**RESEARCH**

**βHB inhibits glucose-induced GLP-1 secretion in GLUTag and human jejunal enteroids**

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**Abstract**

Ingestion of nutrients stimulates incretin secretion from enteroendocrine cells (EECs) of the epithelial layer of the gut. Glucagon-like peptide-1 (GLP-1) is one of these incretins that stimulate postprandial insulin release and signal satiety to the brain. Understanding the regulation of incretin secretion might open up new therapeutic options for obesity and type-2 diabetes mellitus. To investigate the inhibitory effect of the ketone body β-hydroxybutyrate (βHB) on glucose-induced GLP-1 secretion from EECs, *in vitro* cultures of murine GLUTag cells and differentiated human jejunal enteroid monolayers were stimulated with glucose to induce GLP-1 secretion. The effect of βHB on GLP-1 secretion was studied using ELISA and ECLIA methods. GLUTag cells stimulated with glucose and βHB were analysed using global proteomics focusing on cellular signalling pathways and the results were verified by Western blot. Results demonstrated βHB had a significant inhibitory effect on glucose-induced GLP-1 secretion at a dose of 100 mM in GLUTag cells. In differentiated human jejunal enteroid monolayers, glucose-induced secretion of GLP-1 was inhibited at a much lower dose of 10 mM βHB. The addition of βHB to GLUTag cells resulted in decreased phosphorylation of kinase AKT and transcription factor STAT3 and also influenced the expressions of signalling molecule IRS-2, kinase DGKs and receptor FFAR3. In conclusion, βHB displays an inhibitory effect on glucose-induced GLP-1 secretion *in vitro* in GLUTag cells and in differentiated human jejunal enteroid monolayers. This effect may be mediated through multiple downstream mediators of G-protein coupled receptor activation, such as PI3K signalling.

**Introduction**

Detailed understanding of the regulation of glucose homeostasis is important for finding better treatment options for diseases like obesity and type-2 diabetes mellitus. The nutrients we ingest trigger a physiological response already in the gastrointestinal tract (Lu et al. 2021). Nutrients, like carbohydrates and fat, can stimulate hormone secretion from enteroendocrine cells (EECs) residing in the epithelial layer of the gut. Incretins are hormones secreted from EECs in the small intestine that stimulate postprandial insulin release and, in the end, lower blood glucose (Holst & Orskov 2001, Drucker & Nauck 2006). One of the incretins released from EECs in the distal small intestine is glucagon-like peptide-1 (GLP-1) (Egerod et al. 2012, Habib et al. 2012, Haber et al. 2017).

Drugs mimicking incretins have beneficial effects on obesity and type-2 diabetes mellitus (Hansen et al. 2021),
2010, Wilding et al. 2021, Jastreboff et al. 2022). Therefore, understanding the regulation of incretin secretion is important for the development of future therapeutic options for these diseases. Increased levels of GLP-1 secretion are seen already in the days after bariatric surgery, and inhibition of these increases food intake which suggests an important role of gut-secreted incretins in weight loss after bariatric surgery (le Roux et al. 2007, Wallenius et al. 2018). GLP-1 levels in plasma also remain elevated at least 12 months after Roux-en-Y gastric bypass compared to levels before surgery (Wallenius et al. 2018). This elevation of the secretion of GLP-1 after bariatric surgery has been linked to increased glycemic control and increased glucose tolerance after surgery (Jørgensen et al. 2013). Since the changes in postprandial incretin profiles appear already 2 days after bariatric surgery, they cannot be associated with weight loss but must instead be associated with changes mediated by the surgery itself.

