Diabetes Publish Ahead of Print, published online May 13, 2008

Selective Small-Molecule Agonists of G protein-coupled Receptor 40 Promote Glucose-Dependent Insulin Secretion and Reduce Blood Glucose in Mice

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Received 30 January 2008 and accepted 7 May 2008.

Objectives: Acute activation of G protein-coupled Receptor 40 (GPR40) by free fatty acids (FFAs) or synthetic GPR40 agonists enhances insulin secretion. However, it is still a matter of debate if activation of GPR40 would be beneficial for the treatment of type 2 diabetes since chronic exposure to FFAs impairs islet function. We sought to evaluate the specific role of GPR40 in islets and its potential as a therapeutic target using compounds that specifically activate GPR40.

Research Design and Methods: We developed a series of GPR40 selective small molecule agonists and studied their acute and chronic effects on glucose-dependent insulin secretion (GDIS) in isolated islets, as well as effects on blood glucose levels during intraperitoneal glucose tolerance tests (IPGTT) in wild-type (WT) and in GPR40 knock-out mice (GPR40-/-).

Results: Small molecule GPR40 agonists significantly enhanced GDIS in isolated islets and improve glucose tolerance in WT mice, but not in GPR40-/- mice. While a 72-hour exposure to FFA in tissue culture significantly impaired GDIS in islets from both WT and GPR40-/- mice, similar exposure to the GPR40 agonist did not impair GDIS in islets from WT mice. Furthermore, the GPR40 agonist enhanced insulin secretion in perfused pancreata from neonatal streptozotocin-induced diabetic rats, and improved glucose levels in high fat diet-induced obese mice acutely and chronically.

Conclusions: GPR40 does not mediate the chronic toxic effects of FFAs on islet function. Pharmacological activation of GPR40 may potentiate GDIS in humans and be beneficial on overall glucose control in patients with type 2 diabetes mellitus.

Loss of glucose-dependent insulin secretion (GDIS) from the pancreatic β-cell is responsible for the onset and progression of type 2 diabetes (1,2). Oral agents that stimulate insulin secretion, such as sulfonylureas and related K^+-ATP channel blockers, reduce blood glucose and have been used as a first-line T2DM therapy for nearly thirty years (3,4). However, these agents act to force the β-cell to secrete insulin continuously, regardless of prevailing glucose levels, thereby promoting hypoglycemia, accelerating the loss of islet function, and eventual diminished efficacy (5, 6). Despite the availability of a range of agents for type 2 diabetes, many diabetic patients fail to achieve or to maintain glycemic targets (7-9). In addition, stricter glycemic guidelines have been proposed to help define a path towards diabetes prevention through identifying and treating the pre-diabetes state (10). Agents that induce GDIS have great potential to replace sulfonylureas as a first-line therapy for the treatment of T2DM. In particular, agents that have positive effects on arresting or even reversing β-cell demise would represent a major therapeutic advance towards addressing the lack of durability seen with current therapies and perhaps obviate the need for eventual insulin intervention (11-13). The recent emergence of glucagon-like peptide 1 (GLP-1) based GDIS agents (14-16), including inhibitors of Dipeptidyl peptidase-4 (DPP-4) (17) and peptidase-stable analogs such as exendin-4 (18), is undoubtedly a major advance toward such direction. Nevertheless, it remains to be observed whether GLP-1 related agents truly exert beneficial effects on β-cell mass and function.

The molecular pharmacology of lipid and lipid-like mediators which signal through GPCRs has expanded significantly over the past few years. To date, heretofore several orphan GPCRs have been paired with lysophospholipids, bile acids, arachidonic acid metabolites, dioleoyl phosphatidic acid, and short, medium and long chain free fatty acids (FFA) (19-21). From these discoveries, GPR40, GPR119, and GPR120 have been reported to play a role in regulating GDIS and therefore have potential as novel targets for the treatment of type 2 diabetes (22-26). GPR40 is a G_q-coupled, family A GPCR highly expressed in β-cells of human and rodent islets. Several naturally-occurring medium to long-chain FFAs and some thiazolidinedione PPARγ agonists specifically activate GPR40 (27,28). Activation of GPR40 by FFAs (29-32) or synthetic compounds (23,33) enhances insulin secretion through the amplification of intracellular calcium signaling.

The pleiotropic effects of FFAs on the pancreatic β-cell are well known. The fact that FFAs are in vitro ligands for GPR40 is suggestive of the link to the wealth of existing literature data on the acute, stimulatory effects of FFAs on insulin release (34,35). However, FFAs also exert suppressive or detrimental effects on β-cells. Lipotoxicity of β–cells, a condition observed with chronic exposure to high FFA levels, results in impairment in their function and a resulting diminution in their insulin secretory capacity (36,37). Currently, there is an ongoing debate if GPR40 mediates the deleterious effects of FFAs on islet function (lipotoxicity), and whether an antagonist of GPR40 is more preferable than an agonist for the treatment of type 2 diabetes (38; 39). Since FFAs can be both metabolized within cells to act as intracellular signaling molecules (35), and activate more than one receptor (20) they cannot be used as specific and selective tools to unravel the role that GPR40 plays in the β-cell. It is therefore necessary to identify small molecules that specifically activate GPR40.

In the following discussion, we will detail the identification and in vitro pharmacology of a novel series of synthetic GPR40 agonists.

Using isolated islets from wild type and homozygous GPR40 knockout (GPR40-/-) mice (to confirm the on-target activity of small molecule activators), we not only extended previous findings that acute activation of GPR40 enhances GDIS in pancreatic β-cells, but also showed that long-term exposure to the GPR40 agonist, in contrast to FFA, did not impair β-cell function, thus dissociating the activation of GPR40 from β-cell lipotoxicity. Finally, acute and subchronic dosing of the GPR40 agonist robustly reduced the blood glucose excursion during an intraperitoneal glucose tolerance test (IPGTT) in wild type, but not GPR40-/- mice.

RESEARCH DESIGN AND METHODS

Generation of GPR40 stable cell lines. Human and mouse GPR40 stable cell-lines were generated either in CHO cells stably expressing NFAT BLA (Beta-lactamase), or in HEK293 cells. The expression plasmids were transfected using lipofectamine (Invitrogen) following manufacturer's instructions. Stable cell lines were generated following appropriate drug selection.

Fluorometric imaging plate reader (FLIPR) based intracellular calcium assay. GPR40/CHO NFAT BLA cells were seeded into black-wall-clear-bottom 384-well plates (Costar) one day before the assay. The cells were incubated with 20 µl / well of the HBSS buffer with 0.1 % BSA, 2.5 mM probenecid and 8µM Fluo-4-AM at room temperature for 100 minutes. Compounds were dissolved in DMSO and diluted to desired concentrations with assay buffer and added to the cells as 5X solution (13.3 µl/well).. Fluorescence output was measured using FLIPR^{II} (Molecular Devices) 10 sec prior to compound addition.

Measurement of inositol 1,4,5-triphosphate (IP_3) production. Human GPR40-HEK293 stable cells were plated at 16,000 cells/well on 96-well poly-D-lysine coated plates and cultured for 72 h in DMEM medium (25 mM glucose) with 10% FBS, 25 mM HEPES, and selection antibiotics. Cells were then washed with HBSS buffer and further incubated for 18 hours in 150 ul of ^{3}H-inositol labeling media (inositol-and serum-free DMEM), to which ^{3}H-myo-inositol (NEN/PerkinElmer, Waltham, MA) was added to a final specific radioactivity of 1 uCi/150 microliter. Agonist titrations have typically been performed by half-log dilutions run in duplicate in 11-point curves. The plates were counted in the MicroBeta instrument (PerkinElmer, Waltham, MA).

