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Long term exposure to sucralose alters the sense of sweet tastants in an intestinal enteroendocrine cell model

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Abstract

Artificial sweeteners can bind to the sweet taste receptors T1R2/T1R3 on the cell membrane of taste receptor cells, including intestinal enteroendocrine cells, and induce an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). Acute adaptation or a reduction in receptor signalling was observed after prolonged exposure to a taste stimulus in an animal model. However, at the cell level, it remains unclear whether the expression level of T1R2/T1R3 will be changed by pre-exposure to sweetener molecules, and whether the corresponding change in $[Ca^{2+}]i$ induced by the altered expression of sweet taste receptors varies. In the present work, western blotting and qPCR analysis showed that the expression level of T1R2/T1R3 in NCI-H716 cells was upregulated by sucralose stimulation. In the sucralose-treated cells, the accumulation of intracellular Ca^{2+} in the sweetener pre-treated cells was significantly enhanced as compared to that in cells without sweetener pre-treatment, although the sweet response differed between acesulfame K and sucralose with the same sweetness. Collectively, the increased expression of T1Rs in sweetener pre-incubated cells enhanced the sweet taste signal response, which implies that long-term administration of artificial sweeteners in beverages or foods could alter the sensitivity of taste receptor cells to sweetener molecules.

Introduction

Sweet taste receptors found in oral and extra-oral tissues play important roles in the regulation of many physiological functions. Sweet taste receptors are composed of two distinct G protein-coupled receptors (GPCRs), T1R2 and T1R3, which are responsive to sweet tastants (Jiang *et al.,* 2005). At present, the sweet taste signal pathway mainly includes the GPCR Gs-cAMP and GPCR-Gq/Gβγ-IP3 pathways. It is clear that the change in $[Ca^{2+}]i$ play an important role in the sweet response of the intestinal tract, while the role of cAMP in the sweet response of the intestinal tract remains unclear (Margolskee, 2002; Young, 2011). In the GPCR-Gq/Gβγ-IP3 pathway, sweet tastants bind to T1R2/T1R3, and are then coupled to the heterotrimeric G-protein, including α-gustducin, Gβ 3, and Gγ13 (Höfer *et al.,* 1996; Margolskee *et al.,* 2007; Tolhurst *et al.,* 2012). The G-protein subunits are separated into α-gustducin and βγ-units (Gβ3-Gγ 13); subsequently, an intracellular cascade is activated, eliciting Ca^{2+} release from internal stores, leading to membrane depolarisation and increasing the intracellular Ca^{2+} concentration, which prompts the release of GI peptides (GLP-1, GIP, PYY, CCK, etc.) (Margolskee, 2002; Meyer-Gerspach *et al.,* 2016). T1R2 and T1R3 can be inhibited by lactisole, an inhibitor of the sweet taste receptor, leading to attenuation of sucralose-induced increase in $[Ca^{2+}]i$ and significantly decreased GLP-1 secretion induced by artificial sweeteners (Hamano *et al.,* 2015).

It has been shown that increasing $[Ca^{2+}]i$ promotes the release of GI peptides (Margolskee, 2002). GLP-1 secretion was dose-dependent with $[Ca^{2+}]i$ in NCI-H716 cells, whereas $[Ca^{2+}]i$ changed in a dose-dependent manner following the induction of both calorie and artificial sweeteners (Nakagawa *et al.,* 2009; Fujiwara *et al.,* 2012). Recently, the identification of sweet taste receptors in the

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gastrointestinal tract in regulating GLP-1, PYY, and CCK release in humans has played an important role (Sternini *et al.,* 2008; Gerspach *et al.,* 2011).

Most studies have considered that the expression of GPCR would not be regulated by exposure to the corresponding ligand; however, some have reported that the expression of GPCR is influenced by exposure to the corresponding ligand. In 2013, it was reported that T1R2/T1R3 expression in the taste buds on the tongue and soft palate epithelia was increased after exposure to the artificial sweetener acesulfame K in mice at 49 days (Li *et al.,* 2013). In 2016, it was shown that the expression of T1R3 and Gα in the middle- and low-dose saccharin exposure groups was increased as compared to the control groups after exposure for five weeks (Gong *et al.,* 2016). In our previous study, we also detected enhanced expression of T1Rs by acesulfame K or saccharin exposure in an animal model (Shi *et al.,* 2019).

