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Research Paper

Atractylodes macrocephala Koidz promotes intestinal epithelial restitution via the polyamine—Voltage-gated K^+ channel pathway

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ABSTRACT

Ethnopharmacological relevance: *Atractylodes macrocephala* Koidz (AMK) has been used widely as a digestive and tonic in traditional Chinese medicine. AMK has shown noteworthy promoting effect on intestinal epithelial cell migration, which might represent a promising candidate for the treatment of intestinal mucosa injury. The aim of this study was to investigate the efficacy of AMK on intestinal mucosal restitution and the underlying mechanisms via IEC-6 cell migration model.

Materials and methods: A wounding model of IEC-6 cells was induced by a single-edge razor blade along the diameter of six-well polystyrene plates. The cells were grown in control cultures and in cultures containing spermidine (5 μmol/L, SPD, reference drug), alpha-difluoromethylornithine (2.5 mmol/L, DFMO, polyamine inhibitor), AMK (50, 100, and 200 μg/mL), DFMO plus SPD and DFMO plus AMK for 24 h. The membrane potential (MP) and cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) were detected by flow cytometry, and polyamines content was determined via high-performance liquid chromatography (HPLC). The expression of Kv1.1 mRNA and protein levels were assessed by RT-qPCR and Western blot analysis, respectively. Cell migration assay was carried out using the Image-Pro Plus software. All of these indexes were used to evaluate the effectiveness of AMK.

Results: (1) Treatment with AMK caused significant increases in cellular polyamines content, membrane hyperpolarization, an elevation of $[Ca^{2+}]_{cyt}$ and an acceleration of cell migration in IEC-6 cells, as compared to control group. (2) AMK not only reversed the inhibitory effects of DFMO on the polyamines content, MP, and $[Ca^{2+}]_{cyt}$ but also restored IEC-6 cell migration to control levels. (3) The Kv1.1 mRNA and protein expression were significantly increased by AMK treatment in control and polyamine-deficient IEC-6 cells.

Conclusions: The results of our current studies revealed that treatment with AMK significantly stimulates the migration of intestinal epithelial cells through polyamine-Kv1.1 channel signaling pathway, which could promote the healing of intestinal injury. These results suggest the potential usefulness of AMK to cure intestinal disorders characterized by injury and ineffective repair of the intestinal mucosa.

1. Introduction

The mucosal epithelium of the alimentary tract represents a crucial barrier to a broad spectrum of noxious and immunogenic substances within the intestinal lumen. An impairment of the integrity of the mucosal epithelial barrier is observed in the course of various intestinal disorders including inflammatory bowel diseases (IBD), celiac disease, intestinal infections, and various other diseases (Dignass, 2001). Intestinal mucosal injury occurs commonly in response to stresses ranging from physiologic daily digestive trauma to severe insults associated with ischemia, chemicals, infection and radiation. When damaged, the intestinal mucosa has the ability to repair itself very rapidly-almost entirely in 24 h (Ray et al., 2003, 2011).

Early epithelial restitution is an important repair modality in the intestinal mucosa and happens as a consequence of epithelial cell migration over the damaged area after superficial injury, a process that is independent of cell proliferation (Dignass et al., 1994; Rao et al., 2007). This rapid mucosal reepithelialization after superficial wounds is a complex process that is highly regulated by numerous extracellular and intracellular factors. Increasing evidence has

Abbreviations: AMK, *Atractylodes macrocephala* Koidz; Kv1.1 channel, voltage-gated K^+ channel a-subunit; MP, membrane potential; $[Ca^{2+}]_{cyt}$, cytosolic free Ca^{2+} concentration; PUT, putrescine; SPD, spermidine; SPM, spermine; MFI, mean fluorescence intensity; TCM, traditional Chinese medicine

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demonstrated that the cellular polyamines play an important role in the regulation of mucosal restitution and are essential for the maintenance of intestinal mucosal integrity (Wang et al., 2000; Rao et al., 2007; Ray et al., 2011).

Polyamine, including spermidine (SPD), spermine (SPM), and their precursor putrescine (PUT), are found in all tissues of almost all living species. The intracellular levels of polyamines are highly regulated and primarily depend on the activity of ornithine decarboxylase (ODC), which catalyzes the first rate-limiting step in polyamine synthesis, forming putrescine from the amino acid ornithine. Putrescine is then converted to spermidine and spermine through the sequential addition of propylamine groups. α-Difluoromethylornithine (DFMO) inhibits ODC and prevents the formation of polyamines (Wang et al., 2000).

The IEC-6 cell line is one of the most accessible models appropriate for studying the process of cell migration involved in the early restitution of mucosal erosions (Elias et al., 2010; Ray et al., 2011; Rao et al., 2012). The polyamine-voltage-gated K^+ channel a-subunit (Kv1.1) signaling pathway is known to play an important role in regulating epithelial restitution and is regarded as a potential therapeutic target for the impairment of the integrity of the mucosal epithelial barrier (Wang et al., 2000; Dignass, 2001; Rao et al., 2001, 2002, 2006; Elias et al., 2010; Ray et al., 2011). Polyamines stimulate expression of Kv1.1 channels in intestinal epithelial cells, the polyamine-induced activation of Kv1.1 channels causes membrane hyperpolarization, enhances Ca^{2+} entry by increasing the driving force for Ca^{2+} influx, raises cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), and promotes cell migration during restitution. Depletion of cellular polyamines by inhibition of ODC with DFMO decreases Kv1.1 channel expression, causes membrane depolarization, reduces $[Ca^{2+}]_{cyt}$, and decreases cell migration.

