Imeglimin Normalizes Glucose Tolerance and Insulin Sensitivity and Improves Mitochondrial Function in Liver of a High-Fat High-Sucrose Diet Mice Model

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ABSTRACT

Imeglimin is the first in a new class of oral glucose-lowering agents currently in phase 2b development. Although imeglimin improves insulin sensitivity in humans, the molecular mechanisms are unknown. This study used a model of 16-week high-fat, high-sucrose diet mice (HFHSD) to characterize its anti-diabetic effects. 6-week imeglimin treatment significantly decreased glycemia, restored normal glucose tolerance and improved insulin sensitivity, without modifying organs, body weights and food intake. This was associated with an increase in insulin-stimulated PKB phosphorylation in the liver and muscle. In liver mitochondria, imeglimin redirects substrate flows in favor of CII, as illustrated by increased respiration with succinate and by the restoration of respiration with glutamate/malate back to control level. In addition, imeglimin inhibits CI and restores CIII activities, suggesting an increase in fatty acid oxidation, which is supported by an increase in hepatic 3-hydroxyacetyl-CoA dehydrogenase activity and acylcarnitine profile, and the reduction of liver steatosis. Imeglimin also reduces ROS production and increases mitochondrial DNA. Lastly, imeglimin effects on mitochondrial phospholipid composition could participate in the benefit of imeglimin on mitochondrial function. In conclusion, imeglimin normalizes glucose tolerance and insulin sensitivity by preserving mitochondrial function from oxidative stress and favoring lipid oxidation in liver of HFHSD mice.
Type 2 diabetes is a worldwide threat that has been labelled as a great challenge to human health in the 21st century. The total number of people with diabetes is estimated to rise from 382 million today to 582 million by 2035 (1), and the prevalence of type 2 diabetes is expected to rise in children and adolescents around the world in all ethnicities (2). In 2013, there were 5.1 million deaths due to diabetes (1). These alarming statistics highlight that only a few effective treatment strategies exist to fight this multifactorial disease. Currently, pharmacological therapy consists of combination therapy to achieve glycemic control in the management of the disease. Therefore, development of anti-diabetic agents with improved safety and effectiveness is urgently needed. Imeglimin is the first in a new tetrahydrotriazine-containing class of oral glucose-lowering agents – the glimins – and is currently in phase 2b clinical development (US/EU EudraCT number 2012-004045-33). Several clinical trials have shown imeglimin to be well tolerated and exhibit benefits on HbA1c as mono and add-on therapy (3-5). Imeglimin acts on the liver, muscle and the pancreas (6), three key organs involved in the pathophysiology of type 2 diabetes through suspected mechanisms targeting the mitochondria and reduced oxidative stress. Imeglimin decreases hepatic glucose production and increases muscle glucose uptake (6). Recently, imeglimin demonstrated increased insulin secretion in response to glucose in diabetic patients during a hyperglycemic clamp study (7). To further elucidate the mechanism of action of imeglimin and its capacity to improve insulin sensitivity, we treated diabetic mice fed with a high-fat, high-sucrose diet (HFHSD). This model, characterized by insulin resistance, glucose intolerance, liver steatosis and mitochondrial dysfunction, is commonly used to study the mechanisms of insulin resistance and the effects of drugs.
RESEARCH DESIGN AND METHODS

High-fat high-sucrose diet (HFHSD)-induced diabetic mice

Male C57BL/6JOlaHsd mice, purchased from Harlan (France) at 4 weeks’ old, were housed at 22°C with a 12-h light/dark cycle. Procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals and were approved by a regional ethics committee. After 1 week of acclimatization, 5-6 week old mice were divided into two groups: one group with free access to a standard chow diet (SD; Harlan), the other with free access to a pelleted HFHSD diet (TD99249; Harlan) during 16 weeks as previously described (8). Animals received imeglimin by oral gavage twice a day (200mg/kg b.i.d.) during the last 6 weeks of HFHSD feeding. Control SD and HFHSD mice were treated by oral gavage with methylcellulose 0.5% as a vehicle for drug treatment (5ml/kg). Food intake was measured every day during the first week, and twice a week until the end of experiment. Results are expressed in g/day/mouse.

Blood sample collection and biochemical analysis

Blood glucose was taken weekly from tails in a fed state until week 11. From weeks 11 to 16, blood glucose was taken each week from tails in a fed state 2 hours post-gavage. Blood glucose was measured using a glucometer (Roche Diagnostics). Blood samples were collected by retro-orbital sampling (fed state and 2-h post-gavage) at 65 and 112 days after starting the HFHSD and at the study end.

Intraperitoneal glucose tolerance test (ipGTT) and intraperitoneal insulin tolerance test (ipITT)

Glucose and insulin tolerance tests were performed on 6-h–fasted mice and 2 hours post-gavage. Glucose (2 mg/g body weight) or insulin (0.75 mU/g body weight) were injected
intraperitoneally; blood glucose levels were monitored using a glucometer at the indicated time points. During ipGTT, plasma insulin and C peptide were quantified before and 15 minutes after glucose injection. C peptide and insulin levels were measured by ELISA methodology (ALPCO, 80 INSMSU.E10, 80-CPTMS-E01, respectively). Relative insulin clearance was estimated 15 minutes after glucose injection as the ratio ([C-peptide]-[Insulin])/[C-peptide].

