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Blockade of multiple monoamines receptors reduce insulin secretion from pancreatic β-cells

Mao Nagata[1], Tomoharu Yokooji[1,2], Tomoe Nakai[1], Yumika Miura[1], Takashi Tomita[1], Takanori Taogoshi[1], Yumi Sugimoto[3] & Hiroaki Matsuo[1*]

Clinical use of olanzapine frequently causes severe hyperglycemia as an adverse effect. In this study, we elucidated mechanisms by which olanzapine reduced insulin secretion using the hamster pancreatic β-cell line HIT-T15. Reverse transcriptional-PCR analysis revealed expression of dopamine (D_2, D_3 and D_4), serotonin (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, and 5-HT_6), and histamine (H_1 and H_2) receptors in HIT-T15 cells. Olanzapine decreased insulin secretion from HIT-T15 cells at clinically relevant concentrations (64–160 nM). A dopamine D_2 agonist, D_3 antagonist, and D_4 antagonist suppressed insulin secretion, whereas a D_2 antagonist and D_3 agonist increased it. A serotonin 5-HT_{2B} agonist slightly increased insulin secretion, while a 5-HT_{2C} antagonist slightly decreased it. Other agonists and antagonists for serotonin receptors did not affect insulin secretion. A histamine H_1 agonist increased insulin secretion, whereas an H_1 antagonist and H_2 agonist suppressed it. Our results suggest that dopamine (D_2, D_3 and D_4), serotonin (5-HT_{2B} and 5-HT_{2C}), and histamine (H_1 and H_2) receptors, which are expressed on pancreatic β-cells, directly modulate insulin secretion from pancreatic β-cells. Thus, olanzapine may induce hyperglycemia in clinical settings by suppressing insulin secretion from pancreatic β-cells through inhibition of dopamine D_3, serotonin 5-HT_{2B} and 5-HT_{2C}, and histamine H_1 receptors.

Schizophrenia is a mental disorder that often appears in adolescence or early adulthood. According to an epidemiological study, the lifetime prevalence of schizophrenia is estimated at 0.7% worldwide[1]. Patients with schizophrenia typically present with various psychiatric symptoms including positive symptoms, negative symptoms, and cognitive symptoms, which result from dysregulated dopaminergic and non-dopaminergic modulation of the mesocorticolimbic system[2]. Positive symptoms such as delusions and hallucinations often develop during the acute phase and improve over time. In contrast, negative symptoms such as abulia, autism, dullness, and avolition, as well as cognitive deficits, generally become worse and chronic.

Pharmacotherapy with antipsychotic agents is a basic treatment for schizophrenia. Antipsychotic agents can be classified into two types: typical and atypical. Atypical antipsychotic agents such as risperidone, olanzapine, and aripiprazole are currently used as first-line agents to treat schizophrenia because they exhibit lower incidence of extrapyramidal symptoms and hyperprolactinemia compared with typical antipsychotic agents. Olanzapine, an atypical antipsychotic agent, exhibits effectiveness on positive symptoms, negative symptoms, and cognitive deficits by inhibiting multiple receptors such as dopamine, serotonin, histamine H_1, α-adrenergic, and muscarinic acetylcholine receptors. According to a prescription survey conducted in 2013, olanzapine was the most frequently used among atypical antipsychotic drugs in Japan and Canada[3]. However, use of olanzapine has reportedly caused serious diabetic ketoacidosis and associated deaths as a result of hyperglycemia. In 2002, the Japan Ministry of Health, Labour, and Welfare issued Emergency Safety Information about the risk of serious hyperglycemia associated with olanzapine administration. However, mechanisms underlying olanzapine-induced hyperglycemia remain incompletely understood. Previous reports have suggested that repeated use of olanzapine caused increasing appetite, weight gain, and obesity, resulting in the development of type 2 diabetes[4,5]. However, olanzapine-induced hyperglycemia has been also observed in patients independent of weight gain[6]. Several reports have shown that olanzapine induced hyperglycemia by induction of apoptosis in insulin-secreting pancreatic β-cells[7], insulin resistance[8], increased glucose production in the liver[9], and/or increased epinephrine

[1]Department of Pharmaceutical Services, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan. [2]Department of Frontier Science for Pharmacotherapy, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan. [3]Department of Pharmacology, Faculty of Pharmaceutical Sciences, Himeji Dokkyo University, Himeji, Japan. *email: hmatsuo@hiroshima-u.ac.jp

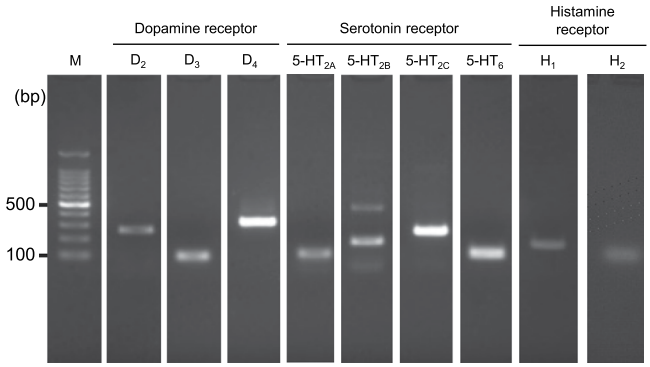

Figure 1. mRNA expression of dopamine (D_2, D_3, and D_4), serotonin (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, and 5-HT_6), and histamine (H_1 and H_2) receptors in HIT-T15 cells. Total RNA extracted from HIT-T15 cells was reverse-transcribed, and first-strand cDNA was synthesized. Target genes were amplified with a set of specific primers (shown in Table 2). For expression analysis of dopamine D_3 and D_4, all serotonin, and histamine H_2 receptors, two-step PCR was performed using nested primers (shown in Table 2). PCR products were separated by electrophoresis using a 2% agarose gel and stained with ethidium bromide. M, 100-bp ladder size marker.

