Oleoyl-Lysophosphatidylinositol Enhances Glucagon-Like Peptide-1 Secretion from Enteroendocrine L-Cells through GPR119

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Running Title: Oleoyl-LPI induces GLP-1 secretion in L-cells

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Abstract

The gastrointestinal tract is increasingly viewed as critical in controlling glucose metabolism, because of its role in secreting multiple glucoregulatory hormones, such as glucagon like peptide-1 (GLP-1). Here we investigate the molecular pathways behind the GLP-1- and insulin-secreting capabilities of a novel GPR119 agonist, Oleoyl-Lysophosphatidylinositol (Oleoyl-LPI). Oleoyl-LPI is the only LPI species able to potently stimulate the release of GLP-1 *in vitro,* from murine and human L-cells, and *ex-vivo* from murine colonic primary cell preparations. Here we show that Oleoyl-LPI mediates GLP-1 secretion through GPR119 as this activity is ablated in cells lacking GPR119 and in colonic primary cell preparation from GPR119$^{-/-}$ mice. Similarly, Oleoyl-LPI-mediated insulin secretion is impaired in islets isolated from GPR119$^{-/-}$ mice. On the other hand, GLP-1 secretion is not impaired in cells lacking GPR55 *in vitro* or in colonic primary cell preparation from GPR55$^{-/-}$ mice. We therefore conclude that GPR119 is the Oleoyl-LPI receptor, upstream of ERK1/2 and cAMP/PKA/CREB pathways, where primarily ERK1/2 is required for GLP-1 secretion, while CREB activation appears dispensable.

Keywords:

Lysophosphatidylinositol (LPI); Glucagon-like peptide-1 (GLP-1); GPR119; GPR55; L-cells; mixed colonic preparation

Abbreviations: Glucagon-like peptide-1, GLP-1; G protein-coupled receptor, GPCR; Oleoyl-lysophosphatidylinositol, Oleoyl-LPI; type 2 diabetes, T2D

1. Introduction

The hormone glucagon-like peptide-1 (GLP-1) is released from the intestine under postprandial conditions and it stimulates insulin secretion from the pancreatic β-cell in a glucose-dependent manner. Although GLP-1 mimics are already being used in the clinic to treat type 2 diabetic (T2D) patients, these have raised several safety issues such as risk of developing pancreatitis and medullary carcinoma of the thyroid [1]. Therefore, an alternative strategy, based on the possibility to increase endogenous GLP-1 secretion rather than administering exogenous GLP-1, is an attractive therapeutic option. It has been reported that glucose-stimulated insulin secretion (GSIS) of GLP-1 can be further modulated by amino acids, fatty acids and lipids [2-4]. Interestingly, most of the endogenous GLP-1 secretion is suggested to be mediated by G protein-coupled receptors (GPCRs) such as GPR119 [5, 6], GPR40 [7] and GPR120 [8]. Of these three, GPR119 seems to be the most important inducer of gastric inhibitory polypeptide or glucose-dependent insulinotropic peptide (GIP) and GLP-1 secretion after fat ingestion [9]. GPR119 is a member of class A GPCRs, which can bind to long-chain fatty acids including oleoylethanolamide (OEA), 2-oleoylglycerol (2-OG) and lysophosphatidylcholine (LPC) [6]. The expression of GPR119 mRNA has been reported in a number of tissues including brain, heart, spleen and stomach [10, 11]. The highest level of GPR119 expression has been reported in islets of Langerhans, pancreatic β-cell lines, and intestinal L-cells [12, 13]. Activation of GPR119 has been shown to positively modulate incretins and insulin secretion in humans [4, 14]. In rodents, this effect results in reduction of food intake and body weight gain [15, 16]. GPR119 expression in pancreatic β-cells led to the hypothesis that this receptor could play a role in modulating insulin secretion. Therefore, GPR119 agonists, which have been shown to raise intracellular cyclic AMP (cAMP) levels *in vitro* in different

pancreatic β-cell systems expressing endogenous GPR119, would be expected to potentiate GSIS in a similar manner as GLP-1 and GIP, hormones which also act via GPCRs in β-cells. The insulinotropic actions of GPR119 agonists have been demonstrated in different models of pancreatic β-cells [17]. Although OEA has been identified as the main ligand for GPR119, other phospholipids such as lysophospholipids are potential ligands for this receptor [11]. The lysophospholipid lysophosphatidylinositol (LPI), initially discovered as ligand for GPR55 [18], has multiple GPR55-independent physiological and pathological roles [19].

In this study, we describe the role of a specific species of LPI in the secretion of GLP-1 from enteroendocrine L-cells and primary cell preparations. We further demonstrate the specific role of GPR119 in LPI-dependent GLP-1 secretion. To achieve this, we downregulated GPR119 and GPR55 protein expression *in vitro* through specific siRNAs and we used colonic primary preparations from $GPR55^{-/-}$ and $GPR119^{-/-}$ transgenic mouse models in *ex vivo* experiments. Investigation of the Oleoyl-LPI-induced GLP-1 secretion mechanism revealed that GPR119 activation regulates GLP-1 secretion through a pathway dependent on the phosphorylation of extracellular signal-regulated kinase (ERK)1/2 and stimulation of a cAMP/protein kinase A (PKA)-dependent pathway.

2. Materials and methods

2.1 Reagents

Different LPI species were purchased from Avanti® Polar Lipids, USA. Stocks were dissolved in methanol:chloroform (M:C) and stored at -20^{o}C in nitrogen atmosphere.

Oleoylethanolamide (OEA), DPP-IV inhibitor (KR-62436), CID16020046 and PD98059 from Sigma-Aldrich, UK, were dissolved in DMSO. The OEA used for the cAMP experiment, from Cayman Chemical, USA, and H-89 (from Santa Cruz Biotechnology, USA) were dissolved in DMSO.

GeneJET RNA Purification Kit, Maxima Reverse Transcription Kit and Maxima SYBR Green/ROX qPCR Master Mix Kit were purchased from Thermo Fisher Scientific, USA. Primary antibodies used for immunoblotting were: anti GPR119 from Santa Cruz Biotechnology, USA, anti GPR55 from Cayman Chemical, USA, anti α-Tubulin from Sigma-Aldrich, UK, anti phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) from Cell Signaling Technology, USA, and anti phospho-CREB (Ser133) from R&D Systems, USA.

Animal use for this study was approved by the Animal Ethics Committee of Curtin University, and all procedures were carried out in accordance to NIH guidelines, No. 8023, revised 1978.

2.2 Cell culturing

NCI-H716 cells were maintained in 75 cm^2 tissue culture flasks (Corning® Costar®) in RPMI 1640 media containing 2.0 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES buffer, 1.0 mM sodium pyruvate, and 4.5 g/L glucose and supplemented with 10% FBS. When plating for an experiment, cell adhesion and endocrine differentiation were initiated by growing cells in dishes coated with growth factor reduced Matrigel^{TM} diluted at the ratio of 1:2 (Becton Dickinson, USA) in high-glucose Dulbecco´s modified Eagle medium (DMEM), 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. GLUTag cells were maintained in low glucose DMEM (Sigma-Aldrich, UK) (1g/L) containing 2 mM L-glutamine, 1 mM sodium

pyruvate supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. COS-7 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100IU/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific, USA).

2.3 siRNA Transfection

GLUTag and NCI-H716 cells transfection was performed according to the "Lipofectamine® 2000 transfection protocol" (Thermo Fisher Scientific, USA) and optimised for a 6-well plate format. Briefly, cells were plated in complete growth medium at a density of 3.0 x 10^5 / well in a 6-well plate and incubated at 37°C/5% CO_2. Twenty-four hours later, 300nM of total siRNA pool and 4.0µl of Lipofectamine transfection reagent were diluted in separate tubes in Opti-MEM™ (Thermo Fisher Scientific, USA) and incubated for 5 min at room temperature (RT). The two solutions were then mixed and incubated for further 20 min at RT to form transfection complexes. Cells were washed twice with PBS, and then incubated for 4h with the siRNA/Lipofectamine complexes before the addition of Opti-MEM™ containing 30% FBS without antibiotic. Cells were further incubated at 37°C/5% CO_2 for 48h before the secretion experiment. Cells were harvested at the appropriate time point for protein expression analysis (between 2 and 4 days post transfection). Transfection efficiency was quantified by real-time RT-PCR. Sequences of siRNAs and primers are listed in Supplementary tables 1A and 1B.

2.4 Protein analysis by Western blot

Whole-cell protein preparations were obtained from cells grown in 6-well plates at ~80-90% confluency. After two washes in cold PBS, cells were lysed, on ice, in 150 µl of

Triton X-100 lysis buffer, containing phosphatase and protease inhibitors (Sigma Aldrich, UK). Lysates were collected by scraping, and cleared from cellular debris by centrifugation at 20,000 g, 4°C for 5 min. The supernatants were then collected and total protein content quantified before storage at -20°C. Lysates were then analysed by SDS/PAGE.