**Analysis of insulin signaling in tissues**

A subgroup of mice (SD, HFHSD and HFHSD + imeglimin, n=10) were fasted for 6 hours, NaCl (0.9%) or insulin (10mU/mice in NaCl 0.9%) were injected i.p. and 15 minutes later, animals were sacrificed by cervical dislocation. Liver and gastrocnemius muscle were rapidly removed and frozen until use for Western-blot analysis of PKB phosphorylation.

**Lipid content in liver (DAG, cholesterol, triglycerides, acylcarnitine profiles)**

Total lipids were extracted from tissue with ethanol/chloroform (1:2, v/v). Before extraction, internal standards (1,2-diheptadecanoyl-sn-gycero-3-phosphocholine, 1,2-diheptadecanoyl-sn-gycero-3-phosphoethanolamine, cholesterol ester 17:0, di-17:0 diglyceride, tri-17:0 triglyceride and stigmasterol) were added. The organic phases were evaporated under N₂ and the different lipid classes separated by thin-layer chromatography using the solvent hexane-diethylether-acetic acid (80:20:1 v/v/v) as eluent. Total phospholipids, diglycerides, triglycerides and cholesterol esters were treated with 14% boron trifluoride in methanol (BF3/methanol). The resulting fatty acid methyl esters were analyzed by gas chromatography using a DELSI chromatograph model DI 200 equipped with a SP-2380 capillary column (60 m x 0.22 mm). Cholesterol was derivatized with bis(trimethylsilyl)trifluoroacetamine and analyzed by gas chromatography (GC)-mass spectrometry (MS) operated in positive chemical ionization (PCI) mode.
Acylcarnitine analysis was performed by flow injection tandem mass spectrometry (Api4500 ABSciex) as previously described except that butyl derivatives were used (9).

**Mitochondrial oxygen consumption and ROS production**

Mouse liver mitochondria were isolated using a standard differential centrifugation procedure in 250 mM sucrose, 20 mM Tris-HCl and 1 mM EGTA, pH 7.4 (10). Mitochondrial oxygen consumption rate (JO₂) was measured on freshly prepared mitochondria at 30°C using a Clark-type O₂ electrode (Hansatech Instruments S1, UK) in a 1 mL-chamber filled with respiration buffer: 125 mmol.L⁻¹ KCl, 10 mmol.L⁻¹ Pi-Tris, 20 mmol.L⁻¹ Tris-HCl, 0.1 mmol.L⁻¹ EGTA, pH 7.2 and using 1 mg of mitochondrial proteins.mL⁻¹. Measurements were conducted in the presence of either glutamate (5 mmol. L⁻¹)/malate (2.5 mmol.L⁻¹) or succinate (5 mmol.L⁻¹) as substrates, after the addition of 1 mmol.L⁻¹ ADP (state-3), followed by 1.5 µg.mL⁻¹ oligomycin (state-4) (Supplementary Fig. 3). ROS production was estimated by measuring H₂O₂ release in a stirred 1 mL-chamber containing 0.2 mg mitochondria and filled with a respiration buffer containing 6 IU horseradish peroxidase and 1 µmol.L⁻¹ Amplex Red® (excitation: 560 nm; emission: 584 nm) and the same substrates as for respiration and using a fluorescence spectrophotometer (F-7000FL, Hitachi USA). Measurements were conducted in basal conditions and after sequential additions of various substrates and 2 µmol.L⁻¹ rotenone (Supplementary Fig. 3). Results were expressed in pmol H₂O₂.min⁻¹.mg.Prot⁻¹ using H₂O₂ standard solutions.

**Western-blot analysis**

Liver and gastrocnemius muscle samples were lysed in PBS containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with EDTA (5mM), Na₃VO₄ (1mM), NaF (20mM), DTT (1mM) and protease inhibitor cocktail (Sigma P2714). Proteins were separated by SDS-10% PAGE, transferred to polyvinylidene difluoride membrane, and incubated
overnight with primary antibodies. Primary antibodies used were total Akt/PKB (Cell Signaling, 9272), phospho-Akt/PKB Ser473 (Cell Signaling, 9271), OXPHOS (Abcam, ab110413), PGC1α (Calbiochem, 516557), OPA1 (BD Biosciences, 612606), DLP1 (BD Biosciences, 611112), PARKIN (Abcam, ab77924), PINK1 (Abcam, ab23707), ANT2 (Santa Cruz, SC-9299), UCP2 (BioLegend, 615902), FAT/CD36 (Santa Cruz, SC-9154) and CPT1A (Abcam, ab128568). The signal was detected with a horseradish peroxidase-conjugated secondary antibody (Biorad, 172-10-19) and revealed with an enhanced chemiluminescence system (Pierce). Each protein was normalized by tubulin expression in each sample, with an internal control on each gel to normalize the inter-gels variability. Results are expressed vs the SD group.

Real time quantitative RT-PCR analysis

Total RNA was extracted with the TRI Reagent Solution (Sigma). Target mRNA levels were measured by RT, followed by real-time PCR using a Rotor-GeneTM 6000 (Corbett Research). A standard curve was systematically generated with six different amounts of purified target cDNA and each assay was performed in duplicate. We measured TATA-binding protein (TBP) mRNA as a reference gene; results are expressed as a ratio that refers to TBP expression and is normalized to the SD group.

