GPR119 is essential for oleoylethanolamide-induced glucagon-like peptide-1 secretion from the intestinal enteroendocrine L-cell

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ABSTRACT

Objective: Intestinal L-cells secrete the incretin glucagon-like peptide-1 (GLP-1) in response to ingestion of nutrients, especially long-chain fatty acids. The G\(_\alpha\)-coupled receptor, GPR119 binds the long-chain fatty acid-derivate oleoylethanolamide (OEA), and GPR119 agonists enhance GLP-1 secretion. We therefore hypothesized that OEA stimulates GLP-1 release through a GPR119-dependent mechanism.

Research Design and Methods: Murine (m) GLUTag, human (h) NCI-H716 and primary fetal rat intestinal L-cell models were used for RT-PCR, and for cAMP and GLP-1 RIA. Anesthetized rats received intravenous or intraileal OEA, and plasma bioactive GLP-1, insulin and glucose levels were determined by Elisa or glucose analyzer.

Results: GPR119 mRNA was detected in all L-cell models. OEA treatment (10\(\mu\)M) of mGLUTag cells increased cAMP levels (p<0.05) and GLP-1 secretion (P<0.001) in all models, with desensitization of the secretory response at higher concentrations. GLP-1 secretion was further enhanced by prevention of OEA degradation using the fatty acid-amide hydrolase inhibitor, URB597 (p<0.05-0.001 vs. OEA alone), and was abolished by H89-induced inhibition of PKA. OEA-induced cAMP levels and GLP-1 secretion were significantly reduced in mGLUTag cells transfected with GPR119-specific siRNA (p<0.05). Application of OEA (10\(\mu\)M) directly into the rat ileum, but not intravenously, increased plasma bioactive GLP-1 levels in euglycemic animals, by 1.5-fold (p<0.05) and insulin levels were increased by 3.9-fold (p<0.01) but only in the presence of hyperglycemia.

Conclusion: The results of these studies demonstrate, for the first time, that OEA increases GLP-1 secretion from intestinal L-cells through activation of the novel GPR119 fatty acid-derivate receptor in vitro and in vivo.
Glucagon-like peptide-1 (GLP-1) is an intestinal hormone with potent insulinotropic effects that are essential to the maintenance of normal glucose homeostasis (1). In addition to glucose-dependent stimulation of insulin secretion, GLP-1 shows other favorable effects, increasing β-cell proliferation in rodents, as well as enhancing β-cell survival in both rodent and in human islets (2; 3). Additionally, GLP-1 has been shown to protect cardiomyocytes from ischemia, and GLP-1 infusion improves cardiac function in patients with heart failure (4; 5). Finally, the CNS effects of GLP-1 include inhibition of gastric emptying, reduction of appetite, and promotion of satiety in humans (6; 7) and rodents (8; 9). As a result of its potent anti-diabetic and anorexic effects, GLP-1 analogs and GLP-1 degradation inhibitors have been successfully introduced to the clinic for pharmacologic treatment of patients with type 2 diabetes (10).

While the biological effects of GLP-1 have been well established, the mechanisms underlying GLP-1 secretion are less well understood. GLP-1 is secreted from intestinal endocrine L-cells, localized predominantly in the distal ileum and colon (11). Rapid GLP-1 release after food intake (12; 13) may be regulated by afferent innervation by the vagus nerve (14; 15) as well as, in rodents, by proximal gut hormones, such as glucose-dependent insulinoetric peptide (GIP) from the duodenal K-cells (16). However, L-cells also release GLP-1 in response to direct stimulation by nutrients (16), such as carbohydrates and, most notably, fat (17; 18). Monoinsaturated long-chain fatty acids, as oleic acid, are strong stimulators of GLP-1 secretion from the L-cells, both in vitro and in vivo, through a signaling pathway that requires protein kinase Cζ (PKCζ) (17; 18). Additional studies have indicated roles for the orphan G protein-coupled receptors, GPR40 and GPR120, in the response of the L-cell to saturated fat and α-linolenic acid, respectively (19; 20). Very recently, the fatty acid-derivate receptor, GPR119, was also found to be expressed in a highly tissue-specific fashion, by the intestinal L-cell and the pancreatic β-cell (21; 22). Furthermore, a GPR119-specific pharmacological agonist was demonstrated to increase the plasma levels of both GLP-1 and insulin, in mice. However, the relevance of physiological ligands of GPR119 to GLP-1 secretion by the L-cell currently remains unknown.

