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Stimulatory effect of imeglimin on incretin secretion

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ABSTRACT

Aims/Introduction: Imeglimin is a new antidiabetic drug structurally related to metformin. Despite this structural similarity, only imeglimin augments glucose-stimulated insulin secretion (GSIS), with the mechanism underlying this effect remaining unclear. Given that glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) also enhance GSIS, we examined whether these incretin hormones might contribute to the pharmacological actions of imeglimin.

Materials and Methods: Blood glucose and plasma insulin, GIP, and GLP-1 concentrations were measured during an oral glucose tolerance test (OGTT) performed in C57BL/6JJcl (C57BL/6) or KK-Ay/TaJcl (KK-Ay) mice after administration of a single dose of imeglimin with or without the dipeptidyl peptidase-4 inhibitor sitagliptin or the GLP-1 receptor antagonist exendin-9. The effects of imeglimin, with or without GIP or GLP-1, on GSIS were examined in C57BL/6 mouse islets.

Results: Imeglimin lowered blood glucose and increased plasma insulin levels during an OGTT in both C57BL/6 and KK-Ay mice, whereas it also increased the plasma levels of GIP and GLP-1 in KK-Ay mice and the GLP-1 levels in C57BL/6 mice. The combination of imeglimin and sitagliptin increased plasma insulin and GLP-1 levels during the OGTT in KK-Ay mice to a markedly greater extent than did either drug alone. Imeglimin enhanced GSIS in an additive manner with GLP-1, but not with GIP, in mouse islets. Exendin-9 had only a minor inhibitory effect on the glucose-lowering action of imeglimin during the OGTT in KK-Ay mice.

Conclusions: Our data suggest that the imeglimin-induced increase in plasma GLP-1 levels likely contributes at least in part to its stimulatory effect on insulin secretion.

INTRODUCTION

Imeglimin is a recently launched antidiabetic drug whose chemical structure is similar to that of metformin[1]. Indeed, imeglimin and metformin share common actions including inhibition of glucose production in the liver and stimulation of glucose uptake in muscle[2]. However, despite the structural similarity between the two drugs, imeglimin enhances glucose-stimulated insulin secretion (GSIS) by pancreatic β-cells in humans and rodents as well as by isolated islets of rats and mice[3–7], whereas metformin does not have such an effect. The NAD^+–cyclic ADP-ribose–Ca^{2+} signaling pathway has been implicated in this effect of imeglimin[7], but the detailed mechanism of action *in vivo* remains unclear.

The incretins glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) improve glucose tolerance by stimulating insulin secretion from pancreatic β-cells in a glucose-dependent manner as well as exerting various other clinically beneficial effects through pleiotropic actions[8,9]. Incretin-related drugs such as dipeptidyl peptidase-4 (DPP-4) inhibitors and GLP-1 receptor agonists are administered widely for the treatment of type 2 diabetes as a result of their high

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efficacy and low risk of hypoglycemia induction[10]. Metformin lowers blood glucose levels predominantly by suppressing glucose production in the liver, but is also thought to stimulate incretin secretion by intestinal endocrine cells through various mechanisms[11]. On the other hand, the potential role of incretins in imeglimin action remains unknown.

We have now examined the effects of imeglimin on glucose tolerance, insulin secretion, and incretin secretion in both diabetic and healthy mice. We show here that imeglimin promotes the secretion of incretins, and that this action may contribute in part to the effects of imeglimin on pancreatic β-cells *in vivo*.

MATERIALS AND METHODS

Animals

Male KK-Ay/TaJcl (KK-Ay) mice and male C57BL/6JJcl (C57BL/6) mice, obtained from CLEA Japan (Tokyo, Japan), were maintained under specific pathogen-free conditions at 23° ± 2°C and 55% ± 10% relative humidity and with a 12 h light/12 h dark cycle, and were freely provided with water and a commercial diet (CE-2; CLEA Japan).

This study was approved by the President of Kobe University after the review by Institutional Animal Care and Use Committee (approval number: 30-03-02) and carried out according to the Kobe University Animal Experimentation Regulations. *Glp1r*$^{-/-}$ (*Glp1r* KO) mice on the C57BL/6J background generated as described previously[12] were kindly provided by Dr Daniel J Drucker (University of Toronto, Canada), and experiments with these mice were performed at Kyoto Prefectural University in accordance with Institutional Regulations for Animal Experiments (approval number: KPU040907-4C). All experiments were performed with male mice between 8 and 15 weeks of age.