Artificial sweeteners (ASs) are widely used as sugar substitutes in the formulation of foods and beverages because of their properties, such as providing an intense sweetness with little or no calories (Fernstrom, 2015). It is known that the sweet signal transduction is mediated by the sweet taste receptors, and that intracellular Ca^{2+} functions as the relevant messenger molecule. However, after long-term sucralose exposure, whether the expression of T1R2/T1R3 changes, and whether the corresponding change in $[Ca^{2+}]i$ induced by the altered expression of sweet taste receptors will be changed, was not clear. In the present work, we used the human enteroendocrine NCI-H716 cell line as an intestinal sweet taste receptor expressing model to investigate the influence of long-term sweetener exposure. The expression of *tas1r2/tas1r3* was quantitatively studied, and $[Ca^{2+}]i$ was monitored. The present work provides experimental data on the relationship between the sense of sweet tastants and altered expression of sweet taste receptors.

Materials and methods

Cell culture and sweetener exposure

The human intestinal endocrine cell line NCI-H716 was cultured in suspension at 37°C/5% CO_2 in 1640 medium (Gibco, New York, USA), supplemented with 10% foetal bovine serum (Hyclone, Utah, USA), 1% L-glutamine, and 1% antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). After 24 h of incubation, NCI-H716 cells were exposed to 8.3 mM sucralose (sweetness 100) (Sigma-Aldrich, St. Louis, MO, USA), or left untreated for another 24 h in preparation for the subsequent experiments.

To compare the data of different sweeteners, the sweetness intensities of sucralose and acesulfame K were converted relative to 2% sucrose as sweetness unit 1 (Hall and Guyton, 2011). The relative sweetness of acesulfame K and sucralose was 200 and 600, respectively (Steinert *et al.,* 2011). Unless otherwise stated, the unit of sweetener mentioned below is shown as a sweetness unit.

Quantitative PCR

The total RNA from NCI-H716 pre-treated cells with or without sucralose (sweetness 100) was isolated using the Trizol method (Thermo Fisher, USA), quantified using Nanodrop 2000 (Thermo Fisher, USA), and transcribed into cDNA using HiScript Q RT SuperMix for qPCR (R123-01, Vazyme, China). Quantitative PCR was conducted in a 20 μL reaction system using AceQ qPCR SYBR Green Master Mix (Q111-02/03, Vazyme, China), and used for the amplification of: hT1R2 (forward 5′-TGTGTTCCAAGAGGTGCCAGTCAG-3′, reverse 5′-GTGACGACGACCACCGTATG-TAC-3′); hT1R3 (forward 5′-TGACAAC-CAGAAGCCCGTGTCC-3′, reverse 5′-CGAAACCCCGACAAGCAAGTGG-3′); and β-actin (forward: 5′-TGGCACCCAGCACAAT-GAA-3′, reverse: 5′-CTAAGTCATAGTC-CGCCTAGAAGCA-3′). The relative gene expression ratio between different groups was calculated based on the comparative Ct method (Ct value) using the StepOne Real-Time PCR System (AB, USA). The following three-step PCR procedure was followed: 15 s at 95°C, 20 s at 60°C, and 20 s at 72°C for 40 cycles. β-Actin was used as the internal reference (Dyer *et al.,* 2009). All tests were performed in triplicate.

