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Original Paper

Insulinotropic effects of GPR120 agonists are altered in obese diabetic and obese non-diabetic states

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Abstract

G-protein-coupled receptor 120 (GPR120) is a putative target for obesity and diabetes therapies. However, it remains controversial whether resident GPR120 plays a direct regulatory role in islet β-cell insulin secretion. The present study examined this issue in isolated rodent islets and rat β-cell line INS-1E, and assessed the role of GPR120 in islet insulin secretion in obese non-diabetic (OND) and diabetic states. GPR120 expression was detected in rodent islet β-cells. Docosahexaenoic acid (DHA) and synthetic GPR120 agonist GSK137647 (GSK) augmented insulin release from rat/mouse islets and INS-1E; DHA effects were partially mediated by GPR40. GPR120 knockdown and overexpression attenuated and enhanced DHA effects in INS-1E respectively. DHA and GSK improved postprandial hyperglycaemia of diabetic mice. Inhibition of calcium signalling in INS-1E reduced GPR120 activation-induced insulinotropic effects. The insulinotropic effects of DHA/GSK were amplified in OND rat islets, but diminished in diabetic rat islets. GPR120 and peroxisome proliferator-activated receptor γ (PPARγ) expression were elevated in OND islets and palmitic acid (PA)-treated INS-1E, but reduced in diabetic islets and high glucose-treated INS-1E. PPARγ activation increased GPR120 expression in rat islets and INS-1E. DHA and GSK induced protein kinase B (Akt)/extracellular signal-regulated kinase (ERK) phosphorylation in rodent islets and INS-1E, and these effects were altered in OND and diabetic states. Taken together, the present study indicates that (i) GPR120 activation has an insulinotropic influence on β-cells with the involvement of calcium signalling; (ii) GPR120 expression in β-cells and GPR120-mediated insulinotropic effects are altered in OND and diabetic states in distinct ways, and these alterations may be mediated by PPARγ.

Key words: calcium signalling, insulin secretion, non-diabetic obesity, peroxisome proliferator-activated receptor γ (PPARγ), Type 2 diabetes mellitus (T2DM).

INTRODUCTION

G-protein-coupled receptor 120 [GPR120, also known as free fatty acid receptor 4 (FFAR4)] has garnered attention as a potential therapeutic target for obesity and its associated diseases, such as Type 2 diabetes mellitus (T2DM). In this regard, GPR120 activation results in anti-inflammatory effects, leading to the maintenance of insulin sensitivity in adipose, liver and skeletal muscle [1,2]. Pancreatic islets, and particularly β-cells within islets, are crucial for T2DM; despite being controversial, GPR120 has been reported to be expressed in human and mouse islets [3,4], as well as in rodent β-cell lines [4–7]. Furthermore, GPR120 expression is reduced in islets from hyperglycaemic and T2DM patients [3].

Non-esterified fatty acids (NEFAs) exhibit some of their beneficial physiological effects via activation of their respective receptors, such as GPR120 and G-protein-coupled receptor 40 (GPR40) [8,9]. Some NEFAs, such as docosahexaenoic acid (DHA), can activate both GPR120 and GPR40 [8,10], and thus augment islet β-cell insulin secretion [10]; these insulinotropic effects of NEFAs are only partially attributed to GPR40 activation in β-cells [9,11]. However, it remains to be controversial whether GPR120 is expressed in β-cells and whether GPR120 mediates, at least in part, the stimulatory effects of NEFAs on insulin secretion. In this regard, recent studies with mouse islets arrived at opposite conclusions [6,12]. It was reported that a synthetic GPR120 agonist did not modulate mouse-islet insulin

Abbreviations: Akt, protein kinase B; AH, AH7614; AUC, area under curve; $[Ca^{2+}]_i$, intracellular calcium levels; DHA, docosahexaenoic acid; ERK, extracellular signal-regulated kinase; FFAR4, free fatty acid receptor 4; FSC, forward scatter; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPCR, G-protein-coupled receptor; GPR120, G-protein-coupled receptor 120; GSK, GSK137647A; HFD, high-fat diet; HOMA-IR, homoeostasis model assessment of insulin resistance; IPGTT, intraperitoneal glucose tolerance test; KO, knockout; KRBB, Krebs-Ringer bicarbonate buffer; NC, negative control; NEFA, non-esterified fatty acid; OGTT, oral glucose tolerance test; OND, obese non-diabetic; PA, palmitic acid; PLC, phospholipase C; PPARγ, peroxisome proliferator-activated receptor γ; RZG, rosiglitazone; STZ, streptozotocin; T2DM, Type 2 diabetes mellitus; TPPO, triphenylphosphine oxide; TRPM5, transient receptor potential cation channel subfamily M member 5.

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secretion [12]; conversely, GPR120 agonists exhibited insulinotropic effect in mouse islets and rat β-cell line [6]. Furthermore, previous studies have contrary results regarding the insulinotropic effects of NEFAs in GPR120 knockout (KO) mouse islets [12,13]. Notably, the ability of GPR120 activation to increase intracellular calcium levels ($[Ca^{2+}]_i$) in enteroendocrine cells and taste bud cells is consistent with the possibility that GPR120 activation might modulate β-cell insulin secretory activity [8,14,15], because elevation of $[Ca^{2+}]_i$ can trigger cellular hormone secretion, including insulin release from β-cells [16–18].

Plasma NEFAs have been shown to exert insulinotropic effects in animals and humans [19–21]. These stimulatory effects appear to be differential between obese non-diabetic (OND) compared with T2DM animals/patients [20,22,23]. It has been proposed that mildly elevated plasma NEFAs in OND individuals may promote insulin release to compensate for obesity-related insulin resistance, whereas these insulinotropic effects are diminished, or even abolished, in diabetic animals/patients [22]. The mechanism underlying the pathology-associated alterations in plasma-NEFA effects has not yet been resolved. Strikingly, GPR120 expression levels are altered in adipose, cardiac and skeletal muscle tissues of OND subjects [1,24,25]. It has yet to be determined whether islet GPR120 expression is changed in OND/diabetic states as well, if so, whether such alterations underlie the differential insulin secretory responses to plasma NEFAs.

Apart from the insulinotropic effects, GPR120 activation may exhibit inhibitory effects on β-cell apoptosis. Previous studies demonstrated that GPR120 activation inhibited apoptosis of enteroendocrine cells and bone marrow-derived mesenchymal stem cells via activating protein kinase B (Akt) and/or extracellular signal-regulated kinases (ERKs) signalling pathways [26,27]. Notably, Akt/ERK phosphorylation in β-cells is able to protect against apoptosis and promote survival as well [28,29].

In the present study, we performed *in vitro*, *ex vivo* and/or *in vivo* experiments to investigate the potential regulatory actions of resident GPR120 in β-cell insulin secretion and Akt/ERK activation. Firsty, we estimated GPR120 expression in rodent islet β-cells, and examined how GPR120 knockdown and overexpression affect the insulinotropic effects of DHA in INS-1E cells. Next, we assessed the insulin secretory responses of isolated rodent islets and INS-1E cells to selective GPR120 agonism and antagonism. In addition, we determined the effects of GPR120 agonists on postprandial hyperglycaemia and serum insulin levels of diabetic mice. To study the mechanisms underlying GPR120-mediated insulinotropic effects, we further tested how these effects were influenced by co-treatments affecting calcium signalling pathways and measured GPR120 agonists-induced $[Ca^{2+}]_i$ changes in INS-1E cells. Furthermore, we investigated whether GPR120-mediated insulinotropic effects and islet GPR120 expression are altered in OND and diabetic rats; the effects of PPARγ agonist and antagonist treatments were also analysed as well to determine whether alterations in GPR120 expression/actions are associated with PPARγ. Finally, we examined the effects of GPR120 agonists on Akt/ERK activation and/or glucotoxicity-induced apoptosis in rodent islets and INS-1E cells, and whether these effects were altered in OND and diabetic islets.

MATERIALS AND METHODS

Animal models

Male obese Zucker rats exhibit obesity, hyperlipidaemia and normoglycaemia by 6–8 weeks of age; their blood glucose start to rise from around 10 weeks, and they develop overt hyperglycaemia and diabetes by 18 weeks of age [30,31]. Hence, in the present study, male obese Zucker rats (*fa/fa*) were used as OND models at 6–7 weeks old and as T2DM models at 22 weeks old; age-matched lean Zucker littermates (*fa/FA* or *FA/FA*) were served as controls.

Six-week-old male C57BL/6 mice fed a high-fat diet (HFD) (60% of Kcal from fat; 21.34 kJ/g, Harlan Laboratories) for 4 months were used as an OND mouse model. The protocol for generation of Type 2 diabetic mouse model was modified as previously reported [32]. Briefly, 6-week-old male C57BL/6 mice were fed an HFD for 2 months, given two intraperitoneal injections of streptozotocin (STZ) (once per week, 60 mg/kg body weight in 10 mM sodium citrate buffer; Sigma) and then fed an HFD for an additional 2 months. Control mice were fed a standard diet and/or injected with sodium citrate buffer.

