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Lactate and short chain fatty acids produced by microbial fermentation downregulate proinflammatory responses in intestinal epithelial cells and myeloid cells

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ABSTRACT

The use of short chain fatty acids to modulate gastrointestinal inflammatory conditions such as ulcerative colitis has produced encouraging results either in animal models or also in clinical trials. Identifying the key cellular and molecular targets of this activity will contribute to establish the appropriate combinations/targeting strategies to maximize the efficacy of anti-inflammatory interventions. In the present work, we evaluated *in vitro* the interaction of lactate, acetate, propionate and butyrate on cells relevant for innate immune response of the gastrointestinal tract. All molecules tested regulate the production of proinflammatory cytokines by TLR-4 and TLR-5 activated intestinal epithelial cells in a dose response manner. Furthermore SCFAs and lactate modulate cytokine secretion of TLR-activated bone marrow derived macrophages and also TLR-dependent CD40 upregulation in bone marrow derived dendritic in a dose-dependent manner. Butyrate and propionate have been effective at concentrations of 1 to 5 mM whereas acetate and lactate produced modulatory effects at concentrations higher than 20–50 mM in different assays. Our results indicate that in concentrations similar to found in large bowel lumen, all SCFAs tested and lactate can modulate activity of relevant sentinel cell types activated by TLR signals. Modulatory activity was not inhibited by pertussis toxin treatment indicating that the effects are not related to G_i signaling. The use of these molecules in combined or separately as intervention strategy in conditions where epithelial or myeloid cells are main triggers of the inflammatory situation seems appropriate.

Abbreviations: cAMP, cyclic adenosine monophosphate; BMMs, bone marrow derived macrophages; BMDCs, bone marrow derived dendritic cells; CCL20, chemokine (C–C motif) ligand 20; dbcAMP, dibutyril cyclic adenosine monophosphate; DC, dendritic cells; FliC, flagellin; GPRs, G-protein coupled receptor; IBD, inflammatory bowel disease; IL-1β, interleukin-1β; IL-6, interleukin 6; IL-8, interleukin 8; IL-12, interleukin 12; ITF, intestinal trefoil factor; LPS, lipopolysaccharides; MFI, mean fluorescence intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *PTX*, bordetella pertussis toxin; PBMC, peripheral blood mononuclear cell; SCFAs, short chain fatty acids.

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1. Introduction

The human intestinal microbiota is a diverse community of microbes reaching up to 10^{11} bacteria/mL in the colon and serves essential functions in food digestion, immunomodulation, and establishment of a barrier effect that prevents colonization by pathogens (Tlaskalova-Hogenova et al., 2011). The human colonic microbiota produces an enormous quantity of molecules that impact on the gut homeostasis (Matsuki et al., 2013). Diet strongly influences qualitatively or quantitatively the gut microbial communities and consequently the microbial-derived molecules present in gut lumen (Vinolo et al., 2011). Dietary carbohydrates that are not digested by the host in the small bowel are fermented in the colon into short-chain fatty acids (SCFAs) including acetate, propionate and butyrate (Russell et al., 2013). Furthermore, several microbiota components produce lactate, which is the most common short chain hydroxy– fatty acid in intestinal lumen, which can be converted to other SCFAs by a subgroup of lactate-fermenting bacterial species. In turn, SCFAs have beneficial effects on the host. Particularly, SCFAs are associated with reduced risk of some diseases including the irritable bowel syndrome, inflammatory bowel disease (IBD), cardiovascular disease, and cancer (Hijova and Chmelarova, 2007; Huda-Faujan et al., 2010).

Administration of SCFAs or prebiotics that are known to enhance SCFA production was proposed as treatment in IBD (Breuer et al., 1997; Scheppach, 1996; Vernia et al., 1995). However, due to partial patient compliance or restricted indications, these treatments were not established as a standard of care. Recent studies have renewed the expectations on strategies related to intestinal SCFAs. Thus, dysbiosis in IBD patients was associated with altered butyrate fermentative pathways (Eeckhaut et al., 2013; Galecka et al., 2013; Kumari et al., 2013; Machiels et al., 2014). Interventions in animal models resulting in increased exposure of intestinal tissue to specific SCFAs have shown protective effects in intestinal mucosa (Komiyama et al., 2011; Vieira et al., 2012). In particular, the administration of probiotic bacteria with the capacity to produce butyrate has been shown to improve the symptoms in IBD models (Eeckhaut et al., 2013). These modulatory effects *in vivo* are probably due to the capacity that show different SCFA to downregulate specific inflammatory cell functions that has been partially described so far (Tedelind et al., 2007; Berndt et al., 2012; Liu et al., 2012). SCFAs have effects in different cell types mediated by different mechanisms. Various G protein-coupled receptors (GPR) such as GPR41, GPR43, and GPR109a may mediate SCFAs activities (Offermanns, 2014). Furthermore, several SCFA may mediate biological effects by inhibiting histone deacetylase (Tan et al., 2014).

