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TAK-875, an Orally Available GPR40/FFA$_1$ Agonist Enhances Glucose-Dependent Insulin Secretion and Improves Both Postprandial and Fasting Hyperglycemia in Type 2 Diabetic Rats

Yoshiyuki Tsujihata, Ryo Ito, Masami Suzuki, Ayako Harada, Nobuyuki Negoro, Tsuneo Yasuma, Yu Momose and Koji Takeuchi

Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Osaka, Japan.

Affiliation

Y.T., R.I., M.S., A.H., K.T.: Metabolic Disease Drug Discovery Unit, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Osaka, Japan.

N.N.: Inflammation Drug Discovery Unit, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Osaka, Japan.

T.Y.: Chemical Development Laboratories, CMC Center, Takeda Pharmaceutical Company Limited, Osaka, Japan.

Y.M.: Project Management Office, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Osaka, Japan.

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Corresponding author: Yoshiyuki Tsujihata, PhD

Metabolic Disease Drug Discovery Unit, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, 17-85, Jusohonmachi 2-chome, Yodogawa-ku, Osaka 532-8686, Japan.

TEL: +81-6-6300-6911, FAX: +81-6-6300-6306

E-mail: Tsujihata_Yoshiyuki@takeda.co.jp

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Abbreviations:

CHO, Chinese hamster ovary; FFA, free fatty acid; GPR40/FFA_1, G protein-coupled receptor 40/Free fatty acid receptor 1; IP, Inositol monophosphate; STZ, streptozotocin

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Abstract

GPR40/FFA_1 is highly expressed in pancreatic β cells and mediates free fatty acid-induced insulin secretion. This study examined the pharmacological effects and potential for avoidance of lipotoxicity of TAK-875, a novel, orally available, selective GPR40 agonist. Insulinoma cell lines and primary rat islets were used to assess the effects of TAK-875 *in vitro*. The *in vivo* effects of TAK-875 on postprandial hyperglycemia, fasting hyperglycemia and normoglycemia were examined in type 2 diabetic and normal rats. In rat insulinoma INS-1 833/15 cells, TAK-875 increased intracellular inositol monophosphate and calcium concentration, consistent with activation of the Gqα signaling pathway. The insulinotropic action of TAK-875 (10 μM) in INS-1 833/15 and primary rat islets was glucose dependent. Prolonged exposure of cytokine-sensitive INS-1 832/13 to TAK-875 for 72 h at pharmacologically active concentrations did not alter glucose-stimulated insulin secretion, insulin content, or caspase 3/7 activity, while prolonged exposure to palmitic or oleic acid impaired β cell function and survival. In an oral glucose tolerance test in type 2 diabetic N-STZ-1.5 rats, TAK-875 (1-10 mg/kg, p.o.) showed a clear improvement in glucose tolerance and augmented insulin secretion. In addition, TAK-875 (10 mg/kg, p.o.) significantly augmented plasma insulin levels and reduced

fasting hyperglycemia in male Zucker diabetic fatty rats, while in fasted normal Sprague Dawley rats, TAK-875 neither enhanced insulin secretion nor caused hypoglycemia even at 30 mg/kg. TAK-875 enhances glucose-dependent insulin secretion and improves both postprandial and fasting hyperglycemia with a low risk of hypoglycemia and no evidence of β cell toxicity.

Introduction

Insulin resistance and impaired insulin secretion are major causes of the onset and development of type 2 diabetes (Muoio and Newgard, 2008). Drugs that enhance insulin secretion, such as sulfonylureas and meglitinides, are commonly used for the treatment of type 2 diabetes. However, these drugs enhance insulin secretion by direct closure of the K_{ATP} channel independent of blood glucose levels, thereby causing hypoglycemia (Doyle and Egan, 2003). Hence, patients with diabetes would benefit from the development of a novel anti-diabetic drug that has a low hypoglycemic risk and effectively improves blood glucose control.

Secretion of insulin from pancreatic β cells is stimulated by glucose and other nutrients including free fatty acids (FFAs) (Prentki et al., 1997; Haber et al., 2003). In isolated human and rodent islets, FFAs enhance insulin secretion in a manner that is dependent on glucose concentration (Gravena et al., 2002). Plasma concentrations of FFAs are elevated in the fasted state, and they play a role in enhancement of the postprandial insulin response *in vivo* (Stein et al., 1996; Dobbins et al., 1998). GPR40, a G protein-coupled receptor highly expressed in pancreatic β cells, was recently identified as a receptor for both saturated and unsaturated medium- and long-chain FFAs (Itoh et al., 2003; Briscoe et al., 2003; Kotarsky et al., 2003). In addition, Itoh et

al. demonstrated that suppression of GPR40/FFA_1 mRNA with small interfering RNA inhibited the enhancement of FFA-induced insulin secretion in mouse insulinoma MIN6 cells, indicating that GPR40/FFA_1 is involved in stimulation of acute insulin secretion by FFAs (Itoh et al., 2003). The role of GPR40/FFA_1 in insulin secretion has also been confirmed by the use of selective small molecule GPR40/FFA_1 agonists (Briscoe et al., 2006; Tan et al., 2008).

In pancreatic β cells, elevation of intracellular calcium triggers insulin secretion (Prentki et al., 1997). Generally, activation of Gqα protein-coupled receptors results in phospholipase C activation, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol production, and increases in intracellular calcium concentration (Taylor et al., 1991). Studies have shown that GPR40/FFA_1 is mainly coupled with Gqα in rodent β cell lines, and that agonist stimulation of GPR40/FFA_1 with FFAs enhances intracellular calcium concentration $[Ca^{2+}]_i$ and insulin secretion in these cells, which can be blocked by inhibitors of Gqα signaling (Fujiwara et al., 2005, Shapiro et al., 2005).

While FFAs acutely stimulate insulin secretion, chronic exposure to them causes β cell dysfunction and/or cell death, so-called lipotoxicity (Haber et al., 2003; Morgan, 2009). Since endogenous ligands of GPR40/FFA_1 are medium- and long-chain FFAs, it has been suggested that GPR40/FFA_1 might mediate chronic toxic effects of FFAs

(Steneberg et al., 2005). However, conflicting results obtained from GPR40/FFA_1-deficient mice have also been reported (Latour et al., 2007; Lan et al., 2008; Kebede et al., 2008; Alquier et al., 2009); these did not show the harmful effects of GPR40/FFA_1 in pancreatic β cells. Moreover, Nagasumi, et al. have reported that overexpression of GPR40/FFA_1 in pancreatic β cells of mice results rather in enhanced insulin secretion, improved glucose tolerance, and resistance to impairment of glucose tolerance induced by a high-fat diet (Nagasumi et al., 2009). Therefore, it remains under debate whether GPR40/FFA_1 agonism or antagonism would be more favorable for the treatment of type 2 diabetes and related disorders.

