Imeglimin Lowers Glucose Primarily by Amplifying Glucose-Stimulated Insulin Secretion in High Fat Fed Rodents

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Running head:
Imeglimin raises insulin secretion, not insulin sensitivity
Abstract

Imeglimin is a promising new oral antihyperglycemic agent, which has been studied in clinical trials as a possible mono- or add-on therapy to lower fasting plasma glucose and improve hemoglobin A1c (1-3, 9). Imeglimin was shown to improve both fasting and postprandial glycemia and to increase insulin secretion in response to glucose during a hyperglycemic clamp after 1-week treatment in type 2 diabetic patients. However, whether the β-cell stimulatory effect of imeglimin is solely or partially responsible for its effects on glycemia remains to be fully confirmed. Here we show that imeglimin directly activates β-cell insulin secretion in awake rodents without affecting hepatic insulin sensitivity, body composition, or energy expenditure. These data identify a primary amplification rather than triggering β-cell mechanism that explains the acute, antidiabetic activity of imeglimin.

Keywords

Imeglimin, β-cell, glucose-stimulated insulin secretion

Introduction

Imeglimin is a new oral antihyperglycemic agent, which has been studied in clinical trials as a possible mono- or add-on therapy to lower fasting plasma glucose and improve hemoglobin A1c (2, 3, 9). Imeglimin was shown to improve both fasting and postprandial glycemia (1), and increase insulin secretion in response to glucose during a hyperglycemic clamp after 1-week treatment in type 2 diabetic patients (8). Improvements in glucose tolerance have been reported in both humans and rodents.
treated with imeglimin but have been ascribed to multiple mechanisms: lower hepatic lipids (12), improved liver and muscle insulin signaling (12), and improved β-cell function (3, 12). However, prior studies have not utilized the gold-standard technique to evaluate hepatic insulin sensitivity, the hyperinsulinemic-euglycemic clamp. Of note, although the mechanism of action of imeglimin has been ascribed at least in part to improved insulin sensitivity (12), improved insulin sensitivity would be expected to result in reductions in plasma insulin concentrations, not the increases that have been noted in multiple human (8, 9) and rodent (12) studies. Additionally, previous papers have not included in vitro studies to determine whether imeglimin increases glucose-stimulated insulin secretion directly or indirectly. In addition, most of the prior studies performed with imeglimin were long-term treatment studies (months-long), which do not allow researchers to identify the first beneficial effects of the drug. To this end, we performed intraperitoneal glucose tolerance tests (GTT) and hyperinsulinemic-euglycemic clamps in chow- and high fat fed rats treated with imeglimin for 2 weeks, thereby conclusively demonstrating the mechanism by which imeglimin improves glycemia in the high fat fed rat using a comprehensive toolkit of gold-standard in vitro and in vivo techniques. Furthermore, we assessed glucose-stimulated insulin secretion in isolated rat islets and identified a direct, glucose-dependent, amplifying mechanism of action that would explain the absence of hypoglycemia when used at recommended pharmacologic concentrations in humans.
Methods

Animals

All animal protocols were approved by the Yale University Institutional Animal Care and Use Committee, and all guidelines in the *Guide for the Care and Use of Laboratory Animals, Eighth Edition* were followed. Because metabolic cages could only be used for mice, Male C57BL/6 mice were purchased from Jackson Labs at 8 weeks of age and were placed on chow or lard-based high fat diet. They were treated twice daily with imeglimin (150 mg/kg) mixed into a small amount of peanut butter, or an equal amount of peanut butter vehicle. Mice underwent metabolic cage analysis (Columbus Instruments, Columbus, OH) after 1 and 3 weeks of treatment in the chow fed group, or after 1 and 5 weeks of treatment in the high fat diet (HFD) fed group.

