Evaluation of the systemic innate immune response and metabolic alterations of nonlactating cows with diet-induced subacute ruminal acidosis

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ABSTRACT

Subacute ruminal acidosis (SARA) increases lipopolysaccharide endotoxin in the rumen, which might translocate into the systemic circulation, triggering a cascade of clinical and immunological alterations. The objective of this study was to characterize the clinical immune and metabolic responses to ruminal-derived lipopolysaccharide in nonlactating cows induced with SARA using 2 challenges, a grain-based SARA challenge (GBSC) or an alfalfa-pellet SARA challenge (APSC). Six dry, nonlactating Holstein cows were used in a 3 × 3 Latin square arrangement of treatments with 4-wk experimental cycles. All cows received the control diet containing 70% forage and 30% mixed concentrates (dry matter basis) for 3 wk. In wk 4, cows received a control diet, GBSC (38% wheat-barley pellets, 32% other mixed concentrate, and 30% forages), or APSC (45% mixed concentrate, 32% alfalfa pellets, and 23% other forages). Total plasma proteins and immunology-related proteins, acute phase proteins, blood cells, serum chemistry, mRNA gene expression of peripheral blood cell surface markers, and selected proinflammatory cytokines were evaluated. Ruminal pH was lower in both groups with induced SARA compared with a control group. Ruminal endotoxins were higher in GBSC; however, plasma endotoxin was not detected in any study group. No significant differences in feed intake, rectal temperature, white blood cell counts, or differentials were found between control and SARA challenge groups; changes in glucose, urea, Ca, and Mg were observed in SARA groups. Total plasma proteins were lower in both SARA groups, and acute phase proteins were higher in GBSC. The expression of *CD14*, *MD2*, and *TLR4* mRNA in peripheral blood leukocytes was not affected by SARA induction. The induction of SARA as a result of GBSC or APSC challenge was successful; however, LPS was not detected in plasma.

Changes in clinical, metabolic, and inflammatory responses were not observed in the SARA-challenged cows, suggesting that, in this study, SARA was not associated with a systemic response to inflammation.

Key words: rumen, subacute ruminal acidosis, immune response, inflammation, metabolite

INTRODUCTION

Energy demands associated with milk production have increased the addition of grain into diet composition of dairy cows, which has now become a common practice in milk producers in North America. Similar to lactic acidosis, SARA has been associated with feeding starch-rich diets (Owens et al., 1998; Kleen et al., 2003; Plaizier et al., 2008). Unlike ruminal acidosis, SARA results from continued ingestion of these feeds over a prolonged period rather than sudden exposure without adequate adaptation (Garry and McConnel, 2008). In dairy cattle, which are not well adapted to grain and high-starch diets, the appearance of SARA is common and the ruminal pH is usually low within defined periods (Kleen et al., 2003). The high content of starch in grains increases grain fermentability by rumen microbes, increasing the production of short-chain fatty acids and therefore reducing ruminal pH, which ultimately changes the composition of the rumen microflora (Zebeli and Metzler-Zebeli, 2012). High concentrations of butyric and propionic acids stimulate proliferation of the ruminal papilla epithelium (Garry and McConnel, 2008); if the proliferation of the ruminal papilla epithelium is severe, it can result in parakeratotic changes associated with decreased absorption of VFA and increased susceptibility to damage and inflammation (Steele et al., 2011). Upon induction of SARA, an increased concentration of LPS is observed within several compartments of the ruminant gastrointestinal tract (Gozho et al., 2005; Li et al., 2012). The ruminal wall lesions allow penetration of bacteria and endotoxin with dissemination to the bloodstream (Kleen et al., 2003). Consequently, the presence of systemic LPS may

Received May 3, 2014.

Accepted August 22, 2014.

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result in an immunological challenge to the animal, triggering a cascade of events associated with an immunological response within the host (Gozho et al., 2005). Consequences of SARA include feed intake depression, fluctuations in feed intake, reduced diet digestibility, reduced milk yield, reduced milk fat percentage, gastrointestinal damage, liver abscesses, and lameness (Krause and Oetzel, 2006; Plaizier et al., 2008).

The presence of LPS in the systemic circulation could engage the pattern recognition receptors, affecting leukocyte populations and triggering the production of proinflammatory cytokines and acute phase proteins (APP). Once in systemic circulation, lipopolysaccharides are recognized by a LPS receptor complex. The LPS receptor complex consists of LPS-binding protein (LBP), membrane (m) CD14, the pattern recognition receptor TLR4, and the associated protein myeloid differentiation factor 2 (MD-2; da Silva Correia et al., 2001). When engaged by LPS, such as in infections caused by gram-negative bacteria, this complex transduces a signal detected by the myeloid differentiation primary response gene 88 (*MyD88*), resulting in the activation of nuclear factor κ -B, initiating a transcription of a proinflammatory cytokine cascade (Erridge et al., 2002). It might be expected that the presence of endotoxins in the gastrointestinal tract occurs in SARA and the translocation of endotoxins to the systemic circulation triggers innate immune mechanisms involving leukocyte proliferation, activation of LPS/LBP/CD4 and TLR4/MD2 complexes, upregulation of proinflammatory cytokines and production of APP, which are an integral part of the acute phase response (APR; Cray et al., 2009).

Results of both experimental induced and field SARA cases have indicated that changes in biochemical profile occur in the course of developing SARA, suggesting a possible relationship between SARA and metabolism (Riuzzi et al., 2009). In dairy cattle suffering from SARA, blood glucose and electrolyte abnormalities have been reported to be associated with the presence of LPS in systemic circulation (Khafipour et al., 2009b). However, the association between the presence of LPS in blood, innate immune response, and biochemical abnormalities in cattle with SARA is yet to be determined.