TAK-875 was identified as a potent and selective small molecule agonist for GPR40/FFA_1, which exhibits rapid absorption, high Cmax, and high plasma exposure with high bioavailabilities in rats and dogs (Negoro et al., 2010). TAK-875 was also well tolerated following administration of a single oral dose in healthy volunteers, and has pharmacokinetic characteristics suitable for a once-daily regimen (Naik et al., 2011). The current study was conducted to evaluate the cellular signaling events induced by TAK-875, and the pharmacological effects in various *in vitro* and *in vivo* models, and to determine if TAK-875 affects β cell function and survival via prolonged activation of GPR40/FFA_1, as has been observed with FFAs. Our results suggest that

GPR40/FFA_1 does not mediate the chronic toxic effects of FFAs, and that selective activation of GPR40/FFA_1 with TAK-875 enhances glucose-dependent insulin secretion in a manner consistent with activation of the Gqα-mediated pathway without inducing β cell toxicity.

Methods

Reagents.

TAK-875([(3S)-6-({2',6'-dimethyl-4'-[3-(methylsulfonyl)propoxy]biphenyl-3-yl}methoxy)-2,3-dihydro-1-benzofuran-3-yl]acetic acid hemi-hydrate) (Negoro et al., 2010) was synthesized in the Chemical Development Laboratories at Takeda Pharmaceutical Company Limited. TAK-875 was dissolved in dimethyl sulfoxide (DMSO) and oleic acid (Sigma) was dissolved in 95% ethanol for *in vitro* experiments unless otherwise indicated. For the experiments of 72 h exposure *in vitro*, sodium palmitic acid (Chem Service) and sodium oleic acid (Sigma) were dissolved in hot distilled water, and were added to the equal volume of 20% (w/v) free fatty acid-free BSA (Wako) solution with stirring on ice. TAK-875 dissolved in DMSO was added to 10% BSA solution. Final concentrations of BSA and DMSO in the experiments of sub-chronic treatment were 1% and 0.1%, respectively, in all samples.

Animals. The care and use of the animals and the experimental protocols used in this research were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited and the Guide for the Care and Use of Laboratory Animals were maintained throughout the study (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number 85-23, revised 1996). All rats were fed regular chow CE-2 (CLEA, Japan) and tap water ad libitum with controlled temperature (23°C), humidity (55%) and lighting (lights on from 7:30 to 19:30). Male N-STZ-1.5 rats were generated by subcutaneous injection of 120 mg/kg of streptozotocin (STZ) in male Wistar Kyoto rats 1-2 days after birth. Male Sprague-Dawley (SD) rats, male Zucker diabetic fatty (ZDF) rats and their Zucker lean (ZL) littermates were obtained from Charles River Laboratories Japan, Inc.

Cells. Chinese hamster ovary (CHO, dihydrofolate reductase negative) cells stably expressing human or rat GPR40/FFA_1 and control cells (CHO-mock) (Negoro et al., 2010) were cultured in alpha-minimum essential medium without nucleotide (Invitrogen) supplemented with 10% dialyzed and heat-inactivated FBS (Thermo, Australia), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen). The cell

lines 833/15 and 832/13, derived from INS-1 rat insulinoma cells, were obtained from Dr. Christopher B. Newgard (Duke University, USA). Cells were grown in RPMI-1640 medium containing L-glutamine (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 10 mM HEPES (Invitrogen), 10% heat-inactivated FBS (Thermo, Australia), 55 μM 2-mercaptoethanol (Invitrogen), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere containing 5% CO_2/95% air at 37°C.

Measurement of Intracellular Inositol Phosphate Production. Intracellular inositol monophosphate (IP) measurements were carried out using an IP-One ELISA kit (Cisbio) according to the manufacturer's instruction. Briefly, CHO cells or INS-1 833/15 cells were suspended in the culture medium described above and seeded at a density of 8×10^4 cells/well and 5×10^4 cells/well, respectively, in 96-well plates (Nunc), and the cells were cultured overnight. After discarding of the medium, cells were incubated at 37°C for 1 h with stimulation buffer (146 mM NaCl, 4.2 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 5.5 mM glucose and 50 mM LiCl, pH=7.4) in the absence or presence of stimulators as shown. In the experiments using INS-1 833/15 cells, glucose at 1, 3 or 10 mM concentration was added to the glucose-free stimulation buffer. After the incubation, lysis reagent was added and the

plate was incubated for another 30 min at 37°C, and intracellular IP concentration was measured. EC_{50} values were calculated by logistic regression analysis (SAS).

Measurement of Intracellular Calcium Concentration. INS-1 833/15 cells were seeded at a density of 5×10^4 cells/well in poly-D-lysine coated 96-well black plates (BD Biocoat), and cultured overnight before experiments. Cells were loaded for 30 min at 37°C with 1 μM Fura-2 AM (Dojindo, Japan) in Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 0.025% pluronic F-127 (Invitrogen), 1 mM glucose and 1% FBS (loading buffer), followed by washing with loading buffer without Fura-2 AM. After the washing, KRBH containing 1, 3 or 10 mM glucose and 0.1% DMSO was added, the cells were excited at 340 and 380 nm alternatively, the emission signals at 510 nm were detected every 10 s by a cooled charge-coupled device camera, and the ratio was derived using an AquaCosmos (Hamamatsu photonics, Japan). After monitoring of the glucose-induced calcium response, the equivalent volume of KRBH containing glucose and test agents was added. The average of 340/380 fluorescence ratios was obtained from 30 randomly selected cells.

Acute Insulin Secretion Assay. INS-1 833/15 cells were seeded at a density of 5×10^4

cells/well in a 96-well plate, and the cells were cultured in RPMI medium overnight before experiments. After discarding of the medium, the cells were pre-incubated for 2 h with KRBH containing 1 mM glucose. After discarding of the pre-incubation buffer, KRBH containing glucose and stimulators as indicated was added and the plate was incubated for 2 h at 37°C. After incubation, supernatants from each well were collected and secreted insulin concentration was measured using a rat insulin ELISA (Morinaga, Japan) according to the manufacturer's instruction.

Insulin Secretion and Intracellular Insulin Content After Prolonged Exposure. INS-1 832/13 cells were suspended in RPMI medium, and were seeded in a 96-well plate at a density of 2×10^4 cells/well; 1% BSA and 0.1% DMSO alone (control), palmitic acid (10, 100 and 1000 μM), oleic acid (10, 100 and 1000 μM) or TAK-875 (1, 10 and 100 μM) was added to the plate. After 72 h culture, medium was discarded, and cells were pre-incubated for 2 h with KRBH containing 1 mM glucose and 0.2% BSA at 37°C. After discarding of the pre-incubation buffer, KRBH containing 1 or 20 mM glucose and 0.2% BSA was added and the plate was further incubated for 2h. The insulin concentration in the supernatant was measured as described above. To measure intracellular insulin content, INS-1 832/13 cells were exposed to 1% BSA and 0.1%

DMSO alone (control), palmitic acid (1000 μM), oleic acid (1000 μM) or TAK-875 (100 μM) with 1% BSA and 0.1% DMSO, as described above. After incubation, cells were washed once with phosphate-buffered saline, and acid-ethanol solution was added to each well, followed by sonication on ice. Intracellular insulin was extracted by overnight incubation at -30°C, followed by separation of supernatant by centrifugation at 12,000 rpm x 5 min at 4°C.

