Short-Chain Fatty Acids Stimulate Glucagon-Like Peptide-1 Secretion via the G-Protein–Coupled Receptor FFAR2

Gwen Tolhurst,¹ Helen Heffron,² Yu Shan Lam,¹ Helen E. Parker,¹ Abdella M. Habib,¹ Eleftheria Diakogiannaki,¹ Jennifer Cameron,² Johannes Grosse,² Frank Reimann,¹ and Fiona M. Gribble¹

Interest in how the gut microbiome can influence the metabolic state of the host has recently heightened. One postulated link is bacterial fermentation of “indigestible” prebiotics to short-chain fatty acids (SCFAs), which in turn modulate the release of gut hormones controlling insulin release and appetite. We show here that SCFAs trigger secretion of the incretin hormone glucagon-like peptide (GLP)-1 from mixed colonic cultures in vitro. Quantitative PCR revealed enriched expression of the SCFA receptors ffar2 (gpr43) and ffar3 (gpr41) in GLP-1-secreting L cells, and consistent with the reported coupling of GPR43 to Gq signaling pathways, SCFAs raised cytosolic Ca²⁺ in L cells in primary culture. Mice lacking ffar2 or ffar3 exhibited reduced SCFA-triggered GLP-1 secretion in vitro and in vivo and a parallel impairment of glucose tolerance. These results highlight SCFAs and their receptors as potential targets for the treatment of diabetes.

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Targeting the release of anorectic and antidiabetic gut peptides is the focus of many ongoing drug development programs, as evidence is accumulating that enhanced secretion of glucagon-like peptide (GLP)-1 and peptide YY (PYY) from intestinal L cells may translate into beneficial effects in subjects with diabetes and obesity (1). Already, the diabetes field has witnessed the impact of therapeutic GLP-1 mimetics and dipeptidyl peptidase (DPP)⁴ inhibitors, which, respectively, mimic endogenous active GLP-1 and slow its enzymatic degradation in the circulation (2). The injectable GLP-1 mimetics, in particular, are associated not only with improved blood glucose control and reduced incidence of hypoglycemia but also with significant weight reduction (3). The observed correlation between elevated postprandial GLP-1 levels and improved glucose homeostasis in patients after bariatric surgery indicates that the body has excess capacity in the GLP-1 axis that can be recruited in obese diabetic patients, with downstream beneficial effects on food intake and diabetes control (4). Identifying and validating pharmaceutical strategies to enhance GLP-1 secretion are central to many ongoing L cell–targeting research programs.

L cells are a component of the enteroendocrine system, diffusely located along the length of the intestinal epithelium (5). They make contact with the gut lumen via apical processes and are believed to respond directly to luminal signals. Among the best characterized triggers of GLP-1 secretion are sugars, amino acids, and long-chain fatty acids, which stimulate L cells by a variety of pathways including transporter-associated uptake, metabolism, and G-protein–coupled receptor activation (5,6). L cells are, however, found in highest density in the colonic epithelium, where these nutrients are unlikely to reach significant concentrations (7). Short-chain fatty acids (SCFAs), derived from bacterial fermentation of macrofibrous material reaching the distal gut, by contrast are known to reach high concentrations under physiological conditions in the colons of healthy subjects. Intraluminal concentrations beyond 100 mmol/L, comprising ~60% acetate (C2), 25% propionate (C3), and 15% butyrate (C4), have been reported (8). Plasma concentrations of SCFA are also dominated by acetate but are generally below ~200 μmol/L unless elevated by ethanol metabolism (9,10). Nondigestible and fermentable dietary fiber, as well as SCFAs themselves, has been shown to increase GLP-1 secretion in humans (11,12) and rodents (13,14), and enhanced PYY release has been proposed as a link between luminal SCFAs and altered gut motility (15,16).

