



Characterization of the taste receptor-related G-protein, α -gustducin, in pancreatic β -cells

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Keywords

α -Gustducin, Insulin secretion, Taste receptor

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ABSTRACT

Aims/Introduction: Taste receptors, T1rs and T2rs, and the taste-selective G-protein, α -gustducin, are expressed outside the taste-sensing system, such as enteroendocrine L cells. Here, we examined whether α -gustducin also affects nutrition sensing and insulin secretion by pancreatic β -cells.

Materials and Methods: The expression of α -gustducin and taste receptors was evaluated in β -cell lines, and in rat and mouse islets either by quantitative polymerase chain reaction or fluorescence immunostaining. The effects of α -gustducin knockdown on insulin secretion and on cyclic adenosine monophosphate and intracellular Ca^{2+} levels in rat INS-1 cells were estimated. Sucralose (taste receptor agonist)-induced insulin secretion was investigated in INS-1 cells with α -gustducin suppression and in islets from mouse disease models.

Results: The expression of Tas1r3 and α -gustducin was confirmed in β -cell lines and pancreatic islets. Basal levels of cyclic adenosine monophosphate, intracellular calcium and insulin secretion were significantly enhanced with α -gustducin knockdown in INS-1 cells. The expression of α -gustducin was decreased in high-fat diet-fed mice and in diabetic *db/db* mice. Sucralose-induced insulin secretion was not attenuated in INS-1 cells with α -gustducin knockdown or in mouse islets with decreased expression of α -gustducin.

Conclusions: α -Gustducin is involved in the regulation of cyclic adenosine monophosphate, intracellular calcium levels and insulin secretion in pancreatic β -cells in a manner independent of taste receptor signaling. α -Gustducin might play a novel role in β -cell physiology and the development of type 2 diabetes.

INTRODUCTION

Taste cells aid in the detection of nutrients, such as sugars, sweeteners and umami compounds, through heterodimers of type 1 taste receptor subunits (T1rs). T1r2 and T1r3 form a heterodimer in taste cells, which responds widely to sweetness elicited by compounds, such as glucose, sucrose and artificial sweeteners^{1,2}. A heterodimer of T1r1 and T1r3 recognizes umami compounds, such as glutamate, monosodium glutamate and inosinic acid^{1,3}. Sweet and bitter compounds bind to and activate specific G-protein coupled receptors that interact with G-proteins, such as α -gustducin⁴.

α -Gustducin is also expressed in brush cells of the stomach, duodenum and pancreatic duct in rats^{5–7}. It has been reported that T1rs, T2rs and α -gustducin are expressed in intestinal endocrine L cells, and are involved in glucagon-like peptide-1 (GLP-1) secretion signaling in response to glucose^{8–10}. As pancreatic β -cells secrete insulin in response to glucose and other nutrients, taste receptors and/or α -gustducin could also be implicated in nutrition sensing in these cells. However, just a few studies have explored the expression and function of these taste-related molecules in pancreatic β -cells^{11–14}.

In the present study, we focused on the taste signaling-related molecule, α -gustducin, and examined its role in insulin secretion by pancreatic β -cells. We examined the expression of

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α -gustducin in rat and mouse islets, and the rat pancreatic β -cell line INS-1, and investigated the role of α -gustducin in INS-1 cells. We also examined whether α -gustducin is involved in the regulation of cyclic adenosine monophosphate (cAMP) and intracellular calcium levels within these cells.

METHODS

Cell culture

INS-1 cells were cultured in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 50 μ mol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 100 U/mL penicillin and 100 μ g/mL streptomycin (Invitrogen) at 37°C in a 5% CO₂-enriched atmosphere¹⁵. MIN6 cells were gifted by Dr J Miyazaki (Osaka University, Osaka, Japan). They were cultured at 37°C in a 5% CO₂-enriched atmosphere in Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 15% heat-inactivated fetal bovine serum (Sigma-Aldrich), 50 μ mol/L 2-mercaptoethanol, 100 U/mL penicillin and 100 μ g/mL streptomycin (Invitrogen).

Animals

Male C57BL/6J mice and male BKS.Cg-+ *Lep^{db}/+* *Lep^{db}/Jcl* (*db/db*) mice were purchased from CLEA Japan Inc. (Tokyo, Japan). C57BL/6J mice were maintained under standard conditions and fed with a standard diet (STD; CE-2, 12 kcal%; CLEA Japan Inc., Tokyo, Japan) ad libitum. At 10 weeks-of-age, one group of mice of each strain was fed with a high-fat diet (HFD; D12492, 60 kcal%; Research Diets Inc., New Brunswick, NJ, USA), whereas other strain- and age-matched control groups were fed with STD during the experimental period. All experimental procedures were carried out in accordance with the guidelines of the animal care and experimentation committee at the National Center for Global Health and Medicine. Male Wistar rats were purchased from CLEA Japan Inc.

