Effects of a proinflammatory response on metabolic function of cultured, primary ruminal epithelial cells

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ABSTRACT

Inflammation of ruminal epithelium may occur during ruminal acidosis as a result of translocation and interaction of ruminal epithelial cells (REC) with molecules such as lipopolysaccharide (LPS). Such inflammation has been reported to alter cellular processes such as nutrient absorption, metabolic regulation, and energy substrate utilization in other cell types but has not been investigated for REC. The objectives of this study were to investigate the effects of LPS on metabolism of short-chain fatty acids by primary REC, as well as investigating the effects of media containing short-chain fatty acids on the proinflammatory response. Ruminal papillae from 9 yearling Speckle Park beef heifers were used to isolate and culture primary REC. Cells were grown in minimum essential medium (MEM) for 12 d before use and then reseeded in 24-well culture plates. The study was conducted as a 2 × 2 factorial, where cells were grown in unaltered MEM (REG) or medium containing 2 mM butyrate and 5 mM propionate (SCFA) with (50,000 EU/mL; +LPS) or without LPS (–LPS) for 24 h. Supernatant samples were collected for analysis of glucose and SCFA consumption. Cells were collected to determine the expression of mRNA for genes associated with inflammation (*TNF*, *IL1B*, *CXCL2*, *CXCL8*, *PTGS2*, and *TLR4*), purinergic signaling (*P2RX7*, *ADORAB2*, and *CD73*), nutrient transport [*SLC16A1* (*MCT1*), *SLC16A3* (*MCT4*), *SLC5A8*, and *MCU*], and cell metabolism [*ACAT1*, *SLC2A1* (*GLUT1*), *IGFBP3*, and *IGFBP5*]. Protein expression of TLR4 and ketogenic enzymes (BDH1 and HMGCS1) were also analyzed using flow cytometry. Statistical analysis was conducted with the MIXED model of SAS version 9.4 (SAS Institute Inc., Cary, NC) with medium, LPS exposure, and medium × LPS interaction as fixed effects and animal within plate as a random effect. Cells tended to consume more glucose

when exposed to LPS as opposed to no LPS exposure (31.8 vs. 28.7 ± 2.7), but consumption of propionate and butyrate was not influenced by LPS. Expression of *TNF* and *IL1B* was upregulated when exposed to LPS, and expression of *CXCL2* and *CXCL8* increased following LPS exposure with SCFA (medium × LPS). For cells exposed to LPS, we found a downregulation of *ACAT1* and *IGFBP5* and an upregulation of *SLC2A1*, *SLC16A3*, *MCU*, and *IGFBP3*. Medium with SCFA led to greater expression of *MCU*. *SLC16A1* was upregulated in cells incubated with SCFA and without LPS compared with the other groups. Protein expression of ketogenic enzymes was not affected; however, BDH1 mean fluorescence intensity (MFI) expression tended to be less in cells exposed to LPS. These data are interpreted to indicate that when REC are exposed to LPS, they may increase glucose metabolism. Moreover, transport of solutes was affected by SCFA in the medium and by exposure to LPS. Overall, the results suggest that metabolic function of REC in vitro is altered by a proinflammatory response, which may lead to a greater glucose requirement.

Key words: cell culture, inflammation, metabolism

INTRODUCTION

The rumen is uniquely designed for efficient uptake and metabolism of nutrients, especially for short-chain fatty acids. These functions are facilitated by the ruminal epithelium, which comprises stratified squamous epithelial cells, organized into the stratum corneum, granulosum, spinosum, and basale (Graham and Simmons, 2005). The highly specialized cells of the spinosum and basale strata are responsible for transcellular transport and metabolism of large quantities of short-chain fatty acids (Stumpff, 2018), and short-chain fatty acids serve as a primary substrate for whole-body energy supply but are also important energy substrates for the cells (Britton and Krehbiel, 1993; Sehested et al., 1999).

Ruminal acidosis, a potential consequence of feeding highly fermentable diets, leads to an increase in the concentration of microbe-associated molecular patterns

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(MAMP) in ruminal fluid, of which LPS has been the most widely studied (Plaizier et al., 2012). Previous work has provided evidence that acidotic conditions can lead to translocation of bacteria or MAMP across the ruminal epithelium. These conditions are thought to play a causative role in the induction of liver abscesses, laminitis, and systemic inflammation, and may negatively affect production, whether milk yield (Khafipour et al., 2009; Plaizier et al., 2012) or growth and feed conversion (Castillo-Lopez et al., 2014). In addition, ruminal MAMP may be capable of interacting with ruminal epithelial cells (REC) and initiating a local inflammatory response (Plaizier et al., 2018; Kent-Dennis et al., 2019).

At a whole-body level, inflammation markedly increases glucose (Kvidera et al., 2017) and amino acid cost (McNeil et al., 2016) in ruminants. Although the liver is a central organ that is involved, localized proinflammatory responses have been shown to alter nutrient transport and metabolic function in intestinal mucosa (Peuhkuri et al., 2010; Glover and Colgan, 2011). Inflammation in the intestinal epithelium not only results in increased permeability but also impairs proton-coupled nutrient transport (Thibault et al., 2007). Moreover, in experimental colitis models, butyrate oxidation by colonocytes was decreased and secretion of ketone bodies reduced (Ahmad et al., 2000). Pathologies of the intestinal mucosa, such as inflammatory bowel diseases, have also been reported to increase demands for oxygen and energy substrates, especially glucose, in inflamed mucosa (Glover and Colgan, 2011). Metabolic function of keratinocytes, which bear many similarities to REC, may also be altered by inflammation of the skin (Zhang et al., 2018). The increased energy costs during inflammation have been attributed to an increase in cell proliferation, especially for leukocytes, as well as to increased production of immune-modulatory molecules such as cytokines, which have additional regulatory effects on metabolism (Richardson and Davidson, 2003).

