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Short title: Propionate directly stimulates insulin release

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Abstract

Aims: Diet-derived short chain fatty acids (SCFAs) improve glucose homeostasis in vivo, but the role of individual SCFAs and their mechanisms of action have not been defined. This study evaluated the effects of increasing colonic delivery of the SCFA propionate on β-cell function in humans and the direct effects of propionate on isolated human islets in vitro.

Materials and Methods: For 24 weeks human subjects ingested an inulin-propionate ester that delivers propionate to the colon. Acute insulin, GLP-1 and non-esterified fatty acid (NEFA) levels were quantified pre- and post-supplementation in response to a mixed meal test. Expression of the SCFA receptor FFAR2 in human islets was determined by western blotting and immunohistochemistry. Dynamic insulin secretion from perifused human islets was quantified by radioimmunoassay and islet apoptosis was determined by quantification of caspase 3/7 activities.

Results: Colonic propionate delivery in vivo was associated with improved β-cell function with increased insulin secretion that was independent of changes in GLP-1 levels. Human islet β-cells expressed FFAR2 and propionate potentiated dynamic glucose-stimulated insulin secretion in vitro, an effect that was dependent on signalling via protein kinase C. Propionate also protected human islets from apoptosis induced by the NEFA sodium palmitate and inflammatory cytokines.

Conclusions: Our results indicate that propionate has beneficial effects on β-cell function in vivo, and in vitro analyses demonstrated that it has direct effects to potentiate glucose-stimulated insulin release and maintain β-cell mass through

inhibition of apoptosis. These observations support ingestion of propiogenic dietary fibres to maintain healthy glucose homeostasis.

Introduction

Diets containing high amounts of indigestible components such as complex carbohydrates and fibre increase satiety, promote weight loss and improve glucose homeostasis in both animals and humans (1,2). It is thought that the key event in transducing the beneficial effects of these indigestible foods is through their fermentation by colonic anaerobic bacteria to generate the short chain fatty acids (SCFAs) acetate, propionate and butyrate. The colon contributes substantially to circulating acetate levels in man, with peak concentrations of ~200μM observed after caecal delivery of the fermentable disaccharide lactulose (3) or oral delivery of SCFAs (4). In contrast, circulating levels of propionate and butyrate are reported to be approximately 10-fold lower (2). It is now apparent that these SCFAs can exert their effects through activation of the G-protein coupled receptors (GPCRs) FFAR2 and FFAR3 (1,2,5), either locally in the colon and/or following their absorption into the systemic circulation. FFAR3 is coupled intracellularly to inhibitory pathways via Gαi whilst FFAR2 is coupled to both Gαi and stimulatory signalling via Gαq (6). FFAR2 is expressed by colonic L-cells and SCFAs can stimulate GLP-1 release from these cells (9). We have shown that propionate stimulation of GLP-1 and PYY secretion is dependent on FFAR2 activation (10) and it has been demonstrated that FFAR2 deletion in mice impairs glucose tolerance and reduces glucose-stimulated GLP-1 release (9). These observations fit with a model in which dietary fibre improves glycaemic control through production of SCFAs in the colon, activation of L-cell FFAR2 and the consequent increased GLP-1 release promotes insulin secretion from β-cells.

However, signalling of SCFAs in tissues distinct from L-cells can also contribute to improved glucose homeostasis. For example, FFAR2 is expressed by adipocytes and the pancreas (2), implying important roles in lipid and glucose regulation. Consistent with this, SCFAs act directly at adipocytes to inhibit lipolysis (10) thus reducing levels of adipocyte-derived long chain non-esterified fatty acids (NEFA) such as palmitate that contribute to glycaemic deterioration (2,11). In addition, SCFAs can act directly at islet β-cells to regulate insulin secretion. However, the studies reported to date have largely been performed using mouse islets, which may not appropriately reflect the human situation (12-14). They have focused on the direct effects of acetate on β-cells and there is currently no consensus from these studies on whether insulin release is stimulated or inhibited by this SCFA (12-14), nor on the role of the other circulating SCFA, propionate. We have recently demonstrated that targeted administration of propionate to the colon, in the form of inulin-propionate ester (IPE), acutely reduces energy intake and ameliorates long-term weight gain in overweight adults (15).

We have now extended our studies to investigate the effects of increasing colonic propionate for 24 weeks on glucose homeostasis in humans, with a focus on acute circulating NEFA and insulin levels following a mixed calorie meal test. We have also analysed the effects of propionate on β-cell function in vivo using the oral disposition index. We have complemented the assessment of in vivo delivery of propionate with characterisation of its direct effects in vitro to regulate insulin secretion from human islets and protect them against apoptosis. Our data demonstrate that long-term colonic propionate delivery significantly improves β-cell function, as evidenced by increased circulating insulin without any changes in insulin sensitivity, an effect that

is not secondary to increased GLP-1 levels. In vitro experiments with human islets indicate that propionate acts directly to reversibly stimulate insulin secretion through a protein kinase C-dependent pathway and protect islets from apoptosis induced by cytokines and the NEFA palmitate.

