

Fasiglifam/TAK-875, a Selective GPR40 Agonist, Improves Hyperglycemia in Rats Unresponsive to Sulfonylureas and Acts Additively with Sulfonylureas^[S]

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Received November 25, 2015; accepted January 21, 2016

ABSTRACT

Sulfonylureas (SUs) are widely used insulin secretagogues, but they have adverse effects including hypoglycemia and secondary failure. Fasiglifam/TAK-875, a selective GPR40 agonist, enhances glucose-stimulated insulin secretion and improves hyperglycemia. In the present study, we compared the *in vivo* glucose-lowering effects of fasiglifam with SUs. The risk of secondary failure of fasiglifam and the efficacy in rats desensitized to SUs were also evaluated. Moreover, we assessed whether fasiglifam was effective when combined with SUs. In diabetic neonatally streptozotocin-induced rats 1.5 days after birth (N-STZ-1.5), oral administrations of fasiglifam (3–30 mg/kg) dose dependently improved glucose tolerance; the effect was greater than that of glibenclamide at maximal effective doses (glucose AUC: fasiglifam, –37.6%; glibenclamide, –12.3%). Although the glucose-lowering effects of glibenclamide (10 mg/kg/day) were completely

diminished in N-STZ-1.5 rats after 4 weeks of treatment, effects were maintained in rats receiving fasiglifam (10 mg/kg/day), even after 15 weeks. Fasiglifam (3–10 mg/kg) was still effective in two models desensitized to SUs: 15-week glibenclamide-treated N-STZ-1.5 rats and aged Zucker diabetic fatty (ZDF) rats. Acute administration of fasiglifam (3 mg/kg) and glimepiride (10 mg/kg) in combination additively decreased glucose AUC (fasiglifam, –25.3%; glimepiride, –20.0%; combination, –43.1%). Although glimepiride (10 mg/kg) decreased plasma glucose below normal in nonfasted control rats, fasiglifam (3 mg/kg) maintained normoglycemia, and no further exaggeration of hypoglycemia was observed with combination treatment. These results indicate that GPR40 agonists could be more effective and durable than SUs. Our results also provide new insights into GPR40 pharmacology and rationale for the use of GPR40 agonists in diabetic patients with SU failure.

Introduction

The loss of glucose-dependent insulin secretion from pancreatic β -cells is responsible for the onset and progression of type 2 diabetes (Leahy, 2005; Wajchenberg, 2007). Oral agents that stimulate insulin secretion such as sulfonylureas (SUs) have been widely used for the treatment of type 2 diabetes therapy for nearly 30 years (Rendell, 2004). These agents stimulate insulin secretion by closing ATP-sensitive K^+ channels on β -cells, leading to depolarization and subsequent increase in intracellular Ca^{2+} . However, SUs act on β -cells to continuously secrete insulin independent of blood glucose levels, which may lead to hypoglycemia. In addition, after long-term SU therapy, a number of patients begin to show an

increase in blood glucose levels, necessitating the addition of other drugs with a different mechanism of action (Matthews et al., 1998). This clinical phenomenon has been termed secondary failure of SUs. This is thought to be broadly associated with β -cell exhaustion by long-term overstimulation and glucose toxicity caused by poor glycemic control (Poitout and Robertson, 2002; Rustenbeck, 2002). Therefore, there is a need for agents that induce glucose-dependent insulin secretion through new mechanisms of action, with low risk of the adverse effects associated with SU treatment.

GPR40/FFAR1 is a G-protein-coupled receptor highly expressed in pancreatic β -cells (Briscoe et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003). Long- and medium-chain free fatty acids (FFAs) are endogenous ligands for GPR40, and the activation of GPR40 by FFAs leads to augmentation of insulin secretion through activation of G_{aq} , which promotes phospholipase C-mediated production of inositol 1,4,5-triphosphate, thereby resulting in augmentation of intracellular Ca^{2+} (Fujiwara et al., 2005; Shapiro et al., 2005; Feng et al., 2006; Sakuma et al., 2015). This mechanism is novel and is clearly distinct from other oral insulinotropic drugs, such as

Parts of these studies were presented at the following workshop: Ito R, Tsujihata Y, Suzuki M, Matsuda K, Miyawaki K, Negoro N, and Takeuchi K (2012) TAK-875, a GPR40 agonist, improves postprandial hyperglycemia additively with sulfonylurea and is effective in rats with sulfonylurea failure. 72nd Scientific Sessions of the American Diabetes Association; 2012 Jun 8–12; Philadelphia, PA.

dx.doi.org/10.1124/jpet.115.230730.

[S] This article has supplemental material available at jpet.aspetjournals.org.

ABBREVIATIONS: ANOVA, analysis of variance; AUC, area under the curve; DPP-4, dipeptidyl peptidase-4; FFAs, free fatty acids; N-STZ 1.5, neonatally streptozotocin-induced 1.5 days after birth; STZ, streptozotocin; SUs, sulfonylureas; WKY, Wistar Kyoto; ZDF, Zucker diabetic fatty; ZL, Zucker lean.

SUs (Rendell, 2004) and dipeptidyl peptidase-4 (DPP-4) inhibitors (Drucker, 2007).

Fasiglifam is a selective and orally available GPR40 agonist that improves postprandial and fasting hyperglycemia by potentiating insulin secretion in diabetic rats (Tsujihata et al., 2011; Negoro et al., 2012; Ito et al., 2013). A recent *in vitro* study has shown that fasiglifam acts as an ago-allosteric modulator of GPR40 and exerts the effects cooperatively with endogenous ligand FFAs rather than competing with one another (Yabuki et al., 2013). The most clinically meaningful feature of GPR40 agonists is the increase in insulin secretion solely in the presence of high glucose concentration, in contrast to SUs, which enhance insulin release regardless of glucose concentration (Tsujihata et al., 2011). This glucose-dependent insulin secretion likely leads not only to a low risk of hypoglycemia, but also to a lesser probability of β -cell exhaustion due to mitigating the burden of β -cells through appropriate insulin secretion compared with SUs. However, the detailed comparison of *in vivo* acute and chronic profiles between GPR40 agonists and SUs has not been sufficiently studied.

Because combination therapy with antidiabetic drugs with different mechanism of action is often used (Charpentier, 2002), information on the combinational effects of existing drugs is important. Fasiglifam has previously shown additive glycemic control in diabetic rodents in combination with the peripheral insulin sensitizer metformin (Ito et al., 2013). Interestingly, it has been reported that the glucose dependency of GPR40-mediated insulin secretion is uncoupled by the presence of SUs in rat insulinoma cells (Yang et al., 2010). This implies that GPR40 agonists would enhance insulin secretion even at low plasma glucose levels when administered with SUs, thereby promoting hypoglycemia. From the aspect of safety, as well as the mechanism of underlying insulinotropic action, there is interest in determining whether GPR40 agonists can work cooperatively with SUs.

Fasiglifam is the first GPR40 agonist to significantly improve glycemic control in patients with type 2 diabetes (Kaku et al., 2015). However, the clinical development of fasiglifam was terminated because of concerns about liver safety. Other than fasiglifam, several other GPR40 agonists are currently still in the preclinical and clinical stages of development (Mancini and Poitout, 2015). Given that GPR40 agonists potentially constitute a new class of insulinotropic drugs, further understanding of their differences from and interactions with widely used insulinotropic drugs is necessary. In the present study, we analyzed the acute glucose-lowering effects between fasiglifam and SUs and assessed the risk of secondary failure of fasiglifam in type 2 diabetic rats. We also examined whether treatment with fasiglifam lowers glucose in two rat models desensitized to SU treatment: 15-week glibenclamide-treated neonatally streptozotocin-induced rats 1.5 days after their birth (N-STZ-1.5) and aged Zucker diabetic fatty (ZDF) rats. Finally, we evaluated the effects of fasiglifam in combination with SUs on postprandial hyperglycemia and the risk of hypoglycemia.

