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Olfactory receptor Olfr544 responding to azelaic acid regulates glucagon secretion in α-cells of mouse pancreatic islets

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ABSTRACT

Olfactory receptors (ORs) are extensively expressed in olfactory as well as non-olfactory tissues. Although many OR transcripts are expressed in non-olfactory tissues, only a few studies demonstrate the functional role of ORs. Here, we verified that mouse pancreatic α-cells express potential OR-mediated downstream effectors. Moreover, high levels of mRNA for the olfactory receptors Olfr543, Olfr544, Olfr545, and Olfr1349 were expressed in α-cells as assessed using RNA-sequencing, microarray, and quantitative real-time RT-PCR analyses. Treatment with dicarboxylic acids (azelaic acid and sebacic acid) increased intracellular Ca^{2+} mobilization in pancreatic α-cells. The azelaic acid-induced Ca^{2+} response as well as glucagon secretion was concentration- and time-dependent manner. Olfr544 was expressed in α-cells, and the EC_{50} value of azelaic acid to Olfr544 was 19.97 μM, whereas Olfr545 did not respond to azelaic acid. Our findings demonstrate that Olfr544 responds to azelaic acid to regulate glucagon secretion through Ca^{2+} mobilization in α-cells of the mouse pancreatic islets, suggesting that Olfr544 may be an important therapeutic target for metabolic diseases.

1. Introduction

Olfactory receptors (ORs) are extensively expressed in olfactory as well as non-olfactory tissues [1,2]. The expression and functional information and the related nomenclature of non-olfactory ORs are summarized in our previous publication [1]. ORs on the cilia of the olfactory receptor neurons in the olfactory epithelium recognize volatile odorants and send a chemical signal as an action potential to the brain, resulting in the perception of smells. However, ORs are also involved in cell movement, such as that of sperm [3,4], muscle cell adhesions [5], cytokinesis [6], and another group of non-olfactory ORs are involved in hormone secretion in the gut [7] and the renal juxtaglomerular apparatus [8]. Recently, ORs were reported to play unique functional roles in brain [9,10], skin [11], and prostate cancer cells [12,13]. However, the function of ORs in non-olfactory tissues remains largely elusive.

Microarray and RNA-sequencing (RNA-seq) analyses demonstrate a robust expression of OR profiles in non-olfactory tissues [14,15]. Previous work in our laboratory noted that specific cell types in various non-olfactory tissues express ORs and their potential downstream effectors and suggested that many ORs are expressed in the neuroendocrine system [16]. The function of these ORs in the neuroendocrine system is unknown, but they may play roles in particular signaling events. The pancreas as an endocrine gland secretes hormones into the bloodstream. Although there are several examples of OR expression in non-olfactory tissues, progress in understanding their physiological functions has been slow and there are no reports on the role of the ORs expressed in the pancreas.

Therefore, in the present study, we investigated whether mouse pancreatic α-cells expressed ORs using RNA-seq and quantitative RT-PCR analyses. After we found several ORs expressed, we determined whether the OR Olfr544 stimulated by azelaic acid (AzA) resulted in the control of glucagon secretion.

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2. Materials and methods

2.1. Cell culture

The glucagon-releasing αTC1-9 cells were purchased from the American Type Cultures Collection. They were maintained in Dulbecco's Modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 2 g/L glucose, 1.5 g/L sodium bicarbonate, 10% FBS (HyClone, Logan, UT), 15 mM HEPES, 0.1 mM nonessential amino acids and 0.02% BSA.

