T1R3 homomeric sweet taste receptor negatively regulates insulin-induced glucose transport through Gαs-mediated microtubules disassembly in 3T3-L1 adipocytes

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Abstract. T1R3 is a class C G protein-coupled receptor family member that forms heterodimeric umami and sweet taste receptors with T1R1 and T1R2, respectively, in the taste cells of taste buds. T1R3 is expressed in 3T3-L1 cells in homomeric form and negatively regulates adipogenesis in a Gαs-dependent but cAMP-independent manner. Although T1R3 expression is markedly upregulated during adipogenesis, its physiological role in mature adipocytes remains obscure. Here, we show that stimulation of T1R3 with sucralose or saccharin induces microtubule disassembly in differentiated 3T3-L1 adipocytes. The effect was reproduced by treatment with cholera toxin or isoproterenol but not with forskolin. Treatment with sucralose or saccharin for 3 h inhibited insulin-stimulated glucose uptake by 32% and 45% in differentiated adipocytes, respectively, similar to the inhibitory effect of nocodazole (by 33%). Isoproterenol treatment inhibited insulin-stimulated glucose transport by 26%, whereas sucralose did not affect the intrinsic activity of the glucose transporter, indicating that it inhibited insulin-induced GLUT4 translocation to the plasma membrane. Immunostaining analysis showed that insulin-stimulated GLUT4 accumulation on the plasma membrane was abrogated in sucralose-treated cells, in association with depolymerization of microtubules. Sucralose-mediated inhibition of GLUT4 translocation was reversed by the overexpression of dominant-negative Gαs (Gαs-G226A) or knockdown of Gαs. Additionally, membrane fractionation analysis showed that sucralose treatment reduced GLUT4 levels in the plasma membrane fraction from insulin-stimulated adipocytes. We have identified a novel non-gustatory role for homomeric T1R3 in adipocytes, and activation of the T1R3 receptor negatively regulates insulin action of glucose transport via Gαs-dependent microtubule disassembly.

Key words: T1R3 taste receptor, Adipogenesis, Microtubule disassembly, GLUT4, Glucose transport

T1R3 is a member of the class C G protein-coupled receptor (GPCR) family and forms heterodimeric umami and sweet taste receptors in association with T1R1 and T1R2, respectively, in the taste cells of taste buds [1]. Over the past decade, accumulated evidence has demonstrated that taste receptors are also expressed in non-gustatory cells of organs including enteroendocrine cells, pancreatic β-cells, testis, heart, respiratory mucosa, and hypothalamus, suggesting that these receptors may have additional physiological functions (for review see [2]). In this context, we have previously reported [3] that T1R3 was expressed in 3T3-L1 preadipocytes and primary adipose tissue-derived stromal cells. Further, T1R3 expression was markedly upregulated along with differentiation to mature adipocytes, whereas the expression of its counterparts T1R1 and T1R2 remained at a very low level.

The disproportionate expression profiles of T1Rs indicated that, unlike taste cells, T1R3 may be expressed in a homomeric form in these cells. Functionally, stimulation of this putative T1R3 homomeric receptor significantly attenuated adipogenesis via a Gαs-dependent and cAMP-independent mechanism [3]. On analysis of this unique signal downstream of the T1R3 homomeric receptor, we found that stimulation of the T1R3 homomeric sweet taste receptor leads to microtubule disassembly in a
Gas-dependent mechanism [4]. The disassembly was accompanied by the activation of GEF-H1 (ARHGEF2), a microtubule-bound RhoGEF [5], resulting in activation of the Rho GTPase which is a negative regulator of adipogenesis [6-9]. Based on these observations, we have proposed a model for the signaling cascade downstream of the homomeric T1R3 sweet taste receptor in 3T3-L1 cells [4]; however, the role of T1R3 in mature adipocytes remains unclear.

In this study, we examined whether a similar signaling cascade works in mature adipocytes and whether T1R3 plays a physiological role. Our data showed that T1R3 stimulation caused microtubule disassembly in differentiated 3T3-L1 adipocytes, and attenuated insulin-induced GLUT4 translocation and glucose transport in a Gas-dependent but cAMP-independent manner.

