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Original Article

Atractylenolide I stimulates intestinal epithelial repair through polyamine-mediated Ca^{2+} signaling pathway

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ABSTRACT

Background: An impairment of the integrity of the mucosal epithelial barrier can be observed in the course of various gastrointestinal diseases. The migration and proliferation of the intestinal epithelial (IEC-6) cells are essential repair modalities to the healing of mucosal ulcers and wounds. Atractylenolide I (AT-I), one of the major bioactive components in the rhizome of *Atractylodes macrocephala* Koidz. (AMR), possesses multiple pharmacological activities. This study was designed to investigate the therapeutic effects and the underlying molecular mechanisms of AT-I on gastrointestinal mucosal injury.

Methods: Scratch method with a gel-loading microtip was used to detect IEC-6 cell migration. The real-time cell analyzer (RTCA) system was adopted to evaluate IEC-6 cell proliferation. Intracellular polyamines content was determined using high performance liquid chromatography (HPLC). Flow cytometry was used to measure cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$). mRNA and protein expression of TRPC1 and PLC-γ_1 were determined by real-time PCR and Western blotting assay respectively.

Results: Treatment of IEC-6 cells with AT-I promoted cell migration and proliferation, increased polyamines content, raised cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$), and enhanced TRPC1 and PLC-γ_1 mRNA and protein expression. Depletion of cellular polyamines by DL-a-difluoromethylornithine (DFMO, an inhibitor of polyamine synthesis) suppressed cell migration and proliferation, decreased polyamines content, and reduced $[Ca^{2+}]_c$, which was paralleled by a decrease in TRPC1 and PLC-γ_1 mRNA and protein expression in IEC-6 cells. AT-I reversed the effects of DFMO on polyamines content, $[Ca^{2+}]_c$, TRPC1 and PLC-γ_1 mRNA and protein expression, and restored IEC-6 cell migration and proliferation to near normal levels.

Conclusion: Our data demonstrate that AT-I stimulates intestinal epithelial cell migration and proliferation via the polyamine-mediated Ca^{2+} signaling pathway. Therefore, AT-I may have the potential to be further developed as a promising therapeutic agent to treat diseases associated with gastrointestinal mucosal injury, such as inflammatory bowel disease and peptic ulcer.

Introduction

The epithelium of the gastrointestinal tract serves both as a physical barrier to microorganisms and as a border of the mucosal immune system. Sometimes the intestine suffers insults, due to very concentrated hydrochloric acid, reflux of bile salts, alcohol, drugs, and foodstuffs with relatively high or low temperatures, pH values, or osmolarity (Gao et al., 2013). Impairment of the gastrointestinal surface barrier is characteristic of several diseases and various pathological states, such as peptic and stress ulcers, inflammatory bowel disease (Crohn's disease and ulcerative colitis), non-

Abbreviations: AT-I, Atractylenolide I; $[Ca^{2+}]_c$, Cytosolic free Ca^{2+}; DFMO, DL-α-difluoromethylornithine; ODC, ornithine decarboxylase; PKC, protein kinase C; PLC, Phospholipase C; PUT, putrescine; SPD, spermidine; SPM, spermine; TRPC1, canonical transient receptor potential-1; TCM, traditional Chinese medicine.

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steroidal anti-inflammatory drug-induced mucosal bleeding, and mucosal injury or erosions induced by *Helicobacter pylori* infection (Rathor et al., 2014b).

The repair of impaired epithelial surface occurs at least through two mechanisms (Ray et al., 2003). The first one is the migration of adjacent wound epithelial cells to the damaged area, in order to cover it. This complex phenomenon includes well-established processes such as cytoskeleton reorganization, membrane protrusion formation, and focal adhesion to the extracellular matrix at the front edge and release of adhesion sites at the rear edge of migrating cells. The second mechanism includes replacement of lost cells through proliferation, which depends on DNA synthesis and begins 12 h after the start of healing. This second mechanism as well as the first one require polyamines such as spermidine (SPD), spermine (SPM) and their precursor putrescine (PUT) that either arise from diet (red meat and cheeses) and bacteria or are the extrusion products from villi of sloughed epithelial cells (Johnson and McCormack, 1999). The intracellular polyamine levels are primarily regulated by ornithine decarboxylase (ODC) and α-difluoromethylornithine (DFMO). ODC, a key enzyme of polyamine biosynthesis, catalyzes the first rate-limiting reaction in polyamine biosynthesis to produce PUT that is then converted to SPD and SPM through the sequential addition of polyamine groups. DFMO is an irreversible restrainer of ODC and can specifically inhibit ODC activity, thus preventing the synthesis of cellular polyamines (Ray et al., 2005).

Cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) concentration plays an important role in the regulation of cell migration and proliferation, and increased $[Ca^{2+}]_c$ promotes epithelial healing after wounding both *in vivo* and *in vitro* (Rao et al., 2007). In intestinal epithelial cells (IEC-6), $[Ca^{2+}]_c$ is increased mainly by Ca^{2+} release from intracellular Ca^{2+} stores (endoplasmic reticulum and sarcoplasmic reticulum) and Ca^{2+} influx through Ca^{2+} permeable channels in the plasma membrane (Rao et al., 2006). A number of studies have shown that the canonical transient receptor potential-1 (TRPC1) protein functions as a Ca^{2+}-permeable channel mediating capacitative Ca^{2+} entry which is activated by Ca^{2+} store depletion (Rao et al., 2006, 2010). Phospholipase C (PLC) is an important regulatory enzyme that catalyzes the hydrolysis of phosphatidylinositol (4, 5)-bisphosphate (PIP_2) to generate diacylglycerol (DAG) and inositol (1, 4, 5)-trisphosphate (IP_3). It is well known that DAG functions as a protein kinase C (PKC) activator and that IP_3 acts as a Ca^{2+}-mobilizing messenger, resulting in the release of Ca^{2+} from IP_3-sensitive intracellular Ca^{2+} stores and activation of Ca^{2+} influx via plasma membrane Ca^{2+}-permeable channels (Rao et al., 2007). Polyamines have been demonstrated to be involved in the regulation of Ca^{2+} influx through the plasma membrane and Ca^{2+} release from intracellular Ca^{2+} stores. Moreover, polyamines are necessary for activation of TRPC1 channels and PLC-γ_1 expression in IEC-6 cells.

The rhizome of *Atractylodes macrocephala* Koidz. (AMR, Baizhu in Chinese, family Asteraceae), is one of the most popular medicinal herbs in traditional Chinese medicine (TCM) that has been widely used for thousands of years (Song et al., 2015). AMR is believed to possess action of, in terms of TCM, invigorating the function of the spleen and replenishing vital qi. TCM doctors mostly prescribe AMR for prevention and treatment of digestive disorders such as peptic ulcer and ulcerative colitis, which are mainly caused by gastrointestinal mucosal injury. Atractylenolide I (AT-I, 3,8aβ-dimethyl-5-methylene-2,4,4aα,5,6,7,8,8a-octahydronaphtho[2,3-b]furan-2-one, Fig. 1A) is one of the major bioactive components isolated from AMR and has drawn great attention due to its multiple therapeutic effects, such as antitumor (Liu et al., 2008; Wang et al., 2002; Yu et al., 2016), anti-inflammation (Endo et al., 1979; Wang et al., 2016), anti-atopic (Lim et al., 2012), and bone protective effects (Ha et al., 2013). However, the protective effects of AT-I on gastrointestinal mucosal injury have never been reported and the possible mechanisms underlying the gastrointestinal protection activity remains to be explored.

In this study, we tested the hypothesis that AT-I stimulated intestinal epithelial repair (cell migration and proliferation) after wounding through polyamine-mediated Ca^{2+} signaling by modulating TRPC1 and PLC-γ_1. First, we examined the effects of AT-I on cell migration and proliferation, polyamines content, and $[Ca^{2+}]_c$ in IEC-6 cells after wounding. Second, we determined whether depletion of cellular polyamines by DFMO decreased the rate of cell migration and proliferation, polyamines content, and $[Ca^{2+}]_c$ in injured IEC-6 cells and further investigated whether AT-I restored cell migration and proliferation, polyamines content, and $[Ca^{2+}]_c$ in polyamine-deficient IEC-6 cells. Third, we determined whether AT-I increased TRPC1 and PLC-γ_1 expression in normal and polyamine-depleted IEC-6 cells.

Materials and methods

Materials

Dulbecco's Modified Eagle Medium (DMEM) and dialyzed fetal bovine serum (dFBS) were purchased from Gibco (Carlsbad, CA, USA). PUT, SPD, and SPM were obtained from Sigma-Aldrich (St. Louis, MO, USA). DL-α-difluoromethylornithine (DFMO) was purchased from Merck (Darmstadt, Germany). RNAiso Plus reagent, PrimeScript RT Master Mix, and SYBR Premix Ex Taq II were purchased from Takara (Shiga, Japan). The primary antibody, rabbit polyclonal anti-TRPC1 antibody (ab75322), rabbit polyclonal anti-phospholipase C gamma1 (PLC-γ_1) antibody (ab107455), mouse monoclonal anti-beta actin antibody (ab8226), the specific goat polyclonal secondary antibody to Rabbit IgG (ab7090), and goat polyclonal secondary antibody to mouse IgG (ab97040) were purchased from Abcam (Hong Kong, China). AT-I of 98% purity verified by the HPLC (Supplementary Fig. S1A and B) was purchased from Guangzhou Haokai Reagent Co. Ltd. (Guangzhou, Guangdong, China). E-plate 16 was purchased from Roche (Basel, Switzerland). Other chemicals and biochemicals were obtained from Beyotime Biotechnology (Haimen, Jiangsu, China).

Cell culture

The IEC-6 cell line (CRL 1592) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). IEC-6 cells were seeded in 6-well plates (6.25×10^4 cells/cm^2) and grown for 24 h in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. After 24 h, cells were treated with PUT (positive control, 10 μM), AT-I (5 and 10 μM), DFMO (5 mM), DFMO + PUT, or DFMO + AT-I for 8 h. In the following series of studies, we examined the effects of AT-I on cell migration and proliferation, polyamines content, $[Ca^{2+}]_c$, TRPC1 and PLC-γ_1 expression in normal and polyamine-depleted IEC-6 cells.

Cell migration assay

IEC-6 cells were treated as described in cell culture, and cell migration was measured as described previously (Ray, Guo, et al., 2007). In brief, plates were marked in the bottom by drawing five lines parallel to the diameter, and wounding of the monolayer was performed perpendicular to the marked line using a 1000 μl gel-loading microtip to initiate cell migration. Plates were washed with PBS to remove the damaged cells, and the area of migration was captured with an inverted phase contrast microscope (Olympus IX-71, Tokyo, Japan) at the intersection of the marked lines and the