Isolation of pancreatic islets and the static GDIS assay. Pancreatic islets of Langerhans were isolated from wild-type and GPR40-/- mice (littermates) by collagenase digestion and discontinous Ficoll gradient separation (40). The islets were cultured overnight in RPMI 1640 medium with 11 mM glucose to facilitate recovery from the isolation process. Insulin secretion was determined by 1-h static incubation in Krebs'-Ringer bicarbonate (KRB) buffer in 96-well format as previously described (41). Briefly, islets were first preincubated in KRB medium with either 2 mM glucose for 30 min, and were then transferred to a 96-well plate (one islet/well) and incubated with 200 ul of the KRB medium with 2 or 16 mM glucose in the presence or absence of oleate, palmitate or testing compounds for 60 min. The buffer was removed from the wells at the end of the incubation and assayed for insulin levels using the Ultra-sensitive Rat Insulin ELISA kit (ALPCO, Salem, NH).

Chronic treatment of islets and GDIS. Islets, isolated from wild-type and GPR40-/- mice (littermates) were cultured in RPMI1640 medium (11 mM glucose, 10% FCS) with vehicle, or 125 µM of FFA (a 1:1 mixture of oleate : palmitate) as described previously (42), or 5 µM of GPR40 small molecule agonist for three days. The FFA was added directly to the culture medium from a 100X stock solutions in distilled water (for oleate)

or 95% Ethanol (for palmitate). After the 3-day exposure to oleate, palmitate or GPR40 small molecule agonist, insulin secretion was determined by the 1-h static incubation in KRB buffer with either 2 or 16 mM glucose following a 30 min pre-incubation in the KRB buffer with 2 mM glucose as described above for the Acute GDIS Assay.

Islet Perifusion. For islet perifusion, batches of 25 islets each were perifused in parallel micro-chambers (Biovail International, Minneapolis, MN) with oxygenated KRB medium with 2 or 16 mmol/l glucose at a rate of 0.8 ml/min, and the fractions of the perfusate were collected once per minute for insulin measurement (43). Insulin concentration in aliquots of the incubation or perifusion buffers was measured by the ultra-sensitive rat insulin EIA kit from ALPCO Diagnostics (Windham, NH).

The neonatal streptozotocin (nSTZ) diabetic rat model and Pancreas perfusion. Timed pregnant Wistar rats were purchased from Charles River Laboratories Inc. Pups were dosed with vehicle (0.5 M citrate, pH 4.5) or 100 mg/kg i.p. streptozotocin (Sigma Aldrich) on 48hrs after birth. At three weeks of age, male pups were separated and housed two per cage. Food and water were given *ad libitum*, and rats were maintained on a 12 hours light-dark cycle. Perfusions were performed when rats were eight weeks old as described previously (44). For each surgery, rats were sedated with Nembutal anesthesia (100 mg/kg i.p). The peritoneal cavity was then opened and the coeliac artery was ligated dorsally. A 27G cannula was inserted into the coeliac artery for perfusant afflux, another cannula was inserted to the portal vein for perfusant efflux. Immediately following surgery, rats were placed into a 37^{o}C humidified whole-body perfusion chamber and perfused at 3 ml/min with a modified KRB buffer (O_2 saturated; 37^{o}C). Perfusant buffer contained 2 mM or 16 mM glucose supplemented with vehicle (DMSO), 10µM Cpd B or 30mM L-Arginine. Perfusant (~90% recovery) was collected in one minute intervals and stored frozen at -70^{o}C until analysis. Insulin was determined using a rat-specific insulin RIA kit (Millipore, Billerica, MA). All procedures were approved by the Merck Rahway Institutional Animal Care and Use Committee.

Intraperitoneal Glucose Tolerance Test (IPGTT). Male GPR40$^{-/-}$ and littermate WT C57BL/6N mice (7-11 weeks of age) from Taconic Farms (Germantown, NY) were housed 10 per cage and fed with rodent chow (Teklad 7012) and water ad libitum. On the morning of study, mice (n=5-7/group) were fasted 5 to 6 hours. Animals were then treated orally with vehicle (0.25% methylcellulose, 10 mL/kg) or Cpd B or Cpd C 60 min prior to the IPGTT (dextrose 2 g/kg, i.p). Blood glucose levels were determined from tail bleeds taken at -60, 0, 20, 40 and 60 min after dextrose challenge. The blood glucose excursion profile from t = 0 to t = 60 min was used to integrate an area under the curve (AUC) for each treatment. Percent inhibition values for each treatment were generated from the AUC data after the subtraction of the AUC of the vehicle & water group which was received vehicle at -60 min and water at 0 min. Concentrations of test compound in mouse plasma were determined by liquid chromatography/tandem mass spectrometry in blood samples collected at 60 min of the IPGTT (2 hr after dosing).

Chronic treatment of established diet-induced obesity (eDIO) mice with GPR40 agonist. C57Bl/6N (Taconic Fram, NY) were switched to a high fat diet (60% kcal, R4129, Research Diet) at age of 6 week and continued through out the study. Cpd A was given to the eDIO mice at age of 20 weeks (14 weeks on HF diet) at 10 mg/kg (oral gavage, once a day) for 10 days. On the day 10 of the treatment, an IPGTT was performed as described above.

Calculations and statistics. All data are expressed as means ± SE. Statistical analysis was conducted by using either single-factor ANOVA or Student's t test as appropriate. Statistical significance was defined as $P < 0.05$.

RESULTS

Identification of small molecule GPR40 agonists. The intracellular signal transduction pathway of GPR40 proceeds through the activation of the G_q class of G_a proteins with subsequent phospholipase C activation, generation of IP_3, and intracellular Ca^{+2} release. We confirmed that multiple medium and long-chain FFAs activated human and mouse GPR40 expressed in CHO cells (Fig. 1A and 1B), whereas the short chain FFAs (propionic, butyric, and pentanoic acid) had minimal activation against the mouse and human receptors. There also appeared to be a general increase in potency across the saturated fatty acids with increasing chain length from hexanoic acid (C6:0) to lauric acid (C12:0) as reported previously. A good correlation of potency was observed between human and mouse GPR40 by those fatty acids.

Kotarsky et al (27) first showed that some thiazolidinedione (TZD) ligands of the peroxisome proliferator-activated receptor (PPARγ) also activate GPR40. We thus screened about 2000 TZD compounds from the Merck compound collection using the FLIPR assay in human GPR40-CHO stable cells, and identified a partial agonist (relative to oleate) for GPR40 with an EC_{50} of 1585 nM (Compound-A, Cpd A in Fig. 1C). This compound was inactive in binding assays (at concentrations up to 10 μM) against human PPAR alpha, delta, and gamma isoforms. Subsequent lead optimization (Yang, L. [2008]; in preparation) significantly improved the properties of Cpd A and resulted in a series of specific and high affinity GPR40 agonists exemplified by Cpd B and Cpd C. As shown in Fig. 1 (panels C and D) Cpd B and Cpd C elicited a dose-dependent increase in calcium mobilization (as detected by FLIPR assays) and IP_3 accumulation in GPR40 stable cell lines. Cpd B (and it's des-methyl analog Cpd C) are both full agonists at both the human and mouse GPR40 receptors with EC_{50}s ranging from 15 to 300 nM. In general, a good correlation was observed between both assays with respect to the rank order of potency for a variety of analogs.