Materials and Methods

Animals. Male N-STZ-1.5 rats were prepared by administering 120 mg/kg of streptozotocin (STZ) subcutaneously to male Wistar Kyoto (WKY) rats on day 1.5 after birth (Portha et al., 1989). The N-STZ-1.5

rats and neonatal saline-injected WKY rats (non-diabetic control rats) were obtained from Takeda Rabbits Limited (Osaka, Japan). Male ZDF (lprfa/CrJlCrj fatty fa/fa) rats and their nondiabetic littermates, male Zucker lean (ZL) (lprfa/CrJlCrj lean +/+ or fa/+) rats, were obtained from Charles River Laboratories Japan, Inc (Yokohama, Japan). Male Sprague-Dawley rats were purchased from Clea, Japan, Inc. (Tokyo, Japan). All rats were housed in individual metal cages in a room with controlled temperature (23°C), humidity (55%), and 12 hours dark/12 hours light. Rats were allowed free access to powdered standard laboratory chow diet (CE-2, Clea, Japan, Inc.) and tap water. The care and use of the animals and experimental protocols used in this study were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited (Kanagawa, Japan).

Materials. Fasiglifam ((3S)-6-((2',6'-dimethyl-4'-[3-(methylsulfonyl)propoxy]biphenyl-3-yl)methoxy)-2,3-dihydro-1-benzofuran-3-yl)acetic acid hemihydrate) and alogliptin (Feng et al., 2007) were synthesized at Chemical Development Laboratories, Takeda Pharmaceutical Company Limited. Glibenclamide and glimepiride were purchased from Wako Pure Chemical Industries, Limited (Osaka, Japan). Drugs were suspended in 0.5% methylcellulose solution (Wako Pure Chemical Industries, Limited).

Oral Glucose Tolerance Test (Mono-treatment). At the age of 20 to 27 weeks, N-STZ-1.5 and WKY rats underwent overnight fasting. N-STZ-1.5 rats were divided into 7 groups ($n = 6$) so that plasma levels of glucose, triglycerides, and body weight were not statistically significantly different among groups. Each group of N-STZ-1.5 rats was given vehicle (0.5% methylcellulose), fasiglifam (3, 10, and 30 mg/kg), or glibenclamide (3, 10, and 30 mg/kg) orally in a volume of 5 ml/kg. WKY rats ($n = 5$) were given vehicle orally. Plasma parameters measured for the grouping of the animals were used as baseline data. Sixty minutes after drug administration, all animals received an oral glucose load (1.5 g/kg). Blood samples were collected from the tail vein just before (time 0) and 10, 30, 60, and 120 minutes after glucose load for the determination of plasma glucose and insulin levels.

Evaluation of the Risk of Secondary Failure of Fasiglifam. N-STZ-1.5 rats at 16 weeks of age were divided into 2 groups ($n = 12$) so that plasma levels of glucose, triglycerides, total cholesterol, nonesterified fatty acids, and body weight were not statistically significantly different among groups. Each group was given vehicle or fasiglifam (10 mg/kg) orally in a volume of 2.5 ml/kg once daily for 15 weeks. On day 90, one rat in the vehicle-treated group died (unexpected decrease in body weight); thus, this rat was excluded from data analysis. After 14 weeks of treatment, each group was regrouped into 2 groups ($n = 5$ or 6). After an additional week of treatment, each group underwent overnight fasting and received vehicle or fasiglifam (3 mg/kg) orally in a volume of 2.5 ml/kg 30 minutes before glucose load (1 g/kg). Blood samples were collected from the tail vein just before (time 0) and 10, 30, 60, and 120 minutes after glucose load for the determination of plasma glucose and insulin levels. WKY rats ($n = 6$) were similarly treated with vehicle for the same period and underwent an oral glucose tolerance test. Rats were killed by exsanguination, and the pancreas was isolated for immunostaining. Isolated pancreata were fixed overnight with Bouin's solution (Polysciences, Inc., Warrington, PA) and transferred into 70% ethanol before processing through paraffin. Immunostaining with anti-insulin and anti-glucagon antibodies (DAKO, Glostrup, Denmark) were performed, and images were obtained with an inverted microscope IX71 (Olympus, Tokyo, Japan).

Evaluation of Rats with SU Secondary Failure. N-STZ-1.5 rats (20 weeks of age) were divided into 2 groups ($n = 16$ and $n = 24$) so that plasma levels of glucose and body weight were not statistically significantly different among groups. Each group was given vehicle ($n = 16$) or glibenclamide (10 mg/kg, $n = 24$) orally in a volume of 2.5 ml/kg once daily for 4 weeks. After overnight fasting, the repeated vehicle-treated group was regrouped into 2 groups, whereas the glibenclamide-treated group was regrouped into 3 groups ($n = 8$). The repeated vehicle-treated groups were given vehicle or glibenclamide (10 mg/kg) orally in a volume of 5 ml/kg 1 hour before

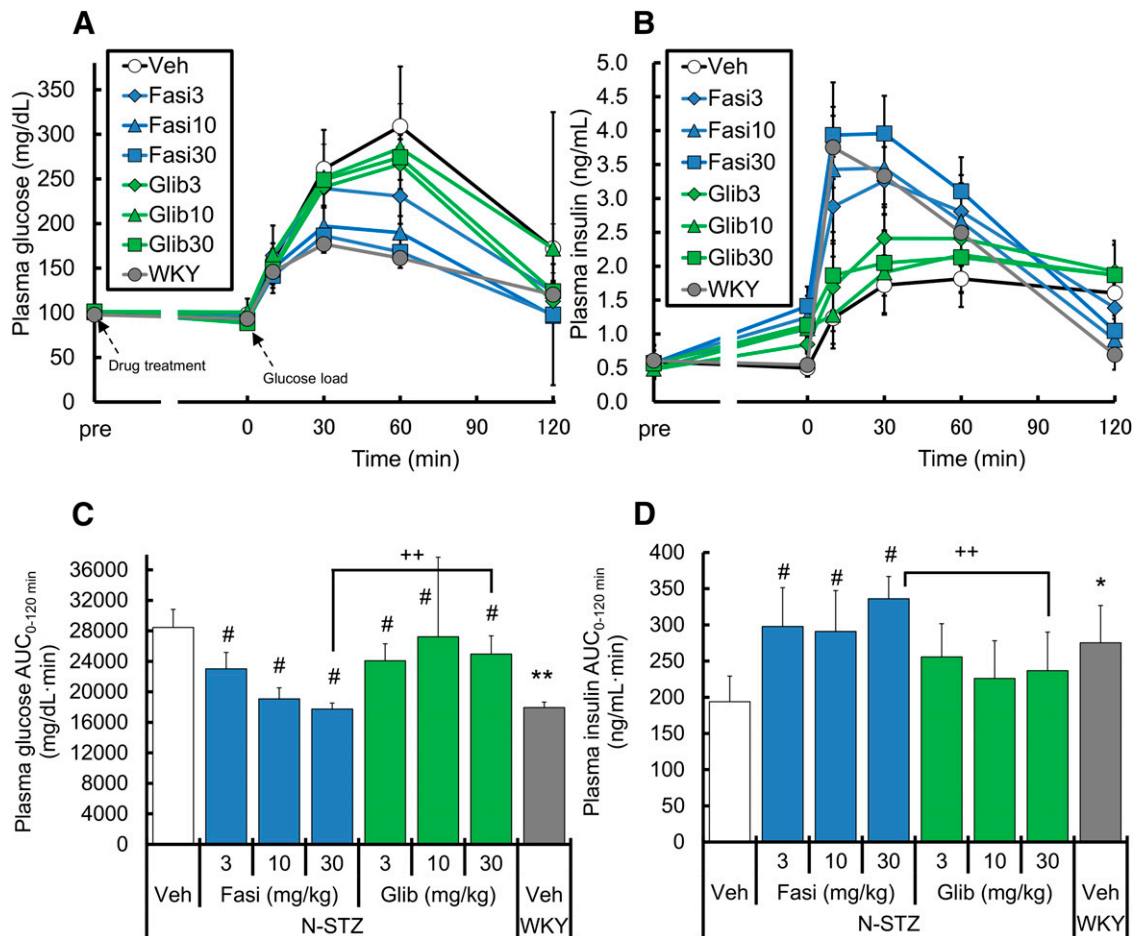


Fig. 1. Fasigliam improves glucose tolerance more effectively than glibenclamide during oral glucose tolerance tests in male N-STZ-1.5 rats. (A) and (B) Time-dependent changes in plasma glucose and insulin levels, respectively, after glucose load (1.5 g/kg) in N-STZ-1.5 rats treated with vehicle (Veh), 3–30 mg/kg of fasigliam (Fasi), or 3–30 mg/kg of glibenclamide (Glib) and in WKY rats (normal controls) treated with vehicle. Data in (C) and (D) represent plasma glucose AUC_{0-120 minutes} and plasma insulin AUC_{0-120 minutes}, respectively. # $P \leq 0.025$ compared with vehicle-treated N-STZ-1.5 rats by one-tailed Williams' test or one-tailed Shirley-Williams test. * $P \leq 0.05$, ** $P \leq 0.01$ compared with vehicle-treated N-STZ-1.5 rats by Student's *t* test or Aspin-Welch test. ++ $P \leq 0.01$ by Student's *t* test or Aspin-Welch test. Values are mean \pm S.D. ($n = 6$ for N-STZ-1.5 rats, $n = 5$ for WKY rats).