Acidotic conditions in the rumen driving a local inflammatory response in the ruminal epithelium have been speculated to alter metabolic function of the REC (Steele et al., 2011; Zhao et al., 2017). However, little direct evidence exists of the effects of inflammation on REC metabolism. Because substantial metabolism of energy substrates occurs in the REC (Britton and Krehbiel, 1993; Penner et al., 2011), it could be speculated that initiation of a proinflammatory response to ruminally-derived MAMP may alter nutrient transport and cell metabolic function. In addition, short-chain fatty acids, especially butyrate, are known to have protective or anti-inflammatory functions in intestinal epithelial cells when exposed to LPS or during active inflammation (Corrêa-Oliveira et al., 2016; Parada et

al., 2019). These functions have not been well characterized in the ruminal epithelium (Zhan et al., 2019). In this experiment, we hypothesized that primary REC would increase utilization of energy substrates when exposed to LPS. In addition, adding butyrate and propionate to the culture medium would have a protective effect, resulting in a suppressed proinflammatory response. Therefore, the aim of this study was to investigate the effects of LPS on nutrient transport and metabolic function of primary REC in culture, as well as to understand how SCFA affect the proinflammatory response, by evaluating energy substrate consumption and by analyzing the expression of genes and proteins involved in inflammation and metabolism.

MATERIALS AND METHODS

This study was preapproved by the University of Saskatchewan (Saskatoon, SK, Canada) Research Ethics Board (protocol no. 20100021) and was conducted in accordance with guidelines of the Canadian Council of Animal Care (Ottawa, ON, Canada).

Rumen Epithelial Cell Isolation and Cultivation

Yearling beef heifers ($n = 9$) were housed in individual pens for 28 d and fed a diet of 40.00% barley silage, 10.00% grass hay, 33.22% dry-rolled barley, 16.44% vitamin and mineral pellet, and 0.34% limestone on a DM basis. Heifers were allowed ad libitum water consumption. On d 28, heifers were euthanized by captive bolt, followed by exsanguination and pithing. Heifers were staggered such that only 1 animal per day was euthanized for sample collection. Ruminal epithelial tissue was excised from the ventral sac and was subsequently washed and transported to the laboratory in ice-cold Ca^{2+} - and Mg^{2+} -free Dulbecco's phosphate-buffered saline (DPBS; Sigma-Aldrich, St. Louis, MO) containing antibiotic-antimycotic (**anti-anti**; final concentration: 400 U/mL penicillin, 400 $\mu\text{g/mL}$ streptomycin, and 1 $\mu\text{g/mL}$ amphotericin B; Thermo Fisher Scientific, Waltham, MA) and nystatin (240 U/mL; Sigma-Aldrich). Ruminal papillae were cut off at the base, washed in DPBS solution containing anti-anti (final concentration: 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 0.25 $\mu\text{g/mL}$ amphotericin B; Thermo Fisher), and approximately 30 g (initial wet weight) of tissue was subjected to serial trypsinization with 50 mL of a trypsin-EDTA solution (0.25% trypsin and 0.02% EDTA; Sigma-Aldrich) at 37°C. Fresh trypsin-EDTA solution was replaced every 30 min. The first 2 fractions of supernatant contained highly keratinized cells and were discarded. The subsequent 4 fractions were each strained through sterile gauze and

centrifuged for 5 min at $200 \times g$ at room temperature. The resulting cell pellet was washed and centrifuged 3 times with sterile DPBS containing Ca^{2+} and Mg^{2+} . Pellets from the 4 fractions were pooled, resuspended, and seeded into 60-mm cell culture plates that were precoated with bovine collagen I (Thermo Fisher). Cells were grown in M199 cell culture medium (M2154; Sigma-Aldrich) with the addition of fetal bovine serum (15% vol/vol; Thermo Fisher), L-glutamine (1.36 mM; Sigma-Aldrich), HEPES (20 mM; Sigma-Aldrich), anti-anti (final concentration: 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 0.25 $\mu\text{g}/\text{mL}$ amphotericin B; Thermo Fisher), nystatin (240 U/mL; Sigma-Aldrich), gentamycin (50 mg/L; Sigma-Aldrich), and kanamycin (100 mg/L; Thermo Fisher) for 2 d, and then the medium was switched to minimum essential medium (MEM) with stable L-glutamine (M4655; Sigma-Aldrich) with the addition of fetal bovine serum (10% vol/vol; Thermo Fisher), HEPES (20 mM; Sigma), and anti-anti (final concentration: 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 0.25 $\mu\text{g}/\text{mL}$ amphotericin B; Thermo Fisher). Fresh medium was replaced every 2 to 3 d, and cells were cultured for 10 to 12 d until 80 to 90% confluency was attained. An anti-CD90 antibody (final concentration: 0.02 mg/mL; Novus Biologicals, Centennial, CO) was used to determine fibroblast contamination (Kent-Dennis et al., 2020). Cells that were <10% positive for CD90 were considered acceptable for experimentation (Kisselbach et al., 2009). Cells from each animal were then reseeded into two 24-well culture plates (Thermo Fisher) at a rate of 10×10^4 cells/mL and used for the experiment.

Experimental Procedure

The day of reseeded was considered d 0 of the experiment (Figure 1). The total experiment for each

batch of REC was conducted over a duration of 5 d. Cells were reseeded in unaltered MEM (**REG**). On day 2, fresh medium was added to the wells with 1 plate per animal receiving REG and the other plate receiving MEM with Na-butyrate and Na-propionate (2 mM and 5 mM final concentrations, respectively; **SCFA**). The concentration of butyrate selected was expected to be sufficient to influence cell functions without causing cytotoxic effects (Gálfi et al., 1991), and the propionate concentration was selected to maintain the butyrate-to-propionate ratio based on Klotz et al. (2001). On d 4, fresh REG and SCFA were added to the respective wells. Subsamples of each medium source, before addition to the cells, were collected and frozen at -20°C . On d 4, cells were exposed to the treatments, which were arranged in a 2×2 factorial, with a total of 4 wells per treatment. Cells were incubated in REG or SCFA and with or without LPS (50,000 EU/mL; *Escherichia coli* 055:B5, Sigma-Aldrich), resulting in the following treatment groups: REG without LPS (control; **REG-LPS**); REG with LPS added (**REG+LPS**); SCFA without LPS (**SCFA-LPS**); and SCFA with LPS added (**SCFA+LPS**). The experiment ended 24 h later, on d 5. Supernatant from each well was collected and frozen at -20°C . Cells from 2 wells per treatment were collected by trypsinization for immediate analysis of TLR4, BDH1, and HMGCS1 staining by flow cytometry. The cell lysate from 2 wells per treatment was also collected and pooled in 1 mL of Trizol (Thermo Fisher). Samples were frozen at -80°C for later RNA extraction.