All other animal studies involved Male Sprague-Dawley rats that were purchased from Charles River at 350 g and were placed on chow or safflower oil-based high fat diet. After 2 weeks on the diet, they were treated twice daily for two weeks with imeglimin (150 mg/kg) in peanut butter, or peanut butter vehicle for two weeks while they were maintained on the diet. After the first week of treatment, they underwent surgery under general isoflurane anesthesia to place polyethylene catheters in the carotid artery and jugular vein, and after a week of recovery and an overnight fast, they underwent intraperitoneal GTT (1 g/kg glucose). The final dose of imeglimin was given 15 minutes prior to the start of the GTT. Blood (200 μl at each draw) was taken from the venous catheter 0, 5, 10, 15, 30, 45, 60, and 90 min after the glucose bolus, immediately placed in heparin-coated tubes, and plasma was isolated after centrifugation for analysis of plasma glucose and insulin concentrations.
In the clamp studies, rats were again studied after a total of four weeks of diet feeding, during the last two of which they were treated with imeglimin (150 mg/kg twice daily in peanut butter, or peanut butter vehicle). Following an overnight fast, a 120 min basal infusion of \([6,6-\text{^2}H_2]\) glucose (1 mg/[kg-min] prime for 5 min, followed by a 0.25 mg/[kg-min] continuous infusion) was administered, with 200 μl whole blood taken after 100, 110, and 120 min of infusion to measure glucose turnover as described below. The final dose of imeglimin was given 15 min prior to the start of the basal infusion. Immediately following the basal infusion, 120 min hyperinsulinemic-euglycemic clamps were performed. Rats received arterial infusions of insulin (prime: 40 mU/kg over 5 min, followed by a continuous dose: 4 mU/[kg-min]) for the duration of the clamp. Euglycemia (100-110 mg/dL) was maintained with a variable infusion of D20 \([6,6-\text{^2}H_2]\) glucose (99% APE). Blood samples (50 μl) were taken every 15 minutes for measurement of glucose using a YSI glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH). After 120 min, 20 mCi of 2-[\text{^14}C] deoxyglucose was injected through the venous line, and glucose uptake was measured in skeletal muscle (quadriceps) as described previously (4). Twenty minutes after administration of the 2-[\text{^14}C] deoxyglucose, rats were sacrificed with intravenous pentobarbital and liver and quadriceps were rapidly freeze-clamped in aluminum tongs pre-cooled in liquid N\(_2\).

**Biochemical analysis**

Plasma glucose concentrations were measured biochemically using a YSI Glucose Analyzer (Yellow Springs Instruments, Yellow Springs, OH) and plasma insulin was measured by radioimmunoassay by the Yale Diabetes Research Core.
Western blots

Pan-acetyl-CoA carboxylase (ACC) phosphorylation and AMPK activation were measured by Western blot (14) in overnight-fasted rat liver two hours following the last imeglimin dose, with antibodies obtained from Cell Signaling. Equal protein loading was confirmed by measuring GAPDH protein content (antibody from Santa Cruz) in each blot.

Insulin secretion in isolated islets

Intact islets isolated from ~300g Sprague-Dawley rats were hand-picked and layered between acrylamide gel column beads (Bio-Gel P4G [156-4124]) in perifusion/static incubation media (DMEM [Sigma D5030], supplemented with NaHCO₃ as per the manufacturer’s instructions, 10 mM HEPES, 4 mM glutamine, 0.2% fatty acid free BSA and 2.5 mM glucose). Eighty islets for each condition were perifused at a rate of 100 μl/min on an 8 or 12-channel BioRep Technologies perifusion device (Miami, FL). Islets were equilibrated on the instrument in basal (2.5 mM) glucose perifusion media with or without imeglimin (100 μM) for 45 minutes prior to sample collection in a 5% CO₂/95% air, 37°C constant environment. For static secretion assays, intact islets were dispersed using accutase (Gibco) and re-aggregated to form single pseudo-islet aggregates. Following dispersion into single cells, the cell suspension was seeded at 5,000 cells per well of a 96-well V-bottom plate and cultured under the same conditions as the intact islets. Pseudo-islet aggregates formed 12-24hrs after incubation at 37°C 5% CO₂/95% air. Rat insulin was measured in the perifusate/incubation media using a
high range rat insulin ELISA (ALPCO, Salem, NH) and normalized to total islet DNA concentration using Quant-it PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA). Secretagogues included the glucokinase activator (MK-0941), tolbutamide, exendin-4 (Tocris), L-leucine, forskolin, and monomethyl ester of succinic acid (SAME) at the indicated concentrations.

