RESEARCH

Short-chain fatty acids as novel therapeutics for gestational diabetes

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Abstract

Gestational diabetes mellitus (GDM) affects up to 16% of pregnant women and is associated with significant long-term health detriments for the mother and her offspring. Two central features of GDM are low-grade inflammation and maternal peripheral insulin resistance, therefore therapeutics which target these may be most effective at preventing the development of GDM. Short-chain fatty acids (SCFAs), such as butyrate and propionate, are metabolites produced from the fermentation of dietary fibre by intestinal microbiota. SCFAs possess anti-inflammatory, anti-obesity and anti-diabetic properties. Therefore, this study aimed to investigate the effect of SCFAs on inflammation and insulin signalling defects in an *in vitro* model of GDM. Human placenta, visceral adipose tissue (VAT) and s.c. adipose tissue (SAT) were stimulated with either the pro-inflammatory cytokine TNF or bacterial product lipopolysaccharide (LPS). The SCFAs butyrate and propionate blocked TNF- and LPS-induced mRNA expression and secretion of pro-inflammatory cytokines and chemokines in placenta, VAT and SAT. Primary human cells isolated from skeletal muscle were stimulated with TNF to assess the effect of SCFAs on inflammation-induced defects in the insulin signalling pathway. Butyrate and propionate were found to reverse TNF-induced increases in IRS-1 serine phosphorylation and decreases in glucose uptake. Butyrate and propionate exerted these effects by preventing ERK activation. Taken together, these results suggest that the SCFAs may be able to improve insulin sensitivity and prevent inflammation induced by sterile or bacterial inflammation. Future *in vivo* studies are warranted to investigate the efficacy and safety of SCFAs in preventing insulin resistance and inflammation associated with GDM.

Key Words

- ▶ short chain fatty acids
- ▶ placenta
- ▶ adipose tissue
- ▶ inflammation
- ▶ insulin resistance
- ▶ gestational diabetes

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Introduction

Gestational diabetes mellitus (GDM) affects up to 16% of pregnant women globally (Yuen *et al.* 2019). Additional to the short-term risks of macrosomia and birthing complications, there are long-term risks for both mother and child such as obesity, metabolic syndrome, type 2 diabetes and cardiovascular disease (Reece 2010, Damm *et al.* 2016). To date, interventions have failed to effectively

reduce the burden of disease (Agha-Jaffar *et al.* 2016). Treatment of GDM with insulin has a short-term focus – it improves pregnancy outcomes, but has little to no impact on the long-term health risks of the next generation (Zhu *et al.* 2019). In addition, there is a daily burden and cost of insulin injections, risk of hypoglycaemia, increased appetite and weight gain (Brawerman & Dolinsky 2018).

There is also a concern about the safety of insulin for mother and her baby (Liang *et al.* 2017). While recent research surrounding lifestyle interventions, such as high plant-based diets and exercise regimes, may mitigate long-term risks, compliance with these regimens is challenging. Accordingly, such interventions do not present a simple, scalable solution that could be applied universally.

GDM is associated with maternal peripheral insulin resistance and low-grade inflammation. Alterations in the insulin signalling pathway, abnormal subcellular localisation of glucose transporters (GLUTs) and reduced insulin-mediated glucose transport have been found in skeletal muscle of women with GDM (Colomiere *et al.* 2010, Catalano 2014, Friedman 2015). This peripheral insulin resistance results in greater substrate availability for foetal growth and adiposity. Chronic low-grade maternal and placental inflammation are also hallmarks of pregnancies complicated by GDM (Pantham *et al.* 2015, Nguyen-Ngo *et al.* 2019). Inflammation is thought to be a driver of the pathophysiology of GDM. Placenta, adipose tissue and skeletal muscle respond to inflammatory insults, such as the cytokine TNF and the bacterial product lipopolysaccharide (LPS), by enhancing the production and expression of pro-inflammatory cytokines and chemokines (Nguyen-Ngo *et al.* 2019). These pro-inflammatory mediators induce further inflammation and promote insulin resistance. Thus, there is a need for new approaches that target both inflammation and insulin resistance.

Short-chain fatty acids (SCFAs) have been touted as potential therapeutic agents in many inflammatory and metabolic disorders (Tan *et al.* 2014, Gill *et al.* 2018). SCFAs can be produced endogenously through metabolic processes or ingested through dietary fibre that is bacterially fermented in the gut. They are found in high mM concentrations in the gut (Bergman 1990) where they are metabolized by gut enterocytes or transported across the gut epithelium into the bloodstream. Human and experimental animal studies demonstrate a protective role of SCFAs in metabolic disease. In non-pregnant animal models of obesity and diabetes, the addition of SCFAs butyrate and propionate to the diet reduces body weight, adiposity, fasting blood glucose, fasting insulin, and insulin tolerance and improved metabolic activity (Gao *et al.* 2009, Henagan *et al.* 2015). In humans, increased gut production of butyrate is associated with improved insulin response after an oral glucose-tolerance test (Sanna *et al.* 2019). Further, abnormalities in the production of propionate is causally related to an increased risk of type 2 diabetes in humans (Sanna *et al.* 2019). In pregnant mice fed a high

fat diet, butyrate supplementation decreases weight gain, blood glucose and total cholesterol as well as reduces maternal inflammation (Li *et al.* 2013). There are, however, no studies that have assessed the anti-inflammatory SCFAs in human placenta and adipose tissue. Likewise, no studies have assessed if SCFAs can improve insulin resistance induced by TNF in human skeletal muscle.