Glucose tolerance test

For an oral glucose tolerance test (OGTT), the mice were deprived of food for 6 h, after which glucose was administered orally at a dose of 1.5 g/kg. Blood samples were obtained from the tail vein, and blood glucose levels were measured with the use of an Antsense Duo glucose analyzer (Horiba, Kyoto, Japan). For measurement of plasma concentrations of insulin, total GIP, and active GLP-1, blood was collected in hematocrit capillaries (Hirschmann, Eberstadt, Germany) and placed into a tube containing an inhibitor cocktail of KR-62436 Sigma-Aldrich (St Louis, MO, USA), EDTA, and aprotinin Fujifilm Wako Pure Chemical Co. (Osaka, Japan). Insulin was measured with an Ultra Sensitive Mouse/Rat Insulin ELISA Kit (Morinaga, Yokohama Japan), total GIP with a rat/mouse GIP (Total) ELISA kit (Sigma-Aldrich), and active GLP-1 with a GLP-1, active form (high sensitivity) assay kit (IBL). Imeglimin (Sumitomo Pharma Co., Ltd) and sitagliptin (Cayman Chemical, Ann Arbor, MI, USA) were administered orally at doses of 200 and 15 mg/kg, respectively, and exendin-9 (Abcam, Cambridge, MA, USA) dissolved in saline was administered intraperitoneally at a dose of 50 μg/body.

Measurement of insulin secretion

Insulin secretion from mouse pancreatic islets was measured as described previously[13]. In brief, islets were isolated from mouse pancreas by collagenase digestion, cultured under 5% CO_2 at 37°C in RPMI-1640 medium (Sigma-Aldrich) for 2 days, washed twice with Krebs-Ringer bicarbonate buffer supplemented with 10 mM HEPES-NaOH (pH 7.4), 0.1% bovine serum albumin, and 2.8 mM glucose (2.8G-KRBH), and incubated in 2.8G-KRBH for 30 min at 37°C. The islets were then washed again with 2.8G-KRBH, transferred to a round-bottom 96-well plate (Corning, Corning, NY, USA) at a density of five islets per well, and incubated for 30 min at 37°C in KRBH containing various stimuli. Insulin released into the medium and remaining in the islets was measured with a homogeneous time-resolved fluorescence assay (Insulin UltraSensitive HTRF assay kit; Cisbio, Codolet, France). The amount of insulin secreted was normalized by islet insulin content.

Statistical analysis

Data are presented as mean ± SEM and were analyzed with the use of GraphPad Prism version 9. The significance of differences between or among groups was evaluated with the two-tailed unpaired Student's *t*-test, by one-way analysis of variance (ANOVA) followed by Dunnett's, Bonferroni's, or Tukey's multiple comparison test, or by two-way ANOVA followed by Tukey's or Bonferroni's multiple comparison test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of a single dose of imeglimin on blood glucose, plasma insulin, and plasma incretin levels during an OGTT

To provide insight into the mechanisms underlying the antidiabetic action of imeglimin, we first investigated the acute effects of the drug in C57BL/6 mice and KK-Ay diabetic mice. Mice deprived of food for 5 h beginning at 09:00 h received a single oral dose of imeglimin or vehicle, and they were subjected to an OGTT after an additional 1 h without food. Imeglimin significantly attenuated the increase in blood glucose levels at all time points examined during the OGTT in both C57BL/6 (Figure 1a) and KK-Ay (Figure 1e) mice, with the reduction in the area under the curve (AUC) being 14% (46,724 ± 796 and 40,032 ± 678 mg/dL min for vehicle vs imeglimin, respectively) for C57BL/6 mice and 22% (101,037 ± 4,796 and 78,759 ± 2,630 mg/dL min for vehicle vs imeglimin, respectively) for KK-Ay mice. Plasma insulin levels 30 min after or both before and 15 and 30 min after initiation of the OGTT were significantly higher in imeglimin-treated C57BL/6 (Figure 1b) or KK-Ay (Figure 1f) mice, respectively, than in vehicle-treated control mice. Whereas plasma levels of GIP were not altered by imeglimin in C57BL/6 mice (Figure 1c), they were significantly increased by the drug at 15 and 30 min after test initiation in KK-Ay mice (Figure 1g). Furthermore, imeglimin treatment increased the plasma concentration of

