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Hesperetin Stimulates Cholecystokinin Secretion in Enteroendocrine STC-1 Cells

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Abstract

Hesperetin (3',5,7-trihydroxy 4'-methoxyflavanone) and its glycoside hesperidin (hesperetin 7-rhamnoglucoside) in oranges have been reported to possess pharmacological effects related to anti-obesity. However, hesperetin and hesperidin have not been studied on suppressive effects on appetite. This study examined that hesperetin and hesperidin can stimulate the release of cholecystokinin (CCK), one of appetite-regulating hormones, from the enteroendocrine STC-1 cells, and then examined the mechanisms involved in the CCK release. Hesperetin significantly and dose-dependently stimulated CCK secretion with an EC_{50} of 0.050 mM and increased the intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) compared to the untreated control. The stimulatory effect by hesperetin was mediated via the entry of extracellular Ca^{2+} and the activation of TRP channels including TRPA1. These results suggest that hesperetin can be a candidate biomolecule for the suppression of appetite and eventually for the therapeutics of obesity.

Key Words: Hesperetin, Cholecystokinin, Intracellular Ca^{2+}, TRP ankyrin 1, Enteroendocrine cells

INTRODUCTION

Peptide hormones released from the gastrointestinal tract communicate information about the current state of energy balance to the brain. These hormones regulate appetite and energy expenditure via the vagus nerve or by acting on key brain regions implicated in energy homeostasis such as the hypothalamus and brainstem. The role of gut hormones and the gut/brain axis in the regulation of appetite has become an area of interest in recent years, owing to the growing global obesity crisis. Peripheral signals controlling appetite may present potential targets for developing novel anti-obesity therapies (Sam *et al.*, 2012). Prominent among gut hormones is cholecystokinin (CCK), a peptide released by enteroendocrine cells in the proximal small intestine. Intestinal CCK has been implicated in the regulation of appetite via a 'gut-brain axis' (Raybould, 2007).

Increases in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) are coupled to CCK secretion from the enteroendocrine cells (Chen *et al.*, 2006). Two mechanisms are thought to be involved in increases in $[Ca^{2+}]_i$ in the cells. One is the mobilization from intracellular Ca^{2+} stores through the activation of G-protein-coupled receptors (GPCRs) such as bitter taste receptors (Chen *et al.*, 2006). And another is the entry from extracellular milieu through the activation of transient receptor potential (TRP) channels (Purhonen *et al.*, 2008). TRP ankyrin 1 (TRPA1) channels, members of TRP superfamily, are calcium permeable non-selective cation channels originally reported to sense noxious cold temperatures (Story *et al.*, 2003). It has been shown that TRPA1 are expressed in mouse duodenal mucosa and that activation of TRPA1 elevates $[Ca^{2+}]_i$ and stimulates CCK release in enteroendocrine STC-1 cells (Purhonen *et al.*, 2008).

Hesperetin (5,7,3'-trihydroxy 4'-methoxyflavanone) is the main aglycone in oranges, and hesperidin (hesperetin 7-rhamnoglucoside) is the main form of hesperetin present in oranges. Both hesperetin and its glycoside hesperidin have been reported to possess pharmacological effects related to anti-obesity, i.e., antioxidant (Cai *et al.*, 2004), anti-inflammatory (Garg *et al.*, 2001), anti-atherogenic (Garg *et al.*, 2001), hypocholesterolemic (Lee *et al.*, 1999), inhibitory in TNF-α-induced adipocyte lipolysis (Yoshida *et al.*, 2010) and regulatory on lipid metabolism (Borradaile *et al.*, 1999). Therefore, hesperetin and hesperidin might possess suppressive effect on appetite, one of mechanisms of action against obesity. However, the effects of hesperetin and hesperidin on appetite, especially CCK release, remain largely unknown.

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In this study, we investigated whether hesperetin and hespridin could stimulate CCK release in the murine CCK-producing enteroendocrine cell line STC-1, and then examined the mechanisms involved in the CCK release. The involement of the increases in $[Ca^{2+}]_i$, external Ca^{2+} entry, TRP and TRPA1 in the hesperetin- or hespridin-induced CCK release was studied in the cells.