Epidemiological evidence suggests that increased dietary fibre consumption is associated with many beneficial health effects including reduced risk of developing cardiovascular disease and diabetes (McNabney & Henagan 2017). Likewise, total dietary fibre, specifically fruit and cereal fibres, is strongly associated with a reduction of GDM risk by 26% (Zhang *et al.* 2006). What is unknown, however, is whether SCFAs contribute to the beneficial effects of a high fibre diet in reducing GDM risk. In keeping with this, no studies have examined the effects of SCFAs on inflammation and insulin resistance associated with GDM using human samples. Thus, the aims of this study were to determine the effect of SCFAs on reducing inflammation in placenta and adipose tissue and skeletal muscle insulin resistance associated with GDM. We hypothesise that SCFAs will reduce inflammation in placenta and adipose tissue induced by TNF and LPS and restore the decrease in glucose uptake in skeletal muscle mediated by TNF.

Materials and methods

Tissue collection

Ethics approval for this project was granted by the Mercy Hospital for Women Research and Ethics Committee (Mercy Health, Ethics approval number R04-29). Written, informed consent was obtained from all participating women. Placenta, SAT, VAT and skeletal muscle (from the rectus pyramidalis) were obtained from healthy normal glucose tolerant (NGT) women with a BMI <30 kg/m² who were delivering healthy, singleton infants at term (37–41 weeks of gestation) via elective Caesarean in the absence of labor. The exclusion criteria included: vascular/renal complication, multiple gestations, GDM, asthma, smokers, preeclampsia, chorioamnionitis, placental abruption, acute foetal distress, and women with any other adverse underlying medical conditions. All tissues were brought to the research laboratory and processed within 15 min of the Caesarean delivery.

Tissue explants

Tissue explants were used to determine the effect of SCFAs on the mRNA expression and protein release of

pro-inflammatory cytokines and chemokines in placenta, SAT and VAT. We also tested the mechanism through which SCFAs act, by examining its effect on MAPK signalling pathway protein ERK.

Tissue explants for placenta (Lim *et al.* 2013b) and adipose tissue (Lappas 2014a) were performed as previously described. Briefly, tissues were washed in PBS and blunt dissected to remove visible connective tissue, vessels and calcium deposits. The processed tissues were then pre-incubated for 1 h in Dulbecco's modified Eagle's medium (DMEM) (containing 100 U/mL penicillin G and 100 µg/mL streptomycin) at 37°C in a humidified incubator of 5% CO₂ and 21% O₂ (adipose tissue) or 8% O₂ (placenta). Tissues were then pre-incubated with 5 mM butyrate or 10 mM propionate (both obtained from Sigma-Aldrich) for 1 h and then treated with 10 µg/mL LPS (derived from *Escherichia coli* 026:B6; Sigma-Aldrich) or 10 ng/mL TNF (PeproTech) for a further 20 h. The final concentration of butyrate and propionate was based on previous studies in non-gestational tissues (Segain *et al.* 2000, Voltolini *et al.* 2012, Hanley *et al.* 2015) and an initial dose response. A dose response for acetate (5 and 20 mM) demonstrated no effect on TNF-induced IL6 or CXCL8 secretion in placenta or adipose tissue and therefore acetate was not used for any subsequent studies. After a 20 h incubation with SCFAs, tissues and conditioned media were collected separately and stored at -80°C for analysis by RT-qPCR or ELISA as detailed subsequently. Experiments were performed in duplicate; the average of the duplicate was used for final data analysis. To determine the effect of treatment on cell membrane integrity, the release of the intracellular enzyme lactate dehydrogenase (LDH) into incubation medium was determined as described previously (Lim *et al.* 2013a). Neither *in vitro* incubation nor experimental treatment significantly affected LDH activity in the incubation medium (data not shown). Explants were performed on placenta and VAT obtained from six patients and SAT from seven patients.

To assess the effects of SCFAs on activation of ERK, tissues were incubated with 5 mM butyrate or 10 mM propionate for 20 h and then treated with 10 ng/mL TNF or 10 µg/mL LPS for 20 min. Tissues were then collected and stored at -80°C for analysis by Western blotting as detailed subsequently. Experiments were performed from placenta and VAT obtained from five patients.

Glucose uptake in skeletal muscle cells

Primary skeletal muscle cells were used to determine the effect of SCFAs on insulin-stimulated glucose uptake.

