The G-Protein–Coupled Receptor GPR40 Directly Mediates Long-Chain Fatty Acid–Induced Secretion of Cholecystokinin

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BACKGROUND & AIMS: Long-chain fatty acid receptors G-protein–coupled receptor 40 (GPR40) (FFAR1) and GPR120 have been implicated in the chemosensation of dietary fats. I cells in the intestine secrete cholecystokinin (CCK), a peptide hormone that stimulates digestion of fat and protein, but these cells are rare and hard to identify. We sought to determine whether dietary fat-induced secretion of CCK is directly mediated by GPR40 expressed on I cells. **METHODS:** We used fluorescence-activated cell sorting to isolate a pure population of I cells from duodenal mucosa in transgenic mice that expressed green fluorescent protein under the control of the CCK promoter (CCK–enhanced green fluorescent protein [eGFP] bacterial artificial chromosome mice). CCK-eGFP cells were evaluated for GPR40 expression by quantitative reverse transcription polymerase chain reaction and immunostaining. *GPR40*$^{-/-}$ mice were bred with CCK-eGFP mice to evaluate functional relevance of GPR40 on long-chain fatty acid–stimulated increases in [Ca^{2+}]i and CCK secretion in isolated CCK-eGFP cells. Plasma levels of CCK after olive oil gavage were compared between *GPR40*$^{+/+}$ and *GPR40*$^{-/-}$ mice. **RESULTS:** Cells that expressed eGFP also expressed GPR40; expression of GPR40 was 100-fold greater than that of cells that did not express eGFP. In vitro, linoleic, oleic, and linolenic acids increased [Ca^{2+}]i; linolenic acid increased CCK secretion by 53% in isolated *GPR40*$^{+/+}$ cells that expressed eGFP. In contrast, in *GPR40*$^{-/-}$ that expressed eGFP, [Ca^{2+}]i response to linoleic acid was reduced by 50% and there was no significant CCK secretion in response to linolenic acid. In mice, olive oil gavage significantly increased plasma levels of CCK compared with pregavage levels: 5.7-fold in *GPR40*$^{+/+}$ mice and 3.1-fold in *GPR40*$^{-/-}$ mice. **CONCLUSIONS: Long-chain fatty acid receptor GPR40 induces secretion of CCK by I cells in response to dietary fat.**

Keywords: Fat Metabolism; Hormone Secretion; FACS Analysis; Digestion.

Lipids and digested lipid products have long been known to stimulate satiety, which is partially mediated by secretion of the neuropeptide hormone cholecystokinin (CCK) from duodenal enteroendocrine I cells. The postprandial satiety effect of CCK is vagally mediated upon binding of CCK_1 receptors on afferent nerve terminals that are closely associated with the enteroendocrine cell; gastrointestinal feedback functions such as inhibition of gastric emptying and gastric acid secretion and stimulation of exocrine pancreas secretion in response to luminal nutrients are also regulated in this manner,[1] whereas CCK regulates gall bladder contraction through a hormonal route.[2]

Products of lipid hydrolysis, particularly long-chain fatty acids (LCFAs) of at least 12-carbon length, are required to stimulate CCK secretion in the murine enteroendocrine cell line STC-1[3] and to elevate plasma CCK and reduce gastric motility in humans.[4,5] The sensing mechanism by which the I cell detects LCFAs and triggers CCK secretion is unknown, but given that the apical membrane of the intestinal I cell communicates with the lumen, it is possible that a direct sensing mechanism exists. Several G-protein–coupled receptors (GPRs) have been identified as fatty acid sensors with nutrient-sensing capabilities by endocrine cells[6,7]; in particular, the LCFA receptors GPR40 and GPR120 have been suggested as possible nutrient detectors mediating CCK secretion.[8,9]

GPR40 is a recently deorphanized GPR that is activated by a range of medium- to long-chain saturated and unsaturated fatty acids of chain lengths >6 carbons.[10] GPR40 is highly expressed in pancreatic islets and has been extensively studied for its role in insulin secretion by mouse pancreatic β cells (MIN6) in response to unsaturated LCFAs, oleic, linoleic, and linolenic acids.[11,12] Multiple other organs, including the brain and intestine in rats and humans, have also been shown to express the transcript for GPR40.[10,12] Using GPR40 reporter mice and in situ hybridization, GPR40 has recently been colocalized with several enteroendocrine cell types through-

Abbreviations used in this paper: apoA-IV, apolipoprotein A-IV; BAC, bacterial artificial chromosome; CCK, cholecystokinin; eGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; FFA, free fatty acid; FI, fluorescence intensity; GPR, G-protein–coupled receptor; LCFA, long-chain fatty acid; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction.

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out the intestine—including glucagon-like peptide 1, glucose insulinotropic peptide, and CCK-expressing cells—and although disruption of GPR40 attenuates incretin secretion in mice fed a high-fat diet,[13] the role of GPR40 on CCK secretion in the native I cell has yet to be evaluated.

The purpose of this study was to elucidate the basis for the chemosensation of dietary fat-stimulated release of CCK by intestinal I cells. Using GPR40$^{-/-}$ mice, we provide both in vitro and in vivo evidence that CCK secretion is stimulated by dietary luminal LCFAs directly sensed by GPR40 expressed on duodenal enteroendocrine I cells.

