Butyrate modulating effects on pro-inflammatory pathways in human intestinal epithelial cells

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RESEARCH ARTICLE

Abstract

Butyrate acts as energy source for intestinal epithelial cells and as key mediator of several immune processes, modulating gene expression mainly through histone deacetylation inhibition. Thanks to these effects, butyrate has been proposed for the treatment of many intestinal diseases. Aim of this study was to investigate the effect of butyrate on the expression of a large series of target genes encoding proteins involved in pro-inflammatory pathways. We performed quantitative real-time-PCR analysis of the expression of 86 genes encoding proteins bearing to pro-inflammatory pathways, before and after butyrate exposure, in primary epithelial cells derived from human small intestine and colon. Butyrate significantly down-regulated the expression of genes involved in inflammatory response, among which nuclear factor kappa beta, interferon-gamma, Toll like 2 receptor and tumour necrosis factor-alpha. Further confirmations of these data, including studies at protein level, would support the use of butyrate as effective therapeutic strategy in intestinal inflammatory disorders.

Keywords: inflammation, membrane carrier, short chain fatty acids

1. Introduction

Among short-chain fatty acids produced by colonic dietary fibre fermentation, butyrate has received most attention for its effects on gut health (Hamer *et al.*, 2008). The functions of butyrate ranges from being an energy source for colonocytes to be a key mediator of anti-inflammatory and anti-tumorigenic effects (Guilloteau *et al.*, 2010). Indeed, a reduced number of butyrate-producing bacteria was found in colon of patients with ulcerative colitis (UC) and colon cancer (Frank *et al.*, 2007; Wang *et al.*, 2012) and experimental and clinical studies demonstrated the beneficial effects of butyrate treatment (mainly by enema) on chronic intestinal inflammatory diseases (Canani *et al.*, 2011; Chen *et al.*, 2014; Felice *et al.*, 2015). In addition, sodium butyrate accelerates maturation and modulates several functions of the small intestine (Gorka *et al.*, 2014). Our group successfully treated with oral butyrate a child affected by the severe congenital chloride diarrhoea (CCD) obtaining a normalisation of stool pattern and of serum and faecal electrolytes balance. This was due to the pro-absorptive effect induced by butyrate on Na^+, Cl^-, and K^+ intestinal transport demonstrated by rectal dialysis and to the trophic effect of butyrate on intestinal mucosa (Canani *et al.*, 2004). Later, we confirmed this result on other patients with CCD, demonstrating that the butyrate effect depends on the solute carrier family 26 member 3 (SLC26A3) genotype, since it enhances the expression of the SLC26A3 disease gene (Canani *et al.*, 2013), and thus, a significant therapeutic effect was obtained in patients bearing mutations that permit the correct topogenesis of the protein at membrane level (Canani *et al.*, 2013). In fact, the

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main molecular mechanism of butyrate is the modulation of gene expression by the inhibition of chromatin-remodelling activity of histone deacetylases (Donohoe *et al.*, 2012). As consequence, butyrate stimulates the proliferation of normal colonocytes whereas it inhibits the proliferation of cancer cells (Guilloteau *et al.*, 2010; Hamer *et al.*, 2008), but its main effect is the anti-inflammatory activity, due to the down-regulation of genes encoding pro-inflammatory modulators (Felice *et al.*, 2015). However, a systematic analysis of genes whose expression is modulated by butyrate has not been performed so far. We analysed gene expression changes induced by butyrate in primary human intestinal epithelial cells either from small intestine and from colon on a series of genes encoding proteins involved in different inflammatory pathways.

