

Short-chain fatty acid sensing in rat duodenum

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Key points

- Luminal lipid in the duodenum modulates gastroduodenal functions via the release of gut hormones and mediators such as cholecystokinin and 5-HT.
- The effects of luminal short-chain fatty acids (SCFAs) in the foregut are unknown.
- Free fatty acid receptors (FFARs) for long-chain fatty acids (LCFAs) and SCFAs are expressed in enteroendocrine cells. SCFA receptors, termed FFA2 and FFA3, are expressed in duodenal enterochromaffin cells and L cells, respectively.
- Activation of LCFA receptor (FFA1) and presumed FFA3 stimulates duodenal HCO₃⁻ secretion via a glucagon-like peptide (GLP)-2 pathway, whereas FFA2 activation induces HCO₃⁻ secretion via muscarinic and 5-HT₄ receptor activation.
- The presence of SCFA sensing in the duodenum with GLP-2 and 5-HT signals further supports the hypothesis that luminal SCFA in the foregut may contribute towards the generation of functional symptoms.

Abstract Intraduodenal fatty acids (FA) and bacterial overgrowth, which generate short-chain FAs (SCFAs), have been implicated in the generation of functional dyspepsia symptoms. We studied the mechanisms by which luminal SCFA perfusion affects duodenal HCO₃⁻ secretion (DBS), a measure of mucosal neurohumoral activation. Free fatty acid receptor (FFAR) 1 (FFA1), which binds long-chain FA (LCFA), and SCFA receptors FFA2 and FFA3 were immunolocalised to duodenal enteroendocrine cells. FFA3 colocalised with glucagon-like peptide (GLP)-1, whereas FFA2 colocalised with 5-HT. Luminal perfusion of the SCFA acetate or propionate increased DBS, enhanced by dipeptidyl peptidase-IV (DPPIV) inhibition, at the same time as increasing GLP-2 portal blood concentrations. Acetate-induced DBS was partially inhibited by monocarboxylate/HCO₃⁻ exchanger inhibition without affecting GLP-2 release, implicating acetate absorption in the partial mediation of DBS. A selective FFA2 agonist dose-dependently increased DBS, unaffected by DPPIV inhibition or by cholecystokinin or 5-HT₃ receptor antagonists, but was inhibited by atropine and a 5-HT₄ antagonist. By contrast, a selective FFA1 agonist increased DBS accompanied by GLP-2 release, enhanced by DPPIV inhibition and inhibited by a GLP-2 receptor antagonist. Activation of FFA1 by LCFA and presumably FFA3 by SCFA increased DBS via GLP-2 release, whereas FFA2 activation stimulated DBS via muscarinic and 5-HT₄ receptor activation. SCFA/HCO₃⁻ exchange also appears to be present in the duodenum. The presence of duodenal fatty acid sensing receptors that signal hormone

release and possibly signal neural activation may be implicated in the pathogenesis of functional dyspepsia.

(Received 10 July 2014; accepted after revision 6 November 2014; first published online 26 November 2014)

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Abbreviations CCK, cholecystokinin; 4-CHCA, α -cyano-4-hydroxycinnamic acid; DPPIV, dipeptidyl peptidase-IV; EC, enterochromaffin; FD, functional dyspepsia; FFAR, free fatty acid receptor; GLP, glucagon-like peptide; GPCR, G protein-coupled; GST, glutathione S-transferase; LCFA, long-chain fatty acid; MCT1, monocarboxylate transporter 1; PV, portal venous; SCFA, short-chain fatty acid; VIP, vasoactive intestinal peptide.

Introduction

Postprandial nutrient sensing in the gastrointestinal mucosa is mediated by nutrient-sensing G protein-coupled receptors (GPCRs) expressed in the apical membranes of hormone-releasing enteroendocrine cells (Engelstoft *et al.* 2008). The presence of nutrients in the foregut lumen activates neuronal and endocrine mechanisms that bolster mucosal protective responses as a possible means to prevent post-prandial mucosal injury (Dockray, 2003; Akiba & Kaunitz, 2011a). Stimulation of nutrient-sensing receptors expressed on enteroendocrine L cells is emerging as a treatment for diabetes because L cells release an incretin, glucagon-like peptide (GLP)-1 (Drucker & Nauck, 2006). L cells also release GLP-2, another proglucagon-derived intestinotrophic hormone of clinical utility (Drucker *et al.* 1996).

We have previously demonstrated that the activation of an amino acid receptor, taste receptor 1 family heterodimer T1R1/T1R3, known as the *umami* receptor, by luminal perfusion of L-glutamate and 5'-inosine monophosphate increases duodenal HCO_3^- secretion via GLP-2 release and GLP-2 receptor activation, followed by nitric oxide and vasoactive intestinal peptide (VIP) release (Akiba *et al.* 2009; Wang *et al.* 2011). These studies suggest that the stimulation of nutrient-sensing GPCRs expressed on L cells by luminal small molecules not only affects glucose homeostasis, but also protects the duodenal mucosa from injury via the GLP-2 pathway. Furthermore, inhibition of dipeptidyl peptidase IV (DPPIV), the principal metabolic enzyme for numerous hormones, including GLP-1 and GLP-2 (Lambeir *et al.* 2003), enhances GLP-2 pathway-induced HCO_3^- secretion (Inoue *et al.* 2012), suggesting that the combination of DPPIV inhibition and luminal nutrient augments GLP-1 and -2 concentrations. Because experimental DPPIV inhibition prolongs the half-life of multiple hormones (Lambeir *et al.* 2003), enhancement of the rate of nutrient-responsive HCO_3^- secretion by DPPIV inhibition strongly supports the mediation of HCO_3^- secretion by peptide hormones. DPPIV inhibition prevents the formation and accelerates the healing of indomethacin-induced intestinal injury via

GLP-2 release, and outcomes are enhanced further by luminal *umami* substances (Inoue *et al.* 2014), suggesting that DPPIV inhibition could be a useful therapeutic for the treatment of intestinal mucosal injury.

Intraduodenal lipid has been implicated in the generation of functional dyspepsia (FD) symptoms, such as epigastric pain, bloating and stomach fullness (Fried & Feinle, 2002; Akiba & Kaunitz, 2011b). These symptoms are attributed to cholecystokinin (CCK) release and vagal afferent activation via the CCK1 receptor (Fried & Feinle, 2002). The orphan GPCRs, GPR40 (also termed free fatty acid receptor 1; FFA1) and GPR120 (FFA4), were identified as long-chain fatty acid (LCFA) receptors (Briscoe *et al.* 2003) that represent attractive candidates for luminal lipid sensors. Activation of FFA1 by luminal LCFA releases CCK from endocrine cell lines or purified enteroendocrine I cells (Liou *et al.* 2011), whereas FFA1 is also expressed in GLP-1 secreting L cells (Edfalk *et al.* 2008). Nevertheless, the involvement of FFA1 has not yet been reported in the context of duodenal HCO_3^- secretion.

Other free fatty acid receptors (FFARs) include FFA2/GPR43 and FFA3/GPR41, which are activated by short-chain fatty acids (SCFAs) (Brown *et al.* 2003). FFA2 and FFA3 are expressed in L cells present in the ileum and colon, colocalised with peptide YY (Karaki *et al.* 2006; Tazoe *et al.* 2009). Luminal SCFAs directly activate FFA2 or FFA3, increasing GLP-1 release from L cells (Tolhurst *et al.* 2012). Although luminal SCFAs exist in the lower small intestine and large intestine in ~ 100 mM concentrations as a consequence of bacterial fermentation of non-digestible fibres (Cummings *et al.* 1987), little is known about foregut SCFA physiology.

Here, we show that luminal perfusion of the selective FFA1 and FFA2 agonists, as well as non-specific ligands for FFA2 and FFA3, differentially stimulates duodenal HCO_3^- secretion. Activation of FFA1, and presumably activation of FFA3, stimulates HCO_3^- secretion via GLP-2 release, whereas HCO_3^- secretion in response to FFA2 activation occurs via muscarinic and 5-HT₄ receptors. Furthermore, the production of SCFAs by gut flora and the observation that luminal lipids activate the muscarinic, serotonergic and CCK pathways implicated in the generation of FD

symptoms provides a novel and plausible mechanism by which small intestinal bacterial overgrowth can aggravate functional gastrointestinal symptoms.

