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Chronic treatment with novel GPR40 agonists improve whole-body glucose metabolism based on the glucose-dependent insulin secretion

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Running title: Novel GPR40 agonists improve whole-body glucose metabolism

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Abbreviations: DPP-IV, Dipeptidyl peptidase-IV; PPAR, Peroxisome proliferator activated receptor; FFA, Free fatty acid; OGTT, Oral glucose tolerance test; HbA1c, Hemoglobin A1c; CHO, Chinese Hamster Ovary

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Abstract

GPR 40 is a free fatty acid receptor that has been shown to regulate glucose-dependent insulin secretion. This study aimed to discover novel GPR40 agonists and investigate the whole body effect on glucose metabolism of GPR40 activation using these novel GPR40 agonists. To identify novel GPR40-specific agonists, we conducted high-throughput chemical compound screening and evaluated glucose-dependent insulin secretion. To investigate the whole body effect on glucose metabolism of GPR40 activation, we conducted repeat administration of the novel GPR40 agonists to diabetic model *ob/ob* mice and evaluated metabolic parameters. To characterize the effect of the novel GPR40 agonists more deeply, we conducted an insulin tolerance test and a euglycemic-hyperinsulinaemic clamp test. As a result, we discovered the novel GPR40-specific agonists including AS2034178 and found that its exhibited glucose-dependent insulin secretion enhancement both *in vitro* and *in vivo*. In addition, the compounds also decreased plasma glucose and HbA1c levels after repeat administration to *ob/ob* mice, with favorable oral absorption and pharmacokinetics. Repeat administration of AS2034178 enhanced insulin sensitivity in an insulin tolerance test and a euglycemic-hyperinsulinaemic clamp test. These results indicate that improvement of glucose-dependent insulin secretion leads the improvement of whole-body glucose metabolism chronically. In conclusion, AS2034178 and other GPR40 agonists may become useful therapeutics in the treatment of type 2 diabetes mellitus.

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Introduction

Type 2 diabetes mellitus, the most common form of diabetes, is mainly caused by dysfunction of pancreatic insulin secretion or peripheral insulin sensitivity. At present, two types of medications are mainly used to treat type 2 diabetes: insulin secretagogues and insulin sensitizers. Sulfonylureas, dipeptidyl peptidase-IV (DPP-IV) inhibitors and GLP-analogs are generally used as insulin secretagogues; however, sulfonylureas increase insulin secretion regardless of glucose level, thereby carrying the risk of promoting hypoglycemia and accelerating the exhaustion of pancreatic β-cells (Pfeifer et al., 1984; Melander et al., 1990), while DPP-IV inhibitors and GLP-analogs exhibit glucose-dependent insulin secretion and carry a low risk of hypoglycemia (Herman et al., 2006; Engel et al., 2010; Bode B., 2011). It is widely reported that long term use of insulin secretagogues lead to reduction in blood HbA1c level in human (Charbonnel et al., 2013; Hanefeld et al., 2007; Sakamoto et al., 2013; Wang et al., 2011).

Among insulin sensitizers, Pioglitazone and Metformin are generally used. Pioglitazone is a peroxisome proliferator activated receptor (PPAR) -gamma agonist that improves insulin resistance by miniaturizing adipocytes and increasing glucose uptake in skeletal muscle (Smith, 2001; Hauner, 2002), while Metformin improves insulin resistance by suppressing gluconeogenesis in the liver and increasing glucose uptake in skeletal muscle and long term use of insulin sensitizers also lead to reduce blood HbA1c levels in humans (Klip and Leiter, 1990; Campbell et al., 1996; Hundal et al., 2000).

GPR40 is a free fatty acid (FFA) receptor and Gq-type, Gq-coupled G protein-coupled

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receptor that is highly expressed in pancreatic β-cells and whose stimulation by FFAs promotes insulin secretion (Briscoe et al., 2003; Itoh et al., 2003). This FFA signaling pathway is believed to be β-cell-specific, as FFA-stimulated insulin secretion was found to be inhibited on treatment with small interfering RNA of GPR40 (Itoh et al., 2003). Stimulation of Gq type G protein-coupled receptors is known to result in phospholipase C activation and inositol 1,4,5-triphosphate production (Taylor et al., 1991), leading to increased levels of intracellular Ca^{2+} and restricted initiation of insulin secretion (Prentki et al., 1997). Because this cascade potentiates glucose-induced insulin secretion, GPR40 activation increases glucose-dependent insulin secretion (Fujiwara et al., 2005; Nagasumi et al., 2009).

A number of chemical compounds reported to be GPR40 agonists have exhibited glucose-dependent insulin secretion *in vitro* and *in vivo* (Christiansen et al., 2008; Tan et al., 2008; Lin et al., 2011; Tsujihata et al., 2011), suggesting that GPR40 agonists can increase glucose-dependent insulin secretion with low risk of hypoglycemia compared with sulfonylureas. We investigated the chronic whole body effect of GPR40 on glucose metabolism, after screening and modifying novel chemical compounds with GPR40-specific agonist activity.

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Materials and Methods

Chemicals

Sodium 3-[2-fluoro-4-({[1-(2-phenoxyethyl)-1,2,3,4-tetrahydroquinolin-5-yl]methyl}amino)phenyl]propanoateMagnesium (AS2031477, Figure 1A), Sodium 2-({4-[(2',6'-dimethyl[1,1'-biphenyl]-3-yl)methoxy]phenyl}methyl)-3,5-dioxo-1,2,4-oxadiazolidin-4-ide (AS1975063, Figure 1B), bis{2-[(4-{[4'-(2-hydroxyethoxy)-2'-methyl[1,1'-biphenyl]-3-yl]methoxy}phenyl)methyl]-3,5-dioxo-1,2,4-oxadiazolidin-4-ide} tetrahydrate (ASP2034178, Figure 1C), and 5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedioneused (Pioglitazone, Ikeda et al., 1990) in the present study were synthesized by Astellas Pharma Inc. (Ibaraki, Japan). Purity of each compounds were over 99 % analyzed by Waters Xbridge C18, 3.5 micron, 4.6x100mm (Waters, Milford, MA, USA) on HITACHI L-7000 HPLC system (Hitachi High-Technologies, Tokyo, Japan), with a mobile phase of acetonitrile:0.05M KH2PO4 aq. (70:30 or 40:60). Metformin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male ICR mice (aged 5 weeks) were purchased from Japan SLC (Shizuoka, Japan) and fed standard rodent chow (CRF-1; Oriental Yeast, Tokyo, Japan). Six-week old *ob/ob* and *ob/lean* mice (litter of *ob/ob* mice) were purchased from Charles River (Yokohama, Japan). Male *ob/ob*

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mice were fed special breeding rodent chow (CMF; Oriental Yeast, Tokyo, Japan), and *ob/lean* mice were fed CRF-1. Male Zucker fatty and Zucker lean rats (aged 11-12 weeks) were purchased from Japan SLC and fed CRF-1. Water was provided *ad libitum*, and all animals were subjected to a standard 12-h light/dark cycle. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc., and the Tsukuba Research Center at Astellas Pharma Inc. was awarded Accreditation Status by the AAALAC International.

Cell-based Ca^{2+} flux assay

Chinese Hamster Ovary (CHO) cells stably expressing human GPR40 were transfected with a pcDNA 3.1(+) expression vector (Invitrogen, Carlsbad, CA, USA) encoding the HEK293-cell-derived *GPR40* cDNA. Lipofectamine 2000 transfection regent (Invitrogen) was used for stable transfection, and G-418 was used as a selection agent. Cells were incubated with a calcium 3 assay kit (Molecular Devices, Sunnyvale, CA, USA) for 2 h, with compounds added as a 5x solution. Fluorescence over time was measured using a fluorometric imaging plate reader (Molecular Devices). This assay was also used for high-throughput screening and modification chemosynthesis screening.

Insulin secretion assay

MIN6 cells were incubated and washed once and then stimulated with compounds containing 2.8 or 22.4 mM glucose for 20 min. Insulin secreted into the supernatant was measured using an

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enzyme immunoassay (Shibayagi, Gunma, Japan).