secretion[10]. We hypothesized that olanzapine can increase hyperglycemia by suppressing insulin secretion from pancreatic β-cells via blockade of multiple monoamine receptors. In this study, we aimed to elucidate the involvement of dopamine, serotonin, and histamine receptors in insulin secretion from pancreatic β-cells.

Results

Expression of dopamine, serotonin, and histamine receptor mRNA in HIT-T15 cells. Expression of dopamine (D_2, D_3, and D_4), serotonin (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, and 5-HT_6), and histamine (H_1 and H_2) receptors were confirmed by reverse transcriptional (RT)-PCR analysis using specific primers and cDNA from hamster pancreatic cells (HIT-T15). A band corresponding to the expected size of each receptor was observed (Fig. 1).

Olanzapine decreased insulin secretion from HIT-T15 cells. Olanzapine decreased insulin secretion from HIT-T15 cells to ~80% of controls at concentrations of 1–1000 nM (Fig. 2). Notably, ~64–160 nM olanzapine has been observed in blood from patients orally administered olanzapine in clinical settings[11,12].

Effects of dopamine receptor agonists and antagonists on insulin secretion from HIT-T15 cells. The involvement of dopamine receptors in insulin secretion was evaluated using HIT-T15 cells (Fig. 3). Dopamine decreased insulin secretion in a concentration-dependent manner (Fig. 3A), consistent with a previous report[13]. Bromocriptine, a dopamine D_2 receptor agonist, decreased insulin secretion in a concentration-dependent manner and this decrease remained constant (~40% of control) at dosages over 100 nM (Fig. 3B). In contrast, haloperidol, a dopamine D_2 receptor antagonist, increased insulin secretion (Fig. 3C). For the dopamine D_3 receptor, the agonist 7-hydorxy PIPAT significantly enhanced insulin secretion (Fig. 3D), whereas the antagonist NGB2904 suppressed it (Fig. 3E). Furthermore, both the dopamine D_4 receptor agonist ABT724 and antagonist sonepirazole significantly suppressed insulin secretion to ~80% of control (Fig. 3F,G). These findings suggest that stimulation of dopamine D_2 and D_4 receptors decreased insulin secretion from pancreatic β-cells, whereas stimulation of D_3 receptors increased it.

Effects of serotonin receptor agonists and antagonists on insulin secretion from HIT-T15 cells. The involvement of serotonin receptors in insulin secretion was evaluated using HIT-T15 cells (Fig. 4). Insulin secretion was decreased to ~80% of control by serotonin in a concentration-dependent manner (Fig. 4A). Neither the serotonin 5-HT_{2A} receptor agonist TCB2 nor antagonist MDL11939 had an effect on insulin secretion (Fig. 4B,C). The serotonin 5-HT_{2B} receptor agonist BW723C86 slightly increased insulin secretion (Fig. 4D), while the antagonist SB204741 exerted no significant effects on secretion (Fig. 4E). Stimulation of serotonin 5-HT_{2C} receptors by agonist Ro60–0175 did not affect insulin secretion, although blockade of serotonin 5-HT_{2C} receptors by antagonist SB242084 slightly decreased secretion (Fig. 4F). Similar to the serotonin 5-HT_{2A} receptor, neither the serotonin 5-HT_6 receptor agonist WAY181187 or antagonist SB399885 had an effect on insulin secretion (Fig. 4G,H). These results suggest that stimulation of serotonin 5-HT_{2B} and 5-HT_{2C} receptors can increase insulin secretion from pancreatic β-cells, although their contributions are much lower than those of dopamine receptors.

Effects of histamine receptor agonists or antagonists on insulin secretion from HIT-T15 cells. We evaluated the involvement of histamine receptors in insulin secretion from HIT-T15 cells (Fig. 5). Histamine decreased insulin secretion at 1–100 nM (Fig. 5A). The histamine H_1 receptor agonist 2-pyridylethylamine (2-PEA) increased insulin secretion in a concentration-dependent manner, whereas trans-triprolidine, a histamine H_1 receptor antagonist, decreased secretion to ~40% of control (Fig. 5B,C). In contrast, the histamine H_2 receptor agonist amthamine decreased insulin secretion to ~75% of control in a concentration-dependent manner. In addition, the histamine H_2 receptor antagonist tiotidine slightly decreased

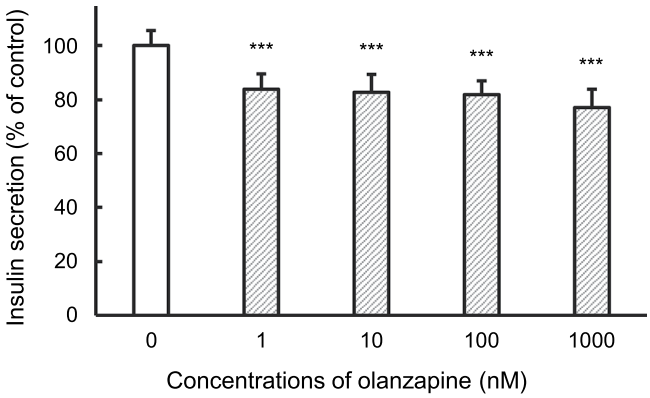

Figure 2. Effect of olanzapine on insulin secretion from HIT-T15 cells. HIT-T15 cells were incubated with medium containing 1% dimethylsulfoxide (control) or olanzapine for 1 h at 37 °C. Concentrations of insulin released into the medium were determined using a rat Insulin ELISA kit. Amounts of insulin secretion were normalized to the total protein content of each well. Each value represents mean ± SD of eight trials. ***$P < 0.001$ with respect to control.