Rats and mice were housed in an animal facility at a constant temperature (25.5 °C) with a 12-h light–dark cycle and water *ad libitum*. All animals were obtained from the Laboratory Animal Services Center of The Chinese University of Hong Kong. The experimental procedures were approved by the Animal Experimentation Ethics Committee at our institution (Ref. # 10/059/GRF-4, 10/064/MIS-5). The methods used in the animal studies were carried out in accordance with the relevant guidelines.

In vivo glucose homoeostasis

For oral glucose tolerance test (OGTT) and intraperitoneal glucose tolerance test (IPGTT), animals were fasted for 6 h, then administrated with 2g/kg glucose via an oral administration and an intraperitoneal injection respectively. Blood glucose and serum insulin levels were measured at each time point. The degree of insulin resistance was determined by homoeostasis model assessment of insulin resistance (HOMA-IR), using the following equation: HOMA-IR = fasting plasma insulin (mU/l) × fasting blood glucose (mM)/22.5 [33].

In vivo effects of GPR120 agonists in Type 2 diabetic mice

The assessments of GPR120 agonist effects on Type 2 diabetic mouse postprandial hyperglycaemia and serum insulin levels were performed as described previously with modifications [34–36]. Briefly, HFD/STZ mice were fasted for 6 h and then orally given with vehicle (0.5% methylcellulose, 0.5% Tween 80; Sigma), DHA (30 mg/kg) or GSK137647A (GSK) (30 mg/kg). One hour later, glucose (2 g/kg) was orally administrated. Blood glucose and serum insulin levels were monitored at −60 (agonist administration), 0, 15, 30, 60, 90 and/or 120 min.

Pharmacological treatments

GPR120 was activated non-selectively with 50 μM DHA (Cayman) [2,8] and selectively with 50 μM GSK (Tocris) [37,38].

GPR120 and GPR40 were antagonized selectively with 100 μM AH7614 (AH; Tocris) [37,39] and 10 μM GW1100 (Cayman) [11,40,41] respectively. Calcium signalling was inhibited with 5 μM U73122 (a phospholipase C (PLC) inhibitor), 100 μM triphenylphosphine oxide (TPPO) (a transient receptor potential cation channel subfamily M member 5 (TRPM5) inhibitor) and 20 μM nifedipine (an L-type voltage-dependent calcium channel inhibitor) (all from Cayman). PPARγ was activated with 10 μM rosiglitazone (RZG) and antagonized with 10 μM GW9662 (Sigma). DHA was dissolved in ethanol to 100 mM and then added to incubation buffer. GSK and AH were dissolved in DMSO to 50 mM before addition to incubation buffer. TPPO was dissolved in absolute ethanol, and other tested substances were dissolved in DMSO.

Islet isolation and culture

Rodent islets were isolated as previously described [42]. Intact isolated rat/mouse pancreatic islets were cultured in 5.6 mM glucose-containing RPMI medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (all materials from Life Technologies).

Islet cell purification by FACS

Rat islet β-cells were purified as described previously with modifications [43]. Healthy male Wistar rats (250–300 g body weight) were obtained from the Laboratory Animal Services Center of The Chinese University of Hong Kong. The isolated intact rat islets were rinsed twice with PBS, then digested with 0.25% trypsin at 37 °C for 10 min. The dispersed cells were washed twice with PBS supplemented with 2% FBS, then re-suspended in Earle's balanced salts (EBSS; 124 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.4 mM KCl, 1.0 mM NaH_2PO_4 and 14.3 mM $NaHCO_3$) with 2.8 mM glucose, 10 mM HEPES and 2% BSA. Before cell sorting, cell clumps were removed by 70 μm filter (BD), and cell viability was assessed by trypan blue test.

The protocol for rat islet β-cell sorting was based on the endogenous FAD auto-fluorescence [43]. The islet β-cells with higher fluorescence were sorted on a BD FACSAria Fusion Flow Cytometer (BD). The FAD content in the dispersed rat islet cells was detected at an excitation wavelength of 488 nm, and the fluorescence was collected at 530 nm.

Determination of sorted islet β-cell purity by flow cytometry

The sorted islet β-cells were fixed and then permeabilized by 4% paraformaldehyde and perm/wash buffer (BD) respectively. Then, the cells were stained with anti-insulin ALEX 647 (BD) and anti-glucagon PE (BD) for 1 h at 4 °C. After washing with perm/wash buffer, the cells were filtered and analysed on a BD FACSAria Fusion Flow Cytometer. Flow cytometric data were analysed by Flow Jo.

Immunofluorescence staining

Optimum cutting temperature compound-embedded pancreatic tissues were sectioned (6 μm) and collected on slides. The sections were then fixed by 4% paraformaldehyde and blocked with 3% BSA (Sigma). To assess islet morphology of diabetic animals, pancreatic sections were labelled with rabbit anti-insulin (1:200, Santa Cruz Biotechnology) and mouse anti-glucagon (1:1000, Abcam). To assess cell purity, the sorted rat islet β-cells seeded on coverslips were fixed and blocked, then labelled with guinea pig anti-insulin.

These slides or coverslips were then incubated with secondary antibodies conjugated with Alexa Fluor 568 (1:200, Life Technologies) or Alexa Fluor 488 (1:200, Life Technologies). Nuclei were counterstained with DAPI (Life Technologies). Digital images were acquired on Olympus FV1200 SIM Confocal System (Olympus) or fluorescence microscope equipped with a DC200 digital camera (Leica Microsystems).

Cell culture and treatments

INS-1E cells were cultured in 11.1 mM glucose-containing RPMI medium supplemented with 10% FBS, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 10 mM HEPES, 100 units/ml penicillin and 100 μg/ml streptomycin (all materials from Life Technologies). In some experiments, INS-1E cells were treated with 33.3 mM D-glucose, 300 μM palmitic acid (PA; Sigma), 10 μM RZG and/or GW9662. PA-supplemented medium was prepared by modification of a previous method [44]. Briefly, PA was dissolved to the final concentration of 100 mM in 50% ethanol (v/v), then NaOH solution was added, PA solution in 200 mM NaOH was incubated at 60 °C for 30 min, then complexed with 10% NEFA-free BSA (v/v) at 1:9 volume ratio, incubated at 37 °C for 30 min, and finally added to cell culture media. For the measurement of Akt and ERK phosphorylation, INS-1E cells were starved in cell culture medium containing 0.5% FBS for 12 h, and subsequently incubated with either 50 μM DHA or GSK for the indicated time.

Determination of cell apoptosis

INS-1E cells were seeded at a density of 1×10^4 per well in 96-well microplates. Two days later, cells were exposed to 33.3 mM glucose with or without 10 μM DHA or GSK for 3 days. Cell apoptosis was then assessed with a cell death detection ELISA plus kit (Roche Applied Science) according to the manufacturer's instructions.

Measurement of insulin secretion

INS-1E cells or isolated islets were allowed to equilibrate in Krebs-Ringer bicarbonate buffer (KRBB, supplemented with 0.1% NEFA-free BSA and 10 mM HEPES) with 1.7 mM glucose for 1 h. They were then incubated in KRBB containing 1.7 mM, 5.6 mM or 16.7 mM glucose with or without DHA, GSK for 1 h. KRBB solution samples were subjected to insulin quantification by an ELISA kit (University of Hong Kong, Hong Kong, China).

siRNA and plasmid transfection

GPR120 mRNA-targeting siRNA and negative control (NC) siRNA were synthesized (Life Technologies) and transfected into INS-1E cells with lipofectamine RNAi Max reagent (Life Technologies). To overexpress GPR120, INS-1E cells were transfected with the plasmid encoding rat-GPR120 or control plasmid (Origene) with lipofectamine 2000 reagents (Life Technologies). Transfected cells with plasmid were grown on coverslips,

incubated with anti-*c-Myc* primary antibody (Cell Signaling Technology), then probed with secondary antibodies conjugated with Alexa Fluor 568 (1:200, Life Technologies) to determine exogenous GPR120 expression.

Intracellular calcium imaging

Dual-wavelength micro-fluorimetry calcium imaging was performed as described previously with modifications [45]. Briefly, INS-1E cells plated on coverslips were loaded with fura 2-acetoxymethyl ester (Life Technologies) in KRBB. The coverslips were transferred to a closed perfusion chamber and superfused by KRBB with or without DHA or GSK, and then irradiated alternately with 340-nm and 380-nm light. Changes in intracellular calcium concentration were indexed by changes in calculated fura-2 340/380-nm ratio.

Quantitative real-time PCR

Total RNA samples were subjected to reverse transcription with iScript Select cDNA Synthesis Kits (Bio–Rad Laboratories). Real-time PCR was performed in an i-Cycler thermal cycler (version 3.1; Bio–Rad Laboratories) with iQ SYBRGreen Supermix (Bio–Rad Laboratories). Relative gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin and calculated by the comparative threshold method ($2^{-\Delta\Delta C}{}_{\mathrm{T}}$).