Results

*Imeglimin treatment does not alter body composition or energetics*

Metabolic cage analysis revealed no difference in any parameter studied in chow- or high fat fed mice treated for one week with imeglimin, with the exception of food intake, which was modestly lower in imeglimin treated mice (Fig. 1-2). Similarly, in chow- and high fat fed (HFD) mice treated chronically (3-5 weeks) with imeglimin, there were no differences seen in body composition, food and water intake, or energetics compared to vehicle-treated controls (Fig. 3-4).

*Imeglimin-treated rats exhibit improved glucose tolerance due to increased insulin secretion in vivo*

In chow fed rats, 2 weeks of imeglimin treatment resulted in a striking improvement in glucose tolerance, with lower plasma glucose concentrations throughout the GTT (Fig. 5A-B). Imeglimin-treated mice exhibited 30-100% increases in insulin secretion at each time point during the GTT, with more than a doubling in the insulin area under the curve during the GTT (Fig. 5C-D). These results were replicated in high fat fed (HFD) rats:
imeglinim improved glucose tolerance by increasing insulin secretion throughout a GTT after a two-week treatment period (Fig. 6A-D).

*Imeglimin does not alter insulin sensitivity in chow- or high fat fed rats*

The improvements in glucose tolerance observed in imeglinim-treated rats were not associated with hepatic AMPK activation in chow fed rats; however, we observed a 50% reduction in ACC phosphorylation as predicted by imeglinim-treated rats' higher plasma insulin concentrations (Fig. 7A-B) (13). In order to determine whether imeglinim treatment was associated with improved hepatic or peripheral muscle insulin sensitivity, we performed hyperinsulinemic-euglycemic clamps and observed no difference in the glucose infusion rate required to maintain euglycemia, in basal or clamped hepatic glucose production, or in peripheral muscle glucose disposal during the clamp (Fig. 7C-G). Similarly in HFD rats, there were no differences in hepatic or muscle insulin sensitivity as indicated by the absence of any differences in glucose infusion rate, hepatic glucose production, or muscle glucose uptake after 2 weeks of imeglinim treatment (Fig. 8A-E).

*Imeglimin amplifies rather than triggers insulin secretion.*

In order to determine whether imeglinim directly stimulates beta-cell insulin secretion, we measured glucose-stimulated insulin secretion by isolated islet perifusion and static incubations. Adding imeglinim to previously untreated rat islets at 100 μM, a dose previously demonstrated to stimulate GSIS (12), caused an immediate increase in glucose-stimulated insulin secretion (Fig. 9A). An area under the curve analysis
identified the mitochondrial metabolism-dependent second phase of insulin secretion accounting for the bulk of the increase (Fig. 9B). In comparison to other islet secretagogues, insulin secretion appeared to be dependent upon glucose metabolism up to a concentration of 1 mM imeglimin (Fig. 9C). The glucose dependency displayed a graded amplifying behavior except at concentrations >5 mM (well above its therapeutic target range) where it triggered insulin release even at low glucose (Fig. 9D,E). To determine whether imeglimin was dependent on glycolytic and/or mitochondrial metabolism, islet perifusions were performed using obligate mitochondrial fuels such as leucine and succinate (Fig. 9F,G,I,J). In the presence of glutamine, 10 mM leucine stimulates insulin secretion by activating glutamate dehydrogenase and supplies both anaplerotic and oxidative pathways of mitochondrial metabolism. Leucine-stimulated secretion was higher than glucose-stimulated and was not further amplified by 1 mM imeglimin (Fig. 9F,G). However, in contrast to the perifusions at increasing concentrations of leucine in static incubations, imeglimin actually amplified secretion suggesting that the lack of an observed difference in the perifusion could be attributed to having reached maximal insulin release (Fig. 9H). Like leucine, the methyl ester of succinate (SAME) has both anaplerotic and oxidative metabolism but showed only a trend toward being amplified by imeglimin (Fig. 9I,J). Taken together, these data imply that the primary mechanism of action of imeglimin is via an amplification of mitochondrial metabolism-dependent signals that stimulate insulin release.
Discussion