2.2. Immunohistochemistry and immunofluorescence staining

All animal procedures were approved by the Daegu Gyeongbuk Institute of Science and Technology's Institutional Animal Care and Use Committee. Immunohistochemistry and immunofluorescence staining were performed according to a previous report [17]. The following antibodies were used: goat anti-olfactory marker protein (OMP, a gift from Frank Margolis at the University of Maryland, Baltimore, MD) along with rabbit anti-glucagon (Millipore, Temecula, CA), rabbit anti-somatostatin (Millipore), or rabbit anti-insulin (Millipore) antibodies. Goat anti-OMP antibodies were applied for double-immunostaining along with rabbit anti-glucagon, rabbit anti-type III adenylyl cyclase (ACIII, Santa Cruz Biotechnology, Santa Cruz, CA.), rabbit anti-olfactory G protein (G_{olf}, Santa Cruz Biotechnology), or rabbit anti-Olfr544 (Abcam, Cambridge, England) antibodies in αTC1-9 cells.

2.3. RNA isolation, RNA-seq, and gene array analyses

To identify ORs expressed in α-cells by RNA-seq and microarray analyses, total RNA and a sequencing library were prepared using a previously described method [18]. For the gene array, total RNA was labeled and amplified using a Low Input Quick Amp labeling kit (Agilent Technologies, Santa Clara, CA). After labeling, the RNA was used with Agilent's SurePrint Mouse Genome 4 × 44K v2 Microarray kit (Agilent Technologies) and analyzed using an Agilent scanner with its associated software.

2.4. RT-PCR and quantitative real-time RT-PCR

Conventional RT-PCR and real-time RT-qPCR were performed with primer sets (Table S1) according to a previous report [18]. The ORs presenting a clear band were used for real-time qRT-PCR and their relative expression values were calculated using a standard concentration curve generated with the pCI-Rho–*olfr544* construct.

2.5. Intracellular Ca^{2+} mobilization measurement

For initial screening of candidate odorants responding in αTC1-9 cells, the concentration of intracellular calcium ($[Ca^{2+}]_i$) in αTC1-9 cells (5×10^5 cells) was determined with the Grynkiewicz method using Fura-2/AM [18]. The various amounts of odorants were tested for Ca^{2+} mobilization. The changes in fluorescence at the dual excitation wavelengths of 340 nm and 380 nm and the emission wavelength of 500 nm were measured using a spectrofluorometer.

2.6. Luciferase reporter assay

The Dual-Glo Luciferase Assay System (Promega, Madison, WI) was used for the luciferase reporter assay. For pairing of Olfr544 or Olfr545 with AzA, CRE-Luc (Stratagene, La Jolla, CA) was used to measure receptor activation using the method described previously [19]. AzA and sebacic acid (SeA) were purchased from Sigma–Aldrich.

2.7. Glucagon measurements

The levels of glucagon secreted from αTC1-9 cells were monitored with a DuoSet ELISA Development System according to the manufacturer's protocol (R&D systems, Minneapolis, MN). The αTC1-9 cells were cultured for 2 days in DMEM. The cells were then incubated at 37 °C in 0.5 mL modified Krebs–Ringer bicarbonate HEPES (mKRBH) buffer (130 mM NaCl, 3.6 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM KH_2PO_4, 2 mM $NaHCO_3$, 10 mM HEPES, 0.1% BSA and 10 mM glucose, pH 7.4) for 2 h. Next, the cells were incubated for 30 min in 400 μL of mKRBH buffer (without glucose) with the samples in a 24-well plate. At the end of the incubation, 350 μL of medium was collected from each well for measurement of the secreted glucagon.

2.8. Small interfering RNA (siRNA) treatment and western blot analysis

To test whether the effects of Olfr544 activation on intracellular Ca^{2+} mobilization and glucagon secretion could be inhibited, RNA interference was performed according to the method provided in a previous report [20]. Briefly, αTC1-9 cells grown in 6-well plates were transfected with an appropriate amount of olfr544 siRNA (#5: GCUCAGAUGUGAUCCGCCA; #10: GAGCAAUGGCACCUACAAU) or non-targeting siRNA (UGGUUUACAUGUCGACUAA, Thermo Fisher Scientific, Waltham, MA) using Lipofectamine 2000, twice sequentially (separated by 24 h). Three days after the transfection, western blot analysis and intercellular Ca^{2+} concentration and glucagon measurements were conducted using standard procedures [18].