MATERIALS AND METHODS

Materials

Hesperetin, hesperidin, allyl isothiocyanate (AITC), ruthenium red (RR) and HC-030031 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and trypsin-EDTA were from Gibco Co. (Grand Island, NY, USA). CCK enzyme immunoassay (EIA) kit was from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). FLIPR Calcium 5 Assay Kit was from Molecular Devices (Sunnyvale, CA, USA).

Cell culture

The STC-1 cell line, a murine enteroendocrine origin, was provided by Professor S.-D. Moon (The Catholic University of Korea College of Medicine) under the permission of Dr. D. Hanahan (University of Califonia, San Francisco, CA, USA). The cells were grown at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin.

CCK secretion studies

For each secretion study, the cells were seeded at a density of 2×10^5 cells/well in 24-well plates for 2-3 days until they reached 80-90% confluency. Cells were washed with HEPES buffer and exposed to test agents dissolved in the same buffer for 60 min at 37°C. The HEPES buffer had the following composition: 4.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, 140 mM NaCl, 20 mM HEPES, 0.1% BSA, pH 7.4. For the secretion study in extracellular Ca^{2+}-free condition, the HEPES buffer without $CaCl_2$ was used. After incubation for 60 min, supernatants from the plates were collected and centrifuged at 800×g for 5 min at 4°C to remove the remaining cells, and then stored at −70°C until CCK concentration measurement with a commercial EIA kit (Nakajima *et al.*, 2010). The concentrations of CCK in the samples were calculated by supplied CCK standard (ng/ml), and the result was expressed as fold of the untreated control. AITC was used as a positive control. A TRP antagonist (RR, 100 μM) and a TRPA1 antagonist (HC-030031, 50 μM) were pre-incubated for 30 min before the addition of the test agents.

Measurement of changes in $[Ca^{2+}]_i$ in STC-1 cells

For the multiwell assays, the cells were seeded onto 96-well plates at 80,000 cells per well. Cells were washed with the assay buffer Hanks' balanced salts solution (HBSS) containing 1.25 mM $CaCl_2$, and 20 mM HEPES, pH 7.4. For the assay in Ca^{2+}-free condition, $CaCl_2$ was omitted. Cells were loaded with a calcium-indicator dye from the FLIPR Calcium 5 Assay Kit by dilution with the assay buffer. The cells were incubated for 60 min at 37°C, after which measurements were made using a Flex Station 3 (Molecular Devices) set at 27°C. Fluorescence changes (i.e., excitation at 485nm and emission at 525 nm with a cutoff at 515 nm) were monitored at 2-s intervals. A 100-μl aliquot of the assay buffer supplemented with 2× the test agents was added at 20 s, and scanning was continued for an additional 100 s. The response of each well was determined as ΔRFU (delta relative fluorescent units), calculated as (maximum fluorescent value) − (minimum fluorescent value) (Fujiwara *et al.*, 2012). A TRP antagonist (RR, 100 μM) and a TRPA1 antagonist (HC-030031, 50 μM) were co-incubated with the calcium-indicator dye for 60 min.

Statistical analysis

The data are expressed as the mean ± standard error of mean (S.E.M.). The groups were analyzed by Student's *t*-test or by one-way ANOVA followed by Duncan's multiple range test using S.A.S. software (S.A.S. Institute, Inc., USA). *p*-Value of less than 0.05 was considered statistically significant.

RESULTS

Effect of hesperetin and hesperidin on CCK secretion in STC-1 cells

At a normal extracellular Ca^{2+} concentration (1.2 mM), exposure to hesperetin for 60 min stimulated significant ($p<0.05$) and dose-dependent CCK secretion in STC-1 cells (Fig. 1). However, hesperidin did not stimulate CCK release in the cells. Hesperetin induced CCK release 16.3-fold by 0.1 mM, 25.1-fold by 0.5 mM, and 46.8-fold by 1 mM compared to the untreated control (Fig. 1). Hesperetin displayed a half maximal effective concentration (EC_{50}) of 0.050 mM when the CCK secretion by 10 mM AITC, a known stimulator of CCK release in STC-1 cells, was regarded as maximum.

