The Orphan G Protein-coupled Receptor GPR40 Is Activated by Medium and Long Chain Fatty Acids*

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GPR40 is a member of a subfamily of homologous G protein-coupled receptors that include GPR41 and GPR43 and that have no current function or ligand ascribed. Ligand fishing experiments in HEK293 cells expressing human GPR40 revealed that a range of saturated and unsaturated carboxylic acids with carbon chain lengths greater than six were able to induce an elevation of [Ca2+]i, measured using a fluorometric imaging plate reader. 5,8,11-Eicosatriynoic acid was the most potent fatty acid tested, with a pEC50 of 5.7. G protein coupling of GPR40 was examined in Chinese hamster ovary cells expressing the Goq9-responsive Gal4-Elk1 reporter system. Expression of human GPR40 led to a constitutive induction of luciferase activity, which was further increased by exposure of the cells to eicosatrynoic acid. Neither the constitutive nor ligand-mediated luciferase induction was inhibited by pertussis toxin treatment, suggesting that GPR40 was coupled to Goq9. Expression analysis by quantitative reverse transcription-PCR showed that GPR40 was specifically expressed in brain and pancreas, with expression in rodent pancreas being localized to insulin-producing β-cells. These data suggest that some of the physiological effects of fatty acids in pancreatic islets and brain may be mediated through a cell-surface receptor.

G protein-coupled receptors (GPCRs)3 not only respond to a large variety of molecules from inorganic ions to peptides but are also critical for a diversity of physiological functions. In the last decade, an increasing number of unliganded receptors, so-called orphan receptors, with unknown function have been identified, warranting much research into their biological role. GPR40 was cloned along with GPR41–43 downstream of CD22 on human chromosomal locus 19q13.1 (1). GPR40–43 belong to a subset of orphan receptors, which are more related to each other than to other liganded receptors, with GPR40 being 30% identical to GPR41 and GPR43. The group of GPCRs of which GPR40–43 are members is thought to have evolved relatively recently and contains several gene duplications, of which GPR41 and GPR42 are examples (2).

Fatty acids play an important physiological role in many tissues. Many effects attributable to fatty acids, such as impairment of insulin-mediated glucose uptake and glycogen synthesis in muscle (3) and potentiation of glucose-stimulated insulin secretion in pancreatic islets (4), have been attributed to their intracellular metabolism to long chain acyl-CoA esters. However, it is also conceivable that fatty acids may act at a cell-surface receptor because receptors for fatty acid derivatives such as prostaglandins and leukotrienes have been identified (5, 6). Moreover, there is significant homology between the GPR40–43 receptor family and the leukotriene receptor family. However, to date, no extracellular receptor for the fatty acids most prevalent in plasma (palmitate, oleate, stearate, linoleate, and linolenate) has been reported.

Using a ligand fishing strategy, previously successful in identifying ligands for other orphan GPCRs (7), we demonstrate that medium and long chain saturated and unsaturated fatty acids can activate GPR40 in a dose-dependent manner. However, short chain fatty acids, shown to activate other family member receptors (GPR41 and GPR43), are inactive in cells expressing GPR40 (2). Pharmacological analysis of GPR40 and its tissue distribution suggests that the receptor may play a role in pancreatic β-cell and neurological function. We have therefore identified a potential novel mechanism by which fatty acids may elicit cellular responses in certain tissues.

EXPERIMENTAL PROCEDURES

Cloning of GPR40—The human GPR40 (hGPR40) receptor (GenBank™/EMBL accession number AF024687) was cloned by PCR from human genomic DNA using nested oligonucleotide sense primers (5'-GATCTGGAGCAAGGGAGGAGTGGTCA-3' and 5'-GGAGGCTGCCAGGGACGAG-3') and antisense primers (5'-CCCTCGAGTTCCTCCGAGACG-3' and 5'-CATGCTCTTCTCCCCCGAGCAG-3') designed based on the genomic sequence of the hGPR40 gene.
Mouse Gpr40 was cloned by hybridization of the 32P-radioiodolated human coding region to a mouse genomic library (Stratagene, La Jolla, CA) under low stringency conditions as previously described (5). Positive clones were plaque-purified, digested with BamH I, and size-fractionated by agarose gel electrophoresis. Southern blot analysis with the hGPR40 probe was used to identify a BamH I fragment that was subcloned into pBluescript (Stratagene). The subclones were sequenced (PerkinElmer Life Sciences); and one clone, designated mouse Gpr40g, contained an open reading frame of 300 amino acids with 83% identity to hGPR40. Oligonucleotides were designed to clone mouse Gpr40 from brain cDNA using PuTurbo (Stratagene). A sequence confirming the cDNA clone (GenBank®/EBI accession number AF358909) was subcloned into the mammalian expression vector pCDN (9).

