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Magnolol and honokiol regulate the calcium-activated potassium channels signaling pathway in *Enterotoxigenic Escherichia coli*-induced diarrhea mice

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ABSTRACT

To explore the regulatory mechanisms of magnolol and honokiol on calcium-activated potassium channels signaling pathway in *Enterotoxigenic Escherichia coli* (ETEC)-induced diarrhea mice, the concentrations of serum chloride ion (Cl^-), sodium ion (Na^+), potassium ion (K^+) and calcium ion (Ca^{2+}) were measured. Additionally, the mRNA expressions of calmodulin 1 (CaM), calcium/calmodulin-dependent protein kinase II alpha subunit (CaMKIIα) and beta subunit (CaMKIIβ), ryanodine receptor 1, inositol 1,4,5-trisphosphate receptors (IP_3 receptors), protein kinases C (PKC), potassium intermediate/small conductance calcium-activated channels (SK) and potassium large conductance calcium-activated channels(BK)were determined. A diarrhea mouse model was established using ETEC suspensions (3.29×10^9 CFU/ml) at a dosage of 0.02 ml/g live body weight (BW). Magnolol or honokiol was intragastrically administered at dosages of 100 (M100 or H100), 300 (M300 or H300) and 500 (M500 or H500) mg/kg BW according to a 3×3 factorial arrangement. Magnolol and honokiol increased the Cl^- and K^+ concentrations, further, upregulated the CaM, BKα1 and BKβ3 mRNA levels but downregulated the IP_3 receptors 1, PKC, SK1, SK2, SK3, SK4 and BKβ4 mRNA expressions. Magnolol and honokiol did not alter the CaMKIIα, CaMKIIβ, ryanodine receptor 1, IP_3 receptor 2, IP_3 receptor 3, BKβ1 and BKβ2 mRNA expressions. These results clarify that magnolol and honokiol, acting through Ca^{2+} channel blockade, inhibit the activation of IP_3 receptor 1 to regulate the IP_3-Ca^{2+} store release, activate CaM to inhibit SK channels, and effectively suppress PKC kinases to promote BKα1 and BKβ3 channels opening and BKβ4 channel closing, which modulates the intestinal ion secretion.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the leading cause of bacterial diarrhea in humans and farming animals worldwide (Fleckenstein et al., 2010; Moxley et al., 1998). The direct explanation is that ETEC easily colonizes the intestine, and secretes exotoxins and endotoxins which disrupt intestinal barrier to induce secretory diarrhea (Fasano, 2002; Kunzelmann and Mall, 2002). The excessive losses of salt and water in secretory diarrhea, stimulated by the activation of transport proteins in luminal [chloride ion (Cl^-) and potassium ion (K^+) channels] and basolateral (NaK_2Cl cotransporter, K^+ channels and Na^+-K^+-ATPase) membranes (Kunzelmann and Mall, 2002; Rosa et al., 2013; Simon et al., 2008). The basolateral membrane calcium-activated potassium channels regulate the Cl^- channels to sustain the electrical driving force for Cl^- extrusion into the lumen (Kunzelmann et al., 2001) to maintain water and electrolytes secretion (Hodges and Gill, 2010), which are stimulated by increments of intracellular calcium ion (Ca^{2+}) (Berkefeld et al., 2010). Calmodulin and calcium/calmodulin-dependent protein kinase II

Abbreviations: CaM, calmodulin 1; CaMKIIα, calcium/calmodulin-dependent protein kinase II alpha subunit; CaMKIIβ, calcium/calmodulin-dependent protein kinase II beta subunit; PKC, protein kinase C; IP_3, inositol 1,4,5-trisphosphate; SK, potassium intermediate/small conductance calcium-activated channels; BKα1, potassium large conductance calcium-activated channel, subfamily M, alpha member 1; BKβ, potassium large conductance calcium-activated channel, subfamily M, beta.

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(CaMK II) can affect the calcium-activated potassium channels (Keen et al., 1999; Liu et al., 2007), while protein kinases C (PKC) and CaMK II play a bidirectional role in regulating these channels (Welie and Lac, 2011). Therefore, the calcium-activated potassium channels may play an important role in electrolyte absorption and secretion during diarrhea.

Magnolol and honokiol are bi-phenolic isomers, which are the main constituents identified in the bark of *Magnolia officinalis* and widely used in traditional Chinese medicine to treat a variety of mental and gastrointestinal disorders (Chen et al., 2009; Maruyama and Kuribara, 2000). Previous studies have described magnolol and honokiol have some pharmacological activities of anti-microbial, anti-inflammatory and anti-oxidant (Chen et al., 2009; Ho et al., 2001; Lin et al., 2007; Maruyama and Kuribara, 2000). Recent study has demonstrated magnolol and honokiol might simultaneously activated functional regulation of the serotonergic and gastroenteric systems (Qiang et al., 2009). Moreover, magnolol and honokiol induce intracellular Ca^{2+} mobilization to increase cytoplasmic free Ca^{2+} in primary cultured rat cortical neurons and human neuroblastoma SH-SY5Y cells (Zhai et al., 2003), and cytosolic free Ca^{2+} in rat neutrophils (Wang and Chen, 1998). Additionally, magnolol may activate CaMK II and extracellular signal-related kinases to increase lipolysis in adipocytes (Huang et al., 2004). Honokiol inhibits PKC activation by attenuating the 4-aminopyridine-induced phosphorylation of PKC in the rat cerebral cortex (Sy et al., 2008), while magnolol inhibits cytosolic PKC in rat neutrophils (Wang et al., 1998). Magnolol is also potent in stimulating BK channel activity in tracheal smooth muscle cells, and the application of magnolol shifts the BK channel activation curve to lessen positive membrane potentials (Wu et al., 2002). Here we hypothesize that magnolol and honokiol might involve a new activation mechanism associated with calcium-activated potassium channels. In the current study, a mouse model of ETEC-mediated diarrhea was established to investigate the regulatory effects of magnolol and honokiol on the concentrations of Cl^-, sodium (Na^+), K^+ and Ca^{2+}, the protein kinases, SK channels and BK channels mRNA expressions to explain the mediated mechanism on the calcium-activated potassium channels.