Effect of hesperetin and hesperidin on changes in $[Ca^{2+}]_i$ in STC-1 cells

To investigate the mechanisms behind hesperetin-mediated CCK release, we examined the effects of hesperetin and hesperidin on the increases in $[Ca^{2+}]_i$, under normal (1.25 mM) extracellular Ca^{2+} conditions. The stimulation of STC-1 cells with AITC, an agonist of TRPA1, induced an increase in $[Ca^{2+}]_i$ (Fig. 2). Hesperetin significantly ($p<0.05$) and dose-dependently stimulated the increases in $[Ca^{2+}]_i$ 5.9-fold by 0.1

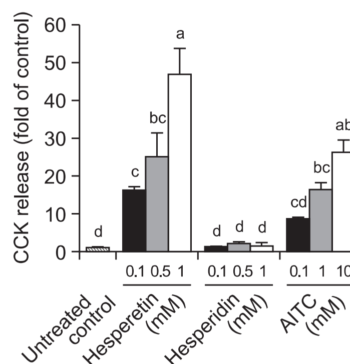

Fig. 1. Effects of hesperetin and hesperidin on CCK secretion. Hesperetin and hesperidin were incubated in HEPES buffer containing 1.2 mM Ca^{2+} at 37°C for 60 min. AITC was used as a positive control. Values are means ± S.E.M. from three separate experiments in triplicate (n=9). Bars not sharing the same letters differ significantly ($p<0.05$) by Duncan's multiple range tests.

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mM, 16.6-fold by 0.5 mM, and 18.4-fold by 1 mM compared to the untreated control (Fig. 2). Meanwhile, hesperidin did not evoke $[Ca^{2+}]_i$ increase (Fig. 2).

Effects of extracellular Ca^{2+}-free conditions on hesperetin-induced responses

To investigate whether extracellular Ca^{2+} affects hesperetin-induced CCK release, the extracellular Ca^{2+}-free conditions were applied to the hesperetin-induced CCK secretion. The extracellular Ca^{2+}-free conditions significantly inhibited hesperetin-induced CCK release by 86.5% at 0.1 mM, 87.7% at 0.5 mM and 72.3% at 1.0 mM hesperetin compared to the CCK release in normal Ca^{2+} conditions (Fig. 3).

To clarify the source of Ca^{2+} responsible for the hesperetin-induced increases in $[Ca^{2+}]$, Ca^{2+} in the extracellular milieu was omitted. Removal of extracellular Ca^{2+} significantly attenuated hesperetin-induced increases in $[Ca^{2+}]$ by 81.6% at 0.1 mM, 92.1% at 0.5 mM and 93.2% at 1.0 mM hesperetin compared to the increases in $[Ca^{2+}]_i$ in normal Ca^{2+} conditions (Fig. 4).

Effects of TRP antagonist and TRPA1 antagonist on hesperetin-induced responses

A TRP antagonist, RR, and a specific TRPA1 antagonist, HC-030031, were used to determine the involvement of TRP and TRPA1 channel in the hesperetin-induced CCK release and increases in $[Ca^{2+}]_i$. In the presence of RR, the CCK release in response to hesperetin was significantly suppressed by 94.5% at 0.1 mM, 30.3% at 0.5 mM and 63.9% at 1.0 mM hesperetin compared to the CCK release without RR (Fig. 5). In the presence of HC-030031, the hesperetin-induced CCK release was significantly inhibited by 93.2% at 0.1 mM, 65.0% at 0.5 mM and 61.9% at 1.0 mM hesperetin compared to the CCK release without HC-030031 (Fig. 5). Also RR significantly inhibited the hesperetin-induced increases in $[Ca^{2+}]_i$ by 67.0% at 0.1 mM, 82.5% at 0.5 mM and 78.4% at 1.0 mM hesperetin compared to the increases in $[Ca^{2+}]_i$ without RR (Fig. 6). HC-030031 significantly inhibited the hesperetin-induced increases in $[Ca^{2+}]_i$ by 50.1% at 0.1 mM, 50.0% at 0.5 mM and 50.0% at 1.0 mM hesperetin compared to the increases in