Clones were screened using oligonucleotides designed in the regions of high homology between the human and mouse genes. A partial fragment of 435 bp was cloned from rat pancreatic and brain cDNAs and sequenced (PerkinElmer Life Sciences). Following confirmation that the clone was the rat ortholog to human and mouse genes, 5'- and 3'-RACE oligonucleotides were designed, and a full-length rat Gpr40 cDNA (GenBank®/EBI accession number AF358910) was cloned using PuTurbo. A sequence confirming the cDNA clone was subcloned into pCDN (9).

**Cell Culture**—CHO cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Invitrogen) containing 5% fetal bovine serum and 2 mM glutamine. MIN6 cells (a gift from Professor M. Furuyama, University of Tokyo, Japan) through Professor J. Miyazaki (University of Tokyo, Japan) were cultured at 37 °C in 5% CO2 in 90% DMEM supplemented with 10% fetal bovine serum and 2 mM glutamine. All cell lines were cultured at 37 °C in 5% CO2 in 90% DMEM supplemented with 10% fetal bovine serum and 2 mM glutamine.

Calcium Mobilization Assays

- Stable Expression of GPR40 in Mammalian Cells—hGPR40 in pCDN was stably expressed in HEK293 cells using LipofectAMINE Plus (Invitrogen) according to the manufacturer's instructions. Stable clonal cell lines were generated by serial dilution into growth medium containing 400 μg/mL G418 (Invitrogen). Stable CHO cells expressing hGPR40-Gal4-Elk1-luc (hGPR40-Gal4-Elk1/luc) (2) were generated using sheared genomic DNA, assuming that 1 ng of cDNA prepared from human tissues (Promega, Madison, WI) essentially as described previously (6), using forward and reverse hGPR40-specific primers and probes (BIOSOURCE, International, Camarillo, CA). Primers and probes were designed using Primer Express software (PE Applied Biosystems, Foster City, CA) and are shown in Table I. Copy number was calculated from a standard curve, generated using sheared genomic DNA, assuming that 1 μg/mL sheared genomic DNA is equivalent to 6 × 106 “single-strand copies.” The data are presented as mRNA copies detected per ng of poly(A)-RNA from four individual donors. For the immune cell samples, TaqMan analysis was performed on 50 ng of total RNA extracted from samples from up to three individuals as described (2). Relative abundance in these samples is expressed as 2^(-ΔΔCt) to reflect the exponential nature of PCR. The ΔCt = 40 cycles – the threshold cycle (Ct). The Ct value is the cycle number at which the reporter fluorescence, generated by cleavage of the probe, passes a fixed threshold above the baseline. The Ct occurs in the exponential phase of PCR when none of the reaction components are limiting. We used an arbitrary cutoff of 40 cycles as a reference. In other words, each cycle represents a 2-fold difference in mRNA abundance.

- Total RNA was isolated from human, rat, or mouse islets or MIN6 cells (a gift from Dr. P. Trziko (Institute of Biotechnology, Warsaw, Poland)) and RNA from four individual donors, including informed consent, was supplied by the Human Islet Facility at the University of Leicester (Leicester, UK).

**In Situ Hybridization Experiments**—A 587-base probe was generated by reverse transcription-PCR from the full-length clone of rat Gpr40 using primers that flank the vector insertion site. After PCR cloning, the vector was linearized with the restriction enzyme SpeI, followed by the generation of radioiodolated antisense transcripts using T7 RNA polymerase and [32P]ribonucleotideUTP (800 Ci/mmol; Amer sham Biosciences). Sense control probes were generated by linearization with NcoI, followed by transcription with SP6 polymerase.

Whole rat pancreas was obtained by rapid necropsy, fixed in neutral buffered 10% Formalin for 24 h, and embedded in paraffin. The tissues were sectioned at 6 μm, mounted on plus slides, deparaffinized, rehydrated, and then pretreated with 0.2 M HCl for 10 min, followed by digestion with 10 μg/mL proteinase K for 20 min. After dehydrating and drying, the sections were prehybridized for 2 h in 50% prehybridization solution containing 200 mM NaCl, 40 mM Tris (pH 8.0), 2 mM EDTA, 0.5% SDS, 20% dithiothreitol, and 0.5 μg/mL RNA. The probes (2 × 10^6 cpm/sample) were mixed with hybridization solution (2 × hybridization mixture and 20% dextran sulfate in formamide, 1:1 (v/v)) and hybridized overnight at 55 °C. Following the overnight incubation, the sections were washed at 55 °C for 15 min in 0.2× SSC at 1 μg/mL proteinase K for 10 min. 0.1 M HCl was then washed with 0.1× SSC at 55 °C for 30 min, washed again with 0.1× SSC at 55 °C for 20 min, and dehydrated. After drying, the slides were dipped in Kodak NTB-2 emulsion, exposed for 4 weeks, developed with Kodak D-19 developer, counterstained with hematoxylin, and examined by both dark-field and light-field microscopy. Immunohistochemical staining was performed using an automated staining device (Ventana Nexus automated stainer with all Ventana reagents (Ventana Medical Systems, Inc., Tucson, AZ)). An enhanced 3,3′-diaminobenzidine kit was used for detection, and sections were counterstained with hematoxylin.
Fatty Acids as Ligands for GPR40