Oral glucose tolerance test (OGTT) and single administration test in normal mice

Male ICR mice and male Zucker fatty rats were fasted for 16 h and then orally administered vehicle (0.5% methylcellulose) alone or containing compounds. After 30 min, glucose was orally administered at 2 g/kg/10ml body weight in the OGTT. Blood was collected for plasma preparation from the tail vein into capillary tubes (Chase Scientific Glass, Rockwood, TN, USA) each 10 μl at baseline and 5, 15, 30, 60, and 120 min after administration of glucose in the OGTT and AS2034178 in the single administration test.

Repeat administration study

Male *ob/ob* mice (aged 7 weeks) were divided 10 animals per group. Group allocation was performed a day before administration to ensure that there were no significant differences in fed plasma glucose levels and body weights. Vehicle (0.5% methylcellulose) alone or with AS2031477, AS2034178, Pioglitazone, or Metformin, was orally administered to mice once or twice daily (at 5 p.m. only or 9 a.m. and 5 p.m.) for 2 or 4 weeks. As a control group, vehicle alone was orally administered to normal mice (litter of *ob/ob* mice) twice daily. Blood samples were collected 16 h after final administration by inferior vena cava each 300 μl under isoflurane anesthesia. Euthanasia was performed by cervical dislocation after blood collection. Pancreatic tissue was also collected, weighed, homogenized, and extracted with 2 ml 1.5% HCl-75% ethanol

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buffer for 2 h, then centrifuged for 10 min at 3000 *g*.

Pharmacokinetic study in vivo

Sixteen hours after administering the final dose of the 2 or 4-week repeat administration of vehicle alone or with AS2031477 or ASP2034178 to *ob/ob* mice, an additional dosing in 0.5% methylcellulose was orally administered to mice at 10 ml/kg body weight (3 animals per group). Blood was collected from the tail vein into capillary tubes each 20 μl at baseline and 0.5, 1, 2, 4, 8, 12, and 24 h after dosing, and plasma concentration of AS2031477 and AS2034178 was measured using an XTerra MS C18 column (Waters) on a TSQ Quantum Ultra system (Thermo Scientific, Waltham, MA, USA), with a mobile phase of 20 mM AcONH4:MeCN (50:50).

Insulin tolerance test

Male *ob/ob* mice (aged 7 weeks) were divided into 6 groups (10 animals per group). Either vehicle (0.5% methylcellulose) alone or with 1 mg/kg AS2034178 or Pioglitazone was orally administered once daily (at 5 p.m.) for 10 days. After final administration, mice were fasted overnight (16 h) and then injected intraperitoneally with biosynthetic human insulin (Novolin R; Novo Nordisk, Bagsvard, Denmark) at 0.5 or 1 U/kg body weight. Blood was collected from the tail vein into capillary tubes each 10 μl at baseline and 30 and 60 min after insulin injection.

Euglycemic-hyperinsulinaemic clamp test

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Male Zucker fatty rats (aged 11-12 weeks) were divided into 4 groups (8-9 animals per group). Either vehicle (0.5% methylcellulose) alone or with 30 mg/kg AS2034178 or 10 mg/kg Pioglitazone was orally administered once daily (at 5 p.m.) for 14 days. After final administration, rats were fasted overnight (16 h) and then a euglycemic-hyperinsulinaemic clamp test was conducted. The clamp procedure was performed according to methods described by previous paper (Minoura et al., 2005) with minor modifications. Rats were anesthetized with pentobarbital given intraperitoneally (50 mg/kg) and maintained by administering additional pentobarbital. Ventilation was performed by inserting a catheter into the respiratory tract. Catheters were inserted in one side of femoral veins for blood sampling and in both sides of jugular veins for insulin and glucose infusions. At 55 and 60 min after infusion of [14C]-glucose (d-[U–14C]glucose, 310 mCi/mmol, GE Healthcare LIFE SCIENCE, Amersham, UK) comprised a priming bolus (10 ACi/ 0.1 ml in saline) and constant infusion (25 ACi/ml in saline 0.4 ml/h) blood sample was taken, then basal plasma glucose level was determined in whole blood sample using ACCU-CHEK Active II (Roche Diagnostics). Basal plasma insulin level and [14C]-glucose radioactivity was determined as described below. Infusion of insulin (Humulin R; Eli Lilly Japan Co., Ltd., Hyogo, Japan) was then performed and the time was defined as 0 min. Insulin was intravenously infused at rates of 90 (0-3 min), 60 (3-6 min), 37.5 (6-10 min) and 30 mU/kg/min (>10 min). Blood samples were drawn at 4-min intervals for determination of plasma glucose level from 10 min after starting the infusion of insulin. Glucose solution (40% [w/v]) was infused at an adequate rate to maintain the plasma glucose level at approximately 100 mg/dl. Three

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consecutive samples of blood were then collected for determination of plasma glucose, insulin levels, and radioactivity before the end of the clamp procedure. Plasma samples (50 µl each) were mixed with 75 µl of 0.15 M Ba(OH)2 and 75 µl of 0.15 M ZnSO4 and then centrifuged after being allowed to sit at room temperature for 20 min to obtain supernatant. A total of 150 µl of supernatant was then applied to AG anion exchange resin (AG1-X8 or AG2-X8; Bio-Rad Laboratories, CA, USA) and eluted twice with 0.75 ml distilled water. Elution was mixed with aqueous scintillant (Aquazol-2; Packard Instrument Company Inc., Meriden, CT, USA) and its radioactivity was counted by using a liquid scintillation analyzer (1900TR or 2200CA; Packard Instrument Company Inc., CT, USA). Hepatic glucose production and glucose disappearance rate (peripheral glucose utilization) were calculated using Steele's equation (Steele, 1959).

PPAR gamma transactivation assay

This assay was performed according to methods described by previous paper (Minoura et al., 2004). CV-1 cells were transfected with an expression and reporter plasmids used in Minoura et al., 2004. After transfection, the cells were harvested and seeded into a 96-well plate at a density of 18000 cells/well in 50 µl of DMEM with 10% delipidated and charcoal-treated fetal calf serum. After the cells had adhered, test compound was added. Cells were lysed and the luciferase activity was measured in a luminometer (MLX Microtiter Plate Luminometer, Dynatech Laboratories, VA, USA) using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

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In vivo sample measurement methods

Plasma glucose levels were measured using glucose CII-test reagent (Wako Pure Chemical Industries, Tokyo, Japan). Plasma and pancreas insulin levels were measured using the murine insulin ELISA kit (Shibayagi). Blood hemoglobin A1c (HbA1c) levels were measured using a DCA2000 Analyzer (Bayer, Leverkusen, Germany). Plasma triglyceride and FFA levels were determined using the Triglyceride E-test (Wako) and the NEFA C-test (Wako).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism5 software (GraphPad Software, San Diego, CA, USA) with data expressed as the mean ± S.E.M. Statistically significant differences between pairs of groups were determined using Student's t-test, while those across multiple groups were assessed using Dunnett's multiple range test. Statistical significance was established as $P < 0.05$.

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Results

Novel GPR40 agonists AS2031477, AS1975063 and AS2034178

We identified novel GPR40-specific agonists via high-throughput screening. Because GPR40 is a Gq-coupled receptor that signals through an increase in intracellular Ca^{2+} levels, we developed GPR40-over-expressing CHO cells and evaluated the increase in intracellular Ca^{2+} concentration caused by each chemical compound. High-throughput screening of a chemical library containing approximately 166,000 low-molecular weight compounds showed that a number of compounds markedly increased Ca^{2+} concentration. To identify agonists with excellent *in vivo* activity, we next narrowed down our selection to those which also demonstrated lengthy persistence in blood on oral administration in mice. We also conducted modification chemosyntheses on approximately 250 compounds, ultimately selecting AS2031477, AS1975063 and AS2034178, which demonstrated the greatest increase in intracellular Ca^{2+} levels dose-dependently (Figure. 2A).

The maximum efficacy of this increase in Ca^{2+} was nearly equal to that of linolenic acid, an endogenous ligand of GPR40 and the potencies of AS2031477, AS1975063 and AS2034178 were much higher than that of linolenic acid (Figure. 2A).