oral glucose load (1 g/kg). The repeated glibenclamide-treated groups were given vehicle, glibenclamide (10 mg/kg), or fasigliam (3 mg/kg) orally in a volume of 5 ml/kg 1 hour before oral glucose load. Blood samples were collected from the tail vein just before (time 0) and 10, 30, 60, and 120 minutes after oral glucose load for the determination of plasma glucose and insulin levels.

Effects on Fasting Hyperglycemia in Severe Diabetic Rats. At the age of 15 weeks of age, male ZDF and ZL rats underwent overnight fasting. ZDF rat were divided into 3 groups ($n = 6$) so that plasma levels of glucose, triglycerides, and body weight were not statistically significantly different among groups. Each group of ZDF rats was given vehicle, fasigliam (10 mg/kg), or glimepiride (10 mg/kg) orally in a volume of 5 ml/kg. ZL rats ($n = 6$) were given vehicle orally. Plasma glucose and insulin levels were determined using blood samples collected from the tail vein at 0, 30, 60, 120, 240, and 360 minutes after drug administration.

Oral Glucose Tolerance Test (Combination Treatment). At the age of 21 weeks, N-STZ-1.5 and WKY rats underwent overnight fasting. N-STZ-1.5 rats were divided into 4 groups ($n = 6$) so that plasma levels of glucose, triglycerides, and body weight were not statistically significantly different among groups. Each group was given vehicle, fasigliam (3 mg/kg), glimepiride (10 mg/kg), or fasigliam (3 mg/kg) in combination with glimepiride (10 mg/kg) orally in a volume of 5 ml/kg. WKY rats ($n = 5$) were given vehicle orally. Sixty minutes after drug administration, all animals received an oral glucose load (1.5 g/kg). Blood samples were collected from

the tail vein just before glucose load (time 0) and 10, 30, 60, and 120 minutes after glucose load for the determination of plasma glucose and insulin levels.

Evaluation of Hypoglycemia Risk. At the age of 10 weeks, nonfasted male Sprague-Dawley rats were divided into 6 groups ($n = 6$) so that body weight was not statistically significantly different among groups. Each group was given vehicle, fasigliam (3 mg/kg), alogliptin (1 mg/kg), glimepiride (10 mg/kg), fasigliam (3 mg/kg) in combination with glimepiride (10 mg/kg), or alogliptin (1 mg/kg) in combination with glimepiride (10 mg/kg) orally in a volume of 5 ml/kg. Plasma glucose and insulin levels were determined using blood samples collected from the tail vein just before drug treatment (time 0) and 0.5, 1, 2, 4, and 6 hours after drug administration.

Measurements of Plasma Parameters. Plasma levels of glucose, triglycerides, total cholesterol, and nonesterified fatty acids were enzymatically measured by Autoanalyzer 7080 (Hitachi, Tokyo, Japan). Plasma insulin was measured using radioimmunoassay kit (Millipore, Bedford, MA). For determination of low levels of plasma insulin, rat insulin enzyme-linked immunosorbent assay kits (Morinaga Institute of Biologic Science, Yokohama, Japan) were used.

Statistical Analysis. To evaluate effects of drugs during oral glucose tolerance test, total plasma glucose area under the curve (AUC) and plasma insulin AUC were calculated. For evaluation of effects under fasted and nonfasted state, change in plasma glucose and change in plasma insulin levels were calculated from the difference between values at indicated time point and initial value, and change in

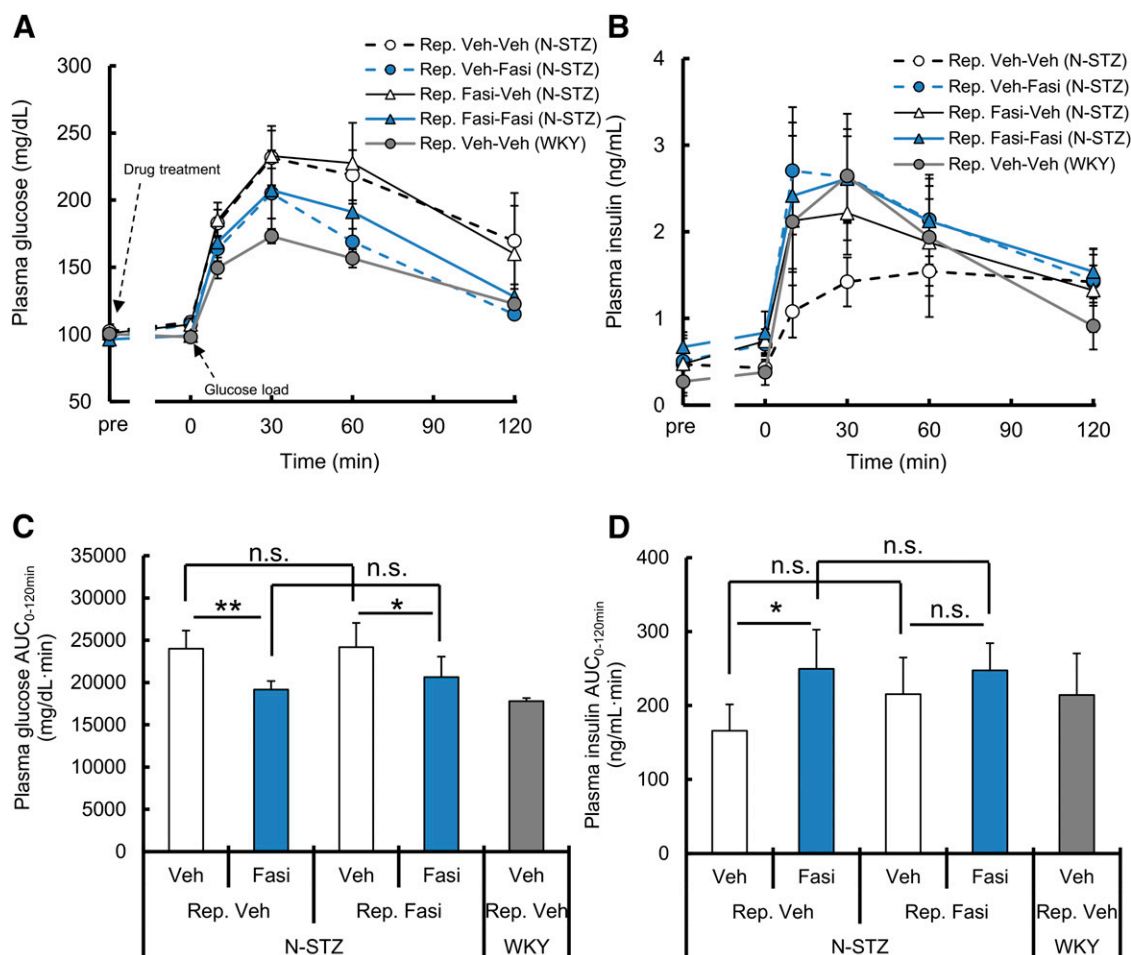


Fig. 2. Chronic treatment with fasiglifam does not cause secondary loss of glucose-lowering efficacy. N-STZ-1.5 rats were treated once daily with vehicle (Rep. Veh) or fasiglifam (Rep. Fasi, 10 mg/kg) for 15 weeks. After fasting overnight, vehicle (Veh) or fasiglifam (Fasi, 3 mg/kg) was given orally to both groups 30 minutes before glucose load (1 g/kg). Similarly, WKY rats (normal controls) were treated with vehicle once daily for 15 weeks, and the same oral glucose tolerance test was performed. (A) and (B) Time-dependent changes in plasma glucose and insulin levels, respectively. Data in (C) and (D) represent plasma glucose AUC_{0-120 minutes} and plasma insulin AUC_{0-120 minutes}, respectively. * $P \leq 0.05$, ** $P \leq 0.01$ by Student's *t* test or Aspin-Welch test. n.s., Not significant. Values are mean \pm S.D. ($n = 5$ or 6).