Chemical	Cell Viability (% of control)
olanzapine	101.2 ± 9.1
bromocriptine	108.2 ± 13.1
haloperidol	105.1 ± 4.7
7-hydroxy PIPAT	94.9 ± 4.1
NGB2904	106.4 ± 3.3
ABT724	104.7 ± 3.3
sonepiprazole	99.7 ± 8.1
TCB2	101.0 ± 9.1
MDL11939	121.4 ± 23.9
BW723C86	94.7 ± 7.9
SB204741	103.2 ± 10.9
Ro60–0175	103.2 ± 16.0
SB242084	99.8 ± 5.0
WAY181187	95.6 ± 12.5
SB39985	113.4 ± 8.6
2-pyridylethylamine	105.2 ± 7.2
trans-triprolidine	102.7 ± 3.4
amthamine	89.1 ± 21.2
tiotidine	93.5 ± 17.2

Table 1. Effects of chemicals on HIT-T15 cell viability.

insulin secretion (Fig. 5D,E). Thus, stimulation of histamine H_1 receptors increased insulin secretion from pancreatic β-cells, whereas stimulation of H_2 receptors decreased secretion.

Olanzapine nor receptor agonists or antagonists affected the viability of HIT-T15 cells. To evaluate whether cytotoxic effects of olanzapine and agonists or antagonists for each receptor occurred, an XTT assay was performed. As shown in Table 1, 1-h exposure of each agent tested did not affect the viability of HIT-T15 cells, indicating that alterations in insulin secretion from HIT-T15 cells induced by these compounds were not the result of cytotoxicity.

Discussion

Several reports have shown that administration of olanzapine could induce hyperglycemia[14,15]. As olanzapine exerts an antipsychotic effect by inhibiting multiple receptors for dopamine, serotonin, histamine, adrenaline, and acetylcholine, we hypothesized that olanzapine could increase hyperglycemia by suppressing insulin secretion from pancreatic β-cells through blockade of multiple receptors. In this study, we investigated the involvement of dopamine, serotonin, and histamine receptors in insulin secretion using HIT-T15 cells.

The effects of olanzapine on plasma insulin levels are incompletely understood because doses of olanzapine used in previous animal experiments were higher than used in clinical settings. Nagata *et al.*[10] reported that serum concentrations of insulin increased following a single intravenous infusion of olanzapine at a dose of 2.5–10 mg/