RESULTS

Activation of hGPR40 Expressed in HEK293 Cells—We transiently transfected HEK293 cells with hGPR40 and screened these cells for their ability to increase intracellular calcium using a FLIPK against a large library of >1500 known and putative natural GPCR agonists. Elaidic acid (C18:1Δ9) was the only ligand found in the library to specifically activate the receptor compared with cells transiently expressing other GPCRs (Fig. 1A). Further testing of other fatty acids in HEK293 cells stably expressing GPR40 demonstrated that a range of medium to long chain saturated and unsaturated fatty acids, e.g. elaidic acid, palmitic acid, and ETA, were able to increase intracellular calcium in a concentration-dependent manner, whereas short chain fatty acids with carbon chain lengths greater than six, e.g. succinic acid and formic acid, produced no response (Fig. 1B). pEC50 values for both saturated and unsaturated fatty acids for the elevation of intracellular calcium in HEK293 cells are shown in Table I. The most potent saturated fatty acids were those with carbon chain lengths of 15 and 16, whereas of all the fatty acids tested, ETA was the most potent, with a pEC50 of 5.71.

Activation of the Gal4-Elk1/5xGal4-luc+ Reporter in Cells Expressing hGPR40—We stably transfected hGPR40 into a stable CHO cell line containing a Gal4-Elk1/5xGal4-luc+ reporter. Following activation of MAPK, the chimeric Gal4-Elk1 transcription factor is phosphorylated and activated and binds to the Gal4 upstream activating sequence, resulting in induction of luciferase expression (14). MAPK activation can occur downstream of signaling pathways initiated by receptors coupled to either Gαs or Gαq/11. The signaling pathways involved can, however, be inferred through the use of PTX, which ADP-ribosylates Gαs, preventing its interaction with receptors while having no effect on Gαq/11 (15). The hGPR40-Gal4-Elk1/5xGal4-luc+ cells exhibited a significantly higher basal luciferase activity compared with host cells lacking hGPR40 (p < 0.01) (Fig. 2A). Pretreatment with PTX decreased basal luciferase activity in both host cells and cells expressing GPR40 to a similar extent (luciferase activity in PTX-treated cells as percent of untreated cells (means ± S.E., n = three experiments): host cells, 52.9 ± 9; and GPR40-expressing cells, 44.2 ± 1.2). These data suggest that some of the basal luciferase activity present in both host cells and hGPR40-Gal4-Elk1/5xGal4-luc+ cells was due to the constitutive activity of endogenous Gαq/11-coupled receptors. Luciferase activity remained significantly higher in hGPR40-Gal4-Elk1/5xGal4-luc+ cells compared with host cells even after PTX treatment. This residual activity may be due to the constitutive activity of GPR40 resulting from coupling to Gαq/11. Similar results in melanophores, showing the constitutive activity of GPR40 resulting from coupling to Gαq/11, support this finding (data not shown).

To investigate the G protein coupling of GPR40 following ligand binding and activation, hGPR40-Gal4-Elk1/5xGal4-luc+ cells were treated with the fatty acid ETA, the most potent ligand for hGPR40 from the initial ligand pairing screen performed in HEK293 cells (Table I). As a positive control, cells were also treated with lysophosphatidic acid (LPA), which binds to an endogenous Gαq-coupled receptor (16). All treatments were performed following an overnight treatment of PTX or vehicle. ETA (3 μM) produced a maximum 3.3-fold response over the control in hGPR40-Gal4-Elk1/5xGal4-luc+ cells (Fig. 2B). Responses to 3 μM ETA in four other hGPR40-Gal4-Elk1/5xGal4-luc+ clones characterized ranged from 3.3- to 7.4-fold (data not shown). No significant induction of luciferase activity was observed in host cells at any concentration of ETA tested (Fig. 2B). LPA in the host cells had a pEC50 of 5.51 ± 0.01 and produced a 13.5-fold response over basal levels at 2.5 μM (Fig. 2C). The response to LPA was essentially abolished by PTX treatment. However, PTX did not inhibit the induction of luciferase activity by ETA in hGPR40-Gal4-Elk1/5xGal4-luc+ cells at any concentration tested, nor did PTX treatment significantly change the pEC50 (without PTX, 5.92 ± 0.05; with PTX, 5.99 ± 0.01). Furthermore, there was a trend for an increase in ETA-stimulated luciferase activity following PTX treatment, suggesting that the Gαq/11-mediated activation of the luciferase reporter was dampened by a Gαq/11 component. Similar potentiation of the response to ETA was observed in three of four other hGPR40-Gal4-Elk1/5xGal4-luc+ clones, whereas in the fourth clone, PTX had no effect (neither inhibitory nor stimulatory) on ETA-stimulated luciferase activity (data not shown).