SCFAs act as a local nutrient source but can also trigger cell-specific signaling cascades by activation of the G-protein–coupled free fatty acid receptor (FFAR)² (GPR43) and FFAR3 (GPR41) (17,18). These two receptors share ~40% amino acid sequence similarity and are conserved across several mammalian species (17–19). Both receptors respond to SCFAs containing two to five carbons, although a preference of FFAR2 for C2 and C3 fatty acids and of FFAR3 for C3–C5 carbon chain lengths has been reported (17,18,20). The receptors differ in their intracellular signaling capabilities, with FFAR2 reportedly coupling to either G₃₉ or G₅₃ and FFAR3 exclusively activating G₅₃ pathways (17,18,20). The finding that both receptors are located in colonic L cells by immunostaining (21–23) suggests that SCFAs may use this pathway to modulate L-cell function. Experimental data in support of this idea are, however, still lacking.

The aim of the current study was to establish and explore the link between SCFAs and GLP-1 secretion, making use of our transgenic mouse model (GLU-Venus) in which L cells are identifiable by their expression of a yellow fluorescent protein derivative, Venus (24).
RESEARCH DESIGN AND METHODS

All animal procedures were approved by the local ethics review committees and conformed to Home Office regulations. Experiments were performed using mice on a C57B/6 background, except in the case of the ffar2<sup>−/−</sup> and ffar3<sup>−/−</sup> mice and littermate controls, which were on a 129/SvEv background.

**Generation of knockout mice.** For generation of the targeting vectors, homology arms (ffar2, 1,250 base pairs [bp] 5' and 3,914 bp 3'; ffar3, 1,761 bp 5' and 4,199 bp 3') were PCR amplified from genomic DNA and cloned into a vector containing internal ribosomal entry site β-gal reporter gene, neo selection marker, and two thymidine kinase negative selection markers (Supplementary Figs. 1 and 2). The linearized vector was electroporated into embryonic stem cells (129/SvEv), and homologous recombination was detected by screening PCR and Southern blot. Chimeras were generated by blastomy injection, bred with 129/SvEv animals, and maintained inbred in this background.

**Glucose-stimulated GLP-1 secretion in vivo.** Experiments were performed on 3- to 4-month-old ffar2<sup>−/−</sup> and ffar3<sup>−/−</sup> mice or wild-type littermate control 129/SvEv mice. No significant differences in body weight were observed between the wild-type and knockout groups. Mice were fasted for 4 h and dosed per os with 20 mg/kg DPP4 inhibitor (cat. no. KR-62436; Sigma). Thirty minutes later, mice were dosed by gavage with 1.5 g/kg glucose solution (Fisher Scientific). For initial GLP-1 measurement, 150 μL blood was collected from awake mice via tail bleed into EDTA-coated capillary tubes (Bilbate) before glucose dosing. Thirty minutes after glucose dosing, mice were killed by CO<sub>2</sub> inhalation and blood was collected via cardiac puncture into tubes containing aprotinin (0.6 trypsin inhibiting units/mL). Blood samples were centrifuged immediately, and plasma was frozen on dry ice before assay in duplicate for active GLP-1 (MesoScale, Gaithersburg, Maryland).

**Oral glucose tolerance test.** Three- to four-month-old ffar2<sup>−/−</sup>, ffar3<sup>−/−</sup>, and wild-type littermate control 129/SvEv mice were fasted for 14 h and then dosed by gavage with 1.5 g/kg glucose. Blood glucose was measured using a handheld glucometer (One Touch Ultra) via tail bleed. Samples for insulin measurements were taken via tail bleed from awake mice into heparinized capillary tubes (Bilbate) and assayed by ELISA (Crystalchem Ultra sensitive mouse insulin ELISA). Mice undergoing the procedure were of similar body weight: ffar2<sup>−/−</sup>, 22.5 ± 0.4 g (n = 11), wild type, 21.5 ± 0.5 g (n = 8); ffar3<sup>−/−</sup>, 22.7 ± 1.5 g (n = 6), and wild type, 20.8 ± 0.6 g (n = 7).

**Insulin tolerance test.** Three- to four-month-old ffar2<sup>−/−</sup>, ffar3<sup>−/−</sup>, and wild-type littermate control 129/SvEv mice were fasted for 4 h and then dosed with 0.75 units/kg insulin i.p. (Actrapid insulin, supplied by our Named Veterinary Surgeon). Blood glucose was measured using a handheld glucometer (One Touch Ultra) via tail bleed from awake mice.