We hypothesized that experimentally induced SARA in dairy cattle triggers changes in feed intake and body temperature and results in alterations of the leukogram, biochemical profile, and innate immune response. We also hypothesized that those changes may be associated with the presence of ruminal-derived LPS. The objectives of this study were to determine whether SARA induces changes in clinical variables, systemic biochemical profile, and systemic immune response

characterized by alteration of the leukogram and expression of CD14 complex on peripheral blood mononuclear cells (PBMC), and increased concentrations of APP. The second objective was to determine whether those changes were associated with the presence of ruminal-derived LPS in bloodstream. Subclinical acute ruminal acidosis was induced using 2 models previously reported (Li et al., 2012), a grain-based SARA challenge (GBSC) and an alfalfa-pellet SARA challenge (APSC).

MATERIALS AND METHODS

Animals, Experimental Design, and Diets

Six nonlactating, nonpregnant, mature (>5 yr) Holstein cows with average initial BW of 620 ± 45.7 kg (mean $\pm$ SD) that were fitted with a ruminal and cecal cannula, were used in a replicated Latin Square experimental design with blocking on cows and periods. Cow pairs were randomly assigned to 1 of 3 treatments in 3 different periods. Each experimental period consisted of 4 wk in which all cows received a diet consisting of 70% (DM basis) of forage in the first 3 wk. In wk 4, cows received 1 of 3 diets. One group remained on the control diet whereas the other 2 groups received 1 of 2 SARA-induction diets, GBSC or APSC. Ingredient and nutrient composition of experimental diets are presented in Table 1; further details on the experimental diets were previously described (Li et al., 2012).

Cows were transitioned from the adaptation diet to SARA-induction diets over 3 d. The cows were housed in individual tie-stalls in the Glenlea Dairy Research Unit at the University of Manitoba (Winnipeg, MB, Canada) in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 1993). On a weekly basis, veterinarians examined cows for heart rate, respiratory rate, and rumen contractions. Rectal temperatures were recorded on sampling days of wk 3 and wk 4. The amounts of TMR offered and refused were recorded daily for each cow.

Blood Collection and Hematological Analyses

Blood samples were collected from the tail vein on d 7 of wk 3, and on d 3 and d 5 of wk 4 of each study period. Blood was collected at 0900 h, before feeding, and at 1500 h, 6 h post feeding. One BD Vacutainer blood-collection tube (BD-Canada, Mississauga, ON, Canada) and one plasma K₂EDTA Vacutainer (Becton Dickinson, Franklin Lakes, NJ) were analyzed by Manitoba Agricultural, Food and Rural Initiatives Vet Diagnostics Services (Winnipeg, MB, Canada) for hematological parameters: red blood cells (RBC), packed

Table 1. Ingredient and nutrient composition of control diet, grain-based SARA challenge (GBSC) diet, and alfalfa-pellet SARA challenge (APSC) diet used for inducing SARA

Item	Control	GBSC	APSC
Ingredient (% of DM)			
Barley silage	40	28	33
Alfalfa hay	30	8	—
Supplement ¹	30	30	30
Wheat-barley pellets ²	—	34	—
Alfalfa pellets	—	—	37
Nutrient composition (% of DM)			
DM (%)	54.3	61.6	69.0
CP	16.1	16.0	16.0
NDF	35.6	22.9	34.5
ADF	23.3	15.2	22.6
NFC ³	34.8	50.4	49.0
Starch	14.2	33.7	15.9
Fat	3.6	3.3	3.2
Ash	9.9	7.4	7.3
Ca	0.85	0.64	1.3
P	0.35	0.41	0.35
Mg	0.35	0.29	0.30
K	0.21	1.25	0.18
Na	0.27	0.28	0.32

¹Supplement composition as nutrient composition (% of DM): DM (%): 90.5; CP: 18.4; NDF: 14.7; ADF: 7.7; NFC: 56.3; starch: 35.6; crude fat: 3.9; ash: 6.7; Ca: 1.05; P: 0.67; K: 0.8; Mg: 0.5; Na: 0.53.

²Consisted of 50% wheat and 50% barley.

³NFC = 100 – (NDF % + CP % + crude fat % + ash %).

cell volume (**PCV**), hemoglobin (**HgB**), mean corpuscular volume (**MCV**), mean corpuscular hemoglobin (**MCH**), mean corpuscular hemoglobin concentration (**MCHC**), white blood cells (**WBC**), differential WBC count (automated hematology analyzer, Cell-Dyn 3500 System, Abbott Laboratories, Abbott Park, IL), fibrinogen concentrations (by refractometry, Leica TS 400, Leica Microsystems Inc., Concord, ON, Canada), and selected parameters of blood chemistry such as urea, creatinine, glucose, CK, aspartate aminotransferase, γ -glutamyltransferase, alkaline phosphatase (**ALKP**), TTP, albumin (**Alb**), globulin (**Glob**), phosphorus, calcium, magnesium; Vitros 250 Chemistry Analyzer, Ortho-Clinical Diagnostics, Markham, ON, Canada).