Acute effects of FFA and GPR40 agonist on GDIS in islets from wild type and GPR40-/- mice. Small molecule agonists of GPR40 have been shown by others (23, 33) to enhance GDIS in insulinoma cell lines, but it has yet to be established that activation of GPR40 with synthetic agonists would enhance GDIS in primary islets. We thus examined the acute effects of FFA and GPR40 agonists (Cpd B and Cpd C) on GDIS in islets from GPR40-/- and wild type (WT) mice in both 1–hour static incubations (Fig. 2A and 2B) and in islet perifusion experiments (Fig. 2C). Insulin secretory responses to glucose were comparable in WT and GPR40-/- islets in the static incubation assay. Fatty acid treatment (200 μM oleate or palmitate) significantly promoted glucose-dependent insulin secretion in wild type islets (Oleate 1.9 ± 0.4 fold, Palmitate 2.4 ± 0.3 fold; n=6, $P<0.01$ for both), but not in the GPR40-/- islets (Oleate 1.2 ± 0.3 fold, Palmitate 1.8 ± 0.5 fold; n=6, $P>0.05$ for both). Likewise, the two small molecule GPR40 agonists, when tested at 10 μM, significantly augmented GDIS in WT islets, but were totally inactive in the GPR40-/- islets (Fig. 2A). The effects of Cpd B on GDIS in WT islets was concentration dependent and a maximal effect was reached at 5 μM (Fig. 2B).

The glucose-dependency of GPR40 mediated insulin secretion was also examined in the islet perifusion system. Islets from WT mice were sequentially stimulated by 2, 6, 8

and 16 mM glucose together with Cpd B and insulin responses to those conditions were monitored at 1-minute intervals. As shown in Fig. 2C, Cpd B significantly enhanced insulin secretion triggered by 16 mM glucose, but not 6 and 8 mM glucose. The AUC of insulin secretion in the presence of Cpd B was significantly greater than that stimulated by glucose alone (32 ± 0.1 vs 19 ± 1.2 ng/25 islet*10 min, P<0.01, n=3).

Chronic effects of FFA and GPR40 agonist in isolated islets from wild type and GPR40-/- mice. To investigate the effects of long-term activation of GPR40 by FFAs or GPR40-selective agonists on islet function, islets were isolated from the GPR40-/- and WT littermates and cultured for 72 hours with or without FFAs (a 1:1 mixture of palmitate and oleate at a total final concentrations of 125 μM) or Cpd B (5 μM). Insulin secretion was measured in 1-hour static incubation assays after the 3 day culture period. There was no difference in glucose or KCl stimulated insulin secretion between GPR40-/- and wild type islets cultured in normal medium. As previous reported (38), the 3-day exposure to FFA equally and significantly inhibited GDIS in WT and GPR40-/- islets (Fig.3A). In addition, insulin secretion in response to membrane depolarization caused by 30 mM KCl and islet insulin content were also diminished identically by the 3-day FFA treatment in islets from wild type and GPR40-/- animals (Fig. 3A, 3B). In contrast, chronic treatment of islets (wild type and KO) with Cpd B did not have any effect on GDIS, indicating that the GPR40 agonism, whether evoked with FFAs or structurally-distinct small molecules, is not involved in the impairment of insulin secretion seen with chronic fatty acid treatment. The 3-day continuous exposure to Cpd B apparently did not cause desensitization of the β-cells to GPR40 activation, as GDIS could be enhanced equally well when fresh compound was added to the islets that had been treated for 3-day by the compounds (data not shown).

Effects of a GPR40 agonist on insulin secretion from the in situ pancreas perifusion of the nSTZ diabetic rat. To begin to explore the potential of GPR40 agonism for the treatment of type 2 diabetes, we tested the efficacy of GPR40 agonists on ex vivo GDIS from the perfused pancreata of neonatal STZ-treated rats (44, 45). Compared to isolated islets, this model is an attractive way to study insulin secretion dynamics in situ as it provides improved resolution and fidelity that approaches the native setting. As shown in Fig. 4A, the pancreata from vehicle (sham) rats exhibited identical biphasic insulin secretory responses to both glucose (16 mM) pulses, which were totally lost in pancreata from the nSTZ rats. When present in the perfusate at 10 μM during the glucose (16 mM) stimulation phase, Cpd B induced a pronounced enhancement of insulin secretion when compared to vehicle-treated pancreata (AUCinsulin: 249 ± 67 vs 29 ± 6 ng/20 min with glucose alone, P<0.01, n=4) . The restoration of insulin secretion by Cpd B mainly occurred to the first phase of β-cell responses. Cpd B also enhanced insulin secretion stimulated by 30 mM arginine at the end of the perifusion experiment.

Effect of GPR40 agonist on IPGTT glucose levels in WT and GPR40-/- mice. To extend the above results to an in vivo setting, we tested the effects of our small molecule GPR40 agonists on glucose excursion during IPGTT in normal lean mice. Cpd C was selected for this experiment based on pharmacokinetic considerations. Cpd C possesses excellent oral bio-availability (~100 %), a plasma half-life of 8 hrs, Tmax (time to reach maximum concentration) of 3 hrs, and Cmax (maximum concentration) of 1.4 μM, following a 2 mg/kg oral dose (data not shown). The oral administration of Cpd C, one hour prior to dextrose challenge in the IPGTT, significantly reduced blood glucose

excursion in a dose-dependent manner from 3 to 100 mg/kg, with maximum efficacy (73% inhibition of AUC_{GLu}) achieved at ~30 mg/kg and a corresponding plasma concentration of 37 μM measured 2 hrs post-dose (Fig. 5, panels A and B). The GLP-1 mimetic exendin-4 was included as a positive control and it completely prevented any glucose excursion at a concentration of 0.0025 mg/kg.

To demonstrate that the observed Cpd C-induced glucose-lowering was GPR40-dependent, the effects of the ligand on blood glucose excursion during an IPGTT was investigated again in a cohort of GPR40-/- mice and littermate WT mice. The administration of 30 mg/kg Cpd C again resulted in a significant suppression of AUC_{GLu} during IPGTT in the WT mice. In contrast, the same dose of the compound exerted no inhibition of blood glucose excursion in the GPR40-/- mice (panel C). The above findings demonstrated that robust glucose lowering in normal WT mice by Cpd C is mediated by GPR40.

Effect of acute and chronic treatment with GPR40 agonist on IPGTT glucose levels in eDIO mice. To further evaluate the potential of GPR40 activation for treatment of type 2 diabetes, we studied the effect of GPR40 agonist (Cpd B) on IPGTT glucose levels in high fat diet (HFD) induced obese (eDIO) mice both acutely and sub-chronically. We induced eDIO in C57BL/6 mice with a 60% HFD for 14 weeks (started at age of 6 weeks). The effects of Cpd B (10 mg/kg, oral gavage) on IPGTT glucose was tested before and after 10 days of continuous dosing (10 mg/kg, daily). The eDIO mice weighed significantly heavier (42 ± 0.6 vs 28 ± 0.4 g, $p<0.001$, n=8) and manifested impaired glucose tolerance compared to mice on regular chow (AUC_{Gluc}: 18311 ± 272 vs 13540 ± 326 mg/dl*60min, $P<0.001$, n=8). Acute treatment of the eDIO mice with Cpd B (10 mg/kg) significantly reduced IPGTT glucose levels by approximately 50% (Fig 6A). The glucose-lowering efficacy of Cpd B was well maintained after a subchronic dosing. As measured by the IPGTT performed on the last day of compound treatment (Fig. 6B), 10 mg/kg Cpd B reduced glucose excursion by 70%, similar to the acute efficacy achieved in these mice before the initiation of the 10-day dosing period. Although we did not measure food intake in this study, the chronic efficacy does not appear to be attributable to any changes in food intake and body weight of the mice. There were no differences in body weight between vehicle and Cpd B treated mice both before (42 ± 0.6 for the vehicle vs 41 ± 0.7 for the Cpd B group, $p>0.05$, n=8) and after the chronic treatment (44 ± 0.8 for the vehicle vs 45 ± 0.8 for the Cpd B group, $p>0.05$, n=8).