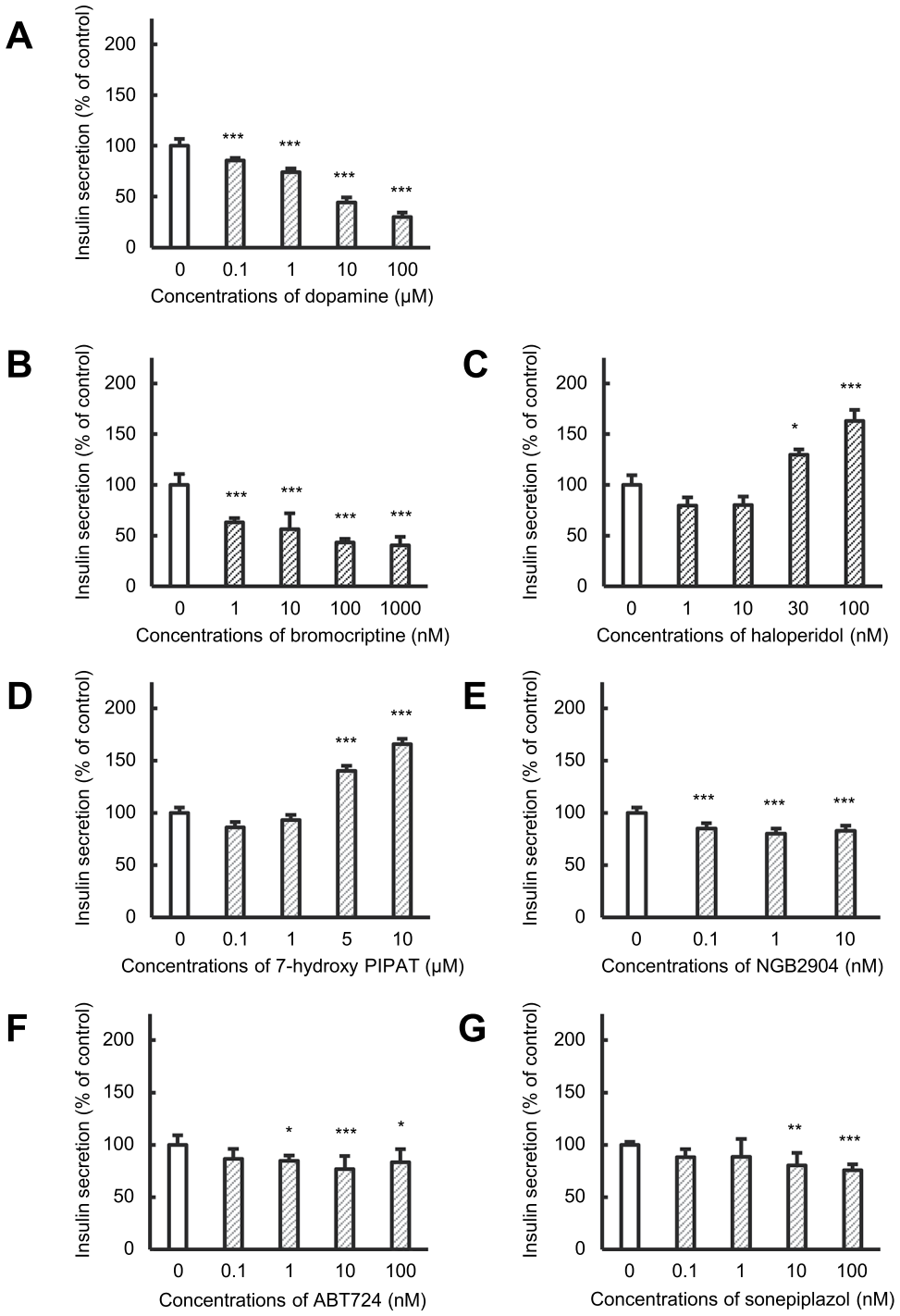

Figure 3. Effects of dopamine receptor agonists and antagonists on insulin secretion from HIT-T15 cells. HIT-T15 cells were incubated with medium containing 1% dimethylsulfoxide (control), dopamine (**A**), bromocriptine (**B**, D_2 agonist), haloperidol (**C**, D_2 antagonist), 7-hydroxy PIPAT (**D**, D_3 agonist), NGB2904 (**E**, D_3 antagonist), ABT724 (**F**, D_4 agonist), or sonepiprazole (**G**, D_4 antagonist) for 1 h at 37 °C. Concentrations of insulin released into the medium were determined using a rat Insulin ELISA kit. Amounts of insulin secretion were normalized to the total protein content of each well. Each value represents mean ± SD of four to eight trials. ***$P < 0.001$ with respect to control.

kg. In their study, serum concentrations of olanzapine were 394–2763 ng/mL (1.26–8.84 μM) at 15 min after olanzapine administration. Simpson *et al.*[13] reported that olanzapine increased insulin secretion from human islets at concentrations of 1–5 μM. In clinical settings, olanzapine is administered orally at a dose of 5–20 mg daily, yielding reportedly therapeutic serum concentrations of 20–50 ng/mL (64–160 nM)[12]. In this study, olanzapine decreased insulin secretion from HIT-T15 cells by ~20% compared with controls at concentrations of 1–1000 nM (Fig. 2). Our preliminary study showed that olanzapine increased insulin secretion from HIT-T15

