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Poncirus trifoliate fruit modulates pacemaker activity in interstitial cells of Cajal from the murine small intestine

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ABSTRACT

Ethnopharmacological relevance: Poncirus fructus (PF) has been widely used as a traditional medicine in Eastern Asia, especially to ameliorate the symptoms of gastrointestinal (GI) disorders related to abnormal GI motility.

Aim of the study: Poncirus fructus (PF), also known as *Poncirus trifoliata* (L.) Raf. (Rutaceae), is widely used as a traditional medicine in Eastern Asia mainly to ameliorate the symptoms of gastrointestinal (GI) disorders related to abnormal GI motility. In a previous study, a methanol extract of PF was found to have particularly potent gastroprokinetic effects. Interstitial cells of Cajal (ICCs) are pacemaker cells in the gastrointestinal tract, but the action mechanisms of PF extract in mouse small intestinal ICCs have not been investigated. Therefore, in the present study, we investigated the effects of a methanol extract of PF (MPF) in mouse small intestinal ICCs. In addition, we sought to identify the receptors involved.

Materials and Methods: Enzymatic digestions were used to dissociate ICCs from small intestines. The whole-cell patch-clamp configuration was used to record potentials (current clamp) from cultured ICCs. In addition, we analyzed intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$).

Results: MPF decreased the amplitudes of pacemaker potentials in ICCs, and depolarized resting membrane potentials in a concentration dependent manner. Y25130 (a 5-HT_3 receptor antagonist) and RS39604 (a 5-HT_4 receptor antagonist) blocked MPF-induced membrane depolarizations, whereas SB269970 (a 5-HT_7 receptor antagonist) did not. Pretreatment with Na^+ or Ca^{2+}-free solution or thapsigargin (a Ca^{2+}-ATPase inhibitor in endoplasmic reticulum) abolished the generation of pacemaker potentials and suppressed MPF-induced activity. $[Ca^{2+}]_i$ analysis showed that MPF increased $[Ca^{2+}]_i$. Furthermore, treatments with PD 98059, SB203580, or JNK II inhibitor blocked MPF-induced membrane depolarizations in ICCs.

Conclusion: These results suggest that MPF modulates pacemaker potentials through 5-HT_3 and 5-HT_4 receptor-mediated pathways via external Na^+ and Ca^{2+} influx, and via Ca^{2+} release from internal stores in a mitogen-activated protein kinase dependent manner. The study shows MPF is a good candidate for the development of a gastroprokinetic agent. In view of the effects of MPF on ICCs, further research is required, particularly to identify the active compound(s) involved and to determine their action mechanisms.

1. Introduction

Poncirus fructus (PF), also known as *Poncirus trifoliata* (L.) Raf. (Rutaceae), is widely used as a traditional medicine in Eastern Asia, especially to ameliorate the symptoms of gastrointestinal (GI) disorders related to abnormal GI motility and gastric secretion (Kim et al., 1997; Yi et al., 2004; Lee et al. 2005). PF extracts continue to occupy an important place as over-the-counter drugs in Korea for the treatment of a variety of GI disorders. Extracts of PF have a variety of pharmacological properties, which include anti-oxidant (Jayaprakasha et al., 2007), anti-platelet (Teng et al., 1992), anti-bacterial (Kim et al., 1999a) and antiallergic (Lee et al., 1996) activities, and are known to contain limonene, linalool, hesperidin, neohesperidin, naringin, poncirin, umbelliferone, auraptene, imperatorin, and synephrine (Kim et al., 1997; Lee et al. 2005).

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PF extracts had no effect on the apparent permeability of ranitidine across the jejunum or the gastric emptying rate (GER) in rat. However, the transit time for charcoal in the intestine was significantly increased by the PF extracts. Therefore, PF extracts have been reported to accelerate small bowel transit in the rat without affecting GER (Lee et al., 2005a), Also, PF extracts have the potential for development as a prokinetic agent that may prevent or alleviate GI motility dysfunctions in human patients (Lee et al., 2005b). In addition, PF extract had been reported to stimulate rat distal colon motility (Choi et al., 2010). Despite the considerable use of PF extracts to treat GI dysfunction, the action mechanisms of these extracts on the regulation of GI motility are not understood.

Of the pathways related to intestinal motility, serotonin (5-hydroxytryptamine, 5-HT; a major neuromodulator) is known to play a critical role in the GI tract. Generally, 5-HT acts as a neurotransmitter in the central nervous system, but most (95%) 5-HT is found in the GI tract (Kim and Camilleri, 2000). Furthermore, although 5-HT is known to interact with seven different 5-HT receptor subtypes, only three of these are found in the interstitial cells of Cajal (ICCs) in the murine small intestine (Shahi et al., 2011).

ICCs are the pacemaking cells in the gastrointestinal (GI) muscles that generate the rhythmic oscillations in membrane potentials known as slow waves (Huizinga et al., 1995; Sanders, 1996). Pacemaker activity in the murine small intestine is mainly caused by the periodic activations of nonselective cation channels (NSCCs) (Koh et al., 2002) or Cl^- channels (Huizinga et al., 2002; Zhu et al., 2009). ICCs also mediate or transduce inputs from the enteric nervous system. Thus, because of the central role played by ICCs in GI motility, loss of function would be extremely detrimental. However, the effects of PF and the action mechanism involved in the GI tract are not investigated.

