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Potential counter regulatory effects of a gut microbiota metabolite in alleviating down-regulation Krüppel-like factor 4 in intestinal inflammation

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POTENTIAL COUNTER REGULATORY EFFECTS OF A GUT MICROBIOTA METABOLITE IN ALLEVIATING DOWN-REGULATION OF KRÜPPEL-LIKE FACTOR 4 IN INTESTINAL INFLAMMATION

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Dr. Richard Egleton, Committee Member

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APPROVAL OF THESIS

We, the faculty supervising the work of Ylva Forslund, affirm that the thesis, *Potential Counter Regulatory Effects of a Gut Microbiota Metabolite in Alleviating Down Regulation of Krüppel Like Factor 4 in Intestinal Inflammation*, meets the high academic standards for original scholarship and creative work established by the Biomedical Research Graduate Program and the School of Medicine. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

Dr. Alip Borthakur, Department of Clinical & Translational Sciences	Committee Chairperson	07/14/2021 Date
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Inflammatory bowel disease (IBD) is a medical condition characterized by chronic inflammation of the intestinal epithelium. Krüppel-like factor 4 (KLF4), a zinc finger transcription factor, is vital for maintaining intestinal epithelial homeostasis. KLF4 promotes differentiation of goblet cells that generate the protective mucus layer. Reduced goblet cell number and defective mucus layer are associated with IBD. Short-chain fatty acids (SCFA) are known to play an important role in the maintenance of a strong and healthy intestinal epithelial layer and also in goblet cell differentiation. However, whether the positive effects of SCFAs on goblet cells are mediated, at least partly, via upregulation of KLF4 is not known. Current studies were designed to investigate if KLF4 expression is altered in inflammation and the role of SCFA in modulating KLF4 expression. We utilized quantitative reverse transcriptase PCR and Western blot, respectively, to measure the mRNA and protein levels of KLF4. Our results showed a significant reduction in KLF4 expression in human intestinal Caco2 cells exposed to cytokines (TNF- α/IFN-γ 10 ng/ml, 24 h) and in a mouse model of dextran sulfate sodium (DSS)-induced colitis (3% DSS 7 days). Of the 3 key SCFAs (acetate, propionate, butyrate), incubation with propionate (2 mM 24 h) maximally upregulated KLF4 mRNA and protein expression in Caco2 and colonic goblet cell-like LS-174T cells. Also, propionate-induced KLF4 expression was abrogated in the presence of the PPAR-γ antagonist GW9662, while the PPAR-γ agonist pioglitazone mimicked and synergized the effects of propionate on KLF4 expression, suggesting the role of PPAR-γ in mediating propionate effects on KLF4. Propionate enhancement of KLF4 could be of importance in correcting goblet cell hypoplasia in IBD.

THE INTESTINAL EPITHELIUM AND INFLAMMATORY BOWEL DISEASE

INTRODUCTION

Structure of the Intestinal Epithelium

The GI tract is a series of hollow organs, reaching from the mouth to the anus, which aids in transporting nutrients into the bloodstream throughout its length (1, 2). The small intestine is approximately 6 meters in length and 2.5-3.0 cm in diameter, while the colon is significantly shorter, only measuring roughly 1.5 meters in length, but has a diameter of 6.0-7.5 cm (1). The lining of both the small and large intestine comprises of intestinal epithelium, which is a single layer of columnar epithelial cells (3, 4). Although the epithelium is only one layer thick, it is not a straight layer of cells. Instead, it consists of inward invaginations called “crypts of Lieberkühn” (5). Intestinal stem cells (ISC) reside at the base of these crypts and give rise to transient amplifying cells. They will further differentiate into different cell types belonging to two lineages of cells, absorptive and secretory cells (6). However, the intestinal epithelial cells (IEC) differ greatly in both architectural structure and cellular composition between the two parts of the intestine. In the small intestine, the epithelium has projections called villi that extend into the lumen. The absorptive IECs then have numerous microvilli further protruding the lumen, which greatly increases the mucosal surface area to improve nutrient absorption. These projections are absent from the colon, giving rise to a flatter mucosal surface, which prohibits potential damage caused by semi-solid stool transiting through the large intestine.

Numerous different cell types are found in the intestinal epithelium, and each differentiated cell type carries out specialized functions. The most prominent cell type is

enterocytes (colonocytes in the large intestine), comprising approximately 80% of the IECs (3, 4, 7). Enterocytes and microfold (M) cells belong to the absorptive lineage and are responsible for water and nutrient absorption, and the uptake of luminal antigens to the immune system, respectively. The secretory cells consist of enteroendocrine cells, Paneth cells, goblet cells, and tuft cells. They produce and secrete gut hormones, antimicrobial peptides, and mucins, respectively, while tuft cells play a key role in the defense against helminths. The majority of all the cell types are found both in the small and large intestine, including enterocytes, enteroendocrine cells, goblet cells, and tuft cells. However, M cells and Paneth cells are unique to the small intestine.

