Subacute Ruminal Acidosis Induces Ruminal Lipopolysaccharide Endotoxin Release and Triggers an Inflammatory Response

G. N. Gozho, J. C. Plaizier, D. O. Krause, A. D. Kennedy, and K. M. Wittenberg

Department of Animal Science, University of Manitoba, Winnipeg, Canada R3T 2N2

ABSTRACT

Subacute ruminal acidosis (SARA) was induced in 3 rumen fistulated Jersey steers by offering them different combinations of wheat-barley pellets and chopped alfalfa hay. Steers were offered 4, 5, and 6 kg/d of pelleted concentrate and 6, 5, and 4 kg/d of chopped alfalfa hay for diets 1, 2, and 3, respectively, during 5-d treatment periods and were fed chopped alfalfa hay between treatment periods. Inducing SARA increased blood concentrations of haptoglobin and serum amyloid-A. Dry matter intake of concentrate and hay decreased from d 1 to 5 in each period. Subacute ruminal acidosis was induced in all steers during d 4 and 5 when concentrate was fed, with ruminal pH remaining below 5.6 for an average of 187 and 174 min/d on these days. Lipopolysaccharide concentration increased significantly during periods of grain feeding compared with times when only hay was fed. Inducing SARA by feeding wheat-barley pellets activated a systemic inflammatory response in the steers. (**Key words:** steers, subacute ruminal acidosis, ruminal pH, acute phase response)

Abbreviation key: **Hp** = haptoglobin, **LAL** = *Limulus* amoebocyte lysate, **SAA** = serum amyloid-A, **SARA** = subacute ruminal acidosis.

INTRODUCTION

Subacute ruminal acidosis (**SARA**) is a metabolic disorder characterized by episodes of low rumen pH between 5.2 and 5.6 (Cooper and Klopfenstein, 1996). Clinical signs of SARA are variable and, thus, it is often difficult to identify animals suffering from the disorder. Affected animals may become anorexic, develop intermittent diarrhea, become dehydrated, have unexplained abscesses, and develop laminitis (Nocek, 1997; Kleen et al., 2003). Some of these signs are not specific to SARA and this may lead to SARA being dismissed as other problems such as poor forage quality or poor bunk man-

agement (Nocek, 1997). Laminitis cannot be used to diagnose SARA because of the long time lapse between SARA and appearance of signs of laminitis.

Lipopolysaccharide from rumen gram-negative bacteria has been implicated in diseases that are related to feeding high concentrate diets such as sudden death syndrome, ruminal acidosis, and laminitis (Dougherty et al., 1975; Nagaraja et al., 1978). There is a paucity of literature on changes in rumen fluid LPS concentration due to feeding high concentrate diets. Nagaraja et al. (1978) demonstrated that steers fed high concentrate diets had considerably higher rumen fluid LPS concentration than steers fed only hay, whereas a more recent study by Andersen et al. (1994) did not find such a relationship. It has been suggested that the acidic rumen environment, changes in osmotic pressure, and ruminal LPS may render the rumen epithelium susceptible to injury (Brent, 1976; Enemark et al., 2002; Kleen et al., 2003). These changes may result in translocation of rumen LPS into the prehepatic bloodstream.

The presence of LPS in the bloodstream results in the production of multiple proinflammatory cytokines, reactive oxygen and nitrogen intermediates, and bioactive lipids, which affect the host's metabolic response to inflammation (Baumann and Gauldie, 1994). When released in large quantities, these mediators lead to an acute phase response (Kushner and Rzewnicki, 1994). Profiles of concentrations of some acute phase proteins are used as markers for inflammatory response (Baumann and Gauldie, 1994). Haptoglobin (**Hp**) and serum amyloid-A (**SAA**) are 2 such proteins that are used as inflammatory markers in cattle (Alsemgeest et al., 1994). Their concentrations are elevated because of tissue damage (Conner et al., 1988) or due to intradermal and intravascular LPS injections (Boosman et al., 1989), bacterial infections (Deignan et al., 2000), or viral infections (Heegaard et al., 2000).