A recent study by our group identified high expression of the ketogenesis rate-limiting enzyme mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2) in the jejunum of obese patients prior to bariatric surgery and a profound decrease of this enzyme after surgery (Wallenius et al. 2020). This decrease was the most prominent proteomics regulation identified in the jejunal mucosa after RYGB. HMGCS2 expression results in the production of substantial amounts of ketone bodies like β-hydroxybutyrate (βHB) in the jejunal mucosa when its substrates, free fatty acids, are available. In the aforementioned study, we also demonstrated that βHB inhibits glucose-stimulated GLP-1 secretion in mouse primary jejunal cell cultures and we suggested that this may be a mechanism contributing to the rapid increase in incretin secretion after bariatric surgery. Ketone production in the alimentary limb should immediately be abolished after bariatric surgery due to substrate unavailability as lipids cannot be degraded to free fatty acids due to the absence of gastric and pancreatic lipases after Roux-en-Y gastric bypass. This is the consequence of the ‘rerouting’ of gastric and pancreatic secretions from the duodenum to the distal part of the jejunum. Therefore, this might be a contributory mechanism to the immediate increase of incretin secretion that has been demonstrated already days after surgery. We recently presented data that jejunal levels of HMGCS2 are increased by high-fat diet also in normal-weight non-diabetic humans (Elebring et al. 2021).

Both glucose and fat are physiological regulators of GLP-1 secretion from the gut (Lim & Brubaker 2006, Lu et al. 2021). Glucose stimulates GLP-1 secretion by entering the EEC along with sodium ions through the glucose transporter SGLT1. This movement of sodium ions produces a small electrical current that depolarizes the cell membrane and triggers an action potential resulting in increased intracellular levels of calcium ions, which in turn trigger GLP-1 secretion. Fats, specifically short-chain fatty acids (SCFAs), can also regulate GLP-1 secretion from EECs through G-protein coupled receptors (GPCRs) of the free fatty acid receptor (FFAR) family (Tollhurst et al. 2012). FFAR2 (previously known as GPR43) is the most potent and is activated by SCFAs with two to four carbon length (Brown et al. 2003, Le Poul et al. 2003). FFAR3 (previously known as GPR41) is activated by SCFAs with a carbon length of three to five and is expressed on EECs in the jejunum of obese patients prior to bariatric surgery (Nøhr et al. 2013, Nøhr et al. 2015). The ketone body βHB is known to inhibit FFAR3 activation in the sympathetic nervous system (Kimura et al. 2011). The mechanism of FFAR signalling in EECs is not clear, but activation of FFAR2 and 3 is known to result in ERK1/2 and AKT activation (Le Poul et al. 2003, Zhou et al. 2021).

In the present study, we employed two different in vitro model systems, murine GLUTag cells and human jejunal enteroid cultures, to study the effect of the ketone body βHB on glucose-stimulated GLP-1 secretion and downstream signalling pathways.

Methods

GLUTag cell culture

GLUTag cells are a stable, immortalized and well-characterized cell line of murine EECs established and provided by Dr Daniel Drucker. Cryopreserved cells were thawed and seeded into T75 flasks with low glucose (5.55 mM) Dulbecco’s Modified Eagle Medium (with pyruvate and l-glutamine, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin-streptomycin (Invitrogen) for expansion. Full change of cell culture media was performed every 2–3 days. When the cultures had reached about 80% confluency, they were split 1:3 or seeded into 24-well plates for stimulation experiments.