Mitochondrial DNA analysis

Total DNA from liver was extracted using phenol/chloroform/isoamyl alcohol (25:24:1) followed by ethanol precipitation. The difference in mitochondrial DNA (mtDNA) was estimated by measuring the level of a mitochondrial-encoded gene (Cox1), versus a nuclear-encoded gene (cyclophilin A (Ppia)) using real-time quantitative PCR, as previously described (8).
Transmission electron microscopy

Liver was dissected and fixed in 2% glutaraldehyde for 2h at 4°C, post fixed in 1% osmium tetroxide for 1h at 4°C, dehydrated and embedded in Epon. The tissue was sliced using a RMC/MTX ultramicrotome (Elexience) and ultrathin sections (60-80nm) mounted on copper grids, contrasted with 8% uranyl acetate and lead citrate, and observed with a Jeol 1200 EX transmission electron microscope (Jeol LTD) equipped with a MegaView II high resolution TEM camera. Analysis was performed with Soft Imaging System (Eloïse SARL).

Histology and oil red O staining

Liver was frozen in TissuTeck and 10µm sections cut with a microtome (MICROM-GmbH-CMT950A). Oil red O and hematoxylin-eosin staining were performed and sections were observed with an optical microscope (Zeiss Axioskop2) linked to a camera (Zeiss AxioCam ERc5s).

Mitochondrial lipids analysis by LC-MS²

Lipids were extracted from mitochondrial preparations (1–2 mg protein) according to the method of Folch et al. (11). Samples were solubilized in 100 µL of chloroform/methanol/water (60/30/4.5). Extracted lipids were further analyzed using a Zorbax Eclipse Plus C18 column plugged on a LC 1200-MS² 6460 QqQ system equipped with an ESI source (Agilent Technologies).

Cardiolipins (2-4 µL) were separated at a flow rate of 0.4 ml/min at 30°C, with a linear gradient of acetonitrile/water/ammonium hydroxide/acetic acid (90/10/0.2/0.5 v/v/v/v) (A) and of isopropanol/water/ ammonium hydroxide /acetic acid (90/10/0.2/0.5 v/v/v/v) (B) as follows : 50% B for 5 min, up to 80% B in 10 min, up to 100% in 15 min, 100 % for 5 min. Acquisition was performed in negative Selected Reaction Monitoring (SRM) ion mode
(source temperature: 325°C, nebulizer gas flow rate 10 L/min, sheath gas flow 12 L/min, temperature 350°C, capillary 3500 V, nozzle 1000 V, fragmentor 280 V, collision energy 76 V). Transitions from molecular ion [M-1]- to linoleic acid (m/z 279.2) or oleic acid (m/z 281.2) were used for cardiolipin quantitation.

Phospholipids (2-4µL) were separated at a flow rate of 0.25 ml/min, 50°C, with a linear gradient of water/methanol (60/40 v/v) 10 mM ammonium acetate, 1 mM acetic acid (A) and of isopropanol/methanol (90/10) 10 mM ammonium acetate, 1 mM acetic acid (B) as follows: 40% B for 1 min, up to 95% B in 15 min and maintained at 100 % for 1 min. Acquisition was performed in SRM ion mode (source temperature: 325°C, nebulizer gas flow rate 10 L/min, sheath gas flow 10 L/min, temperature 400°C, capillary 3500 V, nozzle 1000 V). The transitions used were as follows: [M+1]+→184.1 (fragmentor 160 V, collision energy 20 V), [M+1]+→[M-140] (fragmentor 120 V, collision energy 17 V) and [M-1]→[M-87] (fragmentor 150 V, collision energy 19 V) for phosphatidylcholines, phosphatidylethanolamines and phosphatidylserines, respectively.

**Mitochondrial enzymatic activities**

Rotenone-sensitive NADH-ubiquinone oxidoreductase (EC 1.6.5.3, complex I) was assayed using 100 µmol.L⁻¹ decylubiquinone as electron acceptor and 200 µmol.L⁻¹ NADH as a donor, in a 10 mmol.L⁻¹ KH₂PO₄/K₂HPO₄ buffer, pH 7.5 containing 3.75 mg.mL⁻¹ BSA, 2 mmol.L⁻¹ KCN 7.5 µmol.L⁻¹ antimycin A. NADH oxidation was measured at 340 nm, before and after the addition of 4 µmol.L⁻¹ rotenone to allow the calculation of the rotenone-sensitive specific activity which is characteristic of complex I.

Succinate-ubiquinone reductase (EC 1.3.5.1, complex II) activity was quantified by measuring the decrease in absorbance due to the reduction of 100 µmol.L⁻¹ DCIP at 600 nm. The measurement was performed in 50 mmol.L⁻¹ KH₂PO₄/K₂HPO₄, pH 7.5 in the presence of
30 mmol.L\(^{-1}\) succinate, 100 µmol.L\(^{-1}\) decylubiquinone, 2 µmol.L\(^{-1}\) rotenone and 2 mmol.L\(^{-1}\) KCN.