Atractylodes macrocephala Koidz (AMK) has been used widely as a digestive and tonic in traditional Chinese medicine (Yim et al., 1988). The rhizome of AMK (common name: "Baizhu" in Chinese Medicine), first described in *Prescriptions for Fifty-two Diseases* in Warring States period (476–221 BC), is an important ingredient of several Chinese herbal prescriptions, and has been used for treating diarrhea, abdominal pain, loss of appetite, vomiting and anorexia and other gastrointestinal tract symptoms (Liu et al., 2008). Various components, such as volatile oils, polysaccharides, amino acids, vitamins, resins and other ingredients have been found in AMK up to now. Low molecular weight substances such as atractylenolide III and atractylenolide I, are reported to possess gastroprotective (Wang et al., 2010a), anti-inflammatory (Dong et al., 2008; Wang et al., 2009), anti-carcinogenic (Kang et al., 2011) and anti-microbial (Kim et al., 2007) activities. Recently, there has been increasing interest in studying the pharmacological roles of this drug, and its beneficial effects on intestinal mucosal healing have been reported (Wang et al., 2010b). However, little is known about the mechanism on how AMK works in those diseases.

Based on the important roles of AMK in the treatment of gastrointestinal disorders recorded by the Chinese Pharmacopoeia (China Pharmacopoeia Committee, 2010) as well as some previous studies of our laboratory and others (Luo, 2008; Wen et al., 2012a), the main purpose of this study was to investigate AMK for its efficacy on intestinal mucosal restitution and the underlying mechanisms via IEC-6 cell migration model. In the current study, we proposed to determine the beneficial role of AMK in the cellular pathway leading to increased cell migration by elevated $[Ca^{2+}]_{cyt}$ following the induction of Kv1.1 channel expression by polyamines elevation during restitution in intestinal epithelial cells. First, we observed the effect of AMK on cellular polyamines content, MP, $[Ca^{2+}]_{cyt}$ and cell migration when it was added to control cells. Second, we determined whether AMK restored polyamines content reduction, membrane depolarization, $[Ca^{2+}]_{cyt}$ reduction and cell migration deceleration to normal levels in polyamine-deficient IEC-6 cells induced by DFMO. Finally, we determined whether AMK enhance the expression of Kv1.1 mRNA and protein in control and polyamine-deficient IEC-6 cells.

2. Materials and methods

2.1. Cell lines, chemicals and biochemicals

The IEC-6 cell line was purchased from American Type Culture Collection at passage 13, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), insulin, Penicillin Streptomycin, Fluo-3, AM ester, bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) were purchased from Life technologies (Eugene, Oregon, USA). Putrescine, Spermidine, Spermine, alpha-difluoromethylornithine (DFMO) were purchased from Sigma (St. Louis, MO, USA). RNAiso Plus reagent, PrimeScript™ RT Master Mix and SYBR Premix Ex Taq™ II were obtained from TaKaRa (Dalian, China). Mouse anti-beta actin, rabbit anti-Kv1.1 potassium channel antibodies, goat polyclonal secondary antibody to rabbit IgG, goat polyclonal secondary antibody to mouse IgG were purchased from Abcam (Hong Kong, China). Authentic standards atractylenolide III and atractylenolide I and β-sitosterol were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The HPLC grade acetonitrile, methanol were purchased from Merck (Darmstadt, Germany).

2.2. Plant material and preparation of AMK extracts

Atractylodes macrocephala Koidz (AMK) was purchased from Guangdong Tiantai Pharmaceutical Co., Ltd. (Lot.120303) and identified by Mr. Jia-yun TONG, Guangzhou University of Chinese Medicine. The authenticated voucher specimens (Voucher 12-08-10) were kept in Spleen and Stomach Institute, Guangzhou University of Chinese Medicine.

Rhizomes of AMK were cut into pieces and powdered. A 100 g AMK powder was accurately weighed and ultrasonicly extracted 3 times with 100% methanol (1:5 (w/w) ratio of solid:liquid) at 35 °C, each time 30 min. The resulting methanol extract was filtered through filter paper, concentrated by rotary evaporator at 45 °C and dried by vacuum drying oven, yielding approximately 10.5 g. The extract was then dissolved in sterilized PBS to obtain a concentration of 10 mg of dry weight in 1 mL of PBS, and was stored at −20 °C before use.

2.3. HPLC fingerprint analysis of AMK extract

In order to control the quality and ensure the consistency and stability of AMK, we used an improved method based on official Chinese Pharmacopoeia (China Pharmacopoeia Committee, 2010), which was a accurate and practical HPLC method for chromatographic fingerprint analysis and simultaneous qualitation of three active constituents (β-sitosterol, atractylenolide III and atractylenolide I) in AMK extracts (Jiang et al., 2011).

Three standard compounds β-sitosterol, atractylenolide III and atractylenolide I were accurately weighed and dissolved in 100% methanol, then diluted to 0.1 mg/mL. The AMK sample was prepared in methanol at 1 mg/mL, then filtered through a syringe filter membrane (0.22 μm) prior to HPLC analysis. Analyses were performed on Agilent Series 1200 liquid chromatograph (Agilent Technologies, USA) with UV detector. Detection wavelengths were set at 210 nm. A Waters SunFire C18 (5 μm, 250 × 4.6 mm i.d.) was used with a flow rate of 1.0 mL/min. The injection volume was