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Role of histamine H_3 receptor in glucagon-secreting αTC1.6 cells

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ABSTRACT

Pancreatic α-cells secrete glucagon to maintain energy homeostasis. Although histamine has an important role in energy homeostasis, the expression and function of histamine receptors in pancreatic α-cells remains unknown. We found that the histamine H_3 receptor (H_3R) was expressed in mouse pancreatic α-cells and αTC1.6 cells, a mouse pancreatic α-cell line. H_3R inhibited glucagon secretion from αTC1.6 cells by inhibiting an increase in intracellular Ca^{2+} concentration. We also found that immepip, a selective H_3R agonist, decreased serum glucagon concentration in rats. These results suggest that H_3R modulates glucagon secretion from pancreatic α-cells.

1. Introduction

Histamine has multiple functions; it is released during allergic reactions, affects gastric acid secretion, and impacts the sleep–wake cycle. These functions are mediated thorough interactions with four different subtypes (H_1–H_4), which belong to the G protein-coupled receptor superfamily. Among these subtypes, histamine H_3 receptor (H_3R) is currently a hot topic because of its important role in energy homeostasis [1]. Neuronal histamine has an inhibitory effect on food intake through modulation of hypothalamic neuronal activities [2,3]. H_3R expressed in the central nervous system (CNS) regulates histamine release [4]. Therefore, H_3R is a prospective therapeutic target for obesity, which could potentially lead to reduced body weight [5].

The regulation of glucagon secretion from pancreatic α-cells is fundamental to the maintenance of glucose homeostasis; under normal conditions they secrete glucagon in response to starvation-induced hypoglycemia. Glucagon is a 29-amino acid peptide hormone that elevates blood glucose concentration by glycogenolysis and gluconeogenesis in the liver [6]. Glucagon secretion from pancreatic α-cells has recently become a hot topic as a new therapeutic target for diabetes mellitus [7], as dysfunctional pancreatic α-cells have been reported in diabetic patients [8]. In these patients, blood glucagon concentrations are elevated even in hyperglycemic states [9]. Conversely, glucagon secretion is insufficient in response to critical hypoglycemia [10]. This inappropriate glucagon secretion aggravates diabetes. Therefore, it is crucial to understand the mechanism of glucagon secretion.

The key secretagogue for glucagon is low serum glucose concentration [11]. Low glucose concentrations facilitate glucagon release from pancreatic α-cells through the activation of voltage-dependent Ca^{2+} channels (VDCCs) [12]. In addition to glucose, many paracrine/endocrine products and neurotransmitters are involved in the modification of glucagon secretion [13]. For example, glucagon-like peptide (GLP)-1, an incretin secreted from the small intestine L-cells, decreases glucagon secretion from pancreatic α-cells [14].

There are some biological molecules that modulate pancreatic α-cells and CNS functions to regulate total energy homeostasis. GLP-1 has an important role in regulating eating behavior by modulating CNS activities. Turton et al. reported that administration of GLP-1 into the lateral ventricle resulted in inhibition of eating behavior [15]. This evidence indicates that pancreatic α-cells and CNS are targets of GLP-1. Additionally, leptin, a peptide adipokine secreted from peripheral adipose tissues [16], has inhibitory effects on pancreatic α-cells [17] and food intake [18]. Moreover, ghrelin, a 28-amino acid peptide synthesized in the gastrointestinal tract, directly stimulates glucagon secretion [19] and eating behavior

Abbreviations: CNS, central nervous system; H_3R, histamine H_3 receptor; H_3KO, histamine H_3 receptor-gene knockout; KRB, Krebs–Ringer bicarbonate buffer; GLP, glucagon-like peptide; VDCCs, voltage-dependent Ca^{2+} channels

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[20]. As previously mentioned, hypothalamic H_3R has an important role in food intake; thus, it is interesting to examine whether H_3R is expressed in pancreatic α-cells and involved in the regulation of energy homeostasis.