To determine if acute dosing of GPR40 agonist promotes in vivo insulin secretion in mice, we performed a slightly modified IPGTT experiment using mice that had been fed with the 60% HFD for 4 weeks. In that cohort of HFD fed mice, Cpd B (30 mg/kg, administrated orally 60 min prior to glucose challenge), did not affect the basal insulin levels at 0 min (1.72 ± 0.5 vs 1.26 ± 0.2 ng/ml), but significantly enhanced insulin responses at 5 min during an IPGTT relative to vehicle control (3.8 ± 0.4 vs 2.4 ± 0.4 ng/ml, $p<0.02$, n=8).

DISCUSSION

GPR40 is a Gq-coupled family A GPCR specifically expressed in the pancreatic β-cell (46,47). The discovery of its activation by medium and long-chain FFAs has sparked considerable interest in and experimentation on this receptor from basic research to potential drug discovery efforts. Nevertheless, there are still several important questions that remain to be answered. How much does GPR40 contribute to the acute (stimulatory) and chronic (inhibitory) effects of FFA on islet function? What will be the consequences of acute and chronic activation

of GRP40 with a pharmacophore on islet function and beyond? Can agonists of GPR40 stimulate sufficient GDIS to result in the reduction of blood glucose in normal and diabetic animals? We set out to address some of these questions in this study using potent selective agonists of the receptor in conjunction with GPR40 knockout mice.

GPR40 has been previously shown to mediate part of the enhancement of GDIS by FFAs, i.e. the acute effect of FFAs on insulin secretion (22, 23, 29-33, 38), but not the chronic toxic effects of FFAs in islets (38). The results from this study are largely consistent with those findings. Our data indicate that both oleate and palmitate lost the majority of their actions on GDIS in GPR40-depleted islets, thus suggesting that GPR40 is a major, if not the sole, mediator of the acute stimulatory action of FFAs on GDIS (no attempt was made to calibrate precisely their EC_{50} and maximal activity on insulin secretion). The residual effects of FFAs observed in the GPR40-/- islets could be mediated by the intracellular metabolism/oxidation of FFAs (35), or by additional cell surface receptors such as GPR120 (23).

The potential role of GPR40 in mediating the chronic inhibitory effects of FFAs on islet function is also a matter of debate. Over-expression of GPR40 selectively in pancreatic β-cells caused dramatic disintegration of the islets and severe hyperglycemia in an IPF-GPR40 transgenic line (39). On the other hand, islets from the GRP40 KO mice appear to be as vulnerable as WT islets to the detrimental effects from FFAs in vitro (38). Our study has provided additional support for the latter observation, namely that GPR40 does not mediate β-cell lipotoxicity. Similar to what was shown by Latour et al (38), we found that three days of exposure to FFAs caused comparable inhibition of GDIS in WT and GPR40-/- islets. In addition, we observed significant and identical reductions in islet insulin content in WT and GPR40-/- islets, an important feature of the chronic inhibitory effects exerted by FFAs in β-cells (42). We thus conclude that GPR40 is not responsible for lipotoxicity in β-cells exposed to elevated FFAs in vitro or in vivo.

Small molecule agonists of GPR40 have recently been reported by at least two groups (23, 33). Yet, to the best of our knowledge, the utility of such agents for potentiating GDIS as a novel therapy of type 2 diabetes has yet to be disclosed. Accordingly, we studied the effects of novel GPR40 agonist discovered in our laboratories upon GDIS in several experimental paradigms. First, we showed that the GPR40 agonist enhanced GDIS in mouse islets using both static incubation and islet perifusion methods; the effects were dependent on the presence of GPR40. Second, we demonstrated that the action of our GPR40 agonist was strictly glucose dependent. Third, we found that the GPR40 agonist restored GDIS (at least the first phase) in pancreatic β-cells from the nSTZ rat, a diabetes model that possesses three key traits similar to that seen in the human disease: sustained hyperglycemia, a substantial reduction in β-cell mass (>90% reduction in pancreatic insulin content) and impaired GDIS in the residual β-cell population (44, 45). The significant restoration of GDIS (particularly the first phase of insulin secretion) in this model by Cpd B strongly suggest that such ligands will be efficacious in treating type 2 diabetic patients with compromised β-cell function and mass. Finally, our compound exerted robust glucose-lowering efficacy during an IPGTT in mice. This is the first report of a GPR40 agonist demonstrating glucose control efficacy in pre-clinical animals. Taken together, our findings support the proposition that GPR40 agonists may be beneficial for restoring GDIS and glucose control in type 2 diabetes and therefore, merit further evaluation in clinical studies. Results from

the present study do not support the assertion that chronic activation of GPR40 may harm β-cell function ("lipotoxicity" hypothesis). In contrast, our study has provided strong evidence that activation of GPR40 can enhance GDIS in islet β-cells from normal and diabetic rodents, and thereby improve glucose tolerance.

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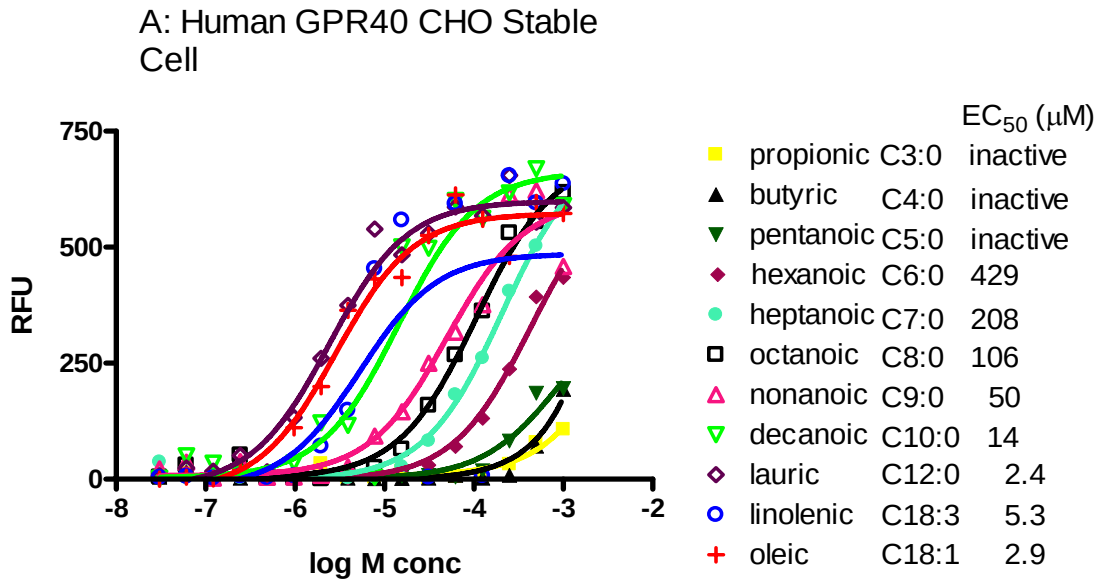
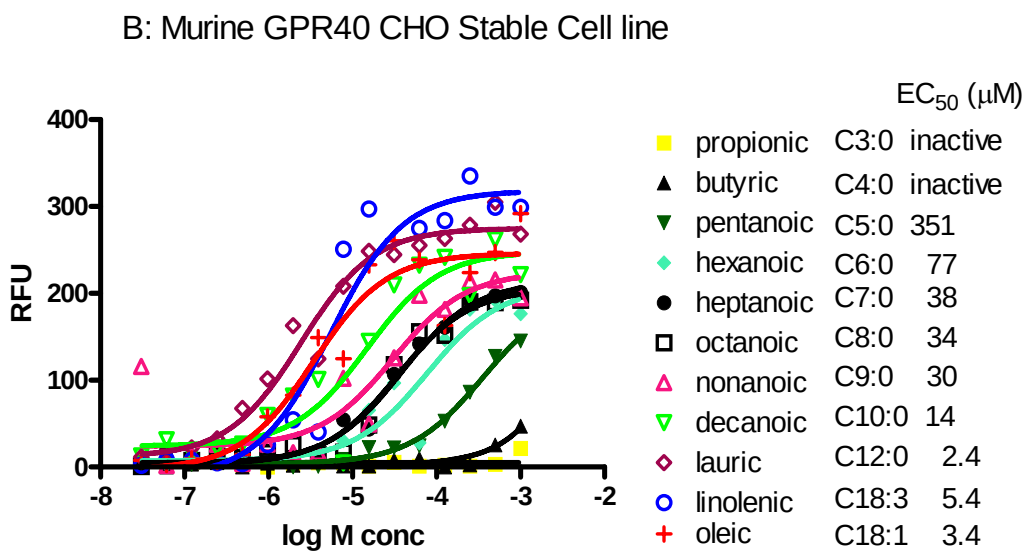
Figure 1