Analysis of Supernatants

Glucose Consumption. Glucose concentrations in the media and supernatants were quantified using an enzymatic assay modified from that used to assess

Figure 1. Timeline of treatments and sample collections of cultured ruminal epithelial cells. Cells were grown with or without short-chain fatty acids (SCFA; 2 mM butyrate and 5 mM propionate) added to the medium starting on d 2 (D2) following reseeded. Cells were incubated with LPS for 24 h starting on d 4.

glucose concentration in plasma. The reaction utilized a peroxidase-glucose oxidase enzyme solution (Sigma-Aldrich) and o-dianisidine (Sigma-Aldrich) as the substrate. Absorbance was determined using a plate reader (Epoch2; BioTek Instruments, Winooski, VT) at a wavelength of 450 nm. Glucose consumption (disappearance) was calculated based on the differences in concentration between the fresh medium and supernatant and the volume of the medium and supernatant.

Butyrate and Propionate Consumption. Concentrations of butyrate and propionate in the media and supernatants were determined as described by Khorasani et al. (1996) using gas chromatography (Agilent Technologies, Santa Clara, CA) equipped with a Zebron capillary column (ZB-FFAP; 30-m $\times$ 0.32-mm inner diameter $\times$ 0.25- μ m film thickness; Phenomenex, Torrance, CA) with minor modifications. For detection of SCFA in cell culture media, a 1:5 split ratio was used with a 1- μ L injection volume. The injector temperature was 225°C. The oven temperature was initially ramped up to 90°C and then increased to 170°C at a rate of 10°C/min, where the temperature was held for 2 min. The detector temperature was held constant at 250°C. Isocaproic acid was used as the internal standard. Consumption (disappearance) of butyrate and propionate by the cells was determined by calculating the difference between concentrations in the medium and the supernatant and corrected to account for well volume and time, as described for glucose.

Cell Staining and Flow Cytometry

Expression of TLR4, BDH1, and HMGCS1, as well as the percent of dead and apoptotic cells, were analyzed using flow cytometry. The staining and analysis were performed as described by Käser et al. (2016). Single-marker staining was conducted using the antibodies detailed in Table 1. Following trypsinization, cells were resuspended in 5 mL of medium to neutralize the trypsin and centrifuged at $200 \times g$ for 5 min at room

temperature, and then the supernatant was decanted. Cells were incubated in 96-well round-bottom plates (Thermo Fisher) for 1 h at 4°C in fixation/permeabilization reagent (3:1 ratio of diluent to concentrate) from the FoxP3/Transcription Factor Staining Buffer Kit (eBioscience, San Diego, CA). Plates were then centrifuged at $500 \times g$ at room temperature, and the supernatant was removed. Cells were blocked with fetal bovine serum (neat) for 15 min at room temperature. Plates were centrifuged again, the supernatant was removed, and cells were incubated with 10 μ L of primary antibody for 10 min at room temperature. Plates were washed twice with 200 μ L of permeabilization buffer provided with the kit (diluted 1:10 with water) and centrifuged, and the supernatant was removed. Cells were then incubated with 10 μ L of secondary antibody for 10 min without light exposure at room temperature. Plates were washed twice, using the same method described above. After the last wash, the cells were resuspended in 200 μ L permeabilization buffer, transferred to a 2-mL microcentrifuge tube (Thermo Fisher), and analyzed using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ) based on a minimum of 5,000 events. The absence of nonspecific binding was evaluated using isotype controls. Data analysis of percent positive and mean fluorescence intensity (**MFI**) were performed using FlowJo Software (BD Biosciences).

A subset of samples ($n = 3$) were used to monitor the effect of the treatments on apoptosis and cell death by staining cells with annexin and propidium iodide markers, respectively (Annexin V-FITC Apoptosis Kit Plus, Biovision Inc., Milpitas, CA). Following trypsinization, cells were resuspended in 100 μ L of binding buffer, provided with the kit, and 1 μ L each of annexin and propidium iodide was added to the cell suspension. Cells were incubated with the markers for 10 min in the dark at room temperature and then analyzed with the flow cytometer. Each marker was incubated and analyzed in separate tubes.

Table 1. Description of antibodies used to evaluate inflammatory and metabolic effects of LPS exposure on cultured ruminal epithelial cells

Antibody	Description	Source ¹	Function	Final concentration
Primary				
Mouse anti-BDH1	IgG1 (1A5)	Thermo	Ketogenic enzyme	0.1 mg/mL
Mouse anti-CD90	IgG1 κ (AF-9)	Novus	Fibroblast marker	0.02 mg/mL
Rabbit anti-HMGCS1	Polyclonal	Thermo	Ketogenic enzyme	0.04 mg/mL
Rabbit anti-TLR4	Polyclonal	Thermo	Pattern-recognition receptor	0.1 mg/mL
Secondary				
Goat anti-mouse IgG	Alexa Fluor 488, preadsorbed	Abcam		4 μ g/mL
Goat anti-rabbit IgG	Alexa Fluor 488, H&L	Abcam		4 μ g/mL

¹Thermo Fisher Scientific, Waltham, MA; Novus Biologicals, Centennial, CO; Abcam, Cambridge, UK.

RNA Extraction and Real-Time Quantitative PCR

Samples of cell lysate, in Trizol, were thawed, and a phenol-chloroform RNA extraction (Thermo Fisher) was used with 2 precipitation steps using isopropanol following phase separation. Linear acrylamide (Thermo Fisher) was used as a co-precipitant (final concentration of 15 $\mu\text{g/mL}$) to visualize the pellet. The concentration of RNA was determined using a Nanodrop (ND2000c; Thermo Fisher). A 1.2% denaturing agarose gel was used to determine RNA integrity. The presence of distinct 18s and 28s bands in all samples was confirmed before RNA was used. Subsequently, 2 μg of RNA was converted to cDNA using the High-Capacity Reverse Transcription Kit (Thermo Fisher). The cDNA was diluted with nuclease-free water to a final concentration of 10 ng/ μL .

