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Ghrelin receptor is activated by naringin and naringenin, constituents of a prokinetic agent *Poncirus fructus*

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ABSTRACT

Ethnopharmacological relevance: *Poncirus fructus* (PF), also known as a dried immature fruit of *Poncirus trifoliata* (L.) Raf. (Rutaceae), has long been traditionally used for the various gastrointestinal disorders in Eastern Asia.
Aim of study: The aqueous extract of PF (PF-W) has the strong prokinetic effect, yet the underlying mechanism is still elusive. The present study investigated whether PF-W has any effect on motilin receptor or ghrelin receptor, since these receptors enhance intestinal motility when activated.
Materials and methods: The effect of PF-W and its components on motilin or ghrelin receptor was determined by calcium imaging and whole-cell patch clamp methods.
Results: PF-W activates the ghrelin receptor, but not the motilin receptor, resulting in a transient increase of intracellular calcium levels. Furthermore, among various constituents of PF, only naringin and naringenin evoked the intracellular calcium augmentation via the ghrelin receptor. Moreover, cortistatin-8 – a ghrelin receptor inhibitor – specifically blocked naringin- and naringenin-induced calcium increases. In addition, naringin and naringenin induced inward currents in ghrelin receptor-expressing cells under whole-cell patch clamp configuration.
Conclusion: PF-W activates the ghrelin receptor, and naringin and naringenin are key constituents responsible for the activation of ghrelin receptor. Therefore, the present study suggests that the ghrelin receptor is a molecular entity responsible for the strong prokinetic activity of PF-W.

1. Introduction

The dried, immature fruit of *Poncirus trifoliata* (L.) Raf. (Rutaceae), also called *Poncirus fructus* (PF), has long been used as a traditional remedy for various gastrointestinal (GI) disorders, especially in Eastern Asia (Benavente-Garcia and Castillo, 2008). Particularly, its aqueous extract form (PF-W) increased GI motor function in both human and rodent, by accelerating the transit of intestinal contents (Lee et al., 2005b). Moreover, PF-W also improved the intestinal transit rate in animal models with GI motility dysfunction (Lee et al., 2005a). Recently, the underlying prokinetic mechanism of PF-W has been investigated, and serotonin receptor subtype 4 (5-HT4R) seems to play a role in this mechanism (Shim et al., 2010). Indeed, activation of 5-HT4R induces changes in GI motility including accelerated gastric emptying, enhanced colonic contractions and transitions (Manabe et al., 2010). In fact, many strong prokinetic agents such as cisapride and mosapride are agonists of 5-HT4R.

However, the 5-HT4R pathway is not the exclusive mechanism that governs GI motility. Motilin and ghrelin – hormones released from cells lining the stomach, pancreas, and small intestine – are well-known endogenous molecules that stimulate GI motilities through their distinctive receptors (Peeters, 2006). In detail, the motilin receptor (MLNR) and the ghrelin receptor (also known as growth hormone secretagogue receptor, GHSR) are typical G protein coupled receptors (GPCR) coupled with the $G\alpha_{q/11}$ pathway (Cruz and Smith, 2008). These receptors release Ca^{2+} from endoplasmic reticulum, stimulating inositol (1,4,5) triphosphate (IP_3) cleavage from phosphatidylinositol 4,5-biphosphate (PIP_2) by phospholipase C (Sato et al., 2012).

Recent advances in this field have revealed motilin/MLNR and ghrelin/GHSR are essentially involved in the intestinal motility (Peeters, 2006). Motilin is considered to be an appetite-regulating hormone following the interdigestive period. The cyclical plasma levels of motilin are synchronized to strong peristaltic contractions initiated from the stomach and migrating to the small intestine,

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modulating interdigestive migrating contractions in the GI tract (Ohno et al., 2010). Similar to motilin, peripheral administration of ghrelin expedites gastric emptying in rodents, enhancing coordinated motor pattern between the antrum and pylorus (antro-pyloric coordination) (Ariga et al., 2008). Ghrelin, when administered intraperitoneally, alleviates symptoms of dyspepsia in relevant animal models (Liu et al., 2006). Moreover, ghrelin regulates the contractions of migrating motor complex which is originating in the stomach and propagating along the small intestine (De Smet et al., 2009). Ghrelin also induced contractions of smooth muscle strip from fundus and antrum by directly activating cholinergic excitatory neurons (Dass et al., 2003). In fact, many GHSR and MLNR agonists, such as TZP-101, TZP-102, mitemcinal (GM-611), and PF-04548043 (KOS-2187), are currently being developed as potential prokinetic agents for the treatment of various GI disorders (Sanger, 2008).