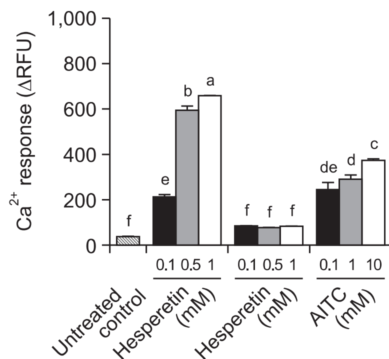

Fig. 2. Effects of hesperetin and hesperidin on changes in ($[Ca^{2+}]_i$. The cells were loaded with a calcium-indicator dye from the FLIPR Calcium 5 Assay Kit for 60 min. After the cells were placed into a Flex Station 3, hesperetin and hesperidin were added at 20 sec. Values are means ± S.E.M. from three separate experiments in triplicate (n=9). Bars not sharing the same letters differ significantly (p<0.05) by Duncan's multiple range tests.

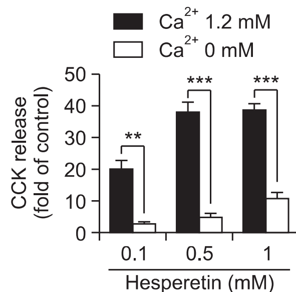

Fig. 3. Effects of extracelluar Ca^{2+}-free conditions on hesperetin-induced CCK release. Cells were exposed to hesperetin in buffer containing 1.2 or 0 mM Ca^{2+} at 37°C for 60 min. Values are means ± S.E.M. from three separate experiments in triplicate (n=9). **p<0.01 , ***p<0.001 vs. CCK secretion induced by hesperetin in 1.2 mM Ca^{2+}-containing buffer.

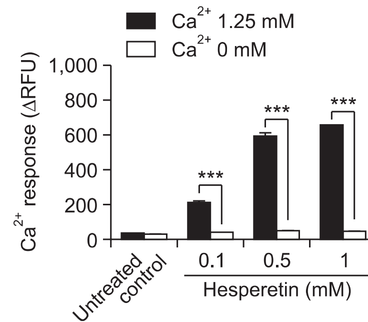

Fig. 4. Effects of extracelluar Ca^{2+}-free conditions on hesperetin-induced Ca^{2+} responses. The cells were loaded with a calcium-indicator dye from the FLIPR Calcium 5 Assay Kit in the presence or absence of 1.25 mM Ca^{2+} for 60 min. After the cells were placed into a Flex Station 3, hesperetin was added at 20 sec. Values are means ± S.E.M. from three separate experiments in triplicate (n=9). ***p<0.001 vs. $[Ca^{2+}]_i$ increases induced by hesperetin in 1.25 mM Ca^{2+}-containing buffer.

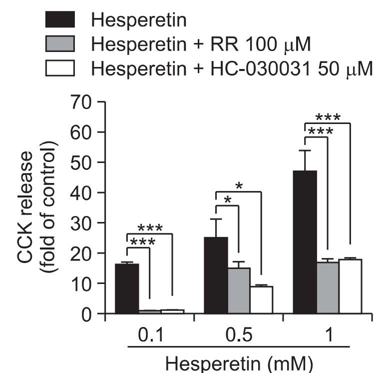

Fig. 5. Effects of ruthenium red (RR) and HC-030031 on hesperetin-induced CCK release. The cells were pretreated with 100 μM RR or 50 μM HC-030031 for 30 min. And then the cells were exposed to hesperetin in HEPES buffer containing 1.2 mM Ca^{2+} at 37°C for 60 min. Values are means ± S.E.M. from three separate experiments in triplicate (n=9). *p<0.05, ***p<0.001 vs. CCK secretion induced by hesperetin alone.