Although acute phase proteins have been used as inflammatory markers in various situations where inflammation is the logical development, no studies have investigated inflammatory responses in cattle with SARA. We hypothesize that SARA leads to increased gram-negative bacterial lysis, which increases ruminal LPS concentration. Rumen wall damage associated with

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Corresponding author: J. C. Plaizier; e-mail: plaizier@ms.umanitoba.ca.

SARA further increases ruminal LPS translocation into the bloodstream, resulting in an inflammatory response. Therefore, the objective of this study was to determine changes in rumen fluid LPS, serum Hp, and plasma SAA concentrations in steers due to SARA induced by feeding wheat-barley pellets.

MATERIALS AND METHODS

Three ruminally fistulated adult Jersey steers were kept in metabolism crates at the Animal Science Research Unit at the University of Manitoba throughout the experiment, in accordance with the guidelines of the Canadian Council of Animal Care. The experimental design was a 3×3 Latin square with 21-d periods that were divided into a 5-d treatment period and 16-d rest period. During the treatment period, SARA was induced in the steers by offering diets 1, 2, and 3. Diets were combinations of wheat-barley pellets and chopped alfalfa hay, respectively, in the following ratios (as-fed basis): diet 1 (4 kg: 6 kg); diet 2 (5 kg: 5 kg); and diet 3 (6 kg: 4 kg). The concentrate consisted of 50% wheat and 50% barley. Concentrate and hay were offered in separate meals. All animals were offered 1 kg of chopped alfalfa hay at 0900 h followed by two-thirds of their allocation of concentrate at 1100 h. The remainder of the concentrate was offered at 1300 h. At 1700 h, concentrate not eaten was removed and the steers were offered chopped alfalfa hay, to which they had access throughout the night. The steers were fed hay ad libitum during the 16-d rest period between treatments. The last 2 d of the rest period, when only hay was fed, were designated d -2 and d -1. Days when concentrate was fed were designated d 1 to 5. Dry matter (%), CP, and NDF (%DM) content were 84.8, 18.9, and 27.3% for chopped alfalfa hay, and 93.0, 14.9, and 45.7% for wheat-barley pellets.

Rumen pH was measured continuously throughout the treatment period by placing one indwelling pH probe into the ventral sac of the rumen of each steer as described by Cumby et al. (2001). Measurements were taken every second and averaged over 60 s. Rumen fluid pH data were summarized as average pH, time below pH 6.0, and time below pH 5.6, area (time $\times$ pH) below pH 6.0, and area (time $\times$ pH) below pH 5.6 for each 24-h period. Rumen fluid samples were collected into sterile plastic tubes from the ventral sac of the rumen at 0900 and 1400 h every day during the treatment period. Samples were mixed thoroughly before 25 mL was transferred into sterilized centrifuge tubes and centrifuged for 30 min at $10,000 \times g$. The supernatant was passed through a disposable $0.22\text{-}\mu\text{m}$ sterile, pyrogen-free filter (Millex, Millipore Corporation, Bedford, MA). Samples were further heated at 100°C for 30 min before being stored at -20°C for the determination of rumen

LPS concentration using the *Limulus* amoebocyte lysate (LAL) assay (Levin and Bang, 1964). The assay was performed using a 96-well microplate (BioWhittaker Inc., Walkersville, MD) with absorbance read at 405 nm using a microplate reader (BioRad model 3550, Hercules, CA). Samples were diluted 1000-fold using pyrogen-free water to determine the noninhibitory dilution, with the final dilution being made of 50% diluted sample and 50% β -glucan blocker (BioWhittaker kit number N190; component number: B50-700). β -Glucan blocker blocks the reactivity of LAL to β -1,3-glucans, conferring increased endotoxin specificity to the LAL test. Noninhibitory dilution is achieved when $75 \pm 25\%$ of spike is recovered in positive control samples. An average recovery rate of $78.0 \pm 8.6\%$ ($n = 8$) was achieved in positive controls with a sample dilution of 1:60,000.