Methods

Chemicals and animals

NVP DPP 728 dihydrochloride (NVP728), GW9508, telnzepine, J104129, GR113808, SR27897 and VIP₆₋₂₈ were obtained from Tocris Bioscience (Ellisville, MO, USA). Phenylacetamide 1 [PA1; (S)-2-(4-chlorophenyl)-3-methyl-N-(thiazol-2-yl)butanamide] was synthesized, purified and verified in the Laboratory of Organic Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, Japan, in accordance with the published

chemical structure (Lee *et al.* 2008). Rat GLP-2(3–33) was synthesized by Bachem Americas, Inc. (Torrance, CA, USA). Atropine sulphate was obtained from Butler (Dublin, OH, USA). Sodium acetate, sodium propionate, ondansetron, Hepes and other chemicals were obtained from Sigma (St Louis, MO, USA). Krebs solution contained (in mM) 136 NaCl, 2.6 KCl, 1.8 CaCl₂ and 10 Hepes at pH 7.0. Osmolality was adjusted to isotonicity by reducing the NaCl concentration. The pH of Krebs solution after dissolving the compound was adjusted to pH 7.0. All solutions were prewarmed at 37°C in a water bath; the temperature was maintained with a heating stirrer or heating pad. All studies were performed with the approval of the Veterans Affairs Institutional Animal Care and Use Committee. Antibody production was performed in accordance with the guidelines for the

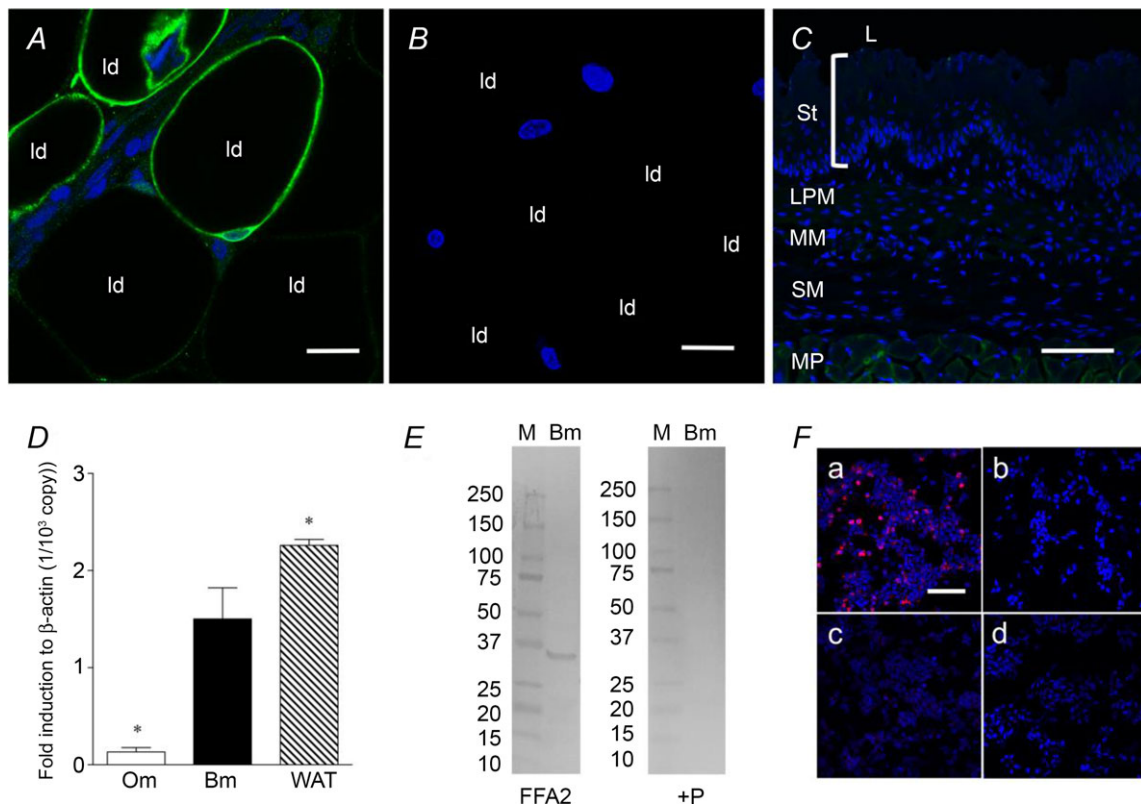


Figure 1. Detection of FFA2 using RK1101 antibody in rat tissues

Whole mount mesenteric white adipose tissue or a cryostat section of oesophagus was incubated with anti-FFA2 antibody RK1101 (green) with or without blocking peptide, counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). Mesenteric adipocytes were positively stained (A), whereas pre-absorption abolished the staining (B). Id, lipid droplet. Internal bars: 20 μ m. C, oesophageal mucosa, consisting of stratum layers (St), lamina propria mucosae (LPM), muscularis mucosae (MM) and submucosa (SM), was negatively stained, with faint staining observed in the muscularis propria (MP). L, lumen. Internal bars: 100 μ m. D, expression of FFA2 in oesophageal mucosa (Om), duodenal bulb mucosa (Bm) and white adipose tissue (WAT) assessed by real time PCR with the Δ C_t method. Each data point represents the mean \pm SEM ($n = 6$ rats). * $P < 0.05$ vs. Bm. E, Western blot for FFA2 using RK1101 in duodenal mucosa (Bm) (left panel; FFA2) and pre-absorption with blocking peptide (right panel; +P). M, molecular marker with size (kDa) on the left. F, FFA2-transfected cells (a) were positively stained with RK1101 (red), whereas FFA2-transfected cells were negatively stained with pre-absorbed antibody (b). FFA3-transfected cells (c) and mock-transfected cells (d) were negatively stained with RK1101. Counterstained with DAPI (blue). Internal bars: 100 μ m.

care and use of laboratory animals of Hokkaido University School of Medicine. Male Sprague–Dawley rats weighing 200–250 g (Harlan, San Diego, CA, USA) were fasted overnight, but had free access to water. Animals were killed by terminal exsanguination under deep isoflurane anaesthesia, followed by thoracotomy.

Antibody production

We produced the affinity-purified primary antibody RK1101, which was raised against rat FFA2. A cDNA fragment, which is preceded with a *Bam*HI site and encodes C-terminal 30 amino acids of FFA2 (301–330 amino acid residues; GenBank NM 001005877), was obtained by PCR. After carrying out thymidine/adenosine cloning using a pGEM-T Easy Vector System I kit (Promega, Madison, WI, USA), the cDNA fragment was subcloned into the *Bam*HI/*Eco*RI site of the pGEX4T-2 plasmid (Pharmacia Biotech AB, Uppsala, Sweden) and *Escherichia coli* BL21 for expression of glutathione *S*-transferase (GST) fusion proteins. GST fusion proteins were purified using glutathione-Sepharose 4B, in accordance with the manufacturer's instructions (Pharmacia Biotech AB). Fusion proteins emulsified with Freund's complete or incomplete adjuvant (Difco, Detroit, MI, USA) were injected s.c. into a female New Zealand White rabbit at intervals of 2 weeks. Antiserum sampled

2 weeks after the sixth injection was affinity-purified using CNBr-activated Sepharose 4B (Pharmacia Biotech AB) coupled with GST-free polypeptides as obtained by in-column thrombin digestion of fusion proteins.

Western blotting

Western blot analysis for FFA2 using RK1101 was performed as described previously (Akiba *et al.* 2008). The scraped mucosa of rat proximal duodenum was homogenized in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Triton X and 1% protease inhibitor cocktail (Sigma). After centrifugation at 10,000 *g* for 10 min at 4°C, supernatant protein samples were reduced and denatured in Laemmli buffer, followed by electrophoresis in a 4–20% gradient gel (Bio-Rad Laboratories, Hercules, CA, USA) and electroblotted onto polyvinylidene difluoride membranes (Thermo Fisher Scientific, Rockford, IL, USA). After blocking with 0.5% skimmed milk at 4°C overnight, the membranes were incubated with rabbit anti-FFA2 antibody (RK1101; 1 μ g ml⁻¹) for 2 h at room temperature, followed by incubation with alkaline phosphatase-conjugated secondary antibody at a dilution of 1:3000 (Chemicon, Temecula, CA, USA). The immunoreaction was visualized with chromogenic substrate solution (Sigma). As a negative control, pre-absorbed RK1101 solution was used after incubation with the