2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose) was used as a fluorescent glucose analogue to monitor glucose uptake. Skeletal muscle cells were isolated from fresh skeletal muscle tissue obtained from pregnant women at the time of term Caesarean section. Skeletal muscle was dissected free of fat and connective tissue before mincing into fine pieces. Finely minced tissue was digested in DMEM containing 0.2% (wt/vol) collagenase type 1 (Worthington Biochemical, Freehold, NJ, USA) for 60 min in a shaking water bath. The isolate was passed through a 70 µm cell strainer and then centrifuged at 500 *g* for 10 min. The supernatant was removed and pellet resuspended in 5 mL of DMEM/F12 (containing 100 U/mL penicillin G and 100 µg/mL streptomycin, 10% heat inactivated FBS). Cells were cultured in flasks coated with 0.2% gelatin and incubated at 21% O₂, 5% CO₂ at 37°C. At 80% confluency, cells were plated into black walled 96-well plates in quadruplicate. Confluent cells (~80%) were differentiated into myotubes by incubating in DMEM containing 2% horse serum for 6 days. Cells were then treated with 5 mM butyrate or 10 mM propionate with or without 10 ng/mL TNF for 20 h. Subsequently, 200 µM of 2-NBDG and 10 nM of insulin were added and incubated for a further 30 min. Media was removed, cells washed three times with PBS and fluorescence intensity quantitated immediately at 540 nm using FLUOstar Omega (BMG Labtech, Victoria, Australia).

siRNA transfection studies in primary human trophoblast cells

Human primary trophoblast cells were used to determine if propionate and butyrate elicit their inflammatory effects via activation of the SCFA receptors FFAR2 or FFAR3. Isolation and purification of primary villous trophoblast cells were performed from fresh placenta from non-obese pregnant women. Placental villous cytotrophoblasts were isolated as previously described (Lappas 2014b) by DNase/trypsin digestion and purified by separation on a Percoll gradient. Briefly, placental villous tissue (~25 g) was dissected and washed in saline and then digested three times in a HEPES-buffered salt solution containing 0.25% trypsin and 0.2 mg/mL DNase. Tissue was shaken at 37°C for 45 min. The cytotrophoblast cells were separated on a Percoll gradient and resuspended in standard cell culture medium (5.5 mM glucose, 44.5% DMEM, 44.5% Ham's-F12, and 10% foetal calf serum supplemented with antibiotics). The cells were plated in 24-well plates at a density of 5 × 10⁵ cells per well. The cells were cultured at

37°C in 8% O₂, 5% CO₂ atmosphere and the cell culture media was changed daily. Transfection of trophoblast cells was performed as previously described (Lappas 2014b) using FFAR2 siRNA (siFFAR2), FFAR3 siRNA (siFFAR3) and negative control siRNA (siCONT) obtained from Ambion (Thermo Fisher Scientific). Cells were transfected with 50 nM siRNA for 48 h, followed by treatment with or without 5 mM butyrate or 10 mM propionate in the presence of 1 µg/mL LPS. After a 20-h incubation, cells and media were collected and stored at –80°C until analysed as detailed subsequently. Experiments were performed on cells isolated from placenta obtained from three patients.

Enzyme immunoassays

The levels of IL6, GM-CSF and CXCL8 in the incubation media were measured using sandwich ELISA from Invitrogen as per the manufacturer's instructions. The levels of CCL2, CXCL1 and CXCL5 in the incubation media were measured by sandwich ELISA from R&D Systems as per the manufacturer's instructions. The release of PGF_{2α} into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit as per the manufacturer's specifications (Cayman Chemical Company). The interassay and intraassay coefficients of variation for all assays were less than 10%.

Quantitative RT-PCR (RT-qPCR)

RNA extractions, cDNA synthesis and RT-qPCR were performed as previously described (Lim *et al.* 2013a) using 100 nM of pre-designed and validated QuantiTect primers (primer sequences not available) (Qiagen). The primers with catalogue number are as follows: GM-CSF, QT00000896; IL1A, QT00001127; IL1B, QT00021385; IL6, QT00083720; CCL2, QT00212730; CCL3, QT01008063; CCL4, QT01008070; CCL8, QT00212639; CXCL1, QT00199752; CXCL2, QT00013104; CXCL5, QT00203686; CXCL8, QT00000322; CXCL10, QT01003065; SDHA, QT00059486; and YWHAZ, QT00087962. Target gene Ct values were normalised to the average YWHAZ and SDHA Ct values of the same cDNA sample. Fold differences were determined using the comparative Ct method.

Western blotting

Western blotting was performed as previously described (Lappas *et al.* 2004). Mouse monoclonal pERK (sc-7383; Santa Cruz Biotechnology), rabbit polyclonal ERK (sc-93;

Santa Cruz Biotechnology), and rabbit polyclonal IRS-1 (sc-560; Santa Cruz Biotechnology) were used at 0.2 µg/mL. Rabbit polyclonal pIRS-1 (Ser307) (07-247; Upstate Biotechnology) was diluted 1:1000. Membranes were viewed and analysed using the ChemiDoc MP system (Bio-Rad Laboratories). Semi-quantitative analysis of the relative density of the bands in Western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad Laboratories).