Western blotting

Total protein samples were extracted from a standardized number of cells with CytoBuster protein extraction reagent (Novagen). The protein was fractionated by SDS/PAGE, transferred to PVDF membranes (Millipore) and then probed with primary antibodies against β-actin (Santa Cruz Biotechnology), GPR120 (Novus), Akt, p-Akt, ERK and p-ERK (Cell Signaling Technology).

Statistical analysis

Results are displayed as means $\pm$ S.E.M. Groups were compared with two-tailed Student's *t* test, one-way ANOVA followed by Tukey's post hoc test or two-way ANOVA followed by Bonferroni post-tests. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

GPR40 partially mediates the insulinotropic effects of DHA in rat/mouse islets and INS-1E cells

As shown in Supplementary Figures S1A and S1B, 50 μM DHA augmented insulin secretion from isolated Wistar rat islets and C57BL/6 mouse islets in the presence of 5.6 mM or 16.7 mM glucose. Meanwhile, DHA exhibited similar stimulatory effects on the insulin release of rat INS-1E β-cells (Supplementary Figure S1C). Meanwhile, GPR40 antagonism with 10 μM GW1100 attenuated, but did not abolish, the insulinotropic effects of DHA in both isolated islets and INS-1E cells (Supplementary Figures S1A–S1C), indicating that islet β-cell GPR40 partially mediates the insulinotropic effects of DHA.

GPR120 is expressed in β-cell lines and rodent islet β-cells

GPR120 mRNA expression was confirmed in INS-1E cells, Wistar rat islets, MIN6 and C57BL/6 mouse islets (Supplementary Figure S2). Moreover, Wistar rat islet β-cells were purified by FACS (Figure 1A), and the purity of the sorted β-cells was assessed by flow cytometry as well as immunofluorescent staining (Figures 1B and 1C). GPR120 mRNA and protein expression were detected in the sorted β-cells (Figures 1D and 1F), and the expression levels are comparable with those in INS-1E cells (Figures 1E and 1F).

GPR120 mediates the insulinotropic effects of DHA in INS-1E cells

To examine the involvement of β-cell GPR120 in insulinotropic actions of DHA, genetic modulations of GPR120 expression were performed in INS-1E cells. siRNA transfection reduced both mRNA and protein expression levels of GPR120 (Supplementary Figures S3A and S3B), whereas plasmid transfection induced expression of exogenous GPR120 (Supplementary Figures S3C, S3D and S3E). Remarkably, siRNA-mediated GPR120 knockdown attenuated (Figures 2A and 2B) whereas plasmid transfection-mediated GPR120 overexpression enhanced (Figures 2C and 2D) the insulinotropic effects of DHA in INS-1E cells. These results indicate strongly that GPR120 in β-cells mediates, at least in part, the insulinotropic effects of DHA.

Selective GPR120 agonism and antagonism regulate insulin release of rat/mouse islets and INS-1E cells

To further prove the regulatory role of islet β-cell GPR120 on insulin release, we employed GSK and AH, which are synthetic agonist and antagonist specific for GPR120 respectively [37–39]. Incubation with 50 μM GSK increased insulin secretion from isolated islets (Wistar rat and C57BL/6 mouse) and INS-1E β-cells in the presence of 5.6 mM or 16.7 mM glucose (Figures 3A–3C). These insulinotropic effects of GSK were blocked by 100 μM AH (Figures 3A–3C).

GPR120 activation-induced insulinotropic effects in β-cells are facilitated via calcium signalling

In INS-1E cells, GPR120 agonism with DHA/GSK produced a rapid rise in $[Ca^{2+}]_i$ in the presence of 16.7 mM glucose (Figure 4A). Moreover, the insulinotropic effect of GSK on INS-1E cells was inhibited by co-treatment with various calcium signalling blockers (Figure 4B), including the PLC inhibitor U73122, the TRPM5 inhibitor TPPO and the L-type calcium channel inhibitor nifedipine.

Validation of OND and T2DM animal models

OND and T2DM rat/mouse models were employed to investigate the regulatory role of GPR120 on insulin secretion under pathological conditions. Compared with age-matched lean Zucker rats, 6- to 7-week-old obese Zucker rats were heavier and had higher insulinaemia and HOMA-IR values, but maintained normoglycaemia (Supplementary Figures S4A–S4D). These observations indicated that the obese Zucker rats were non-diabetic

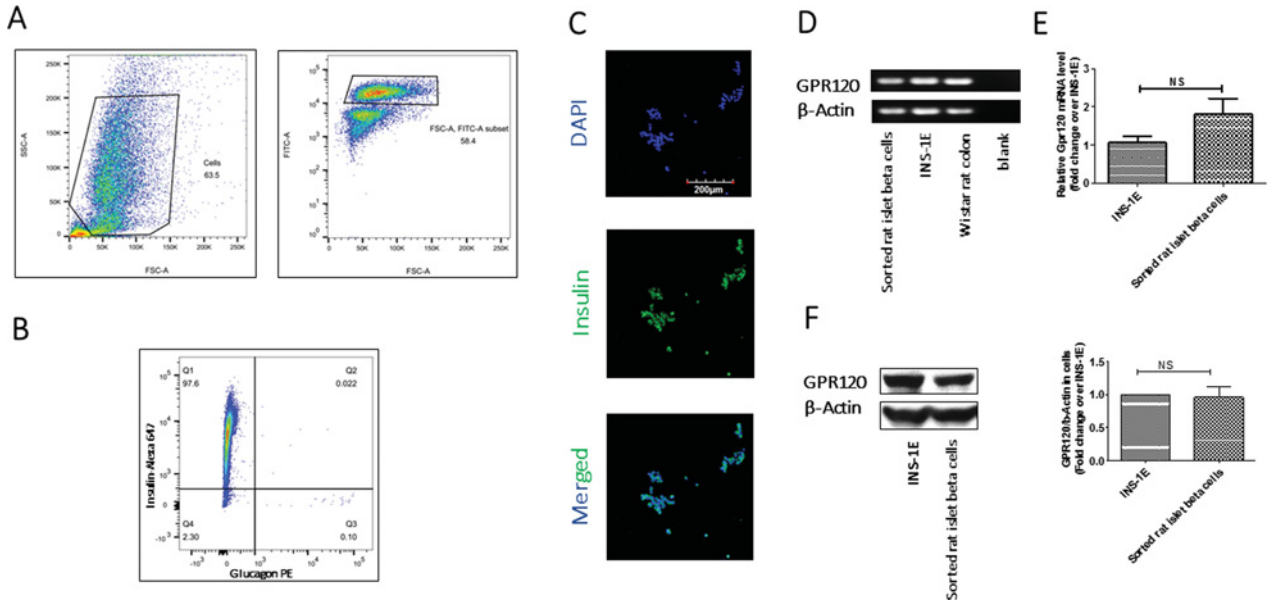

Figure 1 **GPR120 is expressed in rodent islet β-cells**

(**A**) Representative flow cytometric data of Wistar rat islet cells about the auto-fluorescence (FITC) and forward scatter (FSC) intensity and gating strategy for β-cell sorting. (**B**) The insulin and glucagon populations in sorted islet β-cell fraction. (**C**) Immunofluorescence analysis of insulin in sorted islet β-cell fraction; scale bar = 200 μm. (**D**) PCR gel images of GPR120/β-actin amplification products in the sorted islet β-cells, INS-1E and Wistar rat colon. Expression levels of GPR120 mRNA (**E**) and protein (**F**) in sorted β-cells, relative to INS-1E cells (n=3–5 per group); NS, no significant difference, $P > 0.05$.

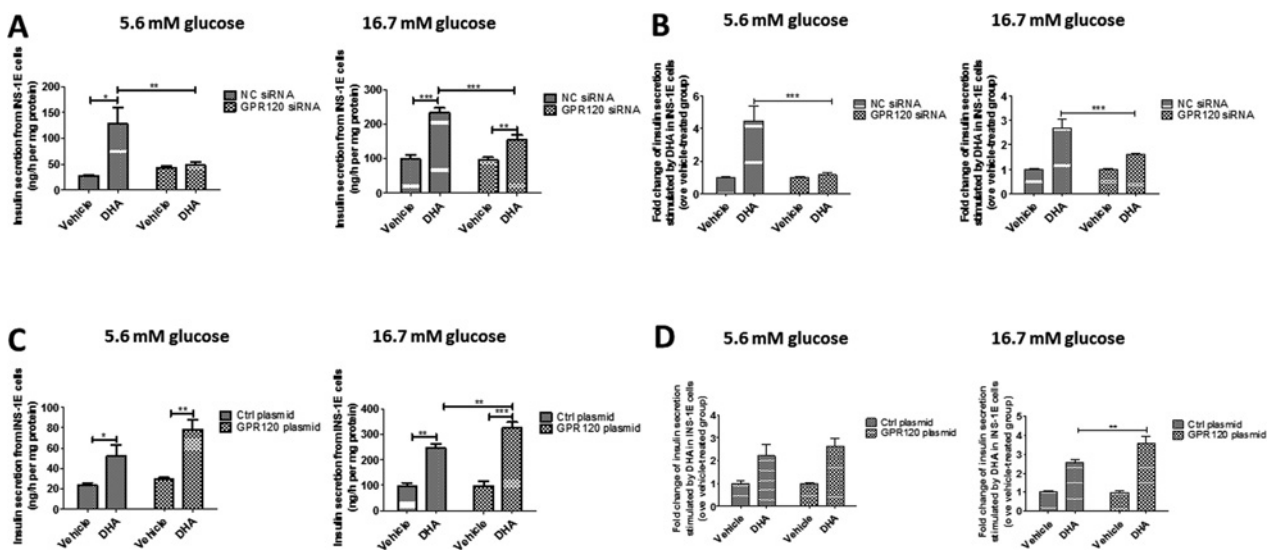

Figure 2 **Genetic modulation of GPR120 expression influences the insulinotropic effects of DHA in INS-1E cells**

