Determining how select SCFAs mechanistically affect IBD

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Abstract

Inflammatory bowel disease (IBD) is a chronic autoimmune condition that affects the gastrointestinal tract, causing debilitating symptoms like chronic abdominal pain, diarrhea, fatigue, and reduced quality of life. Although treatments such as biologics, surgeries, steroids, and antibiotics are available, recent research suggests dietary interventions may offer additional benefits. Specifically, diets like the Specific Carbohydrate Diet and the Crohn's Disease Exclusion Diet have shown promise in reducing IBD symptoms for some patients, although the mechanisms by which diet modulates the immune response remain unclear. This study investigated how molecules derived from dietary fibers, namely short-chain fatty acids (SCFAs), impact inflammation. Using a co-culture system of Caco2 cells (intestinal epithelial cell surrogate) and peripheral blood mononuclear cells (PBMCs, immune cell surrogates), varying concentrations of the SCFAs butyrate and propionate were added to assess their influence on inflammatory markers post lipopolysaccharides (LPS) stimulation. Results indicated that both butyrate and propionate significantly upregulated anti-inflammatory markers while downregulating pro-inflammatory markers, shedding light on the potential mechanisms by which SCFAs can modulate immune activity in IBD. These findings underscore the therapeutic potential of dietary SCFAs and support further exploration of diet as an adjunct treatment for IBD.

Introduction

Approximately 0.7% of the American population is currently diagnosed with Inflammatory Bowel Disease (IBD), a chronic, intestinal-based disease that significantly impacts one's overall health and quality of life (1). IBD can be divided into two main subtypes based on where inflammation occurs; Crohn's disease can affect all parts of the body, but is mainly concentrated along the entire gastrointestinal (GI) tract from the mouth to the anus, while ulcerative colitis is limited to the large intestine and anus. Treatments for IBD include biological medicines, steroids, surgeries, & dietary supplements — many of which target intestinal immune pathways or the gut microbiome.

Physiologically, the intestine is a complex organ that is critical in absorbing nutrients from ingested food. It is home to a population of microbes that help break down certain types of complex nutrients (such as dietary fibers) into metabolites that can be readily absorbed by the intestinal villi/wall. To protect the body from these ingested microbes and/or pathogens, the body must maintain a physical barrier consisting of an epithelial cell wall, mucus, and immune cells. In IBD patients, this wall is inflamed, allowing easier access for bacteria and/or pathogens to invade the tissue. This process can trigger an overt immune response that, if left unchecked, can cause chronic inflammation. While exact mechanisms for why IBD can widely vary, common

factors include genetics, environmental conditions, and/or microbial dysbiosis – a term describing imbalance in the microbial community (2).

Recent breakthroughs and literature have pointed to diet as an effective treatment option for those suffering from IBD. For example, the clinical remission rate for patients suffering from IBD who follow External Enteral Nutrition (EEN) – a diet composed of purely formula drink – is approximately 80%. (3) Similarly, for patients following the Crohn's Disease Exclusion Diet (CDED) or Specific Carbohydrate Diet (SCD), the clinical remission rate is approximately 63% by week 6 and 33% by week 8 of treatment, respectively (4,5). These diets are generally designed to provide patients with consistent and restrictive foods consisting of unprocessed natural foods and complex dietary fibers while eliminating common ingredients such as carbohydrates, refined sugars, artificial ingredients, gluten, and dairy. Nevertheless, while these diets are effective for some patients, doctors typically do not prescribe dietary change when diagnosing and/or treating patients; it is usually not recognized as a suitable treatment plan as it can often risk lifestyle changes, or in extreme cases, could stunt the growth of pediatric patients due to malnutrition (6). If dietary changes are to become a more prevalent form of IBD treatment, more information on the mechanism by which they impact intestinal inflammation is required.

To identify mechanisms in which diet can modulate intestinal inflammation, an *in vitro* PBMCs/Caco2 co-culture model was developed to investigate the effects of fiber-derived short-chain fatty acids (SCFAs), specifically butyrate and propionate. In this model, Caco2 cells (representing intestinal epithelial cells) were co-cultured with PBMCs (representing immune cells) in the presence or absence of lipopolysaccharides (LPS), a component known to trigger inflammation. By simulating inflammatory conditions in this setup, this study aimed to assess how these SCFAs modulate inflammation, providing valuable insights into how diets like the Crohn's Disease Exclusion Diet (CDED) and Specific Carbohydrate Diet (SCD) may influence the immune response and potentially alleviate IBD symptoms.