Primer pairs for reference and target genes were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 2. Primers were designed to span exon-exon junctions, where possible, and were assessed for efficiency [calculated as $-1 + 10(-1/\text{slope}) \times 100$] using a serial dilution of pooled cDNA. Melt curves for primers were assessed for dimer formation to verify the presence of a single product.

Quantitative real-time PCR was performed using 20 ng of cDNA, run in duplicate, using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) on a CFX96 Real-Time PCR Detection System (Bio-Rad). The Ct values for the target genes were normalized to the geometric mean of the 3 reference genes (*ACTB*, *GAPDH*, and *STX5*), and statistical analysis was performed on ΔCt . Data are presented as fold changes, with treatments held relative to the mean value of REG–LPS.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC) with LPS treatment, medium, and treatment $\times$ medium interaction as fixed effects. Animal within plate was considered random. Data were analyzed using mean separations. Differences were considered significant at $P < 0.05$.

RESULTS

Glucose and SCFA Concentrations in Cell Supernatants

The percent of glucose (Table 3) consumed by REC incubated with SCFA was 13.9%, compared with 46.6% when incubated with REG (SEM = 3.7; $P < 0.001$). Cells exposed to LPS tended to consume more glucose than those in medium without LPS (31.8 vs. 28.7 ± 2.7 ;

$P = 0.072$), but the amount of butyrate and propionate consumed was not affected by LPS exposure. Collectively, cells incubated with SCFA consumed 0.42 mmol (21.2%) of the butyrate and 0.38 mmol (7.6%) of the propionate (Table 3).

Cell Staining

The MFI for TLR4, BDH1, and HMGCS1 (Table 4) were not affected by medium or LPS treatment, and no interactions were detected. However, we detected a tendency for MFI of BDH1 to be less in cells exposed to LPS ($P = 0.076$). Compared with REG, exposure to SCFA resulted in a reduced number of cells positive for TLR4 (94.8 vs. $87.0 \pm 2.5\%$; $P = 0.046$) and BDH1 (83.4 vs. $66.4 \pm 4.6\%$; $P = 0.020$), but HMGCS1 was not affected. Cell death (propidium iodide) and apoptosis (annexin) were not affected by SCFA or LPS exposure (Table 4).

Gene Expression

Expression of *TNF* (Figure 2A) and *IL1B* (Figure 2B) was greater ($P < 0.001$) following LPS exposure. Addition of SCFA to the medium also resulted in greater expression of *TNF* and *IL1B* compared with REG ($P < 0.001$). Compared with REG–LPS, REG+LPS and SCFA–LPS increased expression of both *CXCL2* (Figure 2C) and *CXCL8* (Figure 2D), and greatest expression of *CXCL2* and *CXCL8* occurred in cells exposed to LPS with SCFA in the medium (LPS $\times$ medium, $P < 0.001$). A tendency for the same response also occurred for *TNF* (LPS $\times$ medium, $P = 0.068$). Exposure to LPS resulted in increased expression of *PTGS2* (Figure 3A; $P < 0.001$); however, *PTGS2* expression was not influenced by SCFA. Expression of *TLR4* (Figure 3B) also increased with LPS exposure ($P < 0.001$); however, SCFA added to the medium downregulated *TLR4* ($P = 0.008$). Neither LPS nor SCFA influenced *TGFB1* (Figure 3C).

A significant treatment $\times$ medium interaction occurred, such that expression of *P2RX7* (Figure 4A) was less for cells exposed to LPS in REG ($P = 0.006$) compared with the other treatments. Exposure to LPS increased expression of *ADORA2B* (Figure 4B; $P < 0.001$) and decreased expression of *CD73* (Figure 4C; $P = 0.002$).

Although *HIF1A* (Figure 5A) was not affected by LPS or medium, SCFA–LPS had decreased expression of *GLUT1* compared with REG+LPS and SCFA+LPS (Figure 5B; treatment $\times$ medium $P = 0.010$). A treatment $\times$ medium interaction resulted in an upregulation of *MCT1* (Figure 6A; treatment $\times$ medium $P = 0.001$) when SCFA was added to the medium without

Table 2. Descriptions of primers used to evaluate inflammatory and metabolic effects of LPS exposure on cultured ruminal epithelial cells