Statistical analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software). Normality of the data was assessed using the Shapiro–Wilk test. Non-normalised data were logarithmically transformed before analysis by a repeated measures one-way ANOVA (with LSD post-hoc testing to discriminate among the means). Statistical significance was ascribed to a *P* value ≤0.05.

Results

Effect of butyrate and propionate on pro-inflammatory cytokines

The effect of butyrate and propionate on LPS- or TNF-induced expression and secretion of pro-inflammatory cytokines in placenta and adipose tissue (VAT and SAT) is demonstrated in Fig. 1 and 2, respectively. The levels of GM-CSF and IL1B in the incubation media of placenta, VAT and SAT, and the levels of IL1A in the incubation media of VAT and SAT were below the sensitivity of the assay and thus not assessed.

In placenta, LPS and TNF treatment significantly increased GM-CSF and IL1B mRNA expression (Fig. 1A and D) and IL1A and IL6 secretion (Fig. 1C and F). On the other hand, only LPS significantly increased IL1A (Fig. 1B) and IL6 (Fig. 1E) mRNA expression. Pre-treatment with either butyrate or propionate significantly suppressed LPS- and TNF-induced mRNA expression and/or protein release of GM-CSF, IL1A, IL1B and IL6.

In VAT, LPS and TNF significantly increased GM-CSF, IL1A and IL6 mRNA expression (Fig. 2A, B and C) and IL6 secretion (Fig. 2D). Butyrate significantly suppressed LPS- and TNF-induced GM-CSF, IL1A and IL6 mRNA expression (Fig. 2A, B and C). In addition, propionate significantly suppressed LPS-induced GM-CSF (Fig. 2A) and IL6 (Fig. 2C) mRNA expression. There was no effect of butyrate or propionate on LPS- or TNF-induced IL6 secretion (Fig. 2D).

Figure 1

Effect of SCFAs on pro-inflammatory cytokines in placenta. Human placenta was incubated in the absence or presence of 5 mM butyrate (But) or 10 mM propionate (Prop) for 1 h and then treated with 10 μ g/mL LPS or 10 ng/mL TNF for a further 20 h ($n = 7$ patients). (A, B, D and E) GM-CSF, IL1A, IL1B and IL6 mRNA expression was analysed by RT-qPCR and fold change was calculated relative to basal. (C and F) The concentration of IL1A and IL6 in the incubation medium was assayed by ELISA. For all graphs, individual data points represent seven independent experiments and displayed as mean $\pm$ s.e.m. $^aP \leq 0.05$ vs LPS, $^bP \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

In SAT, LPS and TNF significantly increased GM-CSF, IL1A and IL6 mRNA expression (Fig. 2E, F and G) and IL6 secretion (Fig. 2H). Butyrate significantly attenuated LPS-induced GM-CSF, IL1A and IL6 mRNA expression and IL6 secretion. Propionate significantly attenuated LPS-induced GM-CSF and IL6 mRNA expression (Fig. 2E and G); however, there was no effect of propionate on LPS-induced IL1A mRNA expression or IL6 secretion (Fig. 2F and H). Additionally, there was no effect of butyrate or propionate on TNF-induced GM-CSF, IL1A and IL6 mRNA expression and IL6 secretion (Fig. 2E, F, G and H).

Effect of butyrate and propionate on chemokines

The effect of butyrate and propionate on LPS- or TNF-induced expression and secretion of CCL and CXCL chemokines in placenta is demonstrated in Fig. 3 and 4, in VAT in Fig. 5 and 6, and in SAT in Fig. 7 and 8. In all three tissues, the levels of CCL3, CCL8, CXCL2 and CXCL10 in the incubation media were below the sensitivity of the assay and thus not assessed.

In placenta, LPS and TNF significantly increased CCL3, CCL4, and CCL8 mRNA expression (Fig. 3C, D and F) and CCL2 and CCL4 secretion (Fig. 3B and E). Likewise, LPS and TNF treatment significantly upregulated CXCL1, CXCL5 and CXCL8 mRNA expression and secretion (Fig. 4A, B, D, E, F and G). The effect of butyrate and propionate pre-treatment was a significant attenuation of the mRNA expression and secretion of all CCL chemokines (Fig. 3),

CXCL1 (Fig. 4A and B), CXCL5 (Fig. 4D and E) and CXCL8 (Fig. 4F and G). There was, however, no effect of LPS or TNF, or butyrate and propionate, on CXCL2 or CXCL10 mRNA expression (Fig. 4C and H).

In VAT, LPS and TNF significantly increased the mRNA expression and secretion of all CCL (Fig. 5) and CXCL chemokines (Fig. 6). Butyrate and propionate significantly suppressed LPS- and TNF-induced CCL2 mRNA expression and secretion (Fig. 5A and B) and LPS-induced CCL4 secretion (Fig. 5E). Furthermore, butyrate significantly suppressed LPS- and TNF-induced CCL4 (Fig. 5D) and CCL8 (Fig. 5F) mRNA expression. There was no effect of butyrate or propionate on LPS- or TNF-induced CCL3 mRNA expression (Fig. 5C). Butyrate and propionate significantly attenuated LPS-induced CXCL1, CXCL5, CXCL8 and CXCL10 mRNA expression and release of CXCL5 (Fig. 6A, D, E, F, H). Both treatments also significantly decreased TNF-induced CXCL5 and CXCL10 mRNA expression (Fig. 6D and H). Additionally, butyrate significantly decreased LPS-induced CXCL2 mRNA expression (Fig. 6C) and TNF-induced release of CXCL5 (Fig. 6E). There was no effect of either treatment on LPS- and TNF-induced CXCL1 and CXCL8 release (Fig. 6B and G) or on TNF-induced CXCL2 and CXCL8 mRNA expression (Fig. 6C and F).

