 10 mM sodium citrate buffer containing 0.05% Tween-20 (pH 6.0) for 5 min at 55°C for antigen retrieval. The slides were then permeabilized with 10 µg/ml proteinase K in 10 mM Tris/HCl (pH 7.6) for 15 min and incubated with 5% BSA in a humidified chamber for 30 min to block non-specific binding. The slides were incubated with antibody against NF-κB-p65 (1:200 in PBS containing 1% BSA; Santa Cruz Biotechnology, Inc.) in a humidified chamber overnight at 4°C, and with fluorescent-fluorescein isothiocyanate-conjugated donkey anti-rabbit immunoglobulin G (1:500 in PBS) at room temperature for 1 h. The nuclei were counterstained with Hoechst 33258 (1:5,000; 10 min). Fluorescence imaging was assessed under a fluorescence microscope (Fluorescence E-1000 microscopy, Nikon, Japan) and subjected to identical exposure times. Instead of the primary antibody, PBS was used for the negative controls.

**Statistical analysis.** Data were analyzed using SPSS v 11.5 software for Windows (SPSS Inc, Chicago, IL, USA). All values are presented as the means ± standard error of the mean (SEM). Differences between groups were compared using analysis of variance with the Bonferroni correction for multiple comparisons. Values of P<0.05 were considered to indicate statistically significant differences.

**Results**

**Osthole improves the survival rate of mice subjected to intestinal I/R injury.** In order to determine the effect of osthole on intestinal I/R injury, the mice were administered various doses of osthole (5, 10, 25, 50 mg/kg) for 3 days prior to the induction of intestinal I/R injury. The results revealed that osthole preconditioning (25 mg/kg and 50 mg/kg) prior to intestinal I/R injury significantly improved the survival rate at 24 h following reperfusion as compared to the vehicle-treated mice (Fig. 1).

**Osthole attenuates intestinal injury induced by I/R.** To further investigate the effect of osthole on intestinal I/R injury, the morphology of the intestinal epithelium and villi was examined by immunohistochemistry. In the sham group, the results of histopathological examination of the intestinal epithelium and villi were normal. Mucosal necrosis and bleeding were observed in the intestinal mucosa and submucosa in the I/R group which also showed a marked reduction in villus height and destruction of epithelial cells within the villi. In the osthole group, mice pre-treated with osthole (50 mg/kg) demonstrated intestinal architecture with almost normal histological features. Although the median intestinal injury score of the osthole group was higher than the sham group (P<0.001), it was significantly lower than that of the I/R group (P<0.01) (Fig. 2B). Taken together, these data suggest that osthole exerts protective effects against intestinal I/R injury.

**Osthole exerts antioxidant effects in intestinal I/R injury.** To elucidate the underlying mechanisms of action of osthole in protecting against intestinal I/R injury, the antioxidant and oxidant products were evaluated. The levels of SOD, an enzyme occurring naturally in the body which protects cells by cleaning up free radicals, were significantly reduced in the I/R group compared with the sham group (P<0.05). Following pre-treatment with osthole, SOD activity was significantly improved compared with the I/R group (P<0.01) (Fig. 3A). The levels of MDA, an important marker of lipoperoxidation associated with oxidative stress, were decreased following pre-treatment with osthole (P<0.05 vs. I/R group) (Fig. 3B). Following I/R injury, the levels of GSH, a common antioxidant, were slightly decreased; however, following pre-treatment with osthole, these levels increased. However, the difference was not statistically significant among the different groups (Fig. 3C). These results demonstrate that osthole relieves oxidative stress.
through the upregulation of SOD and the downregulation of MDA levels following intestinal I/R injury.

**Osthole reduces neutrophil infiltration following intestinal I/R injury.** Tissue MPO activity is a hallmark of intestinal I/R injury which reflects the infiltration of neutrophils. In order to examine the effect of osthole on inflammation, the activity of MPO was measured. Compared to the sham group, a significant increase in MPO activity was observed in the I/R group, which was alleviated in the osthole-treated group (P<0.01) (Fig. 4).