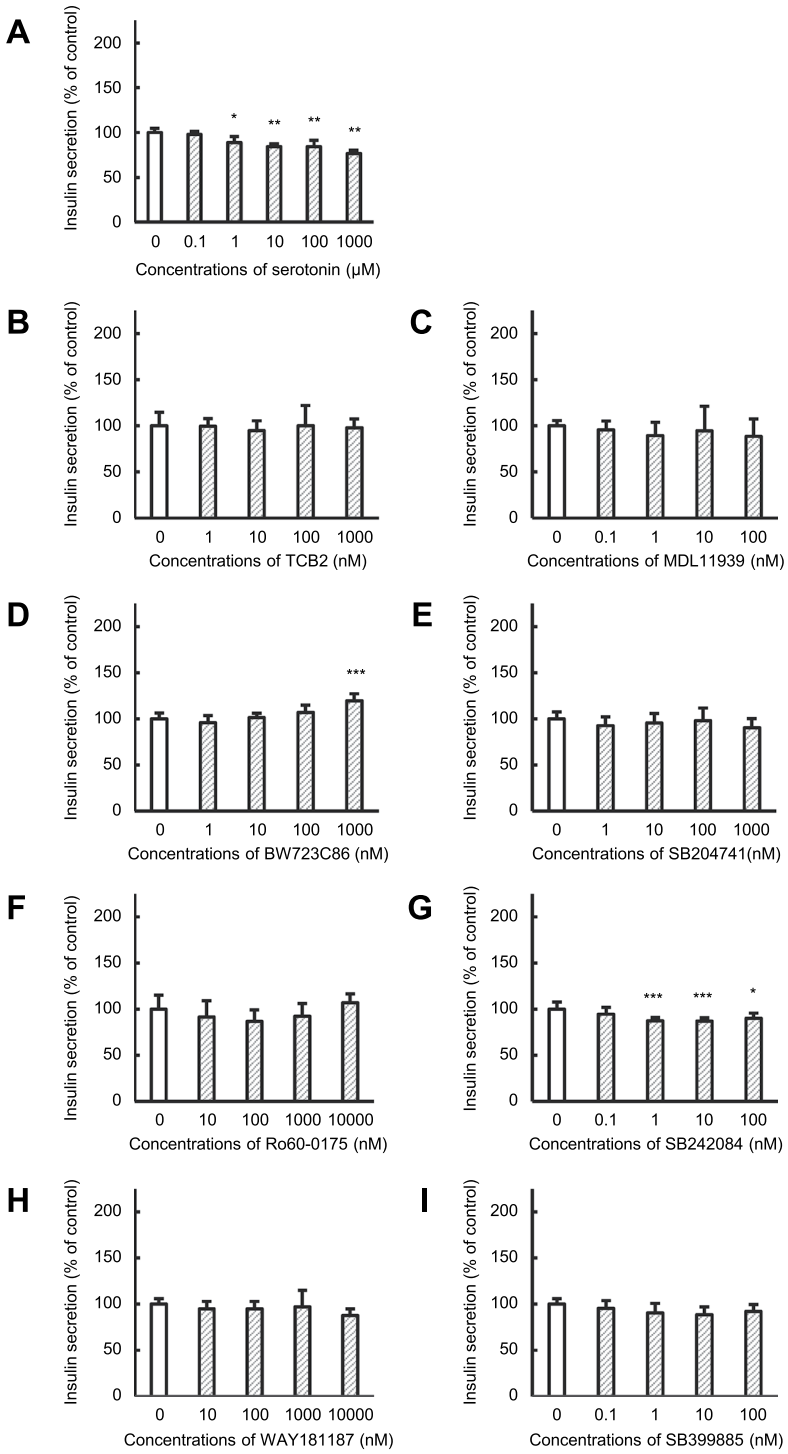

Figure 4. Effects of serotonin receptor agonists and antagonists on insulin secretion from HIT-T15 cells. HIT-T15 cells were incubated with medium containing 1% dimethylsulfoxide (control), serotonin (**A**), TCB2 (**B**, 5-HT_{2A} agonist), MDL11939 (**C**, 5-HT_{2A} antagonist), BW723C86 (**D**, 5-HT_{2B} agonist), SB204741 (E, 5-HT_{2B} antagonist), Ro60–0175 (**F**, 5-HT_{2C} agonist), SB242084 (**G**, 5-HT_{2C} antagonist), WAY181187 (**H**, 5-HT_6 agonist), or SB399885 (**I**, 5-HT_6 antagonist) for 1 h at 37 °C. Concentrations of insulin released into the medium were determined using a rat Insulin ELISA kit. Amounts of insulin secretion were normalized to the total protein content of each well. Each value represents mean ± SD of eight trials. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ with respect to control.

cells at concentrations of 10–30 μM (data not shown). These findings suggest that olanzapine directly suppresses insulin secretion from pancreatic β-cells at clinical concentrations. Blood glucose levels were reportedly altered by 20% when insulin secretion was altered by 25% after feeding[16], suggesting that olanzapine can induce hyperglycemia by suppressing insulin secretion from pancreatic β-cells at clinical concentrations.