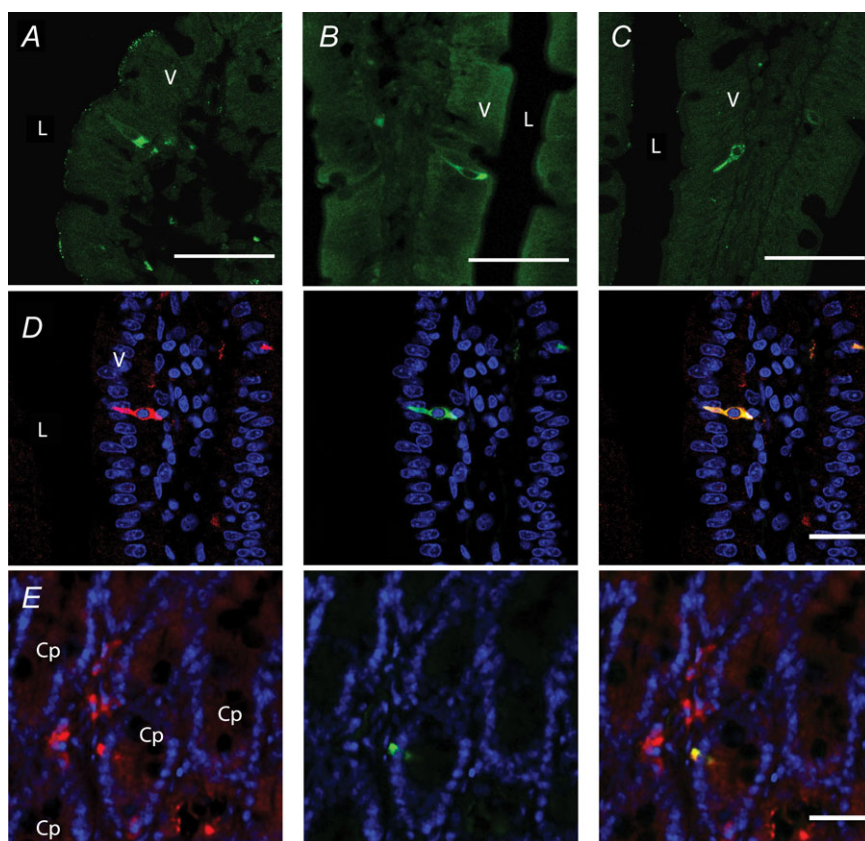


Figure 2. Localisation of FFARs in rat duodenum

Frozen cryostat sections were incubated with the primary antibody for FFA1 (A), FFA2 (B) or FFA3 (C). L, lumen; V, villus. Internal bars: 100 μ m. D, double staining with FFA2 (red; RK1101 antibody, left), 5-HT (green; middle) and merged image (right). Counterstained with DAPI (blue). L, lumen; V, villus. Internal bars: 20 μ m. E, double staining with FFA3 (red; left), GLP-1 (green; middle) and merged image (right). Counterstained with DAPI (blue). Cp, crypt. Internal bars: 20 μ m.

GST-free antigen peptide described above at $100 \mu\text{g ml}^{-1}$ for 30 min.

Localisation of FFARs in rat duodenum

FFA1, FFA2 and FFA3 immunolocalisation was carried out on cryostat sections of Zamboni-fixed tissues incubated with goat anti-FFA1 antibody (dilution 1:100, sc-28417; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit anti-FFA2 antibody (RK1101; $1 \mu\text{g ml}^{-1}$) or rabbit anti-FFA3 antibody (dilution 1:100, sc-98332; Santa Cruz Biotechnology Inc.), followed by incubation with Alexa488 or Alexa594 secondary antibody (Molecular Probes, Eugene, OR, USA). Some were double-labelled with goat anti-GLP-1 antibody (dilution 1:200, sc-7782; Santa Cruz Biotechnology Inc.) or mouse anti-5-HT antibody (dilution 1:100, MCA3190Z; AbD Serotec, Kidlington, UK), followed by incubation with the corresponding Alexa488 secondary antibody (Molecular Probes). Fluorescence was observed with an Axio Observer Z1 microscope (Zeiss, Munich-Harbergmoons, Germany) or a confocal laser microscope (FV300; Olympus, Tokyo, Japan; LSM-710; Zeiss). Negative controls were processed identically, with the omission of the primary antibody or with incubation with primary antibody pre-absorbed with the immunizing peptide ($100 \mu\text{g ml}^{-1}$). Furthermore, rat FFA2 and FFA3 cDNA were amplified by PCR, and inserted into the pTracer-CMV2 mammalian expression plasmid (Invitrogen, Carlsbad, CA, USA). The plasmid was transfected into human embryonic kidney (HEK293T) cells using Lipofectamine 2000 (Invitrogen)

in accordance with the manufacturer's instructions. The transfected HEK293T cells were immunostained with RK1101 antibody.

Expression of FFA2 in the tissues was also analysed by real time RT-PCR as described previously (Akiba *et al.* 2009). The PCR primers of rat FFA2 were sense (5'-CACCGAGAACCAAATCACCT-3') and anti-sense (5'-GAGGGACTCTGCCTCAAGTG-3'), giving rise to a 288 bp PCR product, and β -actin was used as an internal control. The expression level was presented as the fold induction per 10^3 copies of β -actin by the ΔC_t method.

Measurement of duodenal HCO_3^- secretion

Duodenal loops were prepared and perfused under iso-flurane anaesthesia as described previously (Mizumori *et al.* 2006; Akiba *et al.* 2007, 2009). After stabilisation with continuous perfusion of saline (pH 7.0) for ~ 30 min, the time was set as $t = 0$. The duodenal loop was perfused with saline (pH 7.0) from $t = 0$ min until $t = 10$ min (basal period). The perfusate was then changed to Krebs solution (pH 7.0) with or without test compounds from $t = 10$ min until $t = 35$ min (challenge period). At $t = 10$ min, the system was gently flushed to rapidly change the perfusate. Duodenal HCO_3^- secretion was expressed as total CO_2 output, calculated from the measured pH and $[\text{CO}_2]$ in the effluent solution as reported previously (Akiba *et al.* 2007, 2009).

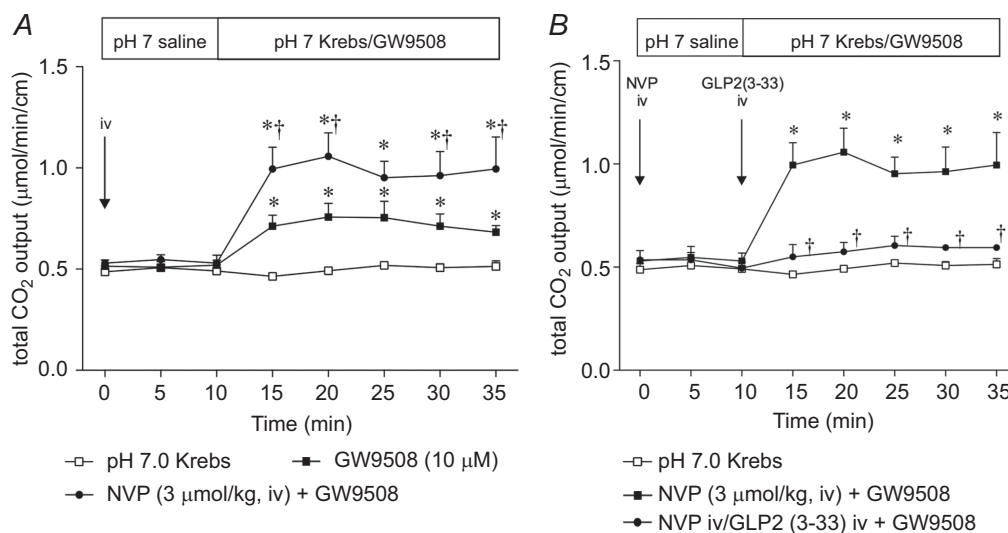


Figure 3. Effect of FFA1 agonist on duodenal HCO_3^- secretion in rats

Duodenal HCO_3^- secretion was measured as total CO_2 output using the flow-through pH and CO_2 electrodes. The duodenal loop was perfused with GW9508 ($10 \mu\text{M}$). The DPPIV inhibitor NVP728 (NVP) was injected i.v. ($3 \mu\text{mol kg}^{-1}$) at $t = 0$ min (A). The GLP-2 receptor antagonist GLP2(3-33) was additionally injected i.v. (3 nmol kg^{-1} , i.v.) at $t = 10$ min (B). Each data point represents the mean \pm SEM ($n = 6$ rats). * $P < 0.05$ vs. pH 7.0 Krebs group. † $P < 0.05$ vs. vehicle i.v. + GW9508 group.