plasma glucose AUC and change in plasma insulin AUC were calculated using these values. Statistical differences between 2 groups were analyzed using the Student's *t* test or the Aspin-Welch test depending on variances between groups. Dose-dependent effects were analyzed using a one-tailed Williams' test or a one-tailed Shirley-Williams test depending on variances between groups. Combination effects were assessed using a two-way analysis of variance (ANOVA) (Moritoh et al., 2009). The results were interpreted as follow: i) when a significant interaction effect ($P \leq 0.05$) was observed, the effect of the combination was considered to be synergistic (when the effect of the combination treatment exceeds the sum of the effect of the monotreatment) and ii) when no significant interaction and significant main effects of both drugs ($P \leq 0.05$) were observed, the effect was considered to be additive (when the effect of the combination treatment equals the sum of the effect of the monotreatment). All data are presented as mean \pm S.D.

Results

Fasiglifam Improves Postprandial Hyperglycemia More Effectively than Glibenclamide. The glucose-lowering effects and insulinotropic action of fasiglifam and SUs were first compared by an oral glucose tolerance test in type 2 diabetic N-STZ-1.5 rats. Compared with vehicle, oral

administration of fasiglifam at doses of 3, 10, and 30 mg/kg dose dependently reduced plasma glucose AUC by 19.1, 33.0, and 37.6%, respectively, and the glucose tolerance in rats given fasiglifam (30 mg/kg) reached a level comparable to that in normal WKY rats (Fig. 1, A and C). Consistent with glucose-lowering effects, fasiglifam at doses of 3, 10, and 30 mg/kg robustly increased plasma insulin AUC 1.5-, 1.5-, and 1.7-fold, respectively (Fig. 1, B and D). Conversely, compared with vehicle, 3, 10, and 30 mg/kg of glibenclamide significantly decreased plasma glucose AUC by 15.3, 4.3, and 12.3%, respectively; the effects reached a plateau at a dose of 3 mg/kg (Fig. 1, A and C). Glibenclamide showed a trend toward increase in plasma insulin AUC after glucose load (Fig. 1, B and D). The similar glucose-lowering effects and insulinotropic action were also observed with glimepiride (3–30 mg/kg; Supplemental Fig. 1). When compared at a maximal dose of 30 mg/kg, the improvement in glucose tolerance and insulin secretion in response to glucose in diabetic rats treated with fasiglifam was significantly greater than those treated with glibenclamide (Fig. 1, C and D). At this dose, both fasiglifam and glibenclamide decreased fasting plasma glucose and increased fasting plasma insulin levels just before glucose load (time 0); however, between drugs,

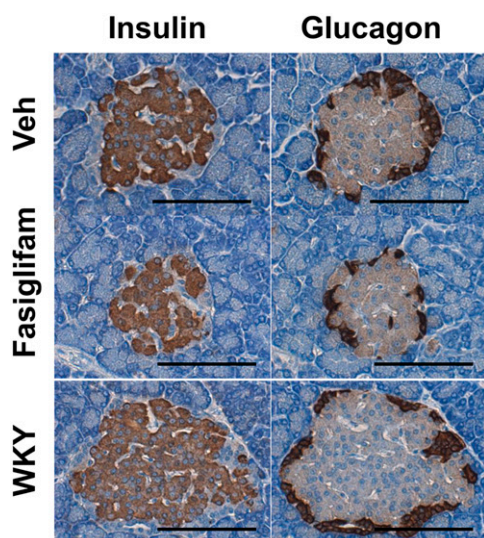


Fig. 3. Pancreatic islet morphology in male N-STZ-1.5 rats after multiple dosing of fasiglifam. N-STZ-1.5 rats were treated with vehicle (Veh) or fasiglifam (10 mg/kg), and WKY rats (normal controls) were treated with vehicle, once daily for 15 weeks. Fixed pancreata were immunostained with anti-insulin and antiglucagon antibodies. Scale bar = 100 μ m.

these effects were not significantly different (fasting plasma glucose: vehicle, 98.1 ± 2.1 mg/dl; fasiglifam, 91.9 ± 4.1 mg/dl; glibenclamide, 88.4 ± 3.7 mg/dl; fasting plasma insulin: vehicle, 0.5 ± 0.1 ng/ml; fasiglifam, 1.4 ± 0.3 ng/ml; glibenclamide, 1.1 ± 0.2 ng/ml; Fig. 1, A and B).

Chronic Treatment with Fasiglifam Does Not Cause Secondary Loss of Glucose-lowering Efficacy. It was previously reported that 4-week treatment with glibenclamide induces the exacerbation of glucose tolerance and the loss of response to glibenclamide in N-STZ-1.5 rats, leading to the development of secondary failure (Asakawa et al., 2009). To determine if chronic treatment with fasiglifam results in the similar secondary loss of its glucose-lowering efficacy, an oral glucose tolerance test was performed in N-STZ-1.5 rats after long-term multiple doses of fasiglifam. Based on the pharmacokinetic profiles in rats, 3 to 10 mg/kg of fasiglifam was selected in the following study; this dose is close to the clinical doses of fasiglifam in humans (Negoro et al., 2010; Naik et al., 2012). After 14 weeks of treatment (1 week before an oral glucose tolerance test), no significant effects on changes in body weight were observed between vehicle and fasiglifam groups (changes in body weight from initial values: vehicle, 71.3 ± 4.8 g; fasiglifam, 68.4 ± 5.7 g). In the N-STZ-1.5 rats given fasiglifam (10 mg/kg) for 15 weeks, glucose tolerance and insulin secretion after glucose load were not significantly different compared with the rats given vehicle for 15 weeks [Fig. 2; repeatedly treated (Rep). Veh-Veh versus Rep. Fasi-Veh]. Moreover, acute administration of fasiglifam (3 mg/kg) 30 minutes before glucose load significantly decreased glucose excursion in rats pretreated with fasiglifam (Fig. 2, A and C; Rep. Fasi-Veh versus Rep. Fasi-Fasi), and the glucose-lowering effects were comparable to that seen with first dosing in rats pretreated with vehicle (Fig. 2, A and C; Rep. Veh-Veh versus Rep. Veh-Fasi). Unexpectedly, because of the increase in early-phase insulin response by vehicle treatment, the significant increase in postprandial insulin secretion in response to fasiglifam was not observed in rats treated with

fasiglifam long term (Fig. 2, B and D). Immunostaining of pancreatic islets with anti-insulin and antiglucagon antibodies showed no apparent differences between the long-term vehicle- and fasiglifam-treated groups (Fig. 3).