The attractive safety and efficacy profile of imeglin in poorly controlled diabetic patients raises the question of how and why it is so safe and effective. Improved glucose tolerance in rodents suggests the possibility of a phenotype in the beta-cell (8); however, definitive studies had not yet been performed to assess hepatic and peripheral muscle insulin sensitivity or whole-body energetics. We determined that short-term imeglin treatment modulates glucose-stimulated insulin secretion (GSIS) in isolated islets and in vivo in rats during a GTT without altering hepatic or peripheral insulin sensitivity, AMPK activity, or energetics. The lack of any difference in hepatic insulin sensitivity in high fat fed rats using the gold-standard technique, the hyperinsulinemic-euglycemic clamp, shows that after 2 weeks of treatment, imeglin's benefit on glucose tolerance in this HFD rat model is due to its ability to increase glucose-stimulated insulin secretion. This result contrasts with the improvement in insulin sensitivity shown by Vial et al. (12) in the high fat, high sucrose diet mouse model (HFHSD) and by Fouqueray et al (1) in streptozotocin-treated diabetic rats. These differences could be explained by the difference in treatment duration (2 weeks in the present study vs. 6-7 weeks in the previous studies) and/or by differences in imeglin effects in the models. Imeglin decreased liver lipid content in HFHSD mice by 30% (12) but had no effect on liver triglycerides in the present study (data not shown).

Treatment of isolated rodent islets with imeglin acutely potentiated insulin secretion indicating that there is a primary and direct effect of the compound on intact islets. Similarly, following the treatment of healthy rodents in vivo for two weeks there was noticeable improvement in insulin secretion consistent with a primary islet effect. It
is not surprising and perhaps anticipated that there are also potential secondary benefits following the improved glucose homeostasis from augmented insulin secretion. These might include improvements in insulin sensitivity after a longer treatment (6 weeks) as observed in a high fat rat model of insulin resistance. It is also possible that chronic imeglimin treatment may secondarily improve liver and muscle insulin sensitivity in diabetic rodents and patients with type 2 diabetes through reversal of glucose toxicity (10).

In isolated islet perifusions from healthy rats, imeglimin displayed a potent metabolism-dependent stimulation that resembled more the amplifying effect observed with cell surface receptors (e.g., GLP-1R or GPR40) rather than triggering by increasing metabolism or directly closing $K_{ATP}$ channels (e.g., glucokinase activators or sulfonylureas) (5, 6). This pharmacologic behavior links the enhanced insulin secretion to metabolic coupling factors. As such, the amplifying effect is minimal when on the low end of the physiologic range. This may help explain imeglimin’s observed efficacy without causing hypoglycemia in humans. As glucose levels climb, there is a progressive increase in secretion as would be needed to re-establish homeostasis following a meal. By only acting when glucose levels are high, the intermittent responsivity prevents basal hyperinsulinism and potentially allows the beta-cell more of a chance to rest between challenges. These may afford a long-term advantage of this novel class of oral agents to protect and/or preserve islet health.

Future studies will be needed to identify the receptor and/or pathway(s) through which this compound acts. In particular, given that both anaplerotic and oxidative mitochondrial metabolism are coupled to insulin secretion, it will be important to
determine if imeglimin’s action is dependent on one and/or the other (7, 11). Regardless, here we demonstrate that the primary mechanism of imeglimin appears to be via islet amplification rather than improving insulin sensitivity in the high fat fed rat.