2.9. Data analysis

Data are shown as mean ± SEM. Student's *t*-test or one-way ANOVA with Dunnett's multiple comparisons test were performed using Microsoft Excel or GraphPad Prism software (GraphPad Software, La Jolla, CA).

3. Results and discussion

3.1. Expression of olfactory signaling downstream effectors in mouse pancreatic α-cells

Many ORs have been identified in non-olfactory systems since OR genes were found in the testis and sperm [1]. Recently, microarray and RNA-seq analyses showed that the principal olfactory signaling components OR, G_{olf}, ACIII, and OMP are also expressed in many non-olfactory tissues [14,15]. Previous work in our laboratory suggested that OMP expression is an indicator of potential OR-associated events in non-olfactory systems [16]. To determine whether ORs and OR-mediated chemoreception existed and functioned, respectively, in the pancreas, we verified the existence of ORs in the islets of Langerhans. In the present study, we first assessed the expression of OMP in the mouse pancreas. We found that OMP was significantly detected in pancreatic tissue (Fig. 1). This staining was most likely specific because blocking the antibody with the recombinant OMP protein completely abrogated the immunopositivity (data not shown). To determine the identity of the cells expressing OMP, double immunofluorescence for OMP with glucagon, insulin, or somatostatin was performed. OMP co-localized with the glucagon-producing α-cells, but not with insulin or somatostatin (Fig. 1 left panel). These results demonstrate that OMP expression is limited to a subset of cells within the islet, mostly likely α-cells, a result supported by the quantitative analysis (Fig. S1). The islets of Langerhans, the main endocrine unit controlling glucose homeostasis, are composed of clusters of four cell