2. Materials and methods

Human small intestine epithelial cells culture

Normal human small intestine epithelial cells (HSIEpiC) from a single donor were purchased by Innoprot (Bizkaia, Spain). Cells were cultured in epithelial pro-conditioned medium (Innoprot) and placed in CELL+ T 25 flasks (Sarstedt, Nümbrecht, Germany). Flasks were coated with collagen type I bovine solution 3 mg/ml (Sigma, St. louis, MO, USA), according to the manufacturer's indications. Medium was changed daily. At confluence of 60%, cells were passed in new flasks after count, using Invitrogen Cell Counters (Invitrogen, Paisley, UK). Trypan blue exclusion test was used to establish total viable cells number and percentage of viability. About 3,000 viable cells/cm^2 per flask were placed. At the sixth day of culture and at the confluence of 60-80%, cells were incubated with 5 mM of sodium butyrate (Sigma) for 24 h. Such conditions had been previously revealed as optimal in modulating the expression of either SLC26A3 and cystic fibrosis transmembrane regulator (CFTR) genes by several human primary cells (Canani *et al.*, 2013). In our previously studies, using primary intestinal cells and patient-derived nasal cells, we performed dose and time dependent curves for some genes responsive to butyrate treatment like the members of SLC family genes SLC26A3 and SLC26A6. We observed that the best response for gene expression modulation was obtained with an exposure of 5 mM of sodium butyrate for 24 h (Canani *et al.*, 2013). In the same study we have administered 100 mg/kg/day to seven children affected by CCD. We demonstrated a genotype-dependency for butyrate therapeutic efficacy in CCD and postulated that the positive effect may be related not only to a SLC26A3 and A6 up-regulation, but also to a modulation of intestinal inflammation. In 2012, Donohoe *et al.* found that a concentration of 5 mM of sodium butyrate is able to explicate the HDAC inhibition, mediated by intracellular accumulation of butyrate on HCT116 colon carcinoma cell nucleus. This dose reflect the physiological amount detected in the colon lumen after colostomy (Luhrs *et al.*, 2002). Starting from these data, we have chosen to study the effect of a concentration of 5 mM of butyrate for 24 h on specific inflammation-related targets.

Human colonic cells culture

Human colon primary cells T150 were purchased by Celprogen (Torrance, CA, USA), and seeding according to the manufacturer's. Cells were placed in CELL+ T 25 flasks (Sarstedt) and maintained in culture with human colon cell culture complete growth serum free medium (Celprogen). Initial seeding density was 3,000 viable cells/cm^2 per flask. Medium was changed daily. At the sixth day of culture and at the confluence of 60-80%, cells were incubated with 5 mM of sodium butyrate (Sigma) for 24 h.

RNA isolation and cDNA preparation

Total RNA was extracted using TRIzol method (Invitrogen). cDNA was transcribed with transcriptor first strand cDNA synthesis kit (Roche, Basel, Switzerland), following manufacture instructions, starting from an amount of 1 µg of total RNA per reaction. Total RNA amount was quantified with Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Real time array design

Real time array was designed according to RealTime ready Custom Panel (Roche). It consists of LightCycler 480 Multiwell Plates 96 containing pre-plated qPCR assays pre tested and validated on human cDNA samples. Each well of a Real Time ready custom panel 96 contains two primers (8 pM of each primer) and one Universal Probe Library probe (4 pM). Each assay includes primers and a Universal Probe Library probe, which is a short fluorescein amidite (FAM)-labelled hydrolysis probe containing locked nucleic acid. Taqman chemistry was adopted for the assay. We analysed a set of transcripts of 83 genes involved in inflammation pathways selecting the most known modulators of inflammation or known targets of short chain acid modulation. A full list of all targets analysed is available in Supplementary Table S1 that reports also the Entrez name of each gene. Three housekeeping genes, i.e. beta actin, glucose-6-phosphate dehydrogenase (G6PD) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were included in the panel as internal control.