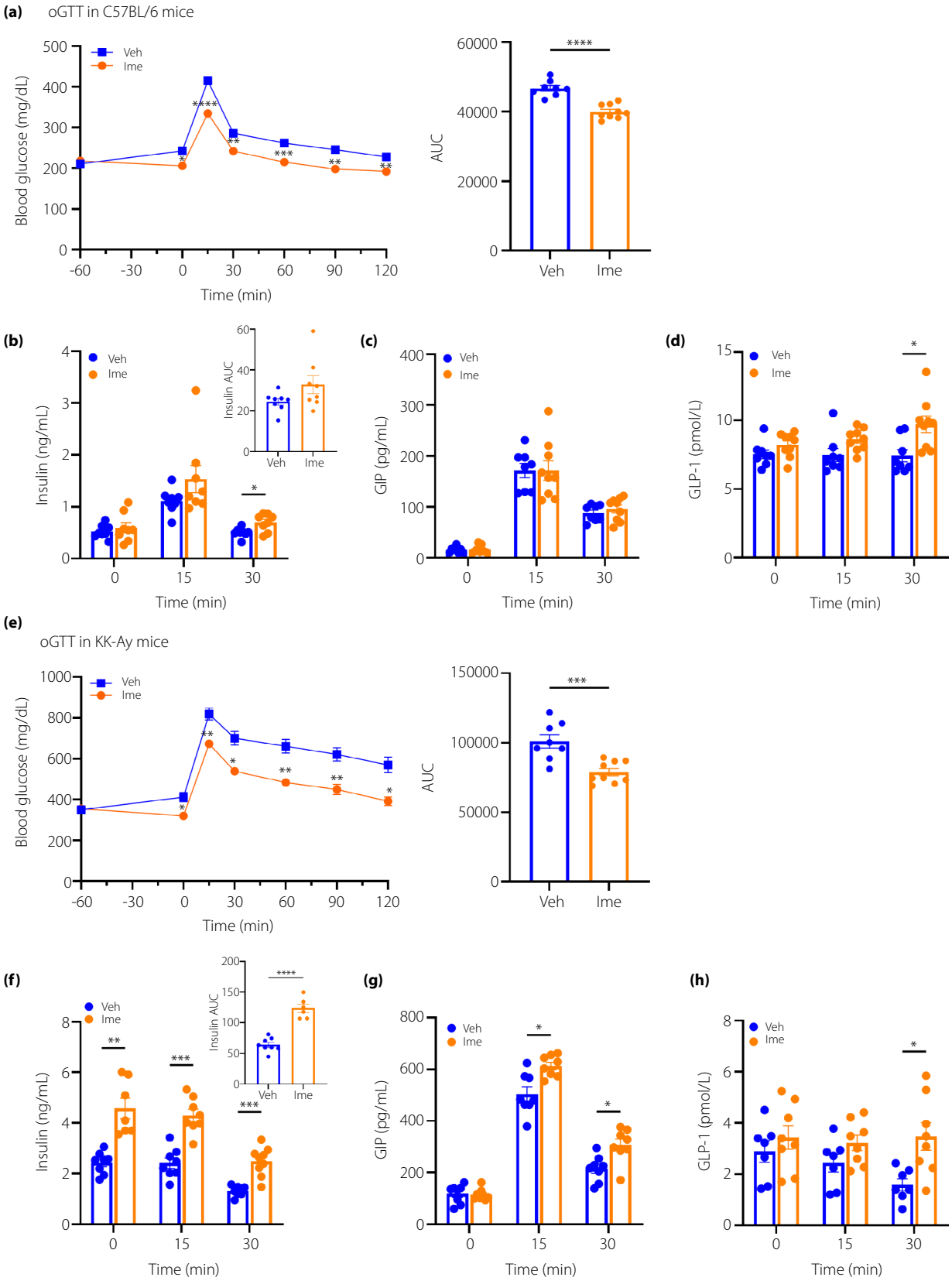


Figure 1 | Effects of a single oral dose of imeglimin on blood glucose, plasma insulin, and plasma incretin levels during an OGTT. (a, e) Effect of imeglimin (Ime) on blood glucose levels in C57BL/6 (a) and KK-Ay (e) mice (n = 8 or 9 per group). The time course (left) and AUC (right) for blood glucose are shown. Imeglimin (200 mg/kg) or vehicle (Veh, deionized water) was administered orally to the fasted mice 1 h before the glucose (1.5 g/kg) challenge. (b, f) Plasma insulin levels at the indicated times after initiation of the OGTT in C57BL/6 (b) and KK-Ay (f) mice (n = 8 or n = 7–9 per group, respectively). The insets show the AUC for plasma insulin. (c, g) Plasma total GIP levels at the indicated times after initiation of the OGTT in C57BL/6 (c) and KK-Ay (g) mice (n = 7–9 or n = 8 per group, respectively). (d, h) Plasma active GLP-1 levels at the indicated times after initiation of the OGTT in C57BL/6 (d) and KK-Ay (h) mice (n = 8 or 9 or n = 7 or 8 per group, respectively). All data are mean ± SEM. Statistical analysis was performed with the two-tailed unpaired Student's t test (AUC in a, b, e, and f) or by two-way ANOVA followed by Bonferroni's multiple comparison test (all other comparisons).*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs the corresponding value for vehicle in the time courses for blood glucose or as indicated.