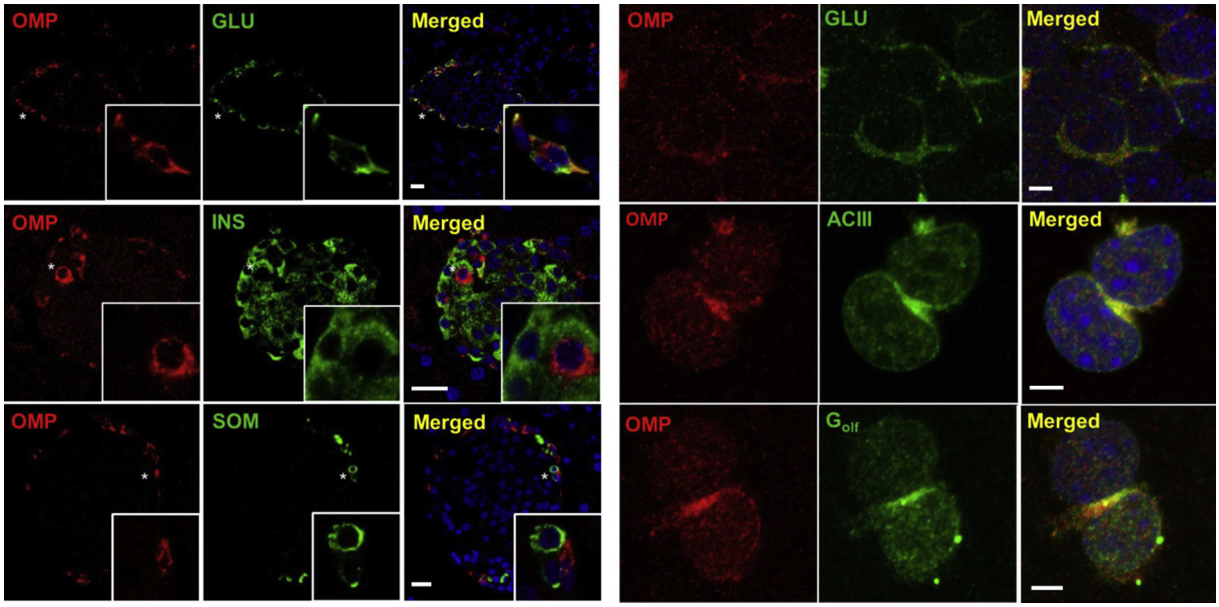

Fig. 1. Mouse pancreatic α-cells express olfactory receptor (OR)-associated signaling components. (Left panel) Olfactory marker protein (OMP)-positive cells in mouse pancreatic islets. Mouse pancreatic tissue sections were processed for double-labeling immunofluorescence of anti-OMP (red) coupled with anti-glucagon (GLU) for α-cells, anti-insulin (INS) for β-cells, or anti-somatostatin (SOM) for δ-cells (green). Boxed areas represent higher magnification of selected cells (white asterisk) in each image. Scale bar = 20 μm. (Right panel) Expression of OR-associated signaling components in the pancreatic α-cell line (αTC1-9). Double-immunofluorescence images of anti-OMP (red) with anti-glucagon, anti-type III adenylyl cyclase (ACIII), or anti-olfactory G Protein (G_{olf}) (green) captured with a confocal laser scanning microscope. The immunoreactivity of OMP, ACIII, and G_{olf} are detected with that for glucagon in αTC1-9 cells, indicating co-expression of three OR-associated signaling components with glucagon in αTC1-9 cells. Scale bar = 5 μm.

types that synthesize various peptide hormones, including glucagon (α-cells), insulin (β-cells), somatostatin (δ-cells) and pancreatic polypeptide (PP-cells) [21]. The simultaneous expression of G_{olf} and ACIII with OMP and the essential OR in specific cells within non-chemosensory tissues suggests that it is plausible for an OR-mediated signaling event to induce a chemical signal. Thus, we surveyed the expression of G_{olf}, ACIII, and OMP with glucagon using immunofluorescence staining techniques in cultured mouse pancreatic α-cells (αTC1-9), which showed positive OMP and glucagon expression (Fig. 1 right panel). Robust staining for G_{olf} and ACIII proteins was apparent in the αTC1-9 cells positive for OMP and glucagon, indicating that the α-cells might use three major components (G_{olf}, ACIII, and OMP) as chemosensory signaling systems (Fig. 1 right panel). These results provide evidence for the co-expression of OMP, G_{olf}, and ACIII in α-cells, and suggest that these cells may use the canonical OR-mediated downstream signaling effectors.

3.2. Identification of candidate OR genes in OMP-positive α-cells

The large number of OR genes can be an obstacle in identifying which cells express ORs. We investigated whether OR genes were expressed in cultured mouse pancreatic α-cells. We first determined OR expression by examining data from various sources, including the results of our microarray data and RNA-seq expression analysis with αTC1-9 cells as well as those from previously published studies [22]. Tables S2 and S3 present the ORs in αTC1-9 cells identified using RNA-seq and microarray analyses. To confirm these results (considered statistically significant when expression values for RNA-seq data were greater than 0.01 and the intensity mean values for gene array data were greater than 100), we performed conventional RT-PCR analysis to determine the expression of the OR genes in αTC1-9 cells, as well as in mouse pancreatic tissue, with specific primer sets (Tables S1). Despite the lack of quantitation using conventional RT-PCR, we detected several OR genes (Fig. S2). Finally, based on results from quantitative real-time RT-PCR, we selected candidate OR genes olfr543, olfr1349, olfr544, and olfr545 (expression values were greater than 1.0 pM) detected in mouse pancreatic αTC1-9 cells (Table 1). Our results indicated that the OR genes existed, allowing for the possibility that their corresponding proteins might function in OR-associated events in pancreatic α-cells and that an OR-mediated chemoreception system in α-cells might play a critical role in the regulation of islet homeostasis.

3.3. Natural OR ligands in mouse pancreatic α-cells

The ORs act through G_{olf} to activate ACIII. The increase in cAMP activates a cyclic nucleotide gated channel, and the resulting Ca^{2+} influx then triggers a Ca^{2+}-activated chloride channel [23]. Based

Table 1
Olfactory receptor expression levels in pancreatic α-cells as determined using real-time RT-qPCR analysis.