We also developed human GPR41-, GPR43-, GPR119-, and GPR120-over-expressing CHO cells and evaluated the increase in intracellular Ca^{2+} concentration caused by AS2034178 using these cells (data not shown). Only GPR40-expressing cells exhibited increased intracellular Ca^{2+} (showing no increase at doses of 10 μM in other cells). Because some GPR40 agonists have been

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reported to exhibit glucose-concentration-dependent insulin secretion, we evaluated this activity using AS2031477, AS1975063 and AS2034178 in pancreas β-cell-derived MIN6 cells. These compounds induced dose-dependent and significant insulin secretion only under high-glucose conditions (22.4 mM), with no increase in secretion under low-glucose conditions (2.8 mM) (Figure. 2B-D). Taken together, these data indicated that these compounds are GPR40-specific agonists which increase glucose-dependent insulin secretion in a pancreatic β-cell-derived cell line.

Effect of a single dose of AS1975063 and AS2034178 on oral glucose tolerance under fasting conditions in normal mice and Zucker fatty rats

To examine the glucose-dependent insulin secretion *in vivo*, first, we conducted an OGTT using normal mice and diabetic model Zucker fatty rats using AS1975063. After 16 h fasting, single-dose oral administration of AS1975063 at 1, 3 or 10 mg/kg 30 min before glucose administration induced dose-dependent suppression of plasma-glucose increase after oral glucose administration in both species (Figure. 3A & 3C). At the same time, plasma insulin levels after oral glucose administration increased dose-dependently in both species (Figure. 3B & 3D).

Next we conducted an OGTT using AS2034178 at 0.3 to 10 mg/kg in normal mice. AS203418 also induced dose-dependent suppression of plasma-glucose increases after oral glucose administration, and the decrease in plasma-glucose AUC level was significant at doses over 1 mg/kg (Figure. 4A & 4B). At the same time, plasma insulin levels after oral glucose

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administration increased, and plasma insulin levels at five minutes after glucose administration were dose-dependently and significantly increased at dosages of AS2034178 over 3 mg/kg (Figure. 4C & 4D).

To clarify the glucose dependency of AS2034178, we then conducted a single-dose administration test of AS2034178 at doses ranging from 1 to 10 mg/kg in fasting normal mice without oral glucose administration. Results showed that plasma glucose levels after AS2034178 administration were unchanged at any dose (Figure. 4E), and plasma insulin levels were also unchanged (Figure. 4F).

Effect of AS2031477 and AS2034178 after repeat administration and plasma concentration transition in diabetic ob/ob mice

To examine the long-term effect of GPR40 agonists on diabetic model mice *in vivo*, first AS2031477 was administered orally twice daily for two weeks to *ob/ob* mice, which are leptin-code-gene deficient and the major mouse model of type 2 diabetes. AS2031477 was administered at 3 to 30 mg/kg. As a result, plasma glucose levels and blood HbA1c level were significantly decreased (Figure 5A & 5B). Further, plasma insulin levels significantly decreased dose-dependently (Figure 5C) and pancreatic insulin content showed a tendency to increase at higher doses (Figure 5D). In this study, body weights of all dose AS2031477 administration groups were unchanged (Figure 5E).

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We sequentially measured plasma concentration of AS2031477 after single (3 mg/kg) or repeated (3 mg/kg twice daily for 2 weeks) administration in *ob/ob* mice. Maximum concentrations exhibited some decrease by repeat administration (1.7 μg/ml vs. 0.84 μg/ml), but plasma half-lives were slightly extended ($t_{1/2}$ = 2.0 h vs. 2.7 h) (Figure 5F).

Next we conducted this repeat administration study in detail using AS2034178 at 0.03 to 1 mg/kg with 1 mg/kg of Pioglitazone, and 200 mg/kg of Metformin as control agents in *ob/ob* mice. As a result, plasma glucose levels significantly decreased in mice receiving AS2034178 and Pioglitazone, and the effect of AS2034178 was dose-dependent and reached maximum efficacy at dosages over 0.1 mg/kg (Figure 6A). Blood HbA1c levels significantly decreased in mice receiving all compounds, and this effect in mice receiving AS2034178 was also dose-dependent (Figure 6B). Plasma insulin levels significantly decreased upon administration of all compounds, and the decrease with AS2034178 was dose-dependent (Figure 6C). Pancreatic insulin content was significantly increased by AS2034178 and Pioglitazone, and the effect of AS2034178 was dose-dependent and reached maximum efficacy at dosages over 0.1 mg/kg (Figure 6D). Plasma FFA levels significantly decreased with Pioglitazone and Metformin (Figure 6E) but plasma triglyceride levels were unchanged in all groups (Figure 6F). With the exception of a significant decrease in body weight at 1 mg/kg dose, all other doses of AS2034178 had no effect on body weight (Figure 6G).

Maximum concentrations of AS2034178 were nearly equal after single or repeated dosing respectively (0.13 μg/ml vs. 0.10 μg/ml), and plasma half-lives were also nearly equal

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respectively ($t_{1/2}$ = 7.1 h vs. 7.4 h). These results indicate that AS2034178 exhibited no plasma concentration accumulation by repeat administration (Figure 6H). And the $t_{1/2}$ of AS2034178 was longer than that of AS2031477 in *ob/ob* mice.

Taken together, these results indicate that repeat administration of GPR40 agonists decreases plasma glucose, insulin, and blood HbA1c levels while increasing pancreatic insulin content. As all effects of repeat administration with GPR40 agonists were observed in the direction expected for the normalization of the diabetic pathology of *ob/ob* mice, these results suggest that GPR40 agonists do indeed improve whole-body glucose metabolism in diabetic *ob/ob* mice.

Insulin resistance improvement effect of AS2034178 on long term treatment

The effects of AS2034178 on repeat administration to *ob/ob* mice resembled those of the insulin sensitizer Pioglitazone, suggesting that AS2034178 may have the potential to improve insulin resistance as a result of whole-body glucose metabolism improvement. To examine this possibility, we conducted an insulin tolerance test and a euglycemic-hyperinsulinaemic clamp test.

In the insulin tolerance test, we first conducted a 10-day trial of once daily repeat administration to *ob/ob* mice of AS2034178 (1 mg/kg), Pioglitazone (1 mg/kg), or vehicle only. After repeat administration and overnight fasting, we conducted an insulin tolerance test via insulin administration (0.5 and 1 U/ml). At both insulin doses, AS2034178 and Pioglitazone caused significant decreases in plasma glucose at 30 and 60 min after insulin administration

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compared to vehicle only (Figures 7A & 7B). These results indicate that repeat administration of AS2034178 can increase insulin sensitivity as a result of chronic treatment.

For the euglycemic-hyperinsulinaemic clamp test, we first conducted a 14-day trial of once daily repeat administration of AS2034178 (30 mg/kg), Pioglitazone (10 mg/kg), or vehicle only to Zucker fatty rats and vehicle only to normal rats. After repeat administration and overnight fasting, plasma glucose levels of AS2034178 administration group was significantly decreased although the decrease was smaller than Pioglitazone administration group (Figure 8A). Plasma insulin levels of AS2034178 administration group was also significantly decreased although the decrease was also smaller than Pioglitazone administration group (Figure 8B). Body weights of the AS2034178 administration group were significantly increased although the increase was smaller than Pioglitazone administration group (Figure 8C). Then, we conducted a euglycemic-hyperinsulinaemic clamp test by infusing d-[U–14C]glucose, insulin, and glucose. After the infusion reached a steady state, we collected plasma samples and calculated peripheral glucose utilization and hepatic glucose production by examining the data of influx d-[U–14C]glucose, influx cold-glucose, clamp-state plasma d-[U–14C]glucose level and plasma cold-glucose level. Results showed that peripheral glucose utilization in the AS2034178 administration group was significantly increased compared to the vehicle administration group (Figure. 8D) and hepatic glucose production in the AS2034178 administration group was significantly decreased compared to the vehicle administration group (Figure. 8E), although these effects were weaker than those observed in the Pioglitazone administration group.

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Discussion

Two types of medications are primarily used to treat type 2 diabetes—insulin secretagogues and insulin sensitizers. Although these two types of medicine have different mechanisms and are prescribed based on the pathology of each patients, both types of medicine can improve whole body glucose metabolism and decrease blood HbA1c levels by long term use.