2.5 Immunoblotting and visualisation

Non-specific antibody binding was prevented by incubating the nitrocellulose membranes in PBS-0.05%Tween-20 (v/v, PBS-T) supplemented with 5% milk for 30 min on a rotating plate. The membranes were then washed with PBS-T followed by ~~an~~ overnight incubation with the primary antibody diluted in PBS-T on a rotating plate at 4°C. After 24h, membranes were washed 5 times for 5 min with PBS-T, and then incubated on a rotating plate with the appropriate secondary antibody for 1 h at RT. The membranes were then washed with PBS-T, and incubated with ECL Prime solution (Amersham Bioscience, UK) or with Immobillon Western Chemiluminescent HRP substrate (Merck Millipore, UK) for 5 min, placed in a dark cassette and exposed to chemiluminescence film, or directly imaged with a ChemiDoc XRS system.

2.6 Real Time quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed according to manufacturer's instructions (Thermo Fisher Scientific, USA). Briefly, 100 ng of cDNA were mixed with 7.5 µl of 2X Maxima SYBR green/Fluorescein qPCR mix, 1µl of forward and reverse primers (10µM stock) to a final volume of 15µl. GADPH cDNAs were also amplified as an internal control. All experiments were performed in triplicate. Data were collected at the extension step.

The relative changes in gene expression quantification were calculated using the relative ddCT analysis mode of the ABI 7500 Real-Time PCR system software.

The primer pairs used for the amplification of GPR119 and GPR55 were based on published sequences [11, 20] and [21] respectively (Supplementary table 1B).

2.7 Primary cell studies

Mice were euthanized and 5 cm of colon were used for isolation of epithelial cells following a previously described protocol [10]. Briefly, colon segments were dissected from 10-week-old C57BL/6 male mice. Segments were cut open longitudinally and washed quickly in DMEM (serum-free) to remove luminal content. Colon was chopped into 1-2 mm pieces and digested with 0.5 mg/ml collagenase-P for 15 min. Resulting cell suspensions were filtered through 70 µm nylon cell strainers (BD Falcon, UK), centrifuged at 300g for 5 min, and pellets were re-suspended in DMEM supplemented with 10% FBS. The purified cells were collected and cultured on 24-well Matrigel-coated plates at 37°C in DMEM (25 mM glucose), 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. On the day of the GLP-1 secretion assay, cells were washed twice with PBS, and then treated with LPI or vehicle M:C for 2 h at 37°C. Supernatants were collected for GLP-1 quantification using the active GLP-1 ELISA kit from Millipore.

After euthanasia, pancreatic islets were isolated as described in detail elsewhere [22]. Following purification, islets were rested overnight at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin.

2.8 Secretion studies

Approximately 80,000 GLUTag cells were plated on poly-L-lysine–coated 24-well culture plates and grown to 80% confluence. NCI-H716 cells were seeded on growth factor reduced Matrigel^{TM} (Becton Dickinson, USA) in DMEM medium, supplemented with 10% FBS the day before the experiment. For NCI-H716 cells, ~25,000 cells were needed for the secretion experiment in order to detect a clear stimulation over low basal GLP-1 secretion. In brief, GLUTag and NCI-H716 cells were washed twice and then incubated for 2h with serum free DMEM containing 1% vehicle (M:C) alone (negative control), 10 µM forskolin as positive control (Sigma-Aldrich, UK), or with different concentrations of Oleoyl- LPI.

For secretion experiments, media and cells were collected separately. In separate studies, GLUTag or NCI-H716 cells were pre-treated for 1h with vehicle (DMSO) alone (negative control), PD98059 (50 µM; Sigma-Aldrich, UK.), H89 (10 µM; Santa Cruz Biotechnology, USA) followed by treatment with Oleoyl-LPI (20µM), OEA (10µM), PMA (10µM) or forskolin (10 µM) in the presence of DPP-IV inhibitors, for 2 h.

Values of GLP-1 detected in the supernatants were normalized to the number of cells/well. Data were then expressed as percentage of basal GLP-1 secretion (GLP-1 detected in supernatants from cells treated with vehicle alone).

Glucose-stimulated insulin secretion (GSIS) was performed on hand-picked islets placed in groups of five into individual micro centrifuge tubes. Islets were washed twice, then incubated with Krebs buffer (KRB, 115 mM NaCl; 4.7 mM KCl; 1.28 mM $CaCl_2 \cdot 2H_2O$; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4 \cdot 7H_2O$; 10 mM $NaHCO_3$; 10 mM HEPES; 0.5% BSA; pH 7.4) supplemented with 1.1 mM glucose for 40 min at 37 °C. Supernatants were discarded and islets were incubated with KRB supplemented with

16.7 mM glucose in the presence or absence of Oleoyl-LPI for 20 min. Supernatants were collected for insulin determination using Mouse Ultrasensitive Insulin ELISA (Mercodia, Sweden) according to manufacturer instructions and data was reported as picograms of Insulin per islet in 20 min (pg/islet/20min).

2.9 cAMP assay

COS-7 cells were transfected with 40 µg GPR119 DNA or pcDNA3 per 175m^2 flask using the calcium-phosphate precipitation method [23]. One day after transfection, 35,000 cells/well were seeded in a 96 well white plate (Perkin Elmer, USA). The cells were washed twice the following day and incubated 30 min at 37°C with HBSS plus 1mM IBMX (Sigma-Aldrich, USA). Agonists were added for 30 min in the assay medium. The Hithunter® cAMP XS+ (DiscoveRx, USA) was carried out following the manufactured description [24].

2.10 Ex-vivo studies

The $GPR55^{-/-}$ mice were kindly provided by Prof. David Baker (Queen Mary University of London) and $GPR119^{-/-}$ were obtained from Taconic. Littermates were used as wild-type controls.

3. Results

3.1 Oleoyl-LPI enhances secretion of GLP-1 from enteroendocrine L-cells

In order to determine which LPI species is able to modulate GLP-1 secretion from enteroendocrine L-cells, murine L-cells GLUTag were stimulated with 20 µM of different species of LPI (Arachidonyl-LPI, Stearoyl-LPI, Heptadecenoyl-LPI and Oleoyl-LPI). OEA was used as a control based on previous studies reporting ~~due to~~ its

role in the secretion of GLP-1 [11, 20]. Forskolin and phorbol 12-myristate 13-acetate (PMA) have been reported to potently enhance GLP-1 release in GLUTag [25] and in human L-cells NCI-H716 [26], hence they were also chosen as positive controls in these experiments. Data indicated that Oleoyl-LPI significantly stimulated GLP-1 secretion in GLUTag (Fig. 1 A). Importantly, no increase of GLP-1 secretion was detected upon stimulation of GLUTag cells with any of the other LPI species used at the same concentration (20μM). Dose response experiments further confirmed increase of GLP-1 secretion upon stimulation with Oleoyl-LPI from both GLUTag (Fig. 1 B) and NCI-H716 (Fig. 1 C) cells.

Interestingly, we observed that Oleoyl-LPI was able to stimulate GLP-1 release much more than OEA in these cells. Dose-response analysis of GLP-1 secretion induced by Oleoyl-LPI and OEA showed similar EC50 values but different maximal efficacies in both GLUTag and NCI-H716 cells (Fig. 1 D and 1 E), suggesting that OEA is only a partial agonist of GPR119 compared to Oleoyl-LPI.

Taken together, these data indicate that Oleoyl-LPI is the specific species of LPI which is responsible for the increased GLP-1 secretion from these L-cells.

3.2 Oleoyl-LPI-induced secretion of GLP-1 is not regulated by GPR55

LPI has been identified as an endogenous ligand for GPR55 [18] with a role in gut motility and satiety [27]. Although GPR55 is abundantly expressed along the gastrointestinal tract, its potential involvement in GLP-1 regulation has never been investigated. Our observation that Oleoyl-LPI potently induced secretion of GLP-1 raised the question of whether GPR55 was involved in this process. To investigate this, GLUTag and NCI-H716 cells were transfected with a specific siRNA targeting GPR55 or a non-targeting siRNA (siControl). Downregulation of GPR55 was assessed at the

protein level by Western blot analysis in both GLUTag and NCI-H716 cells (Fig. 2 A and 2 B), as confirmed by densitometry analysis (Fig. 2 C and 2 D) and further validated at mRNA levels in GLUTag cells by RT-qPCR (Fig. 2 E). Transfected GLUTag (Fig. 2 F) and NCI-H716 (Fig. 2 G) cells were then treated with Oleoyl-LPI to assess GLP-1 secretion. There were no statistically significant differences in Oleoyl-LPI-induced GLP-1 secretion between cells transfected with siControl or siGPR55, indicating that GPR55 was not involved in mediating GLP-1 secretion upon Oleoyl-LPI stimulation. Similarly, treatment of cells with the GPR55 antagonist CID16020046 did not affect the Oleoyl-induced GLP-1 secretion (Fig. S1).