In SAT, LPS and TNF significantly increased the mRNA expression and secretion of all CCL (Fig. 7) and CXCL chemokines (Fig. 8). Butyrate and propionate significantly decreased LPS- and TNF-induced CCL2 and

Visceral Adipose Tissue

Subcutaneous Adipose Tissue

Figure 2

Effect of SCFAs on pro-inflammatory cytokines in VAT and SAT. Human (A, B, C and D) VAT ($n = 6$ patients) and (E, F, G and H) SAT ($n = 6-7$ patients) were incubated in the absence or presence of 5 mM butyrate (But) or 10 mM propionate (Prop) for 1 h and then treated with 10 $\mu\text{g/mL}$ LPS or 10 ng/mL TNF for a further 20 h. (A, B and C, E, F and G) GM-CSF, IL1A and IL6 mRNA expression was analysed by RT-qPCR and fold change was calculated relative to basal. (D and H) The concentration of IL6 in the incubation medium was assayed by ELISA. For all graphs, individual data points represent 6-7 independent experiments and displayed as mean $\pm$ s.e.m. ^a $P \leq 0.05$ vs LPS, ^b $P \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

CCL8 mRNA expression (Fig. 7A and F) and release of CCL2 (Fig. 7B). Butyrate and propionate significantly attenuated LPS-induced CCL4 mRNA expression (Fig. 7D). Butyrate also significantly decreased TNF-induced CCL4 mRNA expression and release (Fig. 7D and E). There was no effect of butyrate or propionate on CCL3 mRNA expression (Fig. 7C) or on LPS-induced CCL4 mRNA expression (Fig. 7E). Butyrate and propionate significantly attenuated LPS- and TNF-induced CXCL5 and CXCL10 mRNA expression (Fig. 8D and H) and LPS-induced CXCL1 mRNA expression (Fig. 8A). Butyrate significantly decreased LPS- and TNF-induced release of CXCL5 (Fig. 8E) and TNF-induced release of CXCL1 (Fig. 8B). There was no effect of butyrate or propionate in SAT on CXCL2 and CXCL8 mRNA expression (Fig. 8C and F) and release of CXCL8 (Fig. 8G).

Effect of butyrate and propionate on glucose uptake in skeletal muscle cells

GDM is characterised by maternal hyperglycaemia due to defective insulin signalling leading to less glucose uptake in skeletal muscle (Lappas *et al.* 2005, Lappas 2014b, Bellamy *et al.* 2009). Studies have previously shown that TNF can cause similar defects in skeletal muscle (Lappas *et al.* 2005). Thus, the next aim was to determine if the SCFAs butyrate and propionate could restore the decrease in SMC glucose uptake by TNF. For these studies, primary SMCs were isolated from skeletal muscle obtained from NGT women at time of Caesarean section, differentiated and then incubated in the presence of TNF with or without butyrate or propionate. The cells were then stimulated with insulin and glucose uptake was measured

Placenta

Figure 3

Effect of SCFAs on CCL chemokines in placenta. Human placenta was incubated in the absence or presence of 5 mM butyrate (But) or 10 mM propionate (Prop) for 1 h and then treated with 10 µg/mL LPS or 10 ng/mL TNF for a further 20 h ($n = 7$ patients). (A, C, D and F) CCL2, CCL3, CCL4 and CCL8 mRNA expression was analysed by RT-qPCR and fold change was calculated relative to basal. (B and E) The concentration of CCL2 and CCL4 in the incubation medium was assayed by ELISA. For all graphs, individual data points represent seven independent experiments and displayed as mean $\pm$ s.e.m. $^aP \leq 0.05$ vs LPS, $^bP \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

Placenta

Figure 4

Effect of SCFAs on CXCL chemokines in placenta. Human placenta was incubated in the absence or presence of 5 mM butyrate (But) or 10 mM propionate (Prop) for 1 h and then treated with 10 µg/mL LPS or 10 ng/mL TNF for a further 20 h ($n = 7$ patients). (A, C, D, F and H) CXCL1, CXCL2, CXCL5, CXCL8 and CXCL10 mRNA expression was analysed by RT-qPCR and fold change was calculated relative to basal. (B, E and G) The concentration of CXCL1, CXCL5 and CXCL8 in the incubation medium was assayed by ELISA. For all graphs, individual data points represent seven independent experiments and displayed as mean $\pm$ s.e.m. $^aP \leq 0.05$ vs LPS, $^bP \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