Here, we discovered the novel GPR40 agonists AS2031477, AS1975063 and AS2034178, and now they can improve not only acute glucose dependent insulin secretion but also chronic whole body glucose metabolism. Although previous reports have demonstrated beneficial effects of GPR40 agonists on acute glucose-dependent insulin secretion, to our knowledge, ours are the first to show such a chronic effect on glucose metabolism improvement.

Insulin secretagogues promote acute insulin secretion and improve acute glucose metabolism by single dosing. However, long term use of insulin secretagogues produce whole body glucose metabolism improvement and decrease blood HbA1c levels (Charbonnel et al., 2013; Hanefeld et al., 2007; Sakamoto et al., 2013; Wang et al., 2011). It is believed that repeat effects of acute glucose metabolism improvement by insulin secretagogues decreases glucotoxity and improves whole body glucose metabolism and insulin resistance improvement secondarily. Our data indicate that long-term treatment with GPR40 agonists improves whole body glucose metabolism and insulin resistance. These effects may be caused secondarily by the decrease of glucotoxity produced by the repeat glucose dependent insulin secretion.

The insulin sensitizer Pioglitazone improves insulin resistance mainly in peripherally

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organized adipocytes and skeletal muscles. Indeed, chronic treatment with Poglitazone resulted in decreased plasma glucose levels and blood HbA1c (Figure. 6A & 6B), increased responsiveness to insulin in an insulin tolerance test (Figure. 7A & 7B), and increased peripheral glucose utilization and decreased hepatic glucose production in the euglycemic-hyperinsulinaemic clamp test (Figure. 8D & 8E)—all effects believed to be due to enhancement of insulin sensitivity. AS2034178 also exhibited similar effects to Pioglitazone (Figure. 6A - D, 7A & 7B, 8D & 8E) although the effects on insulin resistance improvement are weaker than those dose to Pioglitazone. Metformin is one of the insulin sensitizers that suppresses gluconeogenesis in the liver and increases glucose uptake in skeletal muscle, and it exhibited decreases of blood HbA1c and plasma insulin levels (Figure. 6B & 6C). However, because these effects were weaker than Pioglitazone and it was not effective on decreasing plasma glucose or increasing pancreatic insulin content, whole body glucose metabolism improvement effect of Metformin is thought to be limited in this *ob/ob* mouse model.

We also confirmed that AS2031477 and AS2034178 are GPR40-specific agonists, that did not affect the activity of PPAR-gamma (<50% of the maximum efficacy of Pioglitazone up to 10 μM), GPR41, GPR43, GPR119, or GPR120. These findings suggest that AS2031477 and AS2034178 improve chronic glucose metabolism by activating GPR40. Although GPR40 activation has not been shown to have this effect in animal models, the GPR40 agonist TAK-875 has been reported to reduce fasting plasma glucose and HbA1c levels in clinical trials (Araki et al., 2012; Burant et al., 2012). Taken together, these present and previous findings indicate the

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possibility that chronic treatment with GPR40 agonists can improve whole-body glucose metabolism by long term use in animals and humans.

Although both AS2031477 and AS2034178 exhibited the whole body glucose metabolism improvement, the potency of AS2034178 was higher than that of AS2031477. In *ob/ob* mice, the $t_{1/2}$ of AS2034178 was longer than AS2031477 and AS2034178 exhibited no plasma concentration accumulation by repeat administration. Taken together, this difference in potency between agents may depend upon the pharmacokinetic profile of these compounds.

By the repeat administration of AS2034178, some body weight changes were observed. Body weight decrease was observed at 1 mg/kg administration group in *ob/ob* mice, and body weight increases were observed at 30 mg/kg administration group in Zucker fatty rats. The cause of these body weight changes were unknown, however, we think it is not related to the efficacy of whole body glucose metabolism improvement of AS2034178. First, the efficacy of metabolism improvement by AS2034178 was observed from 0.03 mg/kg and significant improvement on plasma glucose levels, HbA1c levels, insulin levels, and pancreatic insulin contents were observed from 0.1 mg/kg in *ob/ob* mice, however body weights were unchanged up to 0.3mg/kg. Second, AS2031477 also exhibited whole body glucose metabolism improvement in *ob/ob* mice with no body weight changes.

Although AS2034178 has the effect of glucose-dependent insulin secretion, chronic treatment with AS2034178 reduced plasma insulin levels and increased pancreas insulin content (Figure. 6C & 6D). In *ob/ob* mice, the animal model for hyperinsulinemia, fed plasma insulin

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concentration reached 234 ng/ml while the value in normal mice was only 1.4 ng/ml (Figure. 6C). So, we consider that plasma insulin level was decreased to normalize whole body glucose metabolism in *ob/ob* mice. Glucotoxity decreases due to the repeat insulin secretion may improve insulin resistance and decrease the necessity of insulin. Although insulin secretagogues have increased risk of pancreatic β-cell exhaustion, AS2034178 may have lower risk of pancreatic β-cell exhaustion because pancreatic insulin content increased in response to AS2034178.

HbA1c levels decreased approximately 1.5% on repeat administration of AS2034178 (Figure. 6B). In type 2 diabetes mellitus patients, slight reductions of plasma HbA1c levels (<1%) are correlated with significant decreases in the incidence of microvascular complications, such as retinopathy and nephropathy, which are often responsible for decreased quality of life ((UK Prospective Diabetes Study (UKPDS) Group 1998). AS2034178 therefore has the therapeutic potential to improve the prognosis of type 2 diabetes patients by potentially reducing microvascular complications.

In conclusion, the novel GPR40 agonists improve whole body glucose metabolism after long term treatment. We believe that GPR40 agonists represent a new class of drugs for treating type 2 diabetes, with AS2034178 as a particularly promising candidate.

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Authorship Contributions

Participated in research design: Tanaka, Yoshida, Oshima, Yamazaki, Matsui, and Shibasaki.

Conducted experiments: Tanaka, Yoshida, Oshima, Minoura, Sakuda, and Yamazaki.

Contributed new reagents or analytic tools: Negoro and Iwasaki.

Performed data analysis: Tanaka, Yoshida, Oshima, Yamazaki, and Sakuda.

Wrote or contributed to the writing of the manuscript: Tanaka

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Footnotes

All authors are currently or were employees of Astellas Pharma Inc. and may own stock or hold stock options in Astellas Pharma Inc.

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Legends for Figures

Figure 1

Chemical structures of (A) AS2031477. (B) AS1975063. (C) AS2034178.

Figure 2

In vitro effects of AS2034178 on Ca^{2+} increase and glucose-stimulated insulin secretion. (A) Ca^{2+} increase in GPR40-over-expressing CHO cells. (B-D) Glucose-stimulated insulin secretion in MIN6 cells. Data are presented as mean ± S.E.M. from (A) three and (B-D) five independent experiments. *P <0.05, **P <0.01 vs. no compound control cells.

Figure 3

Effect of single administration of AS1975063 on glucose dependent insulin secretion in (A, B) normal mice and (C, D) Zucker fatty rats. (A, C) Time course of changes in plasma glucose levels during the OGTT. (B, D) Time course of changes in plasma insulin levels during the OGTT. Data are presented as mean ± S.E.M. of each group of 6-8 animals.

Figure 4

Effect of single administration of AS2034178 on glucose dependent insulin secretion in normal mice. (A) Time course of changes in plasma glucose levels during the OGTT. (B) Area under the

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plasma glucose concentration-time curve (AUC) during the OGTT until 120 min after glucose administration. (C) Time course of changes in plasma insulin levels during the OGTT. (D) Plasma insulin levels 5 min after glucose administration. Time course of changes in the (E) Plasma glucose levels and (F) Plasma insulin levels after only AS2034178 administration. Data are presented as mean ± S.E.M. of each group of 6-8 animals. * *P* <0.05, ** *P* <0.01 vs. vehicle control group.

Figure 5

Effects of repeat administration of AS2031477 on blood and pancreas parameters in diabetic model *ob/ob* mice and normal mice (N). Vehicle alone (N and V) and AS2031477 were orally administered twice daily for two weeks. (A) Plasma glucose levels, (B) Blood HbA1c levels, (C) Plasma insulin levels, (D) Pancreatic insulin levels, (E) Body weight. Data are presented as mean ± S.E.M. for 10 animals. *, *P* <0.05, **, *P* <0.01 vs. vehicle control group. (F) Plasma concentration of AS2031477 at single administration (3 mg/kg) and after 2 weeks of repeat administration (3 mg/kg). Each sample was measured by mixing plasma sample from 3 animals.