**Colonic tissue and cell preparation.** Mice aged 6–26 weeks were killed by cervical dislocation, and colons were collected in ice-cold Leibovitz-15 medium. Cultures for secretion and calcium imaging experiments were prepared as previously described (24). Colonic tissue used for RNA and protein extraction was washed in PBS and placed in RNA later or a protein lysis buffer, respectively, and frozen until processed.
GLUTag cells were cultured in Dulbecco’s modified Eagle’s medium (5.5 mMol/L glucose) supplemented with 10% FBS, 2 mMol/L L-glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin.

RNA extraction and quantitative PCR. Total RNA from cells sorted with fluorescence-activated cell sorter (FACS) prepared from GLUT-1 transgenic mice (24) was isolated using a microscale RNA isolation kit (Ambion). Total RNA from GLUTag cells and murine colonic tissue was prepared following the Tri-Reagent protocol (Sigma). All samples were reverse transcribed according to standard protocols. Quantitative RT-PCR was performed with a 7000 HT Fast-Real-Time PCR system (Applied Biosystems) using Taqman probes for β-actin, pre pro, flk2, and tfr2 from Applied Biosystems. Expression was compared with that of β-actin measured in parallel on the same sample, giving a ΔCt for β-actin minus the test gene. If the test gene was undetectable, it was assigned a Ct value of 40. Means ± SE were calculated and statistics were performed for the ΔCt data and only converted to relative expression levels (2-ΔΔCt) for presentation in the Figures.

Colonc protein analysis. Tissue was mechanically homogenized in lysis buffer. Active GLP-1 was assessed by ELISA (Eliisa Millipore, Watford, U.K.) and expressed relative to total protein content, measured using a Bradford assay (Sigma).

Secretion from primary mixed colonic culture. Secretion studies were performed 24–36 h after culture preparation. Where applicable, cultures were preincubated with 0.2 μg/mL pertussis toxin for 18 h. Cultures were washed with standard saline and incubated with test substances for 20 s. Glucose-secreted and cellular GLP-1 was extracted as previously described (24), and active GLP-1 was quantitated by ELSIA (Millipore). GLP-1 secretion was expressed as a fraction of the total hormone (secreted plus extracted) and normalized to basal secretion measured in the same set of experiments.

Ca2+ imaging. Experiments were performed 4–7 days postplating, using colonic tissue from GLUT-1 transgenic mice with L-cell-specific Venus expression (24). Cells were loaded with 7 μM Fura2-AM (Molecular Probes, Paisley, U.K.), 0.01% pluronic F127, and 300 mMol/L glucose in essential sodium saline solution for 30 min at 37°C. Single-cell imaging was performed using an in vitro fluorescence microscope (Olympus IX71; Southall, U.K.) with a 4× oil-immersion objective. Time-lapse imaging was performed using MetaFluor software (Molecular Devices, Wokingham, U.K.). Emission was captured using an Orca ER CCD camera (Hamamatsu, Welwyn Garden City, U.K.) using a dichroic mirror and a 510-nm long-pass filter. Test substances were added to the bath solution and per fused at 1–10 μM. Fura2 fluorescence measurements were taken every 2 s, background corrected, and expressed as the 340/380 nm ratio. Average fluorescence ratios from individual cells were determined over 20-s periods before addition and during perfusion of a test agent. Peak responses to a test agent were expressed as the mean ratio of the test agent divided by the averaged ratios measured prior to drug application.

Solutions. Standard in vitro saline solution contained (in millimoles per liter) 4.5 KCl, 138 NaCl, 4.2 NaHCO3, 1.2 NaH2PO4, 2.6 CaCl2, 1.2 MgCl2, 10 glucose, and 10 HEPES, pH 7.4 (NaOH). Essentially fatty acid–free BSA (0.1%) was added to solutions used for static secretion experiments. SCFAs were dissolved directly in saline solution and pH corrected if necessary. All other drug solutions were prepared by dissolving the compounds in DMSO as a 1,000 μg/mL mixture (25) and diluted with 100% DMSO just prior to use. The synthetic GPR43 agonist was synthesized, its identity was confirmed by nuclear magnetic resonance analysis, and it was dissolved in DMSO as a 1,000 μg/mL mixture.