Coenzyme Q - cytochrome c - oxidoreductase activity (EC 1.10.2.2, complex III, sometimes called the cytochrome bc1 complex), was quantified by measuring the increase in absorbance due to the reduction of 100 µmol.L\(^{-1}\) cytochrome c at 550 nm. The measurement was performed in 50 mmol.L\(^{-1}\) KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\), pH 7.5 in the presence of 100 µmol.L\(^{-1}\) decylubiquinone previously reduced by dithionite, 50 µmol.L\(^{-1}\) EDTA, 1 mmol.L\(^{-1}\) KCN. The specific activity was calculated by subtracting the activity obtained before and after addition of 5 µg/ml antimycin A.

3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35, HAD) activity was quantified by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH (200 µmol.L\(^{-1}\)) and the reduction of S-acetoacetylCoA (50 µmol.L\(^{-1}\)). The measurement was performed in imidazole (40 mM) and EDTA (60 µmol.L\(^{-1}\)), pH 7.

**Statistical analysis**

All data are presented as mean ± SEM. One-way analysis of variance (ANOVA) was used to determine the global effects of treatment. When appropriate, differences between groups were tested with a PLSD Fisher post hoc test. Statistical significance was accepted at p <0.05 (* = significantly different between SD and HFHSD; # = significantly different between HFHSD and HFHSD + imeglimin). Mann and Whitney tests were applied when values were not normally distributed.
RESULTS

Imeglimin normalizes glucose tolerance and insulin sensitivity in HFHSD mice

HFHSD-fed mice were used as a model of altered glucose homeostasis in rodents (8; 12). Despite a decrease in food intake (g/day) (Supplementary Fig. 1A), HFHSD induced an increase in daily caloric intake compared to SD (SD=11.45 kcal/day vs. HFHSD=13.04 kcal/day). As expected, HFHSD mice were obese, hyperglycemic, glucose intolerant and insulin resistant compared to SD mice (Fig. 1A-C and Supplementary Fig. 1B-C). The mass of liver, gastrocnemius and epididymal fat is increased in HFHSD mice (Supplementary Fig. 1D-G). They also showed higher levels of both insulin and peptide C levels (Fig. 1D) compared to SD mice, indicating a modification of insulin secretion and/or clearance. Whereas C-peptide concentration was five-fold higher than insulin in SD mice, it was only two-fold higher in HFHSD mice (Fig. 1D), indicating a large decrease in insulin clearance under this diet.

Imeglimin was administered orally at 200 mg/kg b.i.d. during the last six weeks of the HFHSD feeding protocol. A slight decrease in body weight and food intake associated with some diarrhea was observed but only during the first few days of treatment (Fig. 1A and Supplementary Fig. 1A). These effects were transient and disappeared after a few days, remaining comparable to control HFHSD mice until the end of treatment. Furthermore, no effect was measured on fat pad weight or on liver and skeletal muscles mass with imeglimin (Supplementary Fig. 1D-G). However, imeglimin significantly decreased hyperglycemia (Fig. 1B), restored normal glucose tolerance (Fig. 1B and Supplementary Fig. 1B), and improved insulin sensitivity (Fig. 1C and Supplementary Fig. 1C). Imeglimin restored insulin secretion during ipGTT (+98% vs. HFHSD mice, p<0.01) and improved C-peptide secretion during ipGTT (Fig. 1D) but did not modify insulin clearance under HFHSD (Fig. 1D). In fact,
relative insulin clearance (([C-peptide]-[Insuline])/[C-peptide]) was decreased by HFHSD (82% in SD group vs 50% in HFHSD group, p<0.001) but was not modified by imeglimin (41% in HFHSD+Imeg group). Together, this data suggests that imeglimin improves glucose homeostasis in HFHSD mice independent of an effect on whole body composition.

Imeglimin improves insulin signaling in both liver and skeletal muscle

HFHSD feeding altered insulin-stimulated PKB phosphorylation in both liver (Fig. 2A) and skeletal muscle (Fig. 2B). Imeglimin improved insulin response in both tissues of HFHSD mice (+157% and +198%, respectively, p<0.05 vs. HFHSD) (Fig. 2A and 2B), indicating an insulin sensitizing effect of the molecule in both organs.

Effect of imeglimin on liver steatosis

HFHSD feeding induced lipid accumulation in the liver compared to SD mice, demonstrated by Oil red O staining (Fig. 3A), electronic microscopy analysis (Fig. 3B) and total lipid quantification (Fig. 3C). HFHSD mice showed higher triglyceride (Fig. 3D), cholesterol (Fig. 3E), and diacylglycerol (Fig. 3F) hepatic contents compared to SD mice. Imeglimin reduced all these lipid parameters in the livers of HFHSD mice (Fig. 3A-3F), indicating that imeglimin decreased hepatic steatosis in HFHSD mice. Biochemical measurements showed that HFHSD significantly increased the mRNA levels of several genes of lipid metabolism (Cpt1a, Acaca, Srebplc, Mlxipl, Acly) (Table 1). However, imeglimin was not associated with a significant improvement in the expression of these genes despite a tendency for decreased expression of the Srebplc gene (p=0.16). In addition, glycogen content was not modified by either the diet or the treatment (data not shown).