Figure 6

Effects of repeat administration of AS2034178 and other anti-diabetic agents on blood and pancreas parameters in diabetic model *ob/ob* mice and normal mice (N). Vehicle alone (N and V), AS2034178, Pioglitazone (Pio), and Metformin (Met) were orally administered once daily for

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four weeks. (A) Plasma glucose levels, (B) Blood HbA1c levels, (C) Plasma insulin levels, (D) Pancreatic insulin levels, (E) Plasma FFA levels, (F) Plasma triglyceride levels, (G) Body weight. Data are presented as mean ± S.E.M. for 10 animals. $*/^{\#}/^{\$}$, *P* <0.05, $**/^{\#\#}/^{\$\$}$, *P* <0.01 vs. vehicle control group from each of group 10 animals. (H) Plasma concentration of AS2034178 at single administration (0.3 mg/kg) and after 4 weeks of repeat administration (0.3 mg/kg). Each sample was measured by mixing plasma sample from 3 animals.

Figure 7

Effect of AS2034178 repeat administration on insulin tolerance test. (A, B) Time course of changes in plasma glucose levels after insulin administration. Biosynthetic human insulin at 0.5 (A) or 1 (B) U/kg body weight was administered intraperitoneally. The change in the plasma glucose levels were exhibited as the delta glucose average from baseline. Data are presented as means ± S.E.M. for 5 animals. */# P <0.05, **/## P <0.01 vs. vehicle control group.

Figure 8

Effects of repeat administration of AS2034178 in diabetic model Zucker fatty rats. Vehicle alone (V), AS2034178, and Pioglitazone were orally administered once daily for two weeks. (A) Plasma glucose levels, (B) Plasma insulin levels, (C) Body weight. (D, E) Effect of AS2034178 repeat administration on euglycemic-hyperinsulinaemic clamp test. (D) Peripheral glucose utilization and (E) Hepatic glucose production at clamp state. Data are presented as means ± S.E.M. for 8-9

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animals. ## P <0.01 vs. normal control group *, P <0.05, **P <0.01 vs. vehicle control group.

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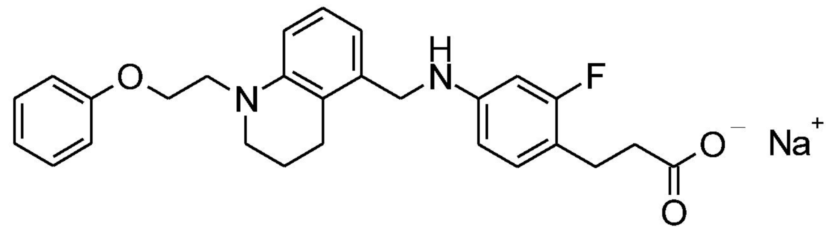
A



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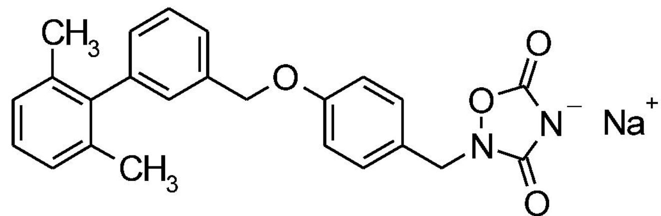

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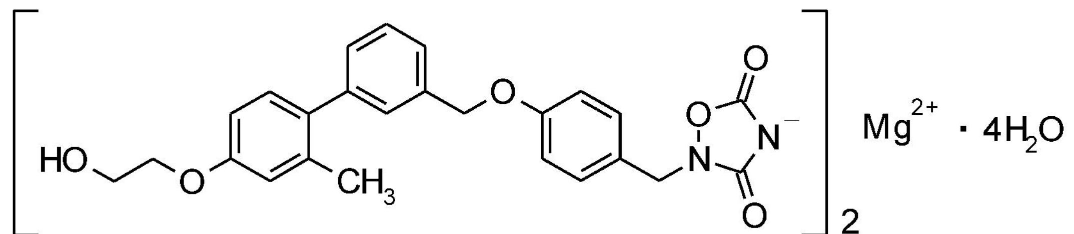

Figure 1

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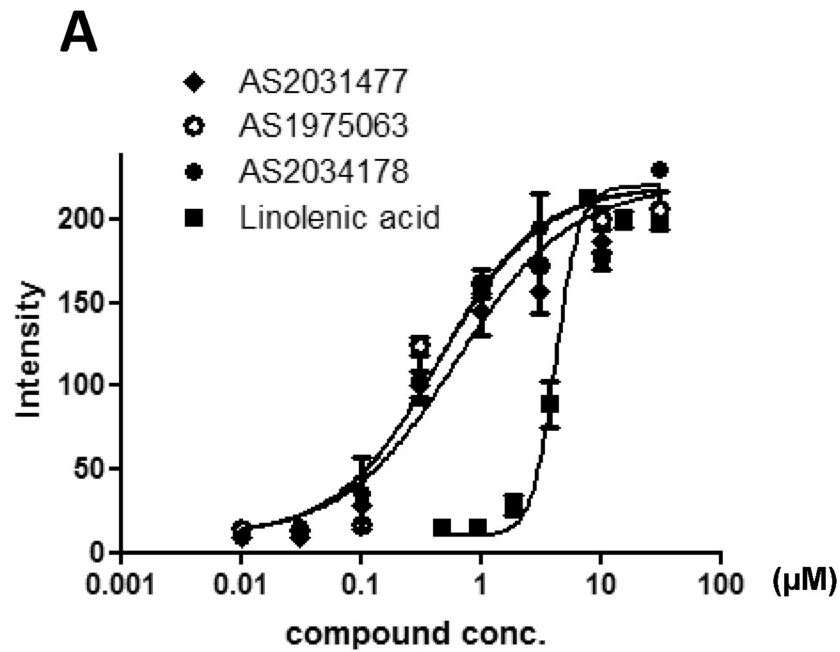

