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Effect and mechanism of evodiamine against ethanol-induced gastric ulcer in mice by suppressing Rho/NF-κB pathway

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ABSTRACT

Evodiamine (EVD), a major alkaloid compound extracted from the dry unripened fruit *Evodia fructus* (*Evodia rutaecarpa* Benth., Rutaceae), has various pharmacological effects. The purpose of the present study was to investigate the possible anti-ulcerogenic potential of EVD and explore the underlying mechanism against ethanol-induced gastric ulcer in mice. Administration of EVD at the doses of 20, 40 mg/kg body weight prior to the ethanol ingestion could effectively protect the stomach from ulceration. The gastric lesion was significantly ameliorated in the EVD group compared with that in the model group. Pre-treatment with EVD prevented the oxidative damage and decreased the levels of prostaglandin E2 (PGE2) content, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). In addition, EVD pretreatment markedly increased the serum levels of glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT), decreased malonaldehyde (MDA) content in serum and activity of myeloperoxidase (MPO) in stomach tissues compared with those in the model group. In the mechanistic study, significant elevation of Rho, Rho-kinase 1 (ROCK1), ROCK2, cytosolic and nucleic NF-κBp65 expressions were observed in the gastric mucosa group, whereas EVD effectively suppressed the protein expressions of Rho, Rho-kinase 1 (ROCK1), ROCK2, cytosolic and nucleic NF-κBp65 in mice. Moreover, EVD showed protective activity on ethanol-induced GES-1 cells, while the therapeutic effects were not due to its cytotoxity. Taken together, these results strongly indicated that EVD exerted a gastro-protective effect against gastric ulceration. The underlying mechanism might be associated with the improvement of antioxidant and anti-inflammatory status through Rho/NF-κB pathway.

1. Introduction

Peptic ulcer is one of the most pervasive gastrointestinal diseases which affect 4–5% people in the society [1]. The gastric ulcer is characterized by the reduction in blood flow, infiltration of neutrophils, induction of oxidative stress and secretion of inflammatory cytokines [2,3]. Various factors including helicobacter pyloriinfection, alcohol consumption, smoking, excessive use of non-steroidal anti-inflammatory drugs (NSAIDs), psychological and physiological stress contribute to gastric ulcer [4]. Especially, alcohol consumption increases the risk for major upper gastrointestinal bleeding [5]. As alcohol is one of the most commonly abused agents, thus, the alcohol-induced peptic ulcer is the main disorders of the gastrointestinal tract [6].

One of the most widely recognized intracellular signaling pathway in inflammatory responses is the nuclear factor kappa B (NF-κB) signaling pathway [7]. Expressed in almost all cells, NF-κB participate in the regulation of various genes involved in immune response and acute phase inflammatory reaction [8]. Many pro-inflammatory stimuli can lead to the activation of NF-κB through the phosphorylation of inhibitors of κB (IκBs) by the IκB kinase (IKK) complex [9]. Afterwards, the freed NF-κB translocate into the nucleus and consequently result in the transcriptional activation of several pro-inflammatory mediators, such as TNF-α, IL-1β and IL-6 [10].

As a member of the Ras super family of small monomeric GTPases, the Rho protein controls a variety of downstream effectors proteins including Rho-kinase. Rho, exerts GDP- and GTP-binding activities, alternates between a GDP-bound inactive state and a GTP-bound active state, which allows Rho to serve as a molecular switch that governs downstream signal transduction [11,12]. Both Rho and Rho-kinase are ubiquitous proteins that have been proved to play critical roles in the mediations of multiple biological pathways including a variety of physiological functions associated with the alterations in the actin cytoskeleton, such as cell adhesion, motility, migration and contraction [13]. Rho and its target protein, Rho-associated coiled-coil forming protein kinase (ROCK) are involved in a calcium-sensitizing signaling pathway implicated in the cytoskeletal contractile reaction via their important influence on myosin ATPase activity [14]. ROCK conduces to the

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phosphorylation of the light-chain of myosin (MLC) on Ser 19 and Thr 18 through the phosphorylation of MLC phosphatase and the suppression of MLC dephosphorylation. The phosphorylated MLC ultimately promotes myosin ATPase activity, resulting in the actin myosin reorganization, tension fiber formation and cell contraction [15]. Therefore, Rho and ROCK are well-established regulators of the permeability between cells. Additionally, it has been proposed that Rho/ROCK pathway plays an essential role in the regulation of inflammatory response [16].

Evodiamine, an alkaloid extracted from the dried unripe fruit of *Evodia rutaecarpa* Bentham (Rutaceae), is a longstanding Chinese herbal medicine traditionally used for the treatment of pain, vomiting and pyresis. Evodiamine exhibits a wide variety of bioactivity including anti-nociceptive, anti-obesity, vasodilatory, anti-tumor and anti-inflammatory effects [17–21]. However, few reports focused on the ethanol-induced gastric ulcer and the effects of EVD on the activation of Rho-kinase. The aim of the study was to investigate the anti-gastric ulcer effects of EVD on ethanol-induced mice and explore its possible mechanisms.