Islet isolation and batch incubation

Islets of Langerhans were isolated from the pancreas of 11-week-old male C57BL/6J mice and 26-week-old STD mice, HFD mice¹⁶ or 8-week-old Wistar rat¹⁷ through collagenase digestion. The isolated islets were then washed and preincubated at 37°C for 30 min in Krebs-Ringer bicarbonate (KRB) buffer (129 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 2 mmol/L CaCl₂, 5 mmol/L NaHCO₃, 10 mmol/L HEPES (pH 7.4) and 0.1% bovine serum albumin [BSA])¹⁸. The islets were incubated in buffer containing 2.8 mmol/L glucose + 10 mmol/L sucralose, 2.8 mmol/L glucose + 0.1 μ mol/L GLP-1 and 16.7 mmol/L glucose for 30 min. Cellular insulin was extracted with acid-ethanol. Insulin secreted into the Krebs-Ringer-HEPES (KRH) buffer and total insulin content was measured using the Ultra-Sensitive Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science Inc., Yokohama, Japan).

Quantitative polymerase chain reaction primers

TaqMan gene expression assay probes for rat guanine nucleotide-binding protein alpha transducing 3 (*Gnat3*; α -gustducin), taste receptor type 1 member 1 (*Tas1r1*), taste receptor type 1 member 2 (*Tas1r2*), taste receptor type 1 member 3 (*Tas1r3*) and β -actin (*Actb*) were obtained from Thermo Fisher Scientific (Waltham, MA, USA; catalog numbers Rn00597619, Rn01516038, Rn01515491, Rn00590759 and 4352931E, respectively). TaqMan gene expression assay probes for mouse *Gnat3* (α -gustducin), *Tas1r1*, *Tas1r2* and *Tas1r3* were obtained from Ambion (Mm01165313, Mm00473433, Mm00499716 and Mm00473459, respectively).

Immunohistochemistry

Pancreata were excised, fixed in 4% paraformaldehyde, embedded in paraffin and immunostained, as described previously¹⁹. Primary antibodies against rabbit anti- α -gustducin (sc395; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and guinea pig anti-insulin (Takara Bio Inc., Shiga, Japan) were used. DyLight 594-conjugated anti-guinea pig immunoglobulin G (1:100) or fluorescein isothiocyanate-conjugated anti-rabbit (1:100; Jackson ImmunoResearch, West Grove, PA, USA) were used as secondary antibodies. TOPRO3 (1:1000) was used to label the nuclei (Molecular Probes, Eugene, OR, USA). Immunofluorescence images were obtained using a Zeiss LSM510 confocal microscope (Carl Zeiss Co., Tokyo, Japan) in confocal mode.

Immunocytochemistry

INS-1 cells were fixed with 4% (w/v) paraformaldehyde for 15 min, permeabilized with 0.25% (w/v) Triton X-100 in phosphate-buffered saline (PBS) for 15 min and immunostained, as described previously^{19,20}. After blocking with PBS containing 10% (w/v) BSA for 30 min, the cells were incubated overnight with the primary antibody in PBS containing 3% (w/v) BSA, washed with PBS and then incubated with secondary antibody in PBS containing 3% (w/v) BSA for 1 h, followed by washing with PBS and mounting. Immunofluorescence images were obtained using a Zeiss LSM510 confocal microscope in confocal mode.

Transfection of small interfering ribonucleic acids

Small interfering ribonucleic acids (siRNA) targeting rat α -gustducin (2 different siRNA), and a non-targeting negative control siRNA were obtained from Thermo Fisher Scientific (*Gust* siRNA1: s141856, *Gust* siRNA2: s141857 and AM4642, respectively).

siRNA was transiently transfected with Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. α -Gustducin messenger RNA (mRNA) expression was assayed 48 h after siRNA transfection, and transcriptional silencing was validated using at least three independent experiments.

RNA preparation and quantitative real-time polymerase chain reaction

Total RNA was extracted from INS-1 cells or mouse pancreatic islets using the RNeasy Mini Kit (Qiagen, Cologne, Germany).

Complementary deoxyribonucleic acid was prepared from 1 μg of total RNA using reverse transcriptase (SuperScript IITM; Invitrogen), according to the manufacturer's instructions. Quantitative polymerase chain reaction amplification was carried out using the TaqMan universal polymerase chain reaction master mix core reagent kit and analyzed using an ABI Prism 7900 (Applied Biosystems). The mRNA levels were evaluated and normalized against Actb expression level using the $2^{-\Delta\Delta\text{Ct}}$ method²¹.

Western blot analysis

Protein fraction of INS-1 cells was extracted using a Subcellular Protein Fractionation Kit (Thermo Fisher Scientific Inc.). The protein samples were resolved using 4–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and gels were transferred to polyvinylidenedifluoride membrane filters (BioRad Laboratories, Hercules, CA, USA). Western blot analyses were carried out using antibodies against α -gustducin (Santa Cruz Biotechnology), Actb, α -tubulin, lamin A/C (Cell Signaling Technology, Danvers, MA, USA; #4967, #2144, #2032, respectively) and E-cadherin (BD Transduction Laboratories, Franklin Lakes, NJ, USA; #610181), and detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore Co., Billerica, MA, USA).

Measurement of insulin level and secretion from INS-1 cells

After the indicated culture periods, INS-1 cells were washed with KRB buffer supplemented with 0.1 mmol/L glucose and 0.5% BSA, preincubated at 37°C for 30 min in KRB buffer containing 3 mmol/L glucose, and incubated at 37°C for 60 min with the indicated concentration of glucose and/or several stimulants. Insulin secreted into the KRB buffer was quantified using the Mouse Insulin ELISA KIT (T-Type; AKRIN-011T; Shibayagi, Gunma, Japan). Cellular insulin was extracted using acid-ethanol overnight at 4 °C, and insulin level was determined using enzyme-linked immunosorbent assay, after dilution.