Taking this evidence into account, we hypothesize that H_3R is expressed and involved in pancreatic α-cell function. We used a mouse pancreatic α-cell line, αTC1.6 cells, to evaluate the function of H_3R in pancreatic α-cells. These αTC1.6 cells have properties similar to those of pancreatic α-cells, and they are widely used in research on glucagon secretion [21]. We discovered inhibitory effects of H_3R on glucagon secretion *in vitro* and *in vivo* using pharmacological assays.

2. Methods

2.1. Animals

Wild-type (WT) and histamine H_3 receptor-gene KO (H_3KO) C57BL/6 [22] mice were bred in our laboratory. Wister rats were purchased from Japan SLC (Hamamatsu, Japan). All mice were maintained on a 12-h light/dark cycle in a humidity- and temperature-controlled room, and they were allowed *ad libitum* access to food and water. This study was approved by the Center for Laboratory Animal Research, Tohoku University, Sendai, Japan. This study had been performed in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2. Cell culture

αTC1.6 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% FCS (Thermo, Waltham, MA, USA), 15 mM HEPES (Gibco, Carlsbad, CA, USA), 0.1 mM non-essential amino acids (Gibco), 0.02% BSA (Sigma, St. Louis, MO, USA), 1.5 g/dL sodium bicarbonate (Sigma), 2 g/L D-glucose (Sigma), 75 mg/mL penicillin G potassium (Wako, Osaka, Japan), and 100 mg/L streptomycin sulfate (Wako) at 37 °C in a 5%-CO_2 humidified incubator.

2.3. RT-PCR

RT-PCR was performed using a previously described protocol [23]. Briefly, total RNA was isolated from αTC1.6 cells and reverse-transcribed. Samples (equivalent to 5 ng total RNA) were amplified by 35 cycles of PCR (10 s at 98 °C, 30 s at 60 °C, and 90 s at 72 °C).

2.4. Western blot

Western blot of H_3R was performed using a previously described protocol [23]. We used the hypothalamic lysate from WT and H_3KO mice as positive and negative controls, respectively. Beta-actin was detected by an antibody (Cell Signaling Technology, Danvers, MA, USA) as a loading control.

2.5. Immunohistochemical analysis

Pancreata were quickly removed from 12-week-old WT and H_3KO mice. Isolated pancreata were fixed in 10% paraformaldehyde and embedded in paraffin. The paraffin sections were deparaffinized and blocked with 10% goat serum at room temperature for 15 min. To detect glucagon, sections were incubated with mouse anti-glucagon antibody (Sigma; 1:2000 dilution) for 2 h at room temperature and then treated with the secondary antibody: Alexa Fluor (Invitrogen) 568 conjugated goat anti-mouse antibody (1:100 dilution). To detect H_3R, samples were incubated overnight at 4 °C with rabbit anti-mouse H_3R antibody (4 μg/mL) [23] and then treated with the secondary antibody: Alexa Fluor 488 conjugated goat anti-rabbit antibody (Invitrogen). For double staining of glucagon and H_3R, samples were incubated with anti-glucagon antibody for 2 h at room temperature, washed thrice with PBS, followed by overnight incubation with anti-H_3R antibody at 4 °C. Specimens from H_3KO mice were used as negative controls for H_3R. Finally, all samples were encapsulated with mounting medium containing DAPI.

2.6. Glucagon secretion assay

αTC1.6 cells were seeded into a 24-well plate at a density of 2.0×10^5 cells per well and then preincubated in 500 μL of Krebs–Ringer bicarbonate buffer (KRB) containing 20 mM glucose for 30 min at 37 °C in 5% CO_2. Subsequently, αTC1.6 cells were incubated in 500 μL of KRB containing 20 or 2.8 mM glucose ± 1 μM histamine (Sigma), 1 and 100 μM of the selective H_3R agonist immepip (Sigma) [24,25], or the selective H_3R inverse agonist JNJ-5207852 (kindly gifted from Dr. Nicholas Carruthers, Johnson & Johnson Pharmaceutical Research and Development, USA) [26,27], or KRB containing 20 mM KCl ± 1 μM immepip, for 1 h at 37 °C in 5% CO_2. Immepip and JNJ-5207852 were dissolved in distilled water. We used a glucagon ELISA kit (Wako) to measure glucagon concentration in KRB.