RESULTS

SCFAs enhance GLP-1 release. In primary murine colonic cultures, acetic and propionic acid (1 mMol/L) significantly stimulated GLP-1 secretion over a 2-h incubation period (Fig. 1A). Secretion was further enhanced in the presence of 100 μM CFMB, a Gq-coupled pathway activator, which is a known stimulator of Ca2+ mobilization in L cells and enhance GLP-1 secretion under these conditions (Fig. 2A). CFMB (25) could also mobilize Ca2+ and found that 30 μMol/L CFMB indeed elevated intracellular Ca2+ in L cells (Fig. 2D). A smaller but significant increase in calcium was also observed in the non–L-cell population. To evaluate any opposing contribution from Gs signaling pathway, we examined GLP-1 release in the presence of the Gs inhibitor pertussis toxin. Whereas SCFAs were stimulatory in the presence of IBMX (Fig. 1D), somatostatin (100 μMol/L), used as a positive control for G_{i}-coupled pathways, abolished GLP-1 secretion under these conditions (n = 3, P < 0.001) (Fig. 4A), an effect that was partially reversed by preincubation with 0.2 μg/mL pertussis toxin (n = 3, P < 0.001). By contrast, pertussis toxin did not have a significant effect on the response to 1 mMol/L propionate (Fig. 4B), suggesting that there is little signaling by SCFA through G_{i}-coupled pathways during 2-h secretion studies.
by 70% (P < 0.001) and the response to acetate was abolished (P < 0.001) in the ffar2 knockout tissue. Knockout of ffar3 also impaired secretory responses to both acetate and propionate (P < 0.01) (Fig. 5A), although to a lesser extent than ffar2 knockout. Similar, but more pronounced, effects were observed in the presence of 100 μmol/L IBMX (Fig. 5A). The higher concentration of mixed SCFA (140 mmol/L, as described above) also failed to enhance GLP-1 secretion significantly in both the ffar2 and ffar3 knockout mouse models (Fig. 5B).

Analysis of mRNA expression in whole colonic tissue extracts from wild-type and knockout mice revealed that, as expected, ffar2<sup>−/−</sup> mice lacked ffar2 mRNA and ffar3<sup>−/−</sup> mice lacked ffar3 (Fig. 5C and D). ffar3 expression was also significantly decreased in ffar2<sup>−/−</sup> mice, but, although we also observed a trend toward a reduced ffar2 expression in ffar3<sup>−/−</sup> mice, this did not reach statistical significance.

**ffar2 knockout decreases GLP-1 content.** To examine whether the receptors have additional effects on L cells operating over a longer time scale, we examined whether colonic tissue from mice lacking ffar2 or ffar3 had altered levels of mRNA for glucagon (gcg) (which includes the coding sequence for GLP-1), pyy, or active GLP-1 peptide. Knockout of ffar3 did not have a significant effect on colonic gcg mRNA or active GLP-1. There was a trend toward reduced colonic gcg and pyy mRNA expression in the ffar2 receptor knockout model that did not reach statistical significance (Fig. 5E and F). ffar3-deficient mice, however, had significantly reduced colonic GLP-1 protein content (n = 6, P < 0.05) (Fig. 5G).
FFAR2 and FFAR3 affect GLP-1 release in vivo. To evaluate whether the reduced in vitro GLP-1 secretory capacity of ffar2–/– and ffar3–/– deficient mice translates into impaired hormone secretion in vivo, we examined plasma GLP-1–level responses to oral glucose administration. Basal levels of active GLP-1 were reduced by ~40% in ffar2–/– deficient mice (P < 0.01) (Fig. 6A) and were also lower, although not significantly, in ffar3–/– deficient mice compared with those in wild-type littermate controls. Following an oral glucose load, more pronounced impairments of plasma GLP-1 responses were observed in both ffar2–/– and ffar3–/– knockout models (n = 5, P < 0.05) (Fig. 6A). Coinciding with the reduced circulating GLP-1 concentrations, ffar2–/– and ffar3–/– deficient mice also exhibited impaired glucose tolerance when tested with gavage administration of 1.5 g/kg glucose (Fig. 6B and C). This correlated with reduced plasma insulin levels, although again this reached significance in mice lacking ffar2 but not ffar3 when compared with their respective littermate controls (Fig. 6D and E). To test for possible differences in insulin sensitivity, ffar2–/– and ffar3–/– mice and littermate controls were injected with 0.75 units/kg insulin after a 4-h fast. No significant differences were found between the different genotypes (Fig. 6F and G).