Role of KLF4 in Goblet Cell Differentiation

The intestinal epithelium is a rapidly self-renewing tissue, and within 4-7 days the epithelial layer is replaced, and the cells are shredded off into the lumen (4, 8, 9). To maintain homeostasis, the events of cell proliferation, differentiation, migration, and apoptosis are under tight regulation (6, 7). Several different studies have identified the zinc finger containing transcription factor, Krüppel-like factor 4 (KLF4), to be involved in the differentiation and migration of goblet cells in the intestinal epithelium (6, 9, 10). KLF4 is normally expressed in post-mitotic, differentiated epithelial cells, and therefore contributes to epithelial homeostasis (6, 9, 10). In 2002, Katz et al. provided evidence of KLF4-null mice showing a reduction of 90% in the number of goblet cells compared to the controls (7). Additionally, the same trend was seen in another *in vivo* study, where KLF4 deletion resulted in overall altered goblet cell maturation and differentiation in the colon (6). Combining the results of these two studies, it is apparent that KLF4 functions as a colonic goblet cell-differentiation factor *in vivo* (6). Lastly, in a study using B-cell-

specific Moloney murine leukemia virus integrin site 1 (BMI1), which is a known reserve intestinal stem cell (rISC) marker, KLF4 deletion in BMI+ ISC mice resulted in significantly lower levels of the goblet cell marker MUC2 compared to control mice (9), again strengthening the evidence of KLF4's role in goblet cell differentiation.

Role of KLF4 in Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD), including Chron's disease (CD) and ulcerative colitis (US), are characterized by inflammation of the gastrointestinal tract (11). The two diseases exhibit recurring inflammation and intestinal epithelial cell damage; however, their clinical symptoms are different. CD pathogenesis is linked to a reduced number of antimicrobial peptides, autophagy, and changes to Paneth cells, causing discontinuous inflammation, while UC patients suffer from goblet cell depletion, which leads to reduced mucin secretion further causing increased intestinal permeability (11). Even if the pathophysiology of IBD is not fully understood yet, the dysfunction of the intestinal epithelial layer is an important part of the development of IBD. Dextran sulfate sodium (DSS)-induced colitis is used to create an inflammatory condition in mice (11). In a recent study, it was found that the steroid receptor coactivator 3 (SRC-3) plays an important role in the development of severe DSS-induced colitis, as SCR-3-null mice displayed colonic goblet cell dysfunction (11). Further, the disruption in goblet cell function was linked to decreased levels of KLF4 in SCR-3-null mice.

Role of KLF4 in Colon Cancer

In the United States, colorectal cancer is the second most commonly diagnosed type of cancer in both sexes, as well as the second leading cause of cancer deaths (10). According to recent epidemiologic research, IBD has been proven to be

associated with increased risks of colorectal cancer (12). In IBD patients, colitis-associated colorectal cancer (CAC) progresses in a stepwise fashion in a sequence referred to as the inflammation-dysplasia-carcinoma pathway (12). Inflammation-induced environmental changes in the gut are thought to play important roles in the induction of CAC. However, the exact mechanism for the development and progression of CAC is unclear. Several factors have been identified to play important roles in the formation of colorectal cancer like Wnt signaling, abnormality in the APC gene or beta-catenin, and mutations in tumor suppressor genes (K-ras and p53) (10). Also, the transcription factor KLF4 has been found to be downregulated in a variety of cancers, including colorectal cancer (6, 10, 12). In an *in vivo* study done, using DSS and azoxymethane (AOM) treatments to induce CAC in a murine mouse model, it was found that KLF4-null mice developed significantly higher amounts of adenomatous polyps and carcinomas *in suit* compared to the control mice (12). Further, they also found that KLF4-null mice suffered shorter colon length, indicating increased inflammation severity. This suggests that depletion of the transcription factor, KLF4, from the intestinal epithelium, together with increased intestinal inflammation, could potentiate early development of adenomas and carcinomas in KLF4-null mice treated AOM/DSS compared to control mice.

Role of SCFAs in Goblet Cell Differentiation

SCFAs are produced in the intestines by anaerobic fermentation of non-digestible carbohydrates (13). SCFAs are important in terms of maintaining intestinal homeostasis and IECs turnover, as well as providing sufficient energy for the epithelial cells (13, 14). Further, SCFAs have been shown to promote mucus secretion, inhibit

inflammation and tumor growth, and enhance cell differentiation (13). A study shows that LS174-T cells, treated with SCFAs, influence MUC2 mRNA and protein levels by increasing both (14). As MUC2 is an indicator for goblet cells, increased MUC2 expression would mean increased goblet cells, and thereby SCFAs affect intestinal protection (15).

Hence, since KLF4 is required for goblet cell differentiation, and SCFAs have an effect on goblet cell differentiation as well, we chose to look at whether SCFA effects on goblet cell differentiation could occur via upregulation of KLF4.

MATERIAL AND METHODS

Chemicals and Antibodies

Acetate, propionate, butyrate, pioglitazone, TNF-α, IFN-γ were purchased from Sigma Aldrich (St. Louis, MO), GW9662 was from Tocris Biosciences (Minneapolis, MN). KLF4 antibody was from Thermo Fisher Scientific. All reagents were of analytical or molecular biology grade.

Cell Culture

Human intestinal epithelial cell line Caco-2 was obtained from American Type Culture Collection (ATCC, Manassas, CA). The Caco-2 cells were grown in cell culture media MEM supplemented with 20% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml gentamicin. The cells were grown at 37°C in a 5% CO_2-95% air environment. LS174-T cells were also obtained from ATCC. LS174-T cells were grown in DMEM media with 10% serum. Cells were seeded on 6, 12, or 24-well plates or 12-well transwell insert plates and grown to confluency. Confluency was reached after 10 days for LS174-T cells and 12 days for Caco-2 cells.