2.7. Intracellular Ca^{2+} concentration measurement

αTC1.6 cells were seeded into a 96-well plate (Greiner, Frickenhausen, Germany) at a density of 2.0×10^4 cells per well and were then preincubated in KRB containing 20 mM glucose, 5 μM fluo-4/AM, 5 mM probenecid, and 1 × Power load (all from Invitrogen) for 30 min at 37 °C. Preincubated KRB was replaced with KRB containing 20 mM glucose ± 1 μM immepip and the 96-well plate was placed into a microplate reader FlexStation 3 (Molecular Devices, Sunnyvale, CA, USA) to record changes in fluorescence (excitation: 495 nm/emission: 518 nm). Cells were excited at 495 nm and the light emitted through a 518-nm filter was detected. After a 1-min measurement of baseline fluorescence, glucose concentration in KRB in each well was diluted to 2.8 mM by adding KRB ± 1 μM immepip from injectors, or KRB containing high KCl ± 1 μM immepip was added from injectors to adjust KCl concentration to 20 mM. Fluorescent intensity before fluo-4/AM loading was measured as background.

2.8. Measurement of serum glucagon concentration in rats

We used rats instead of mice to evaluate the effect of immepip on serum glucagon concentration because a glucagon ELISA kit required 100 μL of serum to measure glucagon correctly. 10-week-old Wister rats were fasted for 24 h prior to the experiment and were anesthetized with isoflurane. Immepip (30 mg/kg) dissolved in normal saline or just normal saline was administered intraperitoneally. Blood samples were collected from the caudal vein before injection as the baseline and at 15 min after injection. Samples were centrifuged for 30 min at 5000 rpm at 4 °C and then the supernatant plasma was collected. Plasma glucagon concentration was measured using a glucagon ELISA kit (Wako).

2.9. Data analysis and presentation

All experiments were performed in triplicate. Statistical analysis was performed using Student's *t*-test or one-way ANOVA. Data

are expressed as mean ± SE. *P*-values of <0.05 were considered significant.

3. Results

3.1. H_3R were expressed in αTC1.6 cells

First, we examined the expression of histamine receptors in αTC1.6 cells using RT-PCR and western blot. H_3R mRNA was predominantly expressed in αTC1.6 cells among the four histamine receptor subtypes (Fig. 1A). Histamine H_1 receptor mRNA was slightly detected, but histamine H_2 and H_4 receptor mRNAs were not detectable. H_3R protein expression in αTC1.6 cells was confirmed by western blotting (Fig. 1B). Immunohistochemical analysis of pancreatic islets showed that glucagon-positive (stained red) expressed H_3R protein (stained green), indicating that H_3R is expressed in glucagon-positive mouse pancreatic α-cells (Fig. 1C). H_1R protein expression was not detectable during western blot of αTC1.6 cells (data not shown).