To further validate these results, we investigated the effects of Oleoyl-LPI on a mixed colonic primary culture from wild type and GPR55$^{-/-}$ mice, isolated as described previously [28-30]. Forskolin was used as positive control in these experiments. Firstly, we observed that two hours treatment with Oleoyl-LPI stimulated GLP-1 secretion in this *ex vivo* system, confirming our *in vitro* results.

Importantly, no difference in the Oleoyl-LPI-stimulated GLP-1 secretion was detected between primary cells isolated from wild type or GPR55$^{-/-}$ mice (Fig. 2 H). Taken together, these data indicate that the GLP-1 secretion induced by Oleoyl-LPI is not regulated by GPR55.

3.3 GPR119 mediates Oleoyl-LPI-induced GLP-1 secretion from L-cells and Insulin secretion from pancreatic β-cells

We next determined whether GPR119 was required for the Oleoyl-LPI-stimulated GLP-1 release. To this end, GLUTag were transiently transfected with a specific siRNA targeting GPR119 before stimulation with Oleoyl-LPI. Downregulation of GPR119 completely inhibited the Oleoyl-LPI-induced GLP-1 secretion (Fig. 3 A). Western blot,

and the correspondent densitometric analysis, indicated efficient downregulation of GPR119 at protein level in GLUTag cells, which was further confirmed by transcript quantification (Fig. 3 B). Similarly, our data indicate that Oleoyl-LPI-mediated GLP-1 secretion was dependent on GPR119 in NCI-H716 cells (Fig. S2 A). Efficient downregulation of GPR119 in these cells was confirmed by Western blot (Fig. S2 B) and RT-qPCR (Fig. S2 B) analysis. We next investigated the effects of Oleoyl-LPI on primary cultures of mixed colonic preparation from wild type and GPR119$^{-/-}$ mice. Oleoyl-LPI was unable to induce GLP-1 release in cultures derived from GPR119$^{-/-}$ mice whereas it potently stimulated GLP-1 secretion in wild type mice (Fig. 3 C). These data demonstrate that downregulation of GPR119 in GLUTag and NCI-H716 cells, and genetic ablation of GPR119 in mice, impair the Oleoyl-LPI-induced GLP-1 secretion.

LPI has been shown previously to stimulate insulin secretion from pancreatic β-cells [31] directly through a GPR55-independent mechanism [32]. We therefore evaluated the ability of pancreatic islets isolated from wild type and GPR119$^{-/-}$ mice to secrete insulin in response to Oleoyl-LPI in the presence of glucose. We observed that Oleoyl-LPI acutely enhanced insulin secretion by approximately 50% in the presence of 16.7 mM of glucose. Importantly, no secretion of insulin was detected in GPR119$^{-/-}$ islets upon stimulation with Oleoyl-LPI (Fig. 3 D), indicating that expression of GPR119 in pancreatic islets is necessary for Oleoyl-LPI stimulation of insulin secretion.

3.4 Intracellular ERK1/2 activation is involved in Oleoyl-LPI-induced GLP-1 secretion

It has been reported that phosphorylation of ERK1/2 is involved in GLP-1 secretion mediated by several ligands [8, 33, 34]. In order to determine whether Oleoyl-LPI activated ERK1/2 in the enteroendocrine L-cells we assessed its phosphorylation state

over a time course experiment. Oleoyl-LPI stimulation increased phosphorylation of ERK1/2 at residues Thr202 and Tyr204 within 10 min (Fig. 4 A). To determine whether GPR119 was involved in this Oleoyl-LPI-mediated ERK1/2 activation, GLUTag cells transfected with siGPR119 or siControl were stimulated for up to 10 min with Oleoyl-LPI, and phosphorylation of ERK1/2 was assessed by Western blot. Data indicated a clear inhibition of ERK1/2 activation in cells transfected with siGPR119, as confirmed by densitometric analysis (Fig. 4 B). Similarly, downregulation of GPR119 inhibited ERK1/2 phosphorylation upon stimulation with Oleoyl-LPI in NCI-H716 cells (Fig. S2C). These data indicate that Oleoyl-LPI stimulates ERK1/2 activation in L-cells through GPR119. To determine whether the detected activation of the mitogen activated protein kinase (MAPK) signalling pathway was involved in regulation of GLP-1 secretion, GLUTag (Fig. 4 C) and NCI-H716 (Fig. 4 D) cells were pre-treated with the mitogen-activated protein kinase 1 and 2 (MEK1/2) inhibitor PD98059 for 1h prior to Oleoyl-LPI stimulation. The inhibitor was kept in the medium throughout the secretion experiment.

Treatment of GLUTag and NCI-H716 cells with PD98059 partially but significantly reduced Oleoyl-LPI-induced ERK1/2 phosphorylation and GLP-1 secretion (Fig. 4 C and 4 D). These data indicate that the Oleoyl-LPI-dependent, GPR119-mediated secretion of GLP-1 is partially regulated through activation of ERK1/2.

3.5 Oleoyl-LPI stimulates cAMP production in COS-7 cells and activates CREB in enteroendocrine L-cells

Previous studies have demonstrated that activation of PKA is able to stimulate proglucagon gene transcription and GLP-1 synthesis in GLUTag cells and primary foetal rat intestinal cultures [35, 36]. Furthermore, ligand binding to GPR119 activates

stimulatory G proteins that signal via adenylate cyclase to increase cAMP levels and activate PKA [15]. To determine whether Oleoyl-LPI was able to increase intracellular cAMP levels through GPR119, we used COS-7 cells overexpressing GPR119. Treatment with Oleoyl-LPI increased the production of cAMP similarly to OEA with comparable EC_{50}s (Oleoyl-LPI 236 nM and OEA 217 nM, Fig. S 3). To determine whether the Oleoyl-LPI-mediated secretion of GLP-1 was dependent on increased cAMP levels, GLUTag and NCI-H716 cells were pre-treated with the PKA inhibitor, H-89 (10 μM) before stimulation. PKA inhibition suggested a hampered Oleoyl-LPI-induced GLP-1 secretion, although data were not statistically significant (Fig. 5 A and 5 B). On the other hand, simultaneous inhibition of MEK1/2 (using PD98059) and PKA (using H-89) completely abolished the Oleoyl-LPI-induced GLP-1 release in GLUTag cells (Fig. S 4). Interestingly, we observed that inhibition of PKA reduced partially the Oleoyl-LPI-dependent ERK1/2 phosphorylation (Fig. S5), suggesting a contribution of the cAMP/PKA pathway on ERK1/2 activation. Taken together these data indicate that total inhibition of Oleoyl-LPI mediated ERK1/2 signalling (as achieved by treatment with PD98059) only partially inhibits GLP-1 secretion whereas partial inhibition of ERK1/2 (as achieved by treatment with H-89) is not sufficient to appreciably affect it. On the other hand, combination of full ERK1/2 inhibition (by PD98059) and PKA inhibition (by H-89) completely blocks the Oleoyl-LPI-dependent GLP-1 secretion.

It is known that the increase of proglucagon gene transcription in L-cells is regulated by a cAMP-response element (CRE) in the 5'-flanking sequence of the gene [37]. CREB is a cellular transcription factor that binds to CRE to regulate the transcription of proglucagon.

Interestingly, we noticed that phosphorylation of CREB at its residue Ser133 was stimulated by treatment with Oleoyl-LPI in both GLUTag (Fig. 5C) and NCI-H716 (Fig. 5D) cells.

Taken together these data suggest that Oleoyl-LPI stimulates cAMP production that subsequently triggers PKA activation and phosphorylation of CREB through GPR119. Considering that the single blockade of PKA does not effectively modulate exocytosis, but on top of a MEK1/2 inhibition completely abolishes any Forskolin or Oleoyl-LPI mediated GLP-1 secretion, a complex form of synergism between the pathways can be postulated.

4. Discussion

It has been reported that both pancreas and gastrointestinal tract express high levels of GPR55, and GPR119. Recently, the lysophospholipid LPI was identified as a ligand for GPR55 and we hypothesized that GPR119 could be another potential LPI receptor. For instance, it has been reported that LPI can activate GPR119 in RH7777 rat hepatoma cells stably expressing human GPR119 [11]. Activation of GPR119 in the pancreas is correlated with enhanced glucose stimulated insulin secretion and activation of this receptor in the gut results in increased secretion of incretin hormones GLP-1 and GIP [4, 38]. These observations suggest that GPR119 activation may reduce blood glucose levels by acting on L-cells to stimulate GLP-1 release that in turn stimulate β-cells to promote glucose stimulated insulin secretion. Therefore, activation of this receptor by Oleoyl-LPI could be an interesting therapeutic target for treatment of T2D. The overall aim of this study was to explore the potential role of LPI in the secretion of

GLP-1 by enteroendocrine L-cells through the activation of GPR119, and the mechanism underlying this process.