Expression Profile of Human, Mouse, and Rat mRNAs—To evaluate the physiological role of GPR40, we performed real-time quantitative reverse transcription-PCR using TaqMan in a variety of human tissues from one to four individuals. Specific
Receptor activation was measured from dose-dependent changes in 
$[\text{Ca}^2+]$, using FLIPR. pEC$_{50}$ values of active saturated (Part A) and unsaturated (Part B) fatty acids are listed. Values are mean pEC$_{50}$ from two or three experiments, with each point determined in duplicate. S.E. is given where three independent experiments were performed. DHETE, dihydroxyeicosatetraenoic acid.

| Fatty acid                        | pEC$_{50}$ | Fatty acid                        | pEC$_{50}$ | Fatty acid                        | pEC$_{50}$ |
|----------------------------------|------------|----------------------------------|------------|----------------------------------|------------|
| **A.**                           |            |                                  |            |                                  |            |
| n-Capric acid (C$_6$H$_{12}$O$_2$) | 4.33 | Lauric acid (dodecanoic acid (C$_{12}$H$_{24}$O$_2$) | 4.92 ± 0.09 | Stearic acid (C$_{18}$H$_{36}$O$_2$) | 4.78 ± 0.04 |
| Heptanoic acid (C$_7$H$_{14}$O$_2$) | 4.28 | Tridecanoic acid (C$_{13}$H$_{26}$O$_2$) | 4.93 ± 0.07 | Nonadecanoic acid (C$_{19}$H$_{38}$O$_2$) | 4.52 |
| Caprylic acid (C$_8$H$_{16}$O$_2$) | 4.42 | Myristic acid (C$_{14}$H$_{28}$O$_2$) | 4.84 ± 0.16 | Arachidic acid (C$_{20}$H$_{40}$O$_2$) | 4.21 |
| Nonanoic acid (C$_9$H$_{18}$O$_2$) | 4.40 | Pentadecanoic acid (C$_{15}$H$_{30}$O$_2$) | 5.18 ± 0.11 | Heneicosanoic acid (C$_{21}$H$_{42}$O$_2$) | 4.49 |
| Capric acid (C$_{10}$H$_{20}$O$_2$) | 4.85 ± 0.06 | Palmitic acid (C$_{16}$H$_{32}$O$_2$) | 5.30 ± 0.12 | Behenic acid (docosanoic acid (C$_{22}$H$_{44}$O$_2$) | 4.30 |
| Undecanoic acid (C$_{11}$H$_{22}$O$_2$) | 4.70 ± 0.07 | Heptadecanoic acid (C$_{17}$H$_{34}$O$_2$) | 4.99 ± 0.07 | Tricosanoic acid (C$_{23}$H$_{46}$O$_2$) | 4.31 |
| **B.**                           |            |                                  |            |                                  |            |
| Mead acid (10:3 9-9)              | 5.6 ± 0.10 | All-trans-retinal (vitamin A aldehyde (C$_{20}$H$_{20}$O) | 4.16 | Eicosa-11Z, 14Z-dienoic acid (20:2n-6) | 4.97 ± 0.18 |
| 9Z-Palmitoleic acid (C$_{16}$H$_{32}$O$_2$) | 4.86 ± 0.05 | All-trans-Retinoic acid (vitamin A acid, tretinoin (C$_{20}$H$_{32}$O$_2$) | 5.58 ± 0.04 | Eicosa-11Z, 14Z, 17Z-trienoic acid (20:3n-3) | 4.95 ± 0.03 |
| 9Z, 12Z, 15Z-Linolenic acid (C$_{18}$H$_{30}$O$_2$) | 4.90 ± 0.13 | 9-cis-Retinoic acid (9-cis-tretinoin (C$_{20}$H$_{32}$O$_2$) | 4.40 | Eicosa-5Z, 8Z, 11Z, 14Z, 17Z-pentaenoic acid (20:5n-3) | 5.17 ± 0.08 |
| γ-Linolenic acid (18:3 n-6)       | 5.05 ± 0.12 | 5Z,8Z,11Z,14Z-Arachidonic acid (C$_{20}$H$_{32}$O$_2$) | 4.92 ± 0.12 | Dihomo-γ linolenic acid (20:3n-6) | 5.14 ± 0.12 |
| 9Z,12Z-Linoleic acid (C$_{18}$H$_{32}$O$_2$) | 5.02 ± 0.16 | (14R,15S)-DHETE | 4.63 | Docosa-13Z, 16Z, 19Z-trienoic acid (22:3n-3) | 5.17 ± 0.05 |
| 9E-Elaidic acid (C$_{18}$H$_{32}$O$_2$) | 5.16 ± 0.07 | 17-Octadecynoic acid (C$_{18}$H$_{32}$O$_2$) | 5.12 ± 0.34 | Docosa-7Z,10Z,13Z,16Z,19Z-pentaenoic acid (22:5n-3) | 5.33 ± 0.17 |
| 9Z-Oleic acid (C$_{18}$H$_{32}$O$_2$) | 4.39 | 5,8,11-Eicosatriynoic acid (C$_{20}$H$_{32}$O$_2$) | 5.71 ± 0.11 | cis-4,7,10,13,16,19-Docosahexaenoic acid (C$_{22}$H$_{32}$O$_2$) | 5.37 ± 0.10 |
| 6Z-Petroselinic acid (C$_{18}$H$_{30}$O$_2$) | 5.00 ± 0.02 | Eicosa-5Z,8Z-dienoic acid (20:2n-12) | 5.11 ± 0.04 | Adrenic acid (22:4n-6) | 4.87 ± 0.22 |
expression in the substantia nigra and medulla oblongata. Analysis of GPR40 expression in immune cells from one to three individuals showed that, in contrast to hGPR43, no expression of GPR40 was detected in peripheral blood mononuclear cells, B-lymphocytes, or neutrophils (2). Expression of GPR40 was detected in monocytes, although at relatively low levels compared with that of GPR43. Because the analysis of GPR40 and GPR43 expression was performed in the same immune cell samples, the data show that there is clear differential expression between these two receptors in immune cells.