Materials and Methods

Materials

Culture media and supplements, general laboratory chemicals: Sigma-Aldrich (Dorset, UK). Alexa-fluor secondary antibodies: GE Healthcare Life Sciences (Little Chalfont, UK). Tissue culture flasks, cover slips, BCA protein assay, 10% polyacrylamide gels, NuPAGE® sample buffer, transfer buffer: Thermo Fisher Scientific (Paisley, UK). FFAR2 antibodies, X-ray film: Santa Cruz Biotechnologies (Middlesex, UK). Insulin antibody: DAKO (Cambridge, UK). HRP-conjugated secondary antibody, ECL western blotting reagents, Cell Titer-Glo 3D assay, Caspase3/7 Glo assay: Promega (Hampshire, UK). Rainbow molecular weight markers and nitrocellulose membrane: Millipore (Watford, UK). Cyclic AMP HiRange cell-based assay: Cisbio assays (Codolet, France). TNFα, IL-1β and IFNγ: PeproTech (London, UK). Plasma glucose assay: Abbot Diagnostics (IL, USA). Insulin radioimmunoassay for in vivo samples: Millipore (MA, USA). NEFA assay: Randox Laboratories (WV, USA).

Colonic propionate delivery in vivo

We have developed inulin-propionate ester (IPE) as a vehicle to efficiently deliver propionate to the colon (15). We have previously reported the impact of IPE on appetite regulation and body weight maintenance (16), and here we analyse its

effect on glucose homeostasis. Recruited subjects (age: 40-65; BMI: 25-40 kg/m^2; 19 male, 30 female) provided informed, written consent prior to participation. Participants were randomised to receive a daily dietary supplement (sachet) of 10g inulin as a control (n=24) or 10g IPE (n=25) over 24 weeks. Overnight fasting and postprandial blood samples (-10 min, 0 min, 15 min, 30 min, 60 min, 90 min and 120 min) were collected before dietary supplementation (week 0) and at the end of the study (week 24) after ingestion of a mixed calorie meal test (398 kcal; 71.2g carbohydrate, 7.9g fat, 10.3g protein), which also contained 10g inulin or 10g IPE at week 24, as appropriate. Levels of glucose, insulin and GLP-1 in blood samples were quantified as previously described (13). Total NEFA levels were analysed on an ILAB 650 Clinical Chemistry Analyser (Diamond Diagnostics, MA, USA). The clinical trial (NCT00750438) was approved by the Hammersmith and Queen Charlotte's Research Ethics Committee (08/H0707/99).

Human Islets

Human islets were isolated from 22 non-diabetic donors (age: 43±2; BMI: 29.7±1.2 kg/m^2; 7 male, 15 female) at the King's College Hospital Islet Transplantation Unit, with appropriate ethical approval (LREC 01-082) (15). Islets were maintained in CMRL medium supplemented with 2% human albumin, 4mM glutamine, 2mM HEPES (pH 7.2-7.4), and 10mM nicotinamide at 37°C, 5% CO_2 prior to functional analyses.

Insulin secretion in vitro

For static incubation assessment of insulin secretion groups of 5 human islets were incubated for 30 minutes at 37°C in buffer (18) supplemented with agents of interest,

after which insulin release was quantified by radioimmunoassay (19). For perifusion experiments groups of 50 human islets were perifused with a physiological salt solution (18) and perifusate samples were collected at 2 minute intervals for quantification of secreted insulin (19-21). Islet PKC can be selectively down-regulated by maintenance for 20 hours in the presence of 4β phorbol 12-myristate 13-acetate (PMA) (20), so in some experiments the role of PKC was investigated by perifusing islets that had been treated with PMA to deplete PKC. Sodium propionate was dissolved in the physiological salt solution (18) immediately before experimental use.

Western Blotting

Groups of 200 human islets were lysed and protein was quantified by the BCA method. 50μg of islet protein extracts were fractionated by denaturing SDS polyacrylamide gel electrophoresis (10% gel, 45 minutes, 200V), transferred onto 0.2μm nitrocellulose and probed overnight at room temperature with rabbit anti-FFAR2 antibody (1:500). The membrane was then incubated for 1h at room temperature with anti-rabbit HRP-conjugated secondary antibody (1:10,000) and exposed to X-ray film after addition of ECL substrate.

Fluorescence Immunohistochemistry

Non-pathological human pancreas 5μm sections were boiled in 0.01M citric acid buffer (pH 6.0) for 2.5 minutes for antigen retrieval, blocked (0.1% donkey serum in PBS with 0.02% triton X-100, 1h, room temperature), then incubated at 4°C overnight with goat anti-FFAR2 (1:50) and guinea pig anti-insulin (1:250) antibodies. Sections were then exposed to Alexa-fluor secondary antibodies (1:250) for 1h at

room temperature, nuclei were stained with DAPI and images were acquired using a Nikon Eclipse TE2000-U microscope.

Calcium Microfluorimetry

Groups of 100,000 dispersed human islet cells on glass coverlips were incubated for 15 minutes with 5μM Fura-2 AM. The coverslips were placed in a steel chamber that was mounted onto a heating platform of an Axiovert 135 inverted microscope. Cells were perifused (1 ml/min) with a physiological salt solution (18) containing test agents. Real-time changes in $[Ca^{2+}]_i$ were determined by illuminating cells alternately at 340nm and 380nm, with the emitted light being filtered at 510nm and recorded with a CCD camera. Data were collected every 3 seconds from multiple cells in a field of view (23).

Cyclic AMP

Groups of 5 human islets were incubated for one hour at 37°C in physiological salt solution containing 20mM glucose and supplemented with 2mM IBMX to inhibit phosphodiesterases. Islets were then lysed and cAMP levels were quantified using a cAMP fluorescence assay, according to the manufacturer's instructions. Data were acquired using a PHERAstar FS microplate reader (100μs delay time, 100μs integration time, 50 flash read time).