Our study demonstrated that Oleoyl-LPI increases GLP-1 secretion *in-vitro* and in *ex-vivo* preparations of enteroendocrine L-cells. Previous reports have demonstrated that fatty acids containing an oleoyl chain such as Oleoylethanolamide (OEA) and 2-Oleoyl-glycerol (2-OG) can enhance secretion of GLP-1 by enteroendocrine cells [4, 20]. Oleoyl-LPI is an endogenous phospholipid with an inositol head and an acylated Oleoyl chain (18:1). A heterogeneous mixture of different species of LPI, with distinct acyl chains, primarily saturated, was initially described as the endogenous ligand for GPR55 [18]. This LPI-GPR55 binding, similarly to multiple cannabinoids, is reported to activate a downstream Gα13/RhoA/ROCK-dependent Calcium signalling [39]. More recently, the presence of the cation channel TRPV2 was found to be necessary for the activation of this pathway [40]. Nonetheless, the stimulation of GPR55 with the synthetic agonist O-1605, fails to mimic the reported LPI activity, indicating the involvement of other molecular players.

Indeed, the data reported by the authors, clearly indicate only a minor involvement of GPR55 in the LPI-mediated secretion of GLP-1, and fail to address the composition of the LPI mixture [40].

There is currently no available information regarding the activity of specific LPI species. Here we show that GPR55, contrarily to GPR119, is dispensable for the Oleoyl-LPI-mediated secretion of GLP-1.

Another interesting finding that we report is that Oleoyl-LPI displays a much higher GLP-1 secreting activity than OEA (Fig. 1 D and 1 E), despite similar cAMP raising capabilities (Fig. S 3). These data appeared to be in contrast with those of Lauffer et al. [20] which concluded that OEA induced GLP-1 secretion when tested at the same

concentration (10μM). Noticeably, these authors maintained the GLUTag cells culture in a higher glucose concentration of 25 mM and not at a level (5.5mM) normally used for this cell line [25, 35, 37, 41, 42] therefore the possible interference of high glucose cannot be ruled out. A recent study [43] revealed that GLUTag cells cultured in high glucose concentration had increased total production and release of active GLP-1, reactive oxygen species and upregulated proglucagon mRNA expression. Our data further demonstrated that GPR119 is required for the Oleoyl-LPI-mediated GLP-1 secretion (Fig. 3).

Consistent with previous reports of an association between increased accumulation of cAMP and GLP-1 secretion [25, 44, 45], we hypothesized that Oleoyl-LPI may regulate GPR119 activation and secretion of GLP-1 secretion through the cAMP/PKA/CREB pathway. Pharmacological inhibition of PKA by H-89 was able to reduce Oleoyl-LPI-induced GLP-1 secretion only slightly. Because the reduction of GLP-1 secretion is not statistically significant (Fig. 5), this indicates that Oleoyl-LPI-mediated activation of CREB is partially dispensable for the secretion of GLP-1 in this system.

In addition, our data show that Oleoyl-LPI induced GLP-1 secretion primarily depends on ERK 1/2 activation. Pharmacological blockade of MEK 1/2 with PD98059 significantly inhibited Oleoyl-LPI mediated GLP-1 secretion (Fig. 4 C and 4 D), but synergism with PKA appears essential for maximal GPR119 mediated GLP-1 secretion (Supplementary Fig. 4).

5. Conclusion

This study demonstrated that Oleoyl-LPI is the specific species of LPI that is involved in the regulation of GLP-1 secretion from enteroendocrine L-cells. We have identified

a signalling pathway involving GPR119 activation, that signals downstream through both the ERK1/2 and the cAMP/PKA/CREB pathway (Fig. 5).

The pharmacology of Oleoyl-LPI could be explored, not only as a specific GPR119 agonist, but also for further development of metabolically stable mimics for the enhancement of endogenous GLP-1 secretion, and subsequent improvement of glucose homeostasis in pathologies such as T2D.

Conflict of interest

The authors declare no conflict of interests.

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S.A.A. performed in vitro and ex vivo experiments, S.P. performed experiments, reviewed/edited the manuscript, organized the literature and figures and performed statistical analyses. TM reviewed/edited the manuscript and provided intellectual contribution to the study. J.H.E., P.N. and M.M.R. reviewed/edited the manuscript. R.C. contributed to discussion and reviewed/edited manuscript. I.C. performed experiments and contributed to discussion. M.F. conceived the project, led and supervised the study, performed experiments and wrote the manuscript.

Appendix A. Supplementary data

Fig. S1 Shows that pharmacological inhibition of GPR55 does not affect Oleoyl-LPI-mediated GLP-1 secretion in GLUTag and NCI-H716 cells.

Fig. S2 Displays the ablation of GLP-1 response in NCI-H716 cells transfected with siRNA targeting GPR119. The panel S2B shows a representative Western Blot, and transcript quantification analysis indicating the efficient downregulation of GPR119

expression. S2C further indicates the siGPR119-mediated ~~blockage~~ inhibition of ERK1/2 activation.

Fig. S3 Shows a cAMP dose-response curve in GPR119 expressing COS-7 cells treated with increasing amounts of Oleoyl-ethanolamide (OEA) red circles, or Oleoyl-LPI, black squares. The two drugs display similar Log EC50s.

Fig. S4 Indicates the complete blockage of GLP-1 secretion upon ~~the~~ simultaneous treatment with PKA and MEK1/2 inhibitors.

Fig. S5 are two typical western blots showing that PKA activity is required for a full Oleoyl-LPI mediated ERK1/2 activation

Table. S1 shows the sequences utilized for (S1 A) knockdown and (S1 B) Real Time qPCR experiments.

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Figure legends

Figure 1: **Oleoyl-LPI enhances GLP-1 secretion in vitro.**

(A) GLUTag cells were incubated with the indicated species of LPI (20μM) or vehicle alone for 2h. Alternatively, GLUTag (B) and NCI-H716 (C) cells were incubated with increasing concentrations of Oleoyl-LPI. In these experiments, OEA and Forskolin were used as positive controls. Secretion of GLP-1 was assessed by ELISA as described in the Materials and Methods section. Data are expressed as percentage of values from cells treated with vehicle alone.

(D, E) Dose-response curves of Oleoyl-LPI (closed circles) and OEA (open squares) in GLUTag (D) and NCI-H716 cells (E) normalized to Oleoyl-LPI 100 μM. The two compounds display different maximal efficacies but similar potencies, with EC_{50}s around 30 μM in both GLUTag and NCI-H716. In all panels data are means ± SEM of at least 3 independent experiments. Statistical significance was assessed via One-way ANOVA followed by a post hoc Dunnet analysis for graphs A, B and C, and Two-way ANOVA with post hoc Sidak test for dose-responses in D and E. Statistical significance is indicated as: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 2: **Oleoyl-LPI-induced secretion of GLP-1 is not regulated by GPR55.**

Representative Western blot showing downregulation of GPR55 in GLUTag (A) and NCI-H716 (B) at 48h post transfection with a specific siRNA targeting GPR55 or a non-targeting siRNA (siControl). Tubulin was used as loading control. Corresponding densitometry analysis are shown in (C) and (D) respectively. (E) mRNA levels of GPR55 in GLUTag cells transfected with the indicated siRNAs (48h post transfection). (F, G) GLP-1 secretion from GLUTag (F) or NCI-H716 (G) cells transfected with the indicated siRNAs and stimulated with 20 μM Oleoyl-LPI (+) or vehicle alone (-) 48h

post transfection. Data are expressed as percentage of values from cells transfected with siControl and treated with vehicle. (H) Secretion of GLP-1 by mixed primary colonic culture from wild type (C57BL/6) and C57BL/6 GPR55$^{-/-}$ mice upon stimulation with Oleoyl-LPI or vehicle alone. Forskolin was used as positive control. Results are normalized to the respective vehicle (control). Data are means ± SEM of at least n = 3 independent experiments. Statistical significance for panels C, D, E was assessed with one-sided t student tests, and for F, G and H with Two-way ANOVAs followed by Sidak test: Significance was assumed for *p<0.05, **p<0.01, ***p<0.001

Figure 3: **GPR119 mediates Oleoyl-LPI-induced GLP-1 and insulin secretion in vitro and ex-vivo.**

(A) Secretion of GLP-1 from GLUTag stimulated with 20 μM Oleoyl-LPI at 48h post transfection with the indicated siRNAs. Data are expressed as percentage of values from cells transfected with siControl and treated with vehicle and are means ± SEM of at least n=3 independent experiments performed in duplicates. Two-way ANOVA with Sidak post hoc test ** p<0.01. (B) Results from RT-qPCR (top right panel), ~~and~~ representative Western blot ~~analysis~~ and densitometry (bottom right panel) confirming ~~the~~ downregulation of GPR119 mRNA and protein levels in GLUTag cells at 48 h post transfection. In the blot tubulin was used as loading control. One-tail Student t-test: ** p<0.01, ***p<0.001. (C) 10 μM Oleoyl-LPI-induced secretion of GLP-1 in mixed primary colonic culture derived from wild type and GPR119$^{-/-}$ mice. Results are normalized to data obtained in cultures incubated with vehicle (control). Averages of 3 independent experiments are shown ± SEM.