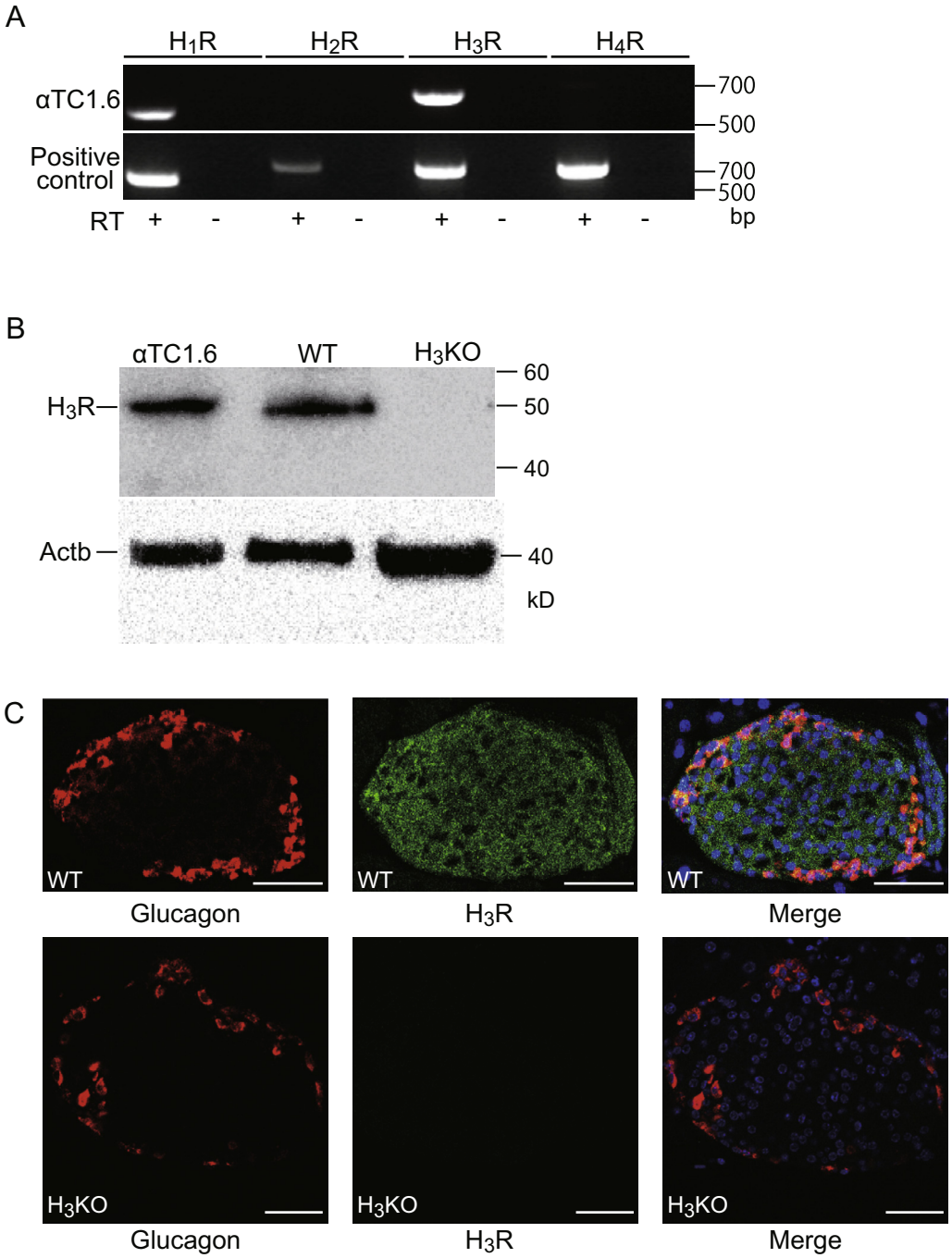

Fig. 1. Histamine H_3 receptor expression in αTC1.6 cells and pancreatic islets. (A) RT-PCR detection of histamine receptors in αTC1.6 cells. RT + and − indicate RT-PCR in the presence or absence of reverse transcriptase, respectively. H_1R: histamine H_1 receptor, H_2R: histamine H_2 receptor, H_3R: histamine H_3 receptor, H_4R: histamine H_4 receptor. (B) Western blot of H_3R in αTC1.6 cells. "WT" is a wild-type mouse hypothalamus lysate used as a positive control. H_3KO is a negative control, a hypothalamus lysate from an H_3R-knockout mouse. Beta-actin (Actb) was used as a loading control (lower panel). (C) Immunohistochemical analysis of H_3R expression in mouse pancreatic islets of WT mice (upper panels) and H_3KO mice (lower panels) as negative controls. Glucagon and H_3R were visualized with Alexa 568 (red) and Alexa 488 (green), respectively. DAPI is depicted in merged images (right panels). Scale bar = 50 μM.