Measurement of Caspase 3/7 Activity. INS-1 832/13 cells were suspended in RPMI medium containing 11 mM glucose and the supplements described above. These cells were seeded at a density of 2×10^4 cells/well in a 96-well black plate coated with poly-D-lysine (BD BioCoat), and 1% BSA and 0.1% DMSO alone (control), palmitic acid (62.5, 125, 250, 500 and 1000 μM), oleic acid (62.5, 125, 250, 500 and 1000 μM) or TAK-875 (6.25, 12.5, 25, 50 and 100 μM) was added to the plate with 1% BSA and 0.1% DMSO, followed by culture for 72 h. After the culture, caspase 3/7 activity was measured with the Apo-one homogeneous caspase 3/7 assay (Promega) according to the manufacturer's instruction. Fluorescence intensity was measured at an excitation of 485 nm and an emission at 535 nm.

Oral Glucose Tolerance Test and Effects on Fasting Normo- and Hyperglycemia. At 18 weeks of age, the N-STZ-1.5 rats were fasted overnight and orally given vehicle (0.5% methylcellulose) or TAK-875 (1, 3, 10 mg/kg). Sixty minutes later, all animals received an oral glucose load (1 g/kg). Blood samples were collected from the tail vein before drug administration (pre), and before glucose load (time 0), and 10, 30, 60 and 120 min after the glucose load. Plasma glucose and insulin levels were measured by Autoanalyzer 7080 (Hitachi, Japan) and radioimmunoassay (RIA) (LINCO Research, USA), respectively. To see the effects of TAK-875 on fasting normo- and hyperglycemia, SD (8 weeks old), or ZDF and ZL (12 weeks old) rats were fasted overnight, and orally given vehicle (0.5% methylcellulose), TAK-875 (10 or 30 mg/kg), nateglinide (50 mg/kg), or glibenclamide (10 mg/kg). Blood samples were collected from the tail vein before drug administration (time 0) and 0.5, 1, 2, and 3 h (SD rats) and 0.5, 1, 2, 4, and 6 h (ZDF and ZL rats) after drug administration, and plasma glucose and insulin levels were measured as described above.

Statistics. Differences between two groups were analyzed by Student’s t-test or the Aspin-Welch test. For the multiple comparisons, differences versus control were tested by Dunnett's test or the Steel test. In the dose-dependent study, statistical significance

versus control was assessed by the one-tailed Williams' test.

Results

Comparison of TAK-875 and Endogenous Ligand Agonist Activity for GPR40/FFA$_1$. It has been demonstrated that TAK-875 increases $[Ca^{2+}]_i$ in Chinese hamster ovary (CHO) cells expressing the human or rat GPR40/FFA$_1$ (Negoro et al., 2010), but the agonist activity has not been compared with that of endogenous ligands. Thus, we first compared the agonist activity of TAK-875 with that of an endogenous ligand, oleic acid, by measuring intracellular inositol monophosphate (IP), a downstream metabolite of IP_3, in CHO cells expressing human GPR40/FFA$_1$ (CHO-hGPR40). TAK-875 (0.01–10 μM) produced a concentration-dependent increase in intracellular IP production in CHO-hGPR40 (Fig. 1A). Oleic acid (3-100 μM) also enhanced intracellular IP production in a concentration-dependent manner, but required much higher ligand concentrations to activate the receptor in comparison to TAK-875. EC_{50} values for TAK-875 and oleic acid were 0.072 μM and 29.9 μM, respectively, demonstrating that TAK-875 is >400-fold more potent at activating hGPR40 than oleic acid. Neither TAK-875 nor oleic acid elicited an IP response in control CHO cells devoid of hGPR40 (Fig. 1B).

TAK-875 Activates the Gqα-Mediated Signaling Pathway in Pancreatic β Cells. We next examined whether TAK-875 activates the Gqα-mediated signaling pathway in pancreatic β cells as observed in CHO cells by measuring the ability of TAK-875 to stimulate IP production and increase $[Ca^{2+}]_i$. Rat insulinoma INS-1 cell clone 833/15 was used as a pancreatic β cell model. It has been reported that INS-1 cells express endogenous GPR40/FFA_1 (Schnell, et al., 2007). Prior studies confirmed that INS-1 833/15 cells highly expressed endogenous GPR40/FFA_1 mRNA to an extent similar to that observed in primary rat islets (data not shown). TAK-875 (0.1-10 μM) dose-dependently augmented intracellular IP production in these cells in the presence of 10 mM glucose, while one of the sulfonylurea drugs, glibenclamide, did not (Table 1). Glucose concentration did not affect TAK-875-induced IP production, and almost equivalent TAK-875-induced IP production was observed in the presence of 1 and 3 mM glucose compared to 10 mM glucose.

As shown in Fig. 2A, 10 mM glucose transiently increased $[Ca^{2+}]_i$ (first peak during measurement) in INS-1 833/15 cells, indicating that the glucose-sensitive $[Ca^{2+}]_i$ response was functional in this model. Addition of vehicle (DMSO) after the first $[Ca^{2+}]_i$ peak did not elevate $[Ca^{2+}]_i$ levels. In contrast, TAK-875 (3-30 μM)

concentration-dependently augmented $[Ca^{2+}]_i$ (Fig. 2B-D). We next examined the glucose dependence of these $[Ca^{2+}]_i$ elevations by TAK-875. As shown in Fig. 3, the TAK-875 (30 μM)-induced increase in $[Ca^{2+}]_i$ was attenuated at glucose concentrations of 1 and 3 mM, compared to the response observed in 10 mM of glucose (Fig. 3A-C). In contrast, glibenclamide (10 μM) augmented $[Ca^{2+}]_i$ independent of glucose concentrations (Fig. 3D-F).

TAK-875 Augments Glucose-Dependent Insulin Secretion in Pancreatic β Cells. The insulinotropic effects of TAK-875 in the presence of different concentrations of glucose were examined. As shown in Fig. 4A, in the presence of 10 mM glucose, TAK-875 (0.001-10 μM) dose-dependently stimulated insulin secretion from INS-1 833/15 cells. TAK-875, at 10 μM, enhanced insulin secretion 1.8-fold, compared with 10 mM glucose alone. Similar to the glucose concentration-dependent effects observed with intracellular calcium mobilization (Fig. 3A-C), TAK-875 significantly augmented insulin secretion from INS-1 833/15 cells only in the presence of glucose at a concentration of 10 mM but not at 1 or 3 mM (Fig. 4B). In contrast, glibenclamide-induced insulin secretions from these cells were independent of glucose concentration.

The effects of TAK-875 on glucose-induced insulin secretion were also evaluated in pancreatic islets isolated from normal Sprague-Dawley rats. TAK-875, at 10 μM, significantly enhanced insulin release at glucose concentrations of 8 and 16 mM, but not at 3 mM (Fig. 4C).

Prolonged Agonist Stimulation of GPR40/FFA$_1$ by TAK-875 Does Not Cause β Cell Dysfunction. The effects of prolonged exposure to TAK-875 on β cell function were examined in cytokine sensitive INS-1 832/13 cells instead of INS-1 833/15 cells, a cytokine resistant clone (Collier et al., 2006). The endogenous ligands for GPR40/FFA$_1$, palmitic acid and oleic acid (Itoh et al., 2003), were used as comparators. Before the experiment, we confirmed that TAK-875 (6.25-100 μM), palmitic acid (62.5-1000 μM) and oleic acid (62.5-1000 μM) showed agonist activities in CHO cells expressing human or rat GPR40/FFA$_1$ in the presence of 1% BSA, corresponding to the BSA concentration to be used in the prolonged exposure experiments (supplemental Fig. 1). Also, we observed that palmitic acid, oleic acid, and TAK-875 dose-dependently augmented insulin secretion in INS-1 832/13 in the presence of 10 mM glucose and 1% BSA (supplemental Fig. 2). These results indicate that TAK-875 sufficiently stimulates GPR40/FFA$_1$ within this dose range, compared to palmitic acid

and oleic acid.