2. Materials and methods

2.1. Reagents

EVD (pure: 99%) were purchased from National Institutes for Food and Drug Control (Beijing, China). As the positive control, omeprazole (OME) was supplied by Shanghai XinyiJiahua Pharmaceutical Company Limited (Shannxi, China). Both EVD and OME were prepared as a stock solution in DMSO and diluted in autoclaved PBS to the appropriate dosage or concentration. It was noteworthy that the final concentration of DMSO was less than 0.1% [v/v] in all the experiment. All biochemical indicator kits were provided by the Institute of Jiancheng Bioengineering (Nanjing, China). The enzyme-linked immunosorbent assay (ELISA) kits for determination of IL-6 and TNF-α were produced by Biolegend (San Diego, CA, USA). All antibodies were supplied by Cell Signaling Technology.

2.2. Animals

A total of 50 female Specific pathogen-free female BALB/c mice, aged 6–8 weeks, were purchased from the Center of Experimental Animals of Jilin University (Changchun, China). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to the experiments and provided with water and standard chow *ad libitum*. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the National Animal Welfare Law of China.

2.3. Induction of gastric ulcer and treatment

Five groups of mice were assigned: groups 1 and 2 were given DMSO plus PBS (vehicle, the final concentration of DMSO was less than 0.1% [v/v]) orally. Group 3 was administered omeprazole (OME) 20 mg/kg, Group 4 and 5 were given EVD (20 mg/kg, 40 mg/kg, respectively). The doses of EVD were selected as previously described by Jiang et al. [22]. After an additional hour, the mice in groups 2–5 intragastrically received ethanol (0.2 ml/kg) while group 1 received PBS instead. Mice were sacrificed after 4 h and their serum and stomach tissues were harvested for the further studies.

2.4. Determinations of GSH, SOD, CAT, MDA in serum and MPO in stomach tissue

At the end of the experiment, the blood was centrifuged at 3000 rpm for 8 min and then the serum samples were stored at −80 °C for pending tests. Stomach tissues were homogenized with cold normal saline and centrifuged at 12,000 rpm for 10 min at 4 °C, the supernatant of the homogenate was collected and stored at −80 °C. The protein content of stomach sample was determined using a BCA protein assay kit. GSH, SOD, CAT and MDA levels in serum and MPO activity in stomach tissue were determined using test kits purchased from Nanjing Jiancheng Bioengineering Institute (China, Nanjing) according to the manufacturer's protocols.

2.5. Determinations of PGE_2, IL-6 and TNF-α

PGE_2, IL-6 and TNF-α level in serum were determined with ELISA kits purchased from Biolegend (San Diego, CA, USA). All procedures were carried out according to the manual.

2.6. Histological analysis

Stomach sample removed from each mouse was fixed in 10% buffered formalin for more than 48 h. After dehydrating in graded alcohol and embedding in paraffin wax, the sections were cut to a thickness of 4 μm and stained with hematoxylin and eosin (H&E) for histological evaluation. Then the pathological changes in the gastric tissues were observed under a light microscope.

2.7. Preparation of nuclear and cytosolic extracts

Nuclear and cytosolic protein extracts were prepared as previously described [23]. The gastric tissues extracted from the animals per experimental group were homogenized in buffer (1 mM EDTA, 10 mM HEPES, 0.25 mM PMSF, 50 mM NaF, 0.5 mM DTT, 2 mM sodium-orthovanadate, 5 mg/ml of leupeptin and pepstatin). The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C and then the supernatant was centrifuged at 100,000 rpm for another 1 h to obtain the cytosolic fraction. After that, the low speed nuclear pellet was washed for two times in homogenization buffer containing 250 mM sucrose. 1 h after the salt extraction, the samples were centrifuged at 8000 rpm for 30 min and the supernatant was taken as the nuclear fraction.

2.8. Western blot of Rho/NF-κB pathway

Then the protein extractions of cytosolic and nuclear fractions were carried out with a cytosolic/nuclei isolation kit (KeyGEN, Nanjing, China) according to the manufacturer's instruction. After the determination of the protein concentration using an enhanced BCA kit (Beyotime, China), the samples were loaded to 10% SDS-PAGE gels and then electrotransferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). The blots were incubated with the appropriate concentrations of specific antibodies at 4 °C overnight. After washing, the blots were incubated with horseradish peroxidase-conjugated second antibody. The membranes were stripped and reblotted to verify the equal loading of protein in each lane. Quantification of protein expression was normalized to GAPDH or Histone H3 using a densitometer (Imaging System).

2.9. Cell culture

The human gastric mucosa cell line GES-1, was obtained from the Beijing Institute for Cancer Research (Beijing, China). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Hyclone, South America), 100 U/ml penicillin and 100 U/ml streptomycin (Amresco, USA) with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.10. MTT assay for cell viability

GES-1 cells were seeded at a density of 1×10^4 cells/well on 96-well culture plates for 24 h. Then the cells were incubated in the presence or