Gene name (official symbol)	Source ¹	Sequence (5'-3') ²	Amplicon size	Efficiency ³
Reference genes				
β Actin (<i>ACTB</i>)	NM_173979.3	F: GAGCTACGAGCTTCTGACGGGC R: AATGCCGAGGATTCCATGCCAG	109	96
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	NM_001034034.2	F: GGTCATCATCTCTGCACCT R: GGAGGCATTGCTGACAATCT	101	91
Syntaxin 5 (<i>STX5</i>)	NM_001075444.1	F: CCATTCAGAGGATCGACGAG R: GGATGTGACCGACTGGAAGT	95	103
Inflammation-related genes				
C-X-C motif chemokine ligand 2 (<i>CXCL2</i>)	NM_174299.3	F: CCGAAGTCATAGCCACTCTCA R: CTCTCTGTTTCCACCTGGTCA	128	99
C-X-C motif chemokine ligand 8 (<i>CXCL8</i>)	NM_173925.2	F: AGAGCTGAGAAAGCAAGATCCA R: ACCCTACACACAGACCCAC	150	104
Interleukin 1 β (<i>IL1B</i>)	NM_174093.1	F: CTGAGGAGCATCCTTTTCATTTC R: GTCTGGAGTTTGCACTTTAT	114	97
Transforming growth factor 1 β (<i>TGFB1</i>)	NM_001166068.1	F: ACTGCTTCAGCTCCACAGAA R: TCCAGGCTCCAGATGTAAGG	148	105
Toll-like receptor 4 (<i>TLR4</i>)	NM_174198.6	F: CCTTGCGTACAGGTTGTTC R: CTCAGGTCCAGCATCTTGGT	104	90
Tumor necrosis factor α (<i>TNF</i>)	NM_173966.3	F: CAAGTAACAAGCCGGTAGCC R: AGATGAGGTAAAGCCCGTCA	153	99
Metabolism-related genes				
Acetyl-CoA acetyltransferase 1 (<i>ACAT1</i>)	NM_001046075.1	F: CGGTGTCAGGAAATAAGGTA R: TAGTGGCTGGCAGAGAGGA	133	103
Adenosine A2b receptor (<i>ADORA2B</i>)	NM_001075925.1	F: CGTCCCGCTCAGGTATAAGA R: TGGCAATTTTCTGTCTGTTTC	128	103
3-Hydroxybutyrate dehydrogenase 1 (<i>BDH1</i>)	NM_001034600.2	F: GACCTGAGAAAGGCTTGTTG R: TCCTTTGAGTCTCCATGCTG	92	96
Hypoxia-inducible factor 1α (<i>HIF1A</i>)	NM_174339.3	F: GCCACTCCCCATAATGTAA R: ATCCAAATCACAGCATCCA	106	98
Insulin-like growth factor binding protein 3 (<i>IGFBP3</i>)	NM_174556.1	F: GCTGAACCACTCAAGTTCC R: CGGCACTGCTTTTCTTTGTA	96	99
Insulin-like growth factor binding protein 5 (<i>IGFBP5</i>)	NM_001105327.2	F: CAAGAGAAAGCAGTGCAAAAC R: AGTCCCCGTCCACGTACTC	107	98
Mitochondrial calcium uniporter (<i>MCU</i>)	NM_001206102.1	F: CACACAGTTTGGCATTTGG R: AACATATCTGGCGTGTCA	139	97
5'-Nucleotidase ecto, aka CD73 (<i>NT5E</i>)	NM_174129.4	F: GGAACAATGGCAGATTACC R: CTTTCAGGGTGGAGCCTTTTA	95	98
Purinergic receptor P2X 7 (<i>P2RX7</i>)	NM_001206516.1	F: CTTCAGAGGAAACAGAGCGTCT R: GGCATCAACGCTGTTTCTA	108	108
Solute carrier family 2 member 1, aka GLUT1 (<i>SLC2A1</i>)	NM_174602.2	F: ACACGACAGTGAAGGCTGTG R: TGCTTAGCAGCAAGTAACA	115	105
Solute carrier family 5 member 8 (<i>SLC5A8</i>)	NM_001164861.1	F: CAGCACACTCATCTCTTGTA R: TCATCGGCTTCTCTATGC	100	98
Solute carrier family 16 member 1, aka MCT1 (<i>SLC16A1</i>)	NM_001037319.1	F: GCTGATAGGACCTCCACCAT R: TGTGGTGAGCTATGCCAAGG	144	95
Solute carrier family 16 member 3, aka MCT4 (<i>SLC16A3</i>)	NM_001109980.3	R: TCAGCGAAGCCGTTGAAGA	183	98

¹National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>).²F = forward; R = reverse.³Efficiency = $-1 + 10^{(-1/\text{slope})} \times 100$.

Table 3. Percent consumption¹ of energy substrates in primary ruminal epithelial cells exposed to 0 (–LPS) or 50,000 EU/mL LPS (+LPS), with or without the addition of SCFA to the culture medium

Item	Medium ²		SEM ³	Treatment			P-value		
	REG	SCFA		–LPS	+LPS	SEM	Medium	Treatment	Medium × Treatment
Glucose, %	46.6	13.9	3.7	28.7	31.8	2.7	<0.001	0.072	0.80
Butyrate, %	ND ⁴	21.2	—	23.8	18.5	2.5	—	0.12	—
Propionate, %	ND	7.6	—	4.0	11.1	3.2	—	0.11	—

¹Calculated by subtracting the amount measured in the supernatants from the amount measured in medium subsamples before use.

²REG = regular (unaltered) culture medium; SCFA = medium supplemented with 2 mM butyrate and 5 mM propionate.

³SEM = standard error of the mean of each factor.

⁴ND = not detected.

LPS exposure. Expression of *MCT4* (Figure 6B) and *SLC5A8* (Figure 6C) were greater ($P = 0.031$ and $P = 0.004$, respectively) following LPS exposure compared with cells incubated without LPS. Exposure to LPS did not affect *MCU* expression (Figure 6D); however, the addition of SCFA increased expression ($P = 0.002$).

Downregulation of *ACAT1* (Figure 7A; $P < 0.001$) and *BDH1* (Figure 7B; $P = 0.001$) occurred following exposure to LPS. However, *BDH1* increased in expression ($P = 0.046$) when SCFA were added to the medium. This also tended to be the case for *ACAT1* ($P = 0.072$). Both LPS and SCFA increased expression of *IGFBP3* (Figure 7C; $P = 0.001$ and $P < 0.001$, respectively), and exposure to LPS resulted in a downregulation of *IGFBP5* (Figure 7D; $P < 0.001$). For *IGFBP5* tendency for a treatment × medium interaction ($P = 0.050$) was detectable.

DISCUSSION

A proinflammatory response at a mucosal surface is initiated, whether by immune cells or nonhemopoietic cell types, by the binding of MAMP to pattern-recognition receptors, activating a signaling cascade that leads to transcription of cytokines, chemokines, and other immune-modulatory molecules (Pott and Horneff, 2012). Recent work has suggested that REC are capable of initiating a proinflammatory response (Zhang et al., 2016; Kent-Dennis et al., 2020). In addition to epithelial cells, large numbers of immune cells, such as neutrophils, macrophages, and lymphocytes, are recruited to the site of the insult and further promote inflammation to eliminate the threat, repair cellular damage, and return the tissue to a homeostatic state (Peterson and Artis, 2014). As a consequence, meta-

Table 4. Percent positive and mean fluorescence intensity for markers in primary ruminal epithelial cells exposed to 0 (–LPS) or 50,000 EU/mL LPS (+LPS)