Two blood samples (7 mL) were collected by tail venipuncture from each steer on d -2, -1, 1, 2, 3, 4, and 5 into serum and plasma tubes at 0900 h. Serum and plasma were harvested by centrifuging samples at 3000 rpm for 30 min. Haptoglobin and SAA were determined in serum and plasma, respectively, using ELISA Tridelta Phase range kits (Tridelta Diagnostics Inc., Cedar Knolls, NJ; catalog numbers TP-801 and TP-802, respectively) (Makimura and Suzuki, 1982; McDonald et al., 1991). For Hp, serum samples were diluted 1:5 with PBS and vortexed. Seventy-five microliters of diluted serum was added to duplicate wells of a 96-well microtiter plate. Stabilized hemoglobin diluted 1:1 with hemoglobin diluent, was added ($100\text{ }\mu\text{L}$) to the diluted sample. Chromogen and substrate, mixed in a ratio of 9:5 ($140\text{ }\mu\text{L}$), were added to the reaction mixture and incubated at room temperature (25°C) for 5 min. Absorbance was read immediately at 630 nm using a microplate spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA). For SAA, plasma samples were diluted 1:500 in diluent buffer and vortexed. Fifty microliters was added to each well of a 96-well plate coated with $50\text{ }\mu\text{L}$ of biotinylated antiserum amyloid-A monoclonal antibody (diluted 1:100 in $1\times$ diluent buffer). The microtiter plate was covered and incubated at 37°C for at least 1 h and then washed 4 times with diluted wash buffer to remove unbound material. Streptavidin-horse-radish peroxidase conjugate was diluted 1:4000 in diluent buffer and added ($100\text{ }\mu\text{L}$) to each well. The plate was incubated at room temperature (25°C) in darkness for 30 min. The microtiter plate was washed (as described above) and tapped dry. Substrate ($100\text{ }\mu\text{L}$) was added to the plate, which was then incubated at room temperature (25°C) in darkness for an additional 30 min. Stop solution was added, and the plate was read in a microplate reader (BioRad model 3550) at 450 nm.

Table 1. Rumen pH variables of steers offered different levels of wheat-barley pellets and chopped alfalfa hay to induce subacute ruminal acidosis.

	Diet ¹				Day					Effect, <i>P</i> value			
	Diet 1	Diet 2	Diet 3	SEM	1	2	3	4	5	SEM	Diet	Day	Diet × Day
Average pH	6.39	6.29	6.26	0.05	6.49 ^a	6.20 ^{ab}	6.27 ^a	6.19 ^b	6.24 ^{ab}	0.10	0.19	0.006	0.057
Time <pH 5.6, min/d	42 ^b	117 ^{ab}	134 ^a	33.1	5 ^b	80 ^{ab}	78 ^{ab}	187 ^a	174 ^a	42.3	0.045	0.013	0.11
Time <pH 6.0, min/d	308	369	404	77.3	9 ^b	471 ^a	425 ^a	589 ^a	480 ^a	88.0	0.64	<0.001	0.14
Area <pH 5.6, min × pH/d	3	25	35	13.6	1	7	10	54	49	18.1	0.17	0.096	0.19
Area <pH 6.0, min × pH/d	71	124	125	28.9	2 ^b	104 ^{ab}	111 ^{ab}	189 ^a	170 ^a	37.5	0.20	0.005	0.13

^{a,b}LSmeans for diets with different letter superscripts within a row were different ($P < 0.05$).

^{c,d}LSmeans for days with different letter superscripts within a row were different ($P < 0.05$).

¹Diet 1 = 4 kg of wheat-barley pellets and 6 kg of chopped alfalfa hay offered daily; Diet 2 = 5 kg of wheat-barley pellets and 5 kg of chopped alfalfa hay offered daily; Diet 3 = 6 kg of wheat-barley pellets and 4 kg of chopped alfalfa hay offered daily.

