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Butyrate Supplementation Affects Mrna Abundance of Genes Involved in Glycolysis, Oxidative Phosphorylation and Lipogenesis in the Rumen Epithelium of Holstein Dairy Cows

[1]Anne Hermen Laarman, [1]Louis Dionissopoulos, [1]Ousama AlZahal, [1]Michael Alexander Steele, [2]Sabrina Louise Greenwood, [3]James Clyde Matthews and [1]Brian William McBride

[1]Department of Animal and Poultry Science,
Ontario Agricultural College, University of Guelph, Guelph ON, Canada
[2]Department of Animal Science,
College of Agriculture and Life Sciences, University of Vermont, Burlington, VT, USA
[3]Department of Animal and Food Sciences,
College of Agriculture, University of Kentucky, Lexington, KY, USA

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ABSTRACT

Energy availability in epithelial cells is a crucial link for maintaining epithelial barrier integrity; energy depletion is linked to impaired barrier function in several epithelia. This study aimed to elucidate the effects of exogenous butyrate on mRNA abundance of genes indirectly involved in rumen epithelial barrier integrity. Sixteen mid-lactation Holstein cows fed a total mixed ration received a concentrate mix to induce Subacute Ruminal Acidosis (SARA). For 7 days, while being fed the concentrate mix, cows were assigned either a control treatment or a butyrate treatment, in which cows were fed butyrate at 2.5% daily dry matter intake in the form of a calcium salt. On days 6 and 7, rumen pH was measured continuously and on day 7, rumen biopsies took place. Rumen pH fell below 5.6 for more than 3 hours per day in both treatments, con-firming the occurrence of SARA. Microarray and pathway analysis, confirmed by real time PCR, showed that exogenous butyrate significantly increased the mRNA abundance of hexokinase 2 (fold change: 2.07), pyruvate kinase (1.19), cytochrome B-complex 3 (1.18) and ATP Synthase, F0 subunit (1.66), which en-code important glycolytic enzymes. Meanwhile, butyrate decreased mRNA abundance of pyruvate dehydrogenase kinase 2(-2.38), ATP citrate lyase (-2.00) and mitochondrial CoA transporter (-2.27), which en-code enzymes involved in lipogenesis. These data suggest exogenous butyrate induces a shift towards energy mobilization in the rumen epithelium, which may aid barrier function in the rumen epithelium during SARA.

Keywords: Rumen Epithelium, Butyrate, Energy Mobilization

1. INTRODUCTION

In cows fed a high grain diet for milk production purposes, Subacute Ruminal Acidosis (SARA) continues to be a costly problem, with one estimate of $400 USD in lost milk production per cow per lactation (Plaizier *et al*., 2008). The accumulation of Short Chain Fatty Acids (SCFA) leads to SARA and strains the integrity of the rumen epithelium (Zebeli and Metzler-Zebeli, 2012). In cases where SARA compromises epithelial integrity, many detrimental health effects can result, such as liver abscesses, lesions and even

Corresponding Author: Anne Hermen Laarman, Department of Animal and Poultry Science, Ontario Agricultural College, University of Guelph, Guelph ON, Canada

laminitis (Krause and Oetzel, 2006). Adverse health events are caused by translocation of bacteria across the rumen epithelium, underscoring the importance of epithelial integrity.

Like other epithelia, ruminal barrier function is attained by tight junction proteins such as tight junction protein isoform 3, zonal occludins isoform 1 andclaudin isoform 1. These proteins physically anchor the cells of the stratum granulosum (Graham and Simmons, 2005), effectively eliminating paracellular bacterial translocation. In so doing, the epithelium can facilitate SCFA and proton transport across the rumen epithelium using multiple transport mechanisms, in addition to a smaller fraction of SCFA that passively diffuses across the epithelium (Aschenbach *et al*., 2009). Maintaining epithelial integrity thus plays a crucial role in health maintenance in dairy cows.