Measurement of cAMP levels

cAMP levels in INS-1 cells were determined using the cAMP Biotrak enzyme immunoassay system (GE Healthcare UK Ltd., Amersham, UK), according to the manufacturer's instructions.

Intracellular calcium analysis

Intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) in INS-1 cells was measured with a Calcium kit Fluo-4 (#CS22; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). INS-1 cells were placed on a ϕ 35-mm glass bottom culture dish. Growth medium was replaced with loading buffer containing 3 mmol/L glucose and Fluo4-AM, and the culture dish was incubated at 37°C for 1 h. Loading buffer was then replaced with recording buffer containing 3 mmol/L glucose. The Fluo-4 fluorescent signal over the INS-1 cell area was detected through excitation at 488 nm using an LSM 880 Live microscope (Carl Zeiss, Oberkochen, Germany).

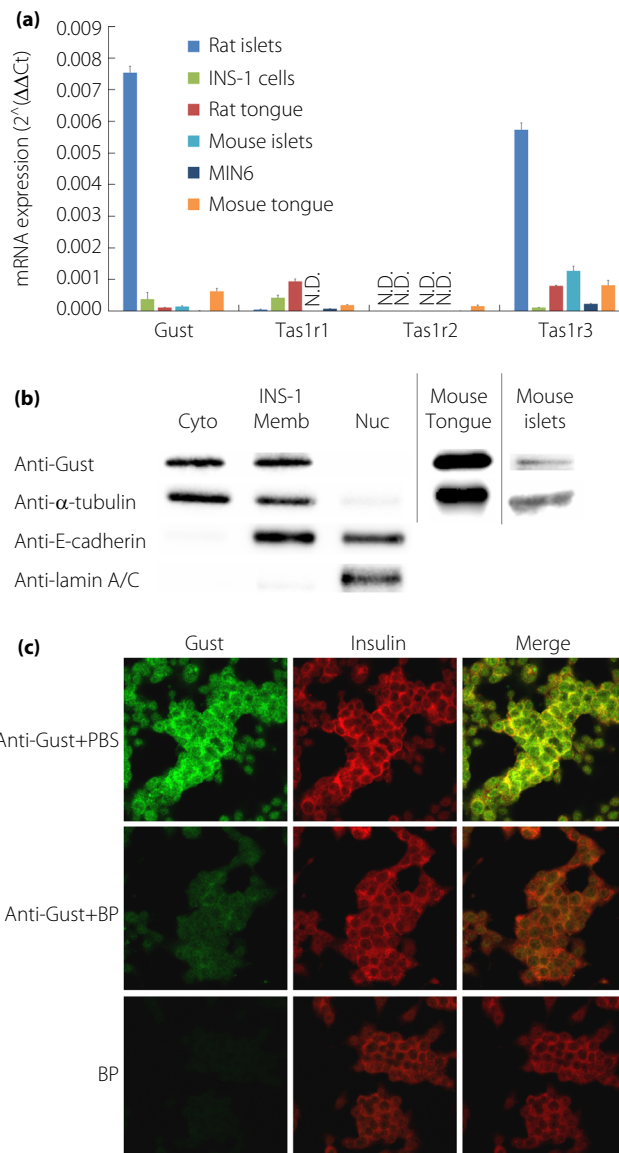


Figure 1 | Expression of taste receptors and α -gustducin (Gust) in islets and cell lines. (a) The expression of taste receptors, T1r1, T1r2 and T1r3, and G-protein, α -gustducin, in isolated rat, mouse islets, and in INS-1 and MIN6 cells were analyzed using quantitative polymerase chain reaction. Values are shown as the mean \pm standard error of the mean ($n = 3$). (b) Portions of each extract in INS-1 cells were analyzed by western blotting using specific antibodies against α -gustducin or endogenous control proteins, including cytoplasm (Cyto; α -tubulin), plasma membrane (Memb; E-cadherin) and nuclear soluble fraction (Nuc; lamin A/C). The expression of α -gustducin in mouse tongue and isolated mouse islets extracted by RIPA buffer was analyzed using western blotting. (c) INS-1 cells were immunostained to detect fluorescein isothiocyanate using anti- α -gustducin antibodies (green) and insulin (red). The specificity of α -gustducin antibody was confirmed using its blocking peptide (BP) in immunocytochemistry; the pre-immune antibody showed no staining at the same concentrations used in these experiments. mRNA, messenger ribonucleic acid; ND, not detected; PBS, phosphate-buffered saline.