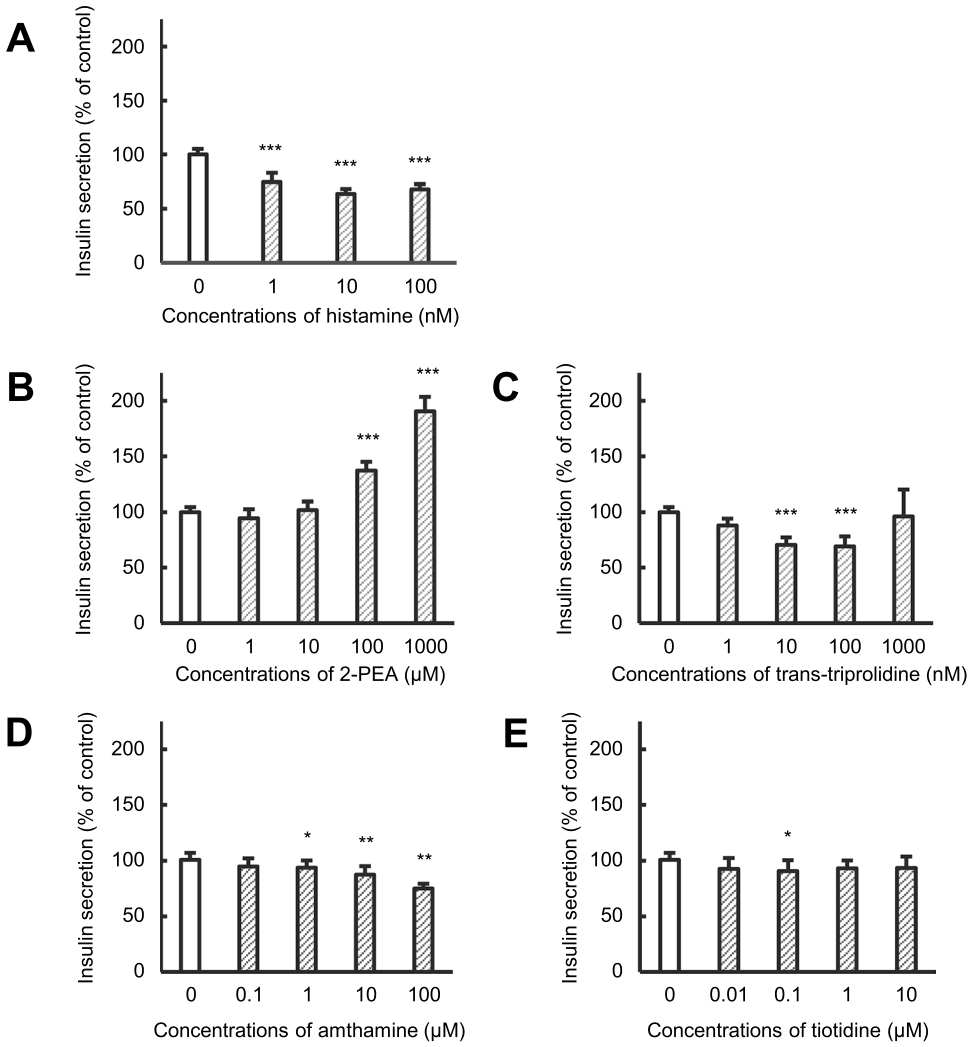

Figure 5. Effects of histamine receptor agonists and antagonists on insulin secretion from HIT-T15 cells. HIT-T15 cells were incubated with medium containing 1% dimethylsulfoxide (control), histamine (**A**), 2-pyridylethylamine (2-PEA) (**B**, H_1 agonist), trans-triprolidine (**C**, H_1 antagonist), amthamine (**D**, H_2 agonist), or tiotidine (**E**, H_2 antagonist) for 1 h at 37 °C. Concentrations of insulin released into the medium were determined using a rat Insulin ELISA kit. Amounts of insulin secretion were normalized to the total protein content of each well. Each value represents mean ± SD of eight trials. ***$P < 0.001$ with respect to control.

We demonstrated that dopamine D_2, D_3, and D_4; serotonin 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, and 5-HT_6; and histamine H_1 and H_2 receptors are expressed by HIT-T15 cells (Fig. 1). A few previous studies reported expression of these receptors in human pancreas[17–19]. Rubí *et al.*[17] reported the detection of dopamine D_2 and D_4 receptor mRNAs in human islets. Bonhaus *et al.*[18] observed mRNAs for serotonin 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors in human pancreas. Although no previous studies reported expression of histamine H_1 or H_2 receptors in human pancreas, Szukiewicz *et al.*[19] reported protein expression of these receptors in pancreatic β-like cells differentiated from human amniotic epithelial cells by nicotinamide treatment. Thus, the involvement of these receptors in insulin secretion may be observed even in human pancreas.

To evaluate the involvement of dopamine, serotonin, and histamine receptor subtypes in insulin secretion, we examined the effects of agonists and antagonists specific for each receptor subtype on insulin secretion from HIT-T15 cells. With regard to dopamine receptors, stimulation of the dopamine D_2 receptor suppressed insulin secretion, whereas its blockade enhanced it (Fig. 3B,C). This result is consistent with a previous report that blockade of dopamine D_2 receptor enhanced insulin secretion from human islets[13]. In contrast, stimulation of the dopamine D_3 receptor enhanced insulin secretion, whereas its blockade enhanced secretion (Fig. 3D,E). Thus, olanzapine can suppress insulin secretion via blockade of the dopamine D_3 receptor. Insulin secretion was increased by either stimulation or blockade of the dopamine D_4 receptor (Fig. 3F,G) using specific dopamine D_4 agonist ABT724 (EC_{50} value for rat dopamine D_4 receptor is 12.4 nM and that for dopamine D_2 is >10 μM) or antagonist sonepiprazole (K_i value for human dopamine D_4 receptor is 10 nM and those for other monoamine receptors are >2 μM), respectively, which seems contradictory[20,21]. However, these findings can be explained by differences in expression levels between dopamine D_2 and D_3 receptors. Dopamine can stimulate both dopamine

D_2 and D_3 receptors when the dopamine D_4 receptor is blocked. Expression levels of dopamine D_2 receptor were higher than those of dopamine D_3 receptor, as expression of mRNA for dopamine D_3 could be detected by two-step PCR with nested primers (Fig. 1). We considered decreased insulin secretion via blockade of the dopamine D_4 receptor to arise from stimulation of the dopamine D_2 receptor.

For serotonin receptors, stimulation of the 5-HT_{2B} receptor slightly increased insulin secretion from HIT-T15 cells, whereas blockade of the 5-HT_{2C} receptor decreased secretion (Fig. 4C,F). In contrast, a 5-HT_{2B} antagonist, 5-HT_{2C} agonist, and both an agonist and antagonist of 5-HT_{2A} and 5-HT_6 did not affect insulin secretion. Bennet *et al.*[22] demonstrated that stimulation of 5-HT_{2B} receptor increased the glucose-stimulated insulin secretion from mouse and human pancreatic β-cells by triggering downstream changes in cellular Ca^{2+} flux that enhance mitochondrial metabolism. These findings suggest that serotonin 5-HT_{2B} and 5-HT_{2C} receptors can modulate insulin secretion from β-cells. Thus, inhibition of serotonin 5-HT_{2B} and 5-HT_{2C} receptors may be involved in olanzapine-reduced insulin secretion, although their contributions may be less than those of dopamine receptors. Interestingly, there is one previous reports showing that 5-HT_3 receptor-mediated insulin secretion was further enhanced in pregnant mice compared to that in normal mice[23]. Thus, it is possible that the contributions of serotonin receptors subtypes to insulin secretion are altered under diseased states.