Item	Medium ¹		SEM ²	Treatment			P-value		
	REG	SCFA		–LPS	+LPS	SEM	Medium	Treatment	Medium × Treatment
TLR4									
% Positive	94.8	87.0	2.5	90.8	91.0	2.7	0.046	0.82	0.60
MFI ³	87,794	94,273	12,953	89,856	92,211	9,299.2	0.73	0.47	0.51
BDH1									
% Positive	83.4	66.4	4.6	76.4	73.4	3.6	0.020	0.31	0.60
MFI	49,199	46,997	5,545.5	50,109	46,086	4,061.6	0.78	0.076	0.49
HMGCS1									
% Positive	66.0	47.9	7.4	56.4	57.5	5.5	0.101	0.72	0.88
MFI	33,872	34,002	5,331.6	32,744	35,129	3,961.0	0.99	0.33	0.62
Annexin ⁴									
% Positive	9.5	10.8	4.7	10.1	10.2	3.4	0.85	0.93	0.73
Propidium iodide ⁵									
% Positive	8.0	7.3	3.8	7.4	7.9	2.7	0.91	0.38	0.54

¹REG = regular (unaltered) medium, SCFA = medium with 2 mM butyrate and 5 mM propionate added.

²SEM = standard error of the mean of each factor.

³Mean fluorescence intensity, arbitrary unit.

⁴Marker indicative of apoptotic cells.

⁵Marker indicative of dead cells.

Figure 2. Gene expression of proinflammatory cytokines, TNF (A) and IL1B (B), and chemokines, CXCL2 (C) and CXCL8 (D), in cultured ruminal epithelial cells exposed to either 0 or 50,000 EU/mL LPS with (SCFA) or without (REG) the addition of short-chain fatty acids (2 mM butyrate and 5 mM propionate) in the culture medium. The ΔC_t was used for statistical analysis ($P < 0.05$), and results are presented as fold change (log), with each individual plotted. The horizontal bar represents the mean. Means with differing letters indicate significant differences ($P < 0.05$).

bolic function of the tissue may be altered; however, effects of a local inflammatory response on ruminal epithelial metabolism have not been explored.

Fiber digestion in the gastrointestinal tract results in the production of short-chain fatty acids by the resident microflora. These end products of fermentation, especially butyrate, have been extensively studied, as they are important energy substrates and may also help to maintain epithelial barrier function, modify cellular processes such as proliferation, and mediate the inflammatory response in the mucosal tissue (Corrêa-Oliveira et al., 2016). In particular, the potential anti-inflammatory effects of short-chain fatty acids on intestinal epithelial cells have been of interest as a possible therapy

for pathologies such as ulcerative colitis and Crohn's disease (Parada et al., 2019). However, the effects of short-chain fatty acids have been inconsistent, with both pro- and anti-inflammatory responses reported (Li et al., 2018). Addition of short-chain fatty acids to culture medium of colon epithelial cells suppressed the production of proinflammatory cytokines (Hung and Suzuki, 2018). Conversely, short-chain fatty acids have been shown to enhance expression of inflammation-induced TLR (Lin et al., 2015; Xiao et al., 2018), increase levels of proinflammatory cytokines (Vancamelbeke et al., 2019), and possibly stimulate recruitment of first-responder cells, such as neutrophils (Corrêa-Oliveira et al., 2016). In the present study, exposure to LPS

Figure 3. Gene expression of a lipid mediator of inflammation, PTGS2 (A), toll-like receptor, TLR4 (B), and growth factor-like cytokine, TGFB1 (C), in cultured ruminal epithelial cells exposed to either 0 or 50,000 EU/mL LPS with (SCFA) or without (REG) the addition of short-chain fatty acids (2 mM butyrate and 5 mM propionate) in the culture medium. The ΔC_t was used for statistical analysis ($P < 0.05$), and results are presented as fold change (log), with each individual plotted. The horizontal bar represents the mean.

and addition of SCFA to the medium independently resulted in increased expression of *TLR4*, *TNF*, and *IL1B*. Moreover, expression of *CXCL2* and *CXCL8*, chemokines that recruit neutrophils during acute inflammation, were enhanced by the combination of LPS and SCFA. Although the reasons for the apparently paradoxical effects of short-chain fatty acids have not been elucidated, the effects observed in the present study suggest that VFA can modulate the inflammatory response to LPS in REC and may have further effects on cellular processes or metabolism. In agreement, Zhan et al. (2019) demonstrated an enhanced immune response in the presence of short-chain fatty acids in cultured REC, providing further evidence that short-chain fatty acids play a regulatory role in the inflammatory response in the ruminal epithelium.

Chemotaxis of neutrophils is intimately linked to purinergic signaling, a process that causes the release of ATP into the extracellular environment as a consequence of cellular stress, injury, or infection (Ferrari et

al., 2016). The free ATP acts as a signaling molecule to fine tune immune cell responses and prevent excessive tissue damage caused by inflammation (Ferrari et al., 2016). Two main types of receptors, P2 and P1, mediate the signaling pathway. The P2 receptors, which are divided into the P2XR and P2YR groups, bind extracellular ATP, inducing a proinflammatory response and leading to secretion of chemokines and recruitment of immune cells (Pastore et al., 2007; Idzko et al., 2014). The ATP is then converted to AMP, facilitated by CD39. Adenosine is produced from the AMP, by the action of CD73, and binds to one of the 4 subtypes (A_1 , A_{2A} , A_{2B} , or A_3) of the P1 receptor group (Antonioli et al., 2013). In the current study, gene expression patterns of CD73, and both a P2 (*P2RX7*) and P1 (*ADORA2B*) receptor were evaluated. Following exposure to LPS, without SCFA added to the medium, REC expression of *P2RX7* was downregulated. Expression of *CD73* and *ADORA2B* (a P1 receptor), however, were upregulated in response to LPS exposure. Production of adenosine

Figure 4. Gene expression of purinergic receptors, P2RX7 (A) and ADORA2B (B), and surface enzyme, CD73 (C) in cultured ruminal epithelial cells exposed to either 0 or 50,000 EU/mL LPS with (SCFA) or without (REG) the addition of short-chain fatty acids (2 mM butyrate and 5 mM propionate) in the culture medium. The ΔC_t was used for statistical analysis ($P < 0.05$), and results are presented as fold change (log), with each individual plotted. The horizontal bar represents the mean. Means with differing letters indicate significant differences ($P < 0.05$).

and binding to P1 receptors is associated with an anti-inflammatory response (Ferrari et al., 2016). The present results are consistent with the literature, whereby activation of P2 and P1 receptors invoke opposing effects (Ferrari et al., 2016) and suggesting that the proinflammatory response in REC may be regulated to prevent cell damage.