Visceral Adipose Tissue

Figure 5

Effect of SCFAs on CCL chemokines in VAT. Human VAT was incubated in the absence or presence of 5 mM butyrate (But) or 10 mM propionate (Prop) for 1 h and then treated with 10 µg/mL LPS or 10 ng/mL TNF for a further 20 h (*n* = 6 patients). (A, C, D and F) CCL2, CCL3, CCL4 and CCL8 mRNA expression was analysed by RT-qPCR and fold change was calculated relative to basal. (B and E) The concentration of CCL2 and CCL4 in the incubation medium was assayed by ELISA. For all graphs, individual data points represent six independent experiments and displayed as mean ± s.e.m. ^a*P* ≤ 0.05 vs LPS, ^b*P* ≤ 0.05 vs TNF; repeated measures one-way ANOVA.

Visceral Adipose Tissue

Figure 6

Effect of SCFAs on CXCL chemokines in VAT. Human VAT was incubated in the absence or presence of 5 mM butyrate (But) or 10 mM propionate (Prop) for 1 h and then treated with 10 µg/mL LPS or 10 ng/mL TNF for a further 20 h (*n* = 6 patients). (A, C, D, F and H) CXCL1, CXCL2, CXCL5, CXCL8 and CXCL10 mRNA expression was analysed by RT-qPCR and fold change was calculated relative to basal. (B, E and G) The concentration of CXCL1, CXCL5 and CXCL8 in the incubation medium was assayed by ELISA. For all graphs, individual data points represent six independent experiments and displayed as mean ± s.e.m. ^a*P* ≤ 0.05 vs LPS, ^b*P* ≤ 0.05 vs TNF; repeated measures one-way ANOVA.

Subcutaneous Adipose Tissue

Figure 7

Effect of SCFAs on CCL chemokines in SAT. Human SAT was incubated in the absence or presence of 5 mM butyrate (But) or 10 mM propionate (Prop) for 1 h and then treated with 10 μ g/mL LPS or 10 ng/mL TNF for a further 20 h ($n = 6-7$ patients). (A, C, D and F) CCL2, CCL3, CCL4 and CCL8 mRNA expression was analysed by RT-qPCR and fold change was calculated relative to basal. (B and E) The concentration of CCL2 and CCL4 in the incubation medium was assayed by ELISA. For all graphs, individual data points represent 6-7 independent experiments and displayed as mean $\pm$ s.e.m. $^aP \leq 0.05$ vs LPS, $^bP \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

Subcutaneous Adipose Tissue

Figure 8

Effect of SCFAs on CXCL chemokines in SAT. Human SAT was incubated in the absence or presence of 5 mM butyrate (But) or 10 mM propionate (Prop) for 1 h and then treated with 10 μ g/mL LPS or 10 ng/mL TNF for a further 20 h ($n = 6-7$ patients). (A, C, D, F and H) CXCL1, CXCL2, CXCL5, CXCL8 and CXCL10 mRNA expression was analysed by RT-qPCR and fold change was calculated relative to basal. (B, E and G) The concentration of CXCL1, CXCL5 and CXCL8 in the incubation medium was assayed by ELISA. For all graphs, individual data points represent 6-7 independent experiments and displayed as mean $\pm$ s.e.m. $^aP \leq 0.05$ vs LPS, $^bP \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

Figure 9

Effect of SCFAs on glucose uptake in skeletal muscle cells. Human SMCs ($n = 5$ patients) were incubated in the presence of 10 ng/mL TNF with or without 5 mM butyrate (But) and 10 mM propionate (Prop) for 20 h, followed by 30 min incubation with 10 nM insulin (Ins). (A) Phosphorylated serine IRS1 (pIRS1) protein expression was normalised to total IRS1. A representative Western blot from one patient is also shown. (B) Glucose uptake was measured by 2NBDG fluorescent assay. For all graphs, the data were calculated relative to the Ins group and displayed as mean $\pm$ S.E.M. with data points representing five individual experiments. $^aP \leq 0.05$ vs Ins, $^bP \leq 0.05$ vs TNF+Ins; repeated measures one-way ANOVA.

using the fluorescent glucose analogue 2NBDG. The data are presented in Fig. 9. As expected, insulin significantly increased glucose uptake by ~50%. This increase in insulin-stimulated glucose uptake was significantly attenuated by pre-incubation with TNF. The effect of co-treatment with butyrate was a significant restoration of glucose uptake by ~40%, back to levels like insulin-stimulated glucose uptake. On the other hand, propionate had no effect on the glucose uptake in the presence of TNF.