Statistical Analyses

Data were analyzed using mixed model analysis with the first-order autoregressive covariance structure in Proc Mixed in SAS (SAS Institute, 1996). The following model was used:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + D_k + (\alpha \times \beta)_{ij} + T_l + (D \times T)_{kl} + e_{ijkl}$$

where Y_{ijkl} = observations for dependent variables; μ = overall mean; α_i = average effect of period i ; β_j = average effect of the animal j ; D_k = average effect of dietary treatment; $(\alpha \times \beta)_{ij}$ = animal × period interaction, which was the main plot error; T_l = average effect of time in days for feed intake, or hours since the beginning of feeding concentrate for blood metabolites or endotoxin concentration; $(D \times T)_{kl}$ = interaction between time and dietary treatment; and e_{ijkl} = subplot error. Lipopolysaccharide concentrations were log transformed because of nonhomogeneous residual error. Orthogonal contrasts were used to compare Hp, SAA, and log-transformed

LPS concentrations during the period when only hay was fed with those when concentrate was fed to induce SARA. Mean differences for rumen pH data and DM intake were separated using Tukey's multiple comparison procedure.

RESULTS AND DISCUSSION

Average rumen fluid pH, time, and area with pH below 6.0 or 5.6 did not differ among the diets (Table 1). However, feeding concentrate decreased average daily pH from d 1 to a nadir on d 4. Both time below pH 6.0 and time below pH 5.6 increased from d 1 to a peak on d 4. Area below pH 6.0 increased from d 1 to a peak on d 4 but area below pH 5.6 showed only numerical increases ($P = 0.096$; Table 1). Because the steers spent 187 and 174 min/d with pH below 5.6 on d 4 and 5, respectively, we concluded that SARA was successfully induced on these days. The severity of SARA was less than that experienced by cows used in studies by Krajcarski-Hunt et al. (2002), who reported time and area below pH 5.6 of 594.4 ± 188.9 min/d and 228.0 ± 88.8 min × pH/d,

Table 2. Feed intake of steers offered different levels of wheat-barley pellets and chopped alfalfa hay to induce subacute ruminal acidosis.¹

	Day					SEM	Effect, <i>P</i> value		
	1	2	3	4	5		Diet	Day	Diet × Day
Concentrate (kg of DM/d)									
Diet 1	3.72	2.68	3.14	2.62	2.84	0.75	0.005	0.01	0.28
Diet 2	4.65	4.65	4.05	3.26	3.45	0.75			
Diet 3	5.61 ^a	4.47 ^a	5.61 ^a	4.26 ^a	1.91 ^b	0.88			
Average across diets	4.66 ^a	3.93 ^a	4.27 ^a	3.38 ^{ab}	2.73 ^{ab}	0.56			
Chopped alfalfa hay (kg of DM/d)									
Diet 1	5.06 ^a	4.69 ^{ab}	2.59 ^b	3.71 ^{ab}	3.50 ^{ab}	0.48	0.085	<0.0001	0.036
Diet 2	4.24 ^a	4.40 ^a	4.08 ^a	2.78 ^b	2.08 ^b	0.48			
Diet 3	3.72 ^a	4.25 ^a	3.35 ^{ab}	1.98 ^{ab}	0.83 ^b	0.59			
Average across	4.34 ^a	4.45 ^a	3.34 ^b	2.82 ^{bc}	2.14 ^c	0.28			

^{a,b,c}Means with different superscripts within each row differed ($P < 0.05$).

¹Diet 1 = 4 kg of wheat-barley pellets and 6 kg of chopped alfalfa hay offered daily; Diet 2 = 5 kg of wheat-barley pellets and 5 kg of chopped alfalfa hay offered daily; Diet 3 = 6 kg of wheat-barley pellets and 4 kg of chopped alfalfa hay offered daily.

respectively. The rumen fluid pH that defines SARA is still a controversial issue, with various threshold values having been arbitrarily set to define SARA. For example, threshold values of 5.5 (Hibbard et al., 1995), 5.6 (Cooper and Klopfenstein, 1996), 5.8 (Beauchemin et al., 2001), and 6.0 (Kriehbiel et al., 1995) have been used to define the upper bound of pH that defines SARA. The duration for which the pH must remain below this threshold has not been properly defined.