Real Time PCR reaction and analysis of results

PCR reaction was performed according to Roche's Real Time ready panel instructions on Light Cycler 480 real time system. Briefly, a mix composed by the Light Cycler 480 probes master (2×), 2 µl of cDNA diluted 1:5 and water, for a total volume of 20 µl of reaction was added in every

well of the plate. PCR program, recommended by Roche, is available in Supplementary Table S2. Real time PCR array analysis was carried out using relative Cp values, calculated from expression levels of the reference genes. Finally, gene expression levels for each sample were normalised to the expression level of the best housekeeping gene within a given sample (ΔCp); the relative expression of each gene before and after butyrate treatment was calculated in order to estimate ΔΔCp (where ΔΔCp corresponds to the increase in the threshold cycle of the target gene respect to the increase in the threshold cycle of the housekeeping gene), according to Pfaffl *et al.* (2001). The final quantification value for each gene indicated the relative change (fold increase) of gene expression in the target gene compared to the control, for each sample.

Statistical analysis

To identify pathways significantly modulated by butyrate, we analysed our data with Ingenuity Pathway Analysis (IPA) (Ingenuity ®Systems; Qiagen Bioinformatics, Aarhus, Denmark). A *P*-value <0.05 and a fold change <2 or >2 was considered significant for expression analysis. Differentially expressed genes before and after butyrate treatment were imported into IPA and the top five canonical pathways were analysed. The canonical pathway analysis identified the molecular pathways from the IPA library of canonical pathways that were most significant to the dataset. Z-score represents the significance of changes in molecular network determined experimentally by observed gene expression levels and function annotation data stored in the Ingenuity Knowledge Base of IPA. These relationships are associated with a direction of change that is either activating (z-score ≥2) or inhibiting (z-score ≤-2).

3. Results

The butyrate treatment of human epithelial cells from colon and from small intestine did not modify the transcription of 55/86 (64%) genes tested (Supplemental Table S3) whereas, it significantly modified the levels of transcription (<2 folds/>2 folds) of 31/86 (36%) genes. For most of such genes, the treatment significantly reduced the expression (from 2 to 32 folds), while it enhanced (from 2 to 8 folds) the expression of only few genes. Figure 1, Table 1 and Supplemental Figure S1 show the effect of butyrate treatment on the 31 genes whose transcription levels were significantly modified.

As shown in Figure 1, in epithelial colon cells butyrate treatment significantly inhibited the expression of 14 genes. For 8/14 of them (i.e. MAPK13, NFKB1A, NFKB1, CXCL1, CXCL2, MAPK3, AKT1, CCL2), we obtained a significant inhibition of expression either in colon and in small intestine cells; for 3/14 of them (i.e. CXCL10, CXCL6, CXCL3), we observed a significant reduction of gene expression in colon cells while, in cells derived from small intestine, we observed a significant enhancement of expression and finally, for the remaining 3/14 (i.e. TRL2, SERPINA1 and TNF) the butyrate caused a significant reduction of gene expression in colon cells while in small intestine cells we did not observe any change of gene expression. Again in colon cells (Figure 1), butyrate treatment significantly enhanced the expression of 2 genes, for 1 of which (i.e. TIMP1) we observed a down-regulation of gene expression in cells from small intestine; for the other (i.e. SERPINA2) we did not observe any change in small intestine cells. In addition, for 13 other genes (i.e. IL-6, CSF3, TLR4, ICAM1, AKT2, VEGFA, IL-12A, AKT3, MAPK10, MVP, CXCL8, IL-1B and