Next we investigated whether the reduction in lipid accumulation could be related to an increase in the oxidation of fatty acids in the liver of imeglimin-treated HFHSD mice. Measurements from indirect calorimetry were not obtained in this study, as a previous study
in rats fed with a standard and high fat diet showed that respiratory quotient was not modified by imeglimin (unpublished data). Nevertheless, the effect of imeglimin on fatty acids oxidation was supported by the measurement of HAD activity and the level of FAT/CD36 protein. Imeglimin increased both parameters (Table 2, +14% and +15% vs. HFHSD group respectively, p<0.05), suggesting an increased lipid oxidation in the liver of HFHSD mice. Moreover, total and free carnitine levels, as well as short chain acyl-carnitine levels, which are decreased by HFHSD (-34%, -41% and -33% vs. SD mice p<0.01), are significantly restored by imeglimin (+45%, +52% and +44% vs. HFHSD mice p<0.01, Fig. 3G). However, imeglimin did not modify CPT1 protein levels in the liver of HFHSD mice (Table 2 and Supplementary Fig. 2).

Imeglimin improves mitochondrial respiration and ROS production in liver of HFHSD mice

Liver mitochondrial function is altered in insulin resistance states (13; 14), therefore, we investigated whether imeglimin affects mitochondria, which are involved in both hepatic glucose and lipid homeostasis. For that, we isolated mitochondria from mouse liver and assessed both the respiration rate and H$_2$O$_2$ production with either glutamate/malate or succinate (Supplementary Fig. 3). Mitochondrial state-3 (phosphorylating) oxygen consumption was higher in HFHSD mice compared to SD, independently of the substrate used (Fig. 4A and 4B). In contrast, state-2 and 4 respiration were not modified by the diet. Imeglimin decreased state-3 respiration with GM (Fig. 4A), but increased respiration with succinate (Fig. 4B). Succinate state-2 and -4 rates were also significantly increased with imeglimin (Fig. 4B).

Liver mitochondria from HFHSD mice displayed an increase in H$_2$O$_2$ production with GM, which was not altered by imeglimin (Fig. 4C). However, HFHSD dramatically increased
mitochondrial H$_2$O$_2$ production with succinate compared to mitochondria isolated from SD mice (Fig. 4D, +69.5% vs. SD mice, p<0.001). Rotenone addition, known to inhibit reverse electron flux from complex II to complex I (15), totally inhibited this production (Fig.4D), suggesting an involvement of complex I in mitochondrial ROS production in HFHSD liver. Imeglimin significantly reduced succinate-induced mitochondrial H$_2$O$_2$ production in HFHSD liver (Fig. 4D, -32% vs HFHSD, p<0.001). HFHSD increased the mRNA levels of Gpx1 and Cybb (subunit P40 of NADPH oxidase), while imeglimin treatment tended to reduce the adaptive increases observed in HFHSD mouse liver, (-9% and -14%, respectively, vs. HFHSD, Table 1). Imeglimin significantly reduced Ncf4 (subunit GP91 of NADPH oxidase) (-19% vs. HFHSD, p<0.05) (Table 1).

**Effect of imeglimin on liver mitochondrial content, composition and enzymatic activities**

HFHSD feeding increased mitochondrial DNA content in liver, as well as PGC1α protein levels, suggesting an increase in mitochondrial density/biogenesis. Imeglimin further increased mitochondrial DNA content without modifying PGC1α expression (Table 2). Measurements of the expression levels of mitochondrial dynamics markers (OPA1, DLP1) and mitophagy markers (PARKIN, PINK1) showed no differences between groups (Table 2). HFHSD feeding significantly reduced the expression and the activity of CIII (Table 2, -25% and -28% respectively, vs. SD mice, p<0.05) without affecting other complexes. Imeglimin totally restored CIII content and activity in HFHSD liver reaching the level of the SD mice (+20% and +40% respectively, vs. HFHSD, p<0.05). In contrast, ATP synthase was overexpressed in HFHSD mice and more intensively in HFHSD mice treated with imeglimin. Lastly, despite a similar CI content in all groups, imeglimin decreased CI activity in liver mitochondria of HFHSD mice (Table 2, -25% vs. HFHSD, p<0.05).

**Effect of imeglimin on mitochondrial phospholipid content**
As mitochondrial phospholipids protect and control the activity of OXPHOS complexes (16-19), liver mitochondrial membrane lipid composition was analyzed. HFHSD induced large changes in mitochondrial phospholipid composition compared to SD mice: total cardiolipin levels increased whereas phosphatidyl-choline (PC), -ethanolamine (PE) -inositol (PI) and -serine (PS) levels were decreased (Table 3). Imeglimin amplified the effects of HFHSD on both cardiolipin and PS content, whereas it tended to restore PC, PE and PI content to normal values in HFHSD mitochondria (Table 3).

Altered phospholipid composition of endoplasmic reticulum (ER) is known to be associated with hepatic ER stress and insulin resistance (20; 21), therefore the effect of imeglimin on hepatic ER stress in HFHSD mice was explored. There was a slight increase in the expression of ER stress markers in liver of HFHSD mice compared to SD mice (Table 1). However, the treatment did not modulate their expression, suggesting that the mechanism of action of imeglimin does not modulate ER homeostasis.