Plasma LPS and APP

Blood tubes containing sodium heparin were centrifuged at $3,000 \times g$ at 19°C for 15 min to isolate plasma. The extracted plasma was used for LPS, LBP, serum amyloid A (**SAA**) and haptoglobin (**Hp**) analyses. Plasma tubes were allowed to clot at room temperature for 45 min before centrifugation at $3,000 \times g$ at 19°C for 15 min. Plasma was separated and stored at –80°C until used for analysis. The concentration of LPS in blood plasma was measured using chromogenic kinetic Limulus amoebocyte lysate (LAL) assay (Kinetic-QCL, Lonza Group, Basel, Switzerland) with a detection

limit of 0.05 endotoxin units (**EU**)/mL (Khafipour et al., 2009b). Concentrations of LBP were analyzed as previously described (Khafipour et al., 2009b) using a commercial kit (HK503, HyCult Biotechnology, Uden, the Netherlands). Serum amyloid A and Hp concentrations were measured using commercial ELISA kits (TP-801 and TP-802, respectively, Tri-Delta Diagnostics Inc., Morris Plains, NJ), as described elsewhere (Gozho et al., 2005).

Cell Preparation and Flow Cytometry Analyses

Blood was collected into Vacutainers containing sodium heparin anticoagulant (Becton Dickinson). Peripheral blood mononuclear cells and PMNL were isolated via density gradient using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and RBC lysis buffer (Fisher Canada, Nepean, ON, Canada) as described by the University of Kansas Medical Center Flow Cytometry Core Laboratory (Kansas City, KS). Blood was first diluted with an equal volume of $1 \times$ PBS (Hyclone Laboratories, Thermo Scientific, Logan, UT), carefully layered on Ficoll-Paque, and centrifuged at $2,000 \times g$ for 25 min at 4°C, discarding the upper supernatant after centrifugation. The buffy coat containing PBMC was extracted and placed in a new microcentrifuge tube. Isolated PBMC were washed several times with $1 \times$ PBS, and then diluted in 1 mL of $1 \times$ PBS for cell counting. The Ficoll-Paque layer was removed to access the granulocyte and erythrocyte layer. To every 1 mL of granulocyte and erythrocyte layer, 3.5 mL of RBC lysis buffer was added and incubated for 5 min at 37°C and centrifuged at $2,000 \times g$ at 4°C for 5 min; the supernatant was discarded. Isolated PMNL were washed several times with $1 \times$ PBS and then diluted in 1 mL of $1 \times$ PBS for cell counting.

Cell viability of PBMC and PMN populations was determined using trypan blue (Gibco, Invitrogen Canada Inc., Burlington, ON, Canada). After counting, approximately 1×10^6 cells/100 μ L were placed into each well of a 96-well plate for antibody staining. Lipopolysaccharide-stimulated PBMC and PMNL were included as positive controls. These cells were stimulated with 1,000 EU/mL of purified *E. coli* O111:B4 LPS (Sigma-Aldrich, St. Louis, MO) and incubated for 1 h at 37°C. The remaining cells were used for total RNA extraction, as described later.

Anti-bovine monoclonal antibodies against CD14 and CD11a (AbD Serotec, Raleigh, NC) were used. Antibody CD11a was used to distinguish the desired cell populations (i.e., granulocytes, lymphocytes, monocytes, and macrophages). Cells were labeled with monoclonal primary or isotype control antibodies at a 1:100 dilution. Labeled cells were incubated in the dark

at 4°C for 30 min, and then centrifuged at 2,000 × *g* for 6 min. Cells were washed twice with 1× PBS to remove any unbound antibodies. Cells were fixed in 1% buffered formalin and left at 4°C overnight. For each sample, 100,000 cells were run through a BD FACScalibur flow cytometer (Becton Dickinson) and analyzed for CD14 expression using Flowjo flow cytometry analysis software (v.7.5, Tree Star Inc., Ashland, OR), and figures were developed using Cyflogic software (v.1.2.1, CyFlo Ltd., Turku, Finland).

RNA Extraction and Reverse Transcription

Allocations of PBMC and PMN cells were left in 500 µL of RNeasy lysis buffer (Qiagen, Applied Biosystems, Foster City, CA) at 4°C overnight. The cells were then centrifuged at 1,500 × *g* at 4°C for 1 min to remove the RNeasy lysis buffer and stored at −80°C until used. Total RNA was extracted, within a week of storage, using TRI Reagent Solution following the manufacturer’s protocol (Ambion, Applied Biosystems). The RNA pellets were resuspended in RNase- and DNase-free water (Invitrogen Canada Inc.) and stored at −80°C. Ten microliters of RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems) according to the manufacturer’s protocol. The cDNA was then stored at −80°C until quantitative real-time PCR analysis.

Quantitative Real-Time PCR and Gene Expression Analyses

The expression of *TLR4* and *MD2* was analyzed and β-actin was included as an endogenous control. The primers used for relative quantification real-time PCR and for cloning plasmid controls were designed with Primer Premier 5 Software (Premier Biosoft, Palo Alto, CA) and synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada). The list of designed primers is located in Table 2. The National Center for Biotechnology Information (NCBI) GenBank was used to identify bovine mRNA sequences of β-actin, *TLR4*, and *MD2*, and NCBI BLASTN (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to verify the specificity of the primers.