Western blotting

The total protein of NCI-H716 cells pre-treated with or without sucralose (sweetness 100) were extracted by shaking and breaking using two 3 mm porcelain beads in 1 mL lysis buffer at 4°C (TissueLyser, LT). The protease inhibitor PMSF and cocktail were added at a working concentration. The cell extract was then lysed using 5× sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer with DTT, and boiled for 5 min. After SDS-PAGE, proteins were transferred to PVDF membranes, and the membranes were incubated with a primary antibody against T1R2 (H90, 1:400, SC-50305; Santa Cruz Biotechnology), T1R3 (M180, 1:800, SC-50352;

Santa Cruz Biotechnology), or β-actin (R1207, 1:2000; HuaAn Biotech, China) at 4°C for 10 h, followed by incubation for 1 h at room temperature with goat anti-rabbit IgG antibodies (GAR007, 1:2000; Multi Sciences, China). The PVDF membrane was treated with BeyoECL Star solution (P0018A; Beyotime, China), and then exposed to an X-ray film in a dark room. Images were scanned to perform densitometric analysis using ImageJ, and β-actin was used as the internal loading control (Cheeseman, 1997; Wu *et al.,* 2002; Margolskee *et al.,* 2007).

Measurement of intracellular Ca^{2+} concentration

Before the analysis, NCI-H716 cells, with or without sucralose pre-treatment, were seeded onto 96-well plates (clear-bottomed black-wall plates; Corning, USA) at approximately 80,000 cells per well. After 20 - 26 h of incubation without sweeteners, cells were loaded with an assay buffer diluted with calcium-indicator dye (100 µL) from the FLIPR® Calcium 6 Assay Kit (Molecular Devices). Approximately 2 h later, measurements were made using Flex Station 3 (Molecular Devices). Fluorescence changes (excitation at 485 nm and emission at 525 nm with a cut-off at 515 nm) were monitored at 2 s intervals. A 50 µL aliquot of assay buffer supplemented with 5× ligands was added at 20 s, and the scanning continued for an additional 120 s. The response of each well was determined as FUL: $\Delta F/F_0 = (F - F_0)/F_0$. The values (F) were normalised to each initial value (F_0) after subtracting the base peak, and the relative fluorescence change was referred to as $(F-F_0)/F_0$ (Fujiwara *et al.,* 2012). Data are reported as the mean ± standard error of the mean of the area under the curve (AUC). The designed concentrations of different assay buffers included sweetness 50 acesulfame K (approximately 25 mM, AK50), sweetness 100 acesulfame K (approximately 50 mM, AK100), sweetness 200 acesulfame K (approximately 100 mM, AK200), sweetness 300 acesulfame K (approximately 150 mM, AK300), sweetness 100 sucralose (approximately 8.3 mM, sucr100), sweetness 200 sucralose (approximately 16.6 mM, sucr200), sweetness 300 sucralose (approximately 25 mM, sucr300), and sweetness 600 sucralose (approximately 50 mM, sucr600).

Statistical analysis

Results were expressed as mean ± standard error (S.E.). All data were analysed using GraphPad Prism software (version 5.0; San Diego, USA). One-way analysis of variance (ANOVA) was performed to analyse differences between samples. Statistical significance was set at $p < 0.05$. Three independent experiments were performed for each result.

Results and discussion

Sucralose exposure up-regulated expression of the sweet taste receptor

To identify the expression of sweet taste receptor, the human enteroendocrine cell line NCI-H716 was pre-incubated in medium with 8.3 mM sucralose (sweetness 100) for approximately 24 h, and the expression levels of T1R2 and T1R3 were detected by qPCR and western blotting. The mRNA level of *tas1r2* increased by approximately 1.97-fold after sucralose exposure, whereas the mRNA level of *tas1r3* increased by approximately 1.50-fold (Figure 1A). Conversely, the protein expression of T1R2 and T1R3 was also significantly enhanced by approximately 1.72- and 3.04-folds, respectively (Figure 1B). These results indicated that pre-incubation with sucralose increased the expression of T1R2 and T1R3, at both the protein and mRNA levels. It is worth noting that the increase in T1R3 was more than that of T1R2 at the protein level; but at the mRNA level, the increase in *tas1r2* was greater than that of *tas1r3*.