Oleylethanolamide (OEA) and lysophosphatidylcholine (LPC) are endogenously-occurring fatty acid-derivates that are specific ligands of GPR119 (23; 24). While LPC is often associated with pathophysiological processes such as atherosclerosis (25), OEA is found in a variety of tissues, including the intestinal epithelium, under physiological conditions (26). OEA is synthesized in vivo from membrane phospholipids through an N-acylphosphatidylethanolamine (NAPE)- phospholipase D (PLD)-dependent pathway (27); OEA can also be degraded into oleic acid and ethanolamide by the naturally-occurring enzyme fatty acid amide hydrolase (FAAH), which is also expressed by the intestinal epithelium (28; 29). Intestinal OEA levels are known to decrease during fasting and increase upon re-feeding, and OEA administration to rats reduces food intake, suggesting a role for OEA in the regulation of satiety (26; 30-32). As OEA was first identified as a ligand for the intra-nuclear PPARα receptor, it has been generally assumed that the appetite reduction is dependent on PPARα activation (33). However, as GLP-1 is known to induce satiety, we hypothesized that OEA may also stimulate GLP-1 secretion from the intestinal L-cells in GPR119-dependent fashion.
MATERIALS AND METHODS

Cell models. The murine (m) GLUTag L-cell line was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 25mM glucose and supplemented with 10%FBS; the medium was changed every 2-3 days and cells were passaged by trypsinization and reseeding at a 1:3 dilution (17; 34; 35). The human (h) NCI-H716 L-cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in suspension in RPMI 1640 supplemented with 10%FBS (35; 36). Fetal rat intestinal cultures (FRIC) were prepared by enzymatic dispersal of term fetal intestines from 19-20 d pregnant Wistar rats and cells were maintained overnight in DMEM containing 25mM glucose, 10%FBS and penicillin-streptomycin, as previously reported (15; 17; 35; 37). All 3 models of the intestinal L-cell have been validated with respect to the regulation of GLP-1 secretion, such that GLP-1 is secreted in response to known secretagogues, including muscarinic/cholinergic agonists, leptin and long-chain fatty acids in all models, as well as glucose-dependent insulinotropic peptide (GIP) in the rodent cells (17; 34-38).

In vitro experiments. mGLUTag cells were plated in poly-D-lysine-coated 24-well culture plates and grown to 80% confluence. For experiments with hNCI-H716 cells, adhesion of the cells was initiated by plating on Matrigel matrix (Becton Dickinson, Bedford, MA) in DMEM medium supplemented with 10%FBS, 2 d before the experiment, as described (35; 38). FRIC cells were investigated the day after cell dispersal and plating. Adenosine 3′,5′-cyclic monophosphate (cAMP) responses to OEA were determined by washing mGLUTag cells with Hanks’ Balanced Salt Solution (HBSS) followed by treatment for 30 min with FBS-free medium containing 1%DMSO alone (negative control), 10μM forskolin (positive control) or OEA (10-15μM), as previously described (39). To prevent cAMP degradation, all media contained 10μM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, Oakville, ON, Canada). Cells were then extracted in ethanol for RIA of cAMP content. Secretion experiments were performed as described (17; 34-36). In brief, mGLUTag, hNCI-H716 and FRIC cells were washed and then incubated for 2 hr with FBS-free DMEM containing 1%DMSO alone (negative control), 10μM forskolin (Sigma-Aldrich) or 1μM GIP (Bachem, Torrance, CA; positive controls) or with different concentrations of oleoylethanolamide (OEA), palmitoylethanolamide (PEA) or lysophosphatidylcholine (LPC; all from Sigma-Aldrich). Some cells were pre-incubated for 30 min with 10μM or 30μM H89 (Sigma-Aldrich) to inhibit PKA or with 1μM URB597 (Calbiochem, Mississauga, ON, Canada), a fatty acid amide hydrolase (FAAH) inhibitor (29). DMSO was used as a solvent to prepare stock solutions of fatty acid derivates and inhibitors. For secretion experiments, medium and cells were collected separately, and peptides were extracted by reversed-phase adsorption using C18 silica cartridges (Sep-Pak, Waters Scientific, Mississauga, ON), as previously described (17; 34-36), for RIA of GLP-1 content. GLP-1-secretion was calculated as total GLP-1 content of medium, normalized for the total amount of GLP-1 in the medium plus cells. Average basal secretion of mGLUTa, hNCI-H716 and FRIC cells was 3.4±0.3% (n=31), 2.7±0.4% (n=28) and 2.86±0.6% (n=4) of total GLP-1, respectively. No changes in total GLP-1 content were found under any of the experimental conditions (data not shown).