Mechanisms by which butyrate and propionate exert their anti-inflammatory actions

Butyrate and propionate do not exert their effects via SCFA receptors

It has been suggested that SCFAs may regulate inflammation by activating their FFAR2 and FFAR3 (Kobayashi *et al.* 2017, Li *et al.* 2018a,b). We then examined the effects of butyrate and propionate in regulating pro-inflammatory mediators in FFAR2 and FFAR3 deficient placental trophoblast cells treated with LPS (Supplementary Fig. 1, see section on supplementary data given at the end of this article). We first confirmed that siFFAR2 and siFFAR3 were effective in knocking down

the targeted SCFAs. There was a 75% decrease in FFAR2 mRNA expression in myometrial cells transfected with siFFAR2 (Supplementary Fig. 1A) and 75% decrease in FFAR3 mRNA expression in myometrial cells transfected with siFFAR3 (Supplementary Fig. 1B). There was no effect of siFFAR2 on FFAR3 mRNA expression and siFFAR3 on FFAR2 mRNA expression. Our results show that the effects of butyrate and propionate are independent of FFAR2 or FFAR3 (Supplementary Fig. 1C and D). That is, the secretion of IL6 and CCL2 was not different in siFFAR2 or siFFAR3 transfected cells compared to siCONT transfected cells that were treated with butyrate and propionate.

Butyrate and propionate inhibit activation of the MAPK protein ERK

Mechanistically, butyrate and propionate have been shown to reduce inflammation via inhibition of key signalling pathways, such as the MAPK protein ERK (Chang *et al.* 2019). Thus, it was of interest to investigate whether butyrate and propionate may exert their anti-inflammatory actions by inhibiting the activation of ERK. In placenta, 20 min treatment with LPS or TNF had no effect on pERK protein expression (Fig. 10A). Similar results were obtained in cells treated with LPS for 5–15 min and 2 and 20 h (data not shown). On the other hand, in omental adipose tissue, 20 min treatment with LPS or TNF significantly increased pERK protein expression (Fig. 10B). In both placenta (Fig. 10A) and omental adipose tissue (Fig. 10B), pre-treatment with butyrate significantly decreased pERK protein expression in the presence of both LPS and TNF. In addition, propionate significantly decreased pERK protein expression in the presence of TNF; there was, however, no effect of propionate on activation of ERK in the presence LPS.

Discussion

To our knowledge, this is the first study that has explored the effects of the SCFAs acetate, butyrate and propionate on key pathways involved in the pathogenesis of GDM. To create a GDM-like state, LPS and TNF were used to treat placenta, visceral and s.c. adipose tissue and skeletal muscle cells in the absence or presence of SCFAs. For the first time, we have shown that the SCFAs propionate and butyrate significantly decrease inflammation-induced expression of pro-inflammatory mediators in human placenta and adipose tissue obtained from pregnant women. Propionate and butyrate were also able to

Figure 10

Effect of SCFAs on ERK activation. Human (A) placenta ($n = 5$ patients) and (B) VAT ($n = 5$ patients) were incubated in the absence or presence of 5 mM butyrate (But) or 10 mM propionate (Prop) for 1 h and then treated with 10 $\mu\text{g/mL}$ LPS or 10 ng/mL TNF for a further 20 min. The protein expression of phosphorylated ERK1 (pERK) and total ERK1 was analysed by Western blotting; pERK protein expression was normalised to total ERK expression. A representative Western blot from one patient is shown also shown. For all graphs, the data were calculated relative to basal and displayed as mean $\pm$ S.E.M. with data points representing five individual experiments. * $P \leq 0.05$ vs LPS, $^bP \leq 0.05$ vs TNF; repeated measures one-way ANOVA.

significantly restore the impaired insulin signalling pathway and insulin-mediated glucose uptake in human skeletal muscle obtained from pregnant women induced by inflammation.

Low-grade maternal inflammation is a key feature of pregnancies complicated by GDM and thought to drive many of the characteristic features that define GDM (Pantham *et al.* 2015, Nguyen-Ngo *et al.* 2019). Sterile or infectious insults can induce low-grade systemic inflammation. Placenta and adipose tissue can respond to sterile and infectious inflammation by increasing expression of various cytokines and chemokines. Chemokines play an important role in the inflammatory response. The two main subfamilies of chemokines, CCL and CXCL, activate maternal peripheral leukocytes (a rich source of cytokines) and induce their infiltration into tissues. These leukocytes are a rich source of cytokines which can alter key pathways involved in the pathophysiology of GDM. They do this by inducing defects in the insulin signalling pathway in adipose tissue and skeletal muscle (Lappas 2014a), causing endothelial cell dysfunction (Lappas 2014c), altering placental nutrient transport (Jones *et al.* 2009, Lager *et al.* 2011),

and increasing oxidative and endoplasmic reticulum stress (Lappas *et al.* 2011, Liong & Lappas 2015).

In this study, placenta and adipose tissue (visceral and s.c.) from normal pregnant women were stimulated with the pro-inflammatory cytokine TNF and the bacterial product LPS to induce an inflammatory state as evidenced by increased expression and production of pro-inflammatory cytokines and chemokines. Butyrate and propionate pre-treatment could significantly suppress LPS- and TNF-induced inflammatory cytokine and chemokine mRNA expression and protein secretion in placenta, VAT and SAT. Collectively, this suggests that the SCFAs butyrate and propionate can attenuate the progression of pro-inflammatory cytokine and chemokine accumulation and limit the recruitment of immune cells to placenta and adipose tissue to prevent the propagation of the inflammatory response.