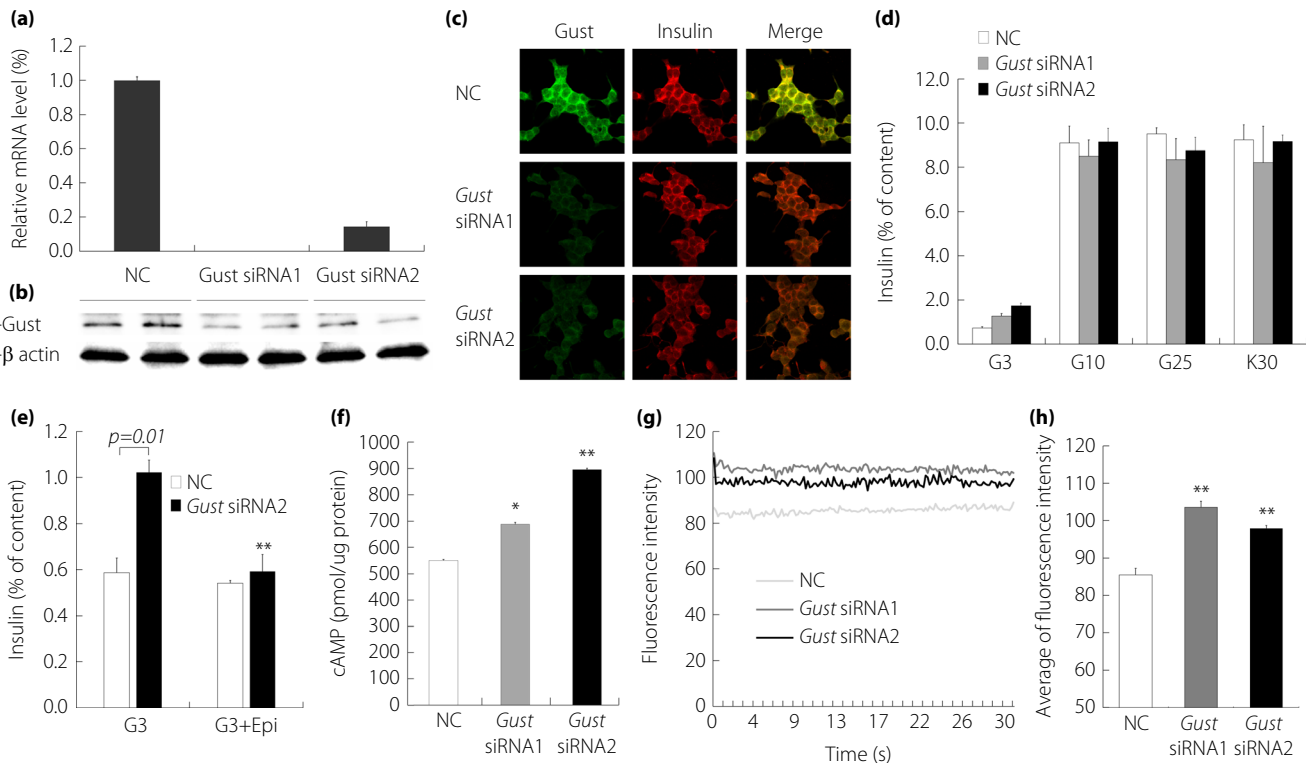


Figure 2 | Effect of α -gustducin knockdown on levels of cyclic adenosine monophosphate (cAMP), intracellular Ca^{2+} and insulin secretion in INS-1 cells. The expression of α -gustducin levels in INS-1 cells at 48 h after the transfection of α -gustducin small interfering ribonucleic acid (siRNA) was examined by (a) quantitative polymerase chain reaction, (b) western blotting and (c) immunocytochemistry. Specific silencing was validated by at least three independent experiments. Values are represented as the mean \pm standard error of the mean ($n = 4$). (d) Insulin secretion from INS-1 cells transfected with negative control siRNA (NC) or two kinds of α -gustducin siRNAs (Gust siRNA1 and Gust siRNA2) was measured after treatment with 3 mmol/L glucose, 10 mmol/L glucose, 25 mmol/L glucose or 30 m/L KCl. The insulin secretion is shown as the percentage of insulin content (secreted insulin / [intracellular insulin content + secreted insulin level]). (e) After transfection of negative control siRNA or α -gustducin siRNA 2, insulin secretion was examined in INS-1 cells stimulated with 0.1 μ mol/L epinephrine plus 3 mmol/L glucose. (f) cAMP in INS-1 cells, cultured in RPMI 1640, was measured after knockdown of α -gustducin. (g) Basal levels of intracellular free calcium [Ca^{2+}]_i were measured in INS-1 cells transfected with Gust siRNA 1 or 2; (h) the average value. ** $P < 0.01$ versus NC. Values are shown as the mean \pm standard error of the mean ($n = 3$). mRNA, messenger ribonucleic acid; ND, not detected.

Statistical analysis

The data are presented as the mean \pm standard error of the mean. Statistical significance was determined using two-tailed unpaired Student's *t*-test. A two-way analysis of variance (ANOVA) was carried out to analyze: (i) insulin secretion data after α -gustducin knockdown and sucralose treatment; and (ii) cAMP concentration data after α -gustducin knockdown and measurement time.