Olfactory receptor	Pancreatic α-cells	Olfactory bulb
	Expression value (pM)	Expression value (pM)
Olfr543	2.986 ± 0.201	6.175 ± 0.403
Olfr1349	1.213 ± 0.057	0.599 ± 0.073
Olfr544	1.107 ± 0.181	1.927 ± 0.227
Olfr545	1.058 ± 0.081	3.779 ± 0.295
Olfr31	0.503 ± 0.030	0.367 ± 0.013
Olfr461	0.387 ± 0.015	0.612 ± 0.027
Olfr12	0.365 ± 0.019	0.593 ± 0.063
Olfr267	0.329 ± 0.004	1.559 ± 0.047
Olfr1420	0.152 ± 0.027	0.893 ± 0.123
Olfr1033	0.098 ± 0.009	0.757 ± 0.124
Olfr317	0.072 ± 0.004	0.727 ± 0.153
Olfr212	0.042 ± 0.005	0.733 ± 0.064
Olfr1410	0.042 ± 0.005	0.123 ± 0.027
Olfr373	0.007 ± 0.001	0.226 ± 0.009
Olfr1229	0.004 ± 0.000	0.302 ± 0.041

on published data [24], we chose eight odorants interacting through potential known ligands with ORs Olfr543, Olfr544, and Olfr545 but not with Olfr1349. We determined the odorant response in αTC1-9 cells by measuring the responses of the cells to known ligands using ratiofluorimetric Ca^{2+} imaging techniques. Of the eight chemical odorants tested in αTC1-9 cells, we identified two as active ligands, the dicarboxylic acids AzA and SeA (Fig. 2A). AzA is a naturally occurring, linear 9-carbon atom dicarboxylic acid, and SeA is a 10-carbon linear dicarboxylic acid, chemically very close to its monocarboxylic linear lipid counterpart (decanoic acid). Because the olfactory receptors Olfr544 (MOR42-3) and Olfr545 (MOR42-1) respond to dicarboxylic acids, including AzA and SeA [24], we focused further study on these ORs. Administration of AzA to αTC1-9 cells demonstrated significant concentration-dependent Ca^{2+} responses, with AzA functioning as a ligand at a concentration of approximately 1.0 mM (Fig. 2B). The Ca^{2+} response in αTC1-9 cells to AzA was better than that for SeA; thus, we selected AzA for further study. Because the OR-mediated signaling components were primarily localized to α-cells of the islets, the effect of AzA on glucagon secretion was examined in αTC1-9 cells exposed to AzA at 0, 15, 30, 60, and 120 min (Fig. 2C). The AzA treatment significantly increased glucagon secretion by a maximum of 2-fold over that in control cells. The AzA was potent and the effect of AzA on the secretion of glucagon was concentration-dependent (Fig. 2D). These data suggested that an OR-mediated chemosensory system may regulate glucagon secretion and that AzA may be a naturally occurring ligand for this OR-mediated chemoreception.

Next, we examined HEK293 cells transiently expressing Olfr544 or Olfr545 with chaperon proteins to improve the surface expression of the ORs according to the procedure described by Zhuang and Matsunami [19]. AzA elicited receptor-induced responses in the Olfr544-expressing but not detectable in the Olfr545-expressing HEK293 cells (Fig. 3A). In untransfected and mock (pcDNA3.1(-))-transfected HEK293 cells, AzA did not induce signals. The EC_{50} value for the AzA-induced response of the recombinantly expressed Olfr544 was 19.97 μM (Fig. 3A). It is the acceptable ranges of AzA endogenous plasma concentration (20–80 μM), which reaches the value of an appropriate 1×10^{-3} M after dietary intake as described in the previous reports [25,26].