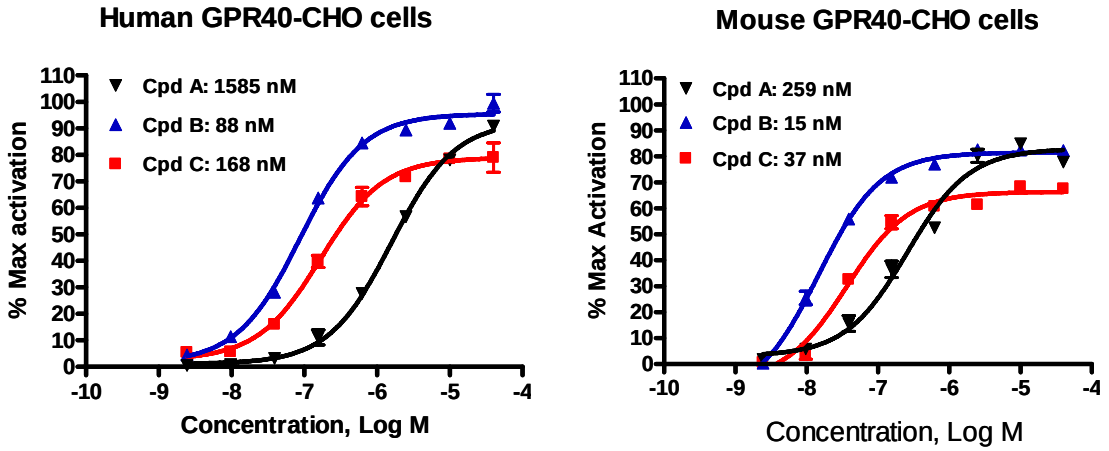
Panel A: Human GPR40/CHO stable cell FLIPR Responses to FFAs

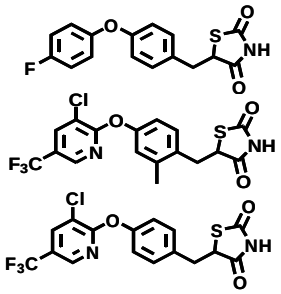


Panel B: Mouse GPR40/CHO stable cell FLIPR Responses to FFAs



Panel C) GPR40/CHO stable cell FLIPR responses to small molecule GPR40 agonists





Cpd A: initial hit from screening

Cpd B: Analogue of Cpd A with improved in vitro potency

Cpd C: Close analogue of Cpd B with improved pharmacokinetic properties

Panel D) Human GPR40/HEK stable cells: IP_3 responses to GPR40 agonists

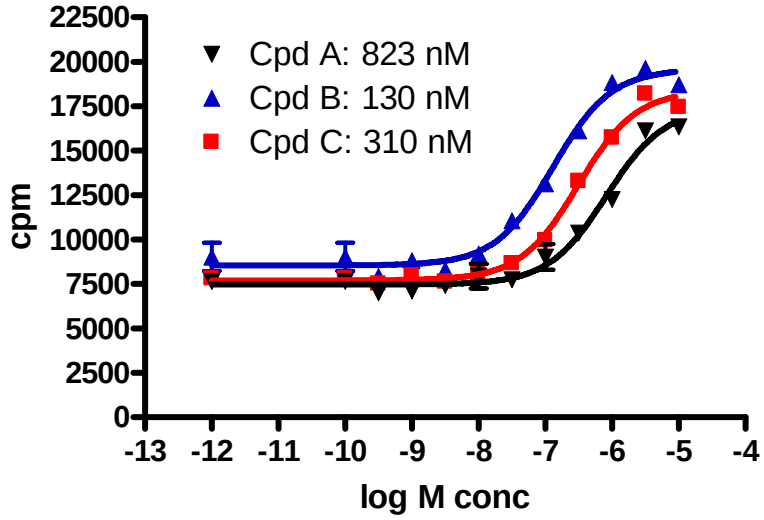
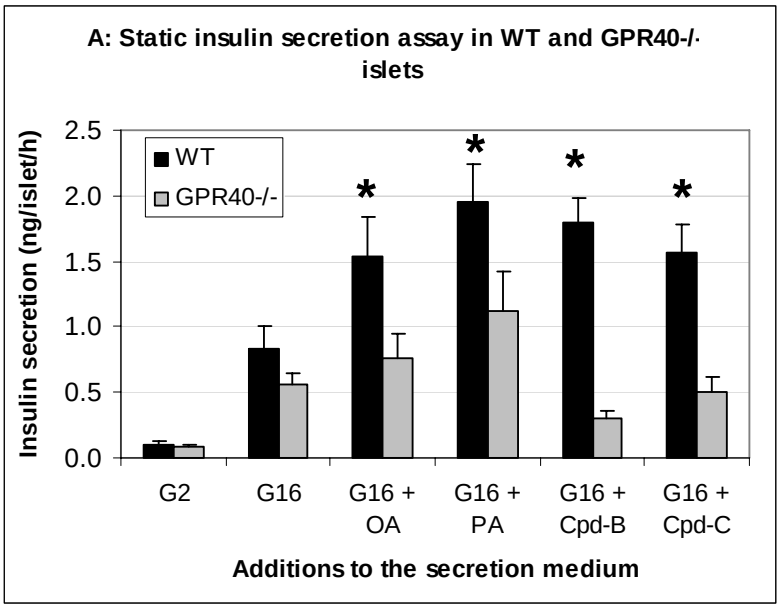


FIG. 1. Activation of human and mouse GPR40 by fatty acids and small molecule agonists.