In INS-1 832/13 cells, 72 h exposure to palmitic acid (1000 μM) together with 1% BSA resulted in a significant reduction in the insulin secretory response to 20 mM glucose (Fig. 5A). Under the same conditions, neither oleic acid (10-1000 μM) nor TAK-875 (1-100 μM) significantly altered glucose-stimulated insulin secretion in these cells. Intracellular insulin content was significantly reduced after 72 h exposure to palmitic acid (1000 μM) or oleic acid (1000 μM), and the deleterious effect was particularly pronounced for palmitic acid compared to oleic acid ($p \leq 0.05$ by Aspin-Welch test) (Fig. 5B). In contrast, prolonged exposure to TAK-875 (100 μM) did not affect intracellular insulin content.

Prolonged Agonist Stimulation of GPR40/FFA1 by TAK-875 Does Not Cause Induction of a Marker of Apoptosis in Pancreatic β Cells. It is well known that chronic exposure to FFAs in pancreatic β cells causes not only impairment of their function but also cell apoptosis (Haber et al., 2003; Morgan et al., 2009). To clarify the effects of prolonged exposure to TAK-875 on apoptotic events in β cells, INS-1 832/13 cells were treated with TAK-875, palmitic acid or oleic acid in the presence of 1% BSA for 72 h, and subsequent caspase 3/7 activity was measured. In these cells, 72 h

exposure to palmitic acid (62.5-1000 μM) and oleic acid (62.5-1000 μM) caused dose-dependent enhancement of caspase 3/7 activity, and statistically significant effects were observed at doses above 250 μM of palmitic acid and 500 μM of oleic acid (Fig. 5C and D). In contrast, TAK-875 (6.25-100 μM) did not show any effect on caspase 3/7 activity under the same conditions (Fig. 5E).

TAK-875 Augments Insulin Secretion and Improves Glucose Tolerance During OGTT in Type 2 Diabetic Rats. Next we performed an oral glucose tolerance test (OGTT) in type 2 diabetic N-STZ-1.5 rats (Portha et al., 1989), to examine the effects of TAK-875 on impaired postprandial glucose tolerance. Single oral administration of TAK-875 (1-10 mg/kg) to these rats 1h prior to an oral glucose load resulted in a potent and dose-dependent reduction of glucose excursion (Fig. 6A and B). The effects on plasma glucose levels were likely mediated through the compound's effects on insulin, since plasma insulin levels, especially during the early phase of the OGTT, increased simultaneously and dose-dependently with TAK-875 (Fig. 6C and D).

Glucose-lowering effects of TAK-875 on normal and elevated fasting plasma glucose. Since TAK-875 showed strictly glucose-dependent insulinotropic effects *in*

vitro (Fig. 4), we speculated that TAK-875 might enhance insulin secretion and reduce blood glucose only when blood glucose levels are elevated. To clarify the hypothesis, the effects of TAK-875 on fasting normoglycemia and hyperglycemia were examined in normal Sprague-Dawley (SD) rats and Zucker diabetic fatty (ZDF) rats, respectively. Two insulin secretagogues that act on the K_{ATP} channel, nateglinide and the sulfonylurea glibenclamide, were included in these studies as comparators. As shown in Fig. 7A and 7B, nateglinide (50 mg/kg) lowered plasma glucose levels below normal fasting levels in SD rats by increasing plasma insulin. Similarly, glibenclamide (10 mg/kg) gradually decreased plasma glucose levels below normal fasting levels with a significant increase in plasma insulin levels. In contrast, TAK-875 at 30 mg/kg, which is a 3- to 10-fold higher dose compared to the dose that improved glucose tolerance in diabetic rats (Fig. 6), did not alter fasting glucose levels in SD rats with normal glucose homeostasis (Fig. 7A). Likewise, TAK-875 did not significantly alter insulin secretion in SD rats with normal fasting glucose levels (Fig. 7B).

The effects of TAK-875, glibenclamide, and nateglinide on fasting hyperglycemia were evaluated in male ZDF rats. As shown in Fig. 7C, fasting plasma glucose levels before drug administration were significantly elevated in ZDF rats compared with the normal Zucker lean (ZL) rats. In ZDF rats, oral administration of TAK-875 (10 mg/kg)

increased plasma insulin levels (Fig. 7D) and lowered plasma glucose levels (Fig. 7C), while nateglinide (50 mg/kg) and glibenclamide (10 mg/kg) did not show statistically significant change.

Discussion

GPR40/FFA_1 is highly expressed in pancreatic islets in mice, rats and humans (Briscoe et al., 2003, Itoh et al., 2003; Tomita T et al., 2006). While it has been reported that GPR40/FFA_1 is expressed not only in pancreatic β cells (insulin-positive cells) but also in α cells (glucagon-positive cells) in mice (Flodgren et al., 2007), expression in insulin-positive cells is dominant in rats and humans (Itoh et al., 2003; Tomita T et al., 2006). In this study, we focused on the function of GPR40/FFA_1 in pancreatic β cells and examined the events caused by pharmacological activation of the receptor using *in vitro* and *in vivo* rat models. Our data indicate that TAK-875 is a potent agonist for GPR40/FFA_1 and activates the PLC pathway, presumably via Gqα in pancreatic β cells. This mechanism of insulinotropic action by TAK-875 is novel among insulinotropic drugs, including sulfonylureas, meglitinides, dipeptidyl peptidase-4 (DPP-4) inhibitors and glucagon-like peptide-1 (GLP-1) analogues, and distinct from those of glucose-dependent insulinotropic polypeptide (GIP) and GPR119

agonists (Drucker, 2007; Winzell and Ahrén, 2007).

We found that TAK-875 enhanced $[Ca^{2+}]_i$ in a glucose-dependent manner in INS-1 cells. Since the increase in $[Ca^{2+}]_i$ is related to enhanced insulin secretion in β cells (Prentki et al., 1997), this mechanism may explain the glucose-dependent insulinotropic effects of TAK-875 through GPR40/FFA_1. The phenomenon was consistent with other reports in which enhancement of $[Ca^{2+}]_i$ in rat primary β cells by stimulation of GPR40/FFA_1 with oleic acid is dependent on glucose concentration (Fujiwara et al., 2005). A specific receptor for IP_3 is present in the endoplasmic reticulum (ER), and the interaction of IP_3 with the IP_3 receptor triggers calcium release from the ER (Berridge, 1993). TAK-875 also enhanced intracellular IP production, thus, an increase in $[Ca^{2+}]_i$ with TAK-875 may, at least in part, result from calcium release from the ER. The interesting observation is that IP production by TAK-875 was not glucose-dependent, while enhancement of $[Ca^{2+}]_i$ and insulin release were strictly dependent on glucose concentration. Similar effects have been shown in Gqα-coupled muscarinic receptors stimulated with the agonist carbachol, in which IP production occurs regardless of glucose concentration, while insulin release is glucose-concentration-dependent in rat islets (Zawalich et al., 1989). Thus, one explanation is that these events might be common phenomena among Gqα-coupled

receptors. Another explanation is that the difference may be caused by the different type of assay employed; real-time and transient measurement of intracellular calcium vs measurement of cumulative IP which is the degradation product of IP_3. Further analysis will be necessary to clarify how GPR40/FFA_1-mediated signals interact with glucose in pancreatic β cells.