Since PF-W is a mixture of various natural compounds, it is assumable that PF-W may possess a fraction that activates MLNR and/or GHSR. Therefore, the present study investigated whether PF-W has an ability to activate MLNR and/or GHSR. Specifically, it was examined whether the increment of intracellular Ca^{2+} ($[Ca^{2+}]_i$) can be induced by PF-W or its constituents, by calcium imaging in cells expressing respective receptors. Moreover, the effects of active compounds were further confirmed by the whole-cell patch clamp technique, and their involvement in 5-HT4R was verified.

2. Materials and methods

2.1. Plant material

Poncirus fructus was obtained from a certified oriental drug store located in the Gyungdong Market (Seoul, Korea). Reference specimens (no. EA239) were deposited at the Herbarium of the College of Pharmacy, Ewha Woman's University (Seoul, Korea).

2.2. Sample preparation

PF-W was prepared with previously described methods (Lee et al., 2005a). 2 kg of PF was boiled in 30 L of distilled water at 100 °C for 2 h, and the aqueous extract was filtered, concentrated *in vacuo*, and lyophilized. The yield was approximately 24.4% (w/w). In order to assure consistent quality, the hesperidin content was determined in each PF-W sample using HPLC. The hesperidin content was calculated to be $0.93 \pm 0.01\%$ (w/w, $n=3$), suggesting a fairly stable content in PF-W.

2.3. Gene cloning

Human full-length MLNR (NM_001507) and GHSR (NM_198407) cDNAs were kindly provided by LG Life Science Ltd. Rat 5-HT4R genes were cloned as mentioned in a previous article (Shim et al., 2010). All sequences were confirmed to be 100% identical to sequences in the Genbank database.

2.4. Cell culture and gene transfection

HEK293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. HEK293T cells were transfected with genes (pcDNA, MLNR, GHSR, 5-HT4R) using a FuGENE® HD transfection reagent (Promega, Wisconsin, USA) according to the manufacturer's protocol. One day after transfection, transfected cells were used for various experiments.

2.5. Measurement of intracellular Ca^{2+}

HEK293T cells were cultured and were transfected with pcDNA, motilin or ghrelin receptors in the lysine coated culture chamber (Lab-Tek™, Thermo Scientific). One day after transfection, the culture media were replaced with normal bath solution [140 mM NaCl, 5.0 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, and 5.5 mM HEPES (pH 7.4)] containing Fluo-3 AM (2 μM, Invitrogen) and 0.1% Pluronic F-127 (Invitrogen), and were then incubated for 40 min at 37 °C. After incubation, the buffer solution containing Fluo-3 AM was washed out with normal bath solution. Analysis of the fluorescent images was performed as described previously (Jang et al., 2012a). F/F_0 indicates a relative ratio of the fluorescent intensity (F) divided by the first fluorescent intensity of the respective cell (F_0). For measuring changes in $[Ca^{2+}]_i$ evoked by PF-W and its constituents, we transfected GHSR or MLNR plasmids in the HEK293T cells, After 1 day of transfection, the HEK293T cells were incubated with Fluo-3 AM. After washing out the medium containing Fluo-3 AM, we confirmed the functional activity of overexpressed-HEK293T cells using GHSR agonists (10 μM L-692,585) or MLNR agonist (10 μM erythromycin). For blocking the receptors, we pre-treated the GHSR antagonist (2 μM cotistatin-8) in the HEK293T cells before applying agonists or test components.

2.6. Measurement of intracellular cAMP

Cells transfected with rat 5-HT4R were washed twice, and 1 mL of 5 mM theophylline was added and incubated for 15 min at 37 °C to inhibit 3′,5′-cyclic-AMP phosphodiesterases. Afterward, a final concentration of 10 μM naringin or naringenin were added and incubated for another 15 min at 37 °C. Media were then quickly removed and the cells were washed twice. Cell lysates were obtained by applying 0.1 N HCl, followed by 20 min incubation at room temperature. The cells were then scraped, put into microtubes, and were centrifuged for 10 min at 1000*g*. After centrifugation, supernatants were obtained and cAMP measurement was performed using a cyclic-AMP EIA kit (Cayman Chemical, Ann. Arbor, MI) following the manufacturer's protocol. Specifically, cAMP levels were obtained by the acetylation method and were normalized by the total protein amount, which was measured by the standard BCA assay method.

2.7. Patch-clamp recording

HEK293T cells were used to record membrane currents and potentials. Resistance of glass pipettes was about 3 MΩ. After forming a whole cell by gentle suction, transient currents were recorded using a patch-clamp amplifier and were filtered at 2 kHz. The output of the amplifier was digitized using a Digidata 1440 (Molecular Devices). The pipette solution contained 118.5 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$. The bath solution contained 140 mM NaCl, 10 mM HEPES, 5 mM KCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$.

2.8. Statistical analysis

All data are presented as the mean ± standard error (SEM). Significances of differences were determined by the Student *t*-test or one-way ANOVA with a Tukey's post hoc test. Differences were regarded as significant when the *p*-values were less than 0.05.