Cell Treatments

For all treatments, model human intestinal Caco-2 and goblet-like LS174-T cells were grown as confluent monolayers on 6-, 12- or 24-well plastic supports or 12-well transwell inserts (for cytokine treatments from basolateral sides). Both cell lines were treated with SCFAs (acetate, butyrate, or propionate, 2 mM, 24 h); propionate (1 mM, 2 mM, and 5 mM); cytokines (IFN-γ 10 ng/ml and 30 ng/mg, TNF-α 10 ng/ml and 50 ng/ml, 24 h); PPAR-γ antagonist GW9662 (5 μM) and PPAR-γ pioglitazone (10 μM). After the desired period of treatments, cells were harvested and stored at -80°C in lysis buffer. RIPA lysis buffer (Santa Cruz Biotechnology) was used for samples prepared for protein extraction, and RLT lysis buffer was used for samples prepared for quantitative Real-Time RT-PCR.

Mice Tissue Samples

Colonic mucosal tissue lysates from normal mice and mice with dextran sulfate sodium (DSS)-induced colitis were obtained from the Department of Gastroenterology and Hepatology, University of Illinois at Chicago on a collaborative basis with the PI (Dr. Borthakur) (16, 17).

RNA Extraction and Real-Time PCR

RNA was isolated from Caco-2 cells, LS174-T cells, or homogenized mouse intestinal mucosal samples using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. An equal amount of RNA (33.3 ng/μl) for each sample was reverse-transcribed and amplified in a one-step reaction using the Brilliant SYBR Green qRT-PCR Master Mix kit (Agilent). The recipe in Table 1 was used to prepare the master mix for the real-time PCR. 2.25 μl of RNA and 7.75 μl of the master mix were

then pipetted into the corresponding low-profile PCR tubes (Bio-Rad). Human KLF4 gene was amplified with gene-specific primers (forward primer 5′-CCACCTTCTTCACCCCTAGA-3′; reverse primer 5′CTTTGTGTAGGTTTTGCCGC-3′), using human GAPDH (forward primer 5′-GAAATCCCATCACCATCTTCC-3′; reverse primer 5′-AAATGAGCCCCAGCCTTCT-3′) as the internal control.

Reagent	**Volume for 1 reaction**
QRT	5 μl
Forward Primer	1 μl
Reverse Primer	1 μl
1:500 SYBR Dye	0.15 μl
Reverse Transcriptase	0.5 μl
DTT	0.1 μl

Table 1. Recipe for 1 Reaction for Real-Time PCR Master Mix

Western Blot Analysis

Lysates from whole cells (Caco-2 cells and LS174-T cells) and mouse colonic mucosa were further prepared after being placed in RIPA buffer. Samples were centrifuged for 10 minutes at 13,000 rpm and 4°C to remove cell material. The supernatants were collected to further use for protein quantification using *DC* Protein Assay (Bio-Rad) protocol (18). Protein samples (50 μg of total protein) were separated using a 10% sodium dodecyl sulfate (SDS) gel and transferred onto a nitrocellulose membrane. Membranes were blocked using 5% milk made with TBST and incubated overnight at 4°C with the following primary antibodies: KLF4, PPAR-γ, IFN-γ, TNF-α,

and internal controls GADPH or villin. The signals were detected using Enhanced Chemiluminescence reagents (ECL Plus; GE Healthcare, London, UK). Quantification of the bands was performed using ImageJ software (US National Institutes of Health) and normalized using GAPDH and villin levels.

Statistical Analyses

Results are presented as means of 3-4 independent experiments, with error bars represented by the standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism (GraphPad Software). The comparison between controls and treated groups was done using one-way ANOVA with Tukey's test or Student's t-test. Statistical significance was considered for $P \leq 0.05$.

RESULTS

KLF4 EXPRESSION IS DOWNREGULATED IN INTESTINAL INFLAMMATION

In the current studies, initially, we examined the effects inflammation had on the protein levels of KLF4, a critical transcription factor involved in maintaining intestinal epithelial homeostasis. In the *in vitro* model, intestinal epithelial Caco-2 cell monolayers grown on transwell inserts were treated from the basolateral side with TNF-α and IFN-γ, two proinflammatory cytokines known to be elevated in IBD, for 24 h. *In vivo*, the protein levels of KLF4 were measured in the colonic mucosa of control mice and mice with DSS-colitis, a widely used animal model of IBD. KLF4 protein in cell lysates or mucosal lysates was determined by SDS-PAGE and immunoblotting with KLF4 antibody. GAPDH or villin was used as the loading control.