3.2. Activation of H_3R decreased glucagon secretion

Glucagon secretion from pancreatic α-cells is vital to enhance serum glucose concentration in hypoglycemia [6]. We investigated effects of histamine on glucagon secretion from αTC1.6 cells. Glucagon secretion increased 1.41-fold over basal concentration in response to low glucose concentrations (Fig. 2A). This secretion associated with low glucose concentrations was remarkably inhibited by 1 μM histamine (0.67 ± 0.05 ng/well/h *versus* 0.96 ± 0.07 ng/well/h, $P < 0.05$), while basal secretion at 20 mM glucose with histamine was maintained at close to control concentrations (0.68 ± 0.06 ng/well/h *versus* 0.68 ± 0.06 ng/well/h, $P > 0.05$; Fig. 2A). These results indicate that histamine negatively regulates glucagon secretion from αTC1.6 cells in response to low glucose concentration.

Subsequently, we examined the role of H_3R expressed in αTC1.6 cells in glucagon secretion using pharmacological assays with the H_3R agonist immepip or the inverse agonist JNJ-5207852. Glucagon secretion associated with low glucose concentrations was remarkably inhibited by 1 μM immepip (0.73 ± 0.08 ng/well/h *versus* 1.01 ± 0.10 ng/well/h, $P < 0.05$; Fig. 2B). Conversely, 1 μM JNJ-5207852 increased glucagon secretion (1.83 ± 0.08 ng/well/h *versus* 1.36 ± 0.21 ng/well/h, $P < 0.05$; Fig. 2C). These results indicate that H_3R inhibits glucagon secretion in αTC1.6 cells.

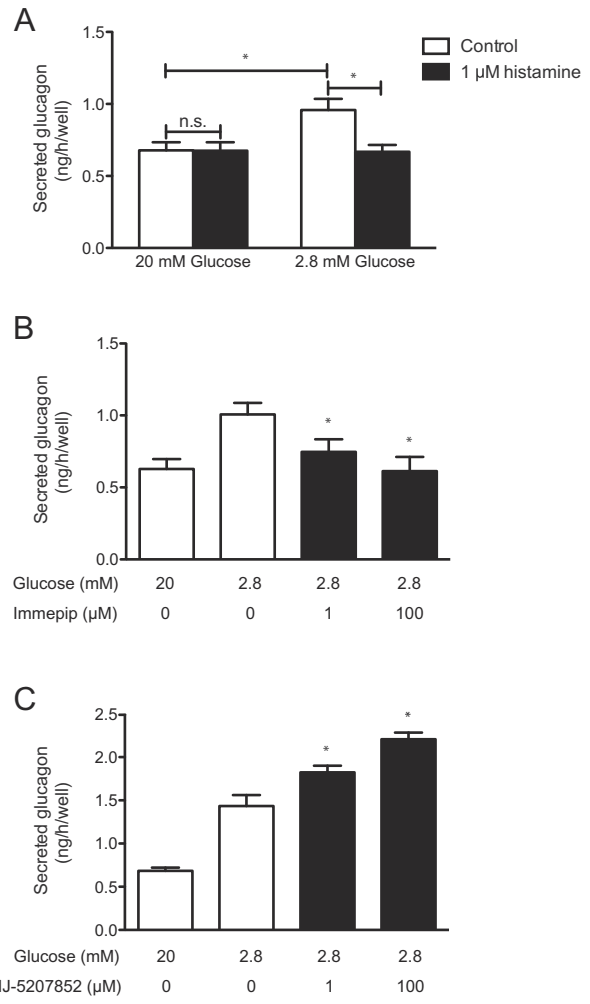

Fig. 2. Effects of histamine, immepip, or JNJ-5207852 on glucagon secretion from αTC1.6 cells. (A) Glucagon secretion from αTC1.6 cells at low (2.8 mM) and high (20 mM) glucose concentrations ± 1 μM histamine. $^*P < 0.05$, $n = 6$. (B) Glucagon secretion from αTC1.6 cells at low (2.8 mM) and high (20 mM) glucose concentrations ± 1 or 100 μM immepip. $^*P < 0.05$, $n = 6$. (C) Glucagon secretion from αTC1.6 cells at low (2.8 mM) and high (20 mM) glucose concentrations ± 1 or 100 μM JNJ-5207852. $^*P < 0.05$, $n = 6$.