As the pancreas is composed of exocrine cells and endocrine islet cells, with the islets making up only 2% of the pancreas, the level of hGPR40 expression in the pancreas was compared with that in human islets. In four independent islet samples, there was a 2–100-fold higher expression of GPR40 than in total pancreas for an equivalent RNA input, with a mean -fold increase in expression of 36.8 ± 25.4 compared with that in total pancreas. These data suggest that pancreatic expression of GPR40 may be localized to human pancreatic islets. Islets contain four cell types (α, β, δ, and PP), of which the insulin-secreting β-cells make up >80% of the islet. To further investigate the localization of Gpr40 in islets, rat and mouse Gpr40 were cloned. At the DNA level, rat and mouse Gpr40 are 94% identical to each other and 75 and 76% identical to hGpr40, respectively. At the protein level, rat and mouse Gpr40 are 95% identical to each other and 82 and 83% identical to the human receptor, respectively. To investigate how expression of Gpr40 changes in a rodent model of obesity and insulin resistance, expression in whole pancreases of ob/ob mice was examined. ob/ob mice are obese, hyperglycemic, and insulin-resistant and exhibit β-cell hyperplasia due to the increased demand on the cells for insulin (17). Using TaqMan primers specific to rat and mouse Gpr40 (Table II), significant increases in expression of Gpr40 mRNA were detected in whole pancreases of ob/ob mice compared with control lean mice. Although the increases in Gpr40 mRNA expression were less than those of insulin (-fold change relative to lean controls (n = two experiments, 5–10 animals/group): Gpr40, 6.5 ± 1.3; and insulin, 10.2 ± 0.9), these data suggest that the increased expression of Gpr40 may be due in part to the increased β-cell number manifest in the pancreas of this animal model. To verify the islet localization of Gpr40, TaqMan RT-PCR was performed in rat and mouse islets and in a mouse insulinoma cell line (MIN6) using species-specific primers. Table III shows that Gpr40 was expressed in both rat and mouse islets and in MIN6 cells. In situ hybridization using riboprobes to rat Gpr40 detected Gpr40 mRNA in sections of rat pancreas in a staining pattern resembling that found in islets (Fig. 4). When parallel sections were immunostained for islet hormones, the pattern of Gpr40 staining was most similar to that of insulin, suggesting that the receptor is in the islet β-cells and not the peripherally staining α-cells. However, whether expression occurs also in δ- and PP-cells cannot be concluded at this stage.