Small interfering (si) RNA transfection. mGLUTag cells were plated in a 24-well plate, as described above. Scrambled siRNA (control) and 2 siRNAs targeting murine GPR119 coding sequences were purchased from Ambion, Austin, TX. Transfection was performed by 5 hr incubation in Opti-Mem.
medium using 20pM siRNA and 1µl Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) as instructed by the manufacturer. Cells were allowed to recover for 2 d prior to cAMP and/or secretion experiments. Transfection efficiency was quantified by real-time RT-PCR. In brief, total RNA was extracted from mGLUTag cells using an RNaseasy kit as instructed by the manufacturer (Qiagen Inc., Mississauga, ON, Canada) and subjected to reverse transcription using SuperScript II and random hexamers (Invitrogen), followed by real-time PCR using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for GPR119 (Mm00731497_s1) and 18S (Hs99999901_s1; endogenous control). Relative quantification of GPR119 mRNA expression was calculated using the ∆∆ cycle threshold method (40).

In vitro assays. Viability of the mGLUTag cells after treatment was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay, as reported (17). Cells were plated in poly-D-lysine coated 96-well plates and treated for 2 hr with medium containing 1%DMSO alone (control), 5mM H2O2 (positive control), or fatty acid derivates or inhibitors at concentrations used for secretion experiment, after which the MTT reaction was carried out. The resulting absorbance was measured at 570nm; higher absorbance correlates with cell viability and lower absorbance with cell death.

cAMP was measured by radioimmunoassay of ethanol extracts as described (39) (Biomedical Technologies, Stoughton, MA). RIA for C-terminal GLP-1 immunoreactivity was conducted using an established lab assay (17; 34-36).

As there is currently no good GPR119 antiserum commercially available, GPR119 expression was determined by RT-PCR. Human jejenum and colon total RNA was purchased from Ambion Inc. (Austin, TX), and human placental RNA was a kind gift from Dr. J.R. Challis (University of Toronto, Toronto, ON, Canada). Total RNA from mGLUTag, hNCI-H716 and FRIC cells, as well as from rodent tissues, was extracted as described above. The primer pairs used for amplification of human GPR119 were described in (23) and for mouse GPR40 and GPR120 in (17); all other primer pairs were designed using PrimerQuest (IDT, Coralville, IA); sequences are shown in Table 1. PCR reactions were carried out at 57°C for 35 cycles, and PCR without RNA template was used as the negative control. Products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide.

In vivo experiments. In vivo animal protocols were approved by the University of Toronto Animal Care Committee. Male Wistar rats (200-300g) were obtained from Charles River Laboratories (St. Constant, QC, Canada), and maintained on a standard laboratory diet with free access to water under a 12 hr light-dark schedule. Following an overnight fast, rats were anesthetized with isoflurane (Baxter Corp., Mississauga, ON, Canada). One hr before blood sampling, some rats were i.p. injected with 3mg/kg URB597 to inhibit FAAH (29; 41). URB597 alone did not affect the levels of glucose, insulin or bioactive GLP-1 in these studies; therefore, data from rats with and without URB597 injection were combined. The carotid artery was cannulated for blood sampling and the jugular vein for injections. To prevent bioactive GLP-1 degradation by dipeptidylpeptidase IV (DPP-IV), rats received 5mg/kg Sitagliptin (Januvia™, MSD Sharp & Dohme, Haar, Germany), a selective inhibitor of DPP-IV(10; 42), i.v. 30 min prior to blood collection. Some rats also received a femoral vein cannula for continuous infusion of 37.5% glucose to maintain glycemia at 13mmol/l for at least 30 min; the glucose infusion rate was adjusted based on frequent (every 5-10 min) blood glucose measurements, as described in (43). Rats
undergoing a hyperglycemic clamp were not pre-treated with Sitagliptin, in order to reduce the number of variables that might affect glycemia. The abdominal cavity of all rats was then opened and a 10 cm-section of the distal ileum was cleansed by perfusion with saline and tied off to create a luminal-distinct compartment that retained vascular perfusion. Subsequent to collection of the basal blood sample at t = 0 min, either the luminal compartment was filled with 2ml of 10μM OEA or vehicle (0.9%saline/10%Tween80; Sigma-Aldrich), or 5mg/kg OEA or vehicle (0.9%saline/10%Tween80) was administered i.v., and additional blood samples were collected at 5, 15, 30 and 60 min. All samples (1ml each) were collected into 10% (vol/vol) Trasylol (5000 Kalikrein Inactivating Units/ml; Bayer Corp., Toronto, ON, Canada) - EDTA (12mg/ml) - Diprotin A (a DPP-IV inhibitor; 68mg/ml, Sigma-Aldrich), and plasma was stored at -80°C until analysis.