Inflammation can result in increased catabolism and increased energy, nitrogen, and micronutrient demands (Lennie et al., 1995). Inflammation has been associated with altered metabolic activity at systemic and tissue levels (Staal-van den Brekel et al., 1995; Hardin et al., 2000). Production of immune-modulatory molecules and increases in cell turnover, both systemically and locally, are implicated in the changes in metabolic demands. Using an intravenous LPS challenge in dairy cows, Kvidera et al. (2017) demonstrated that a systemic inflammatory response increased whole-body glucose requirements. At a tissue level, an ex vivo experiment with rabbit intestinal epithelium showed that proinflammatory cytokines were associated with an increase in metabolic demands and increased glucose absorption (Hardin et al., 2000). In the present experiment, exposure to LPS increased expression of *TLR4*, *PTGS2*, and cytokines *TNF* and *IL1B*, suggesting the REC had initiated a proinflammatory response. Following LPS exposure, REC tended to consume more glucose, indicating that the response may have increased the energetic demand. However, the amount of butyrate and propionate consumed by the cells was not affected

by LPS exposure. The increase in glucose utilization may indicate a metabolic shift toward glycolysis or oxidation. However, we did not assess lactate formation arising from glucose consumption and therefore cannot conclude whether glucose was partially metabolized or completely oxidized. Immune cells (Kominsky et al., 2010), airway epithelial cells (Garnett et al., 2016), and keratinocytes (Wickersham et al., 2017) also favor glycolysis under inflammatory conditions, suggesting that REC may do so as well.

Exposure to LPS increased the expression of *GLUT1* in REC, supporting the increase in glucose consumption observed. Interestingly, the addition of SCFA, without LPS, resulted in a downregulation of *GLUT1*. Ability to alter the energy substrates used, depending on their availability, has previously been demonstrated in ruminal epithelial tissue (Wiese et al., 2013). These data suggest that in the presence of SCFA, a shift away from glucose utilization may occur. In addition to the function of *GLUT1* in glucose transport, previous work has shown that *GLUT1* may be important for modulating inflammation in the skin (Zhang et al., 2018). *GLUT1* was also found to increase during inflammation-induced hypoxia, a result of depleted oxygen and increased glycolysis at sites of inflammation (Corcoran and O'Neill, 2016; Zhang et al., 2019). The present results show that *GLUT1* expression in the SCFA+LPS cells was greater than without LPS, suggesting that the presence of short-chain fatty acids may promote inflammation. Others have also shown that short-chain

Figure 5. Gene expression of glucose transporter, GLUT1 (A), and hypoxia-inducible factor, HIF1A (B), in cultured ruminal epithelial cells exposed to either 0 or 50,000 EU/mL LPS with (SCFA) or without (REG) the addition of short-chain fatty acids (2 mM butyrate and 5 mM propionate) in the culture medium. The ΔC_t was used for statistical analysis ($P < 0.05$), and results are presented as fold change (log), with each individual plotted. The horizontal bar represents the mean. Means with differing letters indicate significant differences ($P < 0.05$).

Figure 6. Gene expression of transporters, MCT1 (A) and MCT4 (B), SLC5A8 (C), and MCU (D) in cultured ruminal epithelial cells exposed to either 0 or 50,000 EU/mL LPS with (SCFA) or without (REG) the addition of short-chain fatty acids (2 mM butyrate and 5 mM propionate) in the culture medium. The ΔC_t was used for statistical analysis ($P < 0.05$), and results are presented as fold change (log), with each individual plotted. The horizontal bar represents the mean. Means with differing letters indicate significant differences ($P < 0.05$).

fatty acids have a modulating effect on REC when exposed to a variety of challenges (Meissner et al., 2017). Inflammation-induced hypoxia is mediated by *HIF1A*. In the present study *HIF1A* was not affected by either treatment; however, *HIF1A* is tightly regulated, with a half-life of only a few minutes (Masoud and Li, 2015); therefore a change in mRNA expression may not have been detectable.

Expression of *IGFBP3* increased in response to SCFA in the medium. Insulin-like growth factor is an important homeorhetic molecule of metabolism that acts as a signal of adequate nutrient availability (Clemmons, 2012). Although tissue-dependent, previous work has shown that IGF1 increases glucose transport into non-

hepatic cells (Simpson et al., 2004) and regulates utilization of nutrients such as oxidation of free fatty acids (Clemmons, 2012). In addition, IGF1 is well known for its effects on proliferation (Kiepe et al., 2005; Zheng et al., 2018). At a systemic level, IGFBP3 and IGFBP5 proteins bind to a tertiary molecule, acid-labile subunit, and together act to increase total serum IGF1 levels by prolonging its half-life (Clemmons, 2018). However, all IGF-binding proteins are pleiotropic and have local tissue-specific functions that are sometimes independent of IGF1 (Baxter, 2001; Zheng et al., 2018). Perks et al. (2000) reported that IGFBP3 and IGFBP5 have opposing functions, which was also observed in ruminal epithelial tissue by Steele et al. (2011), who

found that a high-grain diet increased gene expression of *IGFBP3*, whereas *IGFBP5* was downregulated. In contrast, *IGFBP3* was decreased and *IGFBP5* increased when butyrate was infused into the rumen in neonatal lambs (Liu et al., 2019). In the present study, *IGFBP3* increased with the addition of SCFA; however, *IGFBP5* was not affected by the medium. Previous work has suggested that butyrate, through its regulatory action on growth factors such as IGF, influences cellular proliferation and apoptosis of REC (Penner et al., 2011). Neogrdy et al. (1989) indicated that butyrate may inhibit REC apoptosis in culture. However, in the present study, rates of apoptosis of REC were not influenced by SCFA in the culture medium. The

mechanisms that mediate cell turnover in the ruminal epithelium in response to dietary changes involve many interconnected processes that have not been fully elucidated and warrant further investigation.