**DISCUSSION**

We have identified medium and long chain saturated and unsaturated fatty acids as ligands for the orphan G protein-coupled receptor GPR40 using increases in [Ca\(^{2+}\)]\textsuperscript{c}, measured using FLIPR in HEK293 cells expressing GPR40. Fatty acids have also been previously identified as natural ligands for the nuclear receptor peroxisome proliferator-activated receptor-α (18). Although GPCRs for fatty acid derivatives such as prostaglandins and leukotrienes have been identified (5, 6), to date, no extracellular receptor for the fatty acids most prevalent in plasma (palmitate, oleate, stearate, linoleate, and linolenate) has been reported. The discovery of fatty acids as ligands for GPR40 is in line with the identification of carboxylic acids as ligands for GPR40.
Fatty Acids as Ligands for GPR40

**Fig. 3.** *hGPR40* is specifically expressed in human pancreas and brain, as demonstrated by quantitative reverse transcription-PCR. The cDNA from the reverse transcription of 1 ng of poly(A)+ RNA from multiple tissues from four different non-diseased individuals was analyzed by TaqMan RT-PCR for expression of *GPR40*. Data are presented as the means ± S.E. of four individuals mRNA levels for each tissue, except for the intestine, which is an equal pool of one intestine and another individual’s large intestine. Analyses of mostly peripheral tissues and brain region tissues were run as separate experiments. *PBMC*, peripheral blood mononuclear cells. A, expression of *GPR40* in human tissues; *B*, expression of *GPR40* in human brain regions; *C*, expression of *GPR40* in freshly isolated human immune cells and cell lines. *Raji* is a B-cell line; *Jurkat* is a T-cell line; and *THP1* is a monocytic cell line.

![Graph](image)

**TABLE II**

| Gene               | Forward primer (5' → 3') | Probe (FAM-5' → 3'-TAMRA) | Reverse primer (5' → 3') |
|--------------------|--------------------------|----------------------------|--------------------------|
| *hGPR40*           | caacctggttcggtctttgg     | aagctggtggacccacagaacac   | gtgtggtgatgecgagga       |
| mouse/rat *Gpr40*  | agtctccctggctcatctcata  | aatactctctctacggaccttg    | caagggcgaagaagcgagca     |
| mouse *Gpr40*      | tgcctcaagtgtgctagtt     | aaacctgtcagaggtctgg       | eectgtgatagtccacte       |
| Rat *Gpr40*        | cctataatgcttccaatgtggctagtt | tacataaacggaggtagaggtctgg | eectgtgatgeccaactc       |

* FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Ligands for GPR41 and GPR43, the other members of this receptor family (2). However, in contrast to GPR41 and GPR43, GPR40 is activated by fatty acids with carbon chain lengths of more than six. Although ligand pairing and G protein coupling experiments were performed with the free fatty acid in the absence of bovine serum albumin, no effects were observed in cells not containing GPR40, demonstrating that the response was not merely a detergent effect. Furthermore, the concentrations used are well below those at which fatty acids exert detergent effects on cell membranes (19).

The potency of the saturated fatty acids was chain length-dependent, with pentadecanoic acid (C15) and palmitic acid (C16) being the most potent, with pEC50 values of 5.18 ± 0.11 and 5.30 ± 0.12, respectively. Across the unsaturated fatty acids, potency did not appear to correlate with carbon chain length or degree of saturation. Of all the fatty acids tested, both saturated
and unsaturated, ETA was the most potent, with a pEC_{50} of 5.71 ± 0.11. The potencies for GPR40 of the fatty acids most predominant in plasma (oleate, palmitate, stearate, linoleate, and linolenate) are at reported physiologically relevant concentrations (20). However, whether GPR40 agonism by some of the fatty acids present at much smaller proportions of the total non-esterified fatty acids is important in the body is not clear.

ETA is commonly used as an inhibitor of 5- and 12-lipoxygenases in the range of 2–100 μM. ETA has also been reported to increase intracellular calcium in Madin-Darby canine kidney cells, with an EC_{50} of 20 μM, although treatment with the phospholipase C inhibitor U73122 suggested that this may not be due to production of inositol 1,4,5-trisphosphate (21). Expression of GPR40 in Madin-Darby canine kidney cells is unknown; and hence, whether the reported mobilization of [Ca^{2+}], or inhibition of lipoxygenase activity is mediated through GPR40 activation remains to be elucidated.