We also evaluated the involvement of histamine receptors in insulin secretion (Fig. 5). Histamine decreased insulin secretion at 1–100 nM (Fig. 5A). Stimulation of the histamine H_1 receptor increased insulin secretion, whereas stimulation of the histamine H_2 receptor decreased it (Fig. 5B,D). Thus, insulin secretion from pancreatic β-cells can be modulated by both histamine receptor subtypes, and olanzapine can suppress insulin secretion via blockade of the histamine H_1 receptor.

The roles of endogenous monoamines in the insulin secretion have not been understood completely. Ustione and Piston[24] reported that dopamine was secreted from pancreatic β-cells simultaneously with insulin and caused negative feedback inhibition on insulin secretion. In agreement with their report, we also showed that physiological levels of dopamine suppressed the insulin secretion from pancreatic β-cells (Fig. 3). In contrast, there have been no reports regarding the roles of endogenous serotonin and histamine on insulin secretion. In this study, we used dopamine, serotonin and histamine at concentrations of 0.1–100 μM, 0.1–100 μM and 1–100 nM, respectively. These concentrations of dopamine and serotonin are higher than those in physiological concentrations which in human plasma are reportedly ~6.5 nM[25] and ~0.6 pM[26], respectively. Thus, further studies are necessary to understand the role of endogenous monoamines in the insulin secretion at physiological conditions.

In this study, we did not confirm the expression and involvement of muscarinic acetylcholine receptors on insulin secretion from HIT-T15 cells. Iismaa *et al.*[27] reported that muscarinic acetylcholine receptors were expressed on rat pancreatic β-cells. Furthermore, Henquin *et al.*[28] reported that insulin secretion was enhanced via stimulation of muscarinic receptors. Because olanzapine inhibits the muscarinic acetylcholine receptors, it is speculated that olanzapine can suppress the insulin secretion from pancreatic β-cells via blockade of muscarinic acetylcholine receptors as well as monoamine receptors. Further studies are necessary to clarify the involvement of muscarinic acetylcholine receptors in insulin secretion from pancreatic β-cells.

In conclusion, we demonstrated that olanzapine suppressed insulin secretion from pancreatic β-cells via blockade of dopamine D_3, serotonin 5-HT_{2B} and 5-HT_{2C}, and histamine H_1 receptors at clinical concentrations *in vitro*. Although further studies are necessary using human pancreatic β-cells for *in vitro* and *in vivo* animal studies, these findings shed new light on the mechanisms underlying olanzapine-induced hyperglycemia.

Materials and Methods

Chemicals. Olanzapine and haloperidol were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Dopamine hydrochloride and bromocriptine were purchased from Sigma-Aldrich (St Louis, MO, USA). 7-Hydroxy PIPAT, ABT724, TCB2, BW723C86, Ro60–0175, WAY181187, 2-PEA, NGB2904, sonepiprazole, MDL11939, SB204741, SB399885, trans-triprolidine, amthamine, and tiotidine were from Tocris Bioscience (Bristol, England, UK). SB242084 was obtained from Toronto Research Chemicals (Ontario, Canada). All other chemicals used were of the highest purity available.

Cell culture. HIT-T15 cells were obtained from Sumitomo Dainippon Pharma (Osaka, Japan). Cells were cultured in Ham's F12K medium (Sigma-Aldrich) containing 10% fetal bovine serum, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 10 mM glucose which corresponds to the physiological blood concentrations in human in an atmosphere of 5% CO_2/95% air at 37 °C. Cells were subcultured once a week using 0.25% EDTA and 0.038% trypsin. Fresh medium was replaced every 2 days. Cells were used between passages 80 and 100.

RT-PCR analysis. Total RNA was extracted from HIT-T15 cells using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Next, total RNA was used for reverse transcription to synthesize cDNA using a ReverTra Ace qPCR RT kit (Qiagen). PCR was performed with an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) using KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan). Conditions for PCR were as follows: initial denaturation at 94 °C for 2 min; denaturation at 94 °C for 30 sec; annealing at optimal temperatures for dopamine, serotonin, and histamine receptors for 30 sec; and extension at 68 °C for 1 min (35 cycles). Primers, annealing temperatures, and product sizes for each receptor are summarized in Table 2. To examine expression of mRNA for dopamine D_3 and D_4 receptors, and all serotonin receptors, we performed two-step PCR with nested primers due to their lower expression in HIT-T15 cells. Nested primers for each receptor are summarized in Table 2. Conditions for the second round of PCR were the same as those for the first round. PCR products were electrophoresed with a 2% agarose gel and visualized under ultraviolet light with ethidium bromide.