Peripheral insulin resistance increases as pregnancy progresses in order to provide adequate nutrient transfer to the growing neonate (Catalano *et al.* 1993). In GDM, however, there is exaggerated insulin resistance which contributes to short- and long-term metabolic abnormalities in the offspring (Friedman 2015).

GDM pregnancies display reduced pancreatic β -cell function by 30–70%, indicating β -cells are unable to compensate for the insulin resistant state of normal pregnancy (Xiang *et al.* 1999). This is then further exacerbated by defective insulin signalling in adipose tissue and skeletal muscle (Colomiere *et al.* 2010, Catalano 2014). GDM patients exhibit reduced IRS-1 and increased serine IRS-1 phosphorylation in skeletal muscle (Shao *et al.* 2002, Barbour *et al.* 2007, Colomiere *et al.* 2010) thereby inhibiting glucose uptake. We have previously shown that TNF can decrease skeletal muscle glucose uptake by interfering with proteins involved in the insulin signalling pathway (Tran *et al.* 2017). Thus, it was of interest to determine if the SCFAs butyrate and propionate could restore the defects in the insulin signalling pathways and glucose uptake induced by TNF. In our study, butyrate and propionate pre-treatment reversed TNF-induced increase of IRS-1 serine phosphorylation and decrease in insulin-stimulated glucose uptake in skeletal muscle cells. In support of our findings, butyrate and propionate increase glucose uptake by promoting GLUT4 gene expression in C2C12 myotubes and adipocytes *in vitro* (Han *et al.* 2014, Heimann *et al.* 2015). Several animal studies have also shown that SCFAs can improve insulin sensitivity (Canfora *et al.* 2015, Weitkunat *et al.* 2017). It should be noted that, while skeletal muscle contributes to 75% of glucose uptake that occurs in the body (Stump *et al.* 2006), adipose tissue, and in particular VAT, it also play a role in insulin signalling and diabetes development. Due to technical difficulties, we could not assess glucose uptake and insulin signalling in primary adipocytes isolated from VAT from pregnant women.

SCFAs can elicit their actions by binding to and activating the SCFA receptors FFAR2 and FFAR3 (Li *et al.* 2018b). For example, inhibition of CCL2 expression by SCFAs was suppressed by FFAR2 and FFAR3 siRNA in human renal cortical epithelial cells (Kobayashi *et al.* 2017) and FFAR2 and FFAR3 activators attenuated the inhibitory effects of SCFAs on IL6 secretion in endothelial cells (Li *et al.* 2018a). Other studies have, however, reported that SCFAs act independently of FFAR2 and FFAR3 to suppress inflammation (Andrade-Oliveira *et al.* 2015, Li *et al.* 2018a), protect against diet-induced obesity and regulate gut hormones (Lin *et al.* 2012) and induce neutrophil apoptosis (Aoyama *et al.* 2010). These studies have shown that butyrate and propionate exert their effects by inhibiting histone deacetylases (HDAC). In this study, the inhibitory effects of butyrate and propionate on inflammation are independent of FFAR2 or FFAR3. We found that inhibition of pro-inflammatory cytokine IL6

and the chemokine CCL2 was not suppressed in placental cells deficient in FFAR2 and FFAR3. The ability of butyrate and propionate to act as HDAC inhibitors is an avenue of future research.

Downstream of activating FFAR2 and FFAR3 or inhibiting HDACs, SCFAs exert their anti-inflammatory actions by interfering with the activation of key signalling proteins including the MAPK protein ERK (Chang *et al.* 2019). Likewise, our results indicate that butyrate and propionate inhibit the activation ERK in placenta and VAT and that these are specific. That is, butyrate and propionate significantly decreased activation of ERK in the presence of TNF in both placenta and VAT. On the other hand, butyrate, but not propionate, significantly decreased activation of ERK in the presence of LPS in both placenta and VAT. Taken together, our results suggest that the anti-inflammatory and anti-diabetic effects of butyrate and propionate are mediated by inhibition of ERK activation.

In this study, we found that the SCFAs butyrate and propionate improved maternal skeletal muscle glucose uptake and prevented inflammation in placenta and maternal adipose tissue in an *in vitro* model of GDM. These findings are of relevance given that maternal and placental inflammation (Pantham *et al.* 2015, Nguyen-Ngo *et al.* 2019) and maternal peripheral insulin resistance (Colomiere *et al.* 2010, Catalano 2014, Friedman 2015) are two key features of GDM. Importantly, these studies support the evidence that dietary therapies have protective effects against inflammation and insulin resistance and reduce the risk of developing GDM (Agha-Jaffar *et al.* 2016, Assaf-Balut *et al.* 2017) and that a low fibre diet is associated with an increased risk of developing GDM (Zhang *et al.* 2006). What is not known, however, is if SCFAs can prevent the development of GDM and improve neonatal outcomes. Therefore, further *in vivo* studies are warranted to determine the effectiveness and safety of butyrate and propionate as novel therapeutic agents for preventing GDM.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-20-0094>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M L conceived and designed the study, analysed the data and wrote the initial draft of the manuscript. R R and C N N performed the experiments and edited the manuscript.

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