Materials and Methods

Reagents

Rabbit anti-GLUT4 antibody was raised in our laboratory as described previously [10]. Mouse monoclonal anti-tubulin (clone TUB 2.1) antibody, isoproterenol, and sucralse were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium saccharin and cholera toxin were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Cell culture and differentiation

3T3-L1 cells provided by Howard Green (Harvard Medical School, Boston, MA) [11] were maintained in Dulbecco’s Modified Eagle’s Medium containing 4.5 g/L D-glucose (DMEM-HG) supplemented with 50 μg/mL penicillin, 75 μg/mL streptomycin and 10% calf serum (CS) at 37°C in a humidified atmosphere of 5% CO₂, and were differentiated into adipocytes as described previously [12]. Briefly, 2 days after confluence, the medium was replaced with fresh DMEM containing 1 g/L D-glucose (DMEM-LG) supplemented with 10% fetal bovine serum (FBS), 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 10 μM dexamethasone, and 1.7 μM insulin. Forty-eight hours later, the medium was replaced with fresh DMEM-LG containing 10% FBS and 1.7 μM insulin. After 48 h, insulin was withdrawn from the culture media and the cells were maintained in DMEM-LG containing 10% FBS.

Subcellular membrane fractionation and immunoblotting

Differential and sucrose density gradient centrifugation was performed as described previously [10]. Briefly, cells were washed and homogenized in ice-cold STE buffer (250 mM sucrose, 10 mM Tris-Cl, pH 7.4, and 1 mM EDTA) containing complete protease inhibitor cocktail (Roche Diagnostics) using a Dounce tissue grinder. The homogenate was centrifuged for 2 min at 3,000 × g. The pellet and fat fraction were discarded, and the infranatant solution was centrifuged for 15 min at 20,000 × g. The pellet was suspended in STE buffer (0.5 mL of STE buffer containing complete protease inhibitor cocktail) and layered on top of a linear (15%–32.5%, w/w) sucrose density gradient (~14 × 80 mm in size) and centrifuged for 40 min at 160,000 × g. After centrifugation, the plasma membrane fraction (1–3.5 cm from the bottom of the tube) was collected and pelleted by centrifugation for 60 min at 150,000 × g. The pelleted plasma membrane fraction was resuspended in STE buffer and analyzed using SDS-PAGE and immunoblotting. The blots were visualized using the Amersham ECL detection system (GE Healthcare) and a LAS-4000 luminescent image analyzer (GE Healthcare). The intensity of the band was quantified using a Multi Gauge software (Fuji Photo Film, Tokyo, Japan).

Immunostaining

Differentiated 3T3-L1 adipocytes (day 8) grown on a coverslip were fixed using 100% methanol for 2 min at −20°C. The cells were immunostained using the mouse anti-β-tubulin and rabbit anti-GLUT4 primary antibodies and Alexa Fluor 488 or Alexa Fluor 568-conjugated secondary antibodies (Thermo Fisher Scientific, MA, U.S.A.) to visualize the nuclei. Immunofluorescence images were obtained using a 60x objective lens with a Fluoview FV1000 confocal microscope system (Olympus, Tokyo, Japan).

Glucose transport assay

Differentiated 3T3-L1 adipocytes (day 8) in a 12-well culture dish were incubated in serum-free DMEM-LG for 3 h at 37°C to stabilize the basal glucose transport activity. Cells were subsequently washed twice with KRH buffer (25 mM Krebs-Ringer-Hepes/Na, pH 7.4, containing 0.4% BSA and 3 mM sodium pyruvate) and incubated in the same buffer for 1 h, followed by stimulation with or without 100 nM insulin for 30 min at 37°C. Next, 2-[1,2-3H] deoxy-D-glucose (0.8 μCi/well) was administered to the cells at a final concentration of 0.1 mM and the cells were incubated for 5 min at 37°C. Nonspecific uptake was measured in the presence of 1 mM cytochalasin B. At the end of incubation, cells were washed twice with ice-cold KRH buffer and lysed in 0.4 mL of 0.4% SDS. The radioactivity in the lysate was measured using a scintillation counter.
Transfection of plasmid DNA or siRNA