The inflammatory response has also been found to regulate the expression of IGFBP (O'Connor et al., 2008). The present results suggested that LPS increased expression of *IGFBP3* but decreased *IGFBP5*, supporting the results of Steele et al. (2011) when imposing a rumen acidosis-induction model. Previous work has suggested that IGF may increase in response to stimulation by proinflammatory cytokines (Clemmons, 2012). However, expression of *IGFBP3* is also associated with protective functions in response to

Figure 7. Gene expression of ketogenic enzymes, ACAT1 (A) and BDH1 (B), IGFBP3 (C), and IGFBP5 (D) in cultured ruminal epithelial cells exposed to either 0 or 50,000 EU/mL LPS with (SCFA) or without (REG) the addition of short-chain fatty acids (2 mM butyrate and 5 mM propionate) in the culture medium. The ΔC_t was used for statistical analysis ($P < 0.05$), and results are presented as fold change (log), with each individual plotted. The horizontal bar represents the mean.

inflammation (O'Connor et al., 2008; Kim et al., 2018). Further research is required to elucidate the potential homeorhetic and immune-modulatory effects in REC.

As further evidence that LPS influenced metabolic activity in the present experiment, expression of *ACAT1*, a key mitochondrial enzyme, was downregulated in cells that had been exposed to LPS, which may suggest a shift away from ketogenesis. In the first step in the formation of ketone bodies, *ACAT1* catalyzes the reaction that combines 2 acetyl-CoA molecules to form acetoacetyl-CoA and, depending on substrate availability, also catalyzes the reverse reaction. Moreover, *BDH1*, a ketogenic enzyme that facilitates BHBA formation, was also downregulated in cells exposed to LPS. The MFI of BDH1 protein tended to be less, suggesting that, in addition to mRNA, protein expression was also affected. In agreement with the present results, Memon et al. (1992) demonstrated that LPS-induced inflammation, by way of proinflammatory cytokines *IL1B* and *TNF*, resulted in reduced production of ketone bodies in mice *in vivo*. The inflammation-induced inhibition of ketogenesis was also observed in hepatocytes by Pailla et al. (1998, 2001).

A state of inflammation not only increases metabolic demands, it may also alter nutrient transport by epithelial cells (Peuhkuri et al., 2010). Previous work has demonstrated detrimental effects of experimental colitis on energy substrate uptake and metabolic function of colonocytes (Ahmad et al., 2000). Thibault et al. (2010) suggested that inflammatory bowel disease was associated with dysregulation of *MCT1* and thus uptake of butyrate. In the gastrointestinal epithelia, *MCT1* is an apically-localized short-chain fatty acid transporter (Graham et al., 2007), whereas *MCT4* is thought to be primarily on the basolateral membrane (Kirat et al., 2007), although evidence suggests that these expression patterns may be reversed in ruminal epithelium (Stumpff, 2018). In the present study, REC not exposed to LPS had greater expression of *MCT1* but only in the presence of SCFA. This is in agreement with findings by Villodre Tudela et al. (2015), that *MCT1* also increased following incubation with butyrate. Additionally, in the present study, expression of *MCT4* and *SLC5A8* were both upregulated following exposure to LPS. Increased expression of *MCT4* was also observed in intestinal epithelium afflicted with inflammatory bowel disease by He et al. (2018). The authors suggest that *MCT4* may be increased in response to greater levels of lactate. Lactate production is commonly reported in cultured cells and can indicate metabolic shifts (Hu et al., 1987; Zagari et al., 2013). Although lactate was not measured in the current experiment, the upregulation in *MCT4* may have occurred in an effort to mediate lactate accumulation

due to increased glycolysis. *SLC5A8*, a sodium-coupled monocarboxylate transporter, is involved in the apical transport of short-chain fatty acids as well as lactate and ketone bodies (Sivaprakasam et al., 2017). Martin et al. (2006) suggested that, in addition to transport of solutes, *SLC5A8* may also play a regulatory role in maintaining the energy status in some tissues.

In the REC incubated with SCFA, glucose consumption decreased compared with those incubated with REG. Although not affected by LPS treatment, the addition of SCFA to the culture medium resulted in increased expression of *MCU*, a transporter localized to the inner mitochondrial membrane that controls the influx of calcium (Paupe and Prudent, 2018). Calcium is a regulator of mitochondrial dehydrogenases and plays a key role in ATP production (Finkel et al., 2015). The increase in *MCU* expression in REC further suggests that metabolic function was altered in culture by the presence of SCFA. Together with the expression patterns of *BDH1*, *MCT1*, and *ACAT1*, the results suggest a shift toward butyrate metabolism in cells incubated with SCFA in culture and indicate the ability of REC to utilize short-chain fatty acids in the culture model.

CONCLUSIONS

The current study demonstrated the complexity of the inflammatory response in REC and explored how the response affected nutrient transport and metabolism. The results suggested that REC consumed more glucose following exposure to LPS, presumably as a result of the proinflammatory response. However, the difference was small, and the response did not appear to influence utilization of short-chain fatty acids added to the culture medium. Nonetheless, the results of this study suggested that the inflammatory response to LPS altered the metabolic activity of REC in culture through differential expression of genes associated with nutrient uptake. Although the exact role of short-chain fatty acids on the inflammatory response was not fully elucidated, the results of this study demonstrated evidence for regulatory roles of short-chain fatty acids in REC, especially in the recruitment of immune cells, such as neutrophils. Many questions still remain, and future research should focus on understanding the mechanisms by which the inflammatory response in REC affects nutrient transport and shifts metabolism, including the metabolic fates of glucose. Overall this study provided evidence that, although the effects on energy substrate consumption may be limited, the proinflammatory response, as a result of the interaction between ruminal epithelium and the ruminal MAMP, may bring about consequences that alter cellular processes and metabolism.

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