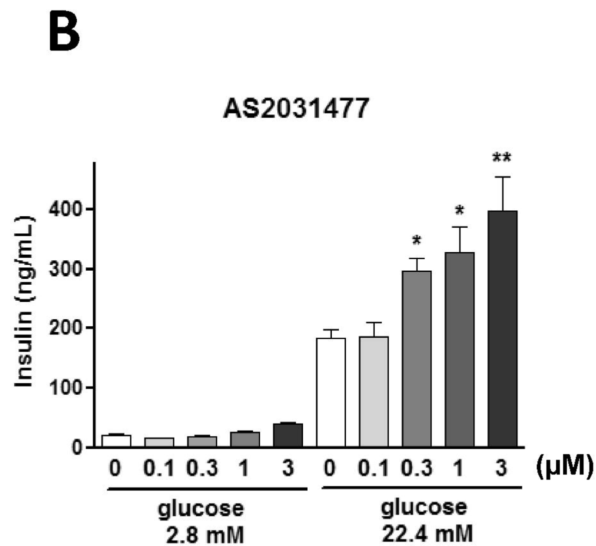

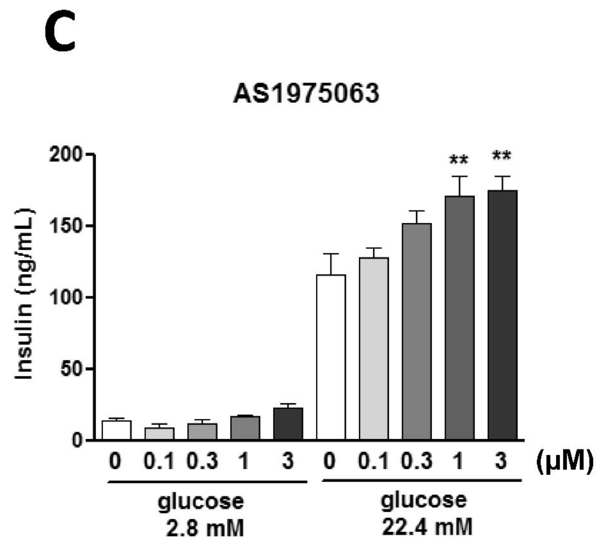

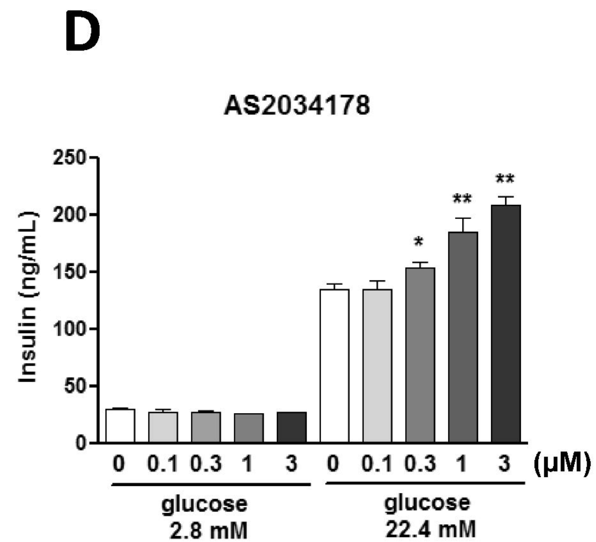

Figure 2

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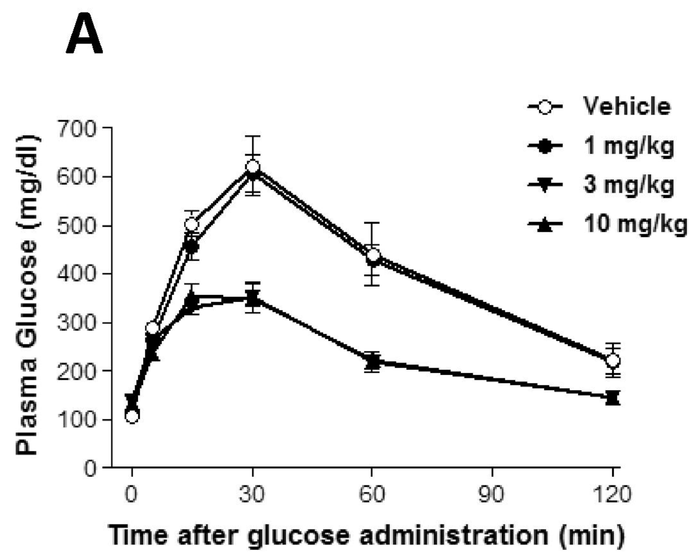

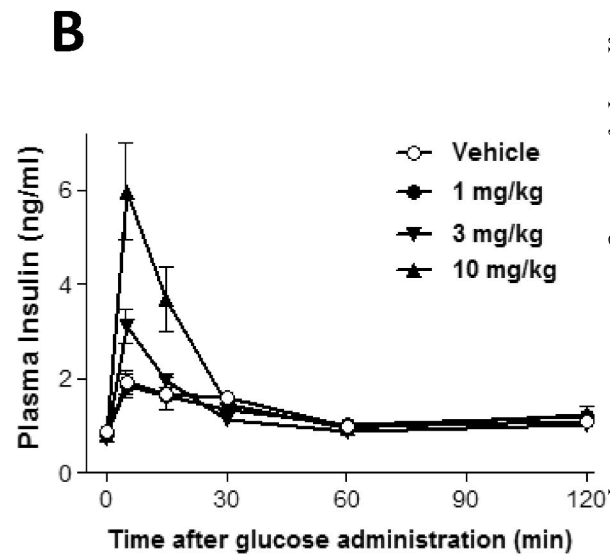

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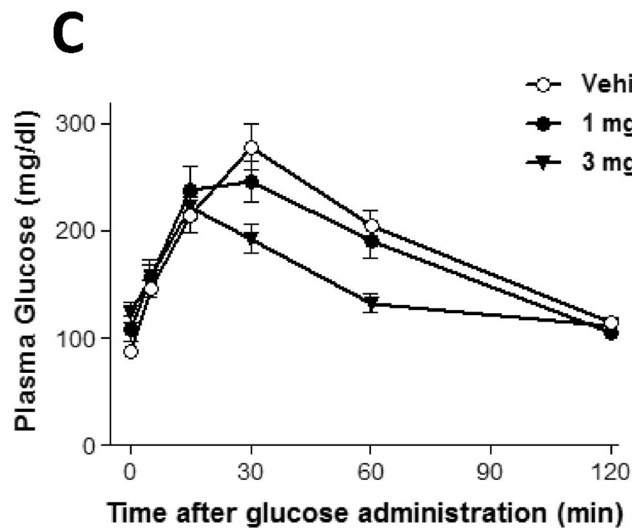