Experimental protocol

We have previously reported that bolus i.v. injection of the DPPIV inhibitor NVP728 ($3 \mu\text{mol kg}^{-1}$), 10-min before perfusion of luminal agonists (at $t = 0$ min), enhances amino acid or bile acid-induced HCO_3^- secretion. Injection of the GLP-2 receptor antagonist, GLP-2(3–33) (3 nmol kg^{-1} , i.v.) at $t = 10$ min, just prior to the perfusion of luminal agonists, inhibits HCO_3^- secretion (Inoue *et al.* 2012). Therefore, to investigate the involvement of GLP-2 in stimulated HCO_3^- secretion, NVP728 ($3 \mu\text{mol kg}^{-1}$) was bolus injected i.v. at $t = 0$ min, with or without GLP-2(3–33) i.v. at $t = 10$ min, followed by the luminal perfusion of FFAR agonists.

To examine the effect of LCFA on duodenal HCO_3^- secretion, the duodenal loop was perfused with the selective FFA1 agonist, GW9508 ($10 \mu\text{M}$) (Briscoe *et al.*

2006) with or without prior NVP728 or GLP2(3–33) injection.

To examine the effect of SCFAs on HCO_3^- secretion, the loop was perfused with acetate or propionate ($10 \mu\text{M}$ to 1 mM), diluted with Krebs solution adjusted to pH 7.0 to eliminate any independent effects of luminal pH, or the selective FFA2 agonist PA1 (0.1 – $10 \mu\text{M}$) (Lee *et al.* 2008) with or without prior NVP728 injection. To inhibit the monocarboxylate transporter 1 (MCT1), α -cyano-4-hydroxycinnamic acid (4-CHCA; 0.1 or 1 mM) (Wang *et al.* 1996) was co-perfused with acetate. To further assess the mechanism of PA1, neurotransmitter receptor antagonists were injected i.v. at $t = 0$: the muscarinic receptor antagonist atropine (0.5 mg kg^{-1}); the M_1 receptor antagonist telenzepine (1 mg kg^{-1}); the M_3 receptor antagonist J104129 (1 mg kg^{-1}) (Mitsuya *et al.* 1999); the 5-HT₃ receptor antagonist ondansetron

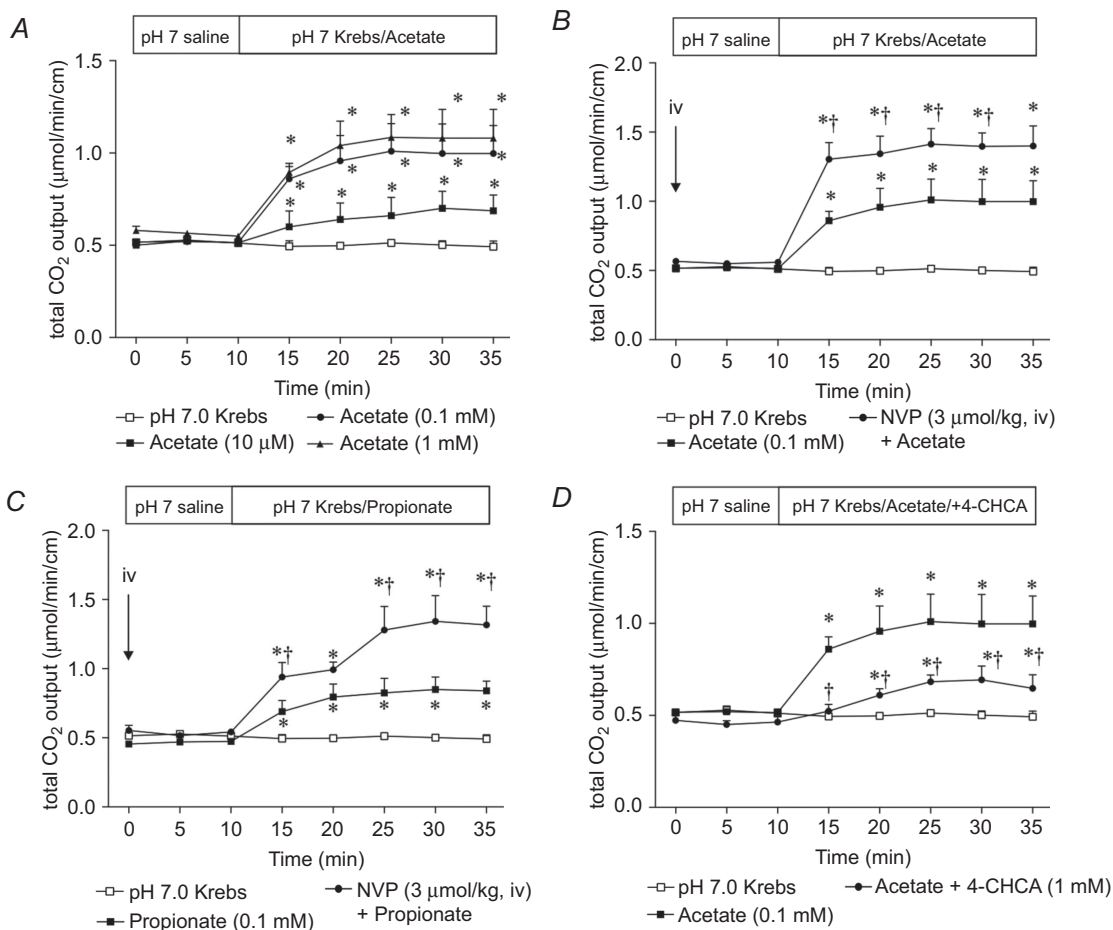


Figure 4. Effect of luminal perfusion of SCFA on duodenal HCO_3^- secretion

A, luminal perfusion of acetate ($10 \mu\text{M}$ to 1 mM) dose-dependently increased HCO_3^- secretion. Each data point represents the mean \pm SEM ($n = 6$ rats). * $P < 0.05$ vs. pH 7.0 Krebs group. B, C, luminal perfusion of acetate (B) or propionate (C) (0.1 mM) increased HCO_3^- secretion, with the effect enhanced by the DPPIV inhibitor NVP pretreatment ($3 \mu\text{mol kg}^{-1}$, i.v.). Each data point represents the mean \pm SEM ($n = 6$ rats). * $P < 0.05$ vs. pH 7.0 Krebs group. † $P < 0.05$ vs. SCFA group. D, co-perfusion of 4-CHCA (1 mM) reduced acetate-induced HCO_3^- secretion. Each data point represents the mean \pm SEM ($n = 6$ rats). * $P < 0.05$ vs. pH 7.0 Krebs group. † $P < 0.05$ vs. Acetate group.

(1 mg kg⁻¹); the 5-HT₄ receptor antagonist GR113808 (1 mg kg⁻¹) (Gale *et al.* 1994); the CCK1 receptor antagonist SR27897 (1 mg kg⁻¹) (Gully *et al.* 1993); or the VIP receptor antagonist VIP₆₋₂₈ (100 nmol kg⁻¹) (Fishbein *et al.* 1994; Wang *et al.* 2011). We also tested the effect of GR113808 or GLP-2(3-33) on acetate-induced HCO₃⁻ secretion.

Measurement of GLP-2 in portal venous (PV) blood

The plasma concentration of GLP-2 was measured in the PV blood samples as described previously (Wang *et al.* 2011; Inoue *et al.* 2012). The samples were collected after a 25 min challenge using a syringe containing 1 μl each of EDTA (0.5 mM) and NVP728 (10 μM). The samples were immediately centrifuged at 5000 g for 5 min; the separated plasma was stored at -80°C. Plasma was diluted with Tris-HCl buffer (50 mM, pH 7.4) containing a protease inhibitor cocktail (1 mg ml⁻¹; Sigma) and NVP728 (10 μM). The total GLP-2 plasma concentration was measured using a rat GLP-2 enzyme immunoassay kit (ALPCO Diagnostics, Salem, NH, USA) in accordance with the manufacturer's instructions.

Statistical analysis

All data are expressed as the mean ± SEM. Data were derived from six rats in each group. Comparisons between groups were made by one-way ANOVA, followed by Fischer's least significant difference test. *P* < 0.05 was considered statistically significant.