The expression plasmids for wild-type rat Gαs and Gαs-G226A were prepared as described previously [4]. Small interfering RNA (siRNA) duplexes targeting mouse Gαs (Table 1) were purchased from Dharmacon siGENOME SMARTpool (Thermo Fisher Scientific Inc., Waltham, MA). 3T3-L1 cells grown on a culture dish were differentiated into adipocytes. Cells were detached using 0.25% trypsin (Sigma-Aldrich, MO, U.S.A.) and 0.5 mg/mL of crude collagenase (Type I, Worthington) in PBS. After three washes with PBS, the cells were resuspended in electroporation buffer (Bio-Rad). A cell aliquot was mixed with the expression plasmid (30 μg) or with the siRNA duplexes (5 nmol) in a cuvette with a 0.4 cm gap before electroporation using the Gene Pulser Xcell (Bio-Rad) set at 200 V and 28 msec in a time-constant mode. Electroporated cells were resuspended in DMEM-LG containing 10% FBS and seeded in a culture dish.

Statistical analysis

Data were analyzed using the Student’s t-test and *p* < 0.05 was considered statistically significant.

Results

To investigate the role of the T1R3 homomeric sweet taste receptor in mature adipocytes, we examined the effects of sucralose or saccharin on microtubules in fully differentiated 3T3-L1 adipocytes. As shown in Fig. 1A, stimulation of differentiated 3T3-L1 cells with 20 mM sucralose for 3 h resulted in depolymerization of the microtubules in a time-dependent manner. Stimulation

| Target | Gene symbol | Target sequence of mRNA |
|--------|-------------|-------------------------|
| Gαs    | Gnas        | 5'-GCUUGAAGGUUCACAAUUU-3' |
|        |             | 5'-GAUCAACACCACGACCUUU-3' |
|        |             | 5'-GGACUACUUUCCAGAGUUC-3' |
|        |             | 5'-GAACAUCCGCCGUCUCUC-3' |

Fig. 1 Sucralose treatment depolymerizes microtubule in 3T3-L1 adipocytes. (A) 3T3-L1 cells grown and differentiated (day 8) on a cover glass were treated with sucralose (20 mM) for 3 h in DMEM-LG. At the indicated time points, wells were fixed and immunostained for β-tubulin (green). Cell nuclei (blue) were visualized with DAPI. (B) Differentiated 3T3-L1 adipocytes (day 8) on a cover glass were treated without (control) or with sucralose (20 mM), saccharin-Na (20 mM), isoproterenol (5 μM), cholera toxin (0.1 μg/mL) or forskolin (20 μM) in DMEM-LG for 3 hours except with cholera toxin (for 6 hours). Cells were then fixed and immunostained for β-tubulin (green). Cell nuclei (blue) were visualized with DAPI.
with saccharin for 3 h also induced microtubules to depolymerize (Fig. 1B). Importantly, the effects of these sweeteners on the microtubules were mimicked via treatment with Gas-activating reagents, cholera toxin (CTX), or isoproterenol, but not with forskolin, a stimulator of adenyl cyclase (Fig. 1B). These findings are consistent with our previous observation that the T1R3 homomeric sweet taste receptor is coupled to Gas in 3T3-L1 cells, and that activation of this receptor causes microtubule disassembly by a Gas-mediated but cAMP-independent mechanism [4].

Because insulin stimulates glucose transport by inducing GLUT4 recruitment to the plasma membrane in adipocytes in a microtubule-dependent manner [13], we investigated the effect of insulin on glucose transport in differentiated 3T3-L1 adipocytes treated with sucralose, saccharin, nocodazole, isoproterenol, or forskolin. As shown in Fig. 2A, insulin administration stimulated glucose uptake by 4-fold in control cells. However, treatment with sucralose or saccharin for 3 h inhibited insulin-stimulated glucose uptake by 32% and 45%, respectively, which was almost equivalent to the inhibitory effect of nocodazole (by 33%). Additionally, isoproterenol treatment inhibited insulin-induced glucose transport by 26%, whereas forskolin, which had little effect on microtubule integrity, showed a profound inhibition of glucose transport (86%). Because forskolin is known to directly bind to and inhibit the activity of facilitative glucose transporters such as GLUT1 and GLUT4 [14], the inhibitory effect of forskolin may result due to its direct inhibition of glucose transporter activity. To exclude the possibility that sweet taste receptor agonists inhibited the insulin-stimulated glucose transporter by direct inhibition of the intrinsic transporter activity, glucose uptake was measured in conditions where subcellular trafficking of glucose transporters was arrested at low temperatures. Differentiated 3T3-L1 adipocytes were stimulated with insulin at 37°C for 30 min to recruit glucose transporters to the cell surface, and the incubation medium was subsequently replaced with fresh medium and the cells were incubated at 10°C to inhibit the subcellular trafficking of glucose transporters, thereby retaining the transporter on the plasma membrane. Next, the uptake of 2-deoxyglucose was measured in the absence or presence of 20 mM sucralose or 5 μM forskolin. As illustrated in Fig. 2B, glucose uptake was not affected by sucralose but was considerably inhibited by forskolin treatment, indicating that sucralose does not inhibit the intrinsic transporter activity. These observations are consistent with the hypothesis that the activation of the T1R3 homomeric sweet taste receptor interferes with insulin action on GLUT4 translocation and glucose transport via the disassembly of the microtubules in 3T3-L1 adipocytes.