Human jejunal enteroid establishment and culture

Human jejunal enteroid cultures were established from biopsies retrieved from the jejunum (approximately 50 cm distal to the ligament of Treitz) from one healthy (BMI <25 kg/m², self-reported good general health and no history of drug abuse) volunteer (23 year old, female). Human biopsy retrieval was performed in accordance with the Declaration of Helsinki and approved by the Regional
Ethical Review Board in Gothenburg, Sweden (ethics application number 049-16). Written informed consent was provided by the study participants. Enteroids were established using the technique developed by Sato et al. (Sato et al. 2011). In brief, crypts were isolated through a series of washes and incubations in chelating and reducing agents. Isolated crypts were seeded, embedded in Matrigel (Corning) in 24-well plates and cultured with Wnt3A-supplemented expansion media (IntestiCult Organoid Growth Media (STEMCELLS, Vancouver, Canada)) with 10 µM Y-27632 (Sigma-Aldrich) and 0.1% gentamycin (Invitrogen) to enrich stem cells. To expand, Matrigel encapsulated cultures were treated with ice-cold Cell Recovery Solution (Corning) on ice for 1 h before being centrifuged and washed. Cystic cultures were broken into smaller fragments by pipetting, before being seeded into Matrigel again. Expanded cultures were recovered as previously described and seeded onto collagen-IV coated (15 µg/cm², Sigma-Aldrich) 24-well Transwell membranes (0.4 µm pore size, Corning) and expanded in expansion media on both sides of the membrane until confluent. When confluent, differentiation was achieved using the air–liquid interface (ALI) technique. With the ALI technique, differentiation medium (IntestiCult Organoid Differentiation Media (STEMCELLS) with 10 µM Y-27632 and 0.1% gentamycin) was only added to the basolateral compartment while the apical compartment was left without medium. Differentiation was carried out for 8 days before the enteroids were ready for stimulation experiments. Transepithelial electrical resistance (TEER) was measured at the start and end of differentiation using a voltmeter (Merck Millipore).

**Immunofluorescence of differentiated monolayers of human jejunal enteroids**

Differentiated monolayers of human jejunal enteroids derived from jejunum were chemically fixed in phosphate-buffered 4% formaldehyde while still on membranes in the Transwell plates. For immunofluorescence evaluation, monolayers were permeabilized and blocked in PBS with 15% FBS, 2% BSA and 0.1% saponin for 30 min before incubation with the primary antibody (chromogranin A, Abcam) overnight at 4°C. After primary antibody incubation, the membranes were washed and incubated with secondary antibody (goat anti-rabbit IgG Alexa 488, Invitrogen) for 1 h in darkness at room temperature. After washing, the membranes were stained with a probe for F-actin (Phallodin 568, Invitrogen) for 1 h in darkness at room temperature. After washing, the membranes were counter-stained with Hoechst (Invitrogen) staining. Membranes were then carefully cut from the inserts and mounted with the apical side up on slides and cover-slipped with ProLong Gold anti-fade reagent (Invitrogen). Blocking buffer instead of the primary antibody was used as a negative control. For a detailed list of primary antibody, secondary antibody and probes, see Supplementary Table 1 (see section on supplementary materials given at the end of this article). The slides were analysed using a fluorescence microscope (Nikon).

**Incretin secretion stimulation of GLUTag and human jejunal enteroid cells**

GLUTag cells at passages 13–15 were seeded at a density of 0.4 million cells per well and expanded until confluent (approximately 3 days). At this stage, they were used for incretin secretion experiments. For method development, GLUTag cell cultures were tested both with and without pre-incubation and with and without the addition of 10 µM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich) and 10 µM forskolin (fsk, Sigma-Aldrich) during incubation. For pre-incubation, wells were treated with saline secretion buffer (138 mM NaCl, 4.5 mM KCl, 4.2 mM NaHCO₃, 1.2 mM NaH₂PO₄, 2.6 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES and 0.1% BSA, adjusted to pH 7.4) (Wallenius et al. 2020) containing 0.5 or 1 mM glucose for 1 h. After pre-incubation, the medium was changed to different incubation media (saline secretion buffer with respective substance added at adequate concentration) and incubated for 2 h. After incubation, media were saved (with the addition of the DPP-4 inhibitor (50 µM, Sigma-Aldrich)) and cells were scraped from the surface in ice-cold RIPA buffer with cOmplete™ Protease Inhibitor Cocktail (Roche) and frozen at −80°C. For dose–response of glucose, the following D-glucose (Sigma-Aldrich) doses were used during incubation: 0, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 mM. For incretin secretion inhibition experiments, 0 mM D-glucose, 1 mM D-glucose and 1 mM D-glucose in combination with 0.01, 0.1, 1, 10 and 100 mM βHB (Sigma-Aldrich) or 0.1 and 1 µM somatostatin (Sigma-Aldrich) were used. To test the effect of βHB on basal GLP-1 secretion, different βHB concentrations (0.1, 1, 10 and 100 mM) were also tested in the absence of glucose. To evaluate the effect on GLP-1 secretion in the presence of high concentrations of other water-soluble molecules, we tested incubation with 100 mM butyrate (Sigma-Aldrich). For human jejunal enteroid monolayer culture, cells from passages 19–20 were differentiated using the ALI technique. For incretin secretion inhibition experiments, after pre-incubation, 0 mM glucose, 10 mM