Results

Localisation of FFARs in rat duodenum

FFA2 immunoreactivity using RK1101 was observed in adipocytes in mesenteric white adipose tissue (Fig. 1A) as a positive control (Kimura *et al.* 2013), whereas pre-absorption with blocking peptide abolished the staining (Fig. 1B). By contrast, the oesophageal mucosa was negatively stained with RK1101 (Fig. 1C), consistent with low FFA2 expression in the oesophageal mucosa compared to the duodenal mucosa or white adipose tissue (Fig. 1D). A 35 kDa band was present in duodenal mucosa detected with RK1101, close to the predicted size of rat FFA2 (37 kDa), whereas pre-absorption abolished the band (Fig. 1E). Furthermore, the RK1101 antibody positively immunostained FFA2-transfected HEK293T

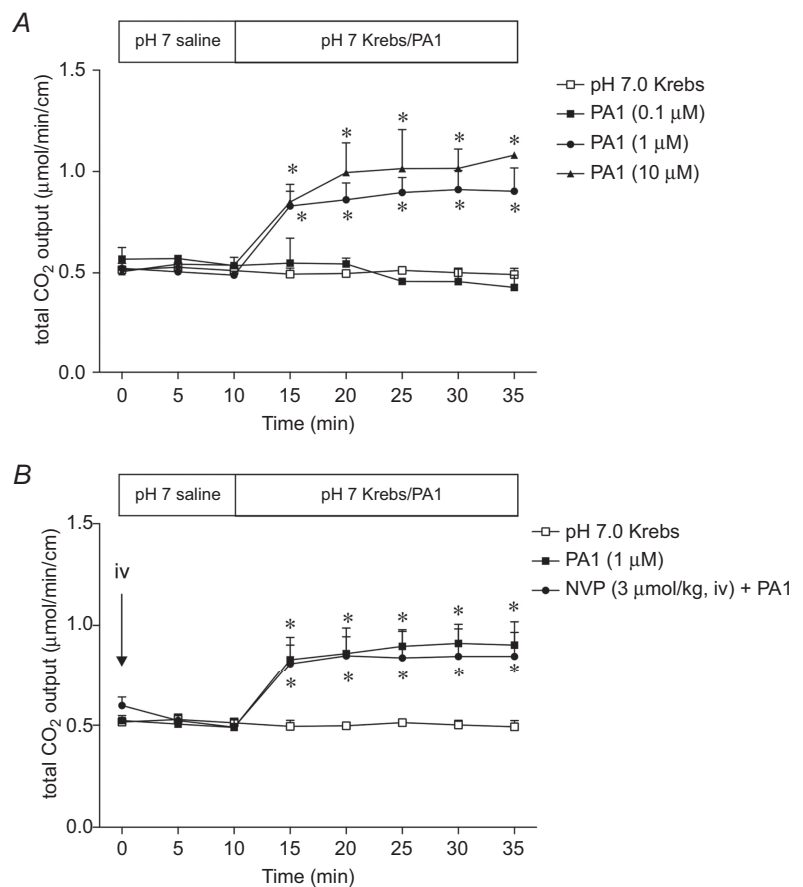


Figure 5. Effect of luminal perfusion of FFA2 agonist on HCO₃⁻ secretion

A, luminal perfusion of PA1 (0.1–10 μM) dose-dependently increased HCO₃⁻ secretion. Each data point represents the mean ± SEM (*n* = 6 rats). **P* < 0.05 vs. pH 7.0 Krebs group. B, PA1 (1 μM)-induced HCO₃⁻ secretion was not affected by the DPPIV inhibitor NVP (3 μmol kg⁻¹, i.v.). Each data point represents the mean ± SEM (*n* = 6 rats). **P* < 0.05 vs. pH 7.0 Krebs group.

cells, whereas immunostaining of FFA2-transfected HEK293T cells with pre-absorbed antibody, or of FFA3-transfected HEK293T cells or of mock-transfected cells, was negative (Fig. 1F).

Immunoreactivity of FFA1, FFA2 and FFA3 was observed in cells of endocrine-like morphology in the duodenal mucosa (Fig. 2A–C). FFA2 colocalised with 5-HT (Fig. 2D) and FFA3 colocalised with GLP-1 (Fig. 2E), consistent with the FFA2 expression on enterochromaffin (EC) cells and FFA3 expression on L cells. These data demonstrate that duodenal endocrine cells express FFA1, 2 or 3.

Effect of an FFA1 agonist on duodenal HCO_3^- secretion

Luminal perfusion of the FFA1 agonist GW9508 ($10 \mu\text{M}$) increased the rate of HCO_3^- secretion, as enhanced by prior injection of the DPPIV inhibitor NVP728

(Fig. 3A), although NVP728 injection had no effect on the basal rate of HCO_3^- secretion. Furthermore, augmented HCO_3^- secretion in response to NVP728 and GW9508 was inhibited by the GLP-2 receptor antagonist GLP-2(3–33) (Fig. 3B). These results support the proposed mechanism that activation of FFA1 in the duodenal mucosa increases HCO_3^- secretion via the GLP-2 pathway.

Effect of SCFAs on duodenal HCO_3^- secretion

Next, we examined the effect of luminal perfusion of SCFAs on duodenal HCO_3^- secretion. Luminal perfusion of acetate dose-dependently increased HCO_3^- secretion (Fig. 4A), as increased by prior injection of NVP728 (Fig. 4B). Similarly, the perfusion of propionate (0.1 mM) increased HCO_3^- secretion, as also enhanced by NVP728

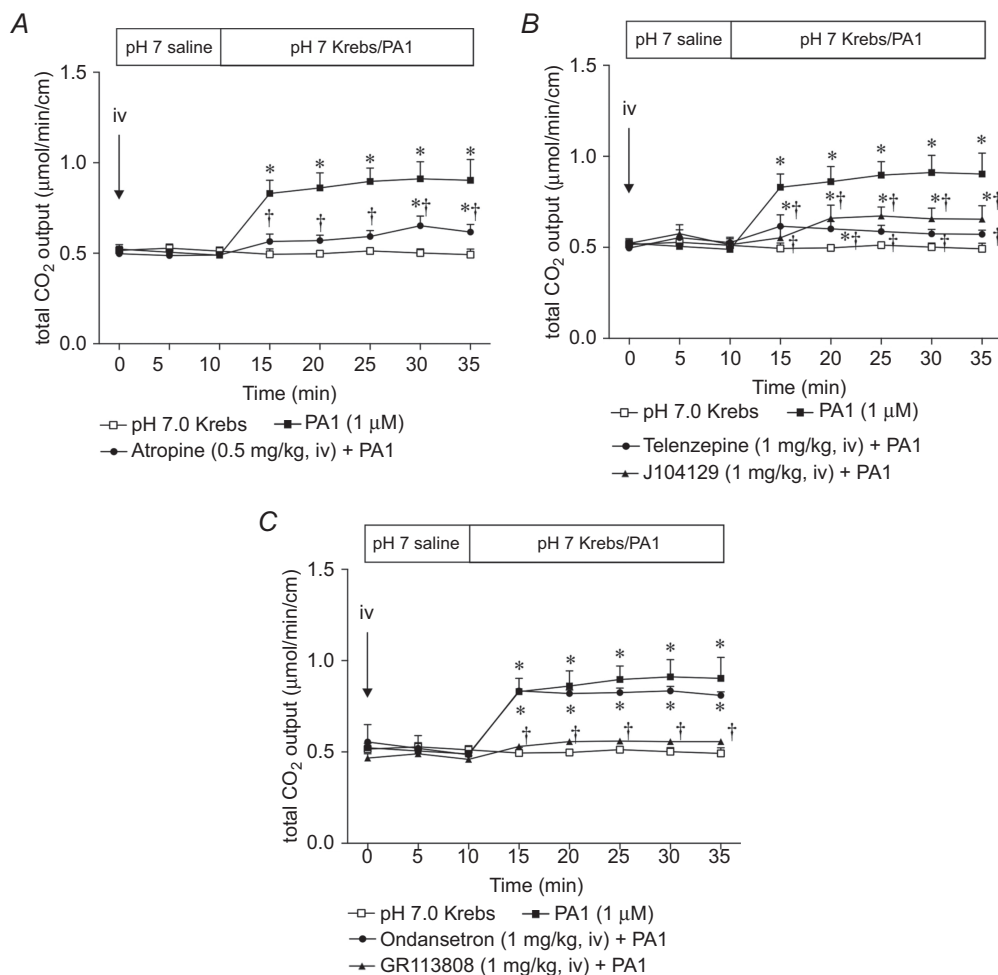


Figure 6. Effect of muscarinic or 5-HT receptor antagonist on PA1-induced HCO_3^- secretion

Muscarinic or 5-HT receptor antagonist was injected i.v. at $t = 0$ min. PA1 ($1 \mu\text{M}$)-induced HCO_3^- secretion was inhibited by atropine (0.5 mg kg^{-1}) (A), telenzepine or J104129 (1 mg kg^{-1}) (B). C, 5-HT₄ antagonist GR113808 (1 mg kg^{-1}) abolished PA1-induced HCO_3^- secretion, whereas 5-HT₃ receptor antagonist ondansetron (1 mg kg^{-1}) had no effect. Each data point represents the mean \pm SEM ($n = 6$ rats). * $P < 0.05$ vs. pH 7.0 Krebs group. † $P < 0.05$ vs. PA1 group.

i.v. (Fig. 4C). Furthermore, acetate-induced HCO_3^- secretion was partially inhibited by the MCT1 inhibitor 4-CHCA (1 mM) (Fig. 4D). These results suggest that luminal SCFAs increase HCO_3^- secretion in part via GLP-2 release, and in part via SCFA transport via MCT1.