DISCUSSION

As Type 2 diabetes is a progressive and multifactorial disease, the combination of several medicines tackling different elements of the pathology are frequently needed for optimal disease management. Recent clinical data demonstrated that in addition to its efficacy as monotherapy, imeglimin could complement the actions of metformin or sitagliptin by significantly improving hemoglobin A1C and fasting plasma glucose in type 2 diabetic patients (3; 4). In this study, we investigated the mechanism of action of imeglimin on glucose homeostasis in HFHSD-induced diabetic mice and demonstrated the efficacy of imeglimin on glucose homeostasis after 6-week treatment in this model. Imeglimin demonstrated benefits on insulin sensitivity with an improvement in insulin signaling in both muscle and liver. We propose that the beneficial effects of imeglimin on liver are mediated at least in part through
an action on mitochondria (Fig. 5). Indeed, imeglimin reduced lipid accumulation in the liver by improving mitochondrial density and function, and increased mitochondrial density by an action independent of PGC1α protein levels and probably without modifying mitochondria dynamics and mitophagy. Imeglimin also modulated the OXPHOS chain activity; it reduced CI activity and ROS produced from this complex when mitochondria oxidize succinate. Furthermore, imeglimin restored the expression of a subunit of CIII and totally restored CIII activity in HFHSD mice. Therefore, this dual effect of imeglimin (CI inhibition and CIII restoration), allows mitochondria to oxidize more CII substrates, and thus potentially more lipids. This assumption is confirmed by the increase in both 3-hydroxyacyl-CoA dehydrogenase activity and FAT/CD36 protein levels, in addition to the restoration of total and free carnitine content and the elevated short chain acyl-carnitine in the liver of imeglimin-treated HFHSD mice. Moreover imeglimin increased the oxidation capacity of liver mitochondria for succinate (or FADH₂), independently of the mitochondrial working state, suggesting an energy waste. The observed energy waste is unconventional as it is not a classical uncoupling. In fact, imeglimin increases mitochondrial respiration in state-2 and -4, only with succinate, but not with glutamate/malate; a classical uncoupling agent or protein (DNP, CCCP or UCP) acts on both substrates. Furthermore, uncoupling is not supported by the reduction in UCP2 protein levels by imeglimin. This energy waste is closer to a slipping of the mitochondrial pump due to the change in efficiency of the complex in pumping electrons when mitochondria used succinate, rather than a change in membrane permeability. This subtle energy waste following imeglimin treatment could participate in the decrease in fatty acid accumulation in the liver. In agreement, liver-targeted mitochondrial uncoupler was recently shown to improve hepatic steatosis and insulin resistance in mice (22). Furthermore, reduction in both liver triglyceride and DAG levels, and oxidative stress, may contribute to improvements in hepatic insulin sensitivity induced by imeglimin. DAG-mediated activation
of PKC and ROS-mediated activation of JNK are well-described contributors to hepatic insulin resistance (22-27). In addition, we cannot exclude a role of imeglimin preventing other deleterious effects of oxidative stress, as previously reported (28; 29).

Cardiolipin content increases in mitochondrial membranes could contribute to the increase in the respiration rate during overfeeding, as cardiolipins are known to induce non-phosphorylating energy wasting in mitochondria (16; 30). Increases in ATP synthase and ANT content could also amplify mitochondrial respiration. While these mitochondrial adaptations in response to overfeeding should lead to increased OXPHOS capacity, this probably failed in HFHSD mice mitochondria as CIII content and activity decreased. Imeglimin reinforced HFHSD induced adaptations by over-increasing cardiolipin, ATP synthase and ANT mitochondrial content and restored CIII content and activity. These effects can explain imeglimin’s capacity to further increase succinate-driven respiration since it is associated with CI inhibition. Specific, tightly bound phospholipids, such as cardiolipins, are essential for the activity of the cytochrome bc1 complex (CIII), an integral membrane protein of the respiratory chain (31), therefore, the modulation of phospholipid composition by imeglimin, through increasing cardiolipin content, could contribute to improved mitochondrial function. Moreover, as cardiolipins modulate the interactions and activity of mitochondrial complexes (32; 33), regulate OXPHOS chain efficacy and reduce ROS production (34), we propose that the imeglimin-induced increase in cardiolipins could participate in inducing CIII activity and reducing ROS production by liver mitochondria. We demonstrate for the first time that an efficient anti-diabetic treatment impacts positively on the liver mitochondrial phospholipid composition of HFHSD mice.

This study demonstrates important positive effects of 6-week imeglimin on glycemia, glucose tolerance, and insulin sensitivity in HFHSD-induced diabetic mice. We propose that the beneficial effect of imeglimin on glucose homeostasis, particularly insulin sensitivity,
involves improvements in hepatic mitochondrial function leading to increased lipid oxidation and reduced ROS production. Imeglimin is the first anti-diabetic compound that induces an increase in mitochondrial phospholipid composition, contributing to improvements in hepatic mitochondrial function. The mitochondrial effects of imeglimin could therefore participate in the imeglimin-mediated improvement of glucose homeostasis in patients with type 2 diabetes. Future studies are required to determine whether improvements in insulin sensitivity in skeletal muscle of HFHSD mice are related to the hepatic effects of imeglimin or whether a similar mechanism could occur directly in skeletal muscle.
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No other potential conflicts of interest relevant to this article were reported.