GLP-1 at 30 min after initiation of the OGTT in both C57BL/6 and KK-Ay mice (Figure 1d,h). These results thus indicated that imeglimin increased plasma GLP-1 levels in diabetic and nondiabetic mice as well as plasma GIP levels in diabetic mice during an OGTT.

Effects of the combination of imeglimin and sitagliptin on blood glucose, plasma insulin, and plasma incretin levels during an OGTT

Given that imeglimin was found to increase the plasma concentrations of GIP and GLP-1 in KK-Ay mice, we next investigated the effects of the combination of imeglimin and a DPP-4 inhibitor on blood glucose and plasma insulin and incretin levels in these mice. The increase in blood glucose levels during an OGTT was significantly or tended to be attenuated by the administration of imeglimin or the DPP-4 inhibitor sitagliptin, respectively, in KK-Ay mice (Figure 2a). The combination of the two drugs reduced blood glucose levels to a significantly greater extent compared with either drug alone at 30–120 min after initiation of the test. The plasma concentration of insulin during the OGTT tended to be higher in imeglimin-treated mice than in vehicle-treated mice, whereas sitagliptin had no substantial effect on insulin levels at the time points examined (Figure 2b). However, combined treatment with the two drugs markedly increased plasma insulin levels compared with treatment with either drug alone. Treatment with imeglimin, sitagliptin, or the combination of the two agents did not increase the plasma concentration of GIP, with sitagliptin and the drug combination actually tending to attenuate the increase in plasma GIP levels during the OGTT (Figure 2c). Plasma levels of GLP-1 tended to be increased in imeglimin-treated mice and sitagliptin-treated mice during the OGTT, and they were significantly higher in mice treated with both drugs than in those treated with either drug alone (Figure 2d). These results indicated that imeglimin and sitagliptin exerted synergistic effects on blood glucose, plasma insulin, and plasma GLP-1 levels.

Effects of imeglimin and incretins on GSIS in mouse islets

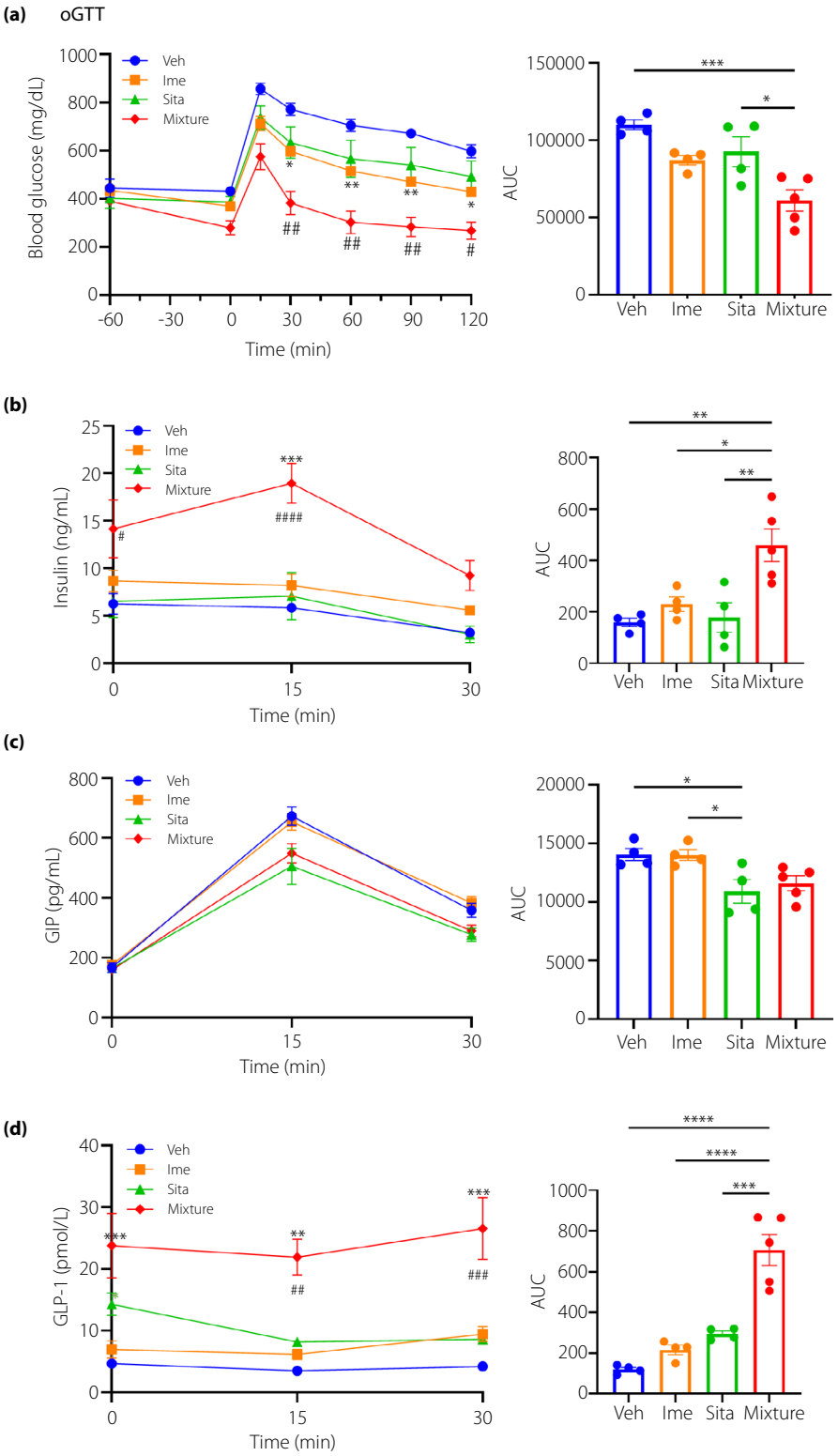
We next investigated the effects of imeglimin and incretins on glucose-stimulated insulin secretion in pancreatic islets isolated from C57BL/6 mice. Exposure of the islets to a high concentration (16.7 mM) of glucose resulted in a marked increase in insulin secretion, and this effect was significantly enhanced by treatment with imeglimin at 5 or 10 mM (Figure 3a). Glucose-stimulated insulin secretion was also significantly, or tended to be, enhanced by treatment of the islets with GIP or GLP-1, respectively (Figure 3b). The addition of imeglimin increased insulin secretion in islets exposed to both a high concentration of glucose and GLP-1, but not in those treated with high glucose and GIP (Figure 3b). These results suggested that imeglimin and GLP-1 enhanced GSIS in an additive manner.