One possible nutraceutical implicated in maintaining barrier function is butyrate, which is shown to genomically regulate several cellular processes and modulate gene expression through Histone Deacetylase Complex (HDAC) inhibition (Ploger *et al*., 2012). In cows, butyrate increases expression of critical barrier function genes, including tight junction proteins and claudins (Baldwin *et al*., 2012). Another example is in Caco-2 cells, where butyrate decreases epithelial permeability and increases transepithelial resistance at low concentrations (Peng *et al*., 2007). On a protein level, butyrate promotes redistribution of tight junction proteins through activation of AMP-Activated Protein Kinase (AMPK) (Peng *et al*., 2009). Activity of AMPK is required for maintaining epithelial cell polarity, but only in a low energetic state, highlighting the importance of energy state in epithelial function (Mirouse *et al*., 2007). In freshly weaned piglets, low energy state adversely affects barrier function in the small intestine, whereas varying dietary inclusions of protein and lactose do not (Spreeuwenberg *et al*., 2001). How butyrate might affect energy status in epithelial cells is unclear.

The objective of this study was to determine how butyrate affects genes involved with energy mobilization in the rumen epithelium. Butyrate's importance in the homeostasis of many processes can clearly be seen in previous studies. Whether genes involved in energy status dynamics are affected by butyrate, however, is unclear. We therefore hypothesized that butyrate would alter gene expression for the purpose of energy mobilization in the rumen epithelium to prevent energetic stress caused by SARA.

2. MATERIALS AND METHODS

2.1. Animals, Treatment and Sampling

This study was carried out as described previously (Dionissopoulos *et al*., 2013). All animal procedures were approved by the Animal Care Committee at the University of Guelph under the guidelines of the Canadian Council for Animal Care (Ottawa, ON, CA). Sixteen fistulated cows on a mid-lactation Total Mixed Ration (TMR) were fed a grain supplement that increased dietary Non-Fibre Carbohydrate (NFC) content to 44.0% on a dry matter basis. Two days before the start of the study, only half the supplement was fed and on the day before the study, the full amount of supplement was fed. Thereafter, cows were divided into a control treatment and a butyrate treatment. Cows on the control treatment received a carrier only, while cows on the butyrate treatment received a carrier containing a butyrate dose (Proformix; Probiotech Inc., Saint Hyacinthe, QC) at the rate of 2.5% of Dry Matter Intake (DMI). Daily, DMI and milk production were measured. On days 1 and 7, blood was sampled and analyzed for serum β-Hydroxybutyrate (BHBA) by the Animal Health Laboratory (Guelph, ON, Canada) using established protocols (Williamson *et al*., 1962). Also on the same day, rumen fluid from the ventral sac of the rumen was strained through 4 layers of cheesecloth and analyzed for SCFA profile by gas chromatography as described previously (Mutsvangwa *et al*., 2002). On days 6 and 7, rumen pH was continuously measured using an in-dwelling pH monitoring system (AlZahal *et al*., 2007). Rumen biopsies were done on day 7 by partial evacuation of the rumen and processed for microarray and quantitative Real-Time PCR (qRT-PCR).

2.2. Microarray and qRT-PCR

Rumen papillae samples were analyzed by microarray as previously described (Dionissopoulos *et al*., 2013). Samples from the control treatment were pooled and the butyrate samples were compared to the pooled control samples using student's t-test (Xue *et al*., 2010). Significance was determined using a pre-screen of 95% confidence and a false discovery rate of 0.1. Through Ingenuity Pathway Analysis (Ingenuity Inc., Redwood City, CA, USA), networks of interconnected differentially expressed genes were created and a subset of differentially expressed genes were identified for confirmation by PCR. Primers were designed using Primer-BLAST (NCBI, Bethesda, MD, USA); most primers were intron-spanning (**Table 1**).