Plasma glucose levels were analyzed on a Beckman Analyzer II (Beckman, Fullerton, CA), and plasma insulin levels were measured by ELISA (Crystal Chem Inc., Downers Grove, IL) in normoglycemic animals and by RIA (Millipore Corp., Billerica, MA.) in hyperglycemic animals, due to the wider insulin detection range. Plasma levels of bioactive GLP-1 were determined by electrochemiluminescence-based detection assay (Meso Scale Discovery, Gaithersburg, MD).

Statistical analyses. All results are expressed as mean ± standard error. Area-under-the-curve (AUC) was determined using the trapezoidal rule and the data is expressed per min. Statistical analysis was performed using SAS software (SAS Institute, Cary, NC). One and two-way analysis of variation (ANOVA) was followed by Student’s t-test or n-1 custom hypotheses post hoc tests, as appropriate. To reduce inter-assay variations, some data were normalized to basal levels. Significance was assumed at p<0.05.

RESULTS

Expression of GPR40, GPR119 and GPR120. RT-PCR for rodent GPR119 mRNA transcripts was performed on total RNA extracted from mGLUTag and FRIC cells, as well as from mouse ileum and rat colon tissue. As shown in Figure 1A, GPR119 mRNA was detected in both rodent L-cell models, as well as in the intestinal samples. Additionally, GPR119 mRNA was detected in the hNCI-H716 cells and in human colon and placental tissue (Figure 1B). In contrast to previous reports (22), we did not detect GPR119 mRNA in human jejunum, possibly due to a low number of L-cells in this tissue (11). Consistent with our previous findings (17), mRNA transcripts for both GPR40 and GPR120 were detected in the mGLUTag cells, and both receptor mRNAs were also found to be expressed in the hNCI-H716 cells (Figure 1C).

OEA induces GLP-1 secretion in vitro. Possible effects of GPR119 receptor activation on GLP-1 secretion were first investigated in mGLUTag cells. Release of GLP-1 was increased to 3.1±0.4-fold of basal values by treatment with forskolin, a direct activator of adenylyl cyclase and strong L-cell secretagogue (34). OEA (5-20μM), a known ligand of GPR119 (24), significantly increased GLP-1 secretion to a maximum of 2.1±0.2-fold of basal levels at 10μM (p<0.001; Figure 2A). The same concentrations of palmitoylethanolamide (PEA), a saturated fatty acid ethanolamide (16:0) that is a very weak agonist of GPR119 (23; 24), did not increase GLP-1 secretion from mGLUTag cells. Importantly, OEA treatment did not affect the viability of the GLUTag cells (Figure 2B). In contrast, although LPC treatment of mGLUTag cells at concentrations reported to activate GPR119 (10-15μM) also enhanced GLP-1 release (to a
maximum of 10.4±0.5-fold of control values, data not shown), LPC was found to markedly reduce cell viability, by up to 60.0±6.8% as compared to control cells (p<0.05 and p<0.001; Figure 2B); LPC was, therefore, not used in further experiments. The effects of OEA on GLP-1 secretion were also confirmed in hNCl-H716 and FRIC cells (Figure 2C-D), wherein OEA treatment significantly increased GLP-1 secretion, to 2.6±0.2-fold and 5.8±2.5-fold of basal levels at 10μM (p<0.001 and p<0.01), respectively. Interestingly, higher concentrations of OEA were associated with diminished GLP-1 release in all cell models, suggestive of desensitization.