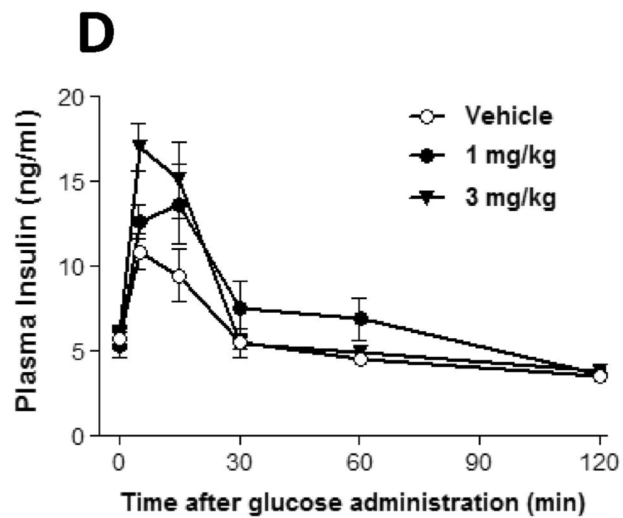

Figure 3

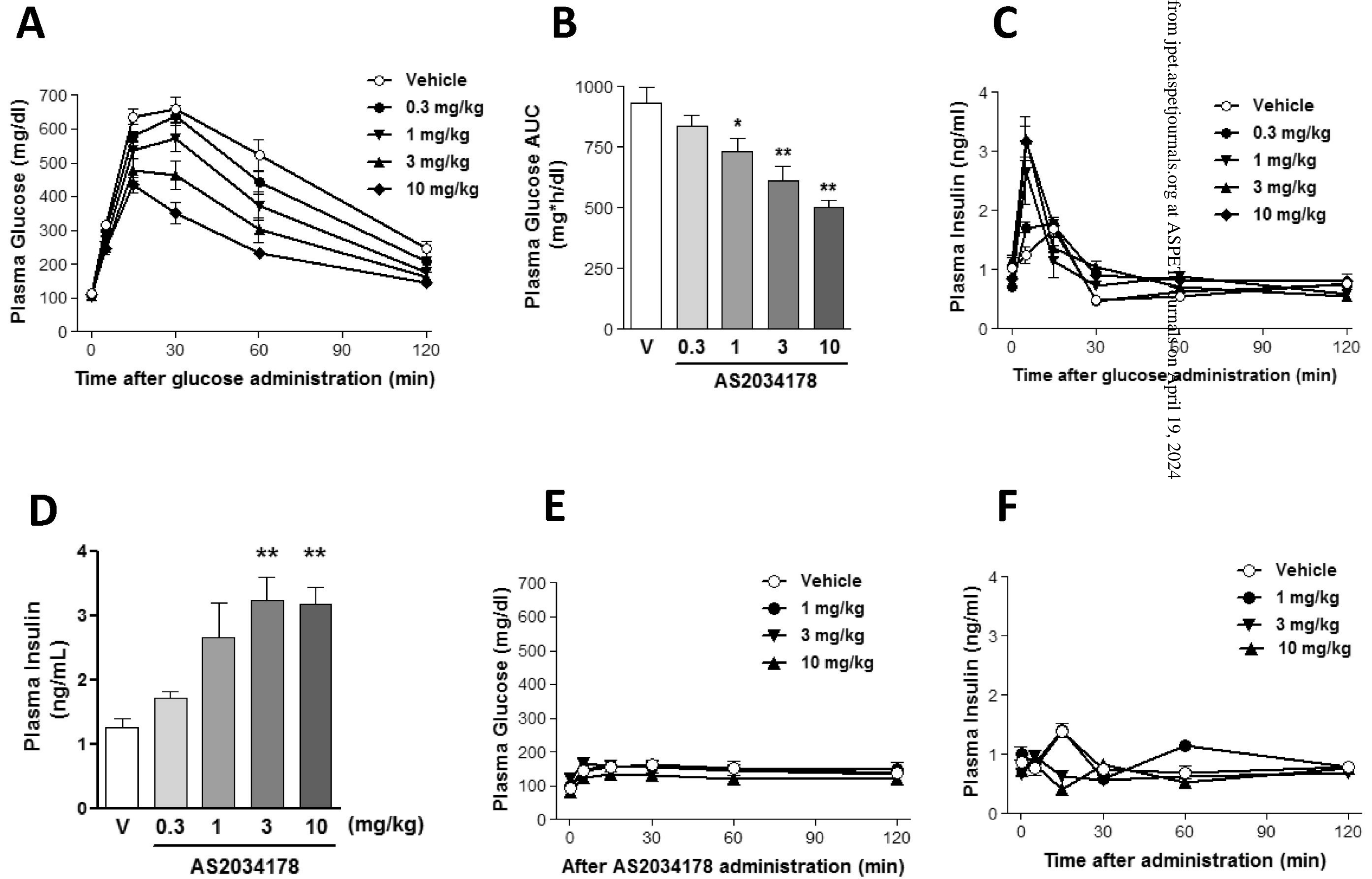

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Figure 4

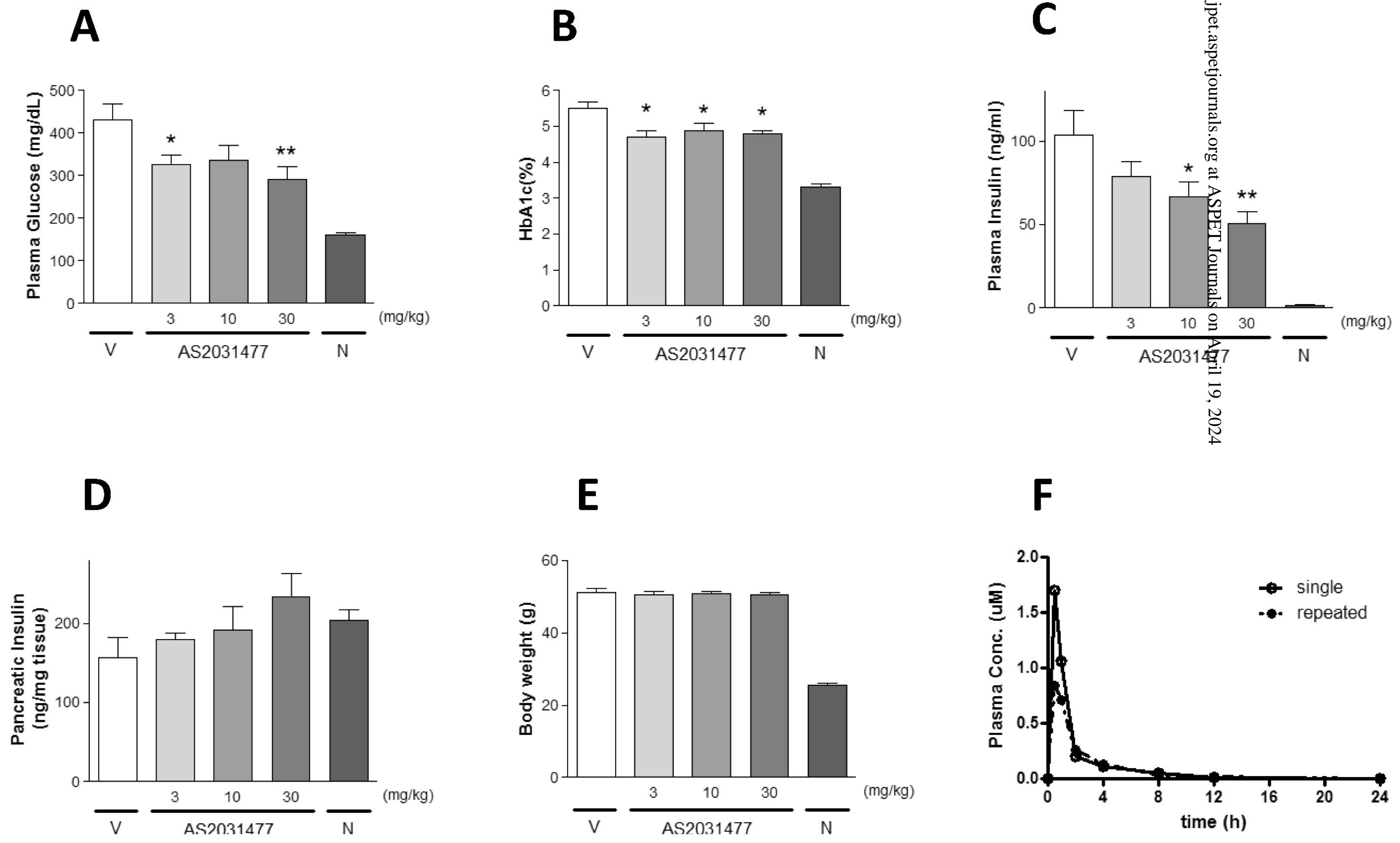

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Figure 5

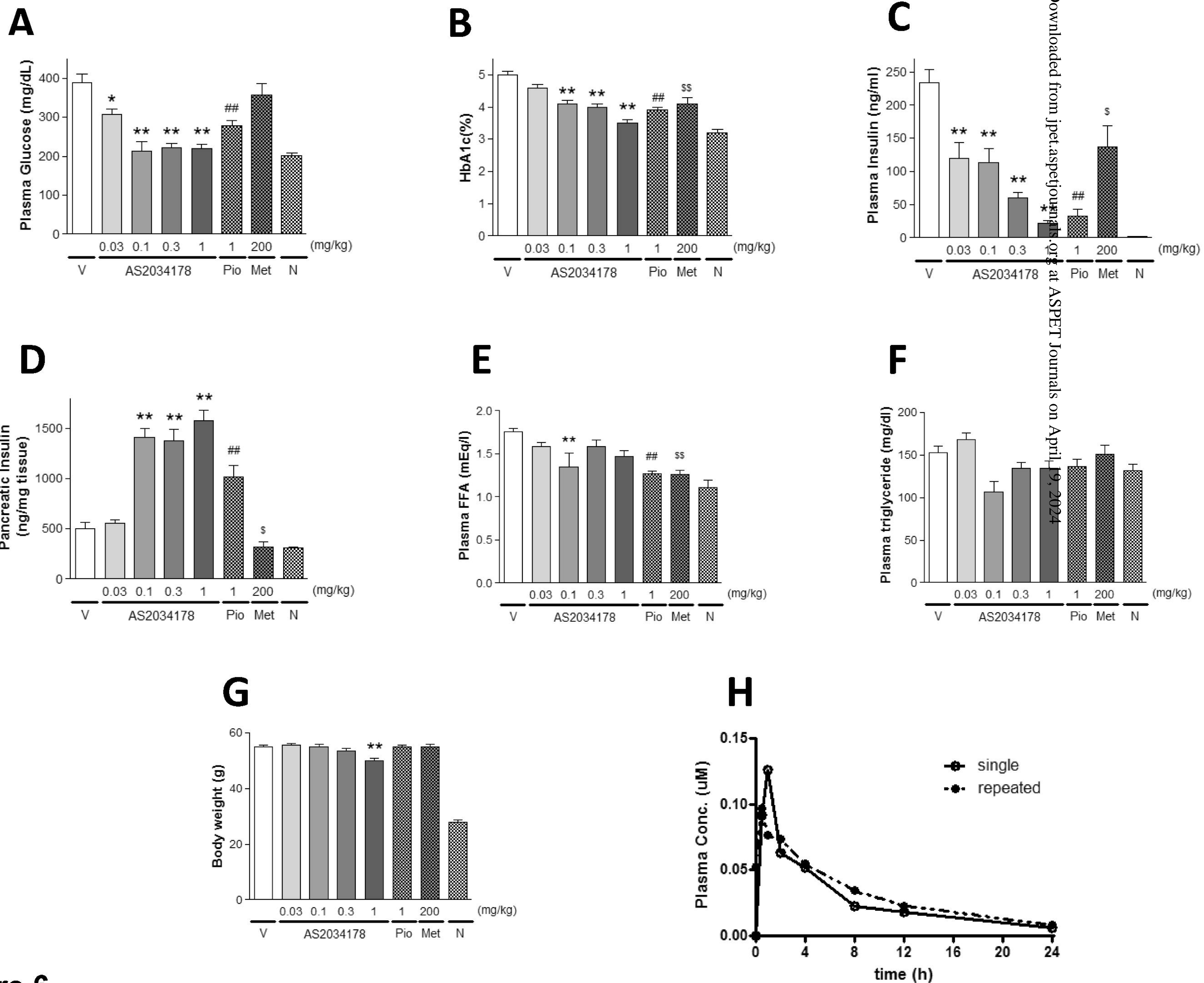