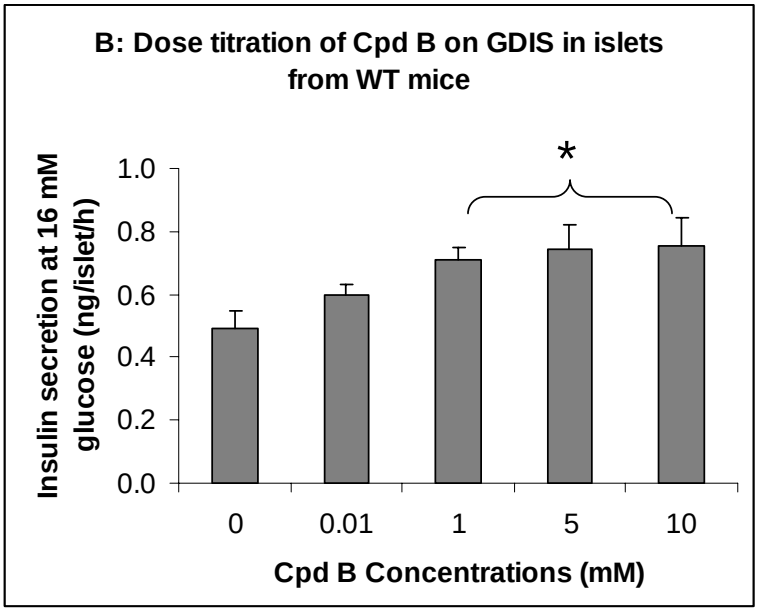
Representative dose-responses of various fatty acids induced calcium mobilization in CHO cells stably expressing human (Panel A) and mouse GRP40 (Panel B) measured by the FLIPR assay. EC_{50} values for various fatty acids are Mean ± SE of three independent titration experiments. C: Chemical structures of the small molecule GPR40 agonists and their dose-responses {EC_{50}} measured in the FLIPR-based calcium mobilization assay. D: IP_3 accumulation assay for GPR40 agonists in human GPR40-HEK293 stable cells.

Figure 2

Panel A: Static incubation of islets form WT and GPR40-/- mice



Panel B: Dose-titration of Cpd B on GDIS in WT islets



Panel C: Glucose-dependency of GPR40 mediated insulin secretion in mouse islets

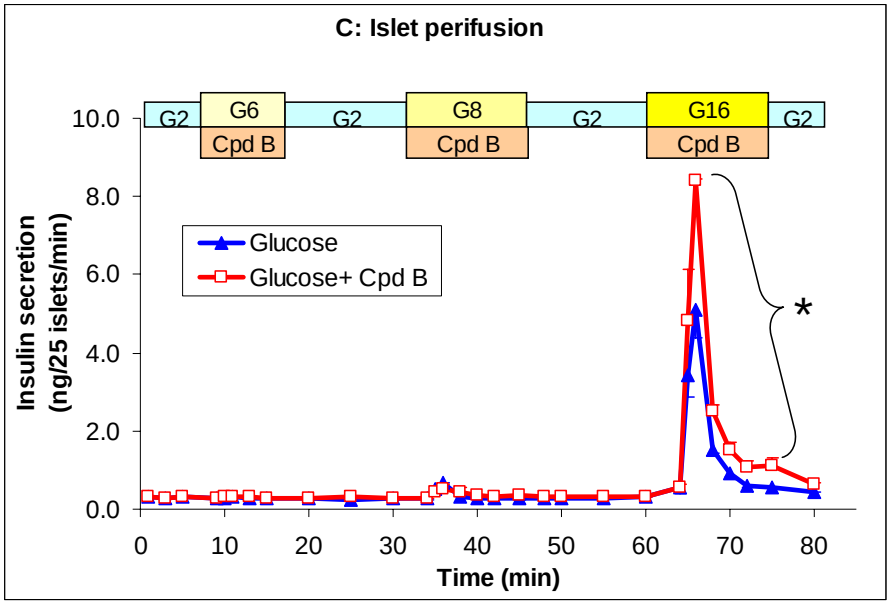


FIG. 2. The acute effect of fatty acid and GPR40 agonists on GDIS in islets isolated from wild type and GPR40-/- mice.

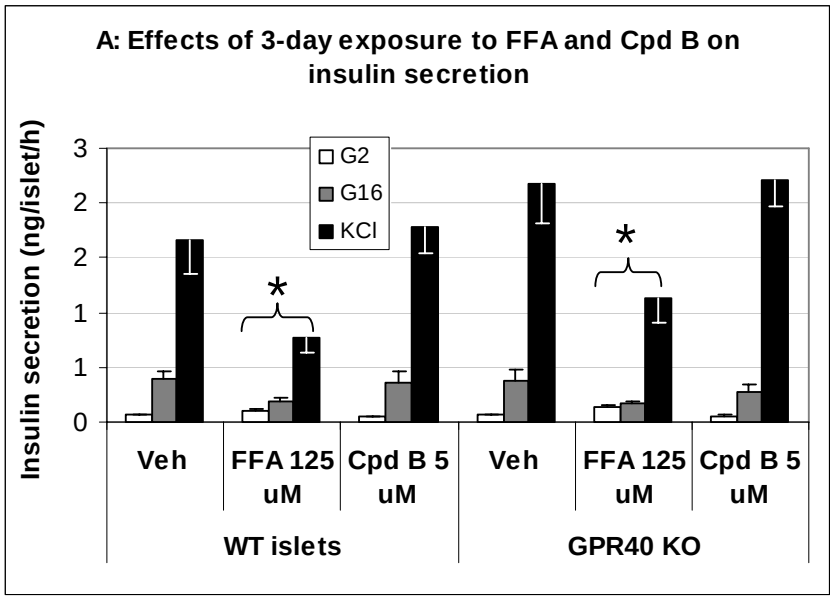
A: Effects of oleate, palmitate and the GPR40 agonists (Cpd B and C) on insulin secretion measured by the static incubation assay in islets from WT and GPR40-/- mice. Following a 30 min preincubation in KRB medium (containing 0.2% BSA) with 2 mM glucose, islets were incubated with either 2 or 16 mM glucose, or 16 mM glucose with oleic acid (OA 200 μM), palmitic acid (PA, 200 μM), Cpd B (10 μM) or Cpd C (10 μM). Data are means ± SE of two independent experiments with six replicates in total. *$p<0.05$ or less compared to G16 of WT islets (with DMSO added as vehicle).

B: Effects of increasing concentrations of Cpd B on insulin response at 16 mM glucose in WT islets measured by the static incubation assay. Data are means ± SE of three experiments. *$p<0.05$ or less compared to vehicle (DMSO) control.

C: Glucose-dependency of GPR40 mediated insulin secretion in mice islets. Batches of islets from C57Bl/6 mice were perifused with KRB medium containing 2, 6, 8 and 8 mM glucose for 10 min each sequentially (with 10 min washout by 2 mM glucose in-between). Insulin released during those stimulation was measured once per minute. Data are means ± SE of three independent experiments. *$p<0.05$ or less compared to 16 mM glucose alone.

Figure 3 Effects of a 3 day exposure to FFAs or Cpd B on insulin secretion and insulin content in islets from WT and GPR40-/- mice