ATP

Groups of 3 islets human islets were incubated for one hour at 37°C, lysed and ATP levels were determined by chemiluminescence with the Cell Titer-Glo assay, according to the manufacturer's instructions (21).

Apoptosis

Human islets were pre-cultured in complete CMRL medium in the absence or presence of propionate for 24h, then groups of 5 islets were exposed to CMRL with 0.2% albumin supplemented either with 500μM sodium palmitate or a cytokine cocktail (5U/μl TNFα , 0.5U/μl IL-1β, 5U/μl IFNγ) in the continued absence or presence of propionate for a further 20h. Islet cell apoptosis was determined using the Caspase3/7 Glo assay according to the manufacturer's instructions (21). Mean luminescence data of each group of islets were normalised to the basal apoptosis levels obtained in the absence of cytokines or sodium palmitate.

Statistical analyses

Changes in postprandial glucose, insulin, NEFA and GLP-1 levels at week 24 were calculated as differences from pre-supplementation values. Between group differences were analysed by repeated measures ANOVA. To determine changes in acute postprandial responses, areas under the curve (AUC) were calculated from 0-30min using the trapezoid method. Postprandial insulin sensitivity (IS) was estimated from the meal test using the Matsuda Index (24) and an oral disposition index, which provides a measure of insulin release adjusted for IS, was calculated as Insulin $AUC_{0–30min}$/Glucose $AUC_{0–30min}$ x IS (25). The change from pre-supplementation values at week 24 was calculated and differences between supplementation groups compared using unpaired t-tests. In vitro data are expressed as mean±SEM for the numbers of experiments and replicates indicated in the Figure legends and statistical analyses were performed by Student's t-tests or one way ANOVA, as appropriate.

Results

Colonic propionate delivery improves β-cell function in vivo

Analysis of $AUC_{0\text{-}30min}$ after the meal test indicated that there was a significant reduction in plasma NEFA levels in subjects that had received the IPE supplement for 24 weeks (Figure 1a) and significant reductions in NEFA were also evident over the 120 minute postprandial period (Supplementary Figure 1a). Long-term colonic propionate delivery was also associated with a significant increase in circulating insulin levels in the first thirty minutes following the meal (Figure 1b) independent of elevations in the early postprandial glucose levels (Figure 1c). There was also a trend for long-term propionate delivery to increase insulin secretion over 120 minutes ($p=0.08$), again without any change in glucose levels (Supplementary Figure 1b-1c). Calculation of the Matsuda Index (24) indicated that the change in IS at week 24 was not different between groups (Figure 1d), but the oral disposition index demonstrated that those subjects taking the IPE supplement had improved β-cell function (Figure 1e), which was not associated with increases in circulating GLP-1 levels (Figure 1f, Supplementary Figure 1d). We have previously reported that IPE supplementation increases propionate concentrations in the peripheral circulation (16), suggesting that the observed increase in acute insulin secretion could be a consequence of circulating propionate acting directly at islet β-cells.

Propionate potentiates dynamic insulin secretion from human islets in vitro.

Static incubation experiments in which human islets were incubated in the presence of 1mM sodium propionate for thirty minutes indicated that it did not significantly affect insulin secretion, despite the islets showing an appropriate secretory response when glucose levels were increased from 2mM to 20mM and when they were

challenged with the muscarinic agonist carbachol, which acts via a Gq-coupled GPCR (Figure 2a). Further experiments were performed by carrying out perifusions, which provide a sensitive dynamic profile of insulin secretion where secretory products are constantly removed to minimise paracrine interactions. Human islets responded to an elevation in glucose from a basal concentration of 2mM to a supraphysiological stimulus of 20mM with a short-lived first phase of insulin secretion that was followed by a sustained plateau (Figure 2b). Exposure of the islets to 1mM propionate in the continued presence of 20mM glucose resulted in a rapid potentiation of insulin secretion that was fully reversible, such that secretion returned to the plateau phase upon removal of propionate. Quantification of AUC data during exposure to propionate indicated that the increase was statistically significant (AUC, 20mM glucose: 324.9±35.5 pg insulin/20min; +1mM propionate: 633.3±95.5, n=4, $p<0.01$). Having established that propionate directly potentiates insulin secretion in vitro, the effects of lower concentrations were also investigated in perifusion experiments. These indicated that propionate concentrations as low as 10μM induced a significant elevation in insulin release at 20mM glucose that was as effective as the stimulation induced by 100μM propionate (Figure 2c).

Human islet β-cells express FFAR2

SCFAs signal through binding to the GPCRs FFAR2 and FFAR3, which show widespread distribution. We have previously detected mRNAs encoding these receptors in human islets (26), but since FFAR3 is coupled to Gαi it is most likely that propionate potentiates insulin secretion through activation of FFAR2, which signals through both Gαq and Gαi. Western blotting with an anti-FFAR2 antibody indicated that an immunoreactive protein of 43kDa was detected in human islets

isolated from two separate donors (Figure 3a). The subcellular localisation of FFAR2 was investigated by fluorescence immunohistochemistry in human pancreas sections. It can be seen from Figure 3b that FFAR2 (red) was readily detected in the endocrine pancreas, where it was co-expressed with insulin (green) in islet β-cells. There was very little FFAR2 immunoreactivity evident in the exocrine pancreas (Figure 3b).