Materials and Methods

Cell culture: Method was adopted and modified from [9]. Caco2 cells, an epithelial cell line, were grown to confluence in 10 cm petri dishes using RPMI1640, 2mM Glutamine, 1% non-essential amino acids, 10% Fetal Bovine Serum (FBS), 5% CO2, 37° C. The media was changed every three days. Once the cells reached 70-80% confluence, they were seeded at 2-4 * 10^4 cells/cm^2 in a 24 well plate and incubated with pooled human-donor PBMCs (plated at a density of 1-2 * 10^6 cells/well). After 24 hrs of incubation, the co-cultured cells were stimulated +/- LPS for 4 hrs and then dosed with 0, 0.5, 1, and 2 mM SCFAs (butyrate, acetate, propionate, and a combination of butyrate & propionate).

Measurement of pro-inflammatory cytokines: Method was adopted and modified from [10,11]. 24 hours post-stimulation and SCFA addition, supernatants from each co-culture well were isolated and stored at -80C. The remaining cells were washed 2X with Phosphate Buffered

Saline (pH 7.4) and then lysed (Qiagen RLT Buffer, Cat. #79216). To quantify TNFa, a sandwich ELISA kit was used (Invitrogen, Cat. #BMS20234). For qPCR, RNA was extracted using an RNeasy kit (Qiagen, Cat. #74181) and a QiacubeHT (Qiagen). A Nanodrop One was used to quantify RNA. cDNA was created using a Reverse Transcriptase Kit (Qiagen Quantitect, Cat. #205311), diluted to ~30 ng/uL, and then analyzed using a QuantStudio 5 Flex machine with SYBR-green (Qiagen QuantiNova, Cat. 208052).

List of Primers used:

- **TNFa**
  - **F 5'-**CCTCTCTCTAATCAGCCCTCTG-3'
  - **R 5'-**GAGGACCTGGGAGTAGATGAG-3'
- **FOXP3**
  - **F 5'-**GTGGCCCGGATGTGAGAAG
  - **R 5'-**GGAGCCCTTGTCGGATGATG
- **IL-22**
  - **F 5'-**GCTTGACAAGTCCAACTTCCA
  - **R 5'-**GCTCACTCATACTGACTCCGT
- **NOD2**
  - **F 5'-**TGGTTCAGCCTCTCACGATGA
  - **R 5'-**CAGGACACTCTCGAAGCCTT
- **GAPDH**
  - **F 5'-**TGTGGGCATCAATGGATTTGG
  - **R 5'-**ACACCATGTATTCCGGGTCAAT
- **PPIA**
  - **F 5'-**CCCACCGTGTTCTTCGACATT
  - **R 5'-**GGACCCGTATGCTTTAGGATGA

Statistics: All ELISA and qPCR measurements were taken at 24 hours. Changes in gene expression levels were determined using the ddCT analysis method with GAPDH and PPIA serving as the normalized housekeeping genes. Statistical analysis was performed using GraphPad Prism. To determine whether gene or protein expression changes were significant, a one-tailed test was performed for all graphs. An alpha value of 0.05 was used to test significance.

Results

Short-chain fatty acids (SCFAs) are byproducts of microbial metabolism of dietary fibers. Previous literature has found that butyrate and propionate, two of the primary SCFAs produced by the microbiome, are readily absorbed by the intestine and can cause beneficial

immunological & physiological effects such as reinforcing the epithelial wall. To determine how certain combinations of SCFAs impact the expression and production of either pro-inflammatory or anti-inflammatory cytokines, Caco2 cells were grown to confluence, mixed with PBMCs, and then incubated with butyrate, propionate, or the combination of both. Cell pellets and supernatants were collected 24-hours post-incubation and used to measure changes to key cytokines related to intestinal inflammation and homeostasis (12-19).

Butyrate and propionate block LPS-induced inflammation *in vitro*

Tumor Necrosis Factor-alpha (TNF-α) is an inflammatory gene that encodes a multifunctional pro-inflammatory cytokine (20). As shown in Fig. 1, expression of TNFa was lowly expressed in un-induced, non-LPS stimulated conditions (RQ 1.01 +/- 0.139) and significantly upregulated in LPS-stimulated conditions (RQ 102.87 +/- 74.16.). Interestingly, when butyrate, propionate, and a combination of the two were added in increasing concentrations (0.5 mM, 1 mM, and 2 mM), TNFa expression decreased significantly when compared to the induced, non-SCFA treated cells (e.g. RQ 1.29 +/- 1.35 for 2mM butyrate combination, p value: 0.0101). Similar trends were observed while directly measuring the protein via ELISA (Figure 2). While the un-induced groups experienced minimal change, levels of TNF-a decreased as butyrate/propionate concentrations increased.The SCFA combination group's expression was the most significant; as concentrations of both SCFAs approached 2 mM, protein expression was similar to un-induced groups.