As discussed above, we surveyed cultured αTC1-9 cells for the expression of G_{olf}, ACIII, and OMP using immunofluorescence staining techniques and found that these three components of the chemosensory signaling system were present. Thus, we next determined whether Olfr544 and OMP were co-expressed in mouse pancreas tissue and in cultured αTC1-9 cells. Dual immunostaining for OMP and Olfr544 showed that these two proteins were indeed co-localized in both mouse pancreas tissue and cultured αTC1-9 cells (Fig. 3B). These results suggest that Olfr544, G_{olf}, ACIII, and OMP may constitute an OR-mediated chemoreception system in mouse pancreatic α-cells.

3.4. The effect of AzA on Ca^{2+} mobilization and glucagon secretion in αTC1-9 cells

The RNA interference (RNAi) method has emerged as a powerful technology for studying gene functions in eukaryotes. RNAi is a post-transcriptional process triggered by the introduction of siRNA that leads to gene silencing in a sequence-specific manner [27]. Two siRNAs specific for Olfr544 (#5 and #10) or non-targeting siRNA were transfected into αTC1-9 cells for evaluating the expression of Olfr544, Ca^{2+} responses, and glucagon secretion. Transfection of the #5 and #10 siRNAs specific for Olfr544 into αTC1-9 cells inhibited the expression of Olfr544 by 80% and 70%, respectively, compared with that of non-targeted siRNA (Fig. 3C). When the same transfected αTC1-9 cells were loaded with Fura-2/AM and stimulated with 1.0 mM AzA, transfection of the #5 and #10 siRNA specific for Olfr544 significantly reduced the intracellular Ca^{2+} response as determined using the ratiofluorimetric Ca^{2+} imaging technique, suggesting that the AzA-stimulated Ca^{2+} response was induced through Olfr544-mediated chemoreception