The stimulatory effects of propionate on insulin secretion from human islets are dependent on protein kinase C activation.

GPCRs that are coupled to Gq signal via elevations in inositol trisphosphate (IP_3) and diacylglycerol (DAG), with IP_3 increasing intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) and DAG activating protein kinase C (PKC). Propionate increased $[Ca^{2+}]_i$ in Fura-2-loaded human islet cells, with an effect that was visible within 30 seconds of exposure (Figure 4a). The potentiation of insulin secretion by propionate was abolished in islets that had been exposed to the phorbol ester 4β-phorbol 12-myristate 13-acetate (PMA) for 24 hours to down-regulate PKC, while islets that had been treated with the inactive phorbol ester 4α-phorbol 12,13-didecanoate (PDD) responded appropriately to propionate (Figure 4b). Potential signalling of propionate through Gi was also investigated by quantification of cAMP generation in human islets. These experiments indicated that forskolin caused a substantial increase in islet cAMP production, which was significantly reduced by the α_2 adrenergic agonist clonidine, but not by propionate (Figure 4c). SCFAs can diffuse through the plasma membrane due to their short carbon skeletons, and it is possible that they potentiate insulin secretion by acting as mitochondrial metabolic substrates. However, measurements of intracellular ATP levels indicated that while increasing the glucose

concentration from 2mM to 20mM resulted in increased ATP, as expected, co-incubation of islets with 100μM or 1mM propionate at 20mM glucose did not result in further increases in ATP production (Figure 4d).

Propionate protects human islets from cytokine- and palmitate-induced apoptosis

In vivo, islets are susceptible to damage by lipolysis-generated fatty acids such as palmitate, and by cytokines that are secreted following islet infiltration by immune cells. The ability of propionate to protect human islets from apoptosis induced by cytotoxic and lipotoxic environments was evaluated by measurement of caspase 3/7 activities in vitro. Human islets exposed for 20 hours to a cytokine cocktail showed elevations in apoptosis, and a significant increase in human islet caspase 3/7 activities was also observed following exposure to 500μM sodium palmitate (Figure 5a, 5b). Maintenance of islets in the presence of 1mM propionate did not affect basal apoptosis, but significantly reduced the pro-apoptotic signalling induced by cytokines (Figure 5a) and palmitate (Figure 5b).

Discussion

There is compelling evidence that diet-derived SCFAs have beneficial effects on fuel homeostasis, but the focus to date has largely been on SCFA-mediated improvements in insulin sensitivity (2) or on indirect effects of SCFAs to increase β-cell function via elevations in GLP-1 release from L-cells (1). In the current study we have demonstrated that subjects undergoing mixed calorie meal tests containing 10g inulin-propionate ester, after 24 week daily supplementation with 10g inulin-propionate ester, show significant improvements in acute insulin secretion and β-cell

function compared to control subjects receiving inulin alone. The lack of effect on GLP-1 levels in the acute meal tests in the propionate subjects at 24 weeks points to the enhanced β-cell function being a direct effect of propionate, rather than secondary to propionate-induced elevations in GLP-1. The limited availability of human islets for research means that the studies to date on the role of SCFAs to directly regulate islet function have largely been carried out using mouse models and/or isolated mouse islets (12-14, 27).

The earlier islet studies have focused on the effects of acetate, and there are reports that this SCFA inhibits (12), stimulates (13) or has no effect (14) on glucose-induced insulin secretion. The reason for the discrepancies between these three studies, all using static incubations with 1mM acetate is not clear, but the dual stimulatory (Gαq) and inhibitory (Gαi) activity downstream of FFAR2 activation may explain, at least in part, different observations on the roles played by these receptors in islet function. In addition, in static incubation experiments paracrine signalling via accumulated products such as somatostatin and GABA, secreted from islet endocrine cells, may affect total insulin release, and static experiments do not provide information on the rate of onset or duration of changes in insulin secretion, nor the reversibility of these effects. Our own studies indicated that static incubation experiments using isolated human islets are not a sufficiently robust experimental model to define the effects of propionate on insulin secretion in vitro. Thus, quantification of insulin secretion after a 30 minute incubation indicated that 1mM propionate did not significantly affect glucose-stimulated insulin secretion, whereas dynamic perifusion experiments revealed that concentrations as low as 10μM were sufficient to reversibly potentiate insulin secretion. Circulating propionate concentrations are approximately 1-13μM

(2) and we have recently reported that IPE supplementation results in elevation of plasma propionate levels above 20μM (16), so these observations of direct stimulatory effects of propionate on insulin secretion from isolated human islets are physiologically relevant.

We have investigated the mechanisms by which propionate potentiates insulin secretion. Numerous studies have indicated that SCFAs, including propionate, activate the fatty acid receptors FFAR2 and FFAR3 (reviewed in 2). Both receptors couple via Gi to inhibit cAMP generation, while FFAR2 also signals via Gq to stimulate PIP_2 hydrolysis. The stimulatory effects of propionate on insulin secretion implicate signalling distinct from Gi coupling in human islets. This was confirmed by the lack of inhibition of cAMP production by propionate, indicating that it does not mediate its effects via Gi-coupled FFAR3 in human islets. Propionate may also enter the TCA cycle, and could potentially increase insulin secretion through enhancing ATP synthesis. However, quantification of ATP production indicated that it did not enhance the ATP generation stimulated by glucose in human islets. Thus, the most likely mechanism through which propionate potentiates insulin secretion is via Gq-mediated signalling consequent to FFAR2 activation. This is supported by our data indicating that FFAR2 is expressed by β-cells in human pancreas, as it is in mouse pancreas (12,14) and by our observations that propionate elevated $[Ca^{2+}]_i$ in human islets and that PKC down-regulation prevented the stimulatory effects of propionate on insulin secretion from human islets.