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glucose or 10 mM glucose in combination with 10 mM βHB was used. Glucose was added only on the apical side of the monolayer and 10 μM IBMX/fsk was added to all groups. Apical and basolateral media were saved separately (with the addition of the DPP-4 inhibitor) and cells were scraped in ice-cold RIPA buffer with cOmplete Protease Inhibitor Cocktail and frozen at −80°C.

Alamar Blue visualization of GLUTag viability

To evaluate the potential toxic effects of βHB, different densities (1000, 5000, 10,000, 50,000, 100,000 and 500,000 cells per well) of GLUTag cells were seeded into 24-well plates and expanded for 2 days. After pre-incubation in saline secretion buffer with 0.5 mM glucose as previously described, the wells were incubated for 2 h with 1 mM glucose, 1 mM glucose with 1 mM βHB or 1 mM glucose with 100 mM βHB. During incubation, 10% Alamar Blue (Bio-Rad) reagent was added to the media. After incubation, the media were transferred to appropriate plates and the absorbance was read at 570 and 600 nm and the percentage reduction was calculated according to the manufacturer’s instructions.

GLP-1 quantification

GLP-1 was quantified with the Multi Species GLP-1 Total ELISA (Merck Millipore) for GLUTag culture media samples and the V-PLEX GLP-1 Total ECLIA (MesoScale, Rockville, MD, USA) for the human jejunal enteroid monolayer culture media samples.

Global proteomics

GLUTag cells stimulated as before with and without 100 mM βHB were analysed using global proteomics. For the complete method used, see Supplementary Methods. In total, 4698 proteins were identified by global proteomics. In order to reduce the data and focus on proteins involved in signalling, three different pathway databases (Reactome Pathways, KEGG Pathways and Wiki Pathways) were used. For each database, terms related to signalling were identified and proteins associated with the selected terms were sorted out for analysis.

Western blot

For protein extraction, stimulated GLUTag cells scraped off in ice-cold RIPA buffer (Sigma-Aldrich) containing cOmplete™ Protease Inhibitor Cocktail were thawed on ice, vortexed, centrifuged (10,000 g, 10 min, 4°C) and the supernatant protein content was quantified with the standard Bradford (Bio-Rad) method. For Western blotting, homogenates were diluted in Laemmli buffer (Bio-Rad) and heated at 95°C for 5 min before being loaded on Criterion TGX Stain-Free Precast gels (Bio-Rad) and electrophoresis was run using tris/glycine/SDS buffer (Bio-Rad). After electrophoresis, proteins were transferred to polyvinyl difluoride membranes (Bio-Rad) using the Trans-Blot Turbo (Bio-Rad) blotting system. Membranes were incubated with primary antibody followed by secondary antibody. Chemiluminescence was developed using Clarity Max Western ECL Substrate (Bio-Rad). Images were captured by the ChemiDoc system (Bio-Rad), and semi-quantification was performed using Image Lab (Bio-Rad) software. Membranes were stripped sequentially using ReBlot Plus Mild (Merck Millipore).

Statistics

Statistical outcomes were analysed with the Wilcoxon test when comparing two paired groups, ordinary one-way ANOVA with Dunnett’s multiple comparison test when comparing multiple unpaired groups with one variable and two-way ANOVA with Dunnett’s multiple comparison test when comparing multiple unpaired groups with two variables. For comparison of groups in global proteomics, Student’s t-test was used. A P-value of <0.05 was considered significant. All analyses were performed using Prism 9 for Mac OS Big Sur (GraphPad).