2. Materials and methods

2.1. Experimental animals

This study was carried out in strict accordance with the recommendations in the Animal Care and the Use Guidelines of the Institute of Subtropical Agriculture (ISA) of the Chinese Academy of Sciences. The protocol was approved by the Animal Care Committee on the Ethics of Animal Experiments of ISA. In this study, all mice were killed with an overdose of sodium pentobarbitone. One hundred and forty male Kunming mice, 6–8 weeks old and live body weight (BW) of 25 ± 2 g, were purchased from Hunan SLAC Laboratory Animal Company (Changsha, China). The animals were maintained in a specific pathogen free room under standard environmental conditions (temperature: 22 ± 2 °C; humidity: 40–60%; 12 h light/12 h dark cycle) and had free access to autoclaved feed and water.

2.2. Reagents

The magnolol and honokiol (the purity was 98%) were purchased from Jinnong Bio. Co. Ltd. (Changsha, China) and kept at −20 °C until use. The magnolol and honokiol were extracted from the bark of *Magnolia officinalis* using 60% edible ethanol for twice in 2.5 h according to the 1:10 ratio of weight to volume. The crude extracts were separated and purified with HP-20 macroporous resin absorbing (Mitsubishi Chemical Holding, Tokyo, Japan), washing with 3 bed volume (BV) water or 65% edible ethanol at a flow velocity of 2 BV/hour, respectively, and then eluting with 3 BV of 78 or 87% edible ethanol at a flow velocity of 2BV/hour, respectively. The 2–3 BV of 78% edible ethanol and 1–3 BV of 87% edible ethanol eluents were collected to depressurize and concentrate at −0.07 MPa and 58 °C to recover ethanol, and then freezed in vacuum to obtain purified magnolol and honokiol. The magnolol and honokiol purity was determined by the high-performance liquid chromatography analysis (Fisher Chemical, Loughborough, UK) by the following conditions: the column used in present study was a Kromasil C18 column (4.6 mm × 200 mm I.D., 5 μm, waters Thermo), the mobile phase composed of methanol–water (80:20, v/v) was eluted at a flow-rate of 1.0 ml/min and the UV detector was set at 294 nm, the temperature was set at 40 °C. The extracted magnolol and honokiol in this study were dissolved in a 2% Tween 80 solution for oral gavage administration.

The powder of ETEC strain O78:K80 (44813) was provided by the China National Institutes for Food and Drug Control, then cultured in liquid Lysogeny Broth medium in a shaking incubator at 180 rpm and 37 °C for 12 h. The ETEC suspensions were further measured at optical density 600 [OD 600=1 corresponding to 1×10^{10} colony forming units (CFU)/ml] using ND-1000 UV–vis spectrophotometer (NanoDrop Ltd., TX). The determined bacterial cell density of the ETEC suspensions was 3.29×10^9 CFU/ml. Loperamide hydrochloride was purchased from the Xi'an-Janssen Pharmaceutical Ltd. (Xi'an, China), and dissolved in a 2% Tween 80 solution (0.1875 mg/ml) for oral gavage administration until use.

2.3. Induction of diarrhea mouse model

A new diarrhea mouse model is needed to study the pathogenesis of diarrhea and provide the therapeutic target. 20 mice were selected to monitor the manifestations of diarrhea induced by ETEC suspensions. After feeding 3 days for acclimatization, the mice were fasted for 6 h, and randomly assigned into two groups, including the normal group and the diarrhea model group. The normal group mice received 0.02 ml/g BW sterile water by gavage, whilst the diarrhea model group mice were orally administered 0.02 ml/g BW of the prepared ETEC suspensions (3.29×10^9 CFU/ml) once. The diarrhea symptoms were initially observed after ETEC infection 40 min later. However, diarrhea developed severe and stable in the infection-process, especially after ETEC infection 3 h later, 10 mice of diarrhea model group almost developed visual diarrhea. Animals were killed humanely as soon as a visual diarrhea appeared in order to measure both stool water content and diarrhea severity, and to avoid causing unnecessary suffering.

2.4. Water content of stool

Stool water content measurement was according to the procedure described by Borenshtein et al. (2009) and Woo et al. (2008). Briefly, twenty 15 ml plastic falcon tubes and weighing papers were weighed prior to the experiment. A 3.5 cm length of the distal colons was excised and placed on a piece of weighing paper after necropsy. The colonic contents were squeezed out using forceps and the weighing paper with colonic contents was placed into a 15 ml plastic falcon tube for weighing (wet weight). The colonic contents were dried in a 37 °C incubator for 48 h and reweighed (dry weight). The differences between the wet versus dry weights were used to calculate the percentage of moisture in the colonic contents.