The insulinotropic effects of 50 μM DHA in INS-1E cells were dampened by GPR120 knockdown (**A** and **B**), and enhanced by GPR120 overexpression (**C** and **D**) in the presence of 5.6 and 16.7 mM glucose (n=6 per group). INS-1E cells transfected with NC siRNA or control (Ctrl) plasmid served as control groups. Insulin secretion is expressed in term of ng/h per mg protein (A and C) and fold change (B and D). Fold change of insulin secretion = insulin secreted with DHA treatment/insulin secreted in respective vehicle group; NS, no significant difference, $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

but developed obesity and insulin resistance. Similar observations were made for C57BL/6 mice subjected to an HFD for 4 months (Supplementary Figures S5A–S5D). Hence, 6- to 7-week-old obese Zucker rats and HFD mice were used as OND models.

Compared with age-matched lean Zucker rats, 22-week-old obese Zucker rats were heavier and had higher insulinaemia and HOMA-IR values, and also exhibited hyperglycaemia (Supplementary Figures S4E–S4H). C57BL/6 mice subjected to an HFD

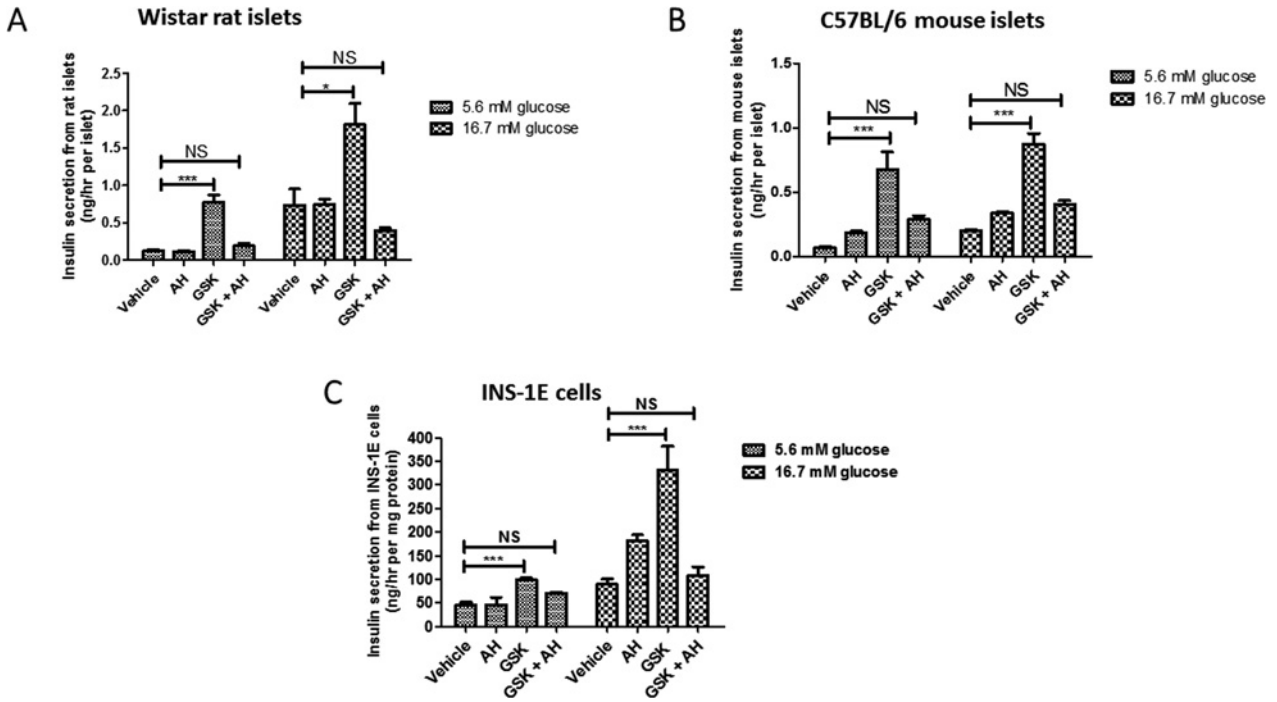

Figure 3 Selective GPR120 agonism and antagonism modulate insulin release from rat/mouse islets and INS-1E cells
GSK (50 μM) exhibited the insulinotropic effects in isolated Wistar rat islets (**A**), C57BL/6 mouse islets (**B**) and INS-1E cells (**C**) at 5.6 and 16.7mM glucose concentrations. The effects of GSK were inhibited by 100 μM AH (n=6–7 per group); NS, no significant difference, $P > 0.05$; $*P < 0.05$, $***P < 0.001$.

plus two low-dose STZ injections exhibited obesity, hyperinsulinaemia, a heightened HOMA-IR index, hyperglycaemia, impaired glucose tolerance and a reduced ratio of islet β-cell to α-cell (Supplementary Figures S5E–S5J). Hence, 22-week-old obese Zucker rats and HFD/STZ mice were validated as T2DM models.

DHA and GSK improve glucose tolerance and augment insulin secretion in Type 2 diabetic mice

We estimated the *in vivo* effects of agonists on the impaired postprandial glucose tolerance of HFD/STZ mice. The oral administration of agonists (30 mg/kg DHA or GSK) to these mice elicited the significant reduction in glucose excursion during the OGTTs (Figures 5A and 5B). Meanwhile, the plasma insulin levels of mice with agonist treatments increased remarkably, especially in the early phase of the OGTTs, when compared with mice in vehicle group (Figures 5C and 5D).

Insulinotropic effects of DHA/GSK are enhanced in OND rat islets but diminished in diabetic rat islets

DHA and GSK amplified insulin secretion from islets of both control and OND rats, with greater secretion occurring in the latter (Figure 6A). Analysis of insulin secretion fold changes in response to DHA/GSK exposure further indicated that the insulinotropic effects of DHA/GSK were enhanced in OND rat islets compared with those in control islets (Figure 6B). On the other hand, the insulinotropic effects of DHA/GSK were reduced in diabetic islets, compared with the control islets (Figures 6C and 6D). To confirm whether the alterations in GPR120 expression and agonist effects observed were independent of age, we compared GPR120 mRNA expression and agonist actions in islets from lean Zucker rats at 6 weeks old and 22 weeks old. Results showed that their respective GPR120 mRNA expression was comparable (Supplementary Figure S6A); consistently, the insulinotropic effects of GPR120 agonists were also comparable (Supplementary Figure S6B).

GPR120 and PPARγ expression are elevated in OND rat islets and PA-treated INS-1E cells but decreased in diabetic rat islets and high-glucose treated INS-1E cells

To examine whether the observed alterations in DHA/GSK effects are relevant to GPR120, the expression levels of GPR120 in rodent islets and β-cells were assessed in OND and diabetic conditions. Relative to levels observed for healthy control islets, the expression levels of GPR120 mRNA and protein were increased in OND rat islets (Figure 7A), but decreased in diabetic rat islets (Figure 7B). In line with the *in vivo* expression profile, GPR120 expression levels in INS-1E cells were elevated following treatment with 300 μM PA (24 h), mimicking the mild hyperlipidaemia of OND (Figure 7C), but reduced in response to chronic 33.3 mM glucose exposure (48–72 h), mimicking diabetic hyperglycaemia (Figure 7D). Meanwhile, the PPARγ mRNA expression pattern was similar to that of GPR120 in OND rat islets, diabetic rat islets, PA-treated INS-1E cells and