Effect of an FFA2 agonist on duodenal HCO_3^- secretion

The FFA2 agonist PA1 dose-dependently stimulated HCO_3^- secretion (Fig. 5A), and was not affected by prior injection of NVP728 (Fig. 5B), suggesting that the prosecretory effect of FFA2 activation does not occur via peptide release. We therefore examined the involvement of cholinergic nerves in PA1-induced HCO_3^- secretion. Atropine injected i.v. inhibited PA1-induced HCO_3^- secretion (Fig. 6A). The M_1 receptor antagonist

telenzipine or the M_3 antagonist J104129 reduced PA1-induced HCO_3^- secretion (Fig. 6B). Furthermore, the 5-HT₄ receptor antagonist GR113808 abolished PA1-induced HCO_3^- secretion, whereas the 5-HT₃ antagonist ondansetron had no effect (Fig. 6C). The CCK1 receptor antagonist SR27897 or the VIP receptor antagonist VIP₆₋₂₈ had no effect on PA1-induced HCO_3^- secretion (Fig. 7A and B). These results suggest that, in contrast to SCFA-induced HCO_3^- secretion, FFA2 activation stimulates HCO_3^- secretion via muscarinic and 5-HT₄ receptor activation, rather than via CCK, VIP or GLP-2 release.

Furthermore, acetate-induced HCO_3^- secretion was reduced by combination of GR113808 and GLP-2(3-33), although each antagonist alone had little effect (Fig. 8A–C). These results support our hypothesis that luminal SCFAs activate both FFA2 and FFA3, followed by 5-HT and GLP-2 release, respectively.

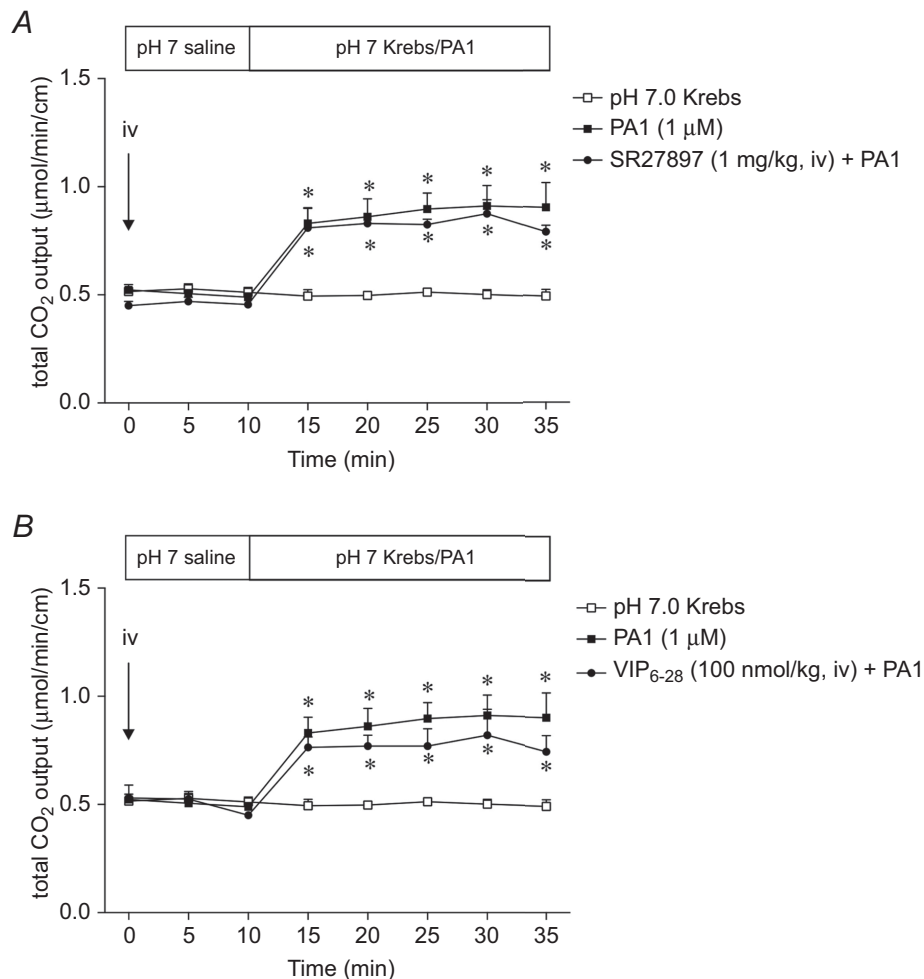


Figure 7. Effect of CCK or VIP receptor antagonist on PA1-induced HCO_3^- secretion

CCK1 receptor antagonist SR27897 (1 mg kg⁻¹) or VIP receptor antagonist VIP₆₋₂₈ (100 nmol kg⁻¹) was injected i.v. at $t = 0$ min. PA1 (1 µM)-induced HCO_3^- secretion was not affected by SR27897 (A) or VIP₆₋₂₈ (B). Each data point represents the mean \pm SEM ($n = 6$ rats). * $P < 0.05$ vs. pH 7.0 Krebs group.

GLP-2 release into PV blood

We measured GLP-2 concentrations in PV blood plasma at baseline and after the challenge period (Fig. 9). Luminal perfusion of GW9508 increased PV GLP-2 concentrations, and was increased further by DPPIV inhibition (Fig. 9A), consistent with GLP-2-mediated HCO_3^- secretion (Fig. 3). Luminal acetate also increased PV GLP-2 concentrations, as enhanced further by NVP i.v. injection, whereas MCT1 inhibition with 4-CHCA or 5-HT₄ receptor antagonism with GR113808 had no additional effect (Fig. 9B), supporting our hypothesis that acetate increases HCO_3^- secretion via GLP-2-dependent and independent pathways, and via SCFA absorption. By contrast, PA1 had no effect on GLP-2 release (Fig. 9C), which is consistent with HCO_3^- secretion via the GLP-2 independent, 5-HT/cholinergic pathway.

Discussion

We have demonstrated that luminal perfusion of fatty acids differentially increased duodenal HCO_3^- secretion via several mechanisms (Fig. 10). LCFA increased HCO_3^- secretion via FFA1 activation that released GLP-2. SCFAs activated FFA2 and FFA3, which in turn activated different mechanisms: FFA3 agonists may release GLP-2, whereas FFA2 agonists activated a 5-HT/cholinergic pathway. Furthermore, SCFAs absorbed by epithelial cells may exchange for secreted HCO_3^- via an apical membrane exchange transporter. This is the first study to demonstrate that SCFA sensors are present in the duodenal mucosa and that activation of SCFA receptors increases the rate of protective HCO_3^- secretion via hormone release and possible neural mechanisms, similar to the mechanism in which duodenal LCFA releases CCK (Holzer *et al.* 1994; Liou *et al.* 2011).