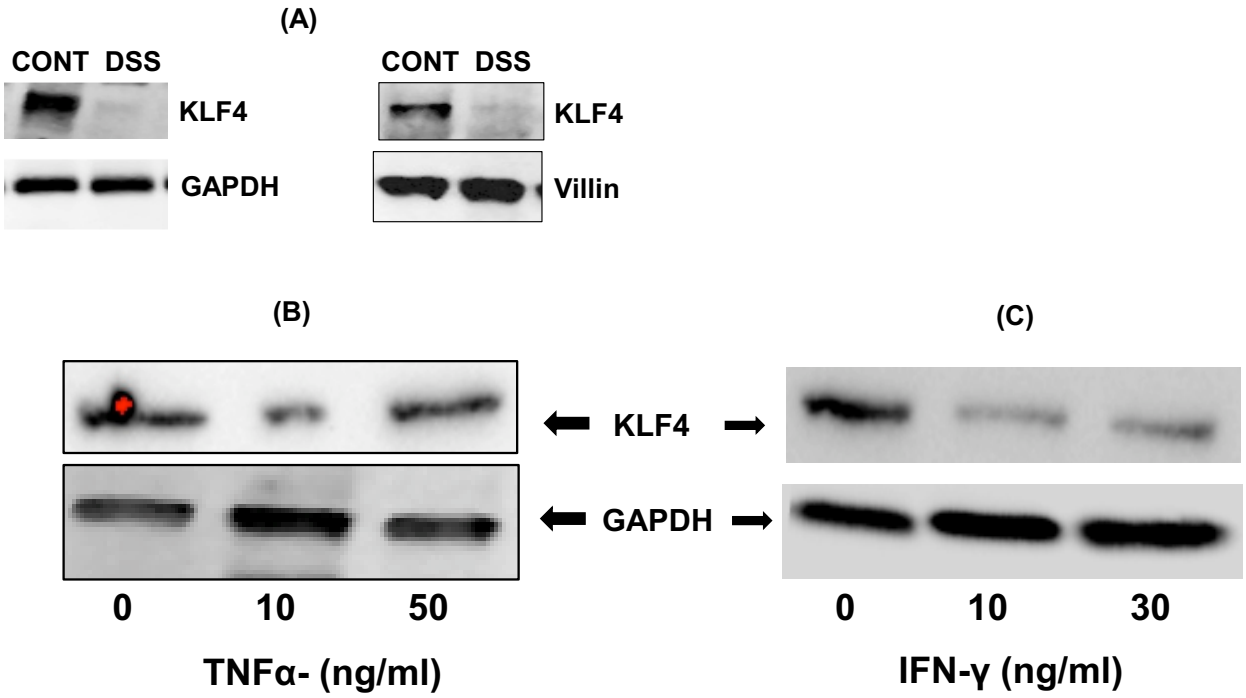

Figure 1. KLF4 Protein Expression in DSS-Colitis Mouse Model and Caco-2 Cells

KLF4 protein expression is decreased *in vivo* in mouse model of DSS-colitis (A) and *in vitro* in Caco-2 cells in response to treatments with TNF-α (B) and IFN-γ (C). Representative blots of 3 independent experiments are shown.

EFFECTS OF SHORT-CHAIN FATTY ACIDS ON KLF4 EXPRESSION *IN VITRO*

SCFAs, primarily acetate, propionate, and butyrate produced by colonic bacterial action on undigested dietary fiber, are known to augment goblet cell differentiation. Since KLF4 is required for goblet cell differentiation, we sought to investigate whether SCFA effects on goblet cells could be via upregulating KLF4. We used model human intestinal cell line Caco-2 and a goblet-like cell line LS174 to examine the effects of SCFAs on mRNA and protein levels of KLF4.

Propionate Treatment Increases KLF4 mRNA Levels in Caco-2 Cells

Confluent cell monolayers of Caco-2 cell line were treated with 2 mM acetate, propionate, or butyrate for 24 h. Total RNA extracted from the cells was used to measure KLF4 mRNA levels by quantitative real-time (qRT)-PCR using human gene specific primers and GAPDH as the internal control. As shown in Figure 2, propionate significantly increased KLF4 mRNA compared to control, whereas acetate and butyrate had no significant effect.

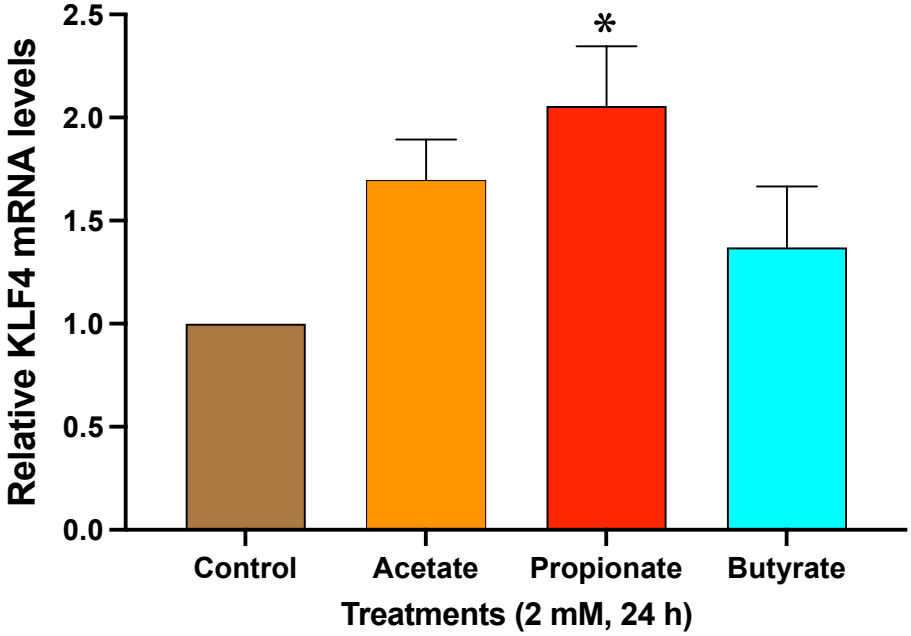

Figure 2. Propionate (2 mM, 24 h) Increases KLF4 mRNA in Caco-2 Cells (N=4, *P<0.001)

Dose Response of Propionate on KLF4 mRNA in Caco-2 Cells

Dose response of propionate was determined by treating the cells with 1, 2, and 5 mM propionate for 24 h, and KLF4 mRNA levels were measured. A maximum increase of KLF4 mRNA was observed with 2 mM propionate (Figure 3). Therefore, in subsequent experiments on propionate effects, 2 mM propionate was used.