Postprandial and fasting hyperglycemia due to insufficient insulin secretion in response to blood glucose is observed in patients with type 2 diabetes. Our results indicate that TAK-875 directly acts on pancreatic β cells but augments insulin secretion only when blood glucose levels are elevated. Indeed, *in vivo* oral administration of TAK-875 (3-10 mg/kg) in type 2 diabetic rats improved both postprandial and fasting hyperglycemia. In terms of future studies, it will be of interest to determine if chronic exposure to TAK-875 in vivo improves type 2 diabetes. Especially, male ZDF rats, in which the single oral dose of TAK-875 improved fasting hyperglycemia, exhibit severe type 2 diabetes with age-dependent decline of plasma insulin levels and β cell mass (Pick et al, 1998). Thus, future studies will focus on the effects of multiple doses of TAK-875 on pancreatic β cell function, apoptosis and islet morphology in this rat model. On the other hand, oral administration of high doses of TAK-875 (30 mg/kg) in normal fasted rats did not induce hypoglycemia. Oral administration of TAK-875

results in rapid absorption of the compound (Tmax=1h) (Negoro et al., 2010), indicating that the absence of hypoglycemic events and the minor insulinotropic effects observed in normal rats receiving high doses of TAK-875 may not be due to the low plasma concentration of the compound. Rather, these results suggest that TAK-875 may present a low risk of hypoglycemia – an adverse effect common to sulfonylureas and meglitinides.

While GPR40/FFA_1 has been considered a possible lipotoxicity mediator (Steneberg et al., 2005), a number of experimental observations do not support a central role for GPR40/FFA_1 in lipotoxicity (Latour et al., 2007; Lan et al., 2008; Kebede et al., 2008; Alquier et al., 2009; Nagasumi et al., 2009). In our experiments, prolonged agonist stimulation to TAK-875 for 72 h in INS-1 cells, at the dose range in which sufficient agonist activity was observed, did not affect subsequent glucose-stimulated insulin secretion, insulin content, or caspase 3/7 activity, while FFAs did affect these parameters. In addition, we did not observe any correlation between these events and agonist activity for GPR40/FFA_1. Our results, therefore, suggest that chronic toxic events induced by FFAs may be independent of GPR40/FFA_1, and that chronic activation of GPR40/FFA_1 by TAK-875 may not lead to either β cell dysfunction or apoptosis. FFAs may induce toxicological effects by other mechanisms, such as

long-chain fatty acyl-coenzyme A accumulation, ceramide synthesis, and ER stress induction (Haber et al., 2003; Morgan, 2009).

Currently, GLP-1 analogues and DPP-4 inhibitors are in clinical use. GLP-1 analogues are glucose-dependent insulinotropic agents, showing excellent efficacy for the treatment of diabetes with a low risk of hypoglycemia. However, these drugs are peptides and currently require administration via injection (Mikhail, 2008). On the other hand, DPP-4 inhibitors are orally available small molecule insulinotropic drugs, with an excellent safety profile. However, the indirect insulinotropic effects dependent on endogenous GLP-1 and/or GIP may limit the efficacy in some patients.

Combination therapy with anti-diabetic drugs is often utilized for the treatment of type 2 diabetes. Our results indicate that TAK-875 is a glucose-dependent insulinotropic agent with a low risk of hypoglycemia. These novel features may allow the use of TAK-875 in combination with insulin-sensitizers (Metformin and thiazolidines) and alpha-glucosidase inhibitors, with a reduced risk of hypoglycemic events. In addition, since TAK-875 has novel insulinotropic effects, combination with insulin secretagogues such as sulfonylureas, DPP-4 inhibitors and GLP-1 analogues may potentiate their glucose-lowering effects.

In conclusion, our results indicate that the GPR40/FFA_1 agonist TAK-875 has the

potential to be a highly effective drug that warrants further investigation for the treatment of type 2 diabetes.

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Authorship Contributions

All authors are employees of Takeda Pharmaceutical Company Limited.

Participated in research design: Tsujihata, Ito, Suzuki, Harada, Takeuchi

Conducted experiments: Tsujihata, Ito, Suzuki, Harada, Takeuchi

Contributed new reagents or analytic tools: Negoro, Yasuma, Momose

Performed data analysis: Tsujihata, Ito, Suzuki, Harada

Wrote or contributed to the writing of the manuscript: Tsujihata, Ito, Takeuchi

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Legends for figures

Fig. 1. TAK-875 shows potent agonist activity in Chinese hamster ovary (CHO) cells expressing human GPR40/FFA_1. Intracellular inositol monophosphate concentrations in CHO cells stably expressing human GPR40/FFA_1 (A) and control vector (B) were measured after stimulation with TAK-875 (0.01–10 μM, closed circles) or oleic acid (3-100 μM, closed triangles). Data shown are the mean + SD of duplicate wells.

Fig. 2. TAK-875 exhibits concentration-dependent augmentation of intracellular calcium concentrations in INS-1 833/15 cells. Changes in intracellular calcium concentration in INS-1 833/15 cells following initial challenge with 10 mM glucose and subsequent challenge with 10 mM glucose and vehicle (DMSO) or TAK-875. Fura-2 loaded INS-1 833/15 cells were treated with 10 mM glucose alone. After treatment with glucose, the cells were stimulated with DMSO (A), or with 3, 10 or 30 μM of TAK-875 (B, C and D, respectively) in the presence of 10 mM glucose. The traces shown are averages of the ratio of fluorescent intensity excited at 340 and 380 nm (average of 30 cells for each fig). Bars indicate the presence of glucose, vehicle, or TAK-875 in the buffer solution.

Fig. 3. TAK-875, but not glibenclamide, exhibits glucose-dependent enhancement of intracellular calcium concentration in INS-1 833/15 cells. Changes in intracellular calcium concentration in INS-1 833/15 cells following initial challenge with various concentrations of glucose and subsequent challenge with each glucose concentration and TAK-875 or glibenclamide. Fura-2 loaded INS-1 833/15 cells were initially treated with 1 mM (A, D), 3 mM (B, E), or 10 mM (C, F) glucose. The cells were subsequently stimulated with 30 μM of TAK-875 (A, B and C) or 10 μM of glibenclamide (D, E and F) in the presence of each glucose concentration. The traces shown are averages of the ratio of fluorescent intensity at 340 and 380 nm (average of 30 cells for each fig). Bars indicate the presence of glucose, vehicle, TAK-875 or glibenclamide in the buffer solution.