To verify the activity of GPR119 in the L-cell, mGLUTag and hNCl-H716 cells were treated with a specific GPR119 agonist, PSN632408 \[10μM, (24)\]. PSN632408 increased GLP-1 secretion by both cell lines to 2.1±0.2- and 2.9±0.5-fold of basal values (p<0.01 for mGLUTag and p<0.05 for hNCl-H716 cells, respectively; Fig. 3), demonstrating that GPR119 is functional and can initiate GLP-1 secretion in all cell models, suggestive of desensitization.

Prevention of OEA degradation with URB597 significantly increased OEA-induced GLP-1 secretion to 3.2±0.4- and 3.3±0.3-fold of control values at 10 and 15μM OEA in mGLUTag cells (Figure 4A; p<0.001 compared to URB597 alone, and p<0.01-0.001, respectively, compared to OEA treatment without URB597), as well as in hNCl-H716 cells (to 3.5±0.2- and 5.5±0.7-fold of control values at 10 and 15μM OEA (Figure 4B; p<0.001 compared to URB597 alone, and p<0.05-0.001 compared to OEA treatment without URB597). These findings indicate that degradation by FAAH limits the effects of OEA on GLP-1 secretion in both of the intestinal L-cell lines utilized.

**OEA signals through a PKA- and GPR119-dependent mechanism.** As ligand binding to GPR119 leads to activation of adenylyl cyclase, increased production of cAMP and enhanced PKA activity (24), mGLUTag cells were first examined for cAMP responses to OEA (10-15μM) and GIP, which is known to signal through cAMP- and PKA-dependent pathway (positive control; (44)). OEA treatment alone caused a small but significant increase in intracellular cAMP to 1.11±0.03- and 1.12±0.04-fold of control values at 10 and 15μM, respectively (Figure 5A, p<0.05). This effect was enhanced when OEA degradation was prevented with URB597, such that cAMP levels increased by an additional 1.3±0.04- and 1.3±0.03-fold, respectively (p<0.001 compared to URB597 alone; p<0.05-0.01 compared to OEA treatment without URB597). Furthermore, pre-treatment of the mGLUTag cells with the PKA inhibitor, H89 (10μM) completely abolished OEA (10-15μM)-induced GLP-1 secretion (Figure 5B). Similar results were found in the hNCl-H716 cells, although an increased concentration of H89 (30 μM) was required to abrogate OEA-induced GLP-1 release (Figure 5C). H89 treatment did not affect GPR40/120-induced GLP-1 secretion (Figure 5C, inset), demonstrating the specificity of this inhibitor for PKA-mediated signaling.

To determine whether the effects of OEA on cAMP production and GLP-1 secretion are dependent upon GPR119, mGLUTag cells
were transfected with specific GPR119 siRNA or scrambled siRNA (control) resulting in 23% knockdown of GPR119 mRNA, as determined by real-time RT-PCR (Fig. 6A, inset). Despite the relatively low level of GPR119 knockdown, OEA (10μM) failed to enhance cAMP levels in cells treated with GPR119 siRNA, whereas the cAMP response to OEA treatment was preserved in the control cells (p<0.05, Figure 6A). Furthermore, GPR119 knockdown led to a 45% reduction in the GLP-1 secretory response to OEA (p<0.05; Figure 5B). These data therefore provide support for a role of GPR119 and the PKA signaling pathway in OEA-induced GLP-1 secretion.

**OEA enhances GLP-1 secretion in vivo.** To establish the effects of OEA on the L-cell in vivo, rats were treated with OEA either intraluminally or intravenously. Intraluminal application of OEA (10 µM; e.g. 20 nmol/rat) to euglycemic rats significantly increased plasma bioactive GLP-1 concentrations, to 1.5±0.2-fold of basal values (p<0.05) within 5 min of administration, and this stimulation was maintained throughout the entire 60 min time-course of the experiment (Figure 7A). Thus, the AUC for the bioactive GLP-1 response was significantly increased, to 1.6±0.1-fold of vehicle-infused rats (Figure 7B, p<0.001). In contrast, intravenous administration of OEA at a 200-fold higher dose (e.g. 4 µmol/250 g rat) than that used intraluminally, demonstrated no effect on bioactive GLP-1 concentrations as compared to rats treated with vehicle alone. Throughout the 60 min experiment, the plasma levels of glucose (Figure 7C) and insulin (Figure 7D) remained stable at basal levels, and did not differ between treatment and control groups.