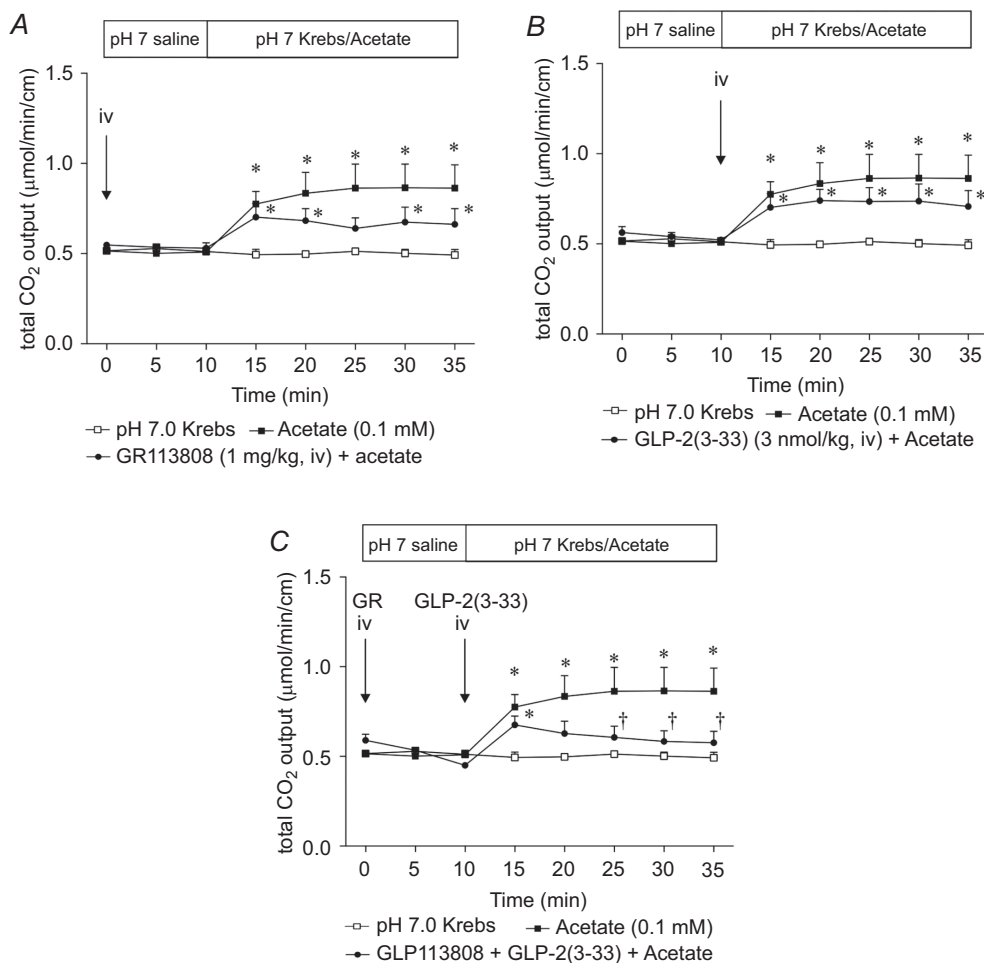


Figure 8. Effect of 5-HT₄ receptor or GLP-2 receptor antagonist on acetate-induced HCO_3^- secretion. GR113808 (1 mg kg⁻¹) was injected i.v. at $t = 0$ min, whereas GLP-2(3–33) was injected i.v. (3 nmol kg⁻¹, i.v.) at $t = 10$ min, followed by luminal acetate (0.1 mM) perfusion. GR113808 (A) or GLP-2(3–33) (B) had little effect on acetate-induced HCO_3^- secretion. Pre-treatment of both antagonists reduced acetate-induced HCO_3^- secretion (C). Each data point represents the mean \pm SEM ($n = 4$ –6 rats). * $P < 0.05$ vs. pH 7.0 Krebs group. † $P < 0.05$ vs. Acetate group.

Intestinal SCFA sensing and uptake has been studied primarily in the colon. The concentration of SCFA in hindgut lumen can reach ~ 100 mM, especially in the cecum and proximal colon, where intestinal flora ferments undigested fibre and carbohydrates (Illman *et al.* 1986; Cummings *et al.* 1987). In the colon, butyrate is consumed *in situ* by the epithelial cells as a source of cellular energy, whereas acetate and propionate traverse the epithelium to enter the circulation (Cummings *et al.* 1987). FFA2 and FFA3 are expressed in L cells in the rat and human colon (Karaki *et al.* 2006, 2008; Tazoe *et al.* 2009; Kaji *et al.* 2011), which is consistent with the presence of abundant SCFA in the colonic lumen.

Few studies have addressed the SCFA content of the foregut lumen, under the assumption that the lack of bacterial flora should be associated with low luminal SCFA concentrations. SCFAs, however, are also present in the foregut lumen at 0.1–1 mM, mainly derived from the fermentation of nutrients by oral flora (Hoverstad *et al.* 1984). Furthermore, condiments, and fermented and preserved foods contain up to a molar range of SCFAs; for example, vinegar contains 4–7% v/v or 0.66–1.17 M acetic acid. Therefore, the foregut mucosa may be exposed to concentrations of SCFAs sufficient to activate cognate sensors such as FFA2 and FFA3. The present study showed that FFA3 was expressed in L cells, whereas FFA2 was expressed in EC cells in the rat duodenum. FFA2 did not colocalise with GLP-1, nor did FFA3 colocalise with 5-HT in the rat duodenum (data not shown), which is an expression pattern different from FFA2 localisation in the colon, and consistent with segmental differences in endocrine cell expression. Foregut SCFA sensing may sense ingested fermented foods, whereas colonic sensors likely sense the products of endogenous fermentation. Therefore, the presence of excessive foregut luminal

SCFA concentrations as a result of bacterial overgrowth may stress homeostatic sensing mechanisms, producing unwanted symptomatology.

SCFAs are an important source of energy, especially for ruminants, although, unlike carbohydrates, SCFAs have a low glycaemic index. SCFA treatment improves glycaemic control in healthy subjects and in type 2 diabetic patients (Brighenti *et al.* 1995; Ostman *et al.* 2005; Liatis *et al.* 2010). Furthermore, colonic SCFA absorption can provide up to 540 kcal day⁻¹ in humans (Ruppin *et al.* 1980), helping to meet nutritional needs with 'nature's after-burner', especially for patients with short bowel syndrome.

FFA2 and FFA3 are expressed in the enteroendocrine cells, myenteric neurons, and leukocytes of transgenic fluorescent protein reporter mice (Nøhr *et al.* 2013). FFA3-positive endocrine cells also express GLP-1, which is consistent with our results, whereas FFA2 is weakly expressed in a fraction of enteroendocrine cells that have not yet been characterized. In the present study, activation of FFA2 expressed in 5-HT-positive EC cells with the selective FFA2 agonist PA1 increased the rate of HCO₃⁻ secretion via 5-HT₄ and muscarinic receptor activation, strongly supporting our hypothesis that FFA2 is functionally expressed in EC cells and that FFA2 activation releases 5-HT, followed by acetylcholine release. The observations that an FFA2 agonist failed to increase GLP-2 release, whereas the mixed FFA2/3 agonist acetate increased GLP-2 release, and that FFA3 is expressed in GLP-1-positive L cells further support our hypothesis that FFA3 activation increases GLP-2 release, followed by GLP-2-mediated HCO₃⁻ secretion. The experimental use of selective FFA3 agonists and antagonists, when available, will help clarify this possibility.

The significance of the HCO₃⁻ secretory response to luminal fatty acids remains unknown. Because the pK_a

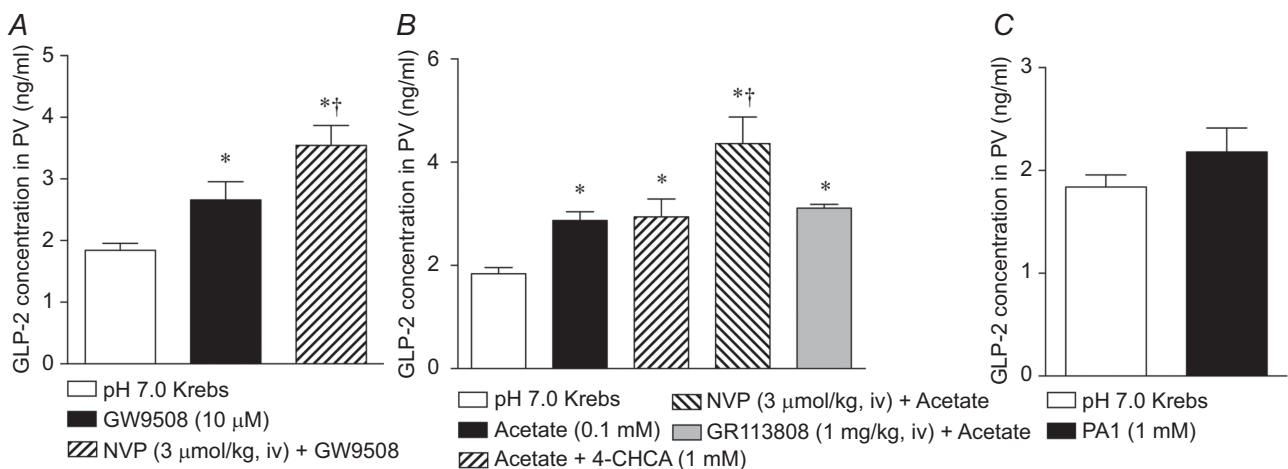


Figure 9. Effect of luminal perfusion of fatty acid receptor agonists on GLP-2 release

GLP-2 concentration was measured in PV plasma. The duodenal loop was perfused with GW9508 (10 μM, A), acetate (0.1 mM, B) or PA1 (1 μM, C) with or without inhibitors. PV blood was collected at $t = 35$ min. Each data point represents the mean \pm SEM ($n = 6$ rats). * $P < 0.05$ vs. pH 7.0 Krebs group.

of SCFA is ~ 5 , SCFAs are mostly dissociated at neutral pH, existing in the anionic form, which may be absorbed via transporters such as the MCT, rather than, as has been historically proposed, absorbed passively in the undissociated form through the colonic epithelium at luminal pH < 6 (Ruppin *et al.* 1980; Umesaki *et al.* 1980). Therefore, the stimulation of HCO_3^- secretion by luminal SCFA may help the duodenal mucosa absorb SCFA anions by raising the luminal pH, and thus increasing the relative concentration of the anionic form.