The Step One Real-Time PCR System (Applied Biosystems) and SYBR Green I dye chemistry were used for relative quantification of gene expression. The SYBR green PCR Master Mix (12.5 µL; Applied Biosystems), 2 µL of primers, 2 µL of diluted cDNA template, and 8.5 µL of water were added into each well. A concentration of 700 nM was used for the β-actin primer and 300 nM was used for *TLR4* and *MD2* primers. All samples were initially diluted 1:25. If the cycle threshold value

Table 2. Sequences of oligonucleotide primers used for plasmid inserts and relative quantification real-time PCR (RT-PCR) during the study

Gene ¹	Sense primer (5' to 3')	Antisense primer (5' to 3')	Size (bp)	Accession no.	Purpose
<i>TLR4</i>	CTGATCCACGACGAGGCGCAT	CCAAAGTGTCTCCAGGTTGGGCA	511	NM_174198	Plasmid insert
	CCATATCAACCAAGATGCTGGACC	CTGGATAGGGTTCCCGTCAG	188		RT-PCR
<i>MD2</i>	GGTCTGCAACTCTCTGAC	CAGTGTCCCTCGATGGCT	360	DQ319076	Plasmid insert
	GCAACTCTCTGACGCCAC	GAGTATTCACGGCTTCCCT	110		RT-PCR
β-Actin	TCGACAAAGGCTCCGCGCATG	GCATTGCGGTGGACGATGGAG	1,094	NM_173979	Plasmid insert
	GTGCCATCTATGAGGGGTACG	GTGGTGAAGCTGTAGCCACGCT	116		RT-PCR

¹*TLR4* = toll-like receptor 4; *MD2* = myeloid differentiation factor 2.

was too low, samples were further diluted to obtain a value between 15 and 30. A plasmid template of each gene was included as a control. Template-free wells were also included. All genes were run on the same plate in duplicate. The run settings were as follows: 95°C for 10 min, and 45 cycles of 95°C for 15 s, 55°C for 30 s, and 60°C for 1 min. This was followed by a 3-step melt curve. Relative quantification of gene expression was calculated using an equation reported by Pfaffl (2001).

Plasmid Control Preparation

Plasmids were prepared for each of the genes analyzed via real-time PCR to be used for primer standardization, as well as a control in real-time PCR data analysis. Partial-length coding sequences of *TLR4*, *MD2*, and β -actin were amplified from PBMC cDNA using PCR Master Mix Kit (Promega Corporation, Fisher Scientific, Nepean, ON, Canada) and a 2720 Thermo Cycler (Applied Biosystems). The PCR thermal profile for *TLR4* and *MD2* was initiated by a 1-min denaturation at 94°C, followed by 35 cycles consisting of 1 min of denaturation at 94°C, 45 s of annealing at 60°C, and 45 s of elongation at 72°C. A final 10-min extension step was included at 72°C followed by a reduction in the temperature to 4°C. The thermal profile for β -actin started with a 1-min denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of elongation at 72°C. A final 15-min extension step was included at 72°C followed by a reduction in the temperature to 4°C. The PCR amplicons were then isolated on a 1.5% agarose gel, stained with ethidium bromide, and purified using Wizard SV Gel and PCR Clean-Up System (Promega Corp., Fisher Scientific). The purified PCR product was ligated into a pGEM-T vector (Promega Corp., Fisher Scientific) according to the manufacturer's protocol and transformed into *E. coli* competent cells (JM109; Promega Corp., Fisher Scientific). Competent cells were added to ligated product, left on ice for 20 min, heat shocked at 42°C for 45 s, and then put back on ice. The transformed cells were plated on Luria-Bertani agar plates containing 50 μ g/mL ampicillin, 100 mM isopropyl thiogalactoside, and 20 mg/mL X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and left at 37°C overnight. Five putative colonies were then transferred to liquid Terrific Broth medium and incubated overnight at 37°C.

A basic alkaline miniprep procedure was used to isolate plasmid DNA (Green and Sambrook, 2012). Cells were centrifuged, supernatant discarded, and pellets resuspended in 50 μ L of Tris-EDTA buffer containing 20 μ g/mL of RNase A. The plasmid DNA concentration was determined at 260 nm (DU800 Spectrophotometer, Beckman Coulter Canada Inc., Mis-

sissauga, ON, Canada). Restriction enzyme digestion (*Bst*Z1; Promega Corporation, Fisher Scientific) and PCR amplification with gene-specific primers (Table 2) were used to verify the gene insert in the vector. Plasmids were stored at -20°C.

Statistical Analysis

The repeated measurements (d 7 of wk 3, and d 5 and d 7 of wk 4) of hematological and blood chemistry parameters, APP, flow cytometry, and quantitative real-time PCR on each cow were analyzed separately using linear mixed models (Dohoo et al., 2009). The overall resulting model to evaluate the outcomes in cow *i* within treatments at period *j* was as follows:

$$y_{ijkl} = \mu + \alpha_i + \beta_j + \delta_k + \gamma_l + A_i + \varepsilon_{ijkl}, \quad [1]$$

where y_{ijkl} corresponds to hematological and blood chemistry parameters, APP, flow cytometry and quantitative real-time PCR; μ is the overall mean; α_i is the treatment effect on *i*th cow; β_j represents the overall effect of *j*th period on cow *i* (period 1, 2, or 3); δ_k represents the effect of sample collection day (d 7 of wk 3, and d 3 and d 5 of wk 4); γ_l is the effect of time of the day *l* (a.m. or p.m.) of day *k*; A_i is the cow random effect; and ε_{ijkl} is the error term.

The statistical models included categorical effects of treatment (control, GBSC, and APSC), period, day, and time of the day; when significant, treatment groups were compared in each period by model-based tests adjusted for multiple testing by the Bonferroni method (briefly, by multiplying the resulting *P*-values by the number of tests). The models were evaluated by standardized residuals, and transformations of the outcome were explored whenever the model assumptions were not fully met (Dohoo et al., 2009). Effects were considered significant at $P < 0.05$, and trends considered at $P < 0.10$. Analyses were carried out using the command *xtmixed* of the statistical package Stata (version 13.1, Stata Corp., College Station, TX).