Fasiglifam Is Effective in Diabetic Rats with SU Secondary Failure. To confirm the effects of fasiglifam in a type 2 diabetic model with SU secondary failure, N-STZ-1.5 rats were given glibenclamide at 10 mg/kg per day for 4 weeks according to methods previously reported (Asakawa et al., 2009). In diabetic N-STZ-1.5 rats that were given vehicle for 4 weeks, single oral administration of glibenclamide (10 mg/kg) significantly improved glucose tolerance, with an increase in insulin secretion (Fig. 4; Rep. Veh-Veh versus Rep. Veh-Glib). In contrast, after 4 weeks of treatment with glibenclamide (10 mg/kg), glucose tolerance was exacerbated (Fig. 4, A and C; Rep. Veh-Veh versus Rep. Glib-Veh), and the response to glibenclamide (10 mg/kg) was completely lost (Fig. 4; Rep. Glib-Veh versus Rep. Glib-Glib), which suggests that these rats developed SU secondary failure. A single dose of fasiglifam (3 mg/kg) significantly improved glucose tolerance with an increase in insulin secretion after treatment with glibenclamide (Fig. 4; Rep. Glib-Veh versus Rep. Glib-Fasi).

Fasiglifam Improves Glycemic Control in Severe Diabetic Rats Unresponsive to SUs. It was previously reported that long-term treatment with SUs are ineffective in lowering blood glucose levels in ZDF rats that develop overt diabetes due to gradually impaired β -cell function (Atkinson et al., 2008). To further evaluate the efficacy of fasiglifam in diabetic rats that are less responsive to SUs, we examined the glucose-lowering effects of fasiglifam in aged ZDF rats. Fasting plasma glucose levels were significantly elevated in 15-week-old ZDF rats compared with age-matched control rats (Fig. 5A; 367.5 ± 58.0 versus 103.8 ± 4.2 mg/dl), which suggests the development of severe diabetes in these rats. As expected, single administration of glimepiride (10 mg/kg) did not result in a significant decrease in fasting plasma glucose and increase in plasma insulin in these rats (Fig. 5, A and B). On the other hand, fasiglifam (10 mg/kg) significantly improved fasting hyperglycemia and enhanced insulin secretion in these rats (Fig. 5).

Combination of Fasiglifam and Glimepiride Actively Improves Glucose Tolerance in Diabetic Rats. We next evaluated combined effects of fasiglifam and glimepiride on glucose tolerance in N-STZ-1.5 rats. Compared with vehicle, oral administration of fasiglifam (3 mg/kg) or glimepiride (10 mg/kg) 1 hour before glucose load decreased plasma glucose AUC by 25.3 or 20.0%, respectively; combination of fasiglifam and glimepiride resulted in a further decrease in plasma glucose AUC by 43.1% compared with either drug alone (Fig. 6, A and C). Two-way ANOVA showed that the combination had additive glucose-lowering effects (fasiglifam, $P \leq 0.01$; glimepiride, $P \leq 0.01$; interaction, not significant). In accordance with the glucose-lowering effects, fasiglifam, glimepiride, or the combination of these drugs enhanced insulin secretion 1.4-, 1.3-, or 1.7-fold, respectively, compared with vehicle (Fig. 6, B and D); the combined effect of the drugs was additive (fasiglifam, $P \leq 0.01$; glimepiride, $P \leq 0.05$; interaction, not significant by two-way ANOVA). In rats given combination treatment with fasiglifam and glimepiride, insulin secretion was restored to a level comparable to control rats (Fig. 6, B and D). These results indicate that fasiglifam and glimepiride improve hyperglycemia and enhance insulin release in a complementary manner.

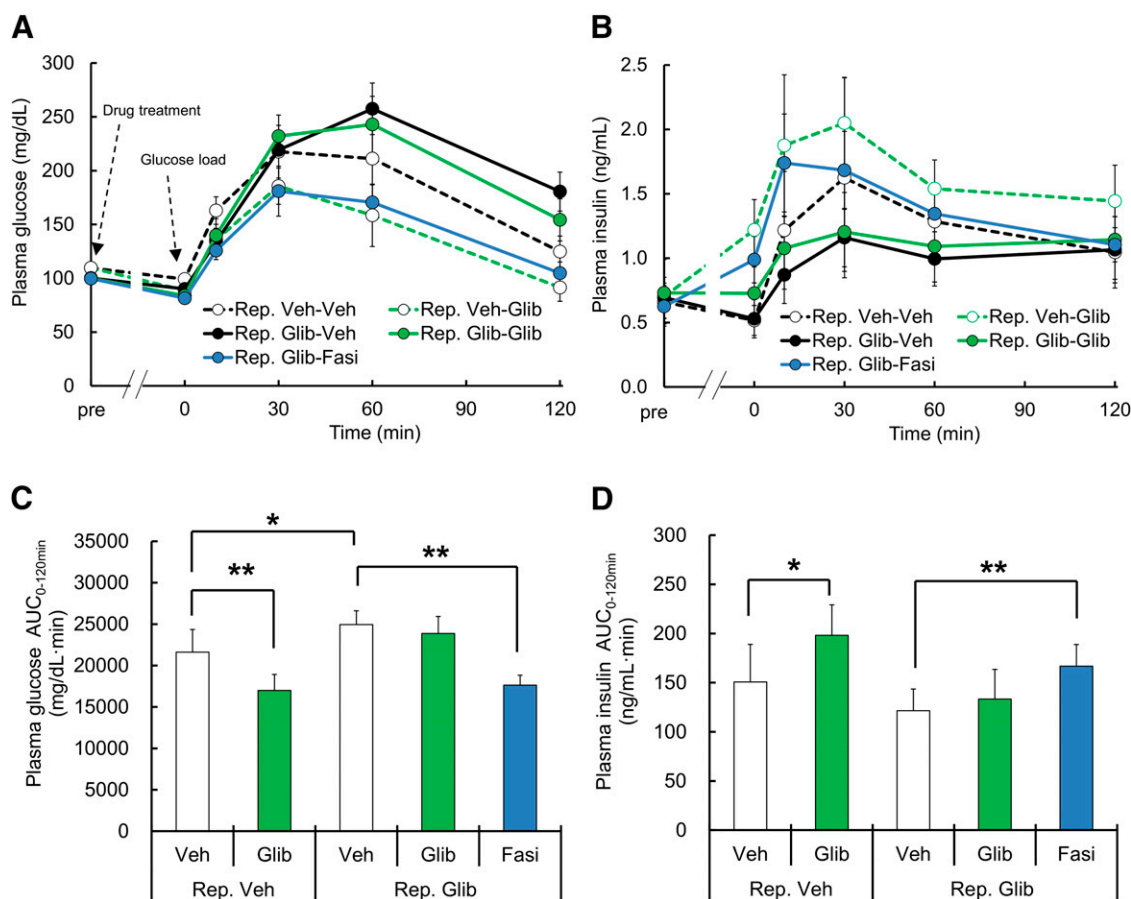


Fig. 4. Fasiglifam is effective in male N-STZ-1.5 rats with SU secondary failure. N-STZ-1.5 rats were treated once daily with vehicle (Rep. Veh) or glibenclamide (Rep. Glib, 10 mg/kg) for 4 weeks. After the treatment, vehicle (Veh) or glibenclamide (Glib, 10 mg/kg) was given orally to the Rep. Veh group, and vehicle, glibenclamide (10 mg/kg), or fasiglifam (Fasi, 3 mg/kg) was given orally to the Rep. Glib group. One hour after dosing, all animals received oral glucose load (1 g/kg). (A) and (B) Time-dependent changes in plasma glucose and insulin levels, respectively. Data in (C) and (D) represent plasma glucose AUC_{0-120 minutes} and plasma insulin AUC_{0-120 minutes}, respectively. * $P \leq 0.05$, ** $P \leq 0.01$ by Student's *t* test or Aspin-Welch test. Values are mean \pm S.D. ($n = 8$).