Effects of exendin-9 on the imeglimin-induced changes in blood glucose and plasma insulin levels during an OGTT

We next examined whether the GLP-1 receptor antagonist exendin-9 might influence the effects of imeglimin on blood glucose and plasma insulin levels during an OGTT. Blood glucose levels at 30–120 min during the OGTT were significantly lower in KK-Ay mice treated with imeglimin than in those treated with vehicle alone, and this effect of imeglimin tended to be inhibited by exendin-9 (Figure 4a). The AUC for blood glucose also tended to be higher in mice treated with both imeglimin and exendin-9 than in those treated with imeglimin alone (Figure 4a). These results suggested that exendin-9 might partially attenuate the glucose-lowering effect of imeglimin. The plasma concentration of insulin at each time point during the OGTT and the AUC for plasma insulin tended to be higher in mice treated with imeglimin than in those treated with vehicle (with the differences not achieving statistical significance in this experiment), whereas they did not differ between mice treated with imeglimin alone and those treated with imeglimin plus exendin-9 (Figure 4b).

Effects of a single dose of imeglimin on blood glucose and plasma insulin levels in *Glp1r* KO mice during an OGTT

Finally, we investigated the effects of imeglimin on blood glucose and plasma insulin levels during an OGTT in GLP-1 receptor knockout (*Glp1r* KO) mice on the C57BL/6 genetic background. Imeglimin significantly attenuated the increase in blood glucose levels in these mice at 15, 60, and 120 min after initiation of the test as well as reduced the AUC for blood glucose by 17% (39,424 ± 1,252 and 32,731 ± 1,244 mg/dL min



for vehicle vs imeglimin, respectively; Figure 4c), with the extent of this effect being similar to that apparent in wild-type C57BL/6 mice (Figure 1a). The plasma levels of insulin (Figure 4d) tended to be increased by imeglimin treatment, but this effect was not statistically significant. Whereas these results do not exclude a possible contribution of GLP-1 to the glucose-lowering effect of imeglimin, they implicate a mechanism independent of GLP-1.