Gene	Primer sequence	Annealing temperature (°C)	Product size (bp)
dopamine D_2	forward: 5′-TCGCCATTTGTCTGGGTCCTG-3′	65	261
	reverse: 5′-TGCCCTTTGAGGGGGGTCTTC-3′		
dopamine D_3 (1^{st} PCR)	forward: 5′-GTCTGGAATTTCAGCCGCATTTGCTGTGA -3′	62	119
	reverse: 5′-ATGACCACTGCTGTGTACCTGTCTATGCTG-3′		
(2^{nd} PCR)	forward: 5′-CAGCCGCATTTGCTGTGATG-3′	62	94
	reverse: 5′-GTACCTGTCTATGCTGATGGCA-3′		
dopamine D_4 (1^{st} PCR)	forward: 5′-GTCCGCTCATGCTACTGCT-3′	60	344
	reverse: 5′-GACTCTCATTGCCTTGCGCTC-3′		
(2^{nd} PCR)	forward: 5′-GCTACTGCTTTACTGGGCCAC-3′	60	329
	reverse: 5′-TCATTGCCTTGCGCTCCCTT-3′		
serotonin 5-HT_{2A} (1^{st} PCR)	forward: 5′-CTGGTCATCATGGCAGTGTCCCTAGAGAA-3′	67	291
	reverse: 5′-GGTTCTGGAGTTGAAGCGGCTATGGTGGA-3′		
(2^{nd} PCR)	forward: 5′-TGATGTCACTTGCCATAGCTG-3′	55	105
	reverse: 5′-AGAGCTTGCTGGGCAAAG-3′		
serotonin 5-HT_{2B} (1^{st} PCR)	forward: 5′-ATGCCGATTGCCCTCTTGAC-3′	67	185
	reverse: 5′-CGGGAGTTGCACTGATTGG-3′		
(2^{nd} PCR)	forward: 5′-GCCGATTGCCCTCTTGACA-3′	62	182
	reverse: 5′-GGGAGTTGCACTGATTGGC-3′		
serotonin 5-HT_{2C} (1^{st} PCR)	forward: 5′-GGGTCCTTCGTGGCATTCTTCATCCCG-3′	65	273
	reverse: 5′-CTTTTCGTTGTTGATAGCTTGCATGGTGCC-3′		
(2^{nd} PCR)	forward: 5′-GTGGCATTCTTCATCCCGTTG-3′	62	254
	reverse: 5′-TTGATAGCTTGCATGGTGCT-3′		
serotonin 5-HT_6 (1^{st} PCR)	forward: 5′-ATGCTGAACGCGCTGTATGG-3′	60	140
	reverse: 5′-GAGAGGATGAGCAGGTAGCG-3′		
(2^{nd} PCR)	forward: 5′-GTATGGGCGCTGGGTGCTA-3′	60	112
	reverse: 5′-GTAGCGGTCCAGGCTGATG-3′		
histamine H_1	forward: 5′-ACTTGAACCGAGAGCGGAAG-3′	60	178
	reverse: 5′-GGGTTCAGCGTGGAGTTGAT-3′		
histamine H_2 (1^{st} PCR)	forward: 5′-CCAGCTCCTGTGACTCCAGA-3′	60	353
	reverse: 5′-GGGTTTGGGAAGGTCTGATG-3′		
(2^{nd} PCR)	forward: 5′-GATCCCTTGCACAAACCCAAC-3′	60	97
	reverse: 5′-TCCTGGTCTGTAGTGTGCGT-3′		

Table 2. Primer sequences, annealing temperatures, and product sizes.

Receptor	Agonist	Antagonist
dopamine D_2	bromocriptine	haloperidol
dopamine D_3	7-hydroxy PIPAT	NGB2904
dopamine D_4	ABT724	sonepiprazole
serotonin 5-HT_{2A}	TCB2	MDL11939
serotonin 5-HT_{2B}	BW723C86	SB204741
serotonin 5-HT_{2C}	Ro60–0175	SB242084
serotonin 5-HT_6	WAY181187	SB399885
histamine H_1	2-pyridylethylamine	trans-triprolidine
histamine H_2	amthamine	tiotidine

Table 3. Agonists and antagonists specific for dopamine, serotonin, or histamine receptors used in this study.

Insulin secretion assay. Insulin secretion assays were performed according to previous reports[29,30]. Briefly, HIT-T15 cells were seeded at a density of 1.0×10^5 cells/well in 24-well plates and cultured for 72 h after seeding. Next, cells were pre-incubated with fresh medium containing 1% dimethylsulfoxide (DMSO) for 30 min at 37 °C. After pre-incubation, cells were incubated with fresh medium for 1 h at 37 °C. To examine the effects of olanzapine or agonists/antagonists for each receptor on insulin secretion, each compound was added to the medium at various concentrations during incubation. Compounds tested are shown in Table 3. After incubation, the concentration of insulin released into the medium was determined using a rat Insulin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan) according to our previously reported method[31,32]. Next, residual cells were

washed with phosphate-buffered saline (pH 7.4), and lysed with 0.3 M NaOH. Concentrations of total protein were determined by Lowry method with bovine serum albumin as the standard. Amounts of insulin secretion were normalized to the total protein content of each well.

XTT assay. HIT-T15 cells were seeded at a density of 1.5×10^4 cells/well in 96-well plates and cultured for 24 h. Next, cells were replaced with serum-free Ham's F12K medium containing optimal concentrations of olanzapine, an agonist or antagonist of each receptor, or 1% DMSO (control). Cells were incubated for 1 h at 37 °C. After washing, 200 µL of Hank's Balanced Salt Solution containing 225 µM XTT and 48 µM 1-methoxy-PMS was added to each well. After incubation for 4 h at 37 °C, absorbance was measured at 450 nm against 630 nm as a reference using a Multiskan GO (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analyses. Data are displayed as mean ± standard deviation of the mean (SD). Differences in mean values between groups were assessed using Kruskal-Wallis or ANOVA tests, followed by post hoc Tukey test, Dunnett's test, Bonferroni test, or Student's t-test. $P < 0.05$ was considered statistically significant.

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Author contributions

M.N., T.Y., T.T. and H.M. conceived and designed the experiments. M.N., T.N. and Y.M. performed the experiments and analyzed the data. M.N., T.Y., T.T., T.T., Y.S. and H.M. wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to H.M.

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