RESULTS

Expression of taste receptor-related protein α -gustducin in islets and pancreatic β -cells

To investigate whether taste receptors and α -gustducin are involved in nutrition sensing in pancreatic β -cells, we first examined mRNA expression of these molecules in rat and mouse islets, and INS-1 and MIN6 cells. As a control, we also measured taste receptors and α -gustducin expression in the

tongue tissue of each species. Quantitative polymerase chain reaction results showed the expression of Tas1r3 and α -gustducin in rat and mouse islets, and in INS-1 and MIN6 cells (Figure 1a). Interestingly, α -gustducin and Tas1r3 expression in rat islets was markedly higher than in the tongue. Expression of Tas1r1 mRNA was detected in rat islets, INS-1 cells, rat tongue, MIN6 cells and mouse tongue, but not in mouse islets. Tas1r2 expression was detected only in the tongue tissue. Furthermore, we examined α -gustducin protein expression by western blot analysis in the cytoplasm, membrane or nuclear extract of INS-1 cells, isolated mouse islets and mouse tongue. The expression of α -gustducin protein was observed in the cytoplasm and membrane fractions of INS-1 cells, mouse isolated islets and mouse tongue (Figure 1b). Fluorescence immunostaining showed that α -gustducin was localized in cytoplasm (Figure 1c). We carried out α -gustducin knockdown experiments using two kinds of siRNAs. Transfection of

α -gustducin siRNA led to a downregulation of α -gustducin mRNA expression to approximately 10%, as compared with the negative control siRNA (Figure 2a). Reduction in α -gustducin protein levels by transfection of siRNA in INS-1 cells was detected by western blotting and fluorescence immunostaining (Figure 2b,c).

Effect of α -gustducin knockdown on levels of cAMP, intracellular Ca^{2+} and insulin secretion in INS-1 cells

We examined whether a reduction in expression of α -gustducin affects insulin secretion from INS-1 cells. Interestingly, basal insulin secretion from INS-1 cells, cultured with 3 mmol/L glucose, was significantly increased by α -gustducin knockdown compared with that in the control siRNA transfected cells (Figure 2d). However, insulin secretion was not affected by 10 mmol/L glucose, 25 mmol/L glucose or 30 mmol/L KCl stimulation (Figure 2d). These results suggested that α -gustducin is involved in basal, but not in high-glucose- or depolarization-stimulated, insulin secretion by INS-1 cells. Next, we determined whether α -gustducin knockdown mediated enhancement of basal insulin secretion was caused by normal exocytosis. Epinephrine stimulation has been reported to suppress insulin secretion by decreasing cAMP through the α -2A-adrenoceptor^{22,23}. Epinephrine stimulation completely abolished the increase in basal insulin secretion by α -gustducin knockdown (Figure 2e). Clapp *et al.* reported that α -gustducin

maintains a tonically low cAMP level in taste cells to ensure adequate Ca^{2+} signaling²⁴. As cAMP and $[\text{Ca}^{2+}]_i$ are also critical for insulin secretion pathway in pancreatic β -cells, we speculated that α -gustducin plays an important role in the regulation of basal insulin secretion by regulating cAMP and calcium levels. First, we examined whether knockdown of α -gustducin causes changes in cAMP concentration. As shown in Figure 2f, the cAMP level in INS-1 cells was significantly increased after α -gustducin knockdown compared with that in cells treated with negative control siRNA. Thus, α -gustducin was found to retain $G\alpha$ -protein-specific functions in pancreatic β -cells. The potentiation of insulin secretion by cAMP is strictly dependent on the extracellular glucose concentration, and the threshold is not reached at 3 mmol/L glucose. We measured $[\text{Ca}^{2+}]_i$ levels in INS-1 cells by α -gustducin knockdown. α -Gustducin knockdown in INS-1 cells significantly increased the amount of basal $[\text{Ca}^{2+}]_i$ (Figure 2g,h). These results suggested that the increase in basal insulin secretion in INS-1 cells by α -gustducin knockdown was caused by an increase in $[\text{Ca}^{2+}]_i$ levels.

Expression of α -gustducin in HFD-fed mice and diabetic *db/db* mice

α -Gustducin is expressed in INS-1 cells, and affects basal insulin secretion by INS-1 cells. However, whether α -gustducin is involved in the pathogenesis of diabetes is still unclear.