The percentage of PBMC of control and SARA-induced cows expressing CD14 on the cell surface after stimulation with 1,000 EU/mL of *Escherichia coli* O111:B4 LPS for 1 h at 37°C ($n = 10$) was compared using a paired Student's *t*-test. Data collected from one cow of APSC group during period 1 were omitted from APP and blood cell differential analyses due to severe gastrointestinal parasitism.

RESULTS

Cows from both diet models developed SARA. Ruminal pH was <5.6 for 298.7 and 225.2 min/d in GBSC and APSC, respectively, compared with 56.4 min/d in

Table 3. Concentrations of LPS [endotoxin units (EU)/mL] and LPS-binding protein (LBP; mg/L) in nonlactating dairy cows fed a control diet or induced (treatment) with SARA by feeding grains (GBSC) or alfalfa pellets (APSC)¹

Item	Treatment				P-value		
	Control	GBSC	APSC	SEM ²	Treatment	Time	Treatment × Time
Rumen LPS	10.405 ^a	168.391 ^c	30.715 ^b	8.738	<0.01	0.84	0.72
Plasma LPS	<0.05	<0.05	<0.05	—	—	—	—
LBP ³	8.9 ^a	12.1 ^b	9.5 ^{ab}	1.3	0.05	0.29	0.89

^{a-c}Means with different superscript letters differed ($P < 0.05$).

¹Results were reported previously (Li et al., 2012).

²SEM for the effect of treatment.

³Statistical analyses were conducted on log₁₀-transformed data. The LBP data are from an earlier unpublished study [A. D. Kroeker, S. Li, S. Shekhar (University of Manitoba, Winnipeg, Manitoba, Canada), A. Ceballos (Cornell University, Geneseo, NY), E. Khafipour (University of Manitoba), D. O. Krause (University of Manitoba), J. C. Plaizier, and J. C. Rodriguez-Lecompte; unpublished data].

control group. A significant increase of LPS in the rumen fluid in both SARA challenge groups was observed (Table 3). However, endotoxins were not detected in plasma of cows in any group (Table 3). The effect of SARA group on ruminal concentration of LPS is fully described elsewhere (Li et al., 2012).

Feed Intake and Rectal Temperature

We detected no decrease in daily feed intake in any of the animals in either of the SARA groups. Dry matter intake increased in both SARA groups (APSC and GPSC were 16.7 and 17.6 kg/d, respectively, compared with the control group was 15.4 kg/d (Li et al., 2012). No difference in rectal temperature was observed at any time point when the control group was compared with the SARA challenge groups. The overall mean ($\pm$ SD) of rectal temperature was $38.4 \pm 0.07^\circ\text{C}$ in the SARA challenges, being comparable across all treatments ($P > 0.05$; data not shown).

Hematological Analysis and Blood Chemistry

The induction of SARA in groups GBSC and APSC compared with the control group did not result in any

significant change in WBC ($P = 0.23$). However, the WBC differential showed a lower percentage of neutrophils and a larger percentage of eosinophils in the APSC group compared with control and GBSC groups. The ratio of percentage neutrophils to percentage lymphocytes of cows induced with either GBSC or APSC was not significantly different from the cows on the control diet ($P = 0.16$). Results of WBC and differentials are shown in Table 4.

Blood glucose concentration was significantly higher in cows of the GBSC group than in cows of the APSC group ($P < 0.01$) or control cows ($P = 0.04$). Cows in GBSC had a significantly decreased blood urea concentration compared with APSC and control cows ($P < 0.01$). Results of blood chemistry are shown in Table 5.

The enzyme activity of aspartate aminotransferase, γ -glutamyltransferase, and creatine phosphokinase upon SARA induction did not result in differences among groups ($P > 0.05$). Plasma Ca concentration was lower in the GBSC group compared with control cows ($P < 0.01$) and APSC cows ($P < 0.01$). Mean ($\pm$ SD) plasma Ca concentration was 2.35 ± 0.02 mmol/L in the control group, whereas in GPSC challenge was 2.21 ± 0.02 mmol/L in APSC was 2.32 ± 0.02 mmol/L

Table 4. Least squares means ($\pm$ SEM) of white blood cell count (WBC) and differentials in nonlactating dairy cows fed a control diet or induced (treatment) with SARA by feeding grains (GBSC) or alfalfa pellets (APSC)

Cell count	Treatment			P-value			
	Control	GBSC	APSC	Treatment	Period	Day	TOD ¹
WBC ($\times 10^9$ cells/L)	8.08 ± 1.13	7.53 ± 1.13	7.57 ± 1.13	0.23	0.03	—	—
Differential (% of WBC)							
Neutrophils (including band cells)	40.8 ± 3.11^a	42.1 ± 3.09^a	36.3 ± 3.10^b	0.02	<0.01	0.06	0.20
Lymphocytes	50.0 ± 3.81	48.5 ± 3.80	48.0 ± 3.80	0.55	0.05	0.11	0.30
Monocytes	5.4 ± 0.57	4.8 ± 0.56	5.3 ± 0.57	0.51	1.00	0.43	0.78
Eosinophils	3.2 ± 1.82^a	4.1 ± 1.81^a	9.4 ± 1.82^b	<0.01	<0.01	0.11	0.85
Neutrophil:lymphocyte ratio	0.90 ± 0.16	1.01 ± 0.16	0.82 ± 0.16	0.16	0.14	0.10	0.20

^{a,b}Means with different superscript letters differed ($P < 0.05$).

¹TOD = time of day (a.m. vs. p.m.).