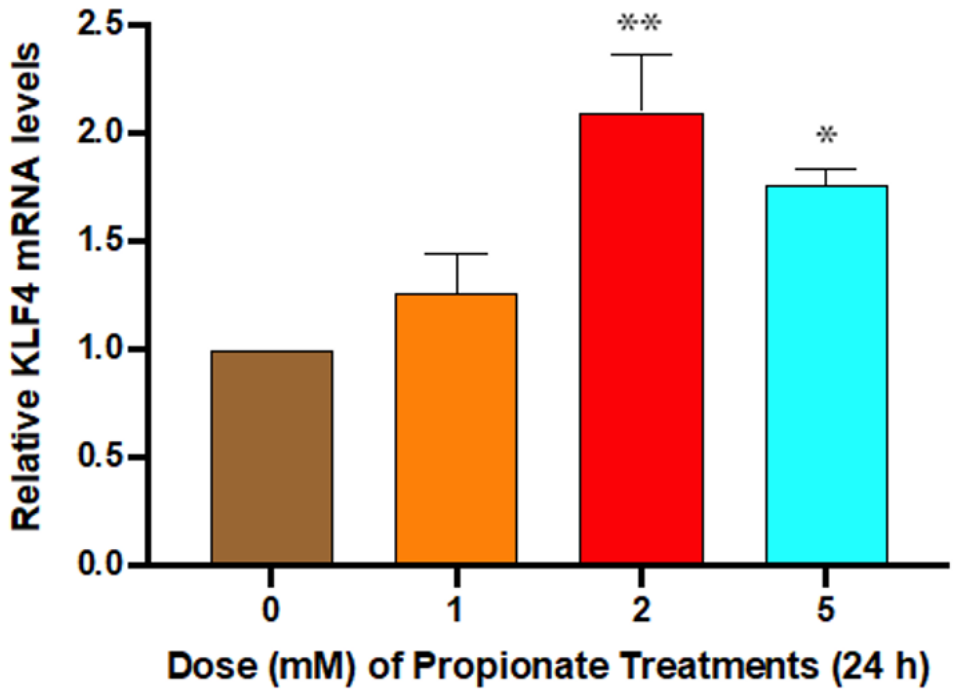

Figure 3. Dose Response of Propionate on KLF4 mRNA in Caco-2 Cells (N=4, **P*<0.05; ***P*<0.001)

Time-Course Effect of Propionate on KLF4 mRNA

The time-course of propionate effect on KLF4 mRNA was next measured by treating the cells with 2 mM propionate for 8, 16, 24, and 48 h. Propionate treatment increased KLF4 mRNA as early as after 8 h, however, maximum increase was observed at 24 h, whereas propionate treatment for 48 h had no effect (Figure 4). Therefore, in all subsequent experiments, 24 h time point was used for propionate treatments.

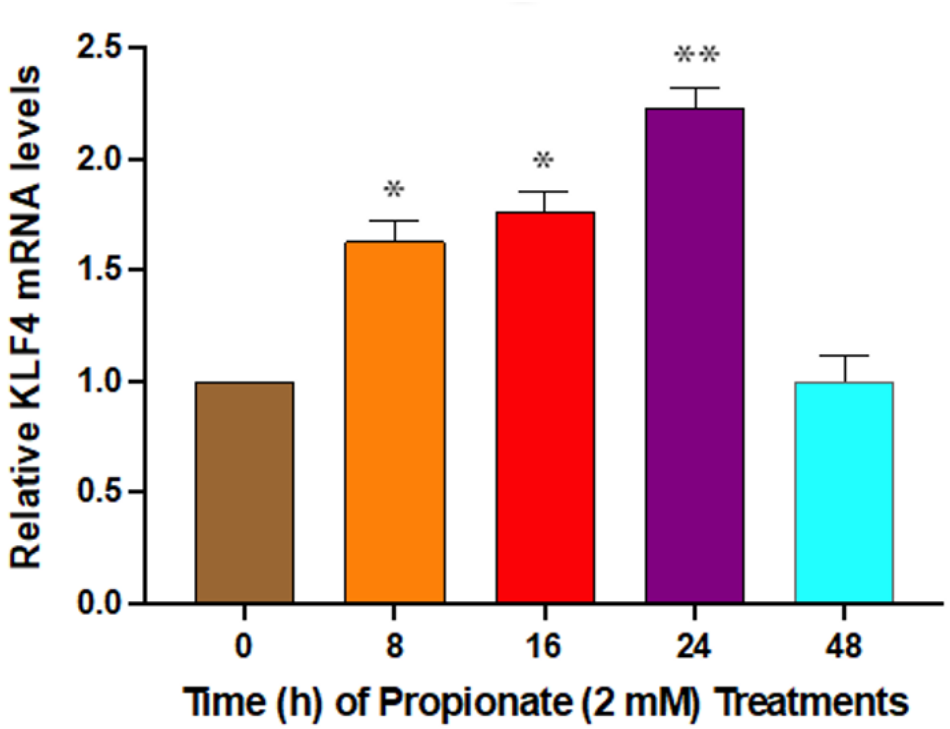

Figure 4. Time-Course Effect of Propionate on KLF4 mRNA in Caco-2 Cells (N=4, **P*<0.05; ***P*<0.001)

Propionate Increases KLF4 Protein Levels in Caco-2 and LS174-T Cells

Confluent monolayers of Caco-2 and LS174-T cells were treated with 2 mM acetate, propionate, and butyrate for 24 h. Total proteins in the cell lysates were separated by SDS-PAGE and probed with KLF4 antibody in immunoblotting. As shown in Figure 5, propionate significantly increased KLF4 protein in Caco-2 cells, whereas acetate and butyrate had no effect (5A). In LS174-T cells, both acetate and propionate significantly increased KLF4 protein, with propionate showing maximum stimulatory effect (5B).