As GLP-1 is known to lose its insulinotropic effects under normoglycemic conditions, changes in insulin levels upon OEA treatment were also measured under hyperglycemic conditions. Glycemia was maintained at 13mmol/L, the upper physiological level in rats, for at least 30 min prior to the start of OEA application and throughout the remainder of the procedure. The basal concentration of insulin before OEA application was 1.4±0.4pg/ml and did not differ between the groups. Intraluminal application of OEA caused a 3.9±0.7-fold increase in insulin plasma levels within 5 min of application (11.2±2.1pg/ml vs. 2.8±2.0pg/ml in control group; p<0.01; Figure 7D, inset). In contrast, intravenous infusion of OEA did not affect insulin levels during the entire treatment period in the hyperglycemic rats.

**DISCUSSION**

Previous studies have indicated that the fatty acid-derivate receptor GPR119 is present on pancreatic β-cells and intestinal L-cells, and its stimulation by a GPR119 agonist increases insulin and GLP-1 secretion, respectively (21; 22; 24). However, the role of physiologically-occurring ligands of GPR119 such as OEA, and the intracellular mechanisms underlying GPR119-dependent GLP-1 secretion from the intestinal L-cell have remained undefined. The results of the present study demonstrate, for the first time, that OEA stimulates GLP-1 secretion from both mouse and human intestinal L-cell lines, as well as from primary rat L-cells in vitro. Additionally, application of OEA directly into the intestinal lumen in rats induced a significant and persistent increase in bioactive GLP-1 levels over 1 hour, supporting the in vitro findings and demonstrating a role for OEA as a GLP-1 secretagogue in vivo.

To further establish the role of OEA in GLP-1 secretion, the intracellular signaling mechanisms underlying its effects on the L-cell were investigated. OEA induced a small, but significant increase in intracellular cAMP concentration in both the mGLUTag and hNCI-H716 cells, comparable with findings in OEA-treated RINm5 and MIN6 pancreatic β-cell lines (45). Furthermore, GLP-1
secretion in OEA-treated cells was strictly dependent on PKA in both the human and mouse L-cells, as indicated by complete abrogation of the response in H89-treated cells. These findings are consistent with studies by our lab and others showing that increased cAMP levels, in response to cAMP analogs as well as to secretagogues such as GIP, stimulate GLP-1 release by the intestinal L-cell (16; 34; 46). Furthermore, while initial studies on OEA identified the intranuclear receptor PPARα and receptor-like ion channel TRPV1 as targets for OEA (27; 33; 47), neither of these receptors has been reported to stimulate the cAMP/PKA-signaling pathway. In contrast, the de-orphanization of GPR119 as a cAMP-linked OEA receptor (24) implicated GPR119 as more likely target for OEA in the intestinal L-cell. Consistent with this hypothesis, treatment of human and mouse intestinal L-cells with a GPR119-specific agonist PSN632408 significantly increased GLP-1 secretion in both cell lines. Furthermore, transfection of mGLUTag cells with GPR119 siRNA significantly diminished both the cAMP and GLP-1 responses to OEA. When taken together, these findings provide support for both the presence of functional GPR119 in the intestinal L-cell and the requirement for this novel G protein-coupled receptor in OEA-induced GLP-1 secretion.

We have previously reported that oleic acid is a strong L-cell secretagogue, increasing GLP-1 secretion both in vivo and in vitro (17; 18; 48). It is therefore interesting that OEA is rapidly degraded to oleic acid and ethanolamide via FAAH-dependent hydrolysis (29), thereby providing a possible GPR119-independent mechanism underlying OEA-induced GLP-1 secretion. Therefore, to exclude the possible effects of oleic acid in the actions of OEA, L-cells were pre-treated with the FAAH inhibitor URB597, to reduce OEA hydrolysis and resultant oleic acid accumulation. The increase in OEA-induced GLP-1 secretion found with URB597 treatment provides support for a direct role of OEA, and not of oleic acid, in OEA-induced GLP-1 secretion.