Sweet taste signals are known to be delivered by intracellular Ca^{2+} (Yarmolinsky *et al.,* 2009; Rasoamanana *et al.,* 2012). Prolonged exposure to a taste stimulus can lead to acute adaptation or a reduction in receptor signalling (Alsio *et al.,* 2012). Repeated oral stimulation with caloric sweeteners has also been reported to result in reduced responsiveness and perceived intensity to both naturally and artificially sweet stimuli (Tonosaki and Funakoshi, 1989). However, whether prolonged exposure to artificial sweeteners can impair the response to sweeteners is still unknown. Our results showed that an increase in $[Ca^{2+}]i$ was induced by the upregulated expression of T1R2/T1R3, which implied an enhanced response to the artificial sweetener stimuli.

Effect of pre-exposure to different sweeteners on intracellular Ca^{2+} release

After the identification of the expression of T1R2 and T1R3, we further examined whether the increase in $[Ca^{2+}]i$ would improve as the sweet receptor increased in the sweetener pre-incubated cells.

We assayed different sweetness tastant liquids including AK50, AK100, AK200, AK300,

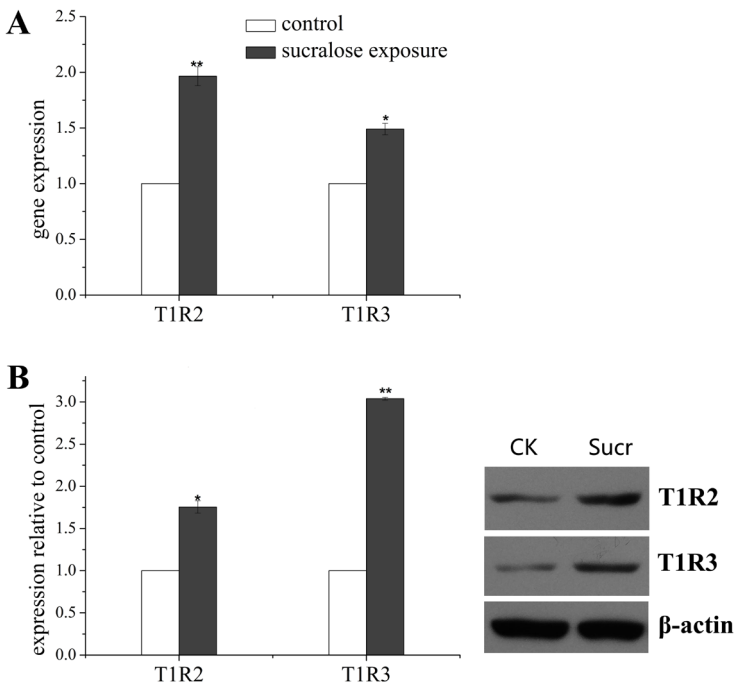

Figure 1. Expression analysis of sweet receptor in sucralose pre-treated cells. NCI-H716 cells were pre-incubated for 24 h with or without 8.3 mM sucralose. (A) The mRNA level of *tas1r2 and tas1r3* was examined by qPCR normalised to β-actin in NCI-H716 cells. (B) The protein expression level of T1R2 and T1R3 was examined by western blotting. Densitometric analysis of western blots normalised T1R2 and T1R3 protein expression to that of β-actin. Values are the mean ± S.E. of three experiments ($n = 3$). *$p < 0.05$, **$p < 0.01$ vs control.

sucr100, sucr200, sucr300, and sucr600 by monitoring $[Ca^{2+}]i$ in the sucralose pre-incubating cell model. All AK groups with different designed sweetness induced a rapid and sustained elevation of $[Ca^{2+}]i$ in NCI-H716 cells without pre-incubation with sweetener (Figure 2A). After the initial peak was achieved, $[Ca^{2+}]i$ remained elevated for at least 120 s. This change in $[Ca^{2+}]i$ had a dose-dependent effect on the sweetness of acesulfame K. Sucralose also induced an increase in $[Ca^{2+}]i$ in a dose-dependent manner with the change in $[Ca^{2+}]i$ (Figure 2B). In contrast, for the pre-incubated NCI-H716 cells, the increase in $[Ca^{2+}]i$ was quicker under the same sweetness acesulfame K stimulation, while the peak valve was higher (Figure 2C). In addition, sucralose induced a larger increase in $[Ca^{2+}]i$ in the pre-incubated cells than in the control cells (Figure 2D). These results indicated that the sweetener pre-incubated cells could increase $[Ca^{2+}]i$ more than control cells under the same stimuli. For further analysis, we calculated the AUC, and found that the value significantly increased for all sweetness of the two tastants (Figures 2E and 2F). These results suggested that the increased expression of sweet taste receptors could promote an increase in artificial sweetener-induced intracellular Ca^{2+}.