To further confirm this hypothesis, we examined the insulin-induced translocation of GLUT4 to the plasma membrane in sucralose-treated 3T3-L1 adipocytes. Additionally, we investigated the role of Gas in sucralose activity by overexpression of dominant-negative Gas (Gas-G226A) and by siRNA-mediated knockdown of Gas. As shown in Fig. 3A, cells overexpressing the wild-type Gas (Gas-WT) showed that the microtubule

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**Fig. 2** Inhibition of insulin-stimulated glucose transport with sweet taste receptor agonists.

(A) Differentiated 3T3-L1 adipocytes (day 8) in a 12-well culture dish were incubated for 3 h serum-free DMEM-LG, and then treated without (control) or with sucrose (20 mM), saccharin-Na (20 mM), nocodazole (5 μM), isoproterenol (5 μM), or forskolin (20 μM) in DMEM-LG for 3 h. At the end of incubation, cells were stimulated without or with 100 nM insulin (Ins) for 30 min, and 2-deoxyglucose uptake over 5 min was measured. Data are shown as the mean ± SE (n = 3). *, p < 0.05, **, p < 0.01, ***, p < 0.0001 (vs. control). (B) Differentiated 3T3-L1 adipocytes (day 8) were stimulated with 100 nM insulin at 37°C for 30 min. The incubation medium was subsequently replaced with fresh medium and the cells were incubated at 10°C. Next, the uptake of 2-deoxyglucose over 5 min was measured at 10°C in the absence (control) or presence of 20 mM sucralose or 20 μM forskolin. Data are shown as the mean ± SE (n = 3).
integrity was preserved and GLUT4 signals accumulated on the plasma membrane after insulin stimulation for 30 min, indicating GLUT4 translocation occurred to the plasma membrane. In contrast, plasma membrane-localized GLUT4 signals were not evident in sucralose-treated cells, in association with microtubule depolymerization. Notably, sucralose-mediated inhibition of GLUT4 translocation was reversed by overexpression of Gαs-G226A [15, 16] and knockdown of Gαs (Fig. 3A). These results indicate that sucralose inhibited GLUT4 translocation via a Gαs-dependent mechanism. The results are consistent with those of our previous study in 3T3-L1 preadipocytes showing that activation of the T1R3 homomeric sweet taste receptor induces microtubule disassembly in a Gαs-dependent manner [4]. In addition, membrane fractionation analysis revealed that sucralose treatment reduced GLUT4 levels in the plasma membrane fraction from insulin-stimulated adipocytes (Fig. 3B). These results supported the immunostaining data showing that insulin-elicited GLUT4 translocation to the plasma membrane was inhibited by sucralose treatment.

![Fig. 3](image)

**Fig. 3** Sucralose inhibits insulin-induced GLUT4 translocation. (A) Differentiated 3T3-L1 adipocytes (day 8) were transfected by electroporation with the expression plasmids (30 μg) for Gαs (wild-type) or Gαs-G226A or with the siRNA (5 nmole) targeting Gαs, and incubated for 24 h. Then, cells were treated without or with 20 mM sucralose for 3 h. After stimulation with 100 nM insulin for 30 min, cells were fixed and immunostained for β-tubulin (green) and GLUT4 (red). Con, control. Suc, sucralose. (B) Differentiated 3T3-L1 adipocytes (day 8) were treated without (control) or with 20 mM sucralose for 30 min, and stimulated with 100 nM insulin for 30 min. Then the plasma membrane fractions were prepared and analyzed by immunoblotting as described in ‘Materials and Methods’. Representative immunoblot data (upper panel) and the relative amount of GLUT4 in the plasma membrane (PM) in control cells (lower panel, left) and sucralose-treated cells (lower panel, right) were shown. Data are shown as the mean of duplicate data.
Discussion