3.3. Immepip inhibited the increase of intracellular Ca^{2+} concentration

H_3R activation modulates cellular functions through several intracellular signaling pathways downstream of H_3R [28,29]. Silver et al. reported that H_3R activation attenuated norepinephrine

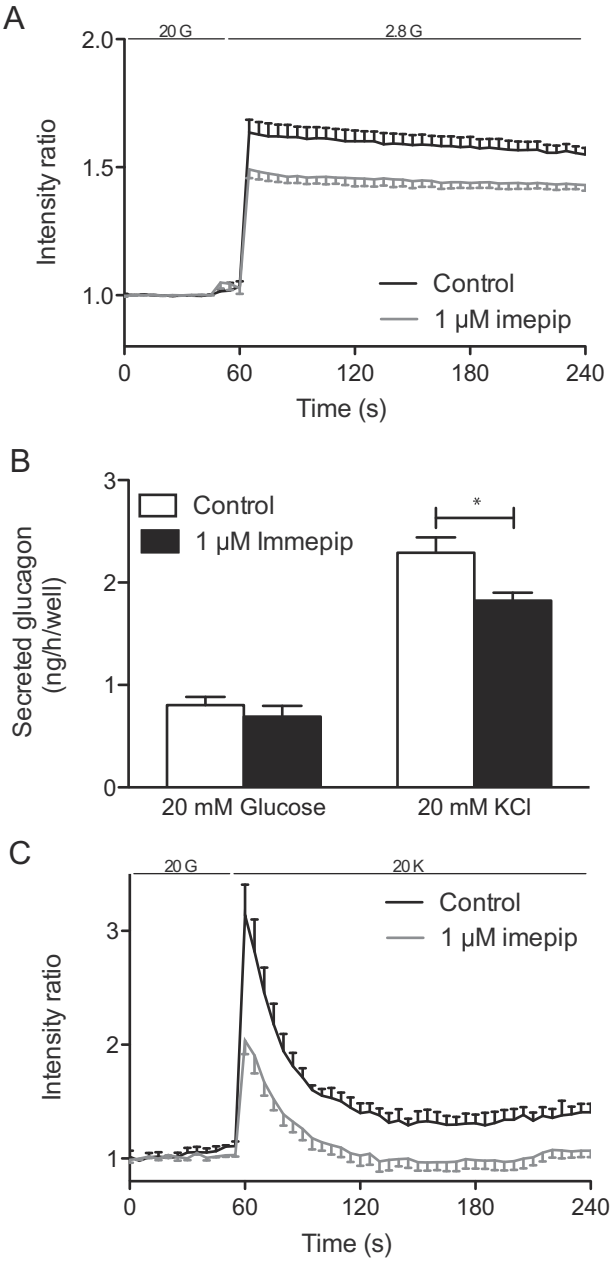

Fig. 3. Analysis of intracellular signaling in αTC1.6 cells. (A) Changes in intracellular Ca^{2+} concentration in response to low (2.8 mM) glucose concentration ± 1 μM immepip. The average of fluorescence intensity from fluo-4 before low glucose stimulation was arbitrarily set as 1. Intensity ratio on *y*-axis relates to the increase of intracellular Ca^{2+} concentration in direct proportion. 20 G: 20 mM glucose, 2.8 G: 2.8 mM glucose, $n = 4$. (B) Glucagon secretion from αTC1.6 cells at high (20 mM) glucose concentration and high (20 mM) KCl concentration ± 1 μM immepip. $^*P < 0.05$, $n = 6$. (C) Changes in intracellular Ca^{2+} concentration in response to high (20 mM) KCl concentration ± 1 μM immepip. The average of fluorescence intensity from fluo-4 before low glucose stimulation was arbitrarily set as 1. Intensity ratio on *y*-axis relates to the increase of intracellular Ca^{2+} concentration in direct proportion. 20 G: 20 mM glucose, 20 K: 20 mM KCl, $n = 4$.