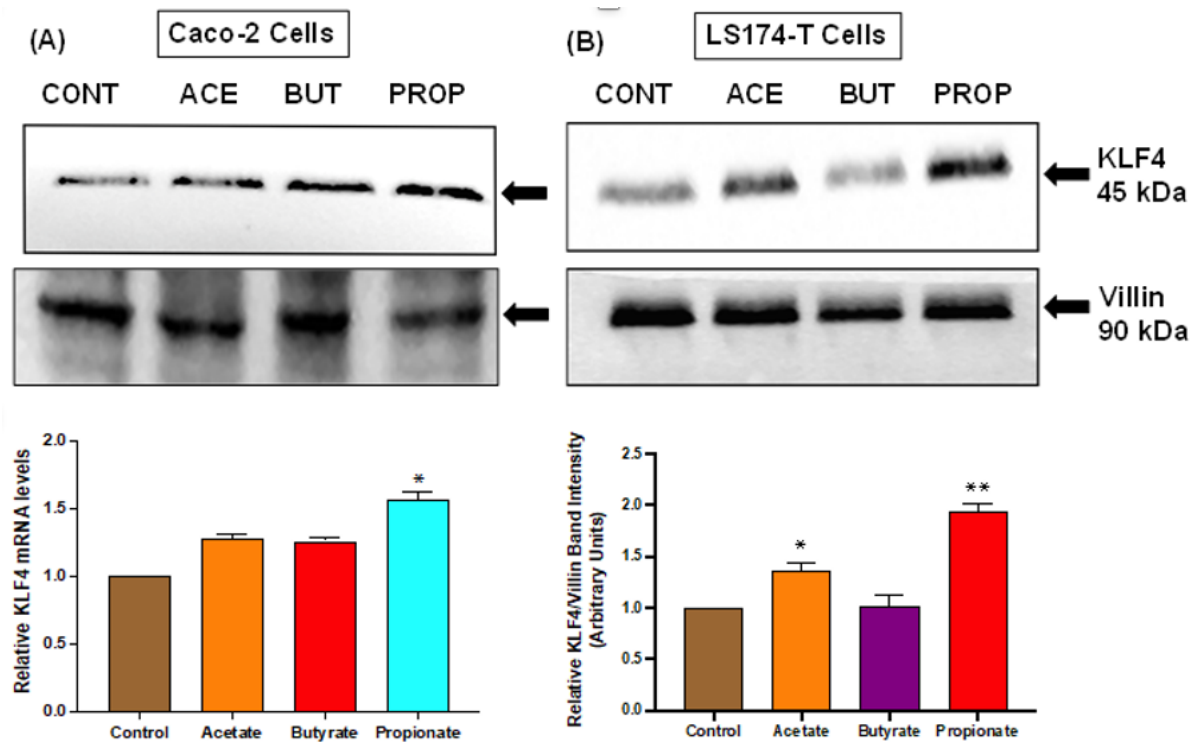

Figure 5. Propionate Increases KLF4 Protein Levels in (A) Caco-2 and (B) LS174-T Cells (N=4, **P*<0.05; ***P*<0.001)

ROLE OF PPAR-γ IN MEDIATING PROPIONATE EFFECTS ON KLF4 EXPRESSION

Previous studies have shown the role of PPAR-γ in mediating SCFA-induced modulation of gene expression (19). Therefore, to elucidate the mechanisms of propionate effects on KLF4 expression, we examined the potential role of PPAR-γ by treating the cells with PPAR-γ agonist and antagonist ± propionate and measuring KLF4 mRNA and protein levels.

Effect of PPAR-γ Agonist Pioglitazone on KLF4 mRNA

Caco-2 and LS174-T cells were incubated with pioglitazone (10 μM) ± propionate (2 mM) for 24 h and KLF4 mRNA was measured. In both cell lines, Caco-2 (A) and LS174-T (B), pioglitazone alone significantly increased KLF4 mRNA, whereas

synergistic/additive effects on KLF4 mRNA were found when cells were treated with both pioglitazone and propionate (Figure 6).

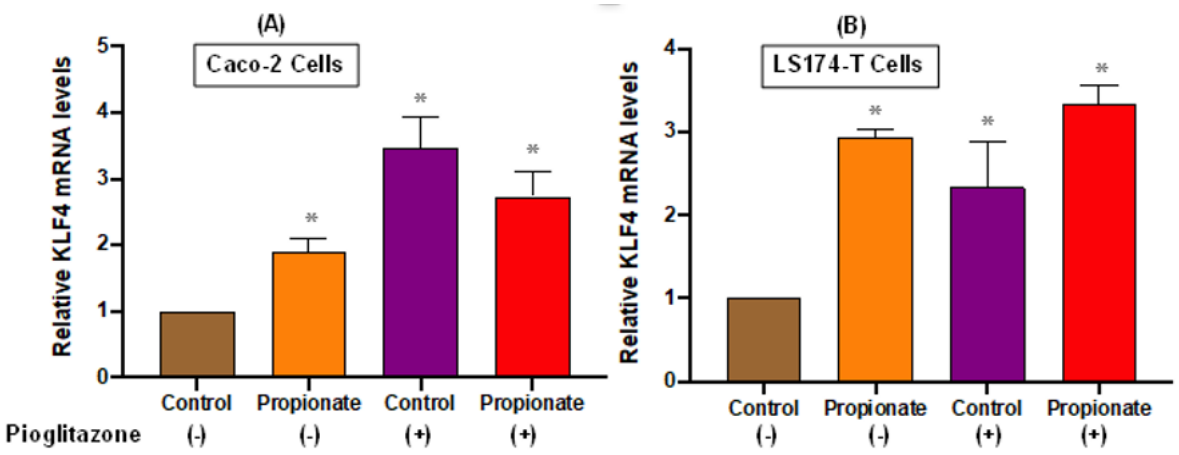

Figure 6. PPAR-γ Agonist Pioglitazone Effect on Caco-2 and LS174-T Cells

PPAR-γ agonist pioglitazone increases KLF4 mRNA and shows synergistic/additive effects with propionate in (A) Caco-2 and (B) LS174-T cells (N=3, *P<0.001)