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Disclosures

This work was supported in part by an investigator-sponsored grant from Poxel SA. Pascale Fouqueray, Sophie Hallakou-Bozec, and Sébastien Bolze are Poxel employees and may own stock in the company. No other potential conflicts of interest relevant to this article were reported.
References


Figure Captions

Fig. 1. One week of imeglimin treatment does not alter body weight, basal energetics, body composition, or food intake in chow fed mice. (A)-(C) Body weight, percent body fat, and percent body muscle. (D)-(E) Food intake during light and dark hours. (F) Food intake time course. In all panels, *P<0.05. (G) Water drinking. (H) 24 hr activity. (I) Activity time course. (J) 24 hr energy expenditure. (K) Energy expenditure time course. (L)-(M) Average VO$_2$ and VO$_2$ time course. (N)-(O) Average VCO$_2$ and VCO$_2$ time course. (P)-(Q) Average respiratory exchange ratio and RER time course. Data are mean ± SEM of n=8 per group.

Fig. 2. Three weeks of imeglimin treatment do not alter body weight, basal energetics, body composition, or food intake in chow fed mice. (A)-(C) Body weight, percent body fat, and percent body muscle. (D)-(E) Food intake during light and dark hours. (F) Food intake time course. In all panels, *P<0.05. (G) Water drinking. (H) 24 hr activity. (I) Activity time course. (J) 24 hr energy expenditure. (K) Energy expenditure time course. (L)-(M) Average VO$_2$ and VO$_2$ time course. (N)-(O) Average VCO$_2$ and VCO$_2$ time course. (P)-(Q) Average respiratory exchange ratio and RER time course. Data are mean ± SEM of n=8 per group.

Fig. 3. One week of imeglimin treatment does not alter basal energetics, body composition, or food intake in high fat fed mice. (A)-(C) Body weight, percent body fat, and percent body muscle. (D)-(E) Food intake during light and dark hours. (F) Food intake time course. In all panels, *P<0.05. (G) Water drinking. (H) 24 hr activity. (I) Activity time course. (J) 24 hr energy expenditure. (K) Energy expenditure time course. (L)-(M) Average VO$_2$ and VO$_2$ time course. (N)-(O) Average VCO$_2$ and VCO$_2$ time course.
course. (P)-(Q) Average respiratory exchange ratio and RER time course. Data are mean ± SEM of n=8 per group, with comparisons by t-test.

**Fig. 4.** Five weeks of imeglimin treatment do not alter basal energetics, body composition, or food intake in high fat fed mice. (A)-(C) Body weight, percent body fat, and percent body muscle. (D)-(E) Food intake during light and dark hours. (F) Food intake time course. In all panels, *P<0.05. (G) Water drinking. (H) 24 hr activity. (I) Activity time course. (J) 24 hr energy expenditure. (K) Energy expenditure time course. (L)-(M) Average VO\textsubscript{2} and VO\textsubscript{2} time course. (N)-(O) Average VCO\textsubscript{2} and VCO\textsubscript{2} time course. (P)-(Q) Average respiratory exchange ratio and RER time course. Data are mean ± SEM of n=8 per group.

**Fig. 5.** Imeglimin increases glucose-stimulated insulin secretion *in vivo* and *in vitro* in chow fed rats. (A)-(B) Plasma glucose and glucose area under the curve during a glucose tolerance test. In panels (A)-(D), *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by t-test. (C)-(D) Plasma insulin and insulin area under the curve during a glucose tolerance test. In panels (A)-(D), n=6 per group. Data are mean ± SEM.

**Fig. 6.** Imeglimin increases glucose-stimulated insulin secretion *in vivo* in high fat fed rats. (A)-(B) Plasma glucose and glucose area under the curve during a glucose tolerance test. (C)-(D) Plasma insulin and insulin area under the curve during a glucose tolerance test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by t-test. Data are mean ± SEM of n=6 per group.

**Fig. 7.** Imeglimin does not alter insulin sensitivity in chow fed rats. (A)-(B) AMPK activation and ACC phosphorylation. (C) Plasma insulin at the end of the clamp. (D) Plasma glucose. (E) Glucose infusion rate to maintain euglycemia. (F) Hepatic glucose
production. ****$P<0.0001$ relative to basal. (G) Skeletal muscle glucose uptake during the clamp. Data are mean ± SEM of n=6 per group, with comparisons by t-test.