While intraluminal application of OEA in vivo significantly increased GLP-1 secretion in normal rats, intravenous injection of OEA at a 200-fold higher dose failed to increase circulating levels of bioactive GLP-1. Additionally, while intraluminal OEA application clearly increased insulin levels in hyperglycemic rats, intravenous injection of OEA did not affect insulin levels in these animals under either euglycemic or hyperglycemic conditions. Taken together, this data suggests that circulating OEA does not cause significant increases in hormone release from either of its known target tissues, the intestinal L-cell and the pancreatic β-cell. There are several possible explanations for these findings. First, the volume of distribution for OEA as a lipophilic substance should be high, therefore increasing the concentration of OEA required for administration into the jugular vein to reach distant target tissues at stimulatory concentrations. However, higher levels of OEA cannot be achieved in the circulation of the rat due to the limited solubility of OEA in solvents. Furthermore, whether OEA is cleared through the liver and/or lungs is unknown and may be significant.

The actions of both OEA and a GPR119 agonist on insulin secretion have previously been reported to be glucose-dependent (21; 45), as further confirmed in the present study. Previous studies have also demonstrated that ~45% of the glucose-lowering effect of an oral GPR119 agonist given concurrently with oral glucose is mediated through enhancement of GLP-1 release (22), suggesting that the insulinotropic effect of GPR119 activation is mediated both directly, through GPR119 expressed on the β-cell, and indirectly, through enhanced release of GLP-1. Nonetheless, although both the L-cell and the β-cell are responsive to OEA, the glucose-
sensitivity of the β-cell response, as compared to the glucose-insensitivity of the L-cell response that we have observed, suggests that oral administration of this fatty acid-derivate alone to enhance GLP-1 secretion, without influencing insulin release, will permit the insulin-independent biological actions of GLP-1. In contrast, administration of OEA in the setting of a glucose-containing meal would facilitate release of both GLP-1 and insulin, thereby also modulating glycemic responses (22; 49).

The observation of apparent desensitization in all of the dose-response curves was unexpected, as this has not previously been reported for GPR119. However, Gαs-coupled receptors are well-established to undergo homologous desensitization (50) and, indeed, preliminary studies in which hNCI-716 cells were pre-treated with the GPR119 agonist, PSN632408 (10μM for 6 hr) demonstrated a 70.9±4.6% decrease in OEA-induced and 50.7±11.7% decrease in PSN632408-induced GLP-1 secretion (data not shown). Collectively, these findings indicate that GPR119 may undergo homologous desensitization, a phenomenon that clearly warrants further investigation.

In summary, the results of this study establish, for the first time, the role of GPR119 in OEA-induced GLP-1 secretion, and adds GPR119 to the growing list of fatty acid-responsive pathways that function to modulate release of GLP-1, including GPR40, GPR120 and PKCζ. When combined with the reported insulinotropic effects of GPR119 agonists, these findings implicate GPR119 as potential pharmacological target, as well as OEA as a nutriceutical approach to enhance GLP-1 in patients with type 2 diabetes.

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**Figure 1. Expression of GPR119 mRNA in L-cell models.**

A Total RNA from mGLUTag cells, Fetal Rat Intestinal Cells (FRIC), and murine intestinal tissues was analyzed for expression of GPR119 mRNA by RT-PCR. B Total RNA from hNCI-H716 cells, human intestinal tissues and human placenta was analyzed for expression of GPR119 mRNA by RT-PCR. C Total RNA from mGLUTag cells and hNCI-H716 cells was analyzed for expression of GPR40 and GPR120 mRNA by RT-PCR. All products were separated on agarose gels and visualized with ethidium bromide, with the molecular size ladder on the left. Negative controls did not include RNA template. The anticipated band sizes of products are indicated in base pairs (bp).
Figure 2. Effects of oleoylethanolamide (OEA) on GLP-1 secretion. A mGLUTag cells (n=9-12), C hNCI-H716 cells (n=8) and D FRIC cells (n=4) were incubated with medium alone (1%DMSO, negative control), forskolin (10µM, positive control), oleoylethanolamide (2-20µM) or palmitoylethanolamide (10-15µM, negative control) for 2 hr. GLP-1 content of media and cells was determined by RIA, *, p<0.05; ***, p<0.01; ****, p<0.001 vs. control.