Effect of PPAR-γ Antagonist GW9662 on KLF4 mRNA

Caco-2 and LS174-T cells were incubated with GW9662 (5 μM) ± propionate (2 mM) for 24 h and KLF4 mRNA was measured. Results are shown in Figure 7. In both the cell lines, Caco-2 (A) and LS174-T (B), GW9662 alone had no effect on KLF4 mRNA. Propionate alone significantly increased KLF4 mRNA, which, however, was abrogated in presence of GW9662. Thus, these studies with PPAR-γ agonist and antagonist suggest that propionate effects on KLF4 could be mediated via PPAR-γ.

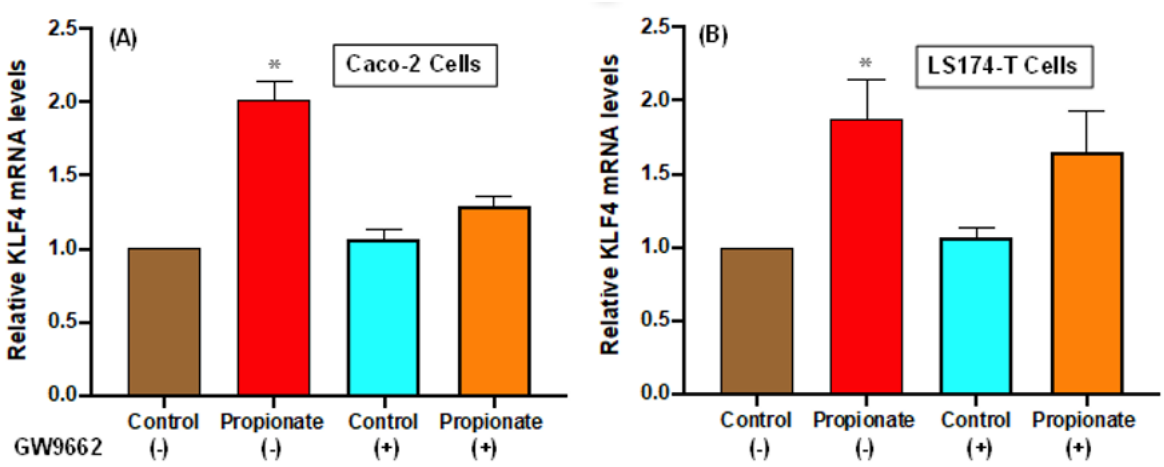

Figure 7. PPAR-γ Antagonist GW9662 Abrogates the Stimulatory Effects of Propionate on KLF4 mRNA in (A) Caco-2 and (B) LS174-T Cells (N=3, *P<0.001)

Effect of PPAR-γ Agonist Pioglitazone on KLF4 Protein Levels in Caco-2 Cells

Caco-2 cells were incubated with pioglitazone (10 μM) ± propionate (2 mM) for 24 h. Cell lysates were prepared and KLF4 protein levels were measured by Western blot. Results are shown in Figure 8. Pioglitazone alone or propionate alone significantly increased KLF4 protein, whereas synergistic/additive effects on KLF4 protein were found when cells were treated with both pioglitazone and propionate. All these results taken together suggest a role of PPAR-γ in mediating the effects of propionate on KLF4 expression.

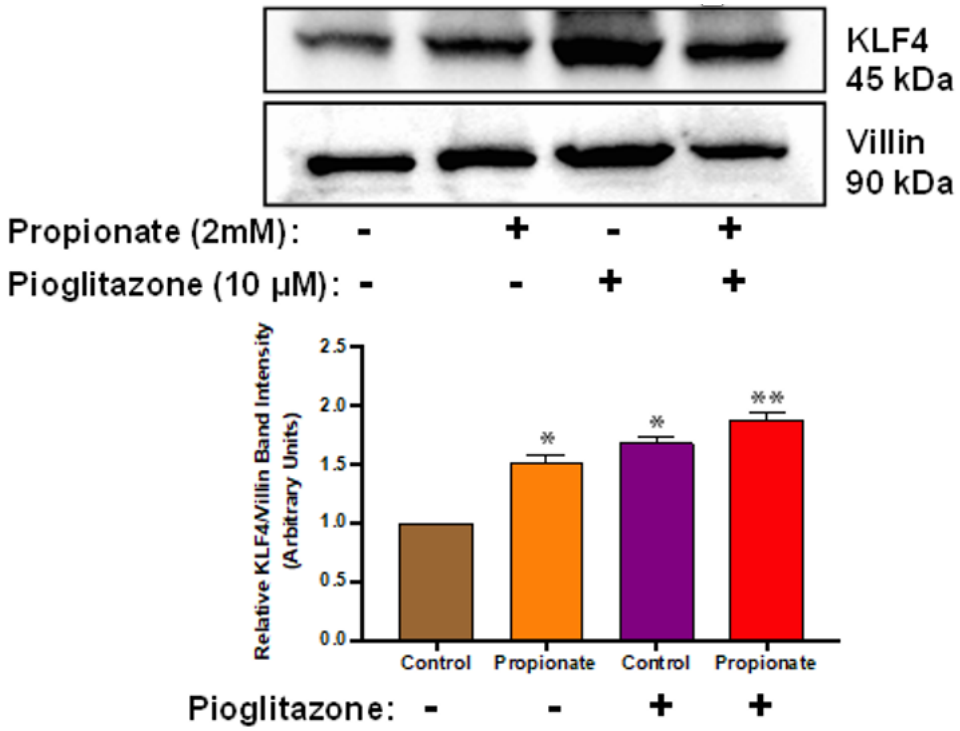

Figure 8. PPAR-γ Agonist Pioglitazone Increases KLF4 Protein and Shows Synergistic/Additive Effects with Propionate in Caco-2 Cells (N=3, *P<0.05; **P<0.001)