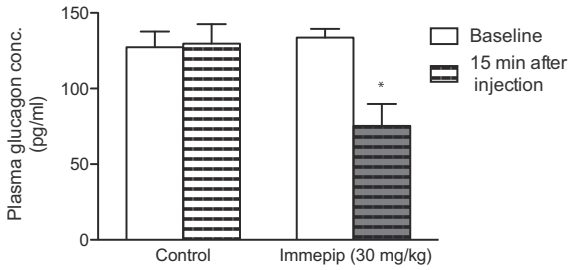

Fig. 4. Effects of immepip on rat plasma glucagon concentration. Serum glucagon concentration of rats before and 15 min after intraperitoneal injection of 30 mg/kg immepip. $^{*}P < 0.05$, $n = 4$.

exocytosis mediated by a decrease in intracellular Ca^{2+} concentration, seemingly because of an impaired entrance of Ca^{2+} through VDCCs [30]. Glucagon exocytosis from pancreatic α-cells also requires an increase in intracellular Ca^{2+} concentration [31,32]. Thus, we examined the effect of activated H_3R on intracellular Ca^{2+} concentration. One micromole of immepip impaired the increase in intracellular Ca^{2+} concentration in response to 2.8 mM glucose (transient changes in Ca^{2+}; 0.36 ± 0.03 *versus* 0.47 ± 0.04; Fig. 3A). This result indicates that H_3R is involved in the regulation of intracellular Ca^{2+} concentration in αTC1.6 cells.

To confirm whether intracellular Ca^{2+} concentration has a direct role in the inhibitory effect of H_3R on glucagon secretion, we stimulated αTC1.6 cells with 20 mM KCl solution, which directly depolarized plasma membranes, followed by Ca^{2+} influx through activated VDCCs [33]. We examined the effect of immepip on glucagon secretion stimulated by 20 mM KCl and found that glucagon secretion in response to 20 mM KCl was reduced by 1 μM immepip (1.82 ± 0.07 ng/well/h *versus* 2.29 ± 0.15 ng/well/h; Fig. 3B). This result suggests that intracellular signaling from H_3R affects the increase in intracellular Ca^{2+} concentration and/or downstream Ca^{2+} signaling, so we examined effects of immepip on the increase in intracellular Ca^{2+} concentration in response to 20 mM KCl. One micromole of immepip impaired the increase in intracellular Ca^{2+} concentration in response to 20 mM KCl (transient changes in Ca^{2+}; 0.13 ± 0.07 *versus* 0.55 ± 0.08; Fig. 3C). These results indicate that H_3R signaling exerted an inhibitory effect on glucagon secretion through the blockage of Ca^{2+} influx.

3.4. H_3R agonist reduced serum glucagon concentration in rats

Finally, we investigated the role of H_3R in glucagon secretion *in vivo*. We examined the effect of immepip on serum glucagon concentration in rats. Serum glucagon concentration after a 24-h fast was remarkably inhibited by 1 μM immepip (75.3 ± 14.5 pg/mL *versus* 130.0 ± 12.8 pg/mL, $P < 0.05$; Fig. 4), indicating that H_3R was involved in the regulation of serum glucagon concentration *in vivo*.

4. Discussion

We demonstrated for the first time that H_3R is expressed in αTC1.6 cells and mouse pancreatic α-cells. Using RT-PCR and western blot analysis, αTC1.6 cells were shown to express H_3R mRNA and protein (Fig. 1A, B). H_3R protein expression in pancreatic α-cells was confirmed by immunohistochemical analysis. Our data showed that activated H_3R inhibited glucagon secretion from αTC1.6 cells in response to low glucose concentrations (Fig. 2).