Table 5. Blood chemistry of nonlactating dairy cows fed a control diet or induced (treatment) with SARA using the grain-pellet or alfalfa-pellet based diet models

Item	Treatment				Effect, <i>P</i> -value			
	Control	Grain-pellet	Alfalfa-pellet	SEM	Treatment	Period	TOD ¹	Treatment × TOD
Carbohydrates								
Glucose (mmol/L)	3.96 ^b	4.19 ^a	3.81 ^b	0.10	<0.01	<0.01	0.03	0.88
Metabolic function								
Urea (mmol/L)	5.51 ^b	4.62 ^a	5.13 ^b	0.31	<0.01	0.17	0.09	<0.01
Creatinine (mmol/L)	72.5	69.7	71.3	2.77	0.10	<0.01	<0.01	0.43
Liver function ²								
CK (U/L)	78.8	75.2	73.2	9.56	0.90	0.90	0.03	0.51
GGT (U/L)	47.7	47.9	46.9	0.87	0.60	0.61	0.44	0.81
AST (U/L)	85.1	95.5	75.9	10.2	0.13	0.76	0.05	0.57
Endocrine function								
Calcium (mmol/L)	2.35 ^b	2.21 ^a	2.32 ^b	0.03	<0.01	0.81	0.71	0.81
Magnesium (mmol/L)	0.86 ^b	0.97 ^a	0.82 ^b	0.04	<0.01	0.79	<0.01	0.30
Phosphorus (mmol/L)	2.25	2.21	2.14	0.10	0.76	0.15	<0.01	0.01
Electrolytes								
Sodium (mmol/L)	138	137	137	0.45	0.44	0.05	<0.01	0.87
Potassium (mmol/L)	4.53	4.45	4.43	0.11	0.37	0.29	<0.01	0.11
Chloride (mmol/L)	100 ^b	102 ^a	101 ^{ab}	0.61	0.04	0.10	<0.01	<0.01

^{a,b}Means within a row with different superscript letters differed ($P \leq 0.05$).

¹TOD = time of day (a.m. vs. p.m.).

²CK = creatine kinase; GGT = γ -glutamyl transferase; AST = aspartate aminotransferase.

($P < 0.01$). Calcium concentration was not affected by period ($P > 0.05$): we observed no differences due to day of sample collection ($P > 0.05$). Magnesium was affected by SARA challenge, with a higher concentration detected in the GPSC group compared with control and ABSC groups ($P < 0.01$). Serum P concentration was similar among groups ($P > 0.05$; Table 5).

Total Plasma Proteins and Acute Phase Proteins

Inducing cows with SARA reduced total plasma proteins compared with control cows (Table 6). Total plasma protein concentrations were lower in both SARA challenge groups due to both hypoalbuminemia

and hypogammaglobulinemia. No differences were identified between SARA groups (Table 6).

Period had a significant effect on the concentration of selected proteins and on the albumin:globulin ratio. Albumin concentration increased slightly from period 1 to period 2 and from period 2 to period 3 ($P < 0.01$). Period had a significant effect on fibrinogen and albumin (Table 6). Albumin concentrations increased significantly from period 1 to period 3 ($P = 0.05$); conversely, fibrinogen decreased significantly from period 1 to period 3 ($P < 0.05$). The total protein to fibrinogen ratios in both SARA induction treatments were reduced compared with the ratio of the control treatment but the difference was not significant (Table 6). The day of

Table 6. Least squares means and standard error of means of the concentration of selected proteins in nonlactating dairy cows fed a control diet or induced (treatment) with SARA feeding grains (GBSC) or alfalfa pellets (APSC)

Item	Treatment				<i>P</i> -value			
	Control	GBSC	APSC	SEM ¹	Treatment	Period	Day	TOD ²
Total plasma proteins (g/L)	83.5 ^a	79.6 ^b	80.4 ^b	1.71	<0.01	<0.01	0.24	0.15
Albumin (g/L)	29.6 ^a	28.7 ^b	28.8 ^b	0.32	<0.01	<0.01	0.06	0.93
Globulin (g/L)	53.9 ^a	51.0 ^b	51.8 ^b	1.56	<0.01	<0.01	0.68	0.19
Fibrinogen (g/L)	6.6	6.8	6.5	0.27	0.62	<0.01	0.04	0.08
Albumin:globulin ratio	0.56	0.57	0.56	0.02	0.52	<0.01	0.77	0.21
Total protein:fibrinogen ratio	12.0	11.2	11.6	0.66	0.20	<0.01	0.01	0.52
Serum amyloid A (mg/L)	38.3 ^b	62.5 ^a	35.6 ^b	1.12	0.002	0.12	0.71	0.41
Haptoglobin (mg/L)	54.4 ^b	83.5 ^a	72.2 ^b	1.21	0.16	0.15	0.02	0.17

^{a,b}Means with different superscript letters differed ($P < 0.05$).

¹Standard error of means for the effect of diet.

²TOD = time of day (a.m. vs. p.m.).

sample collection and the time of day had no effects on either selected proteins or their ratios (Table 6).

Concentrations of serum LBP (Table 3), SAA, and Hp were significantly higher in the GBSC cows compared with the control group ($P < 0.01$; Table 6). Mean plasma concentration of SAA was 38.4 ± 1.12 mg/L in control cows and 62.6 ± 1.12 mg/L ($P < 0.01$) in GBSC cows. The concentrations of LBP, SAA, and Hp of cows in the APSC group were similar to those in the control group (Table 6).

LPS-Associated Recognition Receptors

The expression of endotoxin-related recognition receptors did not change due to the induction of SARA. The percentage of PBMC expressing CD14 on the cell surface did not differ between SARA groups and control treatment ($P = 0.85$). However, CD14 was not detected on the surface of PMNL.