Recent studies in mice have indicated direct signalling via FFAR2 in islets to regulate β-cell mass. Thus, it has been reported that a phenylacetamide derivative agonist of

FFAR2 enhances β-cell proliferation (14), FFAR2 knockout mice show reduced β-cell expansion during pregnancy (27), and a FFAR2 agonist that preferentially couples via Gq promotes mouse islet β-cell proliferation (28). Consistent with SCFAs playing an important role in maintaining β-cell mass, we have demonstrated, for the first time, that propionate significantly reduced human islet apoptosis induced by sodium palmitate and by cytokines. This suggests that propionate can play a key protective role in the context of obesity where β-cells are required to hypersecrete insulin to compensate for insulin resistance, but are compromised by increased apoptosis driven by elevations in circulating NEFA and locally produced cytokines.

In summary, we have demonstrated that increasing colonic propionate improves β-cell function in vivo, and our in vitro observations in human islets indicate that this may occur both via short-term acute effects of propionate to directly stimulate insulin secretion and through its longer term effects to protect β-cells from apoptotic stimuli. These data support the ingestion of propiogenic dietary fibres to maintain healthy glucose homeostasis, both through the established beneficial effects via elevations in GLP-1 and PYY (16) and by the novel signalling identified here, of direct stimulatory effects of propionate at β-cells.

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Author Contributions

The study was designed by AP, EC, TH, GB, GF and SJP. DM, TP, CT and GF designed and produced inulin propionate ester for the intervention study. Data were collected and analysed by AP, EC, TH, IRM, BL and SP. RCG provided human pancreas blocks. GCH and PC provided isolated human islets. The article was drafted by AP and SJP. All authors revised the article critically for important intellectual content. All authors gave their final approval of the current version to be published. GF is an NIHR senior investigator. SJP takes responsibility for the contents of the article.

Figure Legends

Figure 1: Effects of elevating colonic propionate levels for 24 weeks on acute postprandial metabolic responses and β-cell function.

a) NEFA $AUC_{0\text{-}30min}$, b) Insulin $AUC_{0\text{-}30min,}$ c) Glucose $AUC_{0\text{-}30min}$, d) Postprandial insulin sensitivity, e) Oral disposition index, f) GLP-1 $AUC_{0\text{-}30min}$. Individual and mean±SEM change from baseline (week 0); Inulin-control, n=24; Inulin-propionate ester, n=25; *$p<0.05$.

Figure 2: Effect of propionate on insulin secretion from human islets in vitro.

a) Static incubation of human islets. Data are expressed as mean+SEM, n=5-7. ***p<0.001 vs 2mM glucose, ##p<0.01 vs 20mM glucose.

b) and c) Perifused human islets. Data are representative of 3-5 independent experiments with islets from different donors. Each treatment group within individual experiments consisted of 4 independent replicates. *p<0.05, 100μM propionate versus 20mM glucose; **p<0.01, 10μM and 1mM propionate vs 20mM glucose.

Figure 3: Expression of FFAR2 by human islets and its subcellular localisation.

a) Western blotting of isolated human islets lysates with an anti-FFAR2 antibody. The two lanes represent two separate batches of islets and are representative of two independent blots using islets from five donors.

b) Fluorescence immunohistochemistry of human pancreas probed with antibodies directed against FFAR2 (red) and insulin (green). The blue staining indicates nuclei and the right hand panel shows merged images.

Figure 4: Propionate-mediated signalling in human islets.

a) Intracellular Ca^{2+}. Data are expressed as mean±SEM of 5 β-cells and the traces are representative of 3 independent experiments.

b) Insulin secretion from human islets incubated for 20 hours in the presence of 200nM 4β phorbol 12-myristate 13-acetate (PMA, white circles) to down-regulate PKC, 200nM 4α phorbol 12,13-didecanoate (PDD, semi-solid circles), an inactive phorbol ester, or under standard tissue culture conditions (control, solid circles). Data are representative of 3 independent experiments with islets from different donors. Each treatment group within individual experiments consisted of 4 independent replicates.

c) Cyclic AMP generation. Data are expressed as mean+SEM, n=6, representative of 2 independent experiments. ***p<0.001 vs cAMP levels at 20mM glucose, #p<0.05 vs cAMP levels at 1μM forskolin.

d) ATP generation. Data are expressed as mean+SEM, n=4-6, representative of 3 independent experiments. **p<0.001 vs ATP levels at 2mM glucose.

Figure 5: Effects of propionate on human islet apoptosis.

Human islets were maintained for 20 hours in the presence of mixed cytokines (a) or sodium palmitate (b) in the absence or presence of 1mM sodium propionate (SP) and apoptosis was determined by luminescence assay of caspase 3/7 activities. Data are expressed as mean+SEM, n=8, representative of 3 independent experiments. ##p<0.01, ###p<0.001 vs apoptosis with vehicle control in absence of palmitate or cytokines; *p<0.05, ***p<0.001 vs apoptosis induced by palmitate or cytokines.

Supplementary Figure 1: Effects of elevating colonic propionate levels for 24 weeks on postprandial metabolic responses.