Fasiglifam neither Induces Hypoglycemia nor Exacerbates SU-Induced Hypoglycemia in Normal Rats. A previous in vitro report has noted that glucose dependency for GPR40-mediated insulin secretion is uncoupled by the presence of SUs (Yang et al., 2010), which implies that fasiglifam would enhance in vivo insulin secretion independent of plasma glucose levels when administered concomitantly with SUs, thereby promoting hypoglycemia caused by SUs. Thus, we evaluated the risk of hypoglycemia in nonfasted normal Sprague-Dawley rats treated with combination of fasiglifam and glimepiride. Oral administration of glimepiride (10 mg/kg) lowered plasma glucose levels below 65 mg/dL, leading to hypoglycemia (Fig. 7, A and C). Consistent with its glucose-lowering effects, glimepiride significantly enhanced insulin secretion compared with vehicle, with a peak increase 60 minutes after dosing (Fig. 7, B and D). Fasiglifam (3 mg/kg) had little effect on plasma glucose and insulin release (Fig. 7, A and B), although slight but significant decrease in plasma glucose AUC was observed (Fig. 7C). Interestingly, when coadministered with fasiglifam (3 mg/kg) and glimepiride (10 mg/kg), there were neither further decreases in plasma glucose AUC nor further increases in plasma insulin AUC compared with glimepiride alone (Fig. 7, C and D). Under the same conditions, oral administration of the DPP-4 inhibitor

alogliptin (1 mg/kg), a comparator that shows glucose-dependent insulin release, also did not lower plasma glucose levels less than the normal range; in combination with glimepiride (10 mg/kg), the combination did not result in a further reduction of plasma glucose levels compared with glimepiride alone (Fig. 7, A and C). These results indicate that fasiglifam does not exacerbate SU-induced hypoglycemia in combination with SUs in normal rats and that the risk of hypoglycemia with fasiglifam and SU is not higher than combination treatment of a DPP4 inhibitor and SU.

Discussion

Fasiglifam, a potent and selective GPR40 agonist, provides glucose-dependent insulin secretion through activation of the Gαq signaling pathway (Tsujihata et al., 2011), which is a mechanism distinct from other clinically available insulinotropic drugs (Winzell and Ahren, 2007). Fasiglifam shows glucose-lowering effects due to the augmentation of insulin secretion in type 2 diabetic rats (Tsujihata et al., 2011; Ito et al., 2013) and in patients with diabetes (Burant et al., 2012; Kaku et al., 2013). However, the clinical development of fasiglifam was terminated because of concerns about liver safety. This provides a key message: a deeper and more

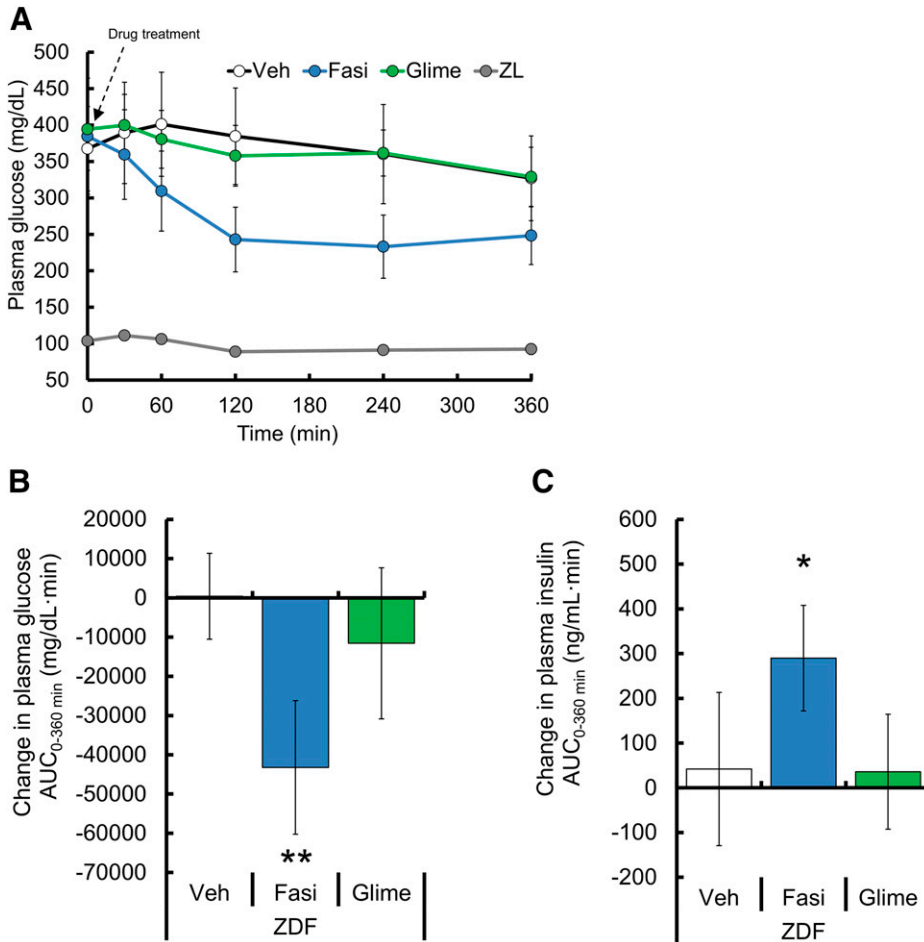


Fig. 5. Fasiglifam improves glycemic control in severe diabetic rats unresponsive to SU. (A) Time-dependent changes in plasma glucose levels in 15-week-old male ZDF and ZL rats (normal controls) after treatment with vehicle (Veh), 10 mg/kg of fasiglifam (Fasi), or 10 mg/kg of glimepiride (Glime). Data in (B) and (C) represent change in plasma glucose AUC_{0-360 minutes} and change in plasma insulin AUC_{0-360 minutes} after drug treatments, respectively. Values are mean \pm S.D. ($n = 6$). * $P \leq 0.05$, ** $P \leq 0.01$ compared with vehicle by Student's t test or Aspin-Welch test.

detailed understanding of its basic biology and pharmacology is required (Mancini and Poitout, 2015). Given in addition to fasiglifam, several other GPR40 agonists are currently still in the preclinical and clinical stages of development (Mancini and Poitout, 2015), GPR40 agonists potentially constitute a new class of antidiabetic drugs. Thus, there is a need to examine their differences from and interactions with widely used insulinotropic drugs. Although comparison of in vitro profiles between GPR40 agonist and SUs have been reported thus far (Zhao et al., 2008; Yang et al., 2010; Tsujihata et al., 2011), the differences in in vivo acute and chronic efficacies between these drugs in diabetic rodents are not yet fully understood. It is also unclear whether these drugs could work cooperatively. In the present study, our data indicated that fasiglifam improved postprandial hyperglycemia more effectively than glibenclamide. Our results showed that chronic GPR40 activation by fasiglifam did not cause secondary loss of the glucose-lowering efficacy, in contrast to glibenclamide, indicating that GPR40 agonists have the better durability compared with SUs. Interestingly, fasiglifam was still effective in two different rodent models that were completely desensitized to SUs. In addition, fasiglifam improved glucose excursion and augmented insulin secretion additively with glimepiride in N-STZ-1.5 rats and did not exacerbate glimepiride-induced hypoglycemia in control rats. These observations suggest that GPR40 agonists could be broadly useful as glycemic rescue agents in patients who have already undergone treatment with SUs.

In the present study, compared at a maximal dose of 30 mg/kg, the glucose-lowering effects and insulinotropic action of fasiglifam after glucose load were significantly greater than those of glibenclamide. Of note, at these doses, the effects on fasting plasma glucose and insulin levels before glucose load were not different between drugs, indicating that fasiglifam exerts robust effects on insulin release especially under the postprandial condition. It is likely that in the postprandial condition, K_{ATP} channel activity in β -cells is already potently inhibited by high glucose, which may preclude the further inhibitory effect of glibenclamide. Another possible explanation is that the interaction of fasiglifam with the gut hormones released from enteroendocrine cells might contribute to the robust postprandial insulin release by fasiglifam treatment in our study. It has been reported that glucagon-like peptide-1 and acetylcholine, which activates the same $G_{\alpha q}$ signaling pathway as GPR40 agonists, exert synergistic effects on insulin secretion in perfused rat islets (Zawalich et al., 1993). In future study, the mechanisms by which these gut hormones interact with GPR40 agonists and SUs should be assessed.