Because the presence of luminal SCFAs can signify the presence of luminal bacterial growth, elevated SCFA concentrations in the foregut lumen may represent bacterial overgrowth, which is implicated in the pathogenesis of FD (Costa *et al.* 2012). We showed that SCFA activation of luminal sensors followed by GLP-2 and 5-HT release with subsequent presumed neuronal activation forms the basis of a plausible pathophysiological mechanism incorporating three well-documented clinical observations: (1) irritable bowel syndrome and FD can be associated with small intestinal bacterial overgrowth

(Costa *et al.* 2012; Ghoshal & Srivastava, 2014); (2) FD symptoms such as nausea, epigastralgia, bloating and fullness can be treated with 5-HT receptor modulators (Beattie & Smith, 2008); and (3) lipids introduced into the duodenal lumen often reproduce dyspeptic symptoms in FD patients (Barbera *et al.* 1995). Therefore, the results of the present study suggest that 5-HT release via FFA2 activation by luminal SCFA derived from bacterial overgrowth may contribute to the generation of FD symptoms. Similarly, the expression of FFA1 in CCK-positive I cells that release CCK in response to LCFA (Liou *et al.* 2011), further supports the hypothesis that luminal fat produces FD symptoms via CCK release and CCK1 activation (Feinle *et al.* 2001).

Controversy still surrounds the existence of FFAR expression and hormone release in response to FAs. FFA1 activation releases CCK, which may increase duodenal HCO_3^- secretion (Flemström *et al.* 1982; Konturek *et al.* 1985), whereas GW9508-induced HCO_3^- secretion was not affected by CCK1 receptor antagonist SR27897 (data not shown). FFA2 activation by PA1 increased GLP-1

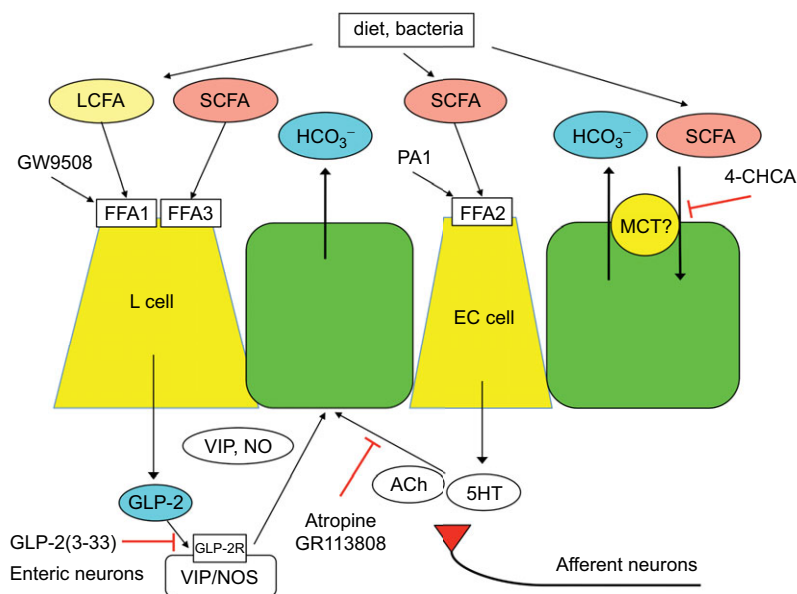


Figure 10. Proposed mechanism of duodenal SCFA sensing

Luminal SCFAs, derived from diet or bacterial metabolism, activate multiple pathways. SCFAs may activate FFA3 on L cells, which release GLP-2, which in turn activates GLP-2 receptors expressed on myenteric neurons (Guan *et al.* 2006), followed by the release of VIP and NO (Wang *et al.* 2011), stimulating epithelial HCO_3^- secretion by established pathways. The actions of GLP-2 are enhanced by DPPIV inhibition (Inoue *et al.* 2012). SCFAs also activate FFA2 on EC cells, which release 5-HT and ACh, activating 5-HT₄ and muscarinic receptors, respectively, with both expressed on enteric and afferent nerves, and on epithelial cells, also stimulating the rate of HCO_3^- secretion. SCFAs might be absorbed by SCFA/ HCO_3^- exchanger, possibly by apical membrane MCT1, increasing the rate of HCO_3^- secretion. By contrast, luminal LCFA increases HCO_3^- secretion via activation of FFA1 expressed in L cells, which releases GLP-2, augmented by DPPIV inhibition, and inhibited by a GLP-2 receptor antagonist GLP-2(3–33). We propose that GLP-2, 5-HT and ACh, which are released by luminal SCFAs, not only activate the local mechanisms involved in duodenal mucosal defences, but also stimulate afferent nerves, which are implicated in the production of pathological symptoms. SCFA, short-chain fatty acid; LCFA, long-chain fatty acid; FFA1, free fatty acid receptor 1; GLP-2, glucagon-like peptide-2; VIP, vasoactive intestinal peptide; NO, nitric oxide; 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; EC, enterochromaffin; MCT, monocarboxylate transporter; DPPIV, dipeptidyl peptidase IV.

release from primary colonic cultures (Tolhurst *et al.* 2012), whereas PA1, which had no effect on GLP-2 release in the duodenum, activated EC cells in the present study. These differences can be explained by the overlapping expression of FFARs and hormones in enteroendocrine cells, presumably dependent upon the endocrine cell lineage (Egerod *et al.* 2012; Habib *et al.* 2012). For example, although EC cells and I cells are assumed to be distinct, 5-HT/CCK double-positive endocrine cells are present in rat duodenum with up to 50% overlap (Cho *et al.* 2014), suggesting that the release of 5-HT or CCK by receptor activation may depend on whether the cells predominantly express FFA1 or FFA2. These findings support the presumed variability of a hormonal and mediator response to luminal FAs, in addition to segmental or species differences, which may underlie the marked variation of clinical irritable bowel syndrome or FD.

The presence of SCFA sensing in the duodenum with the release of GLP-2 and stimulated HCO_3^- secretion suggests that the described pathways differentially activated by FFA1 and FFA3 agonists might be useful clinically. Exogenous potent FFA1 and FFA3 agonists may ameliorate small intestinal injury via GLP-2 release, similar to the previously described mechanism by which nutrient receptor activation combined with DPPIV inhibition accelerates the healing of non-steroidal anti-inflammatory drug-induced enteropathy (Inoue *et al.* 2014) or increases the rate of mucosal proliferation in the treatment of short bowel syndrome (Tappenden *et al.* 2003). Conversely, FFA2 receptor antagonists might be useful in the treatment of FD by kerbing the 5-HT and neutrally mediated responses to foregut SCFAs.

In conclusion, duodenal enteroendocrine cells express functional FFARs, which, when activated, release GLP-2 in addition to the highly bioactive mediators 5-HT and acetylcholine. This novel pathway, with its inherent ability to locally regulate hormone and mediator release, may serve important functions in regard to mucosal protection and repair but, when deregulated, may contribute towards the genesis of FD-related symptoms.

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Additional information

Competing interests

All authors declare that they have no conflicts of interest.

Author contributions

Y.A. and J.D.K. were responsible for the study concept and design. Y.A., T.I., I.K., M.H., K.I. and M.W. were responsible for the acquisition of data. Y.A., T.I., I.K., M.H., P.H.G., E.E., A.K. and J.D.K. were responsible for data analysis. Y.A. and J.D.K. were responsible for manuscript preparation and revision. I.K. and M.W. were responsible for antibody production. K.N. was responsible for the acquisition of additional data. K.I. was responsible for chemical design and synthesis. P.H.G., E.E., A.K. and J.D.K. were responsible for the interpretation of data.

Funding

This work was supported by a Department of Veterans Affairs Merit Review Award, NIH-NIDDK R01 DK54221 (J. Kaunitz), a Grant-in-aid for Japanese Society for the Promotion of Science Fellow (I. Kaji, 24-5317) and the animal core of NIH-NIDDK P30 DK0413 (J. E. Rozengurt).

Acknowledgements

We thank Bea Palileo for her assistance with the preparation of the manuscript.