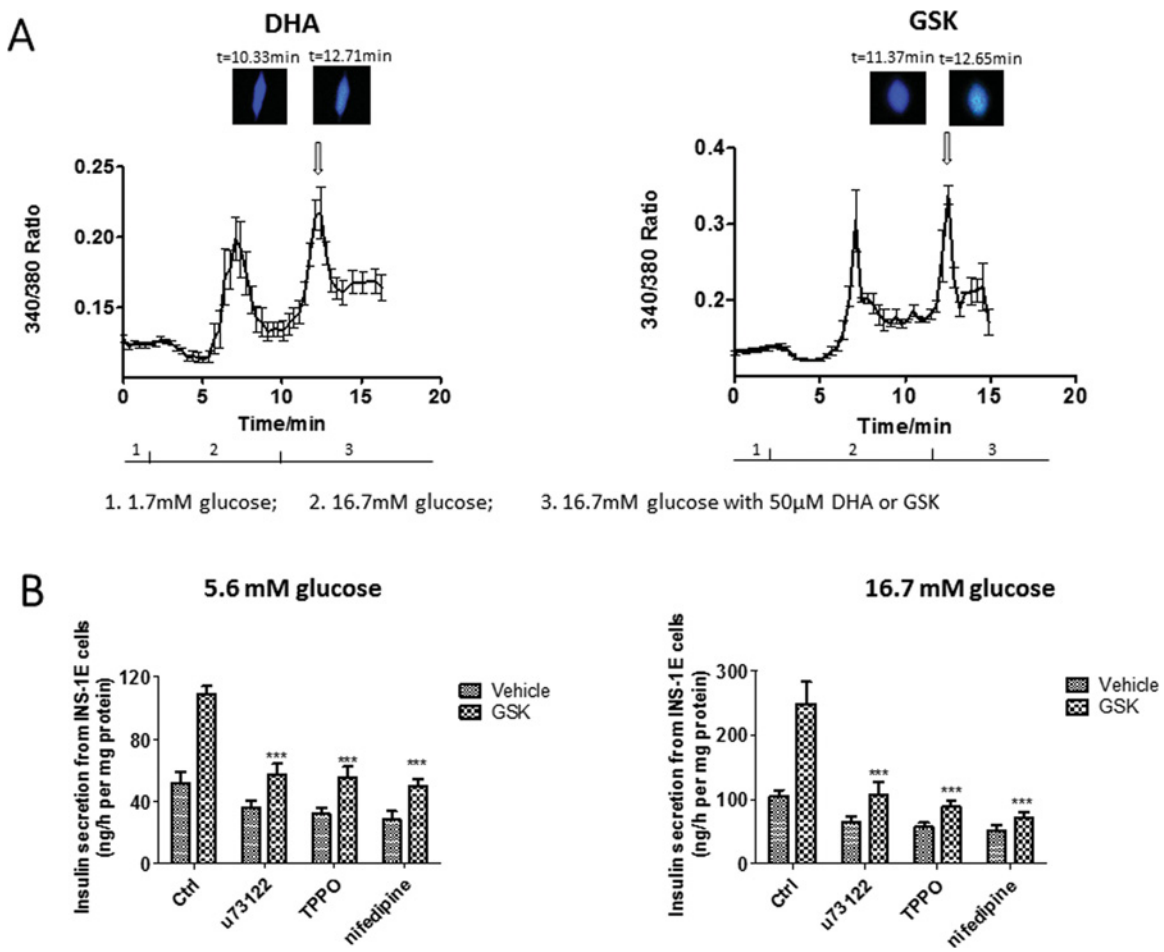

Figure 4 GPR120 agonists elevate $[Ca^{2+}]_i$ in INS-1E cells, and interruption of calcium signalling inhibits the insulinotropic effects of GPR120 activation

(**A**) DHA (left, 50 μM) or GSK (right, 50 μM) elevated $[Ca^{2+}]_i$ (arrows) in INS-1E cells (n=4–5 per group). Representative images for each indicated time point are shown. (**B**) The insulinotropic effects of 50 μM GSK were reduced in the presence of 5 μM U73122 (PLC inhibitor), 100 μM TPPO (TRPM5 inhibitor) or 20 μM nifedipine (L-type calcium channel inhibitor) at 5.6 and 16.7 mM glucose concentrations in INS-1E cells (n=4–5 per group); $***P < 0.001$ compared with GSK-treated INS-1E cells in control (Ctrl) group.

high glucose-exposed INS-1E cells (Figures 7A–7D). We then employed PPARγ agonist and antagonist to investigate whether the observed alterations in GPR120 expression were associated with PPARγ action. Treatment with the RGZ (10 μM, 48–72 h) enhanced GPR120 mRNA and protein expression in both rat islets and INS-1E cells; these effects were counteracted by co-treatment with GW9662 (10 μM) (Figures 7E and 7F).

DHA insulinotropic effects and/or GPR120 expression are enhanced in OND mouse islets but diminished in diabetic mouse islets

In OND mice and diabetic mice, we also observed alterations in DHA effects and/or islet GPR120 expression, which were similar to the changes in rat models. DHA effects in islets from HFD/STZ-induced diabetic mice were reduced relative to controls (Supplementary Figures S7A and S7B). Furthermore, GPR120 mRNA expression was increased in OND mouse islets (Supplementary Figure S7C), but decreased in diabetic mouse islets (Supplementary Figure S7D), compared with their respective controls. Meanwhile, a similar mRNA expression pattern between GPR120 and PPARγ was also observed in islets from OND mice and diabetic mice (Supplementary Figures S7C and S7D).

DHA and GSK activate Akt/ERK in rodent islets/INS-1E cells and inhibit glucotoxicity-induced apoptosis in INS-1E

In addition to amplifying insulin release, we also demonstrate that GPR120 agonists activate Akt and ERK signalling. In this regard, treatments with 50 μM DHA or GSK for 5 to 30 min triggered Akt/ERK phosphorylation in C57BL/6 mouse islets and INS-1E cells (Figures 8A and 8B). In addition, treatments with 10 μM DHA or GSK for 72 h protected against high-glucose-induced apoptosis in INS-1E cells (Figure 8C).

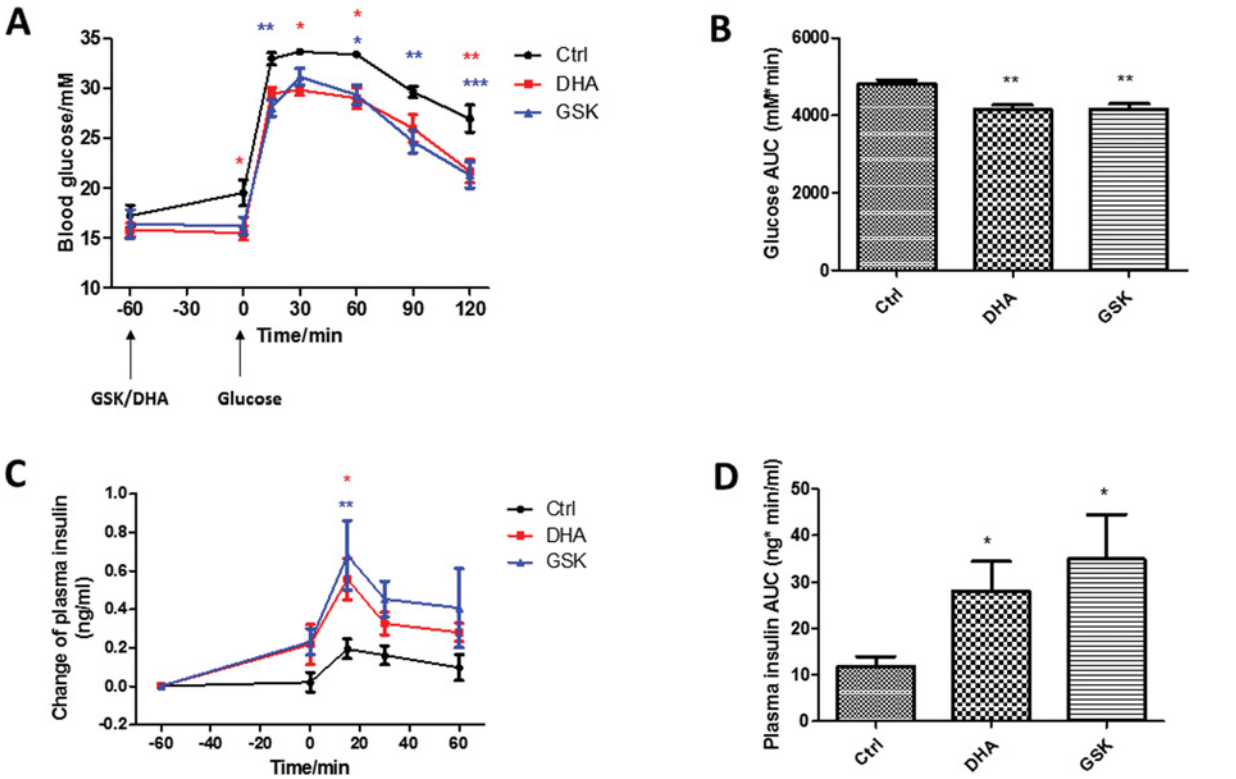

Figure 5 **GPR120 agonists improve postprandial hyperglycaemia and increase serum insulin levels in T2DM mice**
HFD/streptozotoxin-treated mice were fasted for 6 h. DHA (30 mg/kg) or GSK (30 mg/kg) were orally administrated 1 h before an oral glucose load (2 g/kg) for the performance of OGTTs. Blood glucose levels (**A**) and area under curve (AUC) of plasma glucose (**B**, −60 to 120 min). Change in plasma insulin levels from baseline (**C**) and AUC of change in plasma insulin (**D**, −60 to 60 min); n=6 per group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with matched control (Ctrl) group.