Figure 2 | Effects of single doses of imeglimin and sitagliptin on blood glucose, plasma insulin, and plasma incretin levels during an OGTT in KK-Ay mice. (a) Effects of imeglimin (Ime), sitagliptin (Sita), and the combination of the two drugs on the time course (left) and AUC (right) for blood glucose (n = 4 or 5 mice per group). Vehicle (Veh, 0.5% carboxymethylcellulose), imeglimin (200 mg/kg), sitagliptin (15 mg/kg), or a mixture of the two drugs was administered orally to the fasted mice 1 h before glucose (1.5 g/kg) challenge. *P < 0.05, **P < 0.01 vs the corresponding value for vehicle; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 vs the corresponding value for imeglimin in the time course. (b) Plasma insulin levels during the OGTT (n = 4 or 5 mice per group). ***P < 0.001, $^{\#}P$ < 0.05, $^{\#\#\#\#}P$ < 0.0001 vs the corresponding values for imeglimin and sitagliptin, respectively, in the time course. (c) Plasma total GIP levels during the OGTT (n = 4 or 5 mice per group). (d) Plasma active GLP-1 levels during the OGTT (n = 4 or 5 mice per group). **P < 0.01, ***P < 0.001 vs the corresponding value for imeglimin; $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 vs the corresponding value for sitagliptin in the time course. All data are mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test (AUC) or by two-way ANOVA followed by Tukey's multiple comparison test (time courses). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 for the indicated comparisons in histograms.

DISCUSSION

We have here shown that a single dose of imeglimin increased the plasma concentrations of GIP and GLP-1 in KK-Ay mice during an OGTT, and that the combination of imeglimin and sitagliptin increased the plasma levels of insulin and GLP-1 in these mice to a markedly greater extent than did either drug alone. Moreover, imeglimin and GLP-1 enhanced glucose-stimulated insulin secretion by isolated mouse islets in an additive manner. These results suggest that an imeglimin-induced increase in GLP-1 levels contributes to the promotion of insulin secretion by this drug, with this effect of imeglimin on GSIS being thought to play an important role in the antidiabetic action of the drug. Imeglimin-induced increase in GIP was observed in KK-Ay mice but not in C57BL/6 mice. Given that the number of K cells is increased in obese model mice[14], the difference in the response of GIP in the two genotypes of mice is attributable to the number of K cells in the intestine.

A phase 3 trial conducted in Japan showed that the administration of imeglimin to individuals with type 2 diabetes treated with DPP-4 inhibitors or GLP-1 receptor agonists (GLP-1RAs) resulted in a reduction in hemoglobin A_{1c} levels by 0.92 and 0.12 percentage points, respectively[15]. Whereas this trial was not designed to compare the efficacy of each type of combination therapy, its results are consistent with our present observations that the combination of imeglimin and sitagliptin showed marked synergistic effects on the plasma levels of GLP-1 and insulin. Moreover, if the antidiabetic action of imeglimin is largely attributable to its ability to increase the circulating concentration of GLP-1, it is possible that the addition of imeglimin to a GLP-1RA will result in only a small reduction in glycemia, with the increase in endogenous GLP-1 levels likely having only a minor effect in this setting in which GLP-1 signaling is already markedly activated by the GLP-1RA. Additional clinical trials investigating the effects of the combination of imeglimin with either of these incretin-related drug types are warranted to understand not only the clinical benefit but also the mode of action of imeglimin.

The mechanism by which imeglimin stimulates the secretion of GLP-1 remains unknown. Imeglimin is structurally related to metformin and exerts biochemical effects similar to those of metformin in hepatocytes[16,17]. Metformin also increases the secretion of GLP-1 in both rodents[18] and humans[19–21]. Possible mechanisms proposed for the induction