(D) Following isolation from wild type, or GPR119$^{-/-}$ mice, pancreatic islets were rested overnight. Glucose-stimulated insulin secretion analysis was performed by

incubating islets with 16.7 mM of glucose in the presence or absence of Oleoyl-LPI (20 µM) for 20 min. Tissues from 4 different animals were used in independent experiments.

Two-way ANOVA with Sidak post hoc test was used to assess statistical significance in C and D: * $p<0.05$, ** $p<0.01$.

Figure 4: **Activation of ERK1/2 is required for the Oleoyl-LPI-induced GLP-1 secretion**

(A) Quantitative analysis of ERK1/2 phosphorylation upon treatment of GLUTag cells with Oleoyl-LPI for the indicated times. A representative blot is also shown (B). GLUTag were transfected with the indicated siRNAs before stimulation with Oleoyl-LPI for the indicated times. Results from densitometric analysis of ERK1/2 phosphorylation and a representative Western blot are shown. (C, D) GLUTag (C) and NCI-H716 (D) were pre-treated with the MEK 1/2 inhibitor PD98059 (50µM) for 1h prior to the stimulation with Oleoyl-LPI, Forskolin, PMA or vehicle alone always in presence of the inhibitor. "Control" cells were pre-treated with DMSO. GLP-1 secretion was assessed as above. Data are expressed as percentage of values from control cells treated with vehicle and are expressed as means ± SEM of n=3 independent experiments. One-way ANOVA post hoc Dunnett test for data in A, and Two-way ANOVA with post hoc Sidak test for figures B, C and D: *$p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. At the end of the secretion experiments, cells were lysed to confirm efficacy of MEK1/2 inhibitor, assessing ERK1/2 phosphorylation by Western blot analysis. Representative blots are shown. Tubulin was used as loading control.

Figure 5: **Oleoyl-LPI activates cAMP response-element binding (CREB) in enteroendocrine L-cells.**

(A,B) Oleoyl-LPI-, Forskolin- and PMA-induced GLP-1 secretion in GLUTag (A) and NCI-H716 cells (B) treated with the synthetic PKA inhibitor H-89 (10μM). "Control cells" were treated with DMSO. Data are expressed as percentage of "control cells" treated with vehicle and are means ± SEM of n=3 independent experiments performed in duplicates. Student t-test: n.s. $p>0.05$. (C,D) Representative Western blots and corresponding densitometric analysis of CREB phosphorylation at its residue Serine 133 in GLUTag (C) and NCI-H716 (D) cells. Cells were pre-treated with 10 μM H-89 before stimulation with 20 μM Oleoyl-LPI, 10μM Forskolin or 10 μM PMA in the presence or absence of H-89.

Data are from n=3 independent experiments. Two-Way ANOVA with Sidak post hoc test *$p<0.05$, **$p<0.01$.

Supplementary Figure 1: **Pharmacological inhibition of GPR55 does not inhibit the Oleoyl-LPI-mediated GLP-1 secretion**. GLUTag and NCI-H716 cells were pre-treated for 30 min with 5 μM CID16020046, followed by 2h stimulation with 10 μM Oleoyl-LPI in the presence or absence of CID16020046 (5 μM). Secreted GLP-1 levels were quantified via ELISA. Data are from n=2 independent experiments.

Supplementary Figure 2: **Oleoyl-LPI-induced GLP-1 secretion requires GPR119 in human NCI-H716.**

(A) Oleoyl-LPI treatment (20μM) and GLP-1 secretion quantification in NCI-H716 cells transfected with a specific siRNA targeting GPR119. (B) Representative Western Blot and transcript quantification analysis in transfected NCI-H716 cells. (C) siRNA-

mediated downregulation of GPR119 inhibits Oleoyl-LPI mediated phosphorylation of ERK1/2. Densitometric analysis of n=3 independent experiments are shown. Statistical significance was assessed via Two-way ANOVA with post hoc Sidak test in S1A and one-way Student-t test for S1C.

Supplementary Figure 3: **Oleoyl-LPI induces cAMP accumulation in GPR119 expressing COS-7 cells.**

cAMP dose-response curve in GPR119 expressing COS-7 cells treated with increasing concentrations of Oleoyl-ethanolamide (OEA) red circles, or Oleoyl-LPI, black squares. The two drugs display similar Log EC50s.

Supplementary Figure 4: **Effect of MEK and PKA inhibition in GLUTag cells on GLP-1 secretion**

GLUTag were pre-treated for 1h with PD98059 (50 μM) alone (A), or in combination with H-89 (10 μM) (B) before treatment with Oleoyl-LPI (10 μM) or vehicle for 2h. Results of active GLP-1 released are expressed as percentage increase compared to the vehicle (DMSO + M:C). Data are means ± SEM of n=3 independent experiments performed in duplicate.

Supplementary Figure 5: **Immunoblot analysis of ~~H-89~~ PKA-mediated ERK1/2 modulation**. A representative blot is shown for GLUTag and NCI-H716 cells pre-treated with 10 μM H-89 for 1h, and then treated with Forskolin 10 μM, or PMA 10 μM, and Oleoyl-LPI 10 μM for 10 min in presence of H-89 10 μM. PKA blockage inhibits Oleoyl-LPI, or Forskolin mediated, but not PMA induced, PKC mediated, ERK1/2 activation.

Figure 1

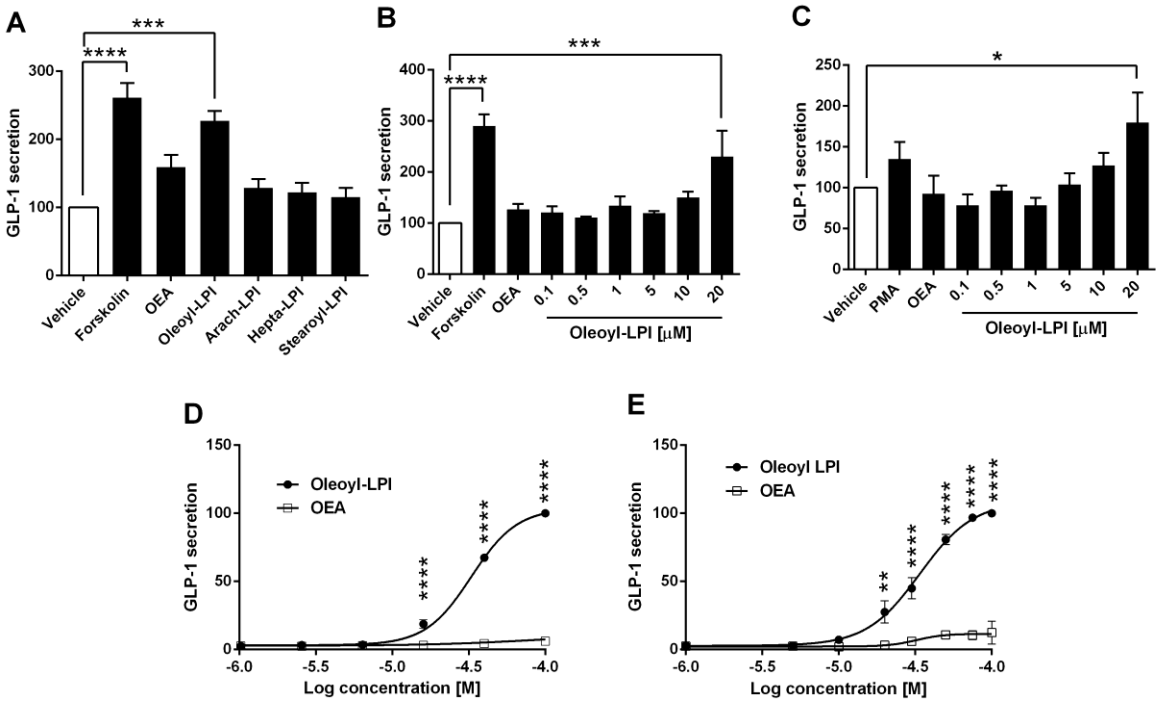

A
B
C
D
E
GLP-1 secretion
Vehicle
Forskolin
OEA
Oleoyl-LPI
Arach-LPI
Hepta-LPI
Stearoyl-LPI
PMA
Oleoyl-LPI [μM]
Oleoyl LPI
Log concentration [M]