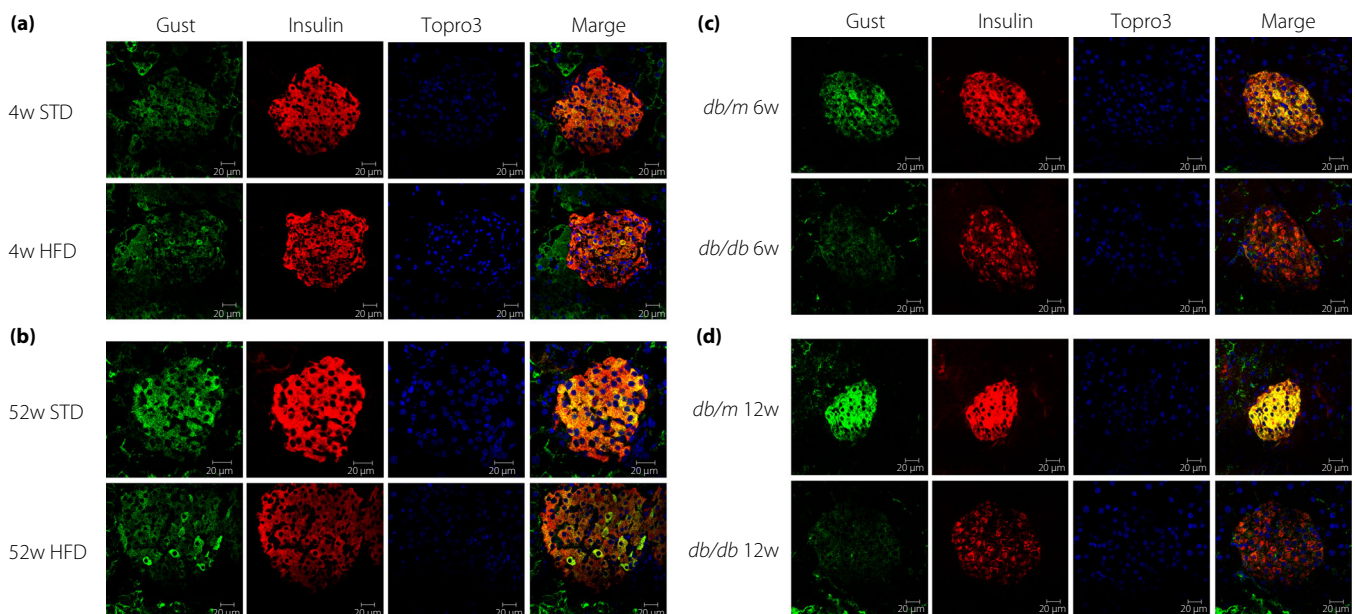


Figure 3 | Expression and localization of α -gustducin (Gust) in high-fat diet-fed (HFD) mice and diabetic *db/db* mice. α -Gustducin was immunostained using anti-gustducin antibodies to detect green fluorescent protein (green), insulin (red) and nuclei (Topro3 blue) in islets of (a) 4-week standard diet-fed mice (4 w STD) and 4-week HFD mice (4 w HFD) and (b) 52-week standard diet-fed mice (52 w STD), and (b) 52-week HFD mice (52 w HFD). Decreasing expression of α -gustducin in the pancreas was observed in diabetic *db/db* mice compared with (c) 6-week-old and (d) 12-week-old control *db/m* mice. Scale bar, 20 μm .

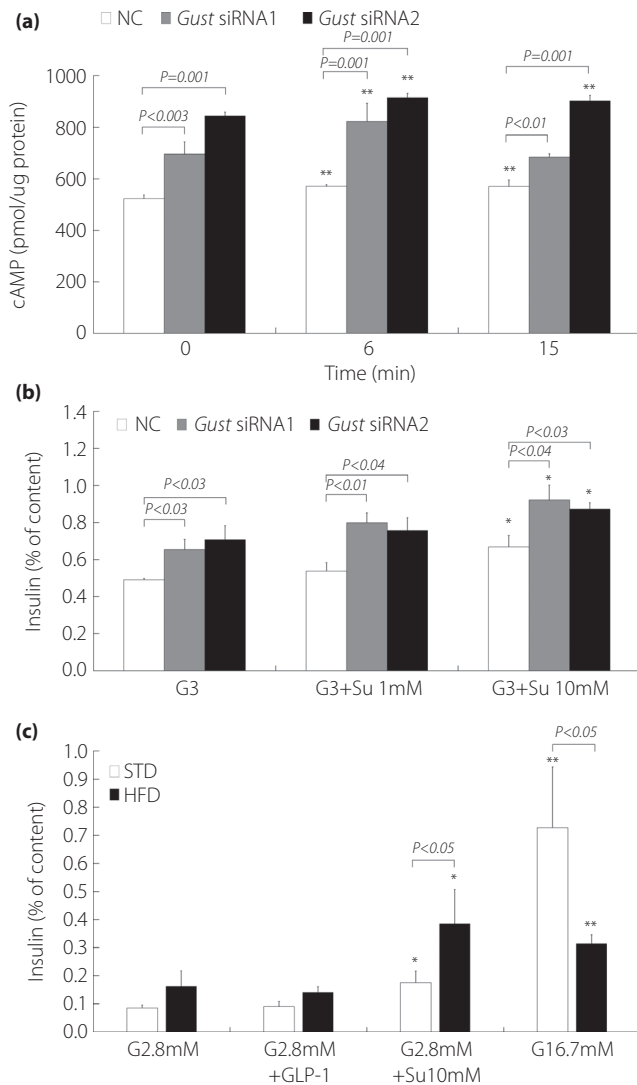


Figure 4 | Functional role of α -gustducin (Gust) involved in the sucralose–taste receptor signaling axis in pancreatic β -cells. (a) After transfection of negative control (NC) small interfering ribonucleic acid (siRNA) or two kinds of α -gustducin siRNAs, cyclic adenosine monophosphate (cAMP) level was examined in INS-1 cells stimulated with 10 mmol/L sucralose plus 3 mmol/L glucose. (b) After transfecting with the negative control siRNA or two kinds of α -gustducin siRNAs, insulin secretion was examined in INS-1 cells treated with 3 mmol/L glucose (G3), 1 mmol/L sucralose plus 3 mmol/L glucose (Su; 1 mmol/L) or 10 mmol/L sucralose plus 3 mmol/L glucose (Su 10 mmol/L). * $P < 0.05$ versus NC. ** $P < 0.01$ versus NC. Values are shown as mean \pm standard error of the mean ($n = 4$). (c) Insulin secretion from isolated islets from mice fed a standard diet (STD) and high-fat diet (HFD) was measured after treating with 2.8 mmol/L glucose (G2.8 mmol/L), G2.8 + 0.1 μ mol/L glucagon-like peptide-(GLP-1), G2.8 + 10 mmol/L sucralose and 16.7 mmol/L glucose. * $P < 0.05$ versus STD. ** $P < 0.01$ versus STD. Values are shown as the mean \pm standard error of the mean ($n = 3$).