**Osthole inhibits the elevation of NO levels following intestinal I/R injury.** The overproduction of NO following intestinal I/R injury has been reported to be closely associated with oxidative damage (11). NO synthesis can contribute to nitrite accumulation. The concentration of nitrite in the intestinal samples was measured to reflect the production of NO. As shown in Fig. 5, pre-treatment with osthole significantly inhibited the elevation of NO levels in the I/R group (P<0.01).

**Osthole protects against intestinal I/R injury by inhibiting the activation of the NF-κB signaling pathway.** It has been reported that NO produced by the iNOS gene is induced during inflammation (24). NF-κB is an important nuclear transcription factor that regulates iNOS gene expression by binding the iNOS promoter region and plays a central role in inflammation through its ability to induce the transcription of pro-inflammatory genes (25). The data mentioned above suggest that osthole reduces neutrophil infiltration and inhibits the NO levels induced by intestinal I/R injury. Thus, we hypothesized that osthole protects against intestinal I/R injury by inhibiting the activation of the NF-κB signaling pathway. To confirm our hypothesis, we examined the effects of osthole on...
NF-κB nuclear translocation. In the sham group, NF-κB was localized in the cytoplasm and translocated to the nucleus in the I/R group. Pre-treatment with osthole inhibited the nuclear translocation of NF-κB compared with the I/R group (Fig. 6). In order to further confirm these results, western blot analysis was performed to examine the nuclear expression of NF-κB and the phosphorylation state of IκBα. Consistent with the immunofluorescence staining results, we observed that the nuclear expression of NF-κB was markedly increased in the I/R group, while pre-treatment with osthole inhibited the nuclear expression of NF-κB (P<0.01 vs. I/R group). We also found that phosphorylated IκBα was increased in the I/R group. In the osthole-treated group, phosphorylated IκBα was significantly inhibited (P<0.01 vs. I/R group) (Fig. 7).

Discussion

Osthole, a Chinese herbal medicine possessing a variety of pharmacological properties, has gained considerable attention over the years and has been used for the treatment of a number of diseases, including carcinoma (14), amnesia (26), metabolic syndromes (27,28), seizures (29) and osteoporosis (30). Recent studies have suggested that osthole is also beneficial in preventing I/R injury. It has been shown that osthole prevents motor impairment, decreases the levels of NO, interleukin (IL)-1β and IL-8, inhibits iNOS and MPO activity, and decreases infarct volume following middle cerebral artery occlusion (31). Similarly, it has been demonstrated that osthole significantly inhibits neuronal cell death in the CA1 area in the hippocampus by decreasing caspase-3 protein expression in
male SD rats subjected to transient global brain ischemia (32). However, no studies have been conducted on the role and potential mechanisms of action of osthole in intestinal I/R injury. In the present study, we demonstrated that pre-treatment with osthole exerted protective effects against intestinal I/R injury. Pre-treatment with osthole improved the survival rates of mice subjected to I/R injury and protected intestinal epithelial cells and villi against I/R injury. Osthole also suppressed stress, neutrophil infiltration and NO overproduction. Furthermore, we found that the underlying mechanisms of action of osthole in preventing intestinal I/R injury involved the inhibition of NF-kB.