Panel A: Insulin secretion measured after 3 day exposure by static incubation



Panel B: Islet insulin content after 3 day exposure to FFAs or Cpd B

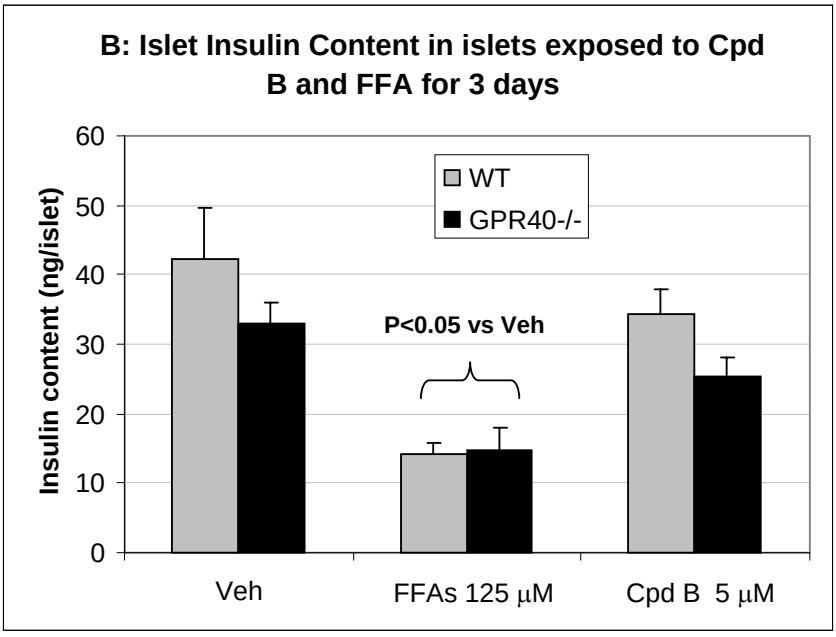
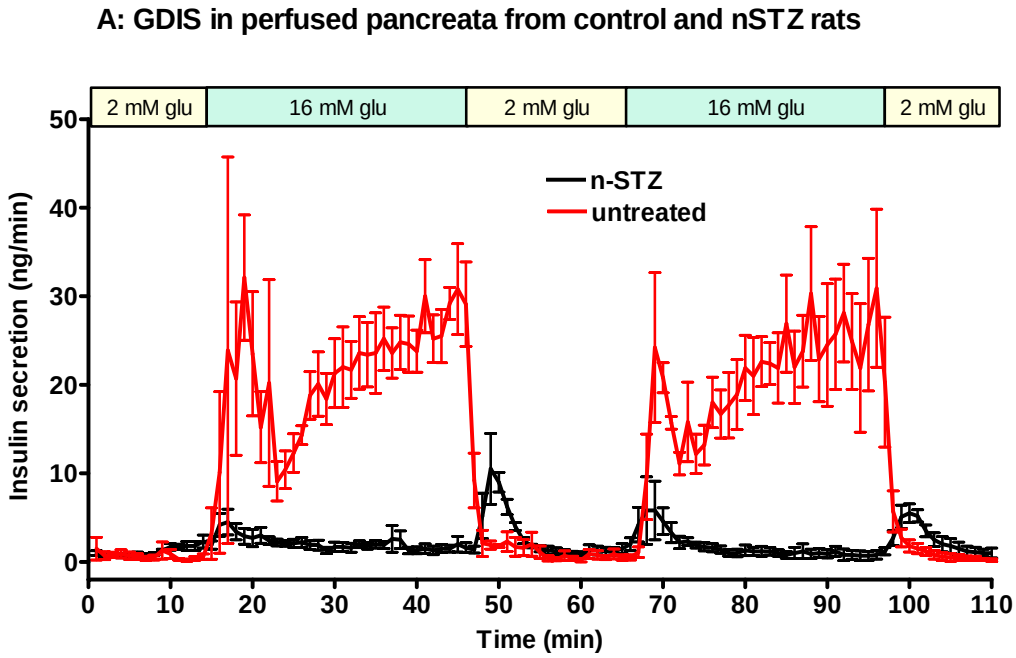


FIG. 3. Effects of a 72-h exposure to fatty acid and Cpd B on islets isolated from wild type and GPR40-/- mice.
Islets from the WT and GPR40-/- mice were cultured for 3 days with or without fatty acids (65 μM of palmitate + 65 μM of oleate) or Cpd B (5 μM). Insulin secretion (Panel A) was measured by the static insulin secretion assay in KRB medium with no FFA or Cpd B present. Similarly treated islets were also used for islet insulin measurement following acid ethanol extraction (Panel B). Data are means ± SE of three separate experiments, *$p<0.05$ or less when compared to vehicle control.

Figure 4

Panel A: Insulin responses to glucose stimulation in pancreata from normal and nSTZ rats perfused in situ



Panel B: Effects of GPR40 agonist (Cpd B) in perfused pancreata from the nSTZ rats

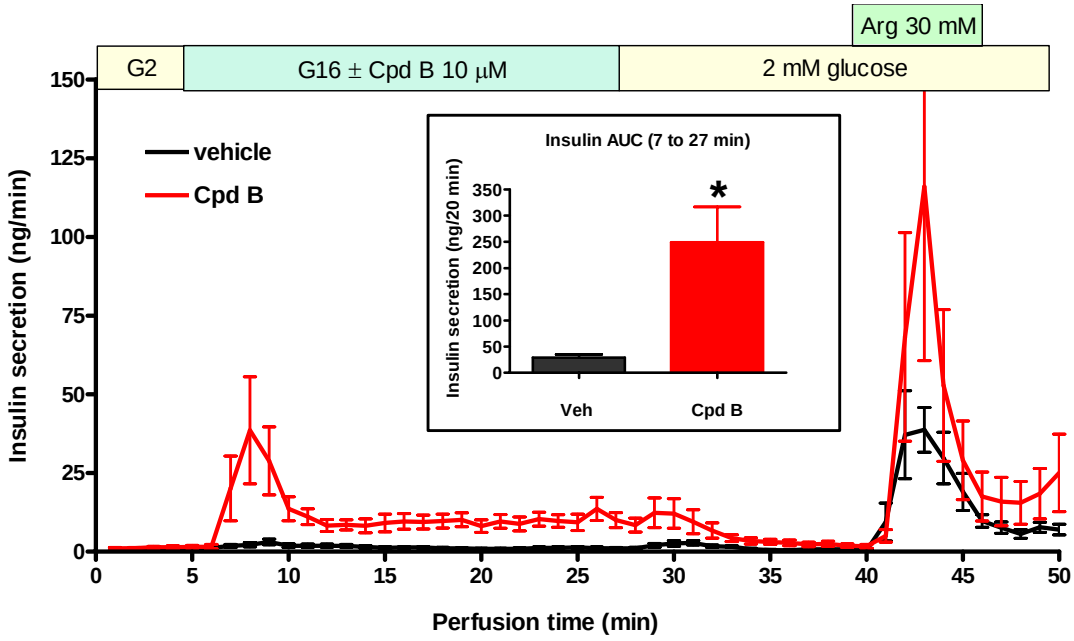
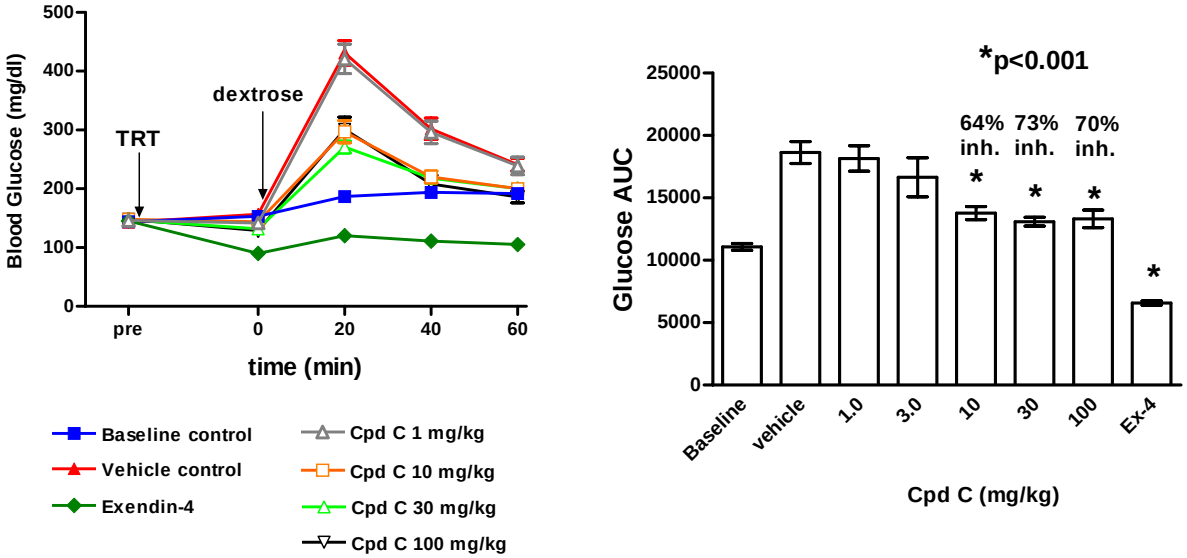


FIG. 4. Effects of GPR40 agonist (Cpd B) on insulin secretion from *in situ* perfused pancreata of STZ-treated rats.
A: Insulin responses to two pulses of 16 mM glucose stimulation in pancreata perfused in situ from normal and nSTZ rats. Data are means ± SE of three preparations for each group. The pancreata from the normal rats exhibited identical biphasic insulin secretory responses to both glucose pulses, which was totally lost in pancreata from the nSTZ rats;
B: Insulin responses to glucose stimulation in the presence or absence of Cpd B in perfused pancreata from the nSTZ rats. Pancreata from the nSTZ rats were challenged first by 16 mM glucose and 30 mM Arginine sequentially (with 15 min washout in-between) with or without 10 uM Cpd B. Data are means means ± SE of five pancreatic preparation for both groups.