Figure 2

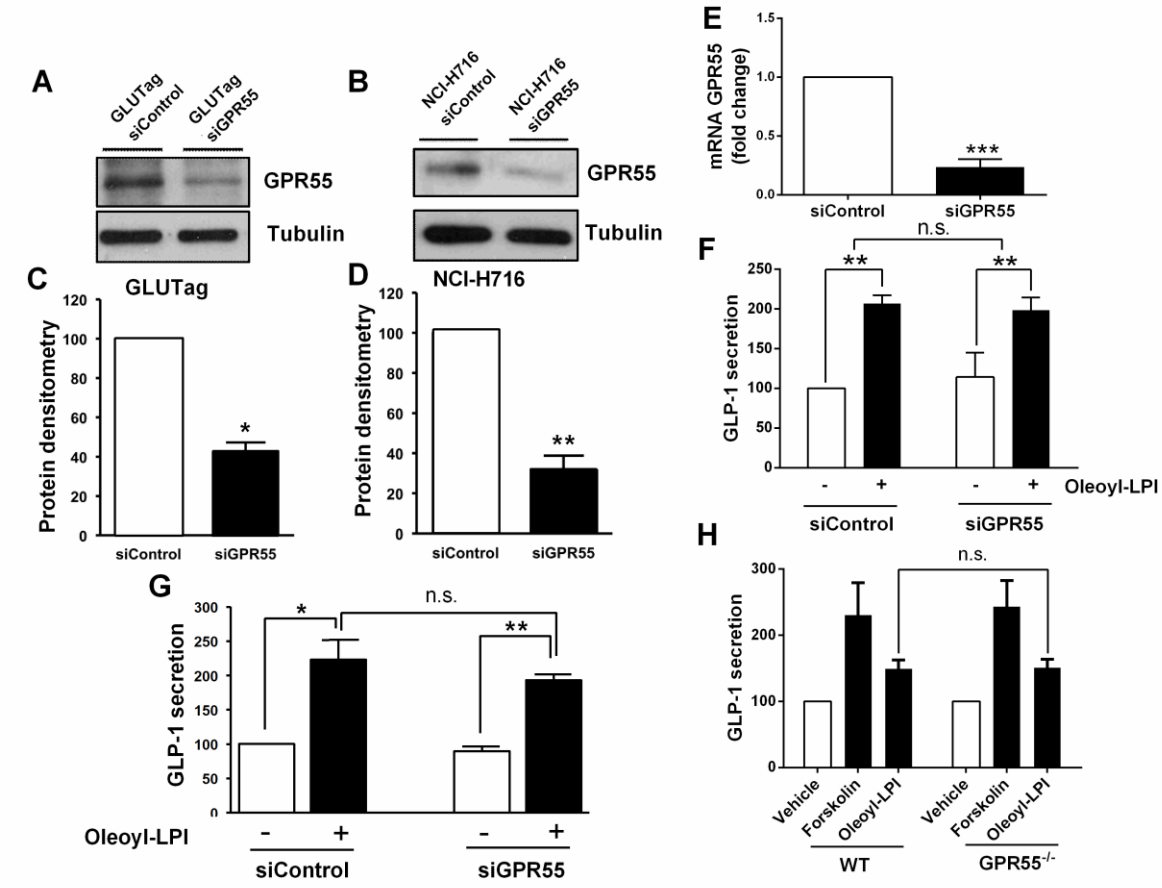

A
GLUTag siControl
GLUTag siGPR55
GPR55
Tubulin
B
NCI-H716 siControl
NCI-H716 siGPR55
GPR55
Tubulin
C
GLUTag
Protein densitometry
siControl
siGPR55
D
NCI-H716
Protein densitometry
siControl
siGPR55
E
mRNA GPR55 (fold change)
siControl
siGPR55
F
GLP-1 secretion
n.s.
Oleoyl-LPI
siControl
siGPR55
G
GLP-1 secretion
n.s.
Oleoyl-LPI
siControl
siGPR55
H
GLP-1 secretion
n.s.
Vehicle
Forskolin
Oleoyl-LPI
WT
GPR55-/-

Figure 3

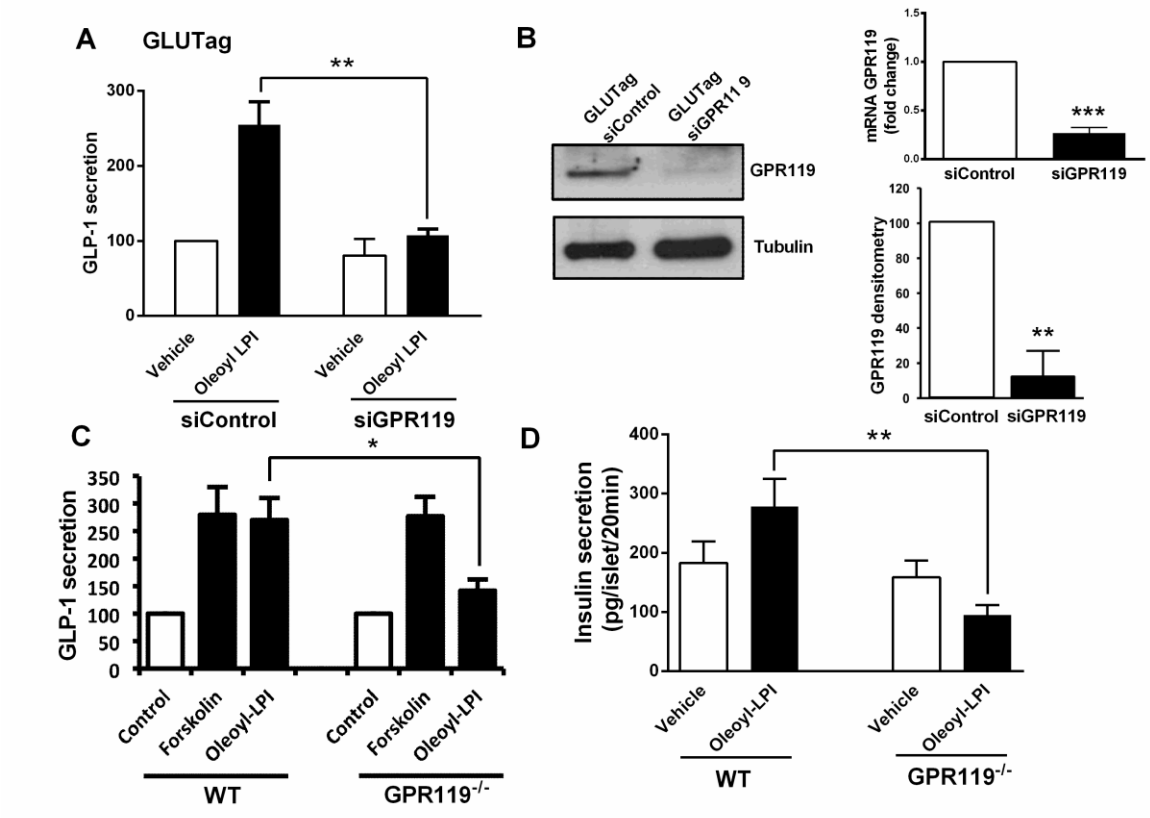

A
GLUTag
**
GLP-1 secretion
300
200
100
0
Vehicle
Oleoyl LPI
Vehicle
Oleoyl LPI
siControl
siGPR119
B
GLUTag siControl
GLUTag siGPR119
GPR119
Tubulin
mRNA GPR119 (fold change)
1.5
1.0
0.5
0.0
***
siControl
siGPR119
GPR119 densitometry
120
100
80
60
40
20
0
**
siControl
siGPR119
C
*
GLP-1 secretion
350
300
250
200
150
100
50
0
Control
Forskolin
Oleoyl-LPI
Control
Forskolin
Oleoyl-LPI
WT
GPR119-/-
D
**
Insulin secretion (pg/islet/20min)
400
300
200
100
0
Vehicle
Oleoyl-LPI
Vehicle
Oleoyl-LPI
WT
GPR119-/-