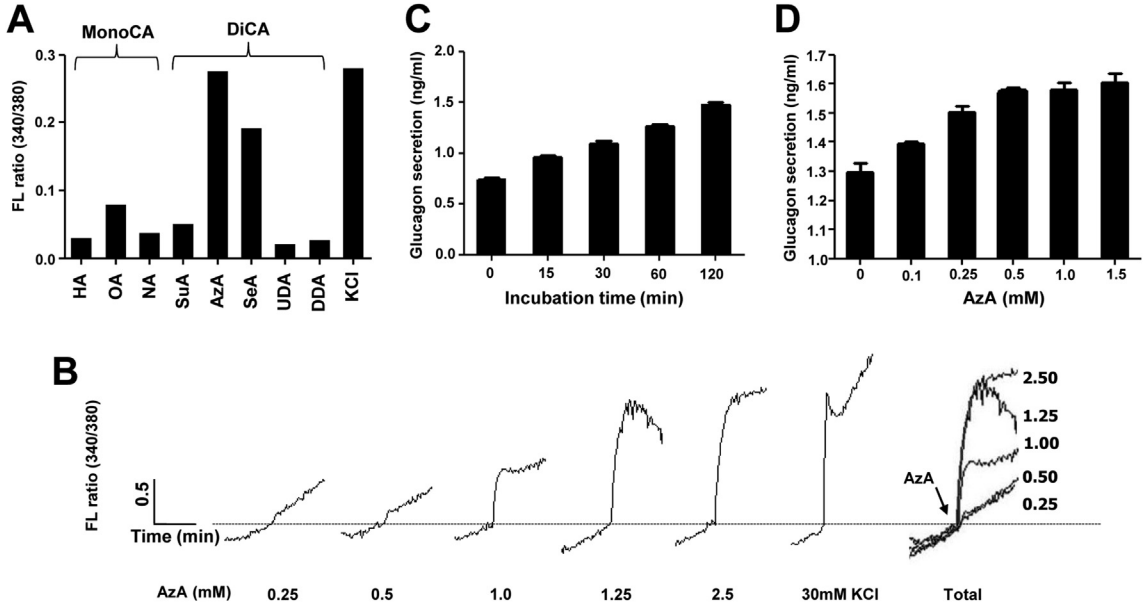

Fig. 2. Azelaic acid evokes Ca^{2+} mobilization and glucagon secretion in mouse pancreatic α-cells. (A) Eight known ligand odorants (MonoCA, monocarboxyic acids; HA, heptanoic acid; OA, octanoic acid; NA, nonanoic acid; DiCA, dicarboxylic acids; SuA, suberic acid; AzA, azelaic acid; SeA, sebacic acid; UDA, undecanedioic acid; DDA, dodecanedioic acid) previously reported to interact with the olfactory receptors Olfr543, Olfr544, and Olfr545 were examined for their ability to cause a Ca^{2+} response in αTC1-9 cells. Cells were loaded with Fura-2/AM and stimulated with 1.0 mM of the odorants. Intracellular Ca^{2+} mobilization was measured using a spectrofluorophotometer. (B) AzA evoked concentration-dependent Ca^{2+} responses (0, 0.25, 0.50, 1.00, 1.25, and 2.50 mM). For a positive control, cells were stimulated with 30 mM KCl before and after the experiment. AzA-induced glucagon secretion is time- (C) and concentration-dependent (D). αTC1-9 cells were pre-incubated with mKRBH buffer containing 10 mM glucose. After 2 h of pre-incubation, the cells were incubated with mKRBH buffer without glucose in the presence of 1.0 mM AzA.