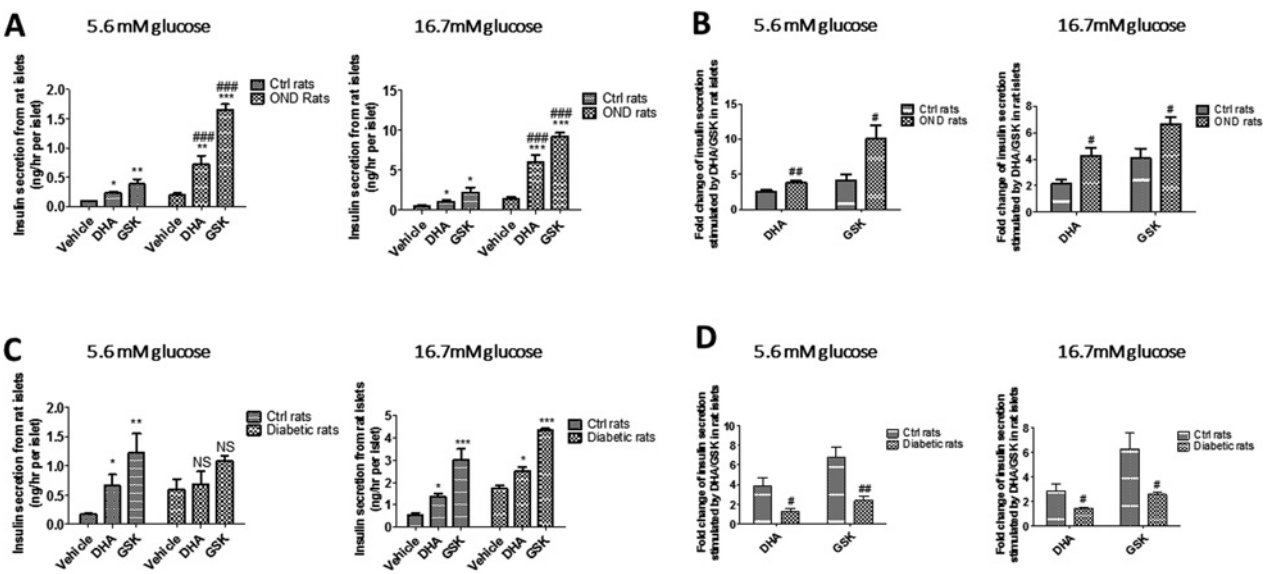

Figure 6 **Insulinotropic effects of DHA and GSK are enhanced in OND rat islets but diminished in diabetic rat islets**
Insulinotropic effects of 50 μM DHA or GSK at 5.6 and 16.7 mM glucose concentrations were altered in OND rat islets (**A** and **B**) and diabetic rat islets (**C** and **D**), compared with their respective healthy controls (n=5–6 per group). (**B** and **D**) Fold change of insulin secretion = insulin secreted with DHA or GSK treatment/insulin secreted in respective vehicle group; NS, no significant difference, $P>0.05$; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with matched vehicle group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with matched control (Ctrl) rat group.

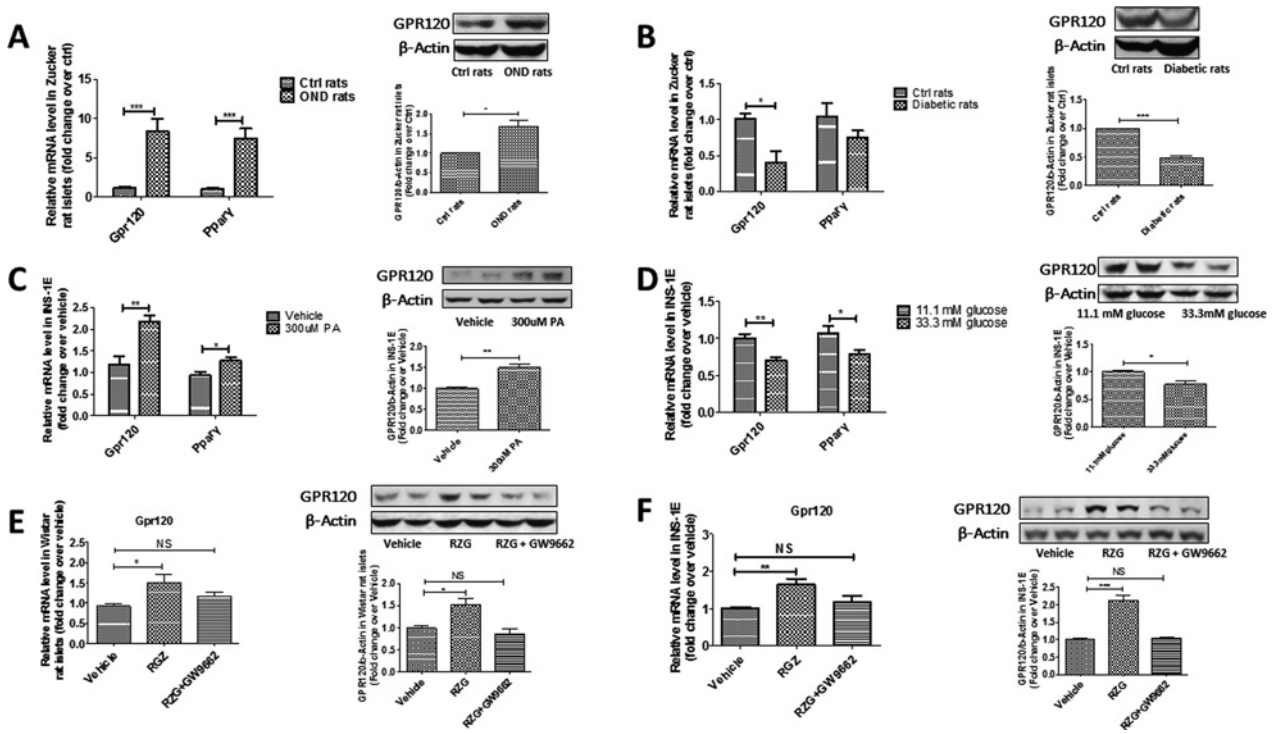

Figure 7 Expression of GPR120 and PPARγ is elevated in OND rat islets and PA-treated INS-1E cells but decreased in diabetic rat islets and high-glucose treated INS-1E cells

mRNA (left) and protein (right) expression of GPR120/PPARγ were altered in OND rat islets (**A**), diabetic rat islets (**B**), 300 μM PA-treated (24 h) INS-1E cells (**C**) and 33.3 mM glucose-treated (48–72 h) INS-1E cells (**D**), compared with the respective control/vehicle groups. Expression of GPR120 mRNA (left) and protein (right) in Wistar rat islets (**E**) and INS-1E cells (**F**) were increased following the treatment with PPARγ agonist (10 μM RZG, 48–72 h); this effect was counteracted by co-treatment with a PPARγ antagonist (10 μM GW9662); n=3–6 per group. NS, no significant difference, $P > 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Stimulatory effects of DHA/GSK on Akt/ERK phosphorylation are enhanced in OND rat islets but diminished in diabetic rat islets

We estimated the effects of GPR120 agonists on Akt/ERK activation in OND rat islets and diabetic rat islets. The effects of both DHA and GSK on Akt phosphorylation were remarkably enhanced in OND islets (Figure 9A), compared with those effects in healthy control islets. Meanwhile, the effects on ERK phosphorylation were also increased in OND islets (Figure 9B). On the other hand, both DHA and GSK failed to stimulate Akt and ERK phosphorylation in diabetic rat islets (Figures 9C and 9D).

DISCUSSION

The present study demonstrates, for the first time, that activation of β-cell GPR120 modulates insulin secretion, and implicates the PLC/Ca^{2+} signalling pathway in this modulation. Our findings extend prior work indicating that DHA, which is detectable in plasma [46,47], can augment insulin release from β-cells [6,10] as well as recent reports characterizing GPR120 as a DHA receptor [8,26]. Meanwhile, our results reveal that GPR120 agonists are able to improve the impaired glucose tolerance and augment insulin secretion in T2DM mice. Furthermore, our results indicate that islet/β-cell GPR120 expression and the insulinotropic effects of GPR120 agonism on islets are altered in OND and diabetic states in distinct ways. These findings help to elucidate the underlying mechanisms for the differential insulin secretory responses to plasma NEFAs between obese and diabetic animals/patients. In addition to the insulinotropic effects, we also find that GPR120 agonists can exhibit stimulatory effects on Akt/ERK phosphorylation in rodent islets/INS-1E cells and inhibit glucotoxicity-induced apoptosis in INS-1E.