G.V. and J.R. designed the experiments, researched data, contributed to discussion, and wrote the manuscript. M.-A.C., N.B., A.-M.M, A.D., E.M., N.B-H., J.-P.P., C.A., researched data. E.F. and H.V. contributed to discussion and reviewed the manuscript. S.H.B and S.B contributed to the design of experiments, the discussions and reviewed the manuscript. G.V. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity and the accuracy of the data analysis.

Prior Presentation

Some of the data in the article were presented as an oral presentation at the 74\textsuperscript{th} Scientific Sessions of the American Diabetes Association, San Francisco, CA, 13-17 June 2014.
References

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Figure Legends

Figure 1: Effects of imeglimin (IMEG) treatment on body mass (A), glucose tolerance (B), insulin sensitivity (C) and insulin secretion (D).

Body mass (A) was measured in the morning twice a week and every day during the first treatment phase of the experiment. ipGTT (B) were performed after a five-week treatment (week 15 of the diet) in a six-hour fasted state and two hours post dose. Blood glucose concentrations were measured before and 15, 30, 45, 60 and 90 min after i.p. injection of glucose (2 g/kg). ipITT (C) were performed after six-week treatment (week 16 of diet) in a six-hour fasted state and two hours post dose. Blood glucose concentrations were measured before and 15, 30, 45 and 60 min after i.p. injection of insulin (0.75 U/kg). During ipGTT, blood samples were collected before and 15 min after i.p. injection of glucose (2 g/kg) for determination of insulin and C-peptide plasmatic concentrations (D). Data are presented as means +/- SEM (n=20).

*P<0.01 vs. SD. #P<0.05 vs. HFHSD.

Figure 2: Effects of imeglimin (IMEG) treatment on insulin signaling pathway.

Representative Western blots showing insulin-stimulated phosphorylation of PKB (Ser473) in the liver (A) or in the muscles (B) of six-week treated mice (week 16 of diet) in a six-hour fasted state and two hours post dose. Mice were rapidly sacrificed after 10 min i.p. insulin (10 mU/g) or NaCl injection. Graphs represent the mean ± SEM of the ratio of phosphorylated protein/total protein or the insulin fold change of P-PKB (n=5).

*P<0.01 vs. SD. #P<0.05 vs. HFHSD.
Figure 3: Effects of imeglimin (IMEG) on liver lipids content.

(A) At the end of treatment, a piece of the liver was frozen in TissuTeck and 10 µm sections were cut with a cryostat and colored with oil red O. Pictures were taken with a Zeiss microscope with x200 magnification. Pictures are representative of each group. (n=3 sections per mouse for 3 mice). (B) For electronic microscopy, liver sections were prepared as described in Materials and Methods and were observed at x4000. Pictures are representative of 3 sections per mouse (n=3 mice per group). (C-F) Liver lipid content was determined after total lipid extraction and analysis with gas chromatography coupled to mass spectrometry. (G) Liver carnitine content (n=10). Data are presented as means +/- SEM (n=7 for SD group and 10 for HFHSD and HFHSD + IMEG groups).

*P<0.01 vs. SD. #P<0.05 vs. HFHSD.

Figure 4: Effect of imeglimin (IMEG) treatment on mitochondrial respiration and ROS production.

Liver mitochondria were isolated as described in Materials and Methods. Respiratory rate was determined at 30°C by incubating mitochondria (1 mg/ml) with glutamate/malate (GM) (A), or succinate (B) without ADP (state-2), in presence of 1 mM ADP (state-3) or 5 µg/mL oligomycin (state-4). H₂O₂ production rate was determined at 30°C by incubating mitochondria (0.2 mg/ml) in a respiration buffer (see Materials and Methods) with 6 IU horseradish peroxidase and 1 µM Amplex Red. Measurements were carried out without substrates and after sequential addition of various substrates and rotenone (Rot): (C) 5 mM glutamate/2.5 mM malate, (D) 5 mM succinate. (n = 10). Representatives traces of both mitochondrial respiration and ROS production experiments are illustrated in Supplementary Fig. 3. Values are expressed as means +/- SEM (n = 10 mice in each group).
*P<0.01 vs. SD. #P<0.05 vs. HFHSD.

Figure 5: Molecular mechanism by which HFHSD leads to hepatic insulin resistance and imeglimin’s (IMEG) action mode.

(A) HFHSD increases intracellular lipids (TG and DAG) leading to alteration of mitochondrial function, which results in inhibition of insulin signaling. (B) Imeglimin improves mitochondrial function by: modulating mitochondrial lipid composition; increasing mitochondrial respiration associated with energy waste in succinate; decreasing ROS production; restoring complex III activity; decreasing complex I activity and reorientating oxidative fluxes to fatty acid oxidation. As a consequence, imeglimin leads to improved insulin signaling and decreased liver steatosis, insulin resistance and glucose intolerance.
Table 1. Effect of HFHSD and imeglimin on gene expression

RT-qPCR was performed as indicated in Methods. Gene expression values were corrected by the mRNA level of TATA box binding protein (TBP) used as a housekeeping gene. Data are expressed as percentage vs. SD condition and are reported as means ± SEM (n=10).