Table 1. Primer sequences for genes verified by PCR

Gene	Name	Accession No.	Primer sequence (F/P)	E (%)
HK2	Hexokinase 2	XM_002691189.2	F AGTGCAGAAGGTTGACCAGT P CCAAAGCACACGGAAGTTGG	106
PKM2	Pyruvate kinase, muscle	BT030503.1	F GCCATGAATGTCGGAAAGGC P GATGGTTTGGGGAAGAGGGG	93
LDHA	Lactate dehydrogenase A	NM_174099	F GTCAGCAGTCTGGCAGCTAT P TAACCAGCCTGGAGTTTGCT	101
LDHB	Lactate dehydrogenase B	NM_174100	F TCGTGCAGCCCTTATCACTC P CGTCAGTCAGAGACTTTCCCA	85
PFKFB4	6-phosphofructo-2-kinase/fructose -2,6-biphosphatase 4	NM_001192835	F ATGACCAACTGTCCAACGCT ATGACCAACTGTCCAACGCT P TGTTGCATCAAAAACCGCCA	99
ACAD9	Acyl-CoA dehydrogenase 9	NM_001078076	F GTCTGGGTCACCAATGGAGG P TGACGCCACCAAAGTCTCTC	93
CYBASC3	Cytochrome B561, member A3	NM_001099149	F TGTTGCCGAGAGTCTGTCAC P CTGACCAGGCAGCCCTTTAT	83
ATP5G1	ATP synthase, F_0 subunit	NM_176649	F CTATGCCAGGAACCCGTCTC P AGGTTAGCACACTCCAGCAC	88
ACLY	ATP citrate lyase	NM_001037457	F TTGGAGAGATAGGGGGCACA P TGGACCTCGGAGGAGAACAT	94
FASN	Fatty acid synthase	NM_001012669	F CTTCCAGTGGGTTGACTCCC P CTCCTCGGGCTTGTCTTGTT	90
PDK2	Pyruvate dehydrogenase kinase 2	NM_001159481	F CCCGAGTCCTAGAAGTGGTC P GGACATACCAGCTCTGTACCA	93
SLC25A42	Mitochondrial CoA transporter	NM_001192032	F GCACGTCTCCTCAAAGAGTG P GCACGTCTCCTCAAAGAGTG	80
ATP1B1	Na/K ATPase	NM_001035334	F GCCCCACCAGGATTAACACA P TGGGATCGTTAGGACGAAAGG	96

Primers were analyzed by Basic Local Alignment Search Tool (NCBI, Bethesda, MD, USA) to confirm specificity to the target gene and to confirm a low risk of non-specific binding. Primers were then further analyzed by OligoAna-lyzer (Integrated DNA Technologies, Coralville, IA, USA) for primer-primer heterodimers, self-dimers and hairpins. After ordering the primers (Sigma-Aldrich, Oakville, ON, Canada), primer fidelity was empirically confirmed through dissociation curves. GAPDH was used as an internal control gene for quantification using previous methods (Pfaffl, 2001).

3. RESULTS

3.1. SCFA, Blood, Rumen pH

Total SCFA concentrations were higher on day 1 in the butyrate treatment (92.76±4.51 Vs 78.87±4.51 mM) but not on day 7 (87.59±4.51 Vs 81.82±4.51 mM). Butyrate concentrations on both days 1 and 7 were higher in butyrate than in control (22.60±0.94 Vs 9.88±0.94 mM and 21.60±0.94 Vs 8.60±0.94 mM; Dionissopoulos *et al*., 2013). Serum BHBA was higher in the butyrate treatment than the control treatment on both days 1 (4201±265 Vs 910±265 μM) and 7 (3262±265 Vs 800±265 μM; Dionissopoulos *et al*., 2013). Rumen pH measurements confirmed the occurrence of acidosis in both butyrate and control treatments, as indicated by the time rumen pH was less than 5.6 (536±89 and 598±97 min/day, respectively; Dionissopoulos *et al*., 2013).

3.2. Microarray and qRT-PCR

Pathway analysis showed arrays of glycolysis, lipogenesis and oxidative phosphorylation genes as being differentially expressed. Of the 31 genes identified by pathway analysis, 13 genes in these 3 processes were confirmed by qRT-PCR (**Table 2**). In cows on the butyrate treatment, glycolytic genes such as hexokinase 2 (fold change: 2.07), pyruvate kinase 2 (FC:1.19), lactate dehydrogenase A (FC:1.45), lactate dehydrogenase B (FC:1.18) were upregulated while the phosphofructokinase inhibitor 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (FC:-2.22) was downregulated. Criticaloxidative phosphorylation genes were upregulated, including acyl-CoA dehydrogenase 9 (FC:-1.30), cytochrome B561, member A3 (FC: 1.11) and ATP synthase F0 subunit (FC: 1.66).