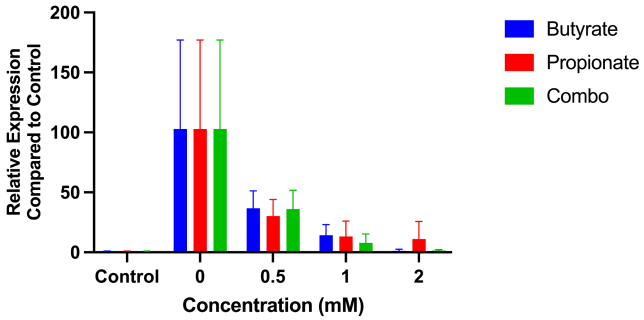

Fig. 1 — TNFa qPCR results from co-cultured cells

As TNF-a is a pro-inflammatory gene, increases in its expression were expected in LPS-stimulated conditions (21). Interestingly, butyrate, propionate, and the combination of the two showed a significant, dose-dependent, decrease in TNF-a expression. Though further studies are required, these results may suggest their presence can downregulate inflammatory genes if present in high concentrations in the intestine.

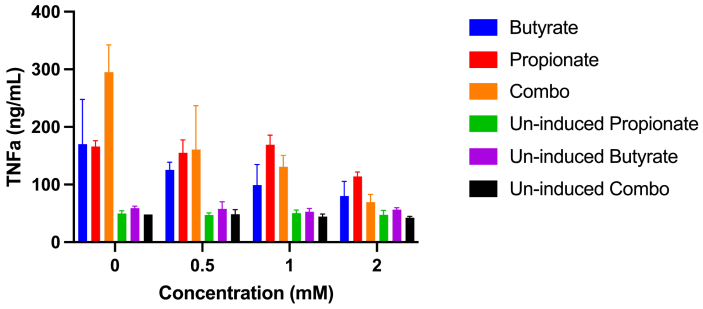

Fig. 2 — TNFa ELISA results from co-cultured cells

FOXP3 expression is not impacted by SCFAs in the PBMCs/CACO2 *in vitro* assay

Previous literature has highlighted the role of SCFAs, specifically butyrate, on the polarization of regulatory T cells (T-regs). T-regs are T cell subsets that play a critical role in

anti-inflammatory responses in the gut and prevent the body from over-reacting to antigens which could contribute to auto-immunity. As butyrate was previously shown to increase T-reg skewing in the gut, Forkhead box protein P3 (FOXP3) – a gene that is used to distinguish T cells – was measured to see how SCFAs impact its expression in the PBMC/CACO-2 co-culture assay (22, 23, 24, 28). As seen in Fig. 3, changes in FOXP3 expression were negligible in un-induced, non-LPS conditions (RQ 1.01 +/- 0.13), versus LPS-induced, non-SCFA treated conditions (RQ 0.75 +/- 0.34). Interestingly, when 0.5 mM of butyrate, propionate, or the combination were added to cells, FOXP3 expression mildly increased. This response was only seen with butyrate at 1mM; at concentrations of 1 and 2 mM, expression decreased, resembling the control cells.

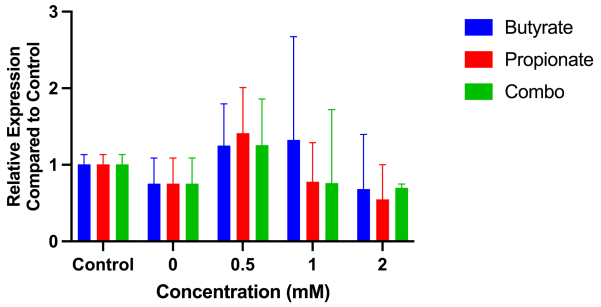

Fig. 3 — foxp3 qPCR results from co-cultured cells

As a whole, these results may suggest that FOXP3 is not significantly affected by LPS and SCFAs in the co-culture setup, possibly due to the low abundance of T-regs in the PBMCs supplied for this assay. While small changes were seen at the lower 0.5mM condition and with butyrate at 1mM, the modest effect suggests this pathway is not the driving force behind the significant results found with the reduction in TNFa levels.