To determine whether the sweet taste receptor was the key molecule for sweet signal transduction, we stimulated NCI-H716 cells (with or without sweetener exposure) with AK300 combined with 2.5 mM lactisole. The results showed that when sweet receptors were inhibited by lactisole, the increase in $[Ca^{2+}]i$ was significantly impaired in NCI-H716 cells with or without exposure to sucralose (Figures 2A and 2C). This suggested that the elevation of $[Ca^{2+}]i$ in response to artificial sweeteners was specifically regulated by the sweet receptor T1R2/T1R3.

Few studies have reported that the expression of GPCRs is altered by prolonged exposure to the associated ligands. In the present work, we found that the expression of T1R2/T1R3 was significantly increased at both the protein and mRNA levels after pre-exposure to sucralose. However, there are discrepancies in the increasing amount of sweet taste receptors between mRNA and protein levels. We found that *tas1r2* mRNA increased to a greater extent than that of *tas1r3*; however, the opposite result was observed at the protein level. The molecular mechanism of the discrepancies remained unknown, but at both levels, the sweet taste receptors were up-regulated.

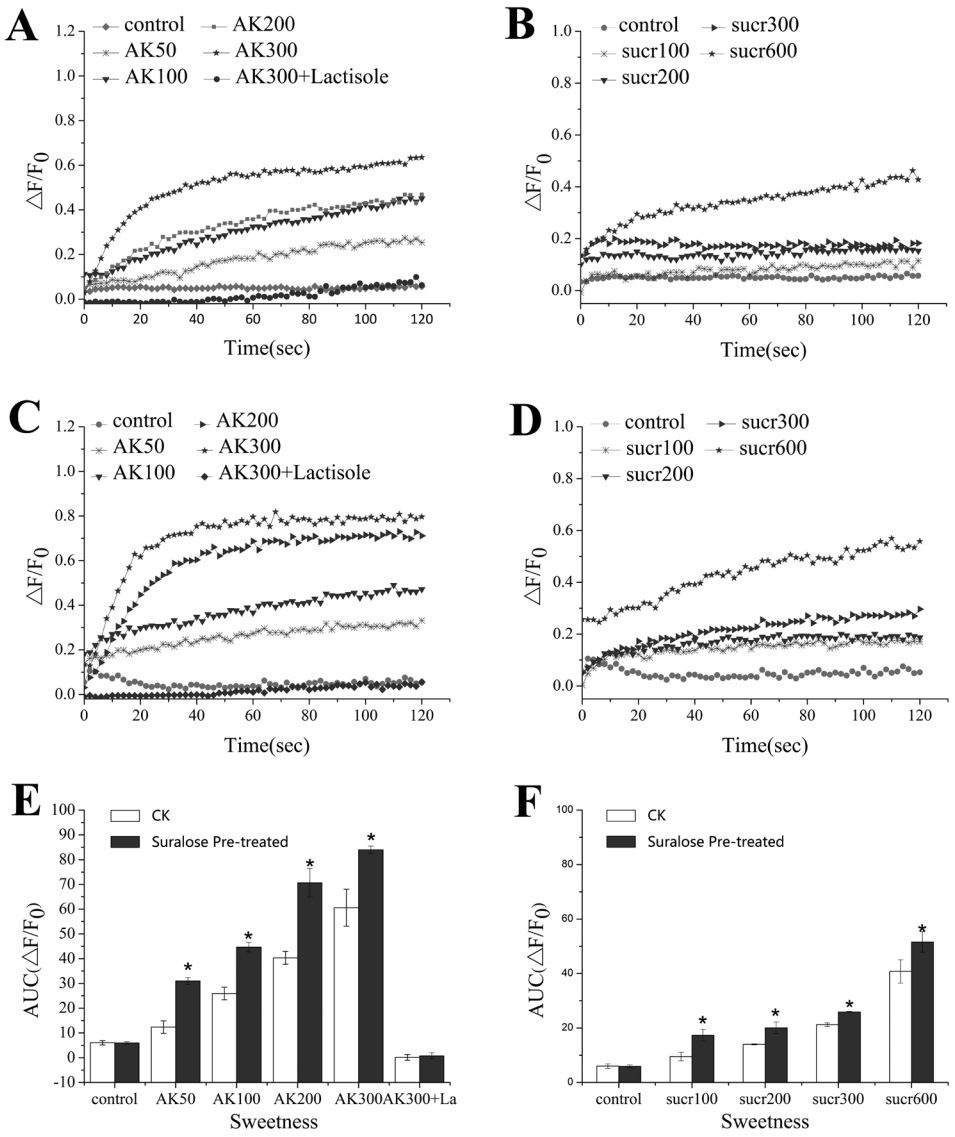

Figure 2. The difference of sweetener induced $[Ca^{2+}]i$ for the NCI-H716 cells with or without sucralose pre-exposure. The changes of $[Ca^{2+}]i$ stimulated by different sweetness AK and sucralose monitored in the NCI-H716 without sucralose pre-treatment (A and B), which was also investigated in the sucralose pre-incubated cells (C and D); Each area under the curve (AUC) from 0 to 120 s was calculated. The comparison of the AUC between cells with or without sucralose pre-treatment was shown. The AUC calculated from the AK (A and C) and sucralose (B and D) were indicated in (E) and (F), respectively. Values are the mean ± S.E. of three experiments ($n = 3$). *$p < 0.05$, **$p < 0.01$ vs control.