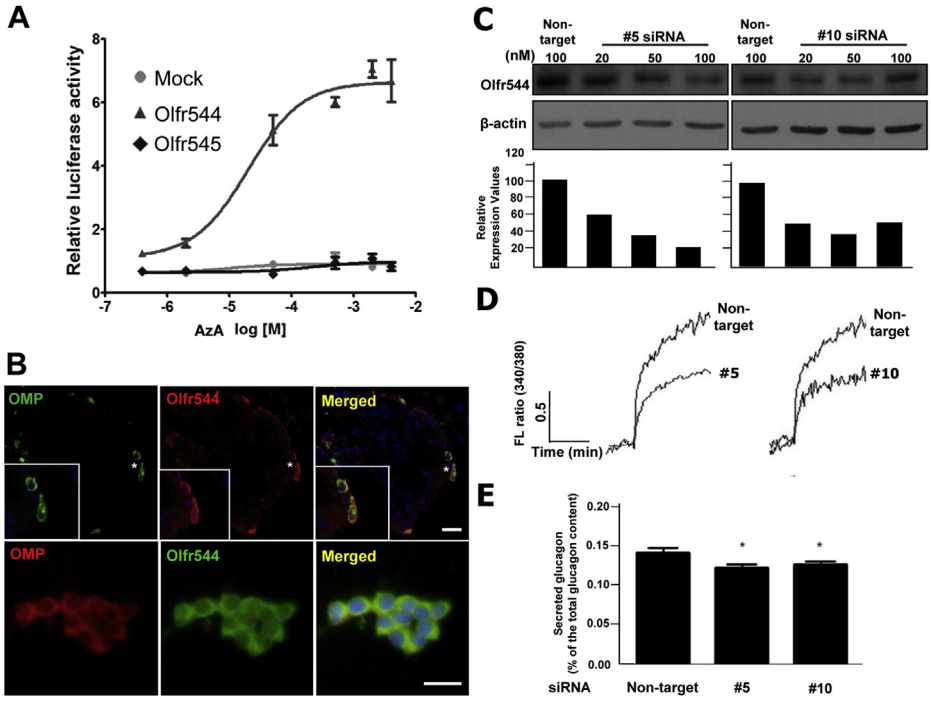

Fig. 3. Azelaic acid regulates Ca^{2+} mobilization and glucagon secretion through an Olfr544-mediated chemoreception system. (A) Concentration-response curves showing that azelaic acid (AzA) significantly activates Olfr544. Olfr544, Olfr545, or Mock was transiently transfected with receptor-transporting protein 1 short (RTP1S) in HEK293 cells, and the transfected cells were measured for their reactivity to AzA. (B) Olfr544 expression in α-cells of mouse pancreatic islets and in αTC1-9 cells. Mouse pancreatic tissue sections (upper row) and αTC1-9 cells (bottom row) were processed for double-labeling immunofluorescence with goat anti-OMP for α-cells and rabbit anti-Olfr544. Boxed areas represent higher magnification of the selected areas (white asterisk) in each image. Scale bar = 20 μm. The effect of AzA on Ca^{2+} mobilization and glucagon secretion in αTC1-9 cells was examined. Two Olfr544-specific (#5 or #10) or non-targeting siRNAs transfected into αTC1-9 cells were evaluated for Olfr544 expression (C), Ca^{2+} responses (D), and glucagon secretion (E). The same transfected cells were loaded with Fura-2/AM and stimulated with 1.0 mM AzA. The #5 and #10 siRNA-treated cells reduce intracellular Ca^{2+} levels. The signal ratio is expressed as fluorescence (FL) ratios. For the effect of AzA and Olfr544 on glucagon secretion, effects of 1 h incubation with 1.0 mM AzA on glucagon secretion after adjusting for total secreted glucagon from siRNA-transfected αTC1-9 cells. Data are expressed as the mean ± SEM (n = 3). *$P < 0.05$ compared with the value obtained from the control.

in αTC1-9 cells, which originate from the mouse pancreatic α-cell (Fig. 3D). Moreover, when we tested the AzA-stimulated secretion of glucagon (as evaluated with an ELISA kit) using siRNAs specific for Olfr544 (#5 and #10), we found that the AzA-stimulated glucagon secretion in the mouse pancreatic αTC1-9 cells was significantly reduced by the Olfr544-specific siRNAs, indicating that the secretion of glucagon stimulated by the naturally occurring ligand AzA is regulated through Olfr544-mediated chemoreception (Fig. 3E). Thus, we showed that all necessary conditions were satisfied to demonstrate the existence of a specific OR-mediated chemoreception system in mouse pancreatic α-cells that could be regulated by a naturally occurring ligand.

The candidate ligand identified for Olfr544 is AzA, which is a 9-carbon atom, linear α,ω-dicarboxylic acid found in whole grains, namely, wheat, rye, barley, oat seeds, and sorghum [28], and is an end product of linoleic acid peroxide degradation [29]. Moreover, cereals such as rice, wheat, and corn make up a major part of most people's diets. During the refining of cereal grains, much of the outer portions of the grains are removed. There is good evidence that consumption of whole grains may reduce the risk of several diseases, including various types of cancer, cardiac diseases, and strokes. Although it is not firmly established, some evidence also suggests that eating a diet rich in whole grains might help protect against diabetes [30].

AzA responding to olfr544 in α-cells of pancreas might result in increased blood glucagon levels. A rise of blood glucagon activates glycogen breakdown in the liver and finally increases blood glucose levels. However, according to two recent reports [31,32], AzA treatment to high-fat diet (HFD)-induced type 2 diabetic animal model showed remarkable reduced blood glucose level and many olfactory receptor genes are induced at the adipose and muscle tissues of a similar animal model. We can speculate on one possibility that Olfr544 may control glucagon secretion reversely at the low and high concentration of glucose in blood. Additional investigation for biological mechanism about this controversial question should be required.

In this study, we investigated molecular components, including Olfr544, G_{olf}, ACIII, and OMP, essential for OR-mediated chemoreception in the islet of Langerhans of mice, particularly in the α-cells. Coupling receptor activation to an optically detectable intercellular Ca^{2+} ion flux and immunological tools allowed concentration-dependent screening of a naturally occurring ligand in αTC1-9 cells, which originate from mouse pancreatic α-cells. We are the first to provide evidence showing that Olfr544 might be functional in the islet of Langerhans, particularly in the α-cells, and that AzA may control glucagon secretion through an Olfr544. These results imply that Olfr544 might be the mediator of metabolic homeostasis to control blood glucose level through glucagon secretion from pancreatic α-cells and suggest that it may be an important target to control glucose homeostasis.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.078.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.078.

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