The production of free radicals from oxygen molecules derived from the electron transport chains of mitochondrial cells, endothelial cells and activated neutrophils elicits oxidative stress and enhances intestinal I/R injury (33). Thus, the antioxidant, SOD, is considered to play a crucial role in relieving oxidative stress during I/R injury. In this study, we found that pre-treatment with osthole upregulated SOD activity which was suppressed in the I/R group. Osthole also downregulated MDA levels following intestinal I/R injury, which are a marker of lipoperoxidation associated with oxidative stress. These results are in agreement with those from previous reports demonstrating that osthole is effective in treating hyperlipidemic and alcoholic fatty liver by the inhibition of ROS production, the enhancement of antioxidative enzyme activity and the reduction of peroxidation (34,35). In addition, it was found that the neuroprotective effects of osthole against acute ischemic stroke and 1-methyl-4-phenylpyridinium ion-induced cytotoxicity in PC12 cells is partly attributed to its antioxidative action (36). Neutrophil infiltration has been proposed as a hallmark of intestinal I/R injury. The activity of MPO which is mainly released by neutrophils can be evaluated as neutrophil infiltration (11). In our study, MPO activity was significantly augmented in the I/R group and was decreased following pre-treatment with osthole. Furthermore, the involvement of NO in intestinal I/R injury has been widely suggested. iNOS can produce excessive amounts of NO following intestinal I/R injury, which is considered responsible for the cytotoxic potential of NO, resulting in oxidative stress and tissue damage (11,37,38). A previous study demonstrated that osthole decreased NO levels and inhibited NOS activity in a rat model of carrageenan-induced hind paw edema (17). In the current study, we found that osthole markedly inhibited the increased NO levels induced by intestinal I/R injury. These results suggest that osthole exerts therapeutic effects against intestinal I/R injury by attenuating oxidative stress, reducing excessive neutrophil infiltration and modulating NO levels.

However, the mechanisms of action of osthole in exerting its protective effects remain to be elucidated. The iNOS promoter region contains binding sites for NF-kB, and its expression during I/R injury requires de novo transcription, which is under the control of NF-kB. NF-kB is usually present in the cytoplasm in an inactive form bound to the inhibitory protein, I-kB. NF-kB is activated by a variety of stimuli, including growth factors, cytokines, oxidative stress and pro-inflammatory stimuli, and the nuclear translocation of NF-kB subunits is induced following intestinal I/R injury (39,40). It has been reported that iNOS is capable of producing abundant NO under certain pathological conditions, which presents beneficial effects but also contributes to the overproduction of cytotoxic radicals (41). In a previous study, it was shown that osthole, isolated from Clausena guillauminii, inhibited the protein expression of iNOS induced by LPS in mouse macrophages (42). Another study also demonstrated that osthole reduced tumor necrosis factor (TNF)-α, NO and cyclooxygenase (COX)-2 expression, inhibited NF-kB activation and ROS release in LPS-stimulated macrophages (43). In this study, pre-treatment with osthole inhibited the translocation of NF-kB to the nucleus and increased I-kB phosphorylation induced by intestinal I/R injury. These data suggest that osthole exerts protective effects against intestinal I/R injury through the NF-kB-iNOS-NO pathway. Together with the free radical scavenging and anti-inflammatory effects, osthole may be a potential candidate for the development of anti-ischemic drugs.

Certain limitations of this study should be noted. Intestinal IR injury has been extensively investigated in animal models and various approaches to diminish the damaging effects of intestinal I/R injury have been investigated in animal models,
as well as in vitro (44.45). However, these models are not a realistic model of the clinical situation and there are a number of obvious differences between animal models and humans during intestinal I/R injury, including antigens of specific proteins and sensitivity to intestinal ischemia and inflammatory responses (46-48). Moreover, advances in the clinical treatment of I/R injury have been minimal. Pre-treatment with osthole prior to ischemia in this study may not be a realistic model of the clinical situation. The mice were pre-treated with osthole prior to the induction of ischemia. Additionally, it was confirmed that osthole preconditioning relieved cerebral ischemic stroke (19). Therefore, although a recent study confirmed that treatment with osthole subsequent to ischemia significantly improved the cognitive deficits induced by chronic cerebral hyperperfusion in rats (49), it can only be speculated whether or not osthole postconditioning would also attenuate intestinal I/R injury. To clarify this important point, treatment with osthole at different starting points following the onset of intestinal ischemia or reperfusion should be investigated.

Taken together, the data from our study demonstrate that pre-treatment with osthole attenuates oxidative stress, reduces neutrophil infiltration and decreases NO levels. The mechanisms behind the protective effects of osthole involve the inhibition of NF-kB nuclear translocation. Thus, the present study suggests that osthole exerts protective and therapeutic effects against intestinal I/R injury.

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