The expression of CD14 on LPS-stimulated PBMC cells, used as a positive control, was downregulated compared with that of control cells (Figure 1). The downregulation of CD14 was accompanied by a change in cell morphology based on cell size (forward scatter) and granularity (side scatter). Both side and forward scatter of the CD14-positive population of cells expanded after LPS stimulation indicated cell differentiation.

The expression of *MD2* and *TLR4* genes was detected in most PBMC and PMNL samples. Nevertheless, SARA induction did not result in differences for either target gene compared with the control diet ($P > 0.05$). All samples expressed β -actin (included as endogenous control).

DISCUSSION

The induction of SARA as a result of GBSC or APSC challenge was successful according to the definition (Kleen et al., 2003; Gozho et al., 2005). A reduction in ruminal pH below 5.6 is an essential characteristic of SARA (Krause and Oetzel, 2006). The stimulus caused by low pH (pH < 5.6) on the ruminal environment lasted 298.7 min/d at most, indicating that cows were able to buffer transient spikes of pH, which may prevent further damage to rumen mucosae and proliferation of the rumen papilla epithelium (Owens et al., 1998; Kleen et al., 2003). The clearance of acids from the rumen is affected by the size and density of the rumen papillae, as these determine how fast acids can be absorbed (Van Soest, 1991). A reduction in absorption (e.g., by inflammation or parakeratosis of the rumen wall due to low rumen pH) increases the risk of SARA in cattle.

Recent studies have reported a coordinated adaptation of many molecular functions and pathways (i.e.,

epithelial proliferation, cellular function, and tissue permeability) in response to pH fluctuations in the rumen (Penner et al., 2010; Taniguchi et al., 2010; Dionissopoulos, 2013). The authors of a previous study concluded that a mild episode of SARA does not affect ruminal epithelial barrier function immediately after the episode; however, a more severe acidification (pH ≤ 5.2) in vitro increases epithelial permeability following the insult (Penner et al., 2010). Furthermore, feeding diets rich in barley grain to goats was shown to decrease ruminal fluid pH to 5.5 and alter electrophysiological properties and permeability of the ruminal wall (Klevenhusen et al., 2013). The results of the present study suggest that the SARA challenges were not prolonged or severe enough to induce a severe lesion on the rumen mucosae to cause massive translocation of bacteria, LPS, or other pathogen-associated molecular patterns (PAMP) from the rumen to the bloodstream. Massive translocation of bacteria, LPS, or PAMP may have triggered severe systemic immune response, resulting in the development of clinical signs, such as fever and altered behavior, and changes in the leukogram parameters (Kleen et al., 2003; Dionissopoulos, 2013). Similar to previous studies (Gozho et al., 2007), the WBC and differentials in SARA-induced cows in the present study were comparable to those of control cows. Throughout the course of the experiment, total plasma proteins, globulins, fibrinogen, and albumin concentrations remained within the reference values (Jones and Allison, 2007; Russell and Roussel, 2007).

A significant increase in rumen LPS, particularly in GBSC SARA, was determined in the current study. Similar to previous studies, multiple-fold increases of

Figure 1. Percentage of peripheral blood mononuclear cells (PBMC) expressing CD14 on the cell surface after stimulation with 1,000 endotoxin units (EU)/mL *Escherichia coli* O111:B4 LPS for 1 h at 37°C (n = 10). Treatment had a significant effect at $P < 0.05$.

endotoxin concentrations in the rumen after SARA induction using either GBSC or APSC were determined (Gozho et al., 2007; Khafipour et al., 2009a; Li et al., 2012). The considerable increase of endotoxins in the rumen has been the focus of previous studies when interpreting APR-related results because LPS is capable of initiating a local immune response (Erridge et al., 2002). It has also been suggested that rumen LPS (simultaneously with other factors affecting the gastrointestinal barrier integrity such as epithelial damage due to acidic nature of the ingesta, proliferation of the ruminal papilla epithelium due to increased concentrations of VFA, and the high rumen osmolality that is seen during SARA) can cause swelling and rupture of ruminal papillae, resulting in local damage and inflammation and LPS translocation, thus leading to a systemic inflammatory response (Khafipour et al., 2009b). Comparable to previous studies (Ametaj et al., 2009; Khafipour et al., 2009b), the concentrations of plasma LBP, SAA, and Hp in the current study were significantly higher in the GBSC cows compared with the control group. The increases in plasma concentrations of the APP such as SAA and Hp do not prove translocation of LPS because these concentrations could increase due to other unspecific inflammatory stimuli. Although an increase in LBP in peripheral circulation may provide evidence of the translocation of LPS (Srisukandan and Altmann, 2008), recent studies have shown that LBP increases in response to a different PAMP (Schröder and Schumann, 2005; Cecilian et al., 2012).

Despite general agreement that grain-induced SARA increases free rumen LPS, contradictory results regarding the presence of LPS in peripheral blood circulation have been reported (Gozho et al., 2007; Khafipour et al., 2009a). Furthermore, the detection of LPS in peripheral circulation is inconsistent during experimentally induced SARA (Khafipour et al., 2009a). Interestingly, in the present study, the plasma concentration of LBP was significantly higher in the GBSC cows but plasma LPS concentrations were not detected. One possibility for our findings is that that plasma concentration of LPS in GBSC cows was lower than the range of detection of the bioassay used in this study. In recent years, the sensitivity and accuracy of bioassays used to detect LPS have been questioned (Khafipour et al., 2009a). Therefore, it is possible that the assay used in the present study was unable to detect low concentration of LPS during the period of study. A second possibility is that translocation of LPS did not occur and that plasma LBP increased in response to translocation of PAMP such as lipoteichoic acid from the outer cell wall of various gram-positive bacteria (Srisukandan and Altmann, 2008). Two recent studies evaluating bovine extrahepatic gene expression of acute phase proteins