Given the number of fatty acids that are agonists for GPR40, it is conceivable that the physiologically relevant fatty acid for GPR40 may vary in a tissue-dependent fashion. Notably, several of the fatty acids shown to activate GPR40 have also been reported to activate a chimera of peroxisome proliferator-activated receptor-α and the glucocorticoid receptor in a reporter gene assay (18). Although there are similarities in the profile of fatty acids that activate the two receptors, including the fact that fatty acids with carbon chain lengths of less than six were inactive, there were also clear differences in relative potencies. For example, linolenic acid at 0.12 mM was the most efficacious activator of the fatty acids tested against the peroxisome proliferator-activated receptor-α-glucocorticoid chimera, whereas 0.12 mM lauric acid was much weaker; in contrast, these two fatty acids both had pEC_{50} values of 4.9 for GPR40 and the same maximum efficacies. Furthermore, the induction of [Ca^{2+}], in HEK293 cells or luciferase activity in CHO cells was clearly dependent on the presence of GPR40, as fatty acids did not elicit a response in cells not expressing the receptor.

Experiments using CHO cells expressing the Gal4-Elk1/5xGal4-luc^{+} reporter, responsive to G_{α_{5i2}} and G_{α_{μi1}}-coupled signaling, demonstrated that expression of GPR40 resulted in increased basal levels of luciferase activity. This reporter is activated following stimulation of MAPK activity, downstream of signals from G_{α_{5}} and G_{α_{μ}}-coupled pathways, which converge at Raf (14). The equivalent decrease in basal luciferase activity in host cells and hGPR40-Gal4-Elk1/5xGal4-luc^{+} cells following PTX treatment suggests that there may be an endogenous receptor present in both CHO-derived cell lines, producing a component of constitutive G_{α_{5}}-coupled activity. The residual elevation in luciferase activity in hGPR40-Gal4-Elk1/5xGal4-luc^{+} cells compared with host cells following PTX treatment suggests that this may be due to the constitutive activity of GPR40 through a G_{α_{μi1}}-coupled pathway. Treatment of the hGPR40-Gal4-Elk1/5xGal4-luc^{+} cells with ETA produced a dose-dependent increase in luciferase activity, with a pEC_{50} of 5.92 ± 0.05. PTX treatment did not alter the pEC_{50} of ETA in hGPR40-Gal4-Elk1/5xGal4-luc^{+} cells (without PTX, 5.92 ± 0.05; and with PTX, 5.99 ± 0.01). However, it did elevate the apparent efficacy of ETA, an effect that reached significance at concentrations of ETA between 1 and 2.25 μM. These data suggest that activation of GPR40 by ETA in hGPR40-Gal4-Elk1/5xGal4-luc^{−} cells is coupled to a G_{α_{q11}} pathway. Moreover, there may be a small PTX-sensitive inhibitory component to the ETA-mediated activation of the Gal4-Elk1/5xGal4-luc^{−} reporter used, the identification of which is unclear at present. Experiments using CHO cells transiently transfected with GPR40 and a luciferase reporter containing cAMP-response elements demonstrated that the receptor was unable to increase cAMP levels through a G_{α_{5}}-coupled pathway in response to ETA (data not shown).

TaqMan RT-PCR showed that GPR40 was specifically expressed in human brain and pancreas, with no expression detected in resting immune cells, apart from low levels in human monocytes. This pattern of expression clearly differentiates GPR40 from the other family members GPR41 and GPR43 (2), suggesting that its function has clearly diverged from that of its related receptors.

The pancreas is composed of exocrine and endocrine tissue. The endocrine cells or islets make up 1–2% of the pancreas. Comparison of mRNA expression abundance by TaqMan analysis illustrated that GPR40 was enriched in islets by 2–100-fold compared with the pancreas and that the receptor was present in isolated islets from mouse and rat pancreases. Islets contain four cell types: glucagon-producing α-cells, insulin-producing β-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-producing cells. The presence of Gpr40 in mouse islet insulinoma MIN6 cells suggested that the receptor may be in β-cells. Furthermore, the increased expression of Gpr40 in pancreases from ob/ob mice compared with control animals may have been due to the β-cell hyperplasia manifest in this animal model of obesity. Whether expression of Gpr40 plays an active role in the development of hyperplasia or is merely a reflection of this event is unclear. Further verification that Gpr40 mRNA was expressed in islet β-cells was accomplished using in situ hybridization. The pattern of Gpr40 expression was characteristic of β-cell rather than α-cell localization compared with immunohistochemistry results using antibodies to insulin and glucagon in parallel pancreatic sections. It remains unclear however, whether Gpr40 may be in pancreatic δ- or PP-cells.