DISCUSSION

In recent years, there has been mounting evidence of the role of gut microbiota and the metabolites, more specifically the SCFA, in maintaining intestinal epithelial homeostasis via varied mechanisms. Both propionate and butyrate, two of the most common SCFAs, are produced from carbohydrate fermentation and amino acid fermentation by different bacterial species. It is known that propionate plays an important role in maintaining homeostasis in the intestinal epithelium, as the SCFA is used as an energy source in intestinal cells and aids in inflammatory responses (14, 20, 21). Therefore, when dysbiosis in the gut microbiota arises, these SCFAs cannot be produced in the same manner, which can cause the intestinal epithelial layer to be

damaged. Such disruption can further cause inflammation of the intestinal epithelium and develop IBD. The SCFA propionate, together with other SCFAs, are considered a promising supplementary treatment for IBD (14). Since IBD is a very individualized disease, with several different side effects, it is difficult to create subtype-specific treatments. Several studies have shown that the use of a SCFAs enema mix has had little effect on both UC and CD patients and animal subjects (14). However, when administering oral treatment containing SCFA-producing bacteria to DSS-colitis mice, the colonic mucosal damage was decreased. This suggests that SCFAs administered by themselves are not as effective as treatments with SCFA-producing bacteria. Therefore, the use of SCFA-producing pre- or probiotics could potentially restore the intestinal mucosa homeostasis in IBD suffering patients.

KLF4 is a zinc finger transcription factor, predominantly expressed in differentiated epithelial cells of the small intestine and colon. KLF4 plays multiple roles in the human body, but recent studies have found the transcription factor to play an important role in regulating intestinal epithelial cell homeostasis (6). Mice with intestine specific deletion of KLF4 exhibited an increase in epithelial proliferation, decreased differentiation, and a reduction in goblet cell number (9, 10, 12). On the other hand, augmenting KLF4 expression has been shown to promote goblet cell differentiation and alleviate DSS-induced colitis (11). Goblet cell hypoplasia and defective synthesis of protective mucus layer are associated with IBD. Therefore, the agents that upregulate KLF4 and/or counteract downregulation of KLF4 in inflammation could correct goblet cell hypoplasia. In this regard, SCFAs are known to enhance epithelial protection via

stimulating mucus synthesis by goblet cells but their effects on KLF4 expression are not studied in detail.

In this study, we first examined the effects of the cytokines TNF-α and IFN-γ, known to be elevated in IBD, on KLF4 expression *in vitro* in Caco-2 cells and also measured KLF4 protein levels in the colonic mucosa of normal versus DSS-colitis mice. In both models, we observed extensive downregulation of KLF4. Indeed, KLF4 was almost diminished in response to DSS-colitis. Secondly, we have observed the SCFA propionate, an important gut microbiota metabolite, as a potential therapeutic agent showing a stimulatory effect on KLF4 expression in both Caco-2 cells and LS174-T cells. Further, we have performed mechanistic studies to show that propionate-induced KLF4 expression was abrogated in the presence of the PPAR-γ-specific antagonist GW9662, while the PPAR-γ-specific agonist pioglitazone mimicked and synergized the effects of propionate on KLF4 expression. This suggests that the propionate-induced KLF4 expression could occur via a PPAR-γ-dependent mechanism. This, in turn, could improve the differentiation of goblet cells, that, via secretion of mucins, exert protective effects on intestinal epithelium. However, these preliminary studies need to be further supported by determining *in vitro* and *in vivo* that propionate treatments could counteract inflammation-induced downregulation of KLF4 expression. Further, to define novel therapeutic targets for IBD, detailed mechanistic studies are needed to establish the link between KLF4 and goblet cell differentiation and function.

CONCLUSION

The results of this study show that the SCFA propionate exerts a stimulatory effect on KLF4 expression in intestinal epithelial cells. Our results also show

downregulation of KLF4 expression in intestinal inflammation. In view of the known role of KLF4 in health and disease, more particularly the experimental evidence from multiple investigators showing its critical role in maintaining intestinal epithelial homeostasis via governing goblet cell differentiation, propionate enhancement of KLF4 could be of importance in correcting the goblet cell hypoplasia in IBD. However, a more detailed knowledge of the mechanism and interplay between the SCFA propionate and the zinc-finger containing transcription factor KLF4 in the pathophysiology of IBD is necessary to define novel therapeutic targets to develop superior treatment modalities for IBD.

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APPENDIX A: APPROVAL LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

July 8, 2021

Ylva Forslund
8 Galleries E
Huntington, WV 25701

Dear Ylva:

This letter is in response to the submitted thesis abstract entitled *"Potential Counter Regulatory Effects of a Gut Microbiota Metabolite in Alleviating Down Regulation of Kruppel Like Factor 4 in Intestinal Inflammation."* After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction, it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director