Many G-protein-coupled receptors involved in insulin secretion in β -cells are known to undergo in vitro rapid desensitization subsequent to repeated stimulation by those specific agonists (DeRubertis and Craven, 1976; Hinke et al., 2000; Baggio et al., 2004). Moreover, because chronic exposure of FFAs, endogenous ligands for GPR40, causes the harmful

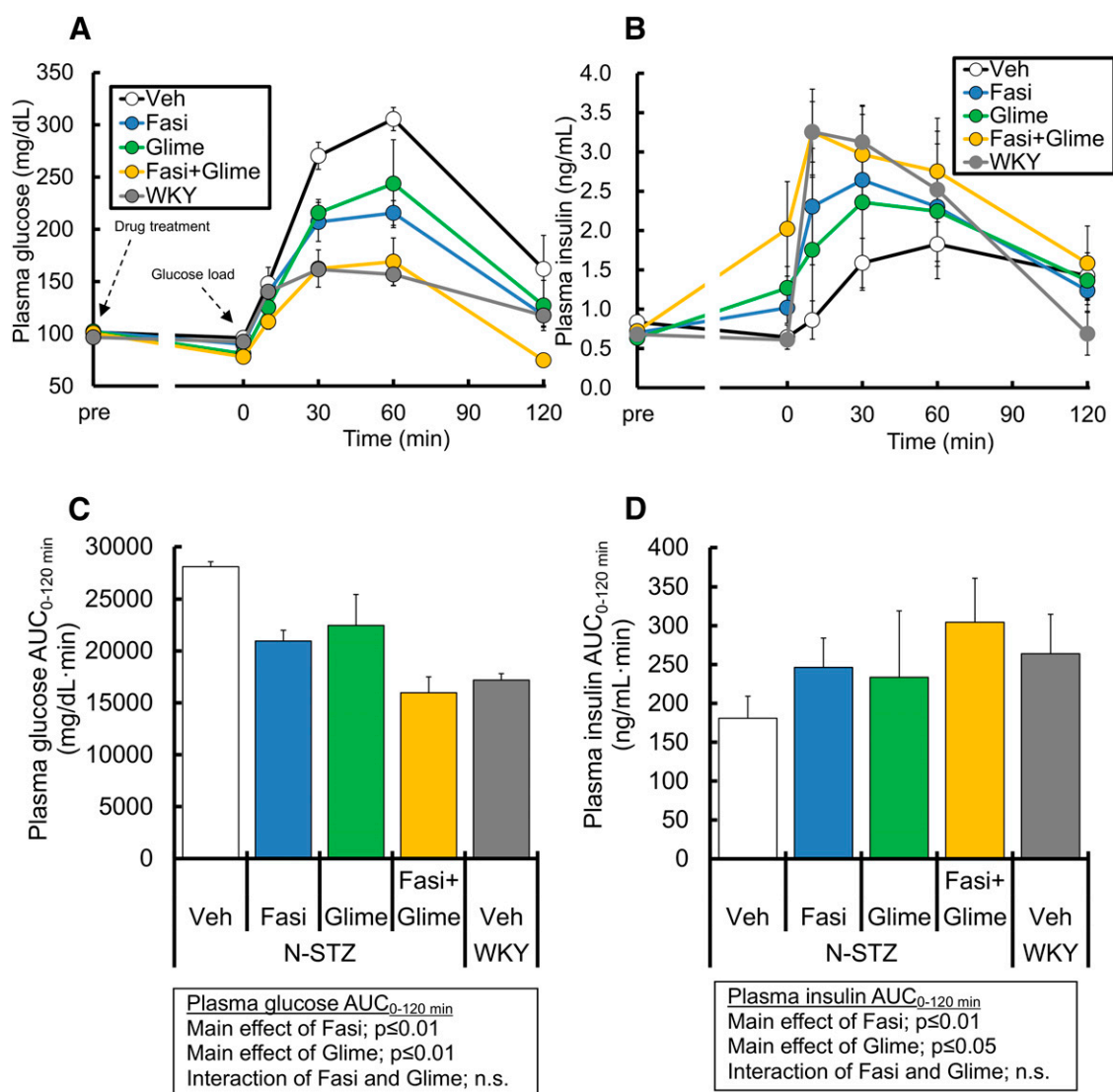


Fig. 6. Combination of fasiglifam and glimepiride additively improves glucose tolerance in N-STZ-1.5 rats. (A) and (B) Time-dependent changes in plasma glucose and insulin levels, respectively, after glucose load (1.5 g/kg) in N-STZ-1.5 rats treated with vehicle (Veh), 3 mg/kg of fasiglifam (Fasi), or 10 mg/kg of glimepiride (Glime) and in WKY rats (normal controls) treated with vehicle. Data in (C) and (D) represent plasma glucose AUC_{0-120 min} and plasma insulin AUC_{0-120 min}, respectively. The results of two-way ANOVA are indicated in insets. n.s., Not significant. Values are mean \pm S.D. ($n = 6$ for N-STZ-1.5 rats, $n = 5$ for WKY rats).

effects on β -cell viability (lipotoxicity), there is great interest in the impact after long-term activation of GPR40 by selective agonists. In this study, in contrast to glibenclamide, even after almost 4 months of treatment in N-STZ-1.5 rats, the glucose-lowering effects of fasiglifam were well preserved to a level comparable to that of the first administration, which is consistent with several short-term studies with other GPR40 agonists (Tan et al., 2008; Lin et al., 2011). These results indicate that GPR40 agonists may have a low risk of receptor desensitization. Furthermore, in our study, there were no apparent differences in islet morphology between the vehicle- and fasiglifam-treated groups. Several experimental observations from genetically modified mice suggest that GPR40 is less involved in lipotoxicity (Latour et al., 2007; Kebede et al., 2008; Nagasumi et al., 2009). In addition, fasiglifam significantly improves glycemic control and is well tolerated after 24 weeks of treatment in patients with type 2 diabetes (Kaku et al., 2013). These findings support that chronic exposure to

GPR40 agonist does not cause β -cell dysfunction. Regarding the low risk of receptor desensitization, it might be explained by the unique binding profile of fasiglifam. It was recently demonstrated that fasiglifam is an ago-allosteric modulator of GPR40, which binds to a different binding site from the orthosteric site (Yabuki et al., 2013; Srivastava et al., 2014). Because it has been shown that some allosteric modulators allow receptor activation with less propensity for receptor desensitization than orthosteric agonists (Gjoni and Urwyler, 2008), it is speculated that fasiglifam may show the similar profile.

After long-term SU treatment, some patients begin to suffer from the secondary failure (Matthews et al., 1998). There are several factors that are involved in the development of SU secondary failure, including β -cell exhaustion by long-term overstimulation and glucose toxicity caused by poor glycemic control (Poitout and Robertson, 2002; Rustenbeck, 2002). In our study, N-STZ-1.5 rats receiving glibenclamide for 4 weeks