Therefore, we examined changes in expression of α -gustducin in diabetic *db/db* mice and HFD-fed mice. α -Gustducin was weakly expressed in both STD-fed mice (4 weeks STD) and HFD-fed mice islets (4 weeks HFD), and co-localized with insulin (Figure 3a). A similar observation was made after comparison between 6-week-old and 12-week-old *db/m* mice (Figure 3c,d). In mice fed with HFD for 52 weeks, the expression of α -gustducin in islets was reduced compared with that in 52-week STD-fed mice (Figure 3b). In addition, in diabetic obesity *db/db* mice, α -gustducin expression was decreased in pancreatic islets compared with that in control *db/m* mice (Figure 3c,d). These results suggested that the expression of α -gustducin in pancreatic islets increases with age and decreased with HFD feeding and obesity.

Involvement of α -gustducin in insulin secretion through the sucralose–taste receptor axis

Although basal insulin secretion levels were enhanced by α -gustducin knockdown in INS-1 cells, glucose-induced insulin secretion was not significantly affected. To elucidate whether α -gustducin is implicated in the detection of sweetness in pancreatic β -cells through taste receptors, we examined levels of basal cAMP and basal insulin secretion under low glucose concentrations induced by the taste signal agonist, sucralose, in pancreatic β -cells. cAMP levels were significantly increased after α -gustducin knockdown in INS-1 cells treated with 10 mmol/L sucralose in the presence of 3 mmol/L glucose (Figure 4a). Two-way ANOVA showed a statistically significant interaction between the effects of α -gustducin knockdown and sucralose treatment time on cAMP ($P < 0.001$). Basal insulin secretion by INS-1 cells was significantly enhanced by 10 mmol/L sucralose treatment, but not by 1 mmol/L sucralose treatment (Figure 4b). Sucralose-stimulated insulin secretion was enhanced by α -gustducin knockdown when compared with that in the control. However, two-way ANOVA showed no statistically significant interaction between the effects of α -gustducin knockdown and sucralose treatment on insulin secretion ($P = 0.056$). These results suggest that α -gustducin is not mainly involved in the sucralose–taste receptor signaling axis in the regulation of insulin secretion.

Next, we investigated whether sucralose stimulation in islets isolated from HFD mice with low α -gustducin expression altered insulin secretion. As previously reported^{25,26}, 16.7 mmol/L glucose stimulation of STD mouse islets significantly increased the insulin secretion compared with 2.8 mmol/L glucose, and this increase was attenuated with HFD exposure; furthermore, GLP-1 stimulation did not increase basal insulin secretion. Meanwhile, we found that sucralose-stimulated basal insulin secretion was significantly increased in HFD mouse islets compared with that in STD mouse islets (Figure 4c). These results suggested that sucralose showed even larger enhancement of basal insulin secretion with lower α -gustducin

expression (Figure 3b,d). These results collectively suggested that α -gustducin is involved in insulin secretion in mouse pancreatic islets through signaling pathways independent of the taste receptor.

DISCUSSION

In the present study, we showed the expression of the taste signaling-related G-protein, α -gustducin, in both mouse islets and the rat pancreatic β -cell line, INS-1, and examined its role in insulin secretion after knockdown experiments in INS-1 cells. Although α -gustducin did not play a major role in glucose- or depolarization-stimulated insulin secretion in INS-1 cells, three interesting observations were made in this study. First, basal insulin secretion, cAMP levels and $[Ca^{2+}]_i$ levels were significantly increased after α -gustducin knockdown in INS-1 cells (Figure 2d–h). The $\alpha 2$ adrenoceptor is coupled with inhibitory G protein and is known to decrease the second messenger cAMP²⁷. The results of stimulation with epinephrine, an $\alpha 2$ -adrenoceptor agonist (Figure 2e) suggested that the increase in basal insulin secretion due to α -gustducin knockdown was not a result of protein leakage, but due to a change in the exocytosis signal. The negative regulation of basal cAMP levels by α -gustducin in INS-1 cells is consistent with the report that the taste cells of α -gustducin-knockout mice show an elevated basal cAMP level²⁴. The suppression of α -gustducin in INS-1 cells significantly increased the basal $[Ca^{2+}]_i$ levels, indicating that in the basal state, α -gustducin contributes to maintain cAMP and $[Ca^{2+}]_i$ at low levels. Second, α -gustducin expressed in mouse pancreatic islets was co-localized with insulin, and the expression levels of α -gustducin were decreased in HFD-fed mice and diabetic *db/db* mice (Figure 4). *db/db* mice at 6–8 weeks-of-age usually present hyperinsulinemia and hyperlipidemia²⁸. At 10–14 weeks-of-age, the *db/db* mice became fully diabetic with significantly higher plasma glucose levels²⁸. We observed that the expression of α -gustducin decreased in 6-week-old or 12-week-old *db/db* mice. Thus, decreased α -gustducin expression levels are speculated to be involved in β -cell dysfunction and the early phase of type 2 diabetes. Third, basal insulin secretion showed no statistically significant correlation with the effects of α -gustducin knockdown and sucralose treatment on insulin secretion ($P = 0.056$). These results suggest that α -gustducin is not involved in the sucralose–taste receptor signaling axis in pancreatic β -cells.