H_3R is coupled to G_i protein [34]. Previous reports revealed that several G_i protein-coupled receptors expressed in pancreatic α-cells inhibited glucagon secretion. For example, group III metabotropic glutamate receptors, coupled with inhibitory G_i protein, are expressed in rat pancreatic α-cells and inhibit glucagon secretion from rat islets under low glucose conditions [35]. Moreover, somatostatin from pancreatic δ-cells also inhibits glucagon secretion through somatostatin receptor 2 coupled to G_i proteins [36]. This evidence corroborates the function of H_3R in pancreatic α-cells.

To elucidate the regulatory mechanism of H_3R on glucagon secretion, we investigated effects of immepip on intracellular Ca^{2+} concentration, which is essential to glucagon secretion [13]. Immepip inhibited increases in intracellular Ca^{2+} concentrations in response to 20 mM KCl stimulation (Fig. 3B). KCl stimulation directly elicits plasma membrane depolarization, leading to the activation of VDCCs. It is possible that H_3R signaling might inhibit the activity of VDCCs in pancreatic α-cells because H_3R in neurons reduced Ca^{2+} influx through VDCCs [37]. However, the inhibitory effect of immepip on Ca^{2+} concentration was more moderate during low glucose conditions than during KCl stimulation (Fig. 3B, C), although immepip inhibited glucagon secretion during low glucose conditions more strongly than during KCl stimulation (Figs. 2B and 3A). This suggests that the intracellular Ca^{2+}-independent signaling pathway might be associated with the inhibitory effect of H_3R on glucagon secretion. Recently, De Marinis showed that the cAMP-PKA pathway increased glucagon secretion by the recruitment of glucagon granules toward the cell membrane [38]. Activated H_3R inhibits the cAMP-PKA pathway [24,39]; thus, any involvement of this pathway might be confirmed by using gene silencing technologies or a pharmacological approach. Further studies are necessary to elucidate the signaling induced by H_3R stimulation in pancreatic α-cells.

Immepip remarkably inhibited plasma glucagon concentration 15 min after intraperitoneal injection, indicating that H_3R has an important role in glucagon concentration *in vivo*. Our data suggests that immepip acts on pancreatic α-cells leading to direct inhibition of glucagon secretion. Additionally, immepip might indirectly modulate glucagon secretion through sympathetic nerves. Sympathetic nerve endings innervate pancreatic α-cells and modulate glucagon secretion [32,40]. Schlicker et al. reported that postganglionic sympathetic nerves expressed H_3R and that this H_3R regulates neurotransmitter release [41]. This evidence indicates that immepip might act on H_3R in sympathetic nerves and indirectly regulate glucagon secretion. However, further studies are needed to elucidate the role of H_3R in pancreatic α-cells or sympathetic nerves in the regulation of serum glucagon secretion.

As previously mentioned, abnormal glucagon secretion from pancreatic α-cells has been observed in diabetic patients [8]. H_3R agonists that decrease glucagon secretion could improve excessive glucagon secretion in hyperglycemic conditions; however, our data only indicates the inhibitory effect of H_3R on glucagon secretion in hypoglycemic conditions. Therefore, further studies are needed to clarify therapeutic effects of H_3R agonists in diabetic animal models, which have abnormal glucagon secretion during hyperglycemia [42].

We found a weak band of H_1R mRNA in αTC1.6 cells (Fig. 1A). Based on our previous study that clearly indicated the absence of H_1R mRNA in pancreatic islets [23], its expression might be undetectably low or absent in pancreatic islets and it may be specific to the cell-line. Additionally, we confirmed that glucagon secretion was not affected by H_1R-specific agonists (data not shown). Accordingly, it is suggested that H_1R is not involved in the regulation of glucagon secretion in pancreatic α-cells.

In this study, we found that H_3R in pancreatic α-cells had an inhibitory effect on glucagon secretion possibly by decreasing intracellular Ca^{2+} concentration. We also found that H_3R was involved in glucagon secretion *in vivo*. It will be interesting to investigate roles of H_3R in glucagon secretion in a diabetic animal model.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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