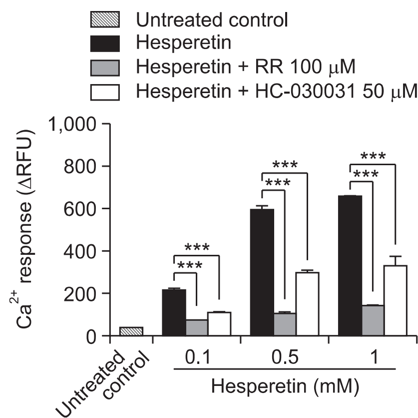

Fig. 6. Effects of ruthenium red (RR) and HC-030031 on hesperetin-induced calcium responses. The cells were loaded with a calcium-indicator dye from the FLIPR Calcium 5 Assay Kit in the absence or the presence of 100 μM RR or 50 μM HC-030031 for 60 min. After the cells were placed into a Flex Station 3, hesperetin was added at 20 sec. Values are means ± S.E.M. from three separate experiments in triplicate (n=9). ****p*<0.001 vs. $[Ca^{2+}]_i$ increases induced by hesperetin alone.

$[Ca^{2+}]_i$ without HC-030031 (Fig. 6).

DISCUSSION

Appetite is in part regulated by the secretion of gastrointestinal hormones such as CCK. Here, using a CCK release and $[Ca^{2+}]_i$ assay in the enteroendocrine STC-1 cells, we demonstrated for the first time that hesperetin stimulates CCK release in the cells. In contrast to the results obtained with hesperetin, hesperidin (a rhamnoglucoside of hesperetin) did not induce any detectable stimulation in CCK release.

Hesperetin mainly occurs in the form of glycoside, hesperidin, in oranges. Deglycosylation of flavonoid glycosides occurs by mammalian β-glycosidases in the small intestine (Walle, 2004; Possemiers *et al.*, 2011). Small intestinal enzyme capable of cleaving flavonoid glycosides are lactase-phlorizin hydrolase, also called lactase (Day *et al.*, 2000), and another, less well-characterized β-glycosidase with a broad substrate specificity (Day *et al.*, 1998). Although the pharmacokinetics of hesperidin has not fully been studied (Garg *et al.*, 2001), orally ingested hesperidin might be hydrolysed to hesperetin, and hesperetin reaches as a intact form in the intestine. Considering that the site in which hesperetin interacts with the enteroendocrine cells is the intestine, our results that hesperetin instead of hesperidin exerts CCK release lead to possibility that hesperetin could show CCK secretion *in vivo*.

Next, we demonstrated that hesperetin induces increases in $[Ca^{2+}]_i$ in a dose-dependent manner, but that hesperidin exhibits no effect in $[Ca^{2+}]_i$ increase. These results suggest that the increases in $[Ca^{2+}]_i$ is coupled to hesperetin-induced CCK release.

The increases in $[Ca^{2+}]_i$ induced by hesperetin were almost completely abolished by the absence of extracellular Ca^{2+} in STC-1 cells, suggesting that the source of Ca^{2+} responsible for the hesperetin-induced increases in $[Ca^{2+}]_i$ comes from extracellular milieu. Also removal of extracellular Ca^{2+} attenuated the hesperetin-induced CCK release. This suggests that hesperetin-induced CCK secretion depends on Ca^{2+} entry from extracellular milieu.

TRP channels, and specifically TRPA1, have been known as channels mediated Ca^{2+} entry in STC-1 cells (Purhonen *et al.*, 2008). A TRP antagonist, RR, and a specific TRPA1 antagonist, HC-030031, were used to elucidate the involvement of TRP and TRPA1 in the hesperetin-induced CCK secretion and increases in $[Ca^{2+}]_i$ in the STC-1 cells. The CCK release mediated by hesperetin was significantly decreased by the presence of RR and HC-030031. The increases in $[Ca^{2+}]_i$ induced by hesperetin were significantly blocked by the presence of RR and HC-030031. This suggests that the activation of TRP channels including TRPA1 on the cell surface plays an important role in Ca^{2+} entry into the STC-1 cells, increases in $[Ca^{2+}]_i$, and then CCK release upon the stimulation by hesperetin.

In conclusion, we demonstrated for the first time that hesperetin stimulated CCK release in the enteroendocrine STC-1 cells. This stimulatory effect was mediated via the entry of extracellular Ca^{2+} and due to the activation of TRP channels including TRPA1. This finding suggests that hesperetin could be a candidate biomolecule for the suppression of appetite and eventually for the therapeutics of obesity.

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