Figure 6

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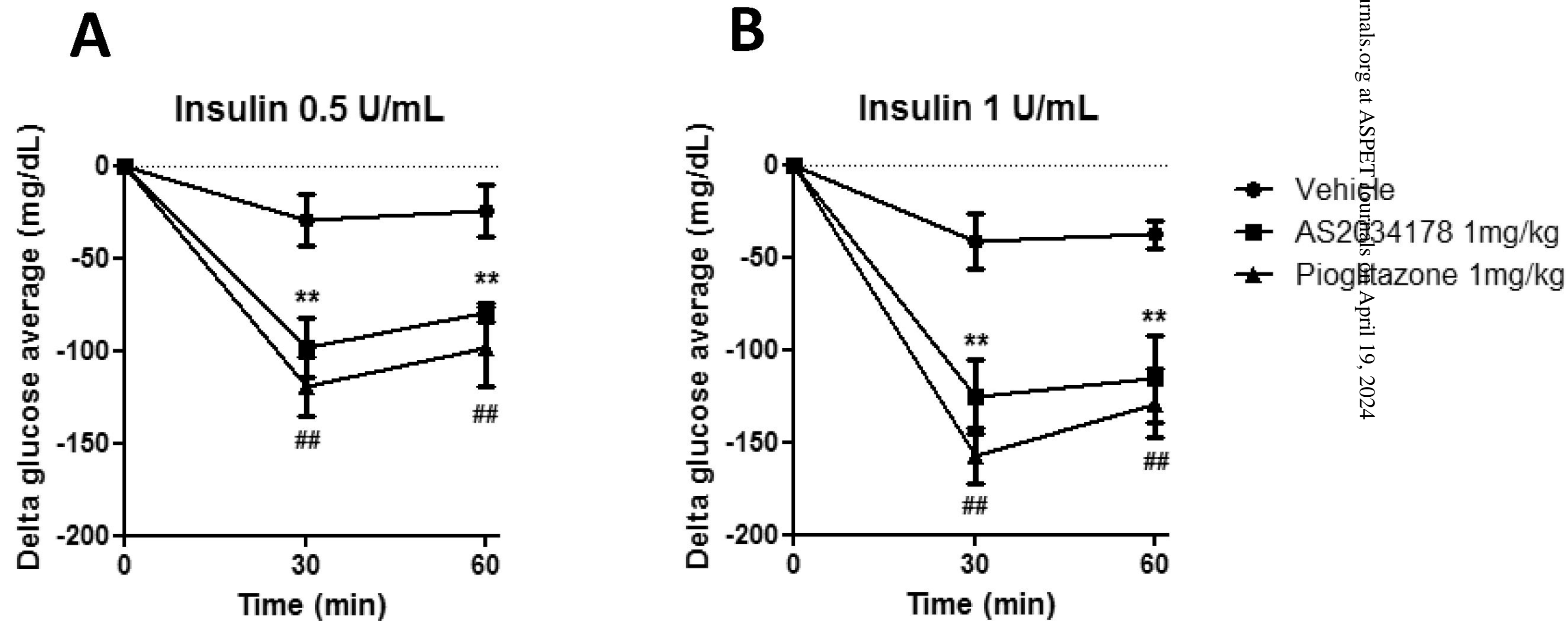

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Figure 7

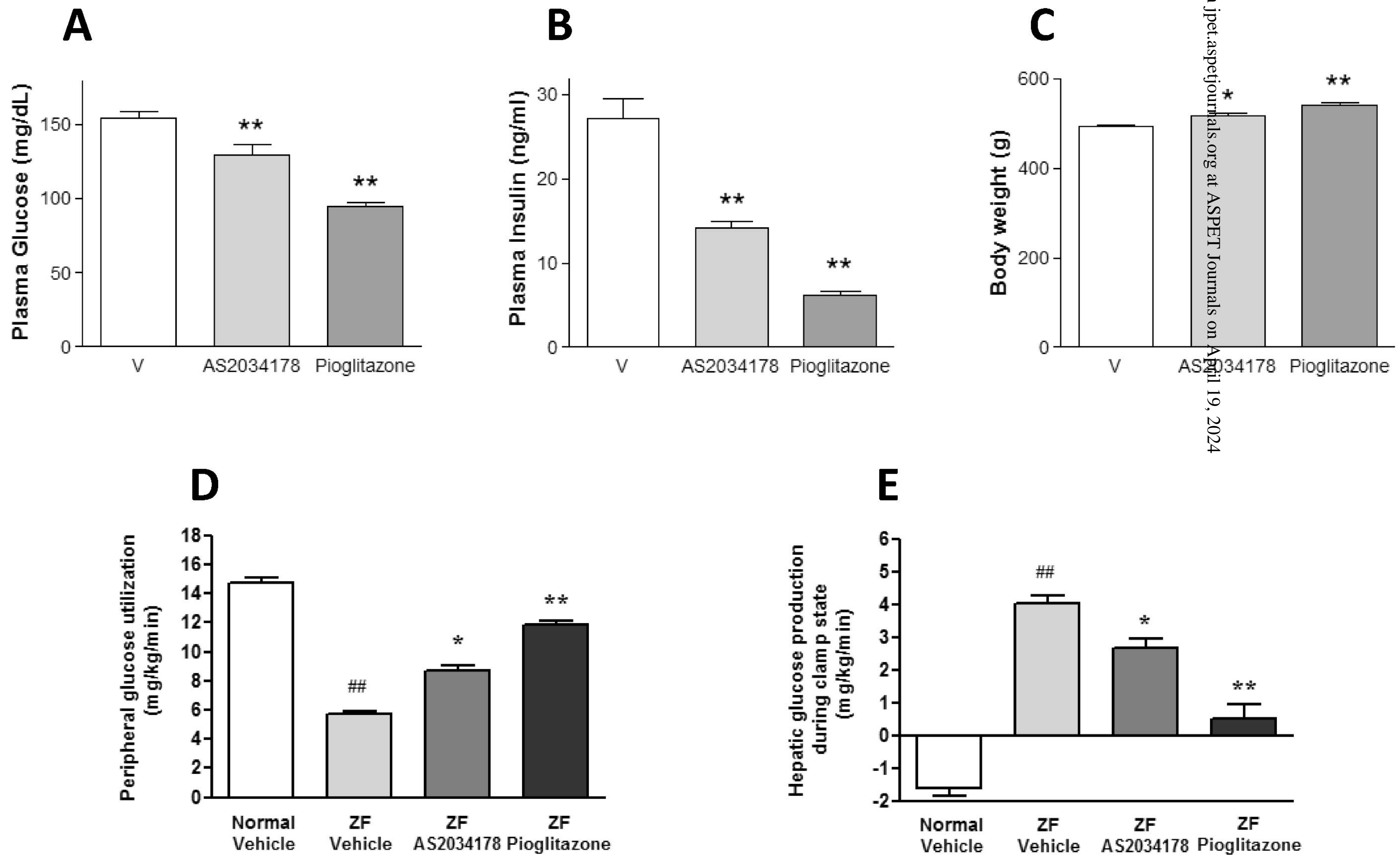

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Figure 8