**DISCUSSION**

Our results demonstrate a direct link between SCFA activation of FFAR2, elevation of intracellular Ca2+ in L cells, and enhanced GLP-1 secretion from primary colonic cultures. A stimulatory role for FFAR2 in vivo is supported by the finding that knockout of ffar2 lowers both basal and glucose-stimulated GLP-1 concentrations.

By quantitative PCR, we could demonstrate that expression of mRNA for ffar2 and ffar3 is enriched in L cells, consistent with the detection of these receptors in PYY- and GLP-1–positive cells in rat and human colon by immunohistochemistry (21–23). Interestingly, ffar2 expression was very low in the GLP-1–secreting cell line GLUTag, perhaps explaining the poor responsiveness of GLUTag cells to SCFAs (data not shown) and emphasizing the importance of studying primary L cells in parallel with cell line models.

FFAR2 reportedly couples to Gq or G12 signaling pathways and FFAR3 exclusively to Gi. The finding that GLP-1 secretion is enhanced, rather than inhibited, by SCFAs suggests that Gq-coupled pathways predominate over any Gi coupling. Indeed, we did not observe any evidence that Gi coupling blunts SCFA-triggered GLP-1 secretion.
Following an overnight fast, mice were given 1.5 g/kg glucose per os, and blood glucose was measured at the time points indicated. No significant differences between genotypes were observed. Data in B-G represent means ± 1 SEM, and statistical significance was assessed by two-way ANOVA with repeated measures: *P < 0.05, **P < 0.01, and ***P < 0.001.

either in secretion studies with added pertussis toxin or after knockout of ffar3. Somatostatin, by contrast, inhibited IBMX-triggered GLP-1 release in a pertussis toxin–sensitive manner, indicating that downstream G_{i}-coupled pathways are globally functional in L cells.

The idea that FFAR2 is coupled to G_{q}-signaling pathways in L cells is suggested by the Ca^{2+} imaging data, which showed that acetate and propionate triggered intracellular Ca^{2+} responses in the L-cell population. In support of this idea, knockout of ffar2 abolished SCFA-triggered GLP-1 secretion in vitro. The explanation for the blunted GLP-1 secretory response to SCFA in cultures from ffar3^{−/−} mice is less clear, as FFAR3 has not been reported to couple to stimulatory G_{q} or G_{s}-signaling pathways. One contributing factor could be the reduced ffar2 expression that was observed in ffar3^{−/−} mice, although this did not reach statistical significance in our samples. Reduced expression of ffar2 has, however, previously been reported in adipose tissue of ffar3 knockout mice (26). Alternatively, there may be a previously unidentified component to FFAR3 signaling in L cells that also contributes to SCFA-triggered GLP-1 secretion. This is not, however, supported by the observation that stimulation of GLP-1 release was not observed in GLUTag cells, which express ffar3 but very little ffar2. A dominant role of FFAR2 over FFAR3 in SCFA-triggered L-cell activation is further suggested by the acute Ca^{2+}-elevations seen in primary L cells in response to the FFAR2-specific agonist CFMB, which reportedly does not have activity against FFAR3 (25).

It is not known whether FFAR2 and FFAR3 reside on the apical or basolateral membrane of L cells or whether they primarily detect luminal or plasma SCFA. SCFA levels in the colonic lumen are in the area of 100 mmol/L. Although this is considerably above the half maximal effective concentrations (EC_{50}s) of FFAR2 and FFAR3 for SCFA (0.5–1 mmol/L) (17,18,20), SCFA concentrations may be lower in the immediate vicinity of the L cells as a result of the diffusional barrier provided by the mucous layer and active uptake by neighboring enterocytes. Circulating plasma SCFA concentrations are in the range of 100–200 μmol/L (10), which is closer to the working range of the receptors, and while most studies have examined how luminally applied SCFAs affect GLP-1 release (14,27,28) it has also been shown that systemically infused acetate can enhance GLP-1 secretion (28). As luminal SCFA concentrations are not predicted to change markedly in response to acute food ingestion, it is possible that SCFAs produced by colonic fermentation provide a chronic stimulatory tone on L cells via apical or basolaterally located SCFA receptors. This could account for the circulating levels of active GLP-1 that are detectable even in the fasting state and amplify the responses to ingested nutrients, mediated, for example, via neurohormonal signals triggered by nutrient arrival higher up the gastrointestinal tract.