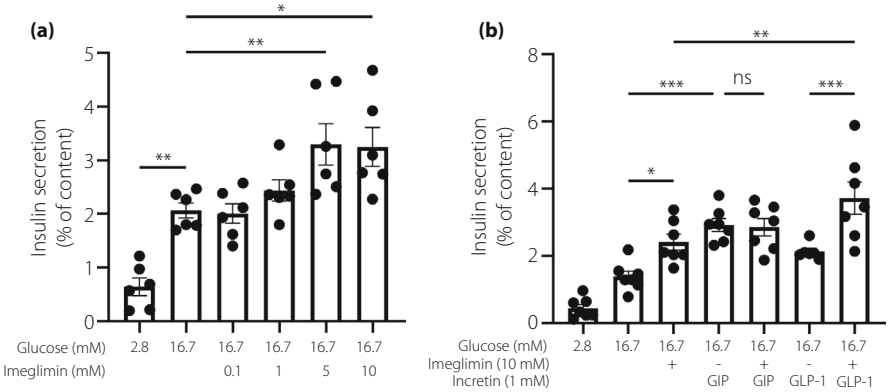
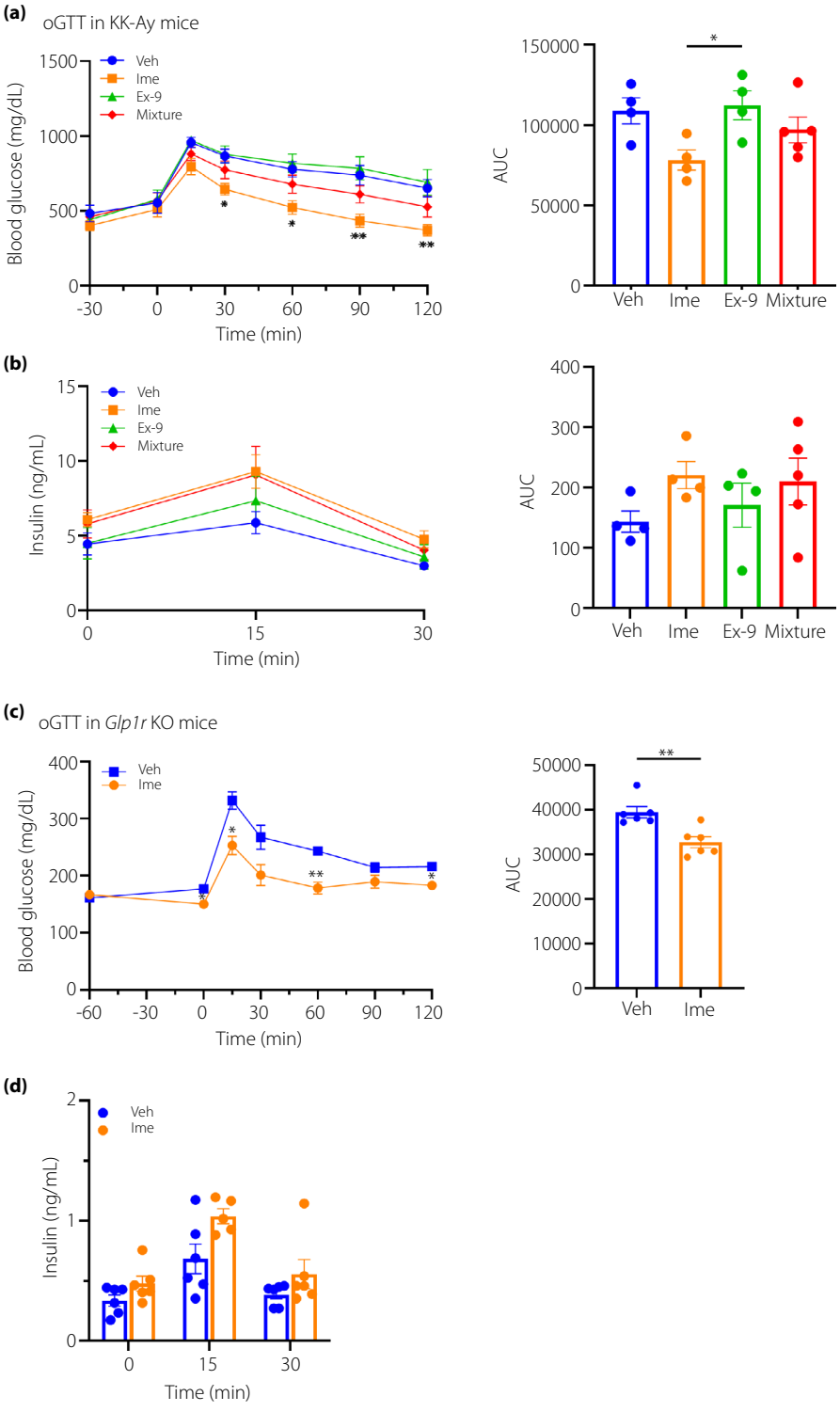


Figure 3 | Effects of imeglimin and incretins on GSIS in mouse pancreatic islets. Pancreatic islets isolated from C57BL/6 mice were incubated for 30 min with the indicated concentrations of glucose and either imeglimin (a) or imeglimin, GIP, or GLP-1 (b), after which insulin secreted into the medium was assayed as a percentage of total islet content. Data are mean ± SEM (n = 6 or 7 independent experiments). Statistical analysis was performed by one-way ANOVA followed either by Dunnett's multiple comparison test (a) or by Bonferroni's multiple comparison test (b). *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant.



of GLP-1 secretion by metformin include a direct effect on L cells[19] and effects mediated *via* changes in bile acids[22,23], or glucose metabolism[24]. It remains to be determined whether such mechanisms also underlie the promotion of GLP-1 secretion by imeglimin.