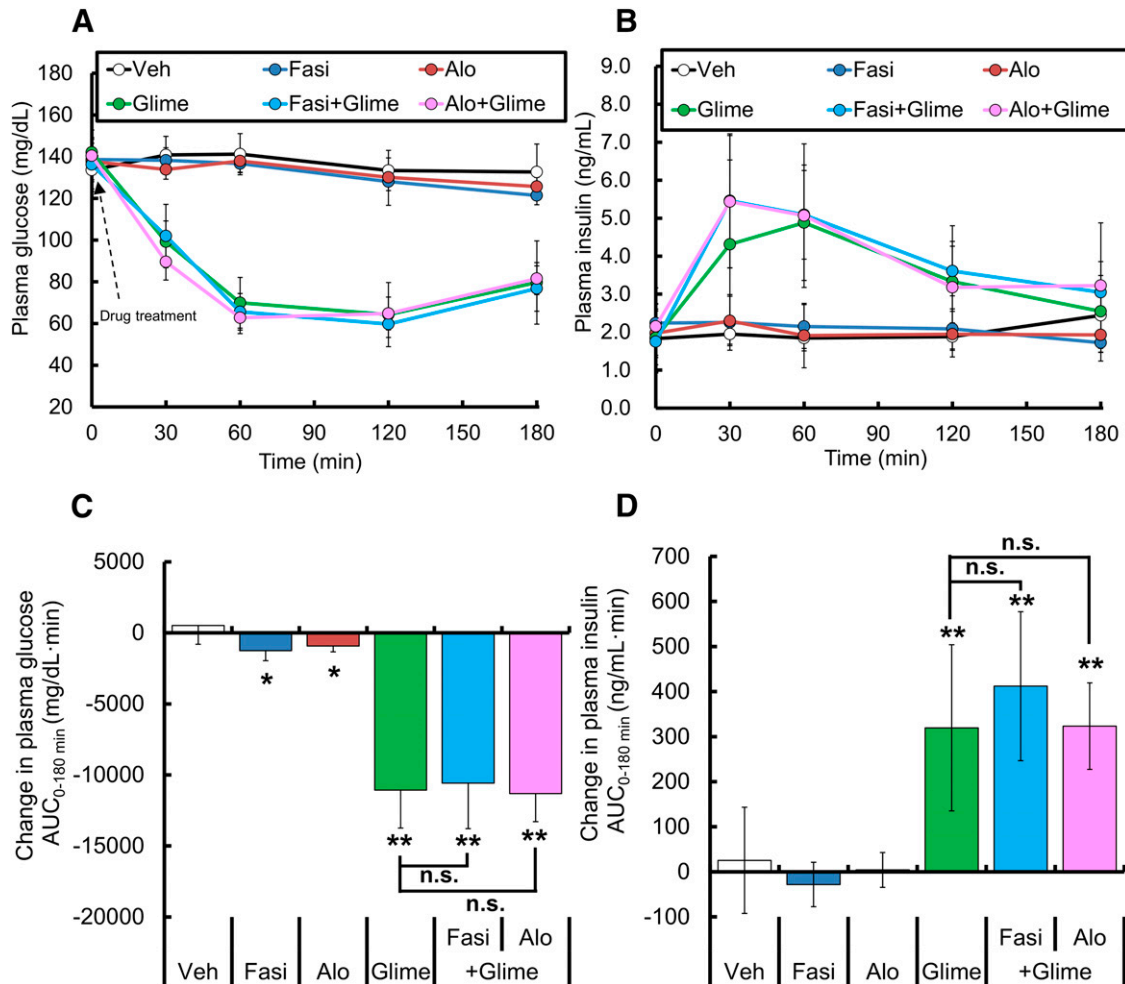


Fig. 7. Fasiglifam does not induce hypoglycemia or exacerbate SU-induced hypoglycemia in normal rats. (A) and (B) Time-dependent changes in plasma glucose and insulin levels, respectively, after treatment with vehicle (Veh), 3 mg/kg of fasiglifam (Fasi), 1 mg/kg of alogliptin (Alo), 10 mg/kg of glimepiride (Glime), 3 mg/kg of fasiglifam in combination with 10 mg/kg of glimepiride, or 1 mg/kg of alogliptin in combination with 10 mg/kg of glimepiride. Data in (C) and (D) represent change in plasma glucose AUC_{0-180 minutes} and change in plasma insulin AUC_{0-180 minutes} after drug treatments, respectively. Values are mean \pm S.D. ($n = 6$). * $P \leq 0.05$, ** $P \leq 0.01$ compared with vehicle by Student's t test or Aspin-Welch test. n.s., Not significant.

exhibited an exacerbation of glucose tolerance and a loss in response to glibenclamide. Considering that these rats exhibited normal fasting plasma glucose levels, the unresponsiveness of β -cells to SUs noted in this model may be mainly due to desensitization of insulin secretion machinery (β -cell exhaustion) rather than glucose toxicity. The previous *in vitro* study indicated that β -cells chronically treated with glibenclamide show reduced functional K_{ATP} channels on plasma membranes, despite elevated intracellular Ca^{2+} (Anello et al., 1999; Kawaki et al., 1999). In the present study, fasiglifam successfully enhanced insulin release in rats after 4 weeks of treatment with glibenclamide. This observation implies that functional K_{ATP} channels may not always be required for triggering insulin secretion via GPR40 activation under the presence of enough Ca^{2+} . Consistent with our finding, it has been shown that GPR40 agonists increase insulin secretion caused by voltage-dependent Ca^{2+} channel activators, which induces direct influx of Ca^{2+} , not via K_{ATP} channels (Yang et al., 2010).

In the present study, the unresponsiveness of β -cells to glimepiride was also observed in aged ZDF rats. This result is inconsistent with the previous observation that glibenclamide

shows a trend toward improved fasting hyperglycemia in ZDF rats (Tsujiyama et al., 2011). The discrepancy may be due to the differences in disease severity and age of the rats used in the two studies. In contrast to glibenclamide-treated N-STZ-1.5 rats, hyperglycemia per se likely contributes to SU desensitization in ZDF rats, probably through increased β -cell death, decreased insulin content, and decreased glucose-sensing genes (Lee et al., 1994; Tokuyama et al., 1995; Harmon et al., 2001). Interestingly, single administration of fasiglifam also robustly increased insulin secretion and improved glycemic control in this model, suggesting the GPR40 and the downstream signaling molecules are sufficiently maintained even in severe diabetic rodents with β -cell dysfunction.

It was previously reported that glucose dependency for GPR40-mediated insulin secretion is uncoupled by the presence of SUs in rat insulinoma cells (Yang et al., 2010), which implies that GPR40 agonists would enhance insulin secretion even at low plasma glucose levels when administered with SUs. In our study, fasiglifam in combination with glimepiride showed an additive effect in the improvement of glucose excursion and augmentation of insulin secretion. Conversely, when administered together at the same doses

in normal rats, fasiglifam did not exacerbate glimepiride-induced hypoglycemia and further increase plasma insulin levels. These results suggest that uncoupling of glucose-dependent effects of fasiglifam may not occur so much in vivo in the presence of SUs. Insulin secretion is intricately regulated by several factors other than glucose, which likely contribute in part to the different observations noted in vitro and in vivo studies. In the present study, a DPP-4 inhibitor, alogliptin, did not also enhance glimepiride-induced hypoglycemia in normal rats. However, there are a few cases of severe hypoglycemia with DPP-4 inhibitor and SU combination in elderly and/or patients with renal insufficiency when relatively higher dose of SUs are administered (Yabe and Seino, 2014). Although it has been reported that fasiglifam alone has very low hypoglycemia risk in the clinical trials (Burant et al., 2012), the reported results of DPP-4 inhibitor and SU combination suggest that careful monitoring should be required in humans especially in the combination therapy with GPR40 agonists and SUs.

In conclusion, the present study clearly indicates that compared with SUs, the GPR40 agonists are effective in lowering glucose, with less risk of secondary failure and hypoglycemia. Moreover, our findings could provide further insights into the pharmacology of GPR40 agonists and the rationale for their use in patients with diabetes with SU secondary failure. GPR40 agonists could also provide further improvement of glycemic control even when used in patients already receiving SU therapy; however, further investigation regarding the clinical risk of hypoglycemia is warranted.

Acknowledgments

The authors thank Drs. Seigo Izumo, Yukio Yamada, Masakuni Noda, and Nobuhiro Nishigaki for offering valuable discussions and helpful suggestions; Hirohisa Miyashita for providing helpful support; and Toshikazu Ando and Shoichi Asano for technical assistance.

Authorship Contributions

Participated in research design: Ito, Tsujihata, Suzuki, Miyawaki, Matsuda, and Takeuchi.

Conducted experiments: Ito, Tsujihata, Suzuki, Miyawaki, Matsuda, and Takeuchi.

Performed data analysis: Ito, Tsujihata, Suzuki, and Miyawaki.

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

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Fasiglifam/TAK-875, a Selective GPR40 Agonist, Improves Hyperglycemia in Rats

Unresponsive to Sulfonylureas and Acts Additively With Sulfonylureas

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Koji Takeuchi

The Journal of Pharmacology and Experimental Therapeutics

Supplemental Figure Legend

Supplemental figure 1

Effect of glimepiride on glucose tolerance and insulin secretion during oral glucose tolerance test in male N-STZ-1.5 rats. (A) and (B) show time-dependent changes in plasma glucose and insulin levels, respectively, after glucose load (1.5 g/kg) in N-STZ-1.5 rats treated with vehicle (Veh) or 3-30 mg/kg of glimepiride (Glime). Values are mean \pm SD (n=6).

Supplemental figure 1