Figure 4

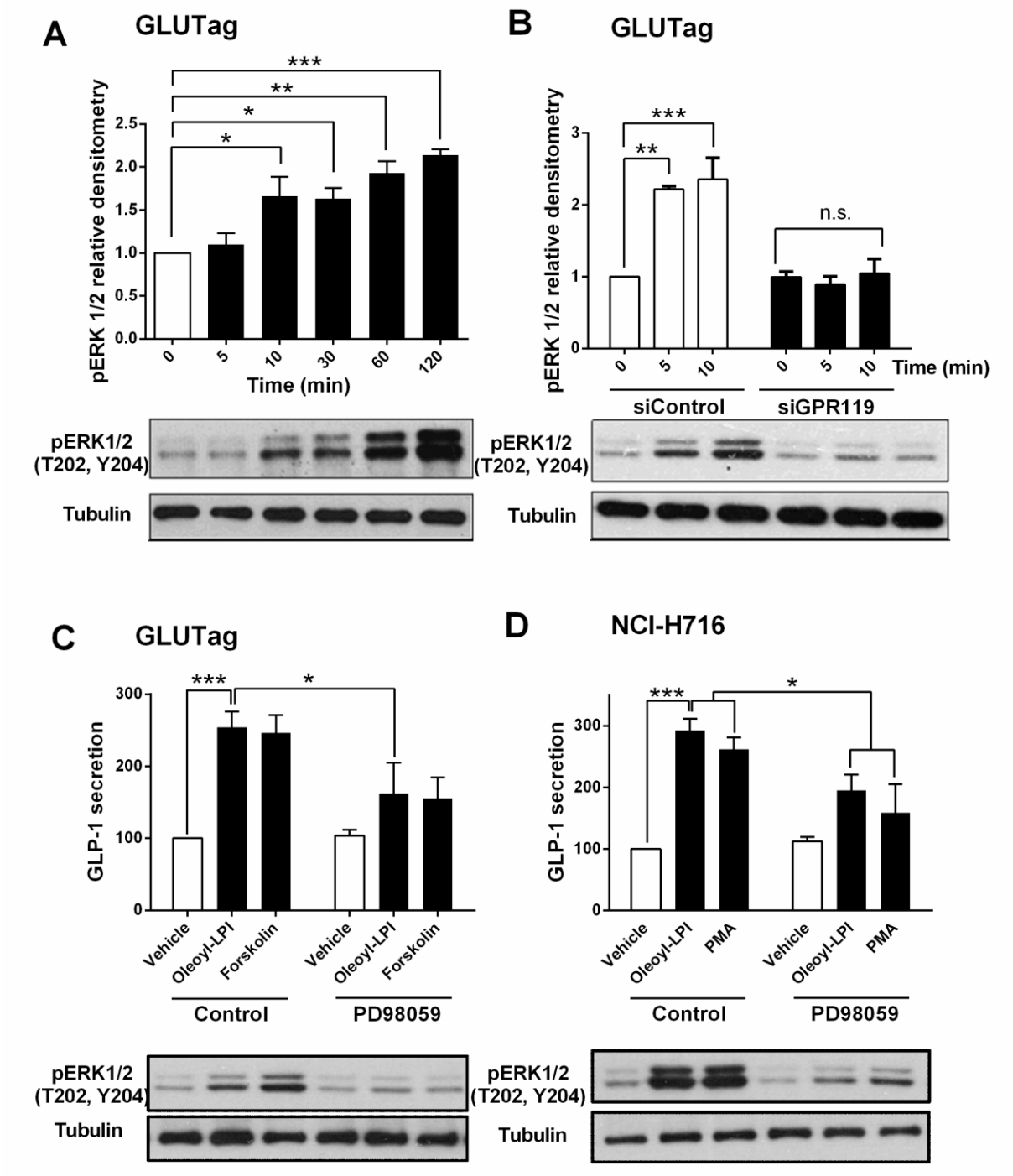
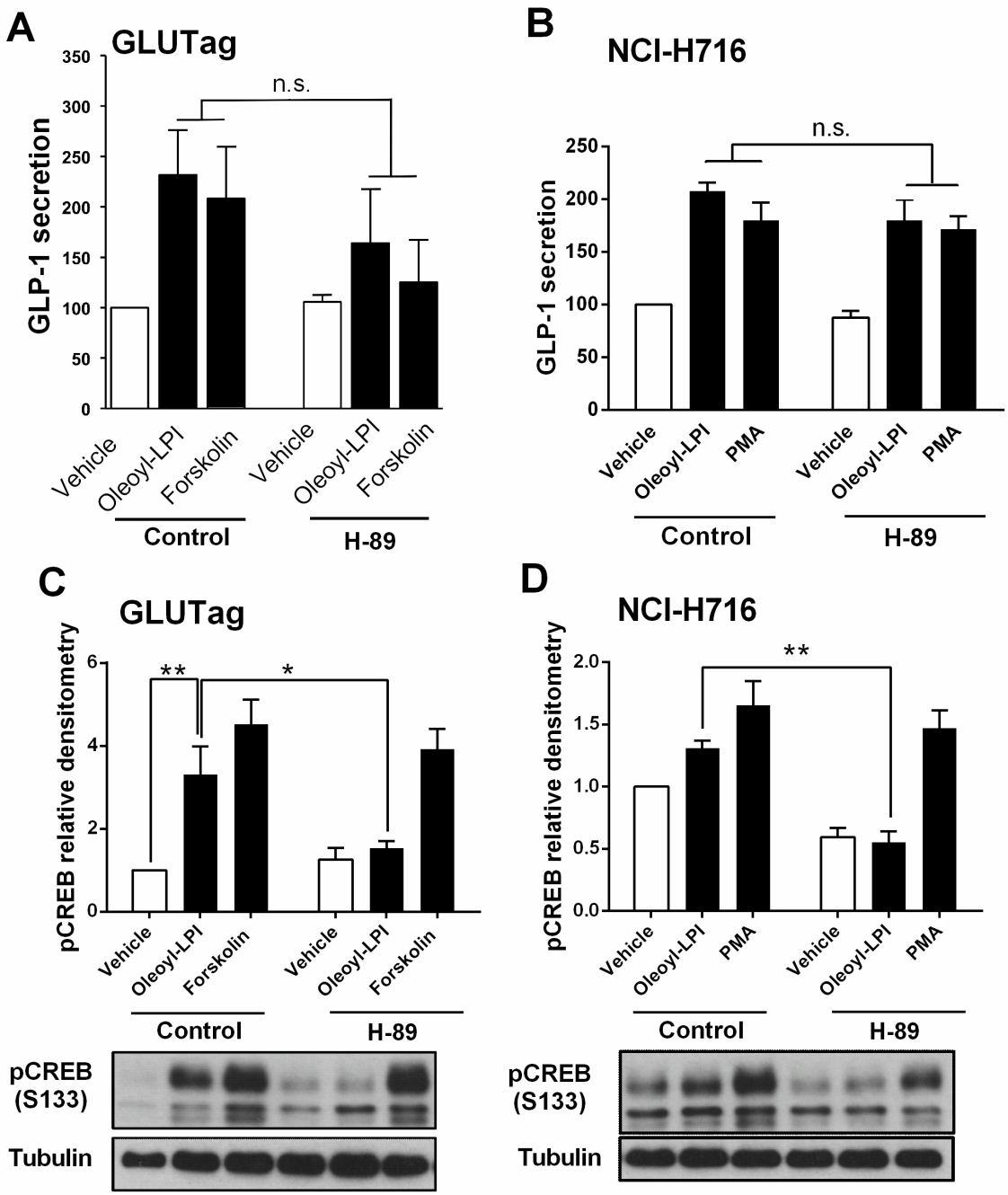
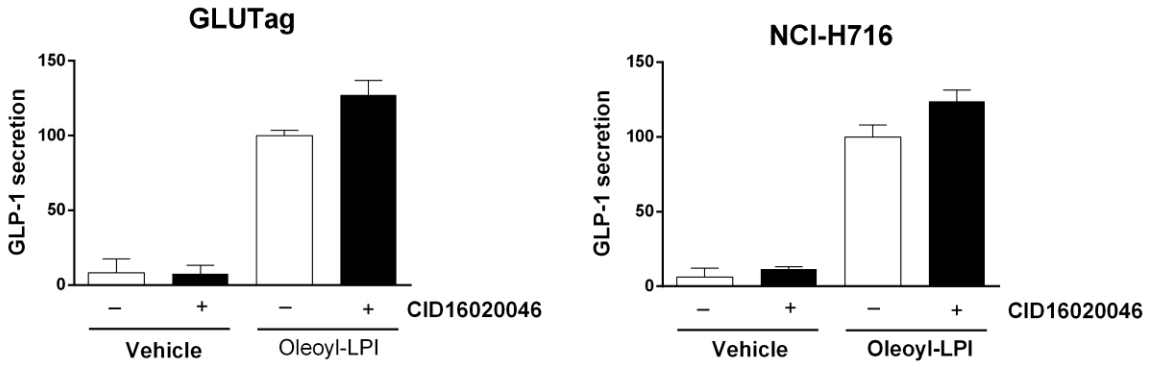

A
GLUTag
pERK 1/2 relative densitometry
Time (min)
0
5
10
30
60
120
pERK1/2 (T202, Y204)
Tubulin
B
GLUTag
pERK 1/2 relative densitometry
n.s.
siControl
siGPR119
Time (min)
C
GLUTag
GLP-1 secretion
Vehicle
Oleoyl-LPI
Forskolin
Control
PD98059
D
NCI-H716
GLP-1 secretion
Vehicle
Oleoyl-LPI
PMA
Control
PD98059