Figure 5

Panel A: Effects of GPR40 agonist (Cpd C) on IPGTT glucose levels in C57Bl/6 mice



Panel B: Effects of GPR40 agonist (Cpd C, 30 mg/kg) on IPGTT glucose levels in WT and GPR40-/- mice

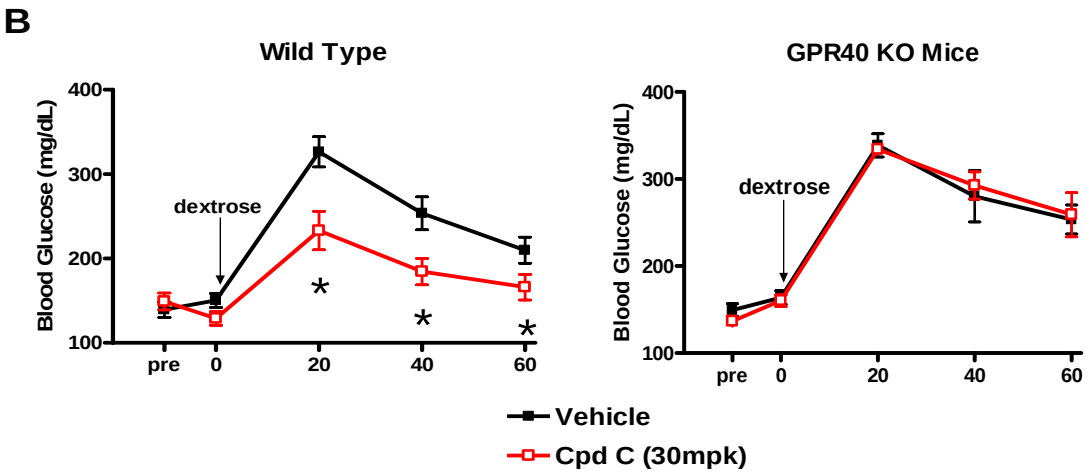


FIG. 5. Glucose-lowering efficacy of Cpd C in wild type C57Bl/6 and GPR40-/- mice.
A: Effects of increasing doses of Cpd C on blood glucose levels during the IPGTT in WT mice. C57BL/6 mice were dosed with vehicle (0.25% methylcellulose) or Cpc C (1 ~ 100 mg/kg) by oral gavage at -60 min, followed by i.p. glucose challenge (2 g dextrose per kg body-weight; same volume of H_2O only for Veh-water group) at 0 min. Blood glucose levels were measured in whole blood samples obtained by tail-sniping at the intervals indicated in the graphs. The % inhibition of glucose levels was calculated based on the glucose AUC during the 60 min IPGTT for each group after subtracting the values from the baseline (no-drug & no-glucose) control group. Data are means ± SE of 7-10 mice per group. *$p<0.01$ or less compared to vehicle treated animals.
B: The GPR40-/- mice and the littermate WT mice were dosed with vehicle or 30 mg/kg Cpd C 60 min prior to the IPGTT as described above in Panel A. Data are means ± SE of 7-10 mice per group. *$p<0.05$ or less compared to vehicle treated animals.

Figure 6 Effects of chronic treatment with GPR40 agonist (Cpd B) on IPGTT glucose levels in C57Bl/6 mice

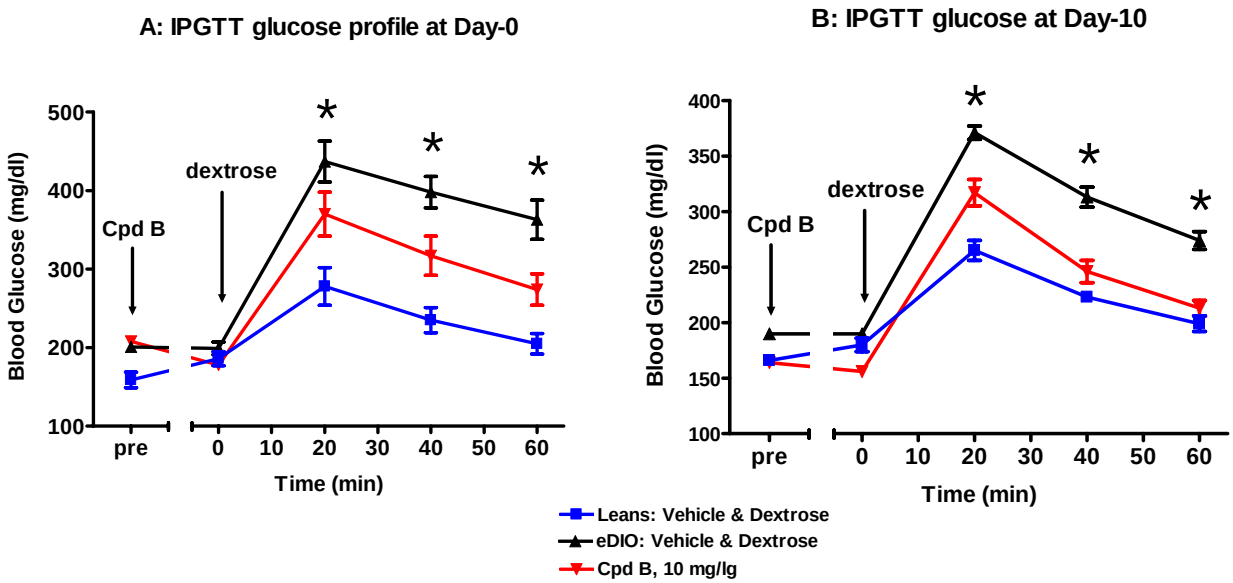


FIG. 6 Effects of GPR40 agonist on IPGTT glucose levels in high-fat (HF) diet induced obesity (eDIO) mice
C57BL/6 mice were switched to a 60% HF diet (D12492i) at age of 6 weeks and kept on the same diet through-out the study. The treatment with the GPR40 agonist (Cpd B, 10 mg/kg, oral gavage, once a day) was started 14 weeks after the initiation of HFD feeding. An IPGTT (1 g dextrose/kg BW) was performed on Day-0 (panel A) and Day-10 (panel B) of the 10-day long treatment with Cpd B to compare the glucose-lowering efficacy of Cpd B before (panel A) and after (panel B) the chronic treatment. The final dose on Day 10 was given one hour prior to glucose challenge. Blood glucose levels were measured in whole blood samples obtained by tail-snipping at the intervals indicated in the graphs. There were 8 mice per group. Single-factor ANOVA analysis was used to compare the difference in glucose AUC among the groups. *P<0.01 compared to vehicle treated DIO mice.