Fig. 4. TAK-875 enhances insulin secretion in INS-1 833/15 cells and primary rat islets with glucose-concentration dependence. (A) Dose-dependent insulinotropic effects of TAK-875 in INS-1 833/15 cells. INS-1 833/15 cells were stimulated with 10 mM glucose in the absence or presence of TAK-875 (0.001-10 μM) for 2 h. Secreted insulin concentration in each supernatant was measured by ELISA. Data shown are mean + SD (n=3). #$p \leq 0.025$ versus vehicle control by one-tailed Williams' test. (B)

Glucose-concentration-dependent insulinotropic effects of TAK-875 in INS-1 833/15 cells. INS-1 833/15 cells were treated with 1, 3 or 10 mM glucose in the absence or presence of TAK-875 (10 μM) or glibenclamide (10 μM) for 2 h. White bars = vehicle, black bars = TAK-875 or glibenclamide. Data shown are mean + SD (n=3). *$p \leq 0.01$ versus vehicle control by Dunnett's test. (C) Glucose-concentration-dependent insulinotropic effects of TAK-875 in isolated rat pancreatic islets. Isolated rat pancreatic islets (10 islets per test) were stimulated with 3, 8 or 16 mM glucose in the absence or presence of TAK-875 (10 μM) for 2h. Secreted insulin concentration was normalized with intracellular DNA content. White bars = vehicle, black bars = TAK-875. Data shown are mean + SD (n=3). * $p \leq 0.05$ and # $p \leq 0.01$ versus vehicle control by Aspin-Welch test and Student's t-test, respectively. G: glucose, Gliben: glibenclamide.

Fig. 5. Prolonged stimulation of GPR40/FFA_1 with TAK-875 does not cause β cell dysfunction and initiate apoptotic signaling in INS-1 832/13 cells. (A) Insulin secretion capacity in response to high glucose concentration after 72 h exposure to palmitic acid, oleic acid or TAK-875 in INS-1 832/13 cells. INS-1 832/13 cells were treated with palmitic acid (10, 100 or 1000 μM), oleic acid (10, 100 or 1000 μM) or TAK-875 (1,

10 or 100 μM) for 72 h, and subsequent insulin secretory capacity in response to 20 mM glucose were examined. White bars = vehicle alone, black bars = palmitic acid, oleic acid, or TAK-875. Data shown are mean + SD (n=3). **p≤0.01 by Aspin-Welch test. #p≤0.025 versus control (20 mM glucose stimulation) by one-tailed Williams' test. (B) Intracellular insulin content in INS-1 832/13 cells after 72 h exposure to palmitic acid (1000 μM), oleic acid (1000 μM) or TAK-875 (100 μM). Data shown are mean + SD (n=3). *p≤0.05 and **p≤0.01 versus vehicle control by Dunnett's test. (C-E) Caspase 3/7 activity in INS-1 832/13 cells after 72 h exposure to (C) palmitic acid (62.5 - 1000 μM), (D) oleic acid (62.5 - 1000 μM) or (E) TAK-875 (6.25 - 100 μM). Data shown are mean + SD (n=3). #p<0.025 versus control by one-tailed Williams' test.

Fig. 6. TAK-875 improves postprandial hyperglycemia in type 2 diabetic rats. Male N-STZ-1.5 rats were fasted overnight and orally given vehicle or TAK-875 (1, 3, 10 mg/kg). One hour later, all animals received an oral glucose load (1 g/kg), and plasma glucose and insulin were monitored for 2h. (A) time-dependent change of plasma glucose, (B) area under the curve of plasma glucose (0-120 min), (C) time-dependent change of plasma insulin, and (D) area under the curve of plasma insulin (pre-30 min).

Data are mean ± SD (n=6). # $p \leq 0.025$ versus control by one-tailed Williams' test.

Fig. 7. TAK-875 improves fasting hyperglycemia without affecting fasting normoglycemia. (A-B) Effects of TAK-875 on fasting normoglycemia. Male Sprague-Dawley (SD) rats were fasted overnight, and orally given vehicle, TAK-875 (30 mg/kg), nateglinide (50 mg/kg) or glibenclamide (10 mg/kg). Plasma glucose (A) and insulin levels (B) were monitored during 3 hours. (C-D) Effects of TAK-875 on fasting hyperglycemia. Male Zucker diabetic fatty (ZDF) and age-matched Zucker lean (ZL) rats were fasted overnight, and orally given vehicle, TAK-875 (10 mg/kg), nateglinide (50 mg/kg) or glibenclamide (10 mg/kg). Plasma glucose (C) and insulin levels (D) were monitored during 6 hours. Data are mean ± SD (n=6). * $p \leq 0.05$ and ** $p \leq 0.01$ versus control by Dunnett's test. # $p \leq 0.05$ versus control by Steel test.

Table 1

TAK-875 enhances intracellular inositol monophosphate production in INS-1 833/15 cells

Compound	Intracellular inositol monophosphate concentration (nM)		
	1 mM glucose	3 mM glucose	10 mM glucose
vehicle	153.9 ± 14.7	151.8 ± 8.8	161.5 ± 15.1
TAK-875 (0.1 μM)	226.0 ± 19.3[a]	236.7 ± 31.7[a]	239.9 ± 14.2[a]
TAK-875 (1 μM)	436.2 ± 30.9[a]	478.4 ± 30.7[a]	415.5 ± 80.8[a]
TAK-875 (10 μM)	486.0 ± 38.5[a]	521.2 ± 67.3[a]	464.2 ± 13.1[a]
Glibenclamide (10 μM)	143.4 ± 19.2	134.5 ± 16.7	128.8 ± 12.2

Data are mean ± SD (n=3). [a] $p \leq 0.025$ vs. control (vehicle with each respective glucose concentration) by one-tailed Williams' test.

Figure 1

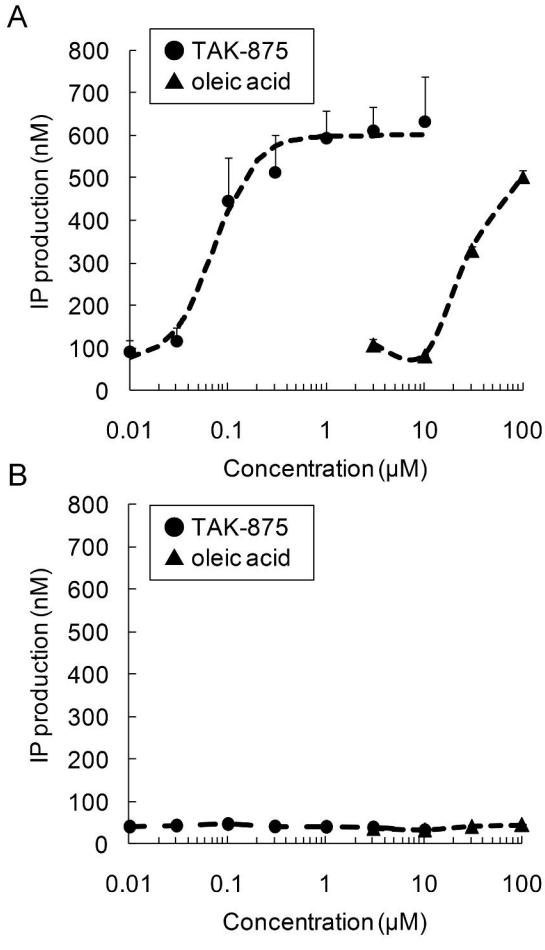

A
TAK-875
oleic acid
IP production (nM)
800
700
600
500
400
300
200
100
0
0.01
0.1
1
10
100
Concentration (μM)
B
TAK-875
oleic acid
IP production (nM)
800
700
600
500
400
300
200
100
0
0.01
0.1
1
10
100
Concentration (μM)