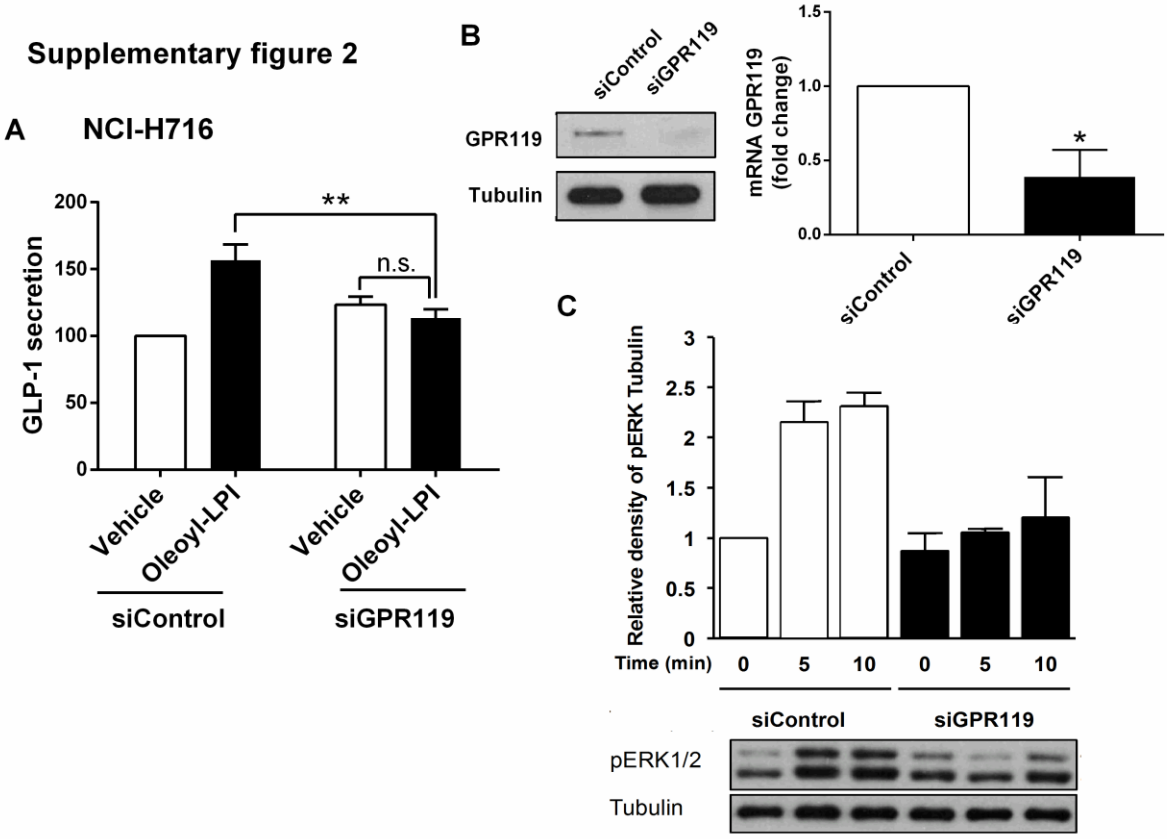
Figure 5


A
GLUTag
GLP-1 secretion
n.s.
Vehicle
Oleoyl-LPI
Forskolin
Control
H-89
B
NCI-H716
n.s.
PMA
C
GLUTag
pCREB relative densitometry
**
*
pCREB (S133)
Tubulin
D
NCI-H716

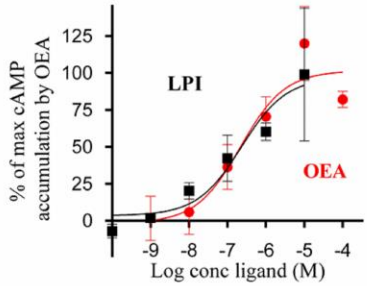
Supplementary figure 1



Supplementary figure 2



Supplementary figure 3



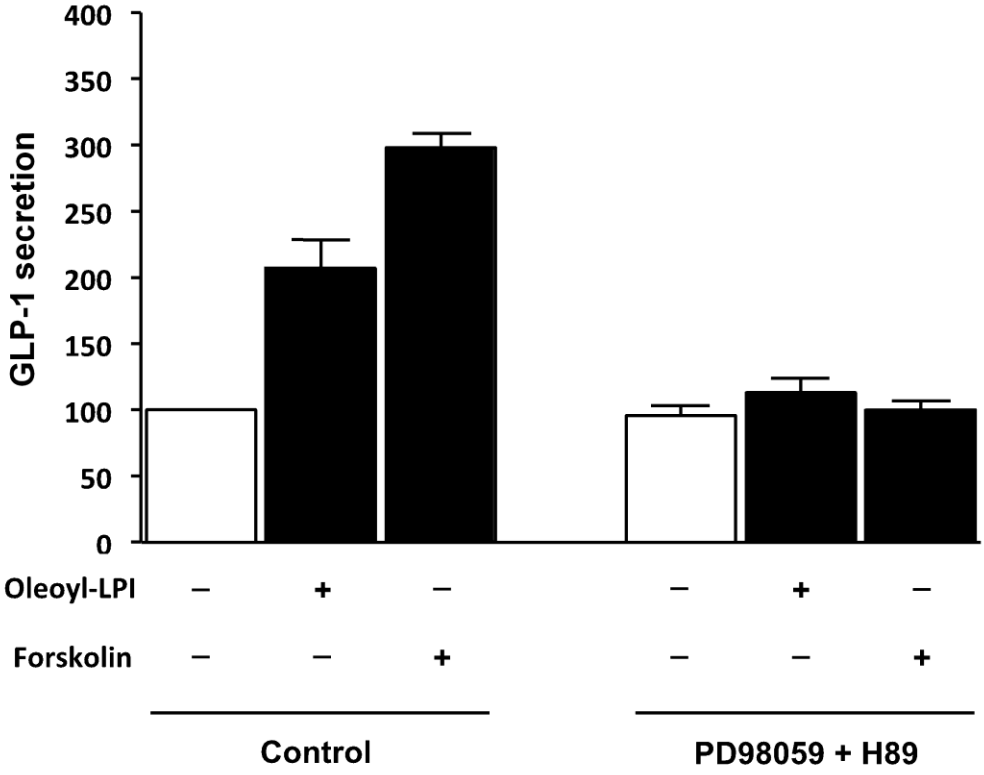
	OEA
Sigmoidal dose-response (variable slope)	
Best-fit values	
Bottom	-1.715
Top	101.4
LogEC50	-6.663
HillSlope	= 0.7500
EC50	2.173e-007
Std. Error	
Bottom	13.64
Top	12.33
LogEC50	0.4415
95% Confidence Intervals	

OEA: EC50=-6,66+/-0,44

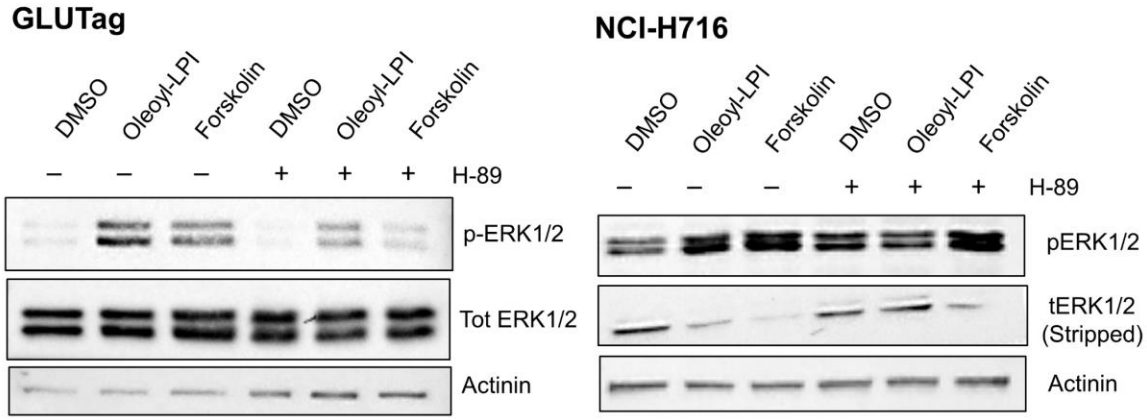
	LPI
Sigmoidal dose-response (variable slope)	
Best-fit values	
Bottom	3.347
Top	96.83
LogEC50	-6.632
HillSlope	= 0.7500
EC50	2.335e-007
Std. Error	
Bottom	14.22
Top	22.92
LogEC50	0.6631

LPI: EC50=-6,63+/-0,66

Supplementary figure 4



Supplementary figure 5



Supplementary Tables

Target gene	siRNA sequences
Human GPR119	UCAAAGCUCUCCGUACUGU,ACAGGUACCUUGCCAUCAA, CCACUCAUCUAUGCCUAUU,ACCUAGUGCUGGAACGGUA
Murine GPR119	CCUAUCACAUCGUCACUAU,UAACUAGCAUUGUGCAGGU UAUCUUACCUUAUCGGCUU,GGCCGUGGCUGAUACCUUG
Human GPR55	GAAUUCCGCAUGAACAUCA
Murine GPR55	ACAGGGAAGUGGAGAGAUA

Supplementary table 1 A: Sequences of siRNAs used in Knockdown experiments

Target gene	Forward	Reverse
Human GPR119	TCTCGGCCCACACAGAAGA	GCTGCGGAGGAAGTGACAAA
Murine GPR119	TGATGGTGTTGGCCTTTGCTTCAC	TGGTAAAGGCAGCATTTGTGGCAG
Human GPR55	GTTTCCATGGGAAAGTGGAA	GGAAGGAGACCACGAAGACA
Murine GPR55	CTATCTACATGATCAACTTGGCTGTTT	TGTGGCAGGACCATCTTGAA
Human GAPDH	AGGGCTGCTTTTAACTCTGGT	CCCCACTTGATTTTGGAGGGA
Murine GAPDH	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA

Supplementary table 1 B: Sequences of Primers used in RT-PCR