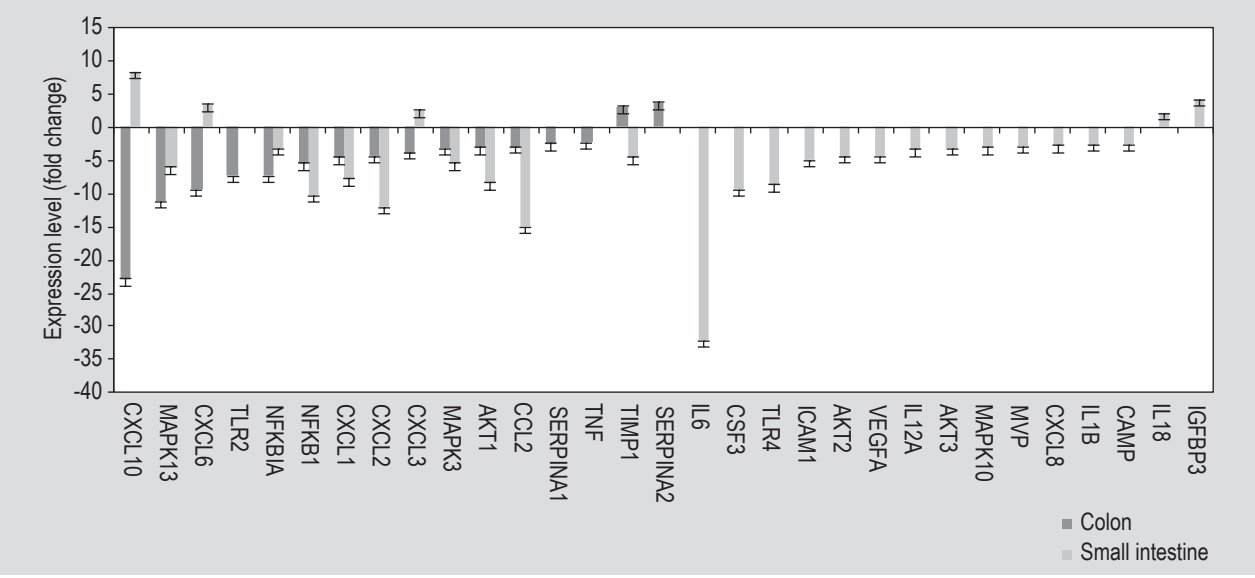

Figure 1. mRNA expression of transcripts related to inflammation modulated by *in vitro* treatment with butyrate in human colon and small intestinal cells.

Table 1. List of 31 transcripts whose expression is modulated by butyrate in human epithelial colon and in small intestinal primary cells (fold change <2 or >2, *P*<0.05).

Symbol	Entrez Gene Name	Fold change Colon	*P*-value	Fold change Small intestine	*P*-value
CXCL10	chemokine (C-X-C motif) ligand 10	-22.9	0.002	8.3	0.001
MAPK13	mitogen-activated protein kinase 13	-11.2	0.003	-6.0	0.001
CXCL6	chemokine (C-X-C motif) ligand 6	-9.5	0.0015	3.4	0.005
TLR2	toll-like receptor 2	-7.4	0.003		
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha	-7.3	0.001	-3.2	0.001
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-5.4	0.002	-10.3	0.0015
CXCL1	chemokine (C-X-C motif) ligand 1	-4.5	0.001	-7.8	0.001
CXCL2	chemokine (C-X-C motif) ligand 2	-4.4	0.002	-12.0	0.003
CXCL3	chemokine (C-X-C motif) ligand 3	-3.9	0.001	2.5	0.0015
MAPK3	mitogen-activated protein kinase 3	-3.2	0.001	-5.4	0.0008
AKT1	v-akt murine thymoma viral oncogene homolog 1	-3.0	0.004	-8.4	0.002
CCL2	chemokine (C-C motif) ligand 2	-3.0	0.001	-15.0	0.003
SERPINA1	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	-2.4	0.0008		
TNF	tumour necrosis factor	-2.4	0.004		
TIMP1	TIMP metallopeptidase inhibitor 1	3.1	0.001	-4.6	0.001
SERPINA2	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 2	3.8	0.001		
IL-6	interleukin 6			-32.2	0.0009
CSF3	colony stimulating factor 3			-9.4	0.008
TLR4	toll-like receptor 4			-8.7	0.001
ICAM1	intercellular adhesion molecule 1			-5.0	0.003
AKT2	v-akt murine thymoma viral oncogene homolog 2			-4.5	0.001
VEGFA	vascular endothelial growth factor A			-4.4	0.001
IL-12A	interleukin 12 A			-3.4	0.001
AKT3	v-akt murine thymoma viral oncogene homolog 3			-3.2	0.003
MAPK10	mitogen-activated protein kinase 10			-3.0	0.0015
MVP	major vault protein			-2.9	0.001
CXCL8	chemokine (C-X-C motif) ligand 8			-2.8	0.0008
IL-1B	interleukin 1, beta			-2.7	0.001
CAMP	cathelicidin antimicrobial peptide			-2.6	0.001
IL-18	interleukin 18			2.2	0.001
IGFBP3	insulin-like growth factor binding protein 3			4.2	0.0008