SCFAs balance the effects of LPS on NOD2 *in vitro*

As mild changes to FOXP3 levels were observed in this experiment, other markers of immune cell pathways implicated in IBD were analyzed. Nucleotide-binding oligomerization domain-containing 2 (NOD2) is a sensor in peripheral leukocytes that detects molecular features associated with certain pathogens, including LPS. Once activated, it triggers the NFKB pathway, a pro-inflammatory response. Mutations, deletions, or other changes that impact NOD2 expression are known to be associated with the development of IBD. (25, 26)

As shown in Fig. 4, NOD2 expression was significantly upregulated in the presence of LPS (RQ 9.35 +/- 19.26, p-value: 0.03). Surprisingly, adding butyrate, propionate, or the combination significantly decreased its expression, bringing it back to baseline, un-induced conditions.

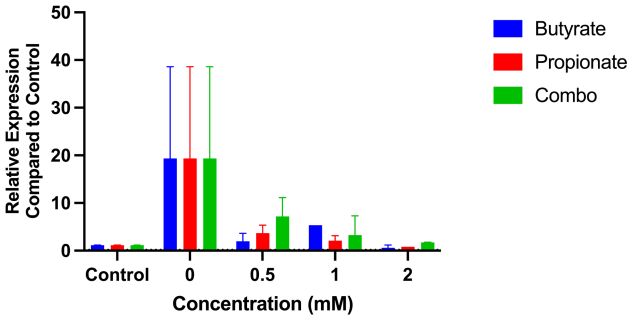

Fig. 4 — Nod2 qPCR results from co-cultured cells

As expression of NOD2 can be stimulated with TNF-a (similar to LPS), future studies should identify whether the decrease in NOD2 observed in this study is due to SCFAs on TNF-a stimulation (as seen in Figure 1) or an independent mechanism of action.

SCFAs stimulate IL-22 gene expression

IL-22 is an IL-10 family cytokine that supports mucosal tissue repair and antimicrobial defenses. While it can have pro-inflammatory effects, in the context of IBD, IL-22 is key to ensuring a healthy gut and reducing the "leaky gut effect" (27).

As shown in Fig. 5, expression of IL-22 in un-induced co-cultures without SCFAs was 1.03 +/- 0.34. Once stimulated with LPS, expression significantly decreased to 0.38 +/- 0.11 (p-value: 0.013), suggesting LPS may negatively regulate IL-22 expression.

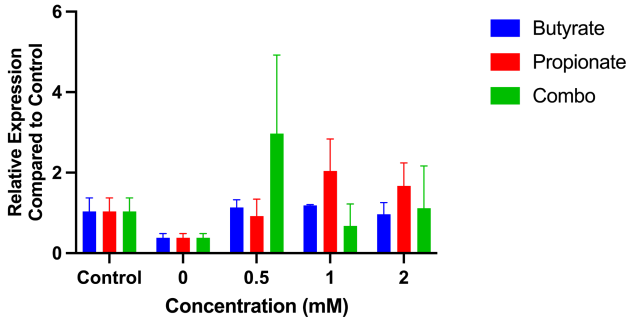

Fig. 5 — IL-22 qPCR results from co-cultured cells

Surprisingly, addition of SCFAs increased IL-22 expression; at all concentrations, gene expression significantly increased compared to the induced non-SCFA treated group. As IL-22 is solely produced by immune cells to act on epithelial cells, these results likely suggest that the PBMCs are the primary cells responding to the SCFAs, not the epithelial cells.

Conclusion

Inflammatory bowel disease (IBD) is a complex, lifelong condition for which optimal treatment options are often difficult to determine. While traditional drug therapies are standard, recent literature underscores the potential role of diet in managing IBD by promoting anti-inflammatory effects. This study investigated the impact of short-chain fatty acids (SCFAs) derived from dietary fibers on inflammatory markers in a Caco-2 and PBMC co-culture model. Results showed that butyrate and propionate significantly modulated TNFα, NOD2, and IL-22 expression, indicating their potential effectiveness in reducing IBD-related inflammation.

Butyrate and propionate are not the only SCFAs produced by the microbiota from dietary fibers, however, as acetate, lactate, and valerate are often present at notable concentrations in the gut. As described in the methods section, acetate was also tested but compromised cell viability, making interpretation challenging. Nevertheless, these findings support the therapeutic potential of butyrate and propionate as dietary-based interventions and further studies should look at the impact of the other SCFAs on inflammation.

Finally, as these results were only generated in cell culture, future research should explore the effects of SCFAs in *in vivo* IBD models to bridge the gap between *in vitro* findings and clinical application. Additionally, further studies are needed to identify foods that exacerbate or mitigate inflammation, guiding dietary recommendations for IBD patients. Given the lack of a definitive cure for IBD, continued research into diet-based treatments holds promise for developing more effective, holistic therapies that could transform patient outcomes.

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