demonstrated that the mucosa of the forestomach produce substantial amounts of LBP (Rahman et al., 2010) and SAA (Dilda et al., 2012). These findings may suggest that the forestomach, and especially the rumen, is able to mount a local acute phase response involving the innate immune system that inhibits systemic dissemination of LPS in this model of SARA. Unfortunately, ruminal concentrations of LBP and SAA were not determined in this study and further conclusions cannot be drawn. The primary biological role of LBP is to bind to free LPS to inactivate it. Subsequently, LBP-LPS complex is recognized by CD14 and TLR4, stimulating leukocyte activation to produce and releasing proinflammatory cytokines (Schröder and Schumann, 2005; Cecilian et al., 2012). However, studies have also shown the involvement of LBP in recognition of lipoteichoic acid from the outer cell wall of various gram-positive bacteria, indicating that LBP activity is not limited to LPS, challenging its specificity (Schröder and Schumann, 2005; Cecilian et al., 2012).

Endotoxins are removed from the circulation by the mononuclear phagocyte system, and the response of mononuclear phagocytes to these LPS plays an important role in determining the severity of clinical disease (Ibeagha-Awemu et al., 2008). In the current study, flow cytometric analysis showed no change in the percentage of monocytes expressing CD14 in nonlactating cows suffering from diet-induced SARA. It has been demonstrated that the anticoagulant used to preserve the sample and processing methods affect the surface expression of mCD14 in bovine neutrophils and monocytes (Ibeagha-Awemu et al., 2012). Interestingly, in the current study, the positive control LPS-incubated cells revealed a downregulation of CD14 expression and an altered morphology associated with size and granulometry of the monocytes. Monocyte-derived macrophages increase in size and granularity compared with monocytes when monocytes are allowed to differentiate, which supports the differentiation of monocytes into macrophages after the detection of endotoxin (Guo et al., 2009). The consistent expression of CD14 on PBMC of SARA-induced and control treatments may suggest that endotoxins were not present or low plasma levels of LPS were insufficient to trigger an immune response.

The induction of SARA in this study did not affect the gene expression of *TLR4* and *MD2* on either PBMC or PMNL. A recent study focusing on the effect of LPS on *TLR4* expression in both monocytes and monocyte-derived macrophages found no changes in gene expression in control samples (Guo et al., 2009). However, an increase in the gene expression levels of *TLR4* in the presence of LPS has been shown in other tissues, such as the mammary gland (Ibeagha-Awemu et al., 2008). It is evident some cells or tissues are refractory

to altered expression of genes associated with LPS in the presence of LPS, suggesting that other factors are required, such as cytokines. The reason for this would be an immunological homeostasis to minimize hyper-responsiveness and constant immune response stimulation (Abreu et al., 2001).

Blood glucose concentration was significantly higher in cows of the GBSC group than in those of the control and APSC groups. The increase in glucose has previously been reported in association with GBSC (Khafipour et al., 2009a; Zebeli et al., 2011) and can be the result of an increase of propionate and butyrate in the rumen (Gozho et al., 2007; Khafipour et al., 2009a; Zebeli et al., 2011). Nevertheless, recent studies suggested that free fatty acids, such as butyrates, might have a protective effect on rumen mucosae in SARA-induced cows (Dionissopoulos, 2013). The changes observed in serum urea concentration might be due to more efficient utilization of rumen ammonia for microbial protein synthesis, as diet protein levels were similar across all the diets used in this study. Nevertheless, these metabolites were also affected by other variables, such as cycle, day of sampling, and time of day when sample was collected. Therefore, the variation in glucose and urea upon SARA induction are associated with metabolic utilization rather than indicating a systemic response to ruminal injuries.

Serum Ca and Mg concentrations were significantly affected in cows with SARA challenges. However, their concentrations remained within accepted reference values. Although the role of macrominerals, such as Ca and Mg, and microminerals in the immunological response of cattle has been recognized for many years (Spears and Weiss, 2008), understanding of the biological roles associated with SARA induction and feeding grain-rich diets is limited and requires further investigation. Nevertheless, strong inverse relationships between ruminal endotoxins and plasma Ca and between SAA and Ca have been recently reported (Zebeli et al., 2010). Although the alterations in serum Ca concentration were statistically significant, the magnitude of those changes was too minor to have clinical significance.

CONCLUSIONS

Our results did not support changes in the health or immune status of the cows, as evaluated by changes in behavior or rectal temperature, WBC population, or total plasma proteins in association with SARA induction. However, mild changes in APP were observed in cows with diet-induced SARA, particularly in the GBSC challenge group. The results of the present study may indicate that the SARA challenges were not prolonged or severe enough to cause a severe lesion on the

rumen mucosae that would allow massive translocation of bacteria, LPS, or other PAMP from rumen to the bloodstream and trigger a systemic immune response, resulting in the development of clinical signs, such as fever and altered behavior, and changes in the leukogram. Nevertheless, the question that remains is whether changes in APP indicate a systemic response or a local immune response. Although LPS concentrations significantly increased in the rumen, the specificity of LBP to LPS is still under debate. Our results indicate that the increase in serum LBP in GBSC was moderate, not necessarily resulting from the presence of LPS in systemic circulation, which might indicate functions of LBP other than binding to LPS. Future research is warranted to determine whether SARA induces systemic inflammatory response depending on the severity and duration of the stimuli on the ruminal epithelium.

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