CAMP), the treatment with butyrate significantly reduced the levels of expression only in epithelial small intestine cells with no effects in colon cells, while for 2 genes (i.e. IL-18 and IGFBP3), the treatment significantly enhanced gene expression in small intestine cells with no effects in colon cells. Then, using the IPA program, we evaluated the global effect of butyrate treatment on several pathways either in colon and in small intestine epithelial cells (Table 2). The results were fully concordant between the two types of cells: in fact, the treatment significantly down-regulated the following 5 pathways in both the types of cells: triggering receptor expressed on myeloid cells (TREM-1) signalling; production of nitric oxide (NO) and reactive oxygen species (ROS); acute phase response signalling; interleukin 6 (IL-6) signalling; high-mobility group box-1 (HMGB1) signalling. While, the treatment significantly up-regulated the peroxisome proliferator-activated receptor alpha (PPARα)/retinoid X receptor alpha (RXR) activation and the liver X receptor (LXR)/RXR activation pathways, again with a concordant effect in the two types of cells.

Table 2. Main pathways regulated by butyrate in in human epithelial colon and in small intestinal primary cells.

Pathway	Effect	Colon z-score	Colon *P*-value	Small intestine z-score	Small intestine *P*-value
TREM1 signalling	downregulated	-2.646	3.76E-11	-2.496	8.19E-26
Production of NO and ROS	downregulated	-2.828	5.28E-10	-3.464	3.27E-16
Acute phase response signalling	downregulated	-2.646	1.17E-8	-2.714	1.55E-13
IL-6 signalling	downregulated	-2.449	3.83E-8	-3.051	6.0E-19
HMGB1 signalling	downregulated	-2.449	4.69E-8	-3.207	2.69E-22
PPARα/RXRα activation	upregulated	2.0	1.87E-5	2.236	2.45E-04
LXR/RXR activation	upregulated	2.236	2.45E-04	2.330	4.19E-12

4. Discussion and conclusions

Butyrate significantly modulates (mainly inhibiting) the expression of 31 genes encoding proteins with pro-inflammatory activity. Although there are some differences between colon and small intestine epithelial cells, the analysis of the data using the IPA program (Zhou *et al.*, 2013) showed a complete overlapping of involved pathways between the two types of cells. Butyrate significantly down-regulates the TREM-1 signalling pathway that during infections leads to the activation of myeloid cells and promotes the synthesis and the release of pro-inflammatory cytokines (Schenk *et al.*, 2007). Either in mouse models of inflammatory bowel disease (IBD) and in humans, such pathway is significantly up-regulated (Mueller, 2012) and its activity is related to disease activity (Goncalves *et al.*, 2013). The down-regulation of TREM-1 pathway explains the beneficial effects of butyrate on IBD (Canani *et al.*, 2011) reinforcing the potential of a novel therapy that seems to be free from toxic effects (Steppe *et al.*, 2014). Furthermore, butyrate down-regulates the NO and ROS pathways, explaining the known effects of butyrate against oxidative stress (Ock *et al.*, 2012). Such pathway is involved in a large series of human chronic diseases, including *Helicobacter pylori*-associated atrophic gastritis and gastric, colon, lung, prostate, bladder and breast cancer (Biasi *et al.*, 2013).