Figure 2

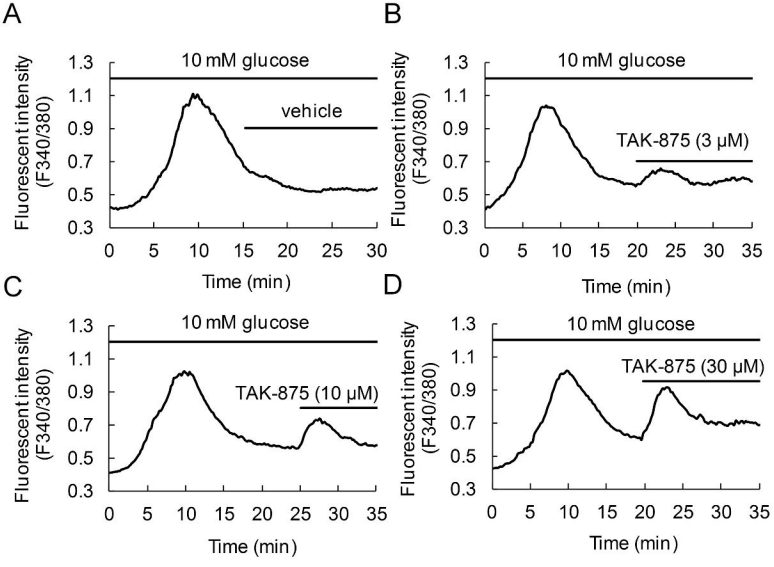

A
10 mM glucose
vehicle
Fluorescent intensity (F340/380)
1.3
1.1
0.9
0.7
0.5
0.3
0 5 10 15 20 25 30
Time (min)
B
10 mM glucose
TAK-875 (3 μM)
Fluorescent intensity (F340/380)
1.3
1.1
0.9
0.7
0.5
0.3
0 5 10 15 20 25 30 35
Time (min)
C
10 mM glucose
TAK-875 (10 μM)
Fluorescent intensity (F340/380)
1.3
1.1
0.9
0.7
0.5
0.3
0 5 10 15 20 25 30 35
Time (min)
D
10 mM glucose
TAK-875 (30 μM)
Fluorescent intensity (F340/380)
1.3
1.1
0.9
0.7
0.5
0.3
0 5 10 15 20 25 30 35
Time (min)

Figure 3

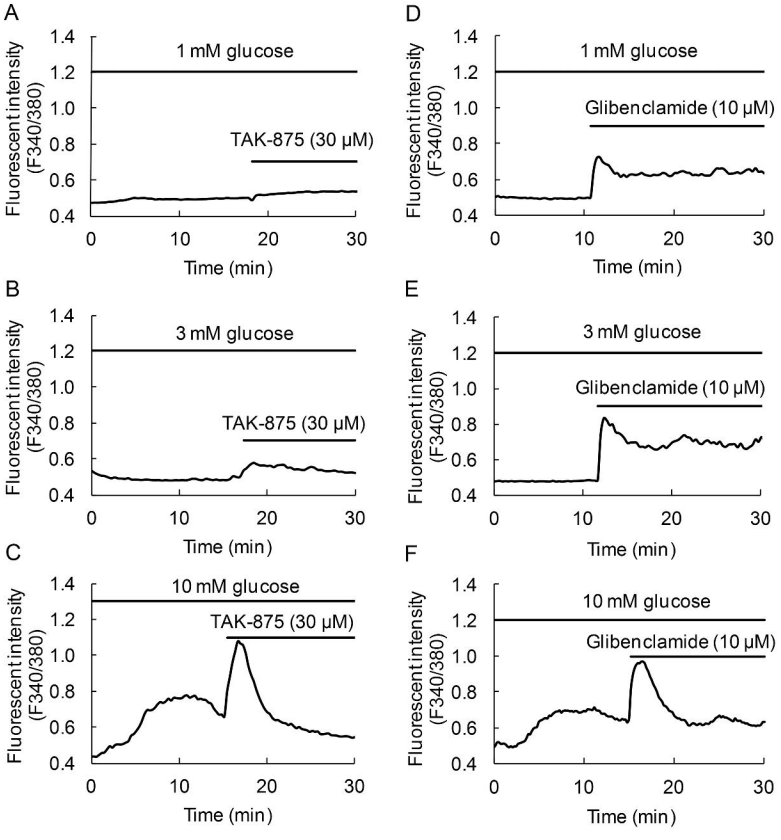

A
1 mM glucose
TAK-875 (30 μM)
Fluorescent intensity (F340/380)
Time (min)
B
3 mM glucose
TAK-875 (30 μM)
C
10 mM glucose
TAK-875 (30 μM)
D
1 mM glucose
Glibenclamide (10 μM)
E
3 mM glucose
Glibenclamide (10 μM)
F
10 mM glucose
Glibenclamide (10 μM)

Figure 4

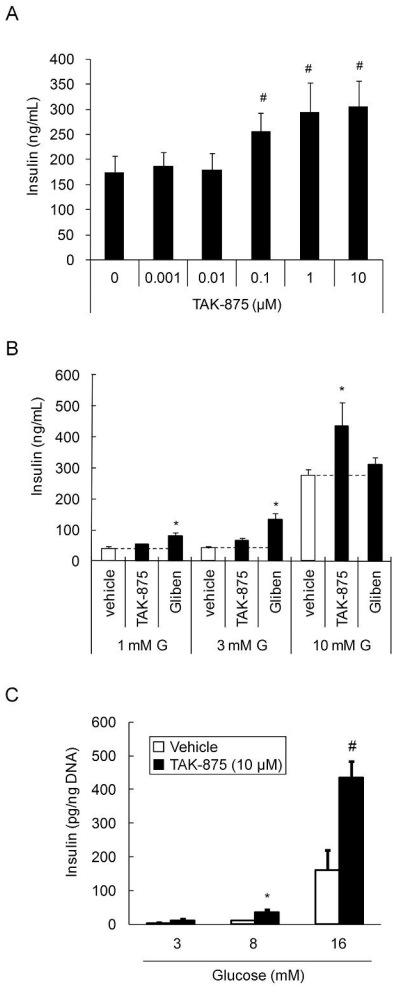
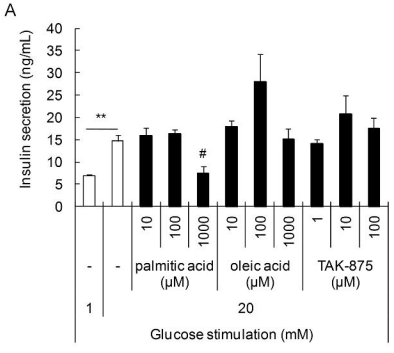
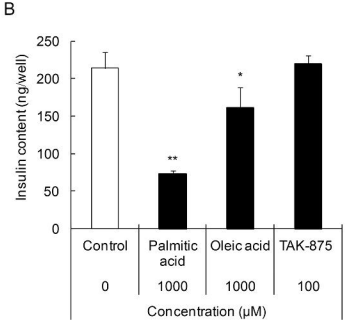
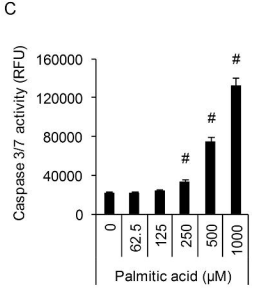
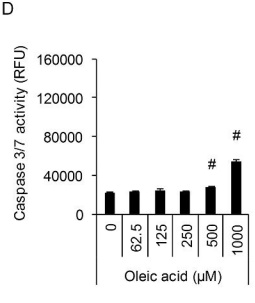