An improvement in our knowledge on the capacity of the different microbial fermentation products to modulate inflammatory activation may contribute to rational design of intervention strategies in situations of altered intestinal homeostasis. In this work, the effects of lactate and various SCFAs on cells that represent the main sentinel cells of the intestine, i.e. epithelial cells, macrophages and dendritic cells were analyzed. Thus, regulation of production of pro-inflammatory responses induced by microbial stimuli or cytokine was investigated.

2. Materials and methods

2.1. Reagents

Racemic DL-Lactic (J. T. Baker, USA), acetic acid (Dorwil, Bs. As., Argentina), propionic and butyric acid (Sigma Chemical Co., USA) were used for preparation of aqueous solutions at 0.5 mol/L, neutralized to pH 7.0 ± 0.2 by the use of solution of NaOH 1 mol/L, filtered through 0.45 μm membrane filter and maintained at −20 °C until used.

Flagellin (FliC) obtaining and purification from *Salmonella enterica* was previously described (Sierro et al., 2001). Human IL-1β was purchased from R&D Systems (Minneapolis, USA). Cyclic adenosine monophosphate (cAMP) analogue (dibutyril-cAMP, dbcAMP) was obtained from Sigma Chemical Co. Ultrapure lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 was from InvivoGen (Toulouse, France). All cell cultures media, serum, and supplements were purchased from Gibco®.

2.2. Culture and stimulation of intestinal epithelial cell lines

Human colonic epithelial cell line Caco-2 were a gift from Dr. J.P. Krahenbühl. Caco-2 cells stably transfected with a luciferase reporter construction under the control of *CCL20* promoter (designated Caco-$2_{CCL20\text{-}luc}$) were previously described (Nempont et al., 2008). Cells were maintained and routinely grown as described (Iraporda et al., 2014). Caco-$2_{CCL20\text{-}luc}$ and Caco-2 cells were used after 8 days of culture at passages between 12 and 22. All experiments were performed in serum-free medium in 48 well plates.

Caco-$2_{CCL20\text{-}luc}$ cells cultured were incubated with aqueous solutions of different concentrations of lactic, acetic, propionic and butyric acid in fetal bovine serum free DMEM (1:1) for 30 min. Cells were then stimulated with FliC (1 μg/mL) or IL-1β (10 ng/mL) and incubated for 5 h at 37 °C in a 5% CO_2 – 95% air atmosphere. All experiments included a basal condition without any treatment. FliC or IL-1β were used respectively as control of 100% induction of pro-inflammatory response. Then, cells were lysed with Lysis Buffer (Promega, Madison, WI, USA). Luciferase activity was measured in a Labsystems Luminoskan TL Plus luminometer (Thermo Scientific, USA) using a Luciferase Assay System (Promega, Madison WI, USA) as previously described (Nempont et al., 2008). Luminescence was normalized to the stimulated control cells and expressed as percentage of normalized average luminescence ± standard deviation (SD) of at least three independent experiments.

For gene expression analysis, cell monolayers of Caco-2, in 24-well tissue culture plates were incubated for 30 min with 500 μL of aqueous solutions of different acids (100 mM) previously neutralized and filtered as described above, in DMEM. Then cells were stimulated with FliC (1 μg/mL) during 2 h.

2.3. Generation and stimulation of primary murine macrophages and dendritic cells

Female C57BL/6J (6–8 weeks old) mice were obtained from Janvier laboratories (St. Berthevin, France). All experiments complied with current national and institutional regulations and ethical guidelines (B59-350009–Institut Pasteur de Lille). Bone marrow (BM) from femurs and tibias were used as source of hematopoietic cell precursors as described (Van Maele et al., 2014). Briefly, BM macrophages (BMMs) were obtained upon 8 days differentiation of BM precursors (5.5×10^5/mL) in DMEM supplemented with 10% FSC, 1% penicillin–streptomycin and 30% of L929 cells-conditioned medium (mM-CSF source), initially seeded Petri culture dishes. Every 3 days, cells were supplemented with fresh medium. BM dendritic cells (BMDCs) were derived upon 9 days of differentiation by culturing 3×10^5/mL precursors in Petri culture dishes with IMDM medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1% penicillin–streptomycin (IMDM complete) and mGM-CSF 20 ng/mL. Every 3 days cells were supplemented with IMDM complete containing fresh mGM-CSF. Differentiation of BMDC was assessed by measurement of CD11c and $I\text{-}A^b$ surface expression by flow cytometry. Cells were stimulated for 18 h with 100ng/mL of LPS in presence of different concentrations of SCFAs. BMDC activation was monitored by changes in surface expression

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