Furthermore, oxidative stress is a key mechanism in the pathogenesis of Crohn's disease (CD) since it dramatically impairs the immune response toward dietary antigens and toward commensal microbiota (Fuyitake *et al.*, 2013). In fact, molecular treatments to contrast the oxidative stress are a primary aim in the field of such diseases (Ito *et al.*, 2004). The acute phase response signalling resulted also down-regulated by butyrate. This pathway has a relevant role in the pathogenesis of either adult UC, for which it has been recently demonstrated the beneficial effect of enteric microbiota (Chen *et al.*, 2014) and in paediatric CD and a massive release of pro-inflammatory cytokines (the cytokine storm) is a typical marker of acute IBD (Biasi *et al.*, 2013). The IL-6 signalling pathway, that in turn is down-regulated by butyrate, has a key role in adaptive immunity and in maintaining chronic inflammation in IBD. In fact, serum IL-6 is a known biochemical marker of IBD activity and outcome and the inhibition of such pathway was successfully used in the treatment of CD (Ito *et al.*, 2004). In addition, both in mice and in humans IL-6 is critical in creating a tumour-promoting microenvironment (Waldner and Neurath, 2014).

Finally, among pathways down-regulated by butyrate there is the HMGB1 one; HMGB1 is a member of the high-mobility group superfamily, with a regulating role in biological processes like transcription, DNA repair, differentiation and development (Czura *et al.*, 2001). In particular, HMGB1 has a cytokine-like role: it is massively released from cells undergoing necrosis and mediates late lethal systemic inflammation (Palumbo and Bianchi, 2004). Recently, a role of HMGB1 in the pathogenesis of IBD in child was revealed and faecal levels of the protein became an effective marker for IBD diagnosis and monitoring (Vitali *et al.*, 2011). Interestingly, our data confirm the observation that butyrate suppresses HMGB1 protein production in liver tissue of rats with acute liver failure (Yang *et al.*, 2014) and in several organs of rats during sepsis (Zhang *et al.*, 2007).

A second group of effects of butyrate involves pathways that regulate lipid absorption and metabolism, in agreement with the known effect of butyrate in providing feeding and energy sources for colonocytes and in regulating some key points of lipid metabolism (Berni Canani *et al.*, 2011). In fact, butyrate up regulates the PPAR alpha/RXR alpha activation and the LXR/RXR activation pathways in either colon and in small intestine cells. Both these pathways have key roles in the regulation of human metabolism. The peroxisome proliferator-activated receptor (PPAR) is a main regulator of the lipid metabolism in peripheral tissue, like liver and skeletal muscle and contributes to enhance fatty acid oxidation in intestinal cells. Such effects dramatically reduce post-prandial lipidemia, mainly triglycerides, so that PPAR is becoming a target for therapy of postprandial

lipidemia in obese subjects (Kimura *et al.*, 2013). Liver X receptors (LXR) are transcription factors that regulate cholesterol, bile acid, triglyceride and glucose homeostasis (Baronowski, 2008) but, more interestingly, LXR seems to modulate also intestinal lipid absorption and intestinal inflammation (Gao *et al.*, 2013). Several studies in mice recognised the role of LXR in protecting from high fat diet-induced obesity and insulin resistance (Stojcev *et al.*, 2013). Thus, the activation of such pathways by butyrate has a relevant impact in the control of lipid metabolism.

Taken together, the effects of butyrate in the regulation of gene expression at intestinal level delineate the role of a substance that contributes to inhibit acute and chronic inflammatory processes (most of which related to chronic intestinal diseases in humans) and maintain the metabolic homeostasis of intestinal cells, confirming the effects observed *in vivo* in several human diseases (Canani *et al.*, 2011; Hamer *et al.,* 2008). Such data encourage further studies (also including the quantitative analysis of proteins) aimed to confirm the anti-inflammatory effects of butyrate and clinical trials on large populations to standardise the use of such molecule in the prevention and treatment of human inflammatory intestinal diseases.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2016.0197.

Table S1. Full list of all targets analysed in this study.

Table S2. Real time PCR program adopted in this study.

Table S3. List of targets whose level of expression was observed as unchanged after butyrate exposure of colon and small intestine.

Figure S1. Heat map of transcripts modulated by 5 mM butyrate exposure of human colon and small intestinal cells for 24 h.

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