Table 2. Microarray and quantitative RT-PCR analysis of genes differentially expressed by microarray

Gene	Name	Role	Microarray fold change	PCR Fold change
HK2	Hexokinase 2	Glycolysis	1.51	2.07
PKM2	Pyruvate kinase, muscle	Glycolysis	1.34	1.19
LDHA	Lactate dehydrogenase A	Glycolysis	1.33	1.45
LDHB	Lactate dehydrogenase B	Glycolysis	1.23	1.18
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	Glycolysis	-1.37	-2.22
GPI	Glucose-6-phosphate isomerase	Glycolysis	1.18	N/A
PFKL	Phosphofructokinase, liver	Glycolysis	1.18	N/A
ACAD9	Acyl-CoA dehydrogenase 9	Oxidative phosphorylation	1.18	-1.30
CYBASC3	Cytochrome B561, member A3	Oxidative phosphorylation	1.18	1.11
ATP5G1	ATP synthase, F_0 subunit	Oxidative phosphorylation	1.15	1.66
ACLY	ATP citrate lyase	Lipogenesis	-1.31	-2.00
FASN	Fatty acid synthase	Lipogenesis	1.27	1.48
PDK2	Pyruvate dehydrogenase kinase 2	Lipogenesis	-1.37	-2.38
SLC25A42	Mitochondrial CoA transporter	Lipogenesis	-1.39	-2.27
ACSS2	Acyl-CoA synthetase short chain	Lipogenesis	-1.47	N/A
SCD	Stearoyl-CoA desaturase (delta9)	Lipogenesis	1.35	N/A
SLC27A2	Long chain fatty acid transporter	Lipogenesis	-2.21	N/A
ATP1B1	Na/K ATPase	Metabolic activity	1.75	1.50

Also, genes involved in lipogenesis were mostly downregulated, including ATP citrate lyase (FC: -2.00), fatty acid synthase (FC: 1.48), pyruvate dehydrogenase kinase 2 (FC: -2.38) and mitochondrial CoA transporter (FC: -2.27). Also, sodium/potassium ATPase was upregulated (FC: 1.50) by butyrate treatment.

4. DISCUSSION

4.1. Metabolic Stress and Barrier Integrity

Energy demands in epithelial tissues can be quite high and potentially very indicative of epithelial barrier integrity status. Infection by rotavirus of Caco-2 cells causes concurrent drops in transepithelial resistance and epithelial ATP concentrations (Dickman *et al*., 2000). In rumen epithelia, low mucosal pH and local ATP depletion are directly linked to increased epithelial permeability (Aschenbach *et al*., 2000). Thus, generating adequate ATP in the epithelium is vital for the maintenance of epithelial integrity. Increased sodium/potassium ATPase mRNA in the butyrate treatment highlights a genomic shift to increased ATP demand. In previous research (McBride and Kelly, 1990), increased sodium/potassium ATPase (ATP1B1) activity was directly associated with increased ATP demand in rumen epithelium. Together, these studies highlight the vital role of ATP in maintaining epithelial integrity.

Energy substrate availability and ATP generation capacity in epithelial tissues are vital for maintaining ATP levels. For example, impairment of glucose transport dynamics through the glucose transporter GLUT1, causes significant impairment of blood-brain barrier function by reducing the expression of occludin in brain microvessels (Muneer *et al*., 2011). In rat pulmonary epithelium, impaired mitochondrial complex I function leads to ATP-depletion and increased endothelial permeability in lung tissue (Bongard *et al*., 2013). In T84 cell lines, inhibition of ATP synthesis through 2,4-dinitrophenol, coupled with *E. coli* exposure, significantly reduces barrier function (Lewis and McKay, 2009). Since the bovine rumen typically has high *E. coli* content, decreased electron transport chain function would likely have an adverse effect on barrier function. In summary, energy substrates must be abundant and metabolizable to ATP for maintenance of barrier function. Impairing the barrier integrity appears to be a direct function of ATP depletion, highlighting the importance of ATP levels in maintaining barrier integrity.