*P<0.05 vs. SD. #P<0.05 vs. HFHSD. ǂP=0.16. ≠P=0.12. §P=0.10

The list of the PCR primers and the quantitative PCR assay conditions are available on request (emmanuelle.meugnier@pop.univ-lyon1.fr).
Table 2. Mitochondrial density, dynamic and content or activities of mitochondrial respiratory chain complex in mouse liver mitochondria.

Content of mitochondrial DNA (mtDNA: Cox1/Ppia) was calculated using real time quantitative PCR. Protein expressions of PGC-1α, OPA1, DLP1, PARKIN, PINK1, Complex I-IV, ATP synthase, ANT2, UCP2, CPT1A and CD36 were determined by Western blotting, normalized to tubulin signal and expressed as percentage to SD groups signals. Representative Western blots are illustrated in Supplementary Fig. 2. Rotenone-sensitive NADH-ubiquinone oxidoreductase (EC 1.6.5.3, complex I), Succinate-ubiquinone reductase (EC 1.3.5.1, complex II), Coenzyme Q - cytochrome c - oxidoreductase activity (EC 1.10.2.2, complex III), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35, HAD) were measured on liver mitochondria using a spectrofluorimeter and specific substrates. Data are presented as mean ± SEM (n=10).

*P<0.05 vs. SD.  †P<0.05 vs. HFHSD.  ‡P=0.07
<table>
<thead>
<tr>
<th>Lipids (nmol/g of mitochondrial proteins)</th>
<th>SD</th>
<th>HFHS</th>
<th>HFHS+IMEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiolipin</td>
<td>2400 ± 95</td>
<td>3089 ± 150*</td>
<td>3493 ± 221#</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>46035 ± 1928</td>
<td>32419 ± 2178*</td>
<td>36890 ± 2372</td>
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<td>Phosphatidylethanolamine</td>
<td>1976 ± 65</td>
<td>1338 ± 118*</td>
<td>1471 ± 111</td>
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<tr>
<td>Phosphatidylinositol</td>
<td>1167 ± 83</td>
<td>880 ± 68*</td>
<td>927 ± 43</td>
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<tr>
<td>Phosphatidylserine</td>
<td>1852 ± 141</td>
<td>1059 ± 85*</td>
<td>779 ± 64#</td>
</tr>
</tbody>
</table>

Table 3. Liver mitochondrial membrane phospholipid composition.

The relative quantitation of phospholipids was performed by high performance liquid chromatography and by calculating the response ratio of the phospholipid considered to respective spiked internal standard. Data are presented as mean ± SEM (n=10).

*P<0.05 vs. SD. #P<0.05 vs. HFHSD.
**A**

Body mass (g) vs. Day for different treatments:
- SD
- HFHSD
- HFHSD + IMEG

**B**

Blood glucose (mg/dL) vs. Time (min) for different treatments:
- SD
- HFHSD
- HFHSD + IMEG

**C**

Blood glucose (mg/dL) vs. Time (min) for different treatments:
- SD
- HFHSD
- HFHSD + IMEG

**D**

Insulin (pM) and C-peptide (pM) levels at T0 min and T15 min for different treatments:
- SD
- HFHSD
- HFHSD + IMEG
Supplementary Figure 1: Effects of imeglimin (IMEG) treatment on food intake (A), area under the curve of glucose tolerance test (B), area under the curve of insulin sensitivity test (C), liver (D), quadriceps (E), gastrocnemius (F) and fat pad (G) mass.

Food intake (A) was measured in the morning twice a week and every day during the first treatment phase of the experiment. Area under the curve of ipGTT (B) were calculated during the ipGTT describe in Figure 1C and were related to t=0. Area under the curve of ipITT (C) were calculated during the ipITT describe in Figure 1B and were related to t=0. Liver, quadriceps, gastrocnemius and epididymal fat pad mass were measured after sacrifice, exsanguination of mice and precise dissection. Data are presented as means +/- SEM (n=20 for food intake and area under the curves of ipGTT and ipITT and n=13 for organs mass).

*P<0.01 vs. SD. #P<0.05 vs. HFHSD.
Supplementary Figure 2: Representative Western Blots of protein expressions described in Table 2.

Representative Western Blots showing PGC-1α, OPA1, DLP1, PARKIN, PINK1, Complex I-IV, ATP synthase, ANT2, UCP2, CPT1A and FAT/CD36 in the liver of six-week imeglimin treated mice (week 16 of diet).
Supplementary Figure 3: Representative traces of mitochondrial respiration and ROS production experiments illustrated in Fig. 4.

Liver mitochondria were freshly prepared as described in Materials and Methods. Respiratory rate was determined at 30°C by incubating mitochondria (1 mg/ml) with glutamate/malate (GM) (A), or succinate (B) without ADP (state-2), in presence of 1 mM ADP (state-3) or 1.5 μg/mL oligomycin (state-4). H$_2$O$_2$ production rate was determined at 30°C by incubating mitochondria (0.2 mg/ml) in a respiration buffer (see Materials and Methods) with 6 IU horseradish peroxidase and 1 μM Amplex Red. Measurements were carried out without substrates and after sequential addition of various substrates and 2 μmol.L$^{-1}$ rotenone (Rot): (C) 5 mM glutamate/2.5 mM malate, (D) 5 mM succinate. Graphs shown representative traces of one experiment per group. Quantitative analysis are illustrated in Fig. 4.