Consistent with the impaired responsiveness of ffar2^{−/−} and ffar3^{−/−} colonic cultures to SCFAs in vitro, we also observed lower GLP-1 levels in the knockout mice in vivo. As ffar2^{−/−} mice exhibited both reduced colonic GLP-1 content and impaired SCFA-triggered secretion in vitro, we cannot conclude from our data which of these components underlies the reduced basal and glucose-triggered GLP-1 secretory responses in vivo. In ffar3^{−/−} mice, however, the colonic GLP-1 content was not reduced, suggesting that the lower plasma GLP-1 levels reflect the impaired SCFA-triggered secretory response.

In addition to having low circulating GLP-1 levels, ffar2^{−/−} and ffar3^{−/−} mice exhibited impaired glucose tolerance. The
reduced GLP-1 concentrations are likely to contribute to the impaired glucose homeostasis but may not be the only cause because ffar2 and ffar3 are not exclusively expressed in intestinal cells. In fact, they are better characterized as mediators of SCFA effects on immune cells and adipocytes (17,18,20), where they have been implicated in modulating neutrophil activation (18,20), reducing inflammation by inhibition of cytokine and chemokine expression, enhancing leptin production (29), stimulating adipogenesis, and inhibiting lipolysis (30,31). They are also expressed in pancreatic β and α cells (G. Tolhurst, H. Parker, A. Habib, F. Reimann, and F. Gribble, unpublished observations). Global knockout of these receptors may therefore affect adipocyte function, inflammation, or pancreatic β-cell function, which would themselves impact on glucose tolerance. The fact that we observed reduced insulin levels in the ffar2fl/fl mice during an oral glucose tolerance test but did not observe differences in insulin tolerance indicates that the observed impaired glucose tolerance in our model reflects an impairment of insulin secretion, possibly in part due to an impaired incretin axis. Another ffar2fl/fl mouse model was recently reported to exhibit increased food intake but had lower body weight and insulin levels compared with wild-type controls after a prolonged period on a high-fat diet (32). No explanation was found for the increased food intake, but our data suggest that it may be related to reduced secretion of L-cell peptides like GLP-1 and PYY. Unlike the findings of our study, those of Bjursell et al. (32) did not show a significant difference in glucose tolerance between chow-fed ffar2fl/fl and control mice. The apparent differences between the two knockout models may reflect the fact that the mice were maintained on different genetic backgrounds (C57B/J6OlHsd vs. 129/SvEv) or that they were studied at different ages.

Interest in the gut microbiome has heightened recently, with the recognition that our complement of colonic bacteria may not simply reflect factors such as diet and illness but can also in return influence food intake and metabolism. Probiotics, prebiotics, and high-fiber diets are promoted for their potential beneficial effects on obesity, diabetes, inflammation, and immunity, as well as local effects on the health and integrity of the gut. One potential link between colonic microflora and systemic activity are SCFAs, which are produced by bacterial fermentation of fiber and which not only act as a local nutrient source but also trigger the release of anorectic hormones like GLP-1 and PYY. Our data suggest that SCFAs produced by bacterial fermentation in the gut can directly influence L cells to enhance the release of peptides such as GLP-1 and PYY. Whether this interrelationship is altered by metabolic conditions such as diabetes and obesity remains to be determined. FFAR2 should perhaps be added to the list of target receptors that may be exploitable for the pharmacological stimulation of the enteroendocrine system.

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G.T., H.H., Y.S.L., H.E.P., A.M.H., E.D., and J.C. researched data. J.G. designed the study. F.R. and F.M.G. designed the study and wrote the manuscript.

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