B To determine potential effects on cell viability, mGLUTag cells were incubated with medium alone (1%DMSO, negative control), H₂O₂ (5mM, positive control), OEA (10-20µM) or lysophosphatidylcholine (LPC, 10-15µM) for 2 hr, followed by MTT assay (n=8-16). *, p<0.05, **, p<0.01, ****, p<0.001 vs. control.
Figure 3. Effects of GPR119 and GPR40/120 agonists on GLP-1 secretion. A mGLUTag (n=4) and B hNCl-H716 (n=4) cells were incubated with medium alone (1%DMSO, negative control), OEA (10µM), the GPR119 agonist PSN632408 (10µM) or the combined GPR40/GPR120 agonist GW9508 (10µM) for 2 hr. GLP-1 content of media and cells was determined by RIA *, p<0.05 and **, p<0.01 vs. control.
Figure 4. Effect of inhibition of OEA degradation on OEA-induced GLP-1 secretion. A mGLUTag (n=6-18) and B hNCl-H716 (n=12) cells were pre-treated for 30 min with URB597 (1µM), to inhibit FAAH and prevent OEA degradation, prior to incubation with medium alone (1%DMSO, negative control), or OEA (10-15µM) for 2 hr. GLP-1 content of media and cells was determined by RIA. ***, p<0.01, ****, p<0.001 vs. control; #, p<0.05; ##, p<0.01; ###, p<0.001 vs. OEA treatment alone.
**Figure 5. Effect of PKA inhibition on OEA-induced GLP-1 secretion.**

A, B mGLUTag (n=6-9) and C hNCI-H716 (n=4-6) cells were pre-treated for 30 min with medium alone (1% DMSO, negative control), H89 (10µM for mGLUTag and 30µM for hNCI-H716) or URB597 (1µM), to inhibit PKA or FAAH, respectively, followed by incubation with medium alone (1% DMSO, negative control), GIP (1µM, positive control), oleoylethanolamide (10-15µM) or GPR40/120 agonist GW9508 (10µM) for 2 hr. C, inset hNCI-H716 cells were pretreated for 30 min with media alone (1% DMSO) or with H89 (30µM), followed by incubation with the GPR40/120 agonist GW9508 (10µM). cAMP content of cells and GLP-1 content of media and cells were determined by RIA. *, p<0.05; ***, p<0.001 vs. appropriate control; #, p<0.05 and ###, p<0.01; ####, p<0.001 vs. paired treatment alone.
Figure 6. Effect of GPR119 knock-down on OEA-induced GLP-1 secretion. mGLUTag cells were transfected with scrambled siRNA (20 pM, control) or GPR119 siRNA (20 pM) 2 days before the experiment. Cells were then incubated with medium alone (1%DMSO, negative control) or oleoylethanolamide (10-15µM) for 2 hr. A, cAMP content of cells (n=6) and B GLP-1 content of media and cells (n=9) were determined by RIA. A, inset GPR119 mRNA transcript levels were determined by qRT-PCR relative to 18S transcript levels. *, p<0.05, ***, p<0.001 vs. control or vs. the delta change in control cells, as indicated by the lines; #, p<0.05 vs. OEA treatment with scrambled siRNA.
Figure 7. In vivo effect of OEA on GLP-1 secretion. Anesthetized rats received intraluminal or intravenous injections of vehicle (saline/10% Tween80; combined controls), intraluminal OEA (2ml of 10µM) or intravenous OEA (5mg/kg) and blood samples were collected over a 1 hr period. Plasma concentrations of A bioactive GLP-1, C glucose and D insulin were determined by Elisa and glucose analyzer, as appropriate (n=5-11). B AUC for the absolute plasma bioactive GLP-1 concentrations was determined using the trapezoidal rule and is expressed per min. D, Inset Rats (n=4-5) were maintained at 13mmol/l plasma glucose (hyperglycemic clamp) for a minimum of 30 min and this was maintained throughout the OEA treatment procedure. Plasma insulin levels were determined by RIA. A To reduce inter-assay variations due to use of separate kits, bioactive GLP-1 concentrations were calculated as fold-increase over basal GLP-1 levels (control: 29.4±8.6pg/ml; intraluminal OEA: 18.9±4.9pg/ml and i.v. OEA: 32.7±3.4pg/ml; p=n.s. between the basal values). *, p< 0.05 vs. control; **, p<0.01 vs. control; ***, p<0.001 vs. control; # p<0.05 and ## p<0.01 vs. basal values.