The increase in $[Ca^{2+}]i$ induced by equi-sweetness acesulfame K and sucralose was inconsistent (Figures 2A and 2C), which could be due to several reasons. The approach of designed sweetness in the present work is to consider the molecular structure and solution properties of the interactions of physicochemical variables. Conversely, the sweetness intensity measurements were still mainly from sensory evaluation panellists; the relative sweetness of acesulfame K and sucralose was 200 and 600, respectively (Chen *et al.,* 2009). However, whether the sweetness of AK and sucralose evaluated by panellists was completely credible was unclear. This could partly explain the existing inconsistency found in the present work, but this specific mechanism requires further study. It has been recognised that artificial sweeteners, which bind to sweet receptors, can elicit increases in $[Ca^{2+}]i$ after a series of cascade reactions, leading to the release of GI peptides including GLP-1, GIP, and PYY (Margolskee, 2002; Kim *et al.,* 2014). GLP-1 secretion is accompanied by an increase in intracellular Ca^{2+} levels in NCI-H716 cells (Kim *et al.,* 2014). Meanwhile, stimulation of NCI-H716 cells with sucrose, glucose, or sucralose led to a concentration-dependent release of GLP-1 into the

medium (Jang *et al.,* 2007). These results implied that in the enteroendocrine cell line NCI-H716, the increased expression of sweet taste receptors could improve GLP-1 secretion when stimulated by artificial sweeteners.

Conclusion

In the present work, we found that long-term sucralose exposure to the enteroendocrine cell NCI-H716 enhances sweet receptor expression. It is clear that intracellular Ca^{2+} increased in the pre-treated group as compared to that in the control group. It is, therefore, reasonable to assume that long-term exposure to artificial sweetener can increase GI peptide secretion and enhance glucose absorption in the small intestine, which may be more harmful to the human body. In addition to acute adaptation or a reduction in receptor signalling when prolonged exposure to a taste stimulus in an animal model, pre-exposure to sucralose altered the sense of sweet tastants in an intestinal enteroendocrine cell model.

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