**Fig. 8.** Imeglimin does not alter insulin sensitivity in high fat fed rats. (A) Plasma insulin at the end of the clamp. (B) Plasma glucose. (C) Glucose infusion rate to maintain euglycemia. (D) Hepatic glucose production. *$P<0.05$, **$P<0.01$ relative to basal. (E) Skeletal muscle glucose uptake during the clamp. Data are mean ± SEM of n=6 per group, with comparisons by t-test.

**Fig. 9.** Imeglimin amplifies insulin secretion in rat islets. (A,B) Insulin release and area under the curve in perifusions from intact islets at 100 μmol/L imeglimin (n=4 replicates per group). (C) Insulin secretion from static rat islet aggregates following 2 hours of stimulation at indicated concentration of glucose and/or stimulus (n=4 replicates per group). (D) Acute static insulin secretion following 2-hour incubations at increasing concentration of glucose as indicated for control and imeglimin (1 mM) (n=3 replicates per group). (E) Acute static insulin secretion following 2-hour incubations at increasing concentration of imeglimin at indicated glucose concentration (n=4 replicates per group). Insulin secretion from islet perifusions at indicated times (F,I) or area under the curve (AUC) (G,J) in the presence of 1 mM imeglimin and the indicated substrate and concentration (SAME: monomethyl ester of succinic acid). Glutamine was present at 4 mM in all perifusions and n=3 replicates per group. (H) Acute static insulin secretion following 2-hour incubations with 1 mM imeglimin at increasing concentrations of leucine at 2.5 mM glucose (n=4 replicates per group). Data are mean ± SEM of n=6 per group, with comparisons by t-test or one-way ANOVA.
Figure 4

A. Body weight

B. Percent fat

C. Percent muscle

D. Food intake - light hours

E. Food intake - dark hours

F. Food intake

G. Drinking

H. Activity

I. Activity

J. Energy expenditure

K. Energy expenditure

L. V\textsubscript{O2}

M. V\textsubscript{O2}

N. V\textsubscript{CO2}

O. V\textsubscript{CO2}

P. Respiratory exchange ratio

Q. Respiratory exchange ratio
Figure 5

A. Plasma glucose

Time (min)

mg/dL

0 15 30 45 60 75 90

Control
Imeglimin

B. Plasma glucose AUC

$P < 1 \times 10^{-6}$

C. Plasma insulin

Time (min)

pM

0 15 30 45 60 75 90

Control
Imeglimin

D. Plasma insulin AUC

$P = 0.0006$
Figure 6

A. Plasma glucose

- **Control** (black line with circles)
- **Imeglimin** (gray line with squares)

B. Plasma glucose AUC

- **Control**
- **Imeglimin**

C. Plasma insulin

- **Control** (black line with circles)
- **Imeglimin** (gray line with squares)

D. Plasma insulin AUC

- **Control**
- **Imeglimin**

*P* values indicated for statistical significance.
Figure 7

A. AMPK activation:
- pAMPK/AMPK (relative units) for Control and Imeglimin.
- Graph showing no significant difference (N.S.).

B. ACC phosphorylation:
- pACC/ACC (relative units) for Control and Imeglimin.
- Graph showing a significant difference (*P = 0.01*).

C. Plasma insulin:
- Graph showing no significant difference (N.S.).

D. Plasma glucose:
- Graph showing a significant increase in glucose levels for Imeglimin compared to Control.

E. Glucose infusion rate:
- Graph showing the infusion rate over time (0-120 min).

F. Hepatic glucose production:
- Graph showing hepatic glucose production during Basal and Clamp conditions.
- No significant difference (N.S.).

G. Skeletal muscle glucose uptake:
- Graph showing glucose uptake in nmol/g-min for Control and Imeglimin.
- No significant difference (N.S.).