Fatty acids are recognized to play an important role in maintenance of basal insulin secretion and potentiation of glucose-stimulated insulin secretion in the fasting state in both rodent and human islets (22–25). To stimulate insulin secretion, glucose enters the β-cell and is metabolized, resulting in an increase in intracellular ATP and a decrease in ADP levels. The increase in the ATP/ADP ratio closes cell-surface K_{ATP} channels, promoting membrane depolarization, opening of voltage-sensitive Ca^{2+} channels, and activation of insulin granule exocytosis. Glucose also augments, as well as initiates, the secretion of insulin through a pathway involving protein kinases A and C (26, 27). The mechanism by which fatty acids exert their potentiating effects has not yet been precisely defined. Some studies have suggested that intracellular metabolism of the fatty acid to its long chain acyl-CoA ester (LC-CoA) is critical for nutrient stimulation of insulin secretion (4, 28). Increases in LC-CoA following glucose administration or exposure of cells to fatty acids have been proposed to potentiate glucose-stimulated insulin exocytosis through a pathway that may involve phospholipid signaling and activation of protein kinase C. Over carbon chain lengths of C16–C18, the relative insulinotropic efficacy of fatty acids is positively correlated with chain length and degree of saturation (22, 23). It remains unclear, however, whether the fatty acid acts as a carboxylic acid or needs to be converted intracellularly into its CoA ester.
to exert its effect (30). The identification of fatty acids as ligands for GPR40 highlights the possibility that fatty acids could also exert some of their effects via an intracellular mechanism. However, the rank order of potency of fatty acids for GPR40 in HEK293 cells was not equivalent to the reported relative efficacy of fatty acids for increasing insulin secretion from perfused pancreas (22) or from islets of fasting rats or from human islets (23). Furthermore, all fatty acids that increased [Ca\(^{2+}\)] in HEK293 cells expressing GPR40 were equally efficacious. It is possible, however, that the fact that our studies were performed in the absence of bovine serum albumin, whereas other studies were performed in the presence of 1% bovine serum albumin, may be important.

We report that the GPR40 agonist ETA activated a G\(_{q}\)-coupled pathway in hGPR40-Gal4-Elk1/5xGal4-luc cells, which would be expected to lead to an increase in [Ca\(^{2+}\)], and protein kinase C and MAPK activation. Ligands (e.g., bombesin or acetylcholine) (31, 32) known to activate G\(_{q}\)-coupled receptors in islets potentiate glucose-stimulated insulin secretion. Thus, if the signaling pathway identified in CHO cells were to be replicated in islets, GPR40 activation may be anticipated to potentiate the effects of glucose on insulin secretion.

Prolonged exposure to elevated free fatty acids, which may occur in obesity or in states of insulin resistance, has, however, been found to be detrimental to \(\beta\)-cell function both in rodents and humans (33, 34). Because signaling pathways downstream of G\(_{q}\)-coupled GPCRs may include activation of MAPK and subsequent stimulation of transcriptional activity, it is also plausible that activation of GPR40 may play a role in the chronic rather than the acute effects of fatty acids. However, recent reports have demonstrated that the ability of fatty acids to cause apoptosis in the rat \(\beta\)-cell line RIN1046-38 and human islets is dependent on the degree of saturation. Whereas palmitic acid and stearic acid were pro-apoptotic when used at 1 mM for 24 h, linoleic acid, palmitoleic acid, and oleic acid did not cause apoptosis and actually protected the \(\beta\)-cells from apoptosis resulting from incubation with saturated fatty acids (35). Because a large range of both saturated and unsaturated fatty acids are agonists for GPR40, the primary role of the receptor may therefore not be mediation of the apoptotic effects of fatty acids.

Further clarification of the role of GPR40 in \(\beta\)-cell function in transgenic mouse models and analysis of receptor expression in islets from models of diabetes such as Zucker diabetic fatty (ZDF) rats, which have high levels of circulating fatty acids due to a defect in the leptin receptor. ZDF rat islets exhibit intrinsic defects in their ability to secrete insulin in response to glucose (36), which deteriorates progressively as fatty acids accumulate in the islets, resulting in apoptosis or “lipotoxicity” (37).

GPR40 is also expressed ubiquitously in human brain, with the highest expression in the substantia nigra and medulla oblongata. Notably, docosahexaenoic acid (DHA), known since the 1960s (38) to be present at a high level in mammalian brain, e.g., in astroglia (37). Also found to have an anti-apoptotic effect on neuronal apoptosis (40). Although apoptosis is a critical part of neural development, it also plays a fundamental role in the neurological disorders of aging, viz. Alzheimer’s disease, Parkinson’s disease, and stroke (41). The mechanism behind the anti-apoptotic effect of DHA is currently unclear, but has been proposed to involve an increase in phosphatidylserine synthesis and gene expression. Although DHA tends to be retained by membrane phospholipids, astro-
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