Postprandial a) NEFA, b) insulin, c) glucose and d) GLP-1 responses at baseline and following 24 weeks of supplementation with inulin-control and inulin-propionate ester. Data are expressed as mean±SEM, Inulin-control, n=24; Inulin-propionate ester, n=25. NEFA levels were significantly reduced over the 120 min postprandial period after 24 weeks of dietary supplementation with IPE compared with inulin-control (mean difference: -0.09±0.03mM, p=0.009. There were no differences between supplementation group on changes in glucose or GLP-1 (mean differences: -0.06±0.22mM, p=0.781 and 1.8±5.0pM, p=0.722, respectively), with a trend for long-

term colonic propionate delivery to increase insulin secretion (mean difference: 7.0±3.9 μU/mL, p=0.081).

References

1. Cani P, Everard A, Duparc T. Gut microbiota, enteroendocrine functions and metabolism. Curr Opin Pharmacol. 2013,13:935-40.

2. Canfora EE, Jocken JW, Blaak EE. Short-chain fatty acids in control of body weight and insulin sensitivity. Nature Rev. Endocrinol. 2015, 11:577-91.

3. Peters SG, Pomare EW, Fisher CA .Portal and peripheral blood short chain fatty acid concentrations after caecal lactulose instillation at surgery. Gut. 1992 Sep;33(9):1249-52

4. Pomare EW, Branch WJ, Cummings JH. Carbohydrate fermentation in the human colon and its relation to acetate concentrations in venous blood. J Clin Invest. 1985 May;75(5):1448-54

5. Sleeth ML, Thompson EL, Ford HE et al. Free fatty acid receptor 2 and nutrient sensing: a proposed role for fibre, fermentable carbohydrates and short-chain fatty acids in appetite regulation. Nutr. Res. Rev. 2010, 23:135-145.

6. Le Poul E, Loison C, Struyf S et al. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J. Biol. Chem 2003, 278:25481-25489.

7. Zhou J, Martin RJ, Tulley RT et al. Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents. Am. J. Physiol. Endocrinol. Metab. 2008, 295:E1160-E1166.

8. Psichas A, Sleeth ML, Murphy KG et al. The short chain fatty acid propionate stimulates GLP-1 and PYY secretion via free fatty acid receptor 2 in rodents. Int. J. Obesity 2015, 39:424-429.

9. Tolhurst G, Heffron H, Lam YS et al. Short-chain fatty acids stimulate glucagon-like peptide-1 secretion via the G-protein-coupled receptor FFAR2. Diabetes 2012, 61:364-371.

10. Ge H, Li X, Weiszmann J et al. Activation of G protein-coupled receptor 43 in adipocytes leads to inhibition of lipolysis and suppression of plasma free fatty acids. Endocrinology 2008, 149:4519-4526.

11. McArdle MA, Finucane OM, Connaughton RM et al. Mechanisms of obesity-induced inflammation and insulin resistance: insights into the emerging role of nutritional strategies. Front. Endocrinol. 2013, 4:52.

12. Tang C, Ahmed K, Gille A et al. Loss of FFA2 and FFA3 increases insulin secretion and improves glucose tolerance in type 2 diabetes. Nature Med. 2015, 21:173-177.

13.Priyadarshini M, Villa SR, Fuller M et al. An Acetate-Specific GPCR, FFAR2, Regulates Insulin Secretion. Mol. Endocrinol 2015, 29:1055-1066.

14.McNelis JC, Lee YS, Mayoral R et al. GPR43 Potentiates β-Cell Function in Obesity. Diabetes 2015, 64 :3203-3217.

15. Polyviou T, MacDougall K, Chambers ES et al. Randomised clinical study: inulin short-chain fatty acid esters for targeted delivery of short-chain fatty acids to the human colon. Aliment Pharmacol Ther. 2016 Oct;44(7):662-72.

16. Chambers E, Viardot A, Psichas A et al. Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults Gut 2015, 64:1744-1754.

17. Huang GC, Zhao M, Jones P et al. The development of new density gradient media for purifying human islets and islet-quality assessments. Transplantation 2004, 77:143-145.

18. Gey GO, Gey MK. The maintenance of human normal cells and tumor cells in continuous culture. Am. J. Cancer 1936, 27:45-76.

19. Jones PM, Salmon DM, Howell SL. Protein phosphorylation in electrically permeabilized islets of Langerhans. Effects of Ca^{2+}, cyclic AMP, a phorbol ester and noradrenaline Biochem. J. 1988, 254:397-403.

20. Pingitore A, Caroleo MC, Cione E et al. Fine tuning of insulin secretion by release of nerve growth factor from mouse and human islet β-cells. Mol. Cell. Endocrinol. 2016, 436:23-32.

21. Persaud SJ, Liu B, Jones PM. Functional analysis of human islets of Langerhans maintained in culture. Methods Mol. Biol. 2012, 806:55-71.

22. Persaud SJ, Jones PM, Sugden D et al. The role of protein kinase C in cholinergic stimulation of insulin secretion from rat islets of Langerhans. Biochem. J. 1989, 264:753-758.

23. Ramracheya RD, Muller DS, Wu Y et al. Direct regulation of insulin secretion by angiotensin II in human islets of Langerhans. Diabetologia 2006, 49:321-331.

24. Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care. 1999, 22:1462-70.

25. Retnakaran R, Qi Y, Goran MI et al. Evaluation of proposed oral disposition index measures in relation to the actual disposition index. Diabet Med. 2009, 26:1198-203.