Materials and Methods

Experimental Animals

Transgenic mice with CCK promoter-driven enhanced green fluorescent protein (eGFP) were developed by the GenSat Bacterial Artificial Chromosomes (BAC) Transgenic project[14] and obtained from the Mutant Mouse Regional Resource Center (Davis, CA).

A GPR40-targeted knockout mouse was developed by replacing the GPR40 coding region with a 21-nucleotide DNA fragment encoding genes for 9 amino acids of influenza hemagglutinin antigen, eGFP, and neomycin (Supplementary Figure 1*A*). eGFP was inserted with the intended use as a reporter for GPR40 expression within the intestine; however, histological examination of GPR40$^{-/-}$ mice did not yield any intestinal eGFP signals by epifluorescent microscopy or by flow cytometric analysis of an intestinal mucosal cell preparation (data not shown). Weakly detectable eGFP in pancreatic β cells suggested that undetectable I cell eGFP was due to lower copy number. Homozygous deletion of GPR40 was confirmed by polymerase chain reaction and Southern blot of genomic tail DNA and by Taqman reverse transcriptase polymerase chain reaction (RT-PCR) of duodenal mucosal scrapings (Supplementary Figure 1*B*–*D*), validating this mouse strain to be a GPR40 knockout model.

The GPR40$^{-/-}$ mice had no obvious phenotype when fed a regular chow diet. Knockout animals were fertile and had body weights and body compositions similar to their wild-type littermates. Metabolically there was no difference in fasting serum glucose, triglyceride, and insulin. In addition, consistent with findings reported by others,[15–18] glucose tolerance and insulin levels were similar between GPR40$^{+/+}$ and GPR40$^{-/-}$ mice fed a regular chow diet.

For functional studies, GPR40$^{-/-}$ mice were bred to CCK-eGFP mice to produce CCK-eGFP^{+} GPR40$^{+/-}$ pups. CCK-eGFP^{+} GPR40$^{+/-}$ mice were bred to produce GPR40$^{-/-}$ and GPR40$^{+/+}$ mice expressing CCK-eGFP cells. See Supplementary Methods for genotyping details. Animals were bred and maintained on regular chow according to the National Institutes of Health Institutional Animal Care and Use Committee guidelines.

Isolation of Intestinal Endocrine Cells

Adult mice were euthanized and the proximal 5 to 6 cm of duodenum collected and confirmed for eGFP expression by epifluorescent microscopy. Intestines were washed with cold Dulbecco's phosphate-buffered saline (PBS) and incubated in 1 mM EDTA-Dulbecco's PBS, followed by 75 U/mL collagenase (CLPSA grade; Worthington Biochemical, Lakewood, NJ,) in a shaking water bath (20′, 37°C each). Cells were resuspended in 10% fetal bovine serum in medium, filtered through 30-μm and 20-μm nylon filters (Spectrum Laboratories, Laguna Hills, CA) to obtain a single-cell population and resuspended in a fluorescence-activated cell sorting (FACS) buffer (5% fetal bovine serum, 50 ug/mL DNase I in phenol-free Dulbecco's modified Eagle's medium). Cells were ~90% viable based on trypan blue exclusion.

The sample underwent FACS using the BD FACS ARIA machine (BD Biosciences, San Jose CA). Cells were sorted based on initial gating for GFP (fluorescein isothiocyanate) with sequential gating based on side and forward scatter to exclude dead cells and doublets. For RT-PCR studies, a non-eGFP cell population (fluorescein isothiocyanate $<10^2$)—comprising a mixture primarily of enterocytes, but also of goblet cells, lymphocytes, and non-GFP endocrine cells—was simultaneously sorted into a separate collection tube. Immediately after FACS, cells were found to be ~78% live using 7-aminoactinomycin D (7-AAD) dead-cell exclusion. Of the live-cell population, post-sorted cells were at least 98.5% eGFP^{+}, which was confirmed by trypan blue exclusion and direct epifluorescent microscopy.

Immunofluorescent Staining

Single-cell preparations obtained before and after FACS were fixed in fresh 4% paraformaldehyde/PBS. Cells were dried on positively charged slides, incubated with rabbit anti-CCK antibody (1:2000; Peninsula Labs, Torrance, CA), and subsequently AlexaFluor 594 goat anti-rabbit IgG secondary antibody (Invitrogen, Carlsbad, CA).

For GPR40 immunostaining, villus-enriched fractions were obtained from the proximal duodenum by incubation in Dulbecco's PBS containing 2 mM EDTA (30 minutes, 4°C) followed by gentle pipetting and centrifugation (250*g*, 2 minutes). Villus samples were immediately incubated with mouse anti-GPR40 monoclonal antibody (1:10; 30 minutes, 4°C; TransGenic Inc, Kumamoto, Japan), washed, incubated with AlexaFluor 633 goat anti-mouse IgG antibody (4°C, 30 minutes; Invitrogen), washed, and fixed with 4% paraformaldehyde/PBS.

For whole tissue sections, duodenum and pancreas tissue were collected from 4% paraformaldehyde/PBS perfused mice. Ten-micrometer cryosections were permeabilized with 0.2% TritonX-100, blocked in 5% bovine serum albumin, and incubated with rabbit anti-CCK (1:1000; Peninsula Labs, Torrance, CA) followed by AlexaFluor 594 goat anti-rabbit IgG antibody (Invitrogen). Slides