Previous findings reported that insulinotropic actions of NEFAs (e.g. PA and linoleic acid) in islets are partially dependent on β-cell GPR40 [9,11]. Accordingly, our results indicate that the observed insulinotropic effects of DHA are mediated partially by GPR40 as well (Supplementary Figure S1). Notably, it remains controversial whether GPR120 is expressed in islet β-cells and whether GPR120 plays regulatory role in islet insulin secretion [6,12]. In addition to GPR40, it was speculated that GPR120 might be another G-protein-coupled receptor (GPCR) that facilitates the insulinotropic actions of NEFAs in islets [4]. Moreover, it was reported that non-selective GPR120 agonists can augment insulin secretion from mouse islets and rat β-cell line [6]. In the present study, we demonstrated convincingly the rodent islet β-cell expression of GPR120 for the first time, as evidenced by

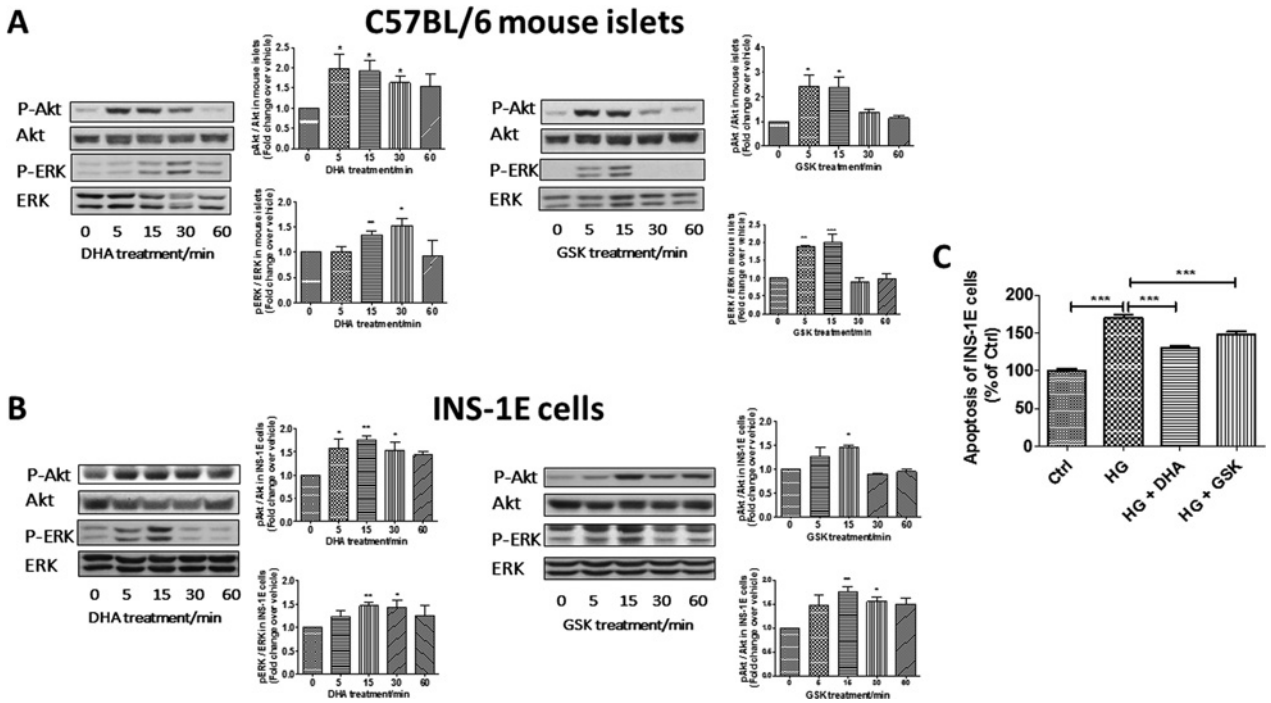

Figure 8 GPR120 activation induces Akt/ERK phosphorylation in mouse islets and INS-1E cells, and protects against glucotoxicity-induced apoptosis in INS-1E cells

Incubation with DHA and GSK (50 μM, 5 to 30 min) induced phosphorylation of Akt and ERK in C57BL/6 mouse islets (**A**) and INS-1E cells (**B**). The densitometry data shown were calculated as pAkt/total Akt or pERK/total ERK (n=3 per group); $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with matched vehicle group. (**C**) Co-treatment of DHA or GSK (10 μM) inhibited high-glucose induced (33.3 mM, 72 h) apoptosis in INS-1E cells (n=4 per group); $***P < 0.001$.

FACS (Figure 1). More importantly, we conducted targeted genetic modulation of β-cell GPR120 with siRNA transfection and plasmid transfection, providing the unambiguous demonstration of a regulatory role of resident GPR120 in β-cell insulin secretion (Figure 2). Using selective agonist and antagonist, we further verified that GPR120 activation can amplify insulin secretion of β-cells and rodent islets (Figure 3).

There were two previous studies reporting the insulinotropic effects of NEFAs in WT mouse islets, but their findings in GPR120 KO mouse islets were obviously opposite [12,13]. In the study of Stone et al. [12], the effects were decreased in KO mouse islets, whereas Suckow et al. [13] did not detect a significant reduction in islets from KO mice they used. These discrepancies may be partly due to the differences between KO mouse strains and/or NEFAs: Stone et al. [12] used the KO mice with no *Gpr120* transcripts and the generally accepted GPR120 agonist DHA. On the other hand, prior studies have suggested that the stimulatory effects of NEFAs on incretin secretion may be facilitated by GPR120-mediated $[Ca^{2+}]_i$ elevation [8,14,15,38]. Consistently, our results showed that GPR120 activation increased $[Ca^{2+}]_i$ within INS-1E cells (Figure 4). It is well established that elevation of $[Ca^{2+}]_i$ in β-cells induces insulin release [16–18]. Our *in vitro* pharmacological experiments implicated the involvement of several Ca^{2+} signalling-related molecules/events in GPR120-mediated insulinotropic effects, including PLC, extracellular calcium influx via L-type calcium channels and TRPM5 cation channels (Figure 4). TRPM5 channels can be activated by surges in $[Ca^{2+}]_i$ and subsequently trigger the opening of calcium ion channels [14,48]. In light of this finding, we speculate that PLC-mediated calcium release from endoplasmic reticulum may stimulate TRPM5, leading to calcium influx, and finally insulin release is triggered by $[Ca^{2+}]_i$ rise from the intracellular/extracellular sources.

In our study, we performed experiments to investigate the regulatory role of GPR120 in insulin secretion in obesity and T2DM models. Our findings showed that a single oral administration of GPR120 agonist can improve the postprandial hyperglycaemia; meanwhile, the agonists augmented insulin secretion of T2DM mice, as evidenced by the early phase of the OGTTs (Figure 5). Therefore, the effects of agonists on blood glucose are probably facilitated by their stimulatory effects on insulin secretion. On the other hand, it is noteworthy that the insulinotropic effects of both the non-selective agonist DHA and the GPR120-selective agonist GSK were enhanced, but reduced in islets from OND and T2DM animals respectively (Figures 6 and Supplementary Figure S7). Consistently, we also observed elevated and reduced expression of GPR120 in islets from OND and T2DM rodents respectively (Figures 7 and Supplementary Figure S7). Moreover, our results showed that GPR120 expression in INS-1E cells was increased following mild PA treatment but decreased following chronic high glucose exposure (Figure 7). Given that the resident GPR120 expression level can affect the regulatory actions