Results

Human jejunal enteroids from stem cells isolated from crypts differentiate spontaneously and result in a subpopulation of enteroendocrine cells

Crypts isolated from human jejunum grow in cystic form (Fig. 1A) when cultivated encapsulated in Matrigel in Wnt3a supplemented media but can also be cultivated in monolayers on Transwell membranes (Fig. 1B). When Wnt3a supplement is withdrawn from media, the expanded stem cell population starts to differentiate spontaneously. Differentiation of monolayers using the air–liquid technique results in a dense cell mat (Fig. 1C) with a significant rise in TEER (P < 0.0001, Fig. 1D).
Differentiated enteroid monolayers from human jejunal crypts characterized using immunofluorescence showed a subpopulation of EECs expressing the enteroendocrine marker Chromogranin A (Fig. 1E).

Pre-incubation in glucose-reduced media and no addition of IBMX/fsk is the optimal setting for GLUTag GLP-1 secretion

GLUTag cells cultured on the bottom of wells (Fig. 1F) display secretion of GLP-1 in a glucose-sensitive manner. The addition of the GLP-1 secretion stimulants IBMX/fsk not only increased the glucose-stimulated secretion but also the basal levels of GLP-1 (Supplementary Fig. 2). Pre-incubation of GLUTag cells in the glucose-reduced medium before the start of the experiments slightly increased the glucose-stimulated compared to the basal ratio of GLP-1 (1.93 vs 1.68).

GLUTag cells secrete GLP-1 in a glucose dose-sensitive manner

GLUTag cells, when cultured in increasing concentrations of glucose, show increasing levels of GLP-1 secretion. Even though individual cell culture experiments showed different absolute values of GLP-1 secretion (Fig. 2A), when normalized, a clear increase of GLP-1 secretion up to the maximum tested glucose dose of 10 mM was seen (Fig. 2B).

Ketone body β-hydroxybutyrate does not affect the viability of GLUTag cells at high cell densities

To ensure that βHB does not affect cell viability, two different concentrations were tested at cell densities ranging from 1000 cells to 500,000 cells per well with the Alamar Blue assay (Fig. 3A). At the highest cell density, neither 1 mM nor 100 mM βHB had any negative effect on
Ketone body β-hydroxybutyrate inhibits glucose-induced incretin secretion in GLUTag and differentiated human jejunal enteroid monolayer cell cultures

The ketone body βHB had a significant ($P < 0.0001$) inhibitory effect on glucose-induced GLP-1 secretion at a dose of 100 mM in GLUTag cells (Fig. 4A). Surprisingly, also the lowest dose tested, 0.01 mM, had a significant effect ($P = 0.0247$, Fig. 4A), while intermediate doses showed no effect. The GLP-1 secretion inhibitor somatostatin displayed significant inhibition at both 0.1 μM and 1 μM ($P < 0.0001$ for both, Fig. 4B). In the absence of glucose, however, the ketone body βHB in different concentrations had no such inhibitory effect on GLP-1 secretion (Supplementary Fig. 1). 100 mM butyrate or 154 mM NaCl did not affect the GLP-1 secretion (Supplementary Fig. 1). Differentiated human jejunal enteroid monolayers from crypts displayed an increased GLP-1 secretion when stimulated with 10 mM glucose compared to culture conditions with no glucose ($P = 0.0336$; Fig. 4C). This glucose-sensitive increase in GLP-1 secretion was completely inhibited when 10 mM βHB was added to the cultures ($P = 0.0361$. Fig. 4C).