Therefore, we undertook to investigate the effects of the methanoic extract of PF (MPF) on the pacemaker potentials of cultured ICCs derived from murine small intestine and to identify the receptors involved.

2. Materials and methods

2.1. Preparation of the MPF

Poncirus fructus (PF) were purchased from Kwangmyungdang Medicinal Herbs (Ulsan, Korea). PF was harvested in Kyungju, Kyungbook province, Korea and in October, 2012. PF was authenticated by Prof. Hyung Woo Kim (Division of Pharmacology, Pusan National University, School of Korean Medicine, Yangsan, Korea). 50 g of powdered dried fruit was then immersed in 1,000 ml of methanol, sonicated for 30 min, and extracted for 24 h. The extract so obtained was filtered through Whatman filter paper (No. 20) and evaporated under reduced pressure using a vacuum evaporator (Eyela, Japan). The condensed extract was then lyophilized using freeze dryer (Labconco, USA). Finally, 6.43 g of lyophilized powder (MPF) was obtained (yield; 12.9%).

2.2. Chromatographic conditions and preparation of standard

Smart LC system comprised a LC800 (GL sciences, Japan) equipped with built-in apparatus including solvent delivery unit, autosampler, column oven and UV-visible detector. The acquired data was processed using EZChrom Elite software (Ver. 3.3.2 SP1). Chromatographic separation was performed on a Inertsil ODS-4 column (2.1 × 50 mm, 2 μm; GL sciences, Japan) with the temperature at 35 °C. The mobile phase consisted of water (A) and acetonitrile (B). A gradient program of mobile phase was used as follows: 5% (B) maintained for 5 min, 5–90% (B) over 5–7 min. The flow rate was set at 0.4 mL/min and the injection volume was 1 μL. Detection wavelength of poncirin was set at 280 nm. Poncirin was used as standard material of Ponciri Fructus Immaturus (PFI), one milligram of poncirin was accurately weighed and dissolved in methanol at the concentration of 100 μg/mL and the solution was 10-fold diluted before the injection.

2.3. Preparation of cells and cell cultures

All animals used were treated ethically according to the Guidelines for the Care and Use of Animals issued by Pusan National University. Balb/c mice (8–13-days-old) of either sex were anaesthetized with ether and sacrificed by cervical dislocation. Small intestines were removed from 1 cm below the pyloric ring to the cecum and opened along the mesenteric border. Luminal contents were washed out with Krebs–Ringer bicarbonate solution, tissues were pinned to the base of a Sylgard dish, and mucosa was removed by sharp dissection. Small strips of intestinal muscle (consisting of both circular and longitudinal muscles) were then equilibrated in Ca^{2+} free Hank's solution containing 5.36 mM KCl, 125 mM NaCl, 0.34 mM NaOH, 0.44 mM Na_2HCO_3, 10 mM glucose, 2.9 mM sucrose and 11 mM *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid (HEPES) for 30 min, and cells were dispersed in a solution containing 1.3 mg/ml collagenase (Worthington Biochemical, Lakewood, NJ, USA), 2 mg/ml bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 2 mg/ml trypsin inhibitor (Sigma-Aldrich) and 0.27 mg/ml ATP. Dispersed cells were plated onto sterile glass coverslips coated with 2.5 mg/ml murine collagen (Falcon/BD, U.S.A.) in 35 mm-diameter culture dishes and cultured at 37 °C in a 95% O_2–5% CO_2 incubator in smooth muscle growth medium (SMGM; Clonetics, San Diego, CA) supplemented with 2% antibiotics/antimycotics (Gibco, Franklin Lakes, NJ, USA) and 5 ng/ml murine stem cell factor (SCF; Sigma-Aldrich). ICCs were identified immunologically by incubation with anti-*c-kit* antibody [phycoerythrin (PE)-conjugated rat antimouse *c-kit* monoclonal antibody; eBioscience, San Diego, CA] at a dilution of 1:50 for 20 min. Because ICCs differed morphologically from other cell types in cultures, they were identified by phase contrast microscopy after incubation with anti-*c-kit* antibody.

2.4. Patch-clamp experiments

The physiological salt solution used to bathe cells (Na^+-Tyrode) contained; 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$ and 10 mM HEPES, adjusted to pH 7.4 with NaOH. The pipette solution contained 140 mM KCl, 5 mM $MgCl_2$, 2.7 mM K_2ATP, 0.1 mM NaGTP, 2.5 mM creatine phosphate disodium, 5 mM HEPES, and 0.1 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), adjusted to pH 7.2 with KOH. Single ICC used in patch clamp experiments were bathed in a solution containing 2.8 mM KCl, 145 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$ and 10 mM HEPES, adjusted to pH 7.4 with NaOH. The pipette solution contained 145 mM Cs-glutamate, 8 mM NaCl, 10 mM Cs-2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid, and 10 mM HEPES-CsOH, adjusted to pH 7.2 with CsOH. The whole-cell configuration patch-clamp technique was used to record membrane potentials (current clamp) of cultured ICC, and an Axopatch I-D (Axon Instruments, Aberdeen, UK) was used to amplify membrane currents and potentials. Command pulses were applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). Data were filtered at 5 kHz, displayed on an oscilloscope and a computer monitor, printed using a Gould 2200 pen recorder (Gould, Valley View, OH, USA), and analyzed using pClamp and Origin (version) software. All experiments were performed at 30 °C.