4.2. Butyrate and Metabolic Stress

Stressed epithelia, such as those in ulcerative colitis and Crohn's disease, are positively impacted by butyrate (Leonel and Alvarez-Leite, 2012; Ploger *et al*., 2012), through several possible cellular mechanisms, such as HDAC inhibition (Davie, 2003). A recent study,

however, suggests butyrate acts as an energy substrate rather than an HDAC inhibitor (Donohoe *et al*., 2011). This may be due to butyrate's extensive metabolism in the epithelium, where 75-90% of absorbed butyrate is metabolized (Bergman, 1990). When mouse embryonic stem cells are exposed to sodium butyrate, however, glycolysis and glucose consumption rates increase (Sharma *et al*., 2006). While butyrate oxidation certainly provides energy to epithelial cells (Bergman, 1990), this energy is made available through its oxidation via the Kreb's Cycle, not through glycolysis. Although increased use of butyrate as an energy substrate cannot be ruled out by our results, the mRNA abundance increases in glycolytic genes in our study, show butyrate acts at the genome level.

Genomically, butyrate inhibits HDAC, resulting in lower deacetylation of lysine residues of histone proteins. Normally, deacytelation of histone proteins is an important and quick-acting buffer of intracellular pH because the released acetate drives proton export through monocarboxylate co-transporters (McBrian *et al*., 2013). With inhibition of HDAC, this source of acetate is unavailable and intracellular pH can drop accordingly. Changes in intracellular pH have pleiotropic consequences, from changes in ion transport, to changes in cell proliferation, to apoptosis (Casey *et al*., 2010), highlighting the importance of HDAC activity.

In glioma cells, HDAC inhibition by butyrate enhances apoptosis, but only when glycolysis is also blocked (Egler *et al*., 2008). Glycolysis, therefore, appears to be a crucial compensatory mechanism for HDAC inhibition. In mice, butyrate decreased HDAC activity and caused a concurrent increase in systemic energy expenditure (Gao *et al*., 2009). With increased systemic energy expenditures comes an increased energy demand, which could be filled by ATP-generating processes like glycolysis. In our study, butyrate increased relative mRNA abundance of hexokinase 2, pyruvate kinase 2, lactate dehydrogenases A and B and decreased mRNA expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4, a phosphofructokinase inhibitor. Together, this suggests butyrate increases mRNA abundance of glycolysis genes, which may lead to a shift towards greater glycolytic activity in the rumen epithelium.

Other than glycolysis, oxidative phosphorylation is another cellular ATP-generator and a far more effective one. Oxidative phosphorylation is increased by butyrate in energy-depleted germ-free mouse colon cells (Donohoe *et al*., 2011), further supporting the concept of butyrate increasing energy supply in epithelial cells. In our study, butyrate increased mRNA expression of acyl-CoA dehydrogenase 9, cytochrome B and ATP synthase, involved in complexes 1, 3 and 5 of the electron transport chain, respectively. The increased mRNA expression shows butyrate induces a genomic shift towards increased oxidative phosphorylation in rumen epithelium.

Coupled with decreased gene expression of lipogenesis genes such as ATP citrate lyase and mitochondrial CoA transporter, our study shows butyrate genomically shifts epithelial metabolism towards energy mobilization in rumen epithelia in SARA cows, in agreement with our hypothesis. Previous studies have highlighted the role of epithelial energy mobilization in preserving barrier integrity and our results show butyrate induces this mobilization, thus may be involved in maintenance of barrier integrity at the genome level during SARA.

5. CONCLUSION

In this study, we found exogenous butyrate modulates epithelial expression of genes involved in glycolysis, oxidative phosphorylation and lipogenesis. Genomically, glycolysis and oxidative phosphorylation appear to be upregulated while lipogenesis is largely downregulated. Overall, the changes in gene expression found in this study indicate butyrate induces a genomic shift towards energy production. As energy production is vital for maintaining proper barrier function, butyrate may be beneficial for barrier function in cows experiencing SARA.

While this study found genomic shifts, the corresponding protein abundance and activity was not studied. In the future, a comprehensive study evaluating mRNA abundance, protein abundance and protein activity would add greatly to our understanding of butyrate's mode of action in the rumen. Further, as this study lasted for 7 days only, we could only focus on the short-term effects of butyrate on gene expression. While short-term effects are certainly useful in studying adaptation to high-grain diets, the long-term effects were outside of this study's scope and could be an appropriate focus of future studies.

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