Ketone body β-hydroxybutyrate influences the expression of proteins in several downstream signalling cascades in GLUTag cells

To investigate the signalling pathways for the inhibitory effect of βHB, global proteomic analysis was performed on GLUTag cells. The proteomes of glucose-treated cells with and without the addition of 100 mM βHB were compared. From this analysis, 20 proteins involved in signalling were found to be significantly down-regulated, while...
Incretin secretion, that is, GLP-1, from the small intestine upon nutrient stimulation is important for glucose homeostasis. In patients with type-2 diabetes mellitus, this secretion is suppressed for unknown reasons (Nauck & Meier 2018). The mechanism for this inhibition of incretin secretion has been elusive. In the present study, we report that the ketone body \( \beta \)HB significantly decreased GLP-1 secretion in murine EECs (GLUTag cells), which we also recently reported in primary mouse jejunal cell cultures (Wallenius et al. 2020). We also report an even more marked inhibitory effect of \( \beta \)HB in human jejunal enteroid cells.

To study the effect of \( \beta \)HB on glucose-stimulated GLP-1 secretion in human jejunal EECs, monolayers of human jejunal enteroid cultures were retrieved and differentiated from crypts isolated from the jejunum of a normal-weight volunteer. These monolayers were differentiated using the ALI technique (no liquid on the apical side of the membrane), which mimics the physiological conditions in the jejunum where the lumen, not all of the time is filled with liquid. During differentiation, TEER increased significantly, indicating successful differentiation. Immunofluorescent characterization of the differentiated human jejunal enteroid monolayers revealed the presence of the EEC marker chromogranin A, indicating the presence of EECs after differentiation. EECs constitute only <0.1%
Figure 5
Global proteomics and Western blot analysis of proteins in glucose-induced GLUTag cells incubated with ketone body β-hydroxybutyrate (βHB). (A) Volcano plot of proteins involved in signalling pathways from global proteomic analysis in GLUTag cells induced with 1 mM glucose and incubated with and without 100 mM βHB (n = 5 for each group). Protein with a P-value (Student's t-test) below 0.05 was considered significant. Blue dots represent significantly down-regulated and red dots represent significantly up-regulated proteins after the addition of βHB. (B–J) Western blot analysis of GLUTag cells incubated with and without different concentrations of βHB (n = 9 for each treatment). (B–D) AKT and phosphorylated AKT. (E–G) STAT3 and phosphorylated STAT3. (H–J) IRS-2, DGK epsilon and FFAR3, respectively. (B–J) Individual intensities were related to total protein intensity for each sample and group data have been related to the glucose control group and are given as means ± s.e.m. *P < 0.05 **P < 0.01 ****P < 0.0001 (one-way ANOVA with Dunnett's multiple comparison test). A full colour version of this figure is available at https://doi.org/10.1530/JME-22-0115.
of the cell population in normal gut (Sternini et al. 2008, Gunawardene et al. 2011), and a similar distribution was seen in differentiated human jejunal enteroid monolayers.

To ensure that the inhibitory effect on GLP-1 secretion seen in GLUTag cells was not due to a toxic effect of βHB, an Alamar Blue assay was used. This assay revealed that at the highest seeding density (500,000 cells per well), no toxic effect of either 1 mM or 100 mM βHB could be seen. The seeding density used for the inhibitory experiments was almost equivalent to the highest tested density in the toxicity assay; thus, the inhibitory effect on GLP-1 secretion was concluded to not be due to the toxic effects of βHB.

βHB at 100 mM exhibited an inhibitory effect on glucose-induced GLP-1 secretion in GLUTag cells close to the effect of the known incretin inhibitor somatostatin. Surprisingly, also the lowest tested dose of βHB (0.01 mM) showed an inhibitory effect on GLP-1 secretion compared to the control group with only glucose added, while the intermediate doses had no effect. This result is a bit puzzling in pharmacological terms but might indicate a dual effect of βHB on two biological systems, one at lower doses and one at higher doses.

βHB at 100 mM might sound like a high and non-physiological dose, but we argue it is not. By a conservative calculation, the local βHB concentration in the mucosal epithelia has been approximated at about 10 mM in starved samples (Wallenius et al. 2020). Moreover, in the GLUTag monocultures, all cells are EECs, so higher doses of ketone bodies are probably needed to reach the inhibitory concentration compared to jejunal mucosa in vivo, or the enteroid cultures, with less than 1% of all cells being EECs.