We found that exendin-9 had only a minor effect on the glucose-lowering action of imeglimin in KK-Ay mice. Moreover, exendin-9 did not significantly inhibit imeglimin-induced insulin secretion in these animals. These results appear inconsistent with the hypothesis that the imeglimin-induced increase

Figure 4 | Effects of imeglimin and exendin-9 in KK-Ay mice as well as of imeglimin in *Glp1r* KO mice. (a) Effects of imeglimin (Ime), exendin-9 (Ex-9), and a mixture of the two agents on the time course (left) and AUC (right) for blood glucose in KK-Ay mice (n = 4 or 5 per group) during an OGTT. Vehicle (Veh, 0.5% carboxymethylcellulose or saline), imeglimin (200 mg/kg, oral), exendin-9 (50 μg/body, intraperitoneal), or both imeglimin and exendin-9 were administered to fasted mice 30 min before glucose (1.5 g/kg) loading. *P < 0.05, **P < 0.01 vs vehicle in the time course. (b) Plasma insulin levels during the OGTT for mice (n = 4 or 5 per group) as in (a). (c) Effect of imeglimin on the time course (left) and AUC (right) for blood glucose in *Glp1r* KO mice (n = 6 per group) during an OGTT. Imeglimin (200 mg/kg) or vehicle (0.5% carboxymethylcellulose) was administered orally to the mice 1 h before glucose (1.5 g/kg) challenge. *P < 0.05, **P < 0.01 vs vehicle in the time course. (d) Plasma insulin levels during the OGTT for mice (n = 5 or 6 per group) as in (c). Data are mean ± SEM. Statistical analysis was performed with the two-tailed unpaired Student's t-test (AUC in c, by one-way ANOVA followed by Tukey's multiple comparison test (AUC in a, b), by two-way ANOVA followed by Tukey's multiple comparison test (time courses in a, b), or by two-way ANOVA followed by Bonferroni's multiple comparison test (time course in c and d). *P < 0.05, **P < 0.01 for histograms.

in circulating GLP-1 levels contributes to the insulin-stimulating and glucose-lowering effects of this drug. However, we cannot exclude the possibility that our experimental conditions were not appropriate for validation of the role of GLP-1 in imeglimin action; we thus do not know whether the dose and timing of exendin-9 administration were sufficient to prevent GLP-1 signaling, although this antagonist was previously shown to be effective in this regard under conditions similar to those of our study[25]. Regardless, our finding that imeglimin lowered blood glucose levels in *Glp1r* KO mice indicates that a GLP-1-independent mechanism contributes to imeglimin action. Further study is required to clarify whether or to what extent GLP-1 signaling plays a role in the glucose-lowering effect of imeglimin.

We selected 200 mg/kg as the dose of imeglimin for the present study, which is much higher than the clinically approved dose in Japan (1,000 mg/body twice daily). Preliminary experiments revealed that a smaller dose (50 mg/kg) did not significantly reduce blood glucose levels in C57BL/6 mice during an OGTT (data not shown). Whereas other investigators also showed that administration of imeglimin to ZDF rats[16] or db/db mice[26] at doses similar or identical to that in the present study (150 and 200 mg/kg, respectively) lowered glycemia during an OGTT, we do not know whether the effects observed in our study also occur in humans treated with imeglimin at the clinical dose.

The current study has several limitations. Whereas the oral glucose load is capable of stimulating GLP-1 secretion, we did not observe an increase in GLP-1 levels in response to OGTT in some experiments. A previous report showed that an increase in GLP-1 secretion was not observed at 30 min after oral glucose administration[27] and the increase in GLP-1 secretion in response to an oral glucose load is dose-dependent[28]. The current experimental condition thus may not be appropriate for observing the increase in GLP-1 secretion in response to an oral glucose load. In addition, the plasma levels of insulin and GLP-1 in KK-Ay mice not treated with imeglimin during OGTT were lower than before the test. Whereas we do not know the mechanism of these phenomena, previous studies also showed that the levels of these hormones during OGTT are not increased, or decreased, in some model animals[27,29–31].

In the current study, the change in the plasma levels of GLP-1 and GIP was not always parallel. We measured total and active proteins for GIP and GLP-1, respectively. Such a difference in the measurements may affect the observed difference in the secretory pattern of the two hormones. Evidence suggests that plasma levels of GLP-1 are increased as body mass increases[32]. However, in some experiments in the current study, GLP-1 levels in KK-Ay mice are lower than that of C57BL/6 mice. We do not know the reason for this apparent inconsistency, but the plasma levels of GLP-1 in KK-Ay mice in this study are consistent with those in a previous study[33].

In summary, we have shown that imeglimin increases plasma GLP-1 levels in mice, and that this action likely contributes at least in part to its stimulatory effect on insulin secretion. Whereas further study is required to validate the clinical relevance of this action of imeglimin, our current findings provide new insight into the pharmacological effects and clinical use of this new antidiabetic drug.

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DISCLOSURE

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Informed consent: N/A.
Registry and the registration no. of the study/trial: N/A.
Animal studies: Approved by the President of Kobe University after the review by Institutional Animal Care and Use Committee (Approval No. 30-03-02), and by Kyoto Prefectural University in accordance with Institutional Regulations for Animal Experiments (approval No. KPU040907-4C).

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