In this study, we investigated the functional role of the T1R3 homomeric receptor in differentiated 3T3-L1 adipocytes. In particular, we focused on whether activation of T1R3 homomeric receptors causes Gαs-dependent microtubule depolymerization in differentiated adipocytes, and whether microtubule depolymerization affects insulin action. Our findings indicate that the T1R3 homomeric receptor negatively regulates insulin action in adipocytes via Gαs-dependent microtubule disassembly. As observed in preadipocytes, activation of T1R3 homomeric receptors with sucralose or saccharin caused time-dependent depolymerization of the microtubules in differentiated adipocytes. This microtubule-depolymerizing effect was reproduced by Gαs activators, isoproterenol, and cholera toxin, but not with the adenylyl cyclase activator forskolin, indicating that Gαs, but not cyclic AMP, is a potential downstream signaling molecule, which is consistent with our previous observations [4]. Further, in conditions where the microtubules were depolymerized after treatment with T1R3 homomeric receptor agonists, insulin action on glucose transport and GLUT4 translocation to the plasma membrane was considerably inhibited by ~40%. Therefore, microtubule integrity is necessary for insulin-stimulated GLUT4 translocation and glucose transport, and there are two intracellular pools of GLUT4 with different microtubule dependencies as also shown in previous research including our analyses [13]. We have shown in a previous study [13] that there are two insulin-sensitive GLUT4 pools with different microtubule dependencies based on the following observations. First, the inhibition of insulin action by microtubule depolymerization is partial (~40%); second, microtubule-dependent and independent pools differ in the rate of translocation by insulin stimulation; third, and importantly, the inhibition of insulin action by microtubule depolymerization is not restored to the level of control cells by inhibition of endocytosis. These data suggest that microtubule depolymerization does not simply slow the translocation of GLUT4 from the entire insulin-sensitive pool; rather, there exists a microtubule-independent subpopulation in the insulin-responsive GLUT4 pool. Notably, the inhibition of GLUT4 translocation in sucralose-treated adipocytes was reversed by overexpression of a dominant-negative Gαs or by knockdown of Gαs. Thus, Gαs may play a negative role in the action of insulin on glucose transport by regulating the integrity of microtubules.

However, the physiological significance of the T1R3 homomeric receptor in the regulation of adipocyte function is unclear. One unresolved issue is that the endogenous ligand for this receptor is unknown. Because the affinity of the receptor for known physiological sweet compounds such as glucose and sweet amino acids present in the blood is ~100 times lower than the artificial sweeteners used in this study [1], endogenous ligands at physiological concentrations do not stimulate the receptor effectively. However, unknown positive allosteric modulators may exist [17] which may synergistically enhance the signal of the endogenous sweet compounds, similar to inosine-5’-monophosphate (IMP) which can strongly potentiate the umami taste intensity of monosodium glutamate [18]. Alternately, in cells such as adipocytes where T1R3 is highly expressed [3], the affinity of homomeric T1R3 receptors for endogenous ligands may approximate their physiological range because of the law of mass action. Despite these issues, in conditions where endogenous ligands activate the homomeric T1R3 receptors, insulin action in adipocytes may be impaired in high glucose conditions prevalent in diabetes. Adipocyte-specific knockout of T1R3 may help in the evaluation of the physiological significance of this receptor.

To summarize, as the expression of T1R3 increases with the differentiation of 3T3-L1 cells, it is likely that the signaling cascade downstream of the T1R3 homomeric sweet taste receptor is also active in differentiated adipocytes. In this study, we identified a new non-gustatory role for the T1R3 homomeric sweet taste receptor in adipocytes, in which activation of the T1R3 homomeric sweet taste receptors negatively regulates insulin action of glucose transport via Gαs-dependent microtubule disassembly.

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Disclosure

The authors have no competing interests to declare.

Author Contributions

Y.M. researched data. J.M. researched data. T.S. analyzed the data. I.K. and T.I. contributed to the discussion. H.S. designed and supervised all experiments and wrote the manuscript. H.S. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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