26. Amisten S, Salehi A, Rorsman P et al. An atlas and functional analysis of G-protein coupled receptors in human islets of Langerhans. Pharmacol. Therap. 2013, 139:359-391.

27. Fuller M, Priyadarshini M, Gibbons SM et al. The short-chain fatty acid receptor, FFA2, contributes to gestational glucose homeostasis. Am J. Physiol. Endocrinol. Metab. 2015, 309:E840-E851.

28. Villa SR, Priyadarshini M, Fuller MH et al. Loss of Free Fatty Acid Receptor 2 leads to impaired islet mass and beta cell survival. Scientific Reports 2016, 6:28159

Figure 1

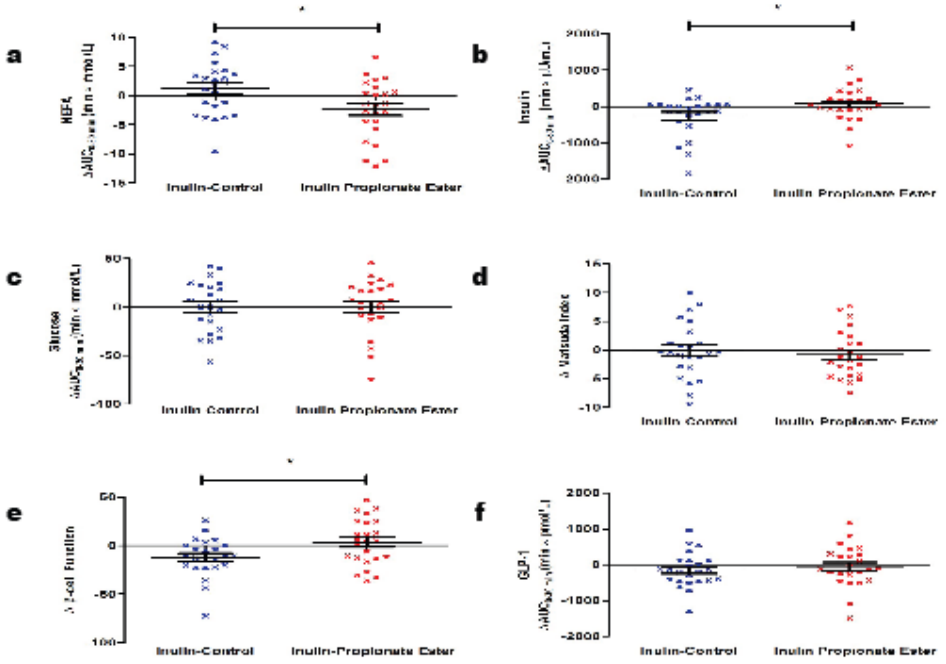

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Figure 2

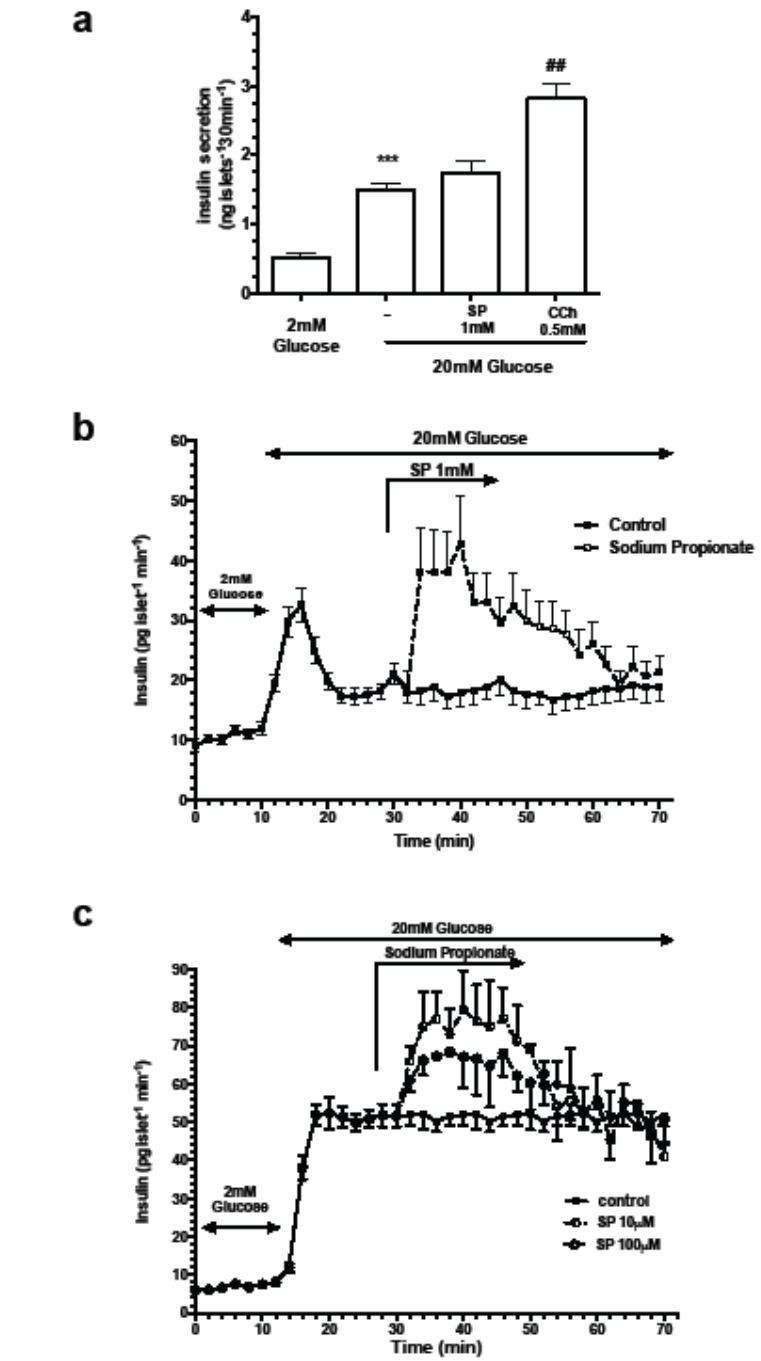

Figure 3

a

b

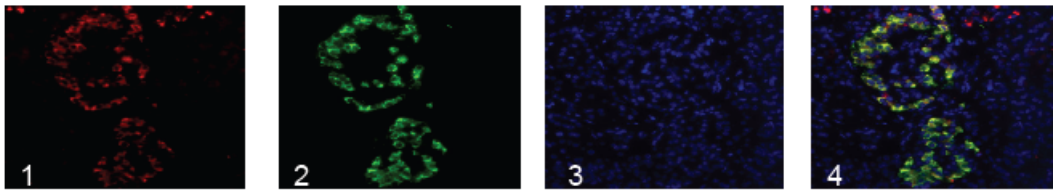

Figure 4

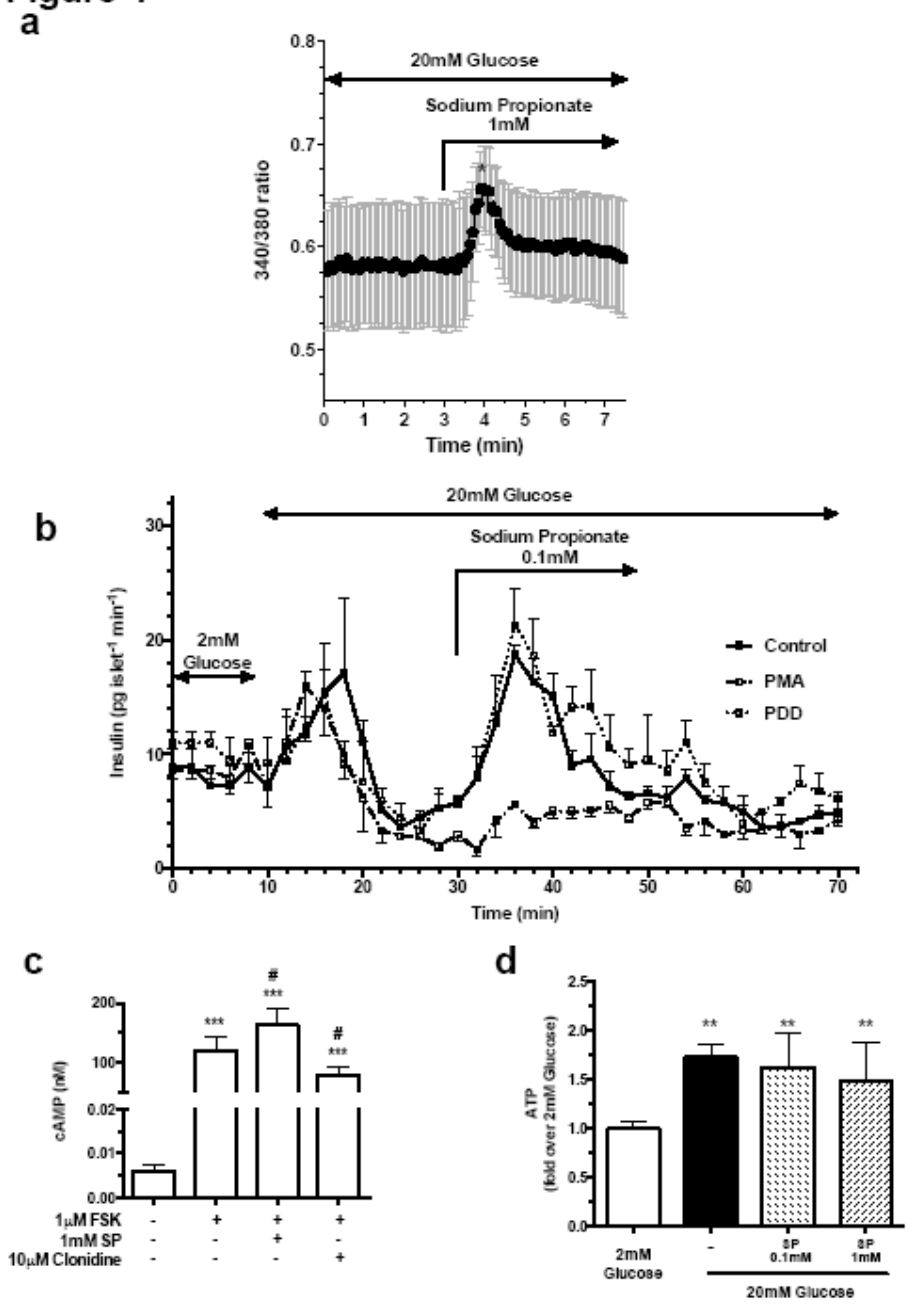

Figure 5