A
Insulin (ng/mL)
400
350
300
250
200
150
100
50
0
#
#
#
0
0.001
0.01
0.1
1
10
TAK-875 (μM)
B
Insulin (ng/mL)
600
500
400
300
200
100
0
*
*
*
vehicle
TAK-875
Gliben
vehicle
TAK-875
Gliben
vehicle
TAK-875
Gliben
1 mM G
3 mM G
10 mM G
C
Insulin (pg/ng DNA)
600
500
400
300
200
100
0
Vehicle
TAK-875 (10 μM)
*
#
3
8
16
Glucose (mM)

Figure 5


A
Insulin secretion (ng/mL)
40
35
30
25
20
15
10
5
0
**
#
10
100
1000
10
100
1000
1
10
100
-
-
palmitic acid (μM)
oleic acid (μM)
TAK-875 (μM)
1
20
Glucose stimulation (mM)


B
Insulin content (ng/well)
250
200
150
100
50
0
**
*
Control
Palmitic acid
Oleic acid
TAK-875
0
1000
1000
100
Concentration (μM)


C
Caspase 3/7 activity (RFU)
160000
120000
80000
40000
0
#
#
#
#
0
62.5
125
250
500
1000
Palmitic acid (μM)


D
Caspase 3/7 activity (RFU)
160000
120000
80000
40000
0
#
#
0
62.5
125
250
500
1000
Oleic acid (μM)

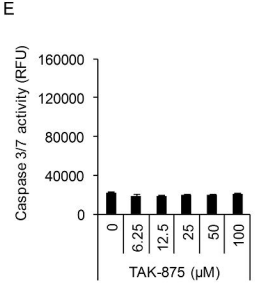

E
Caspase 3/7 activity (RFU)
160000
120000
80000
40000
0
0
6.25
12.5
25
50
100
TAK-875 (μM)

Figure 6

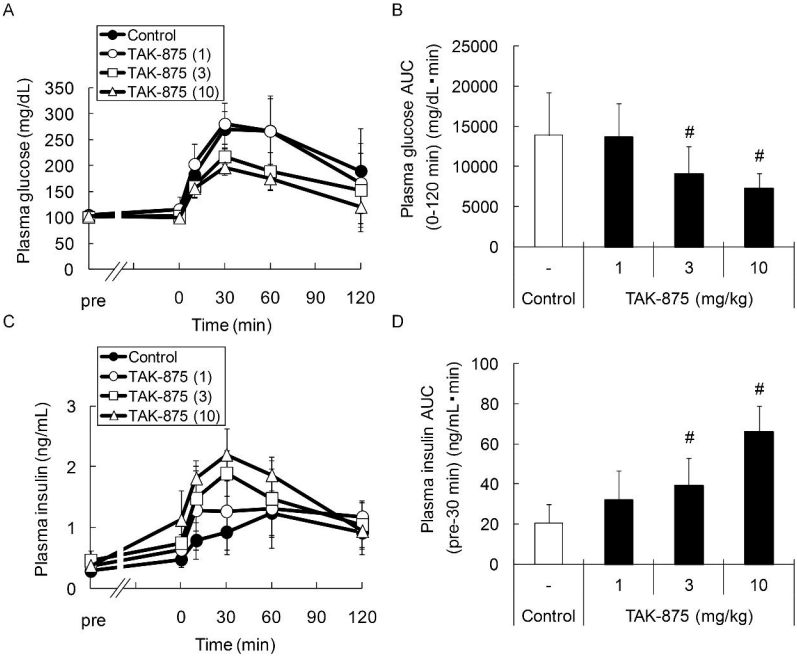
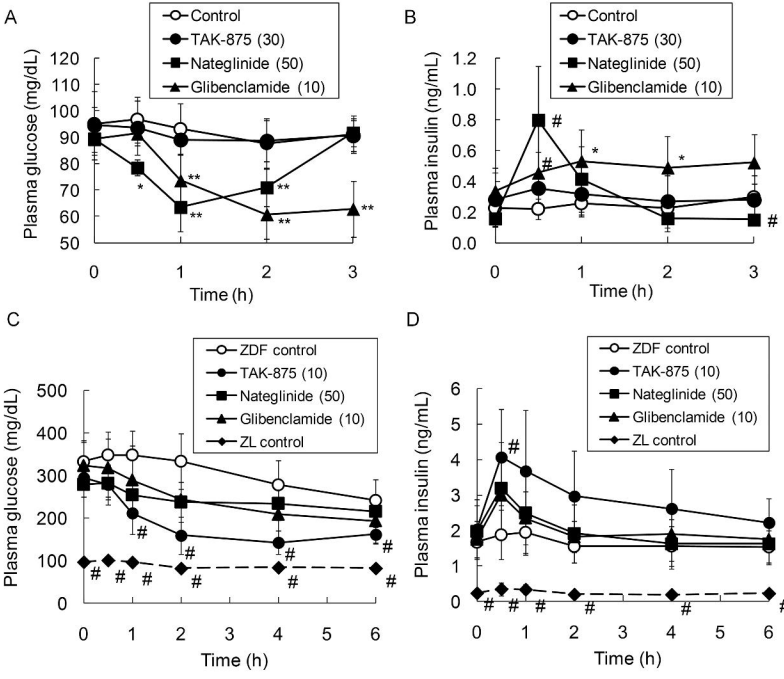

A
Control
TAK-875 (1)
TAK-875 (3)
TAK-875 (10)
Plasma glucose (mg/dL)
350
300
250
200
150
100
50
0
pre
0
30
60
90
120
Time (min)
B
Plasma glucose AUC
(0-120 min) (mg/dL・min)
25000
20000
15000
10000
5000
0
#
#
-
1
3
10
Control
TAK-875 (mg/kg)
C
Control
TAK-875 (1)
TAK-875 (3)
TAK-875 (10)
Plasma insulin (ng/mL)
3
2
1
0
pre
0
30
60
90
120
Time (min)
D
Plasma insulin AUC
(pre-30 min) (ng/mL・min)
100
80
60
40
20
0
#
#
-
1
3
10
Control
TAK-875 (mg/kg)

Figure 7


A
Control
TAK-875 (30)
Nateglinide (50)
Glibenclamide (10)
Plasma glucose (mg/dL)
Time (h)
B
Plasma insulin (ng/mL)
C
ZDF control
TAK-875 (10)
Nateglinide (50)
Glibenclamide (10)
ZL control
D