Offering different amounts of concentrate and hay was intended to induce SARA to different extents. Thus, diets were designed to differ significantly in their DM intake. Intake of both concentrate and hay was variable in all diets. However, offering 6 kg of concentrate resulted in more variable intake response among days compared with the other 2 diets. Dry matter intake of hay was more variable than that of concentrate and may be the reason for the diet $\times$ day interaction (Table 2). Animals went through cycles in which a high intake on one day was followed by low intake the following day. Averaged across diets, the decrease in DM intake was greatest between d 3 and 4 for concentrate and between d 2 and 3 for hay. The steers appeared to reduce hay intake in favor of concentrate on d 2 and 3. Variations in intake could explain the large variation in rumen pH among days that was also observed (Table 1).

Different levels of wheat-barley pellets in the diets did not affect LPS concentration. However, wheat-barley pellets increased (compared with hay-only days) LPS concentration from d 1 to a peak on d 4 ($P < 0.05$; Figure 1). The peak coincided with the day when the time with pH below 5.6 was greatest. Ruminal LPS increased from 3715 endotoxin units per mL for the 0900-h sample on d 1 (before concentrate was fed) to a peak of 12,589 endotoxin units/mL on d 4. This may have been due to an increase in free LPS under acidic conditions. High concentrations of LPS with low rumen fluid pH may be due to increased lysis of dead bacterial cells or shedding of free LPS from rapidly growing gram-negative bacteria (Nagaraja et al., 1978).

Haptoglobin concentration in blood serum was not different in animals fed different diets. However, inducing SARA increased concentrations of Hp from 0.43 ± 0.14 (when only hay was fed) to 0.79 ± 0.14 mg/mL on d 5 of the treatment period ($P < 0.05$; Figure 1). Inducing SARA resulted in higher Hp concentrations on d 3 and 5 ($P < 0.05$). Other researchers have reported that Hp concentrations are undetectable in healthy cattle, with concentrations only becoming detectable when there is an inflammatory response (Deignan et al., 2000). In the present study, low concentrations were detected over the 3 d before feeding wheat-barley pellets. The response in Hp concentration to SARA was low compared with levels

Figure 1. Concentrations of ruminal LPS (log₁₀ endotoxin units/mL; panel A), haptoglobin (Hp; panel B), and serum amyloid-A (SAA; panel C) in steers fed chopped alfalfa hay only (d -2 and d -1) and during concentrate feeding (d 1 to 5). Orthogonal contrasts were used to determine which concentrations differed from baseline values. Lipopolysaccharide concentrations measured in samples collected before feeding concentrate on d 1 were taken as baseline. Bars indicate SE, and significance was declared at the 5% level of significance.

seen in experimentally induced bacterial and virus infections, in which Hp concentrations increased 100-fold (Deignan et al., 2000; Heegaard et al., 2000).

Serum amyloid-A concentrations were not significantly different among the different diets but concentrations in blood plasma increased from 33.6 ± 36.53 (when only hay was fed) to 170.7 ± 36.53 $\mu\text{g/mL}$ on d 5 ($P < 0.001$), when concentrate was offered in addition to hay (Figure 1). Serum amyloid-A is the other major acute phase protein, and is a more sensitive marker of inflammatory challenge than Hp (Horadagoda et al., 1999). The acute phase protein profiles obtained in the present study indicate that as time with pH below 5.6 increased, the intensity of the acute phase response increased (Figure 1). This could be due to formation of extensive microlesions on the ruminal epithelium leading to increased LPS translocation across the ruminal epithelium into the systemic circulation, which would amplify the acute phase response associated with SARA. To our knowledge, this is the first study that has examined an acute phase response due to SARA.

Therefore, in conclusion, offering steers wheat-barley pellets and chopped alfalfa hay successfully induced SARA, particularly on d 4 and 5 of our study, although DM intake declined from d 1 to 5. Inducing SARA increased the concentration of LPS in rumen fluid, plasma SAA, and serum Hp concentrations. Although SAA concentrations increased within 24 h of feeding concentrate, Hp levels did not change until the third day of feeding concentrate. Inducing SARA increased rumen LPS concentration and initiated an acute phase response. Causal factors for inflammatory response may be an increase in LPS and bacterial translocation into the prehepatic circulation. Further work needs to be done under controlled feed intake conditions to remove possible confounding of LPS concentration with variable feed intake.

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