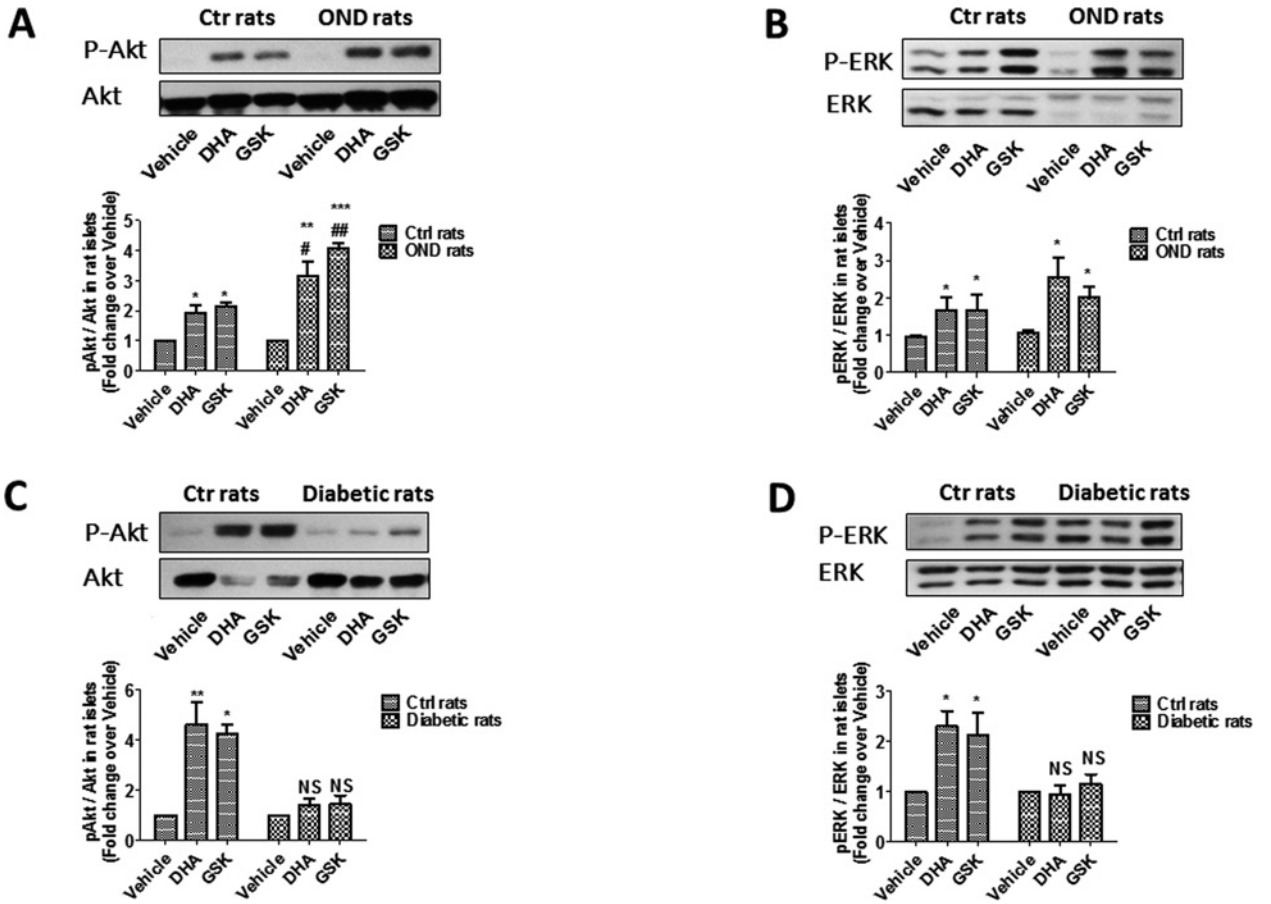

Figure 9 Stimulatory effects of DHA and GSK on Akt/ERK phosphorylation are enhanced in OND rat islets, but diminished in diabetic rat islets

Effects of 50 μM DHA or GSK on Akt/ERK activation were altered in OND rat islets (**A** and **B**) and diabetic rat islets (**C** and **D**), compared with their respective healthy controls (n=3–4 per group); NS, no significant difference, $P > 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with matched vehicle group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with matched control (Ctrl) rat group.

of NEFAs [15,26], it promotes us to propose that changes in the expression of β-cell GPR120 may contribute to the altered insulinotropic effects of DHA in OND and diabetic states. Furthermore, our observations implicate that the alterations in β-cell GPR120 expression may be relevant to the differential insulin secretory responses to plasma NEFAs between OND and diabetic patients/animals [20,22,23].

We speculate that mild hyperlipidaemia-induced β-cell GPR120 expression in the OND state is a compensatory response to obesity-related insulin resistance. The induced up-regulation of GPR120 probably compensates for insulin resistance via enhancing insulinotropic effects of NEFAs. Indeed, GPR120 expression was elevated in adipose and skeletal muscles from OND rodents and humans [1,24,25]. Aside from direct effects on β-cell secretion of insulin, broader GPR120-mediated anti-inflammatory effects in adipose tissues can help counteract obesity-associated insulin resistance [1,2]. We also speculate that chronic hyperglycaemia in diabetes may reduce β-cell GPR120 expression, thereby diminishing the insulinotropic effects of plasma NEFAs, and further exacerbating hyperglycaemia and progression to T2DM [3,20]. On the other hand, PPARγ is a transcription factor expressed in islets and other metabolic tissues [49]. In β-cells, PPARγ regulates the expression of several genes related to cell function and compensatory responses [50,51]. In our study, we obtained evidence in islets from animal models as well as in INS-1E cells subjected to PA or high glucose, suggesting that PPARγ has a role in up-regulating and down-regulating GPR120 expression in OND and diabetic states respectively (Figure 7 and Supplementary Figure S7). Furthermore, we demonstrated that PPARγ agonism, which can enhance expression of PPARγ and its target genes [24,52], induced GPR120 expression in rat islets and INS-1E cells (Figure 7), in line with previous findings that GPR120 expression was influenced by PPARγ modulation during differentiation of 3T3-1L adipocytes [24]. Taken together, our findings imply that mild hyperlipidaemia in an OND state and chronic hyperglycaemia in a diabetic state can affect β-cell PPARγ expression in distinct ways; these changes may be responsible, at least in part, for the concomitant changes in GPR120 expression and actions. We postulate that PPARγ is able to induce GPR120 expression by binding to the PPAR

response element, which has been identified in the proximal promoter of another GPCR, namely G-protein-coupled receptor 81 (GPR81) [53].

Although the insulinotropic effects of NEFAs on islets may be differential between rats and mice as proposed previously [12], our results showed that the insulinotropic effects of GSK were similar between rat and mouse islets, as well as INS-1E and MIN6 cells (Figure 3 and Supplementary Figure S8). We did not see the evidence of species-specific differences in β-cell GPR120-mediated insulinotropic effects, even in OND and diabetic states. Indeed, rat islet architecture and composition are highly similar to those of mouse islets [54], and the sequence identities of rat and mouse GPR120 are highly similar [21,55].

Apart from its insulinotropic effects, GPR120 is proposed to have a regulatory role in β-cell survival. GPR120 activation can induce Akt/ERK phosphorylation in enteroendocrine cells, adipocytes, macrophages and hypothalamic neurons [2,26,56]. Notably, activation of Akt/ERK signalling in β-cells is positively related to cell survival and function [28,29]. In this regard, we observed that GPR120 agonism triggered Akt/ERK phosphorylation in rodent islets/INS-1E cells and protected against glucotoxicity-induced apoptosis in INS-1E (Figure 8). Furthermore, our results showed that the stimulatory effects of GPR120 agonists on Akt/ERK activation were enhanced in OND islets but reduced in diabetic islets, consistent with the alterations in insulinotropic effects in OND and diabetic states (Figure 9). These findings suggest that GPR120 have the regulatory roles in islet β-cell survival as well, especially in the pathological conditions.

In summary, the present study is the first to demonstrate that resident GPR120 plays regulatory roles in β-cell insulin secretion and Akt/ERK signalling pathways. Our mechanistic experiments suggest that β-cell GPR120 mediates insulinotropic effects through a PLC/Ca^{2+} signalling pathway. Meanwhile, we did not obtain any evidence supporting a species disassociation in the insulinotropic effects of GPR120 activation. Our results indicate that the *in vivo* administration of GPR120 agonists is able to augment insulin secretion and improve impaired glucose tolerance of T2DM mouse model. Furthermore, we observed opposing alterations in islet/β-cell GPR120 expression and islet GPR120-mediated insulinotropic effects in OND compared with T2DM animals. Additionally, our findings reveal that GPR120 agonists activate Akt/ERK signalling pathways in rodent islets/INS-1E cells, and the stimulatory effects of Akt/ERK activation are altered in OND and diabetic states as well. Finally, our findings suggest that PPARγ facilitates the pathophysiological changes in GPR120 expression and actions; these alterations may contribute to the distinct insulinotropic effects of plasma NEFAs in obesity and T2DM [20]. If confirmed, pharmacological targeting of GPR120 and PPARγ represents a promising therapeutic approach to T2DM prevention and treatment [52,57].

CLINICAL PERSPECTIVES

- Given its anti-inflammatory effects in metabolic tissues, GPR120 is currently considered a pharmacological target for the management of obesity/T2DM. Whether resident GPR120 has a role in β-cell insulin secretion remains controversial.
- The present results demonstrate that GPR120 activation amplifies insulin secretion and induces Akt/ERK phosphorylation in rodent islets, INS-1E cells and/or diabetic mice, and that islet/β-cell GPR120 expression and its agonism-mediated effects on insulin secretion and Akt/ERK activation are differentially altered in OND and diabetic states, probably via PPARγ.
- Our study findings (i) provide valuable insight into the GPR120 agonists being potential insulinotropic drugs for obesity-associated T2DM therapy; (ii) elucidate the underlying mechanisms for the differential insulin secretory responses to plasma NEFAs between OND and diabetic patients; (iii) imply that manipulation of islet β-cell GPR120 may be an alternative mechanism for PPARγ agonists to prevent and treat obesity/T2DM.

AUTHOR CONTRIBUTION

Dan Zhang designed and performed experiments, analysed and interpreted data and drafted the manuscript. Wing Yan So analysed and interpreted data. Yi Wang analysed and interpreted data. Shang Ying Wu performed experiments. Qianni Cheng analysed and interpreted data. Po Sing Leung designed the experiments, analysed and interpreted data and edited and revised the manuscript. All authors approved the manuscript.

Parts of the present study were presented in abstract form at the 74th Scientific Sessions of the American Diabetes Association in San Francisco, CA, U.S.A., 2014 and at the Japan Diabetes Society's 58th Annual Meeting in Shimonoseki, Japan, 2015.

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