To investigate the downstream regulation resulting in the inhibition of GLP-1 secretion, we used global proteomics analysis of GLUTag cells treated with βHB. The comparison was made between cells treated only with 1 mM glucose and cells treated with 1 mM glucose and 100 mM βHB. To reduce the data from the proteomics analysis and focus on relevant proteins for the regulation of GLP-1 secretion, we employed existing pathway databases to single out proteins involved in signalling pathways. This reduced the number of identified proteins from almost 5000 to 412. Statistical analysis identified 55 of these proteins to be significantly regulated (Supplementary Table 2). Among the most regulated proteins were PEA15, IRS-2, DGKε and STAT3. The list of significantly regulated proteins identified with global proteomics also included a number of kinases. Therefore, we decided apart from the four proteins previously mentioned to also include the signalling mediators AKT and ERK1/2 in the following Western blot analysis.

From Western blot analysis, it could be seen that 100 mM βHB influenced the phosphorylation of AKT and STAT3 in the GLUTag cells. βHB increased the ratio of phosphorylated form to native form for both AKT and STAT3, while ERK1/2 was unchanged. These observations are interesting and give a clue on how βHB might influence GLP-1 secretion. In previous studies, it has been shown that activation of FFAR3 results in activation of the AKT pathway (Zhou et al. 2021). Here, in our present study, the results implicate the same: a high dose of βHB results in changed expression and phosphorylation of AKT. It has previously been reported that GLP-1 production is mediated through STAT3 signalling (Lei et al. 2019).

βHB at 100 mM decreased the expression of DGKε in GLUTag cells according to Western blot, while the same enzyme was identified to be up-regulated in the global proteomics analysis. Why the direction of regulation differs is puzzling, but both techniques have identified this enzyme to be significantly regulated with the high dose of βHB used. DGKs catalyse the conversion of diacylglycerol to phosphatidic acid, thereby influencing phosphoinositide 3-kinase (PI3K) signalling (Shulga et al. 2011). PI3K signalling is associated with GPCR activation and has previously been shown to be activated by FFAR3 activation (Vanhaesebroeck et al. 2012, Zhou et al. 2021). This is interesting and might be a clue to how the ketone body βHB inhibits GLP-1 secretion. For lower doses of βHB (0.01 and 1 mM), Western blot revealed IRS-2 to be up-regulated. IRS-2 also influences PI3K signalling and might further bring evidence to the effect of βHB being mediated via PI3K signalling (Landis & Shaw 2014).

The present study also showed an increased expression of FFAR3 with stimulation of GLUTag cells with the ketone body βHB. This is an interesting observation and might be a cellular response to compensate for the inhibitory action of βHB by expressing more receptors. Since GLP-1 secretion can also be induced by SCFAs through FFARs (Tolhurst et al. 2012), it is of interest in the future to also study the effect of βHB with simultaneous SCFA stimulation of EECs. This is because the inhibitory effect might be even greater since the induction and inhibition may be mediated via the same receptor(s).

In conclusion, this study demonstrates an inhibitory effect of the ketone body βHB on glucose-induced GLP-1 secretion in vitro in murine GLUTag cells. We also show this inhibitory effect, to our knowledge, for the first time, in vitro in differentiated human jejunal enteroid...
monolayers. Further, we demonstrate that the ketone body βHB regulates multiple mediators downstream of GPCR activation, indicating that the inhibitory effect of βHB might be mediated via these pathways.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/JME-22-0115.

Declaration of interest
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Author contribution statement
EE planned the study, performed the cell culture, Western blots, ELISA, analysed the data and wrote the first draft including the statistical analysis; AC planned the study, performed immunofluorescence and ECLIA, analysed the data and revised the manuscript; SP performed Western blots; LF and VW planned the study, provided funding, analysed the data and revised the manuscript; JØ and JD planned the study, performed immunofluorescence and ECLIA, analysed the data and wrote the first draft including the statistical analysis; AC planned the study, performed immunofluorescence and ECLIA, analysed the data and revised the manuscript.

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