Previously, Nakagawa *et al.*¹¹ reported sucralose-stimulated insulin secretion in the mouse pancreatic β -cell line, MIN6, due to higher cAMP and Ca^{2+} levels. They concluded that these effects of sucralose were, at least partially, mediated by the heterodimer of T1r2 and T1r3, the sweet receptor found in taste cells. Furthermore, Nakagawa *et al.* showed that expression levels of α -gustducin were extremely low in MIN6 cells, and that α -gustducin knockdown did not affect $[Ca^{2+}]_i$ responses to various artificial sweeteners¹². Some differences of their results and the present results could be explained either by species difference (between mice and rats) or by the difference between

tumor cell lines. We confirmed high expression of α -gustducin, Tas1r1, Tas1r2 and Tas1r3 mRNA in rat islets as positive controls, but exact reasons for apparent discrepancies between the findings of Nakagawa *et al.* and the present study regarding the roles of α -gustducin in insulin secretion remain unknown. Interestingly, sucralose increased basal insulin secretion even in cellular states with abnormal glucose responsiveness after HFD challenge. In order to determine how this affects damaged β -cells, further studies are necessary.

The previous studies investigated different β -cell lines; the expression of T1r2 mRNA was not detected in the INS-1 cells used by us, and the classical sweet receptor– α -gustducin pathway (mediated by T1r2 + T1r3) involved in insulin secretion might be absent from INS-1 cells. Therefore, taken together, α -gustducin might couple with other G protein-coupled receptors, in addition to the classical taste receptor, T1r3. Currently, we do not know which receptors couple with α -gustducin and mediate the effect of insulin secretion in INS-1 cells. Nakagawa *et al.* suggested that T1r3 acts as a homodimer; however, its association with α -gustducin is not yet clear²⁹. We were not able to verify the association between α -gustducin and T1r3 in INS-1 cells, because we could not silence the T1r3 gene in INS-1 cells. We observed that certain subtypes of T1rs (Figure 1a) and T2rs (data not shown) were expressed in INS-1 cells, in which T1r2 was absent. The types of extracellular factors that couple with α -gustducin in β -cells are yet to be explored. In taste cells, sweet compounds bind to and activate specific G protein-coupled receptors that interact with α -gustducin, G β_3 and G γ_{13} ^{4,30}. The present data showed that during sucralose-stimulated insulin secretion, sucralose is not absorbed from the intestine³¹, and does not affect β -cells directly *in vivo*. Wong *et al.*³² reported that α -gustducin knockout mice showed reduced response to bitter, sweet and umami stimuli. We examined whether α -gustducin participates in insulin secretion stimulated by possible taste receptor ligands other than glucose and sweeteners. Although various substances, such as fructose, denatonium, leucine and monosodium glutamate, and the hormones, GLP-1 and glucagon, induced insulin secretion in INS-1 cells, the effects of α -gustducin knockdown on insulin secretion were not significant (data not shown). However, Kyriazis *et al.*¹⁴ showed that ablation of sweet taste receptor protein, T1r2, obliterates fructose-induced calcium responses and insulin release in the mouse pancreatic β -cell line, MIN6, and mouse islets. In addition, taste receptor complexes of T1r1 and T1r3 regulate the amino acid-induced insulin secretion in the mouse pancreatic β -cell line, MIN6¹³. Li *et al.*³³ showed that α -gustducin coupled with the fatty acid receptor in the colon. Taken together, α -gustducin is speculated to be associated with amino acid or fatty acid signaling in pancreatic β -cells. Further studies are required to identify the ligands and/or receptors that activate α -gustducin *in vivo*.

At the basal state, α -gustducin maintains tonically low cAMP level and insulin secretion in INS-1 cells. It has been reported that cAMP exerts pleiotropic effects and is important not only

for insulin secretion, but also for β -cell proliferation or survival. It would be interesting to explore the possible role of α -gustducin in this respect.

In conclusion, α -gustducin is expressed in rat and mouse islets, and in INS-1 cells and MIN6 cells, and negatively regulates $[Ca^{2+}]_i$ and cAMP levels, and insulin secretion in INS-1 cells. The present results show a potential role for α -gustducin and a novel mechanism regulating insulin secretion in pancreatic β -cells. Furthermore, the change in α -gustducin expression indicates its role in the development of type 2 diabetes, because expression of α -gustducin is reduced in pancreatic islets of HFD-fed mice and diabetic *db/db* mice. Further *in vivo* studies could elucidate the significance of these findings in normal physiology, as well as in pathological states, such as diabetes mellitus.

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DISCLOSURE

The authors declare no conflict of interest.

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