Effects of bacterial lipopolysaccharide injection on white blood cell counts, hematological variables, and serum glucose, insulin, and cortisol concentrations in ewes fed low- or high-protein diets


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ABSTRACT: Bacterial lipopolysaccharide endotoxins (LPS) elicit inflammatory responses reflective of acute bacterial infection. We determined if feeding ewes high-CP (15.5%) or low-CP (8.5%) diets for 10 d altered inflammatory responses to an intravenous bolus of 0 (control), 0.75 (L75), or 1.50 (L150) μg of LPS/kg of BW in a 2 × 3 factorial arrangement of treatments (n = 5/treatment). Rectal temperatures, heart and respiratory rates, blood leukocyte concentrations, and serum cortisol, insulin, and glucose concentrations were measured for 24 h after an LPS bolus (bolus = 0 h). In general, rectal temperatures were greater (P ≤ 0.05) in control ewes fed high CP, but LPS increased (P ≤ 0.05) rectal temperatures in a dose-dependent manner at most times between 2 and 24 h after the bolus. Peak rectal temperatures in L75 and L150 occurred 4 h after the bolus. A monophasic, dose-independent increase (P ≤ 0.023) in serum cortisol occurred from 0.5 to 24 h after the bolus, with peak cortisol at 4 h. Serum insulin was increased (P ≤ 0.016) by LPS in a dose-dependent manner from 4 to 24 h after the bolus. Insulin did not differ between control ewes fed high- and low-CP diets but was greater (P < 0.001) in L75 ewes fed low CP compared with high CP and in L150 ewes fed high CP compared with low CP. Increased insulin was not preceded by increased serum glucose. Total white blood cell concentrations were not affected (P ≥ 0.135) by LPS, but the neutrophil and monocyte fractions of white blood cells were increased (P ≤ 0.135) by LPS, and the lymphocyte fraction was increased (P = 0.037) at 2 h and decreased (P ≤ 0.006) at 12 and 24 h after the bolus. Red blood cell and hemoglobin concentrations and hematocrit (%) were increased (P ≤ 0.022) at 2 and 4 h after the bolus. Rectal temperatures and serum glucose were greater (P ≤ 0.033) in ewes fed a high-CP diet before LPS injection, but these effects were lost at and within 2.5 h of the bolus, respectively. Feeding high-CP diets for 10 d did not reduce inflammation in ewes during the first 24 h after LPS exposure but may benefit livestock by preventing acute insulin resistance when endotoxin exposure is mild.

Key words: cortisol, crude protein, insulin, lipopolysaccharide, white blood cell

INTRODUCTION

Bacterial and viral infections reduce livestock performance, increase morbidity and death loss, and are leading sources of profit loss in livestock (Duff and Galyean, 2007). Exposure to bacterial lipopolysaccharides (LPS) replicates infection in livestock (Steiger et al., 1999; Thorgersen et al., 2010) and allows the study of inflammatory responses without the risk of using a live pathogen. In previous studies, LPS exposure depleted plasma AA and glucose concentrations (Briard et al., 2000; Waggoner et al., 2009) and increased serum cortisol (You et al., 2008; Karrow et al., 2010). These findings indicate that catabolism increases during systemic inflammation (Soto et al., 1998) and may increase CP requirements (Waggoner et al., 2009). The effects of feeding CP above NRC (2007) requirements on immune function and inflammation in livestock are not clear (Duff and Galyean, 2007), but a CP deficiency in mice suppressed viral defenses, which in turn predisposed the mice to bacterial infections (Jakab et al., 1981). In the present study, we sought to determine whether feed-
Materials and Methods

All procedures involving animals were approved by the New Mexico State University Institutional Animal Care and Use Committee.

Animals and Experimental Procedure

Thirty mature (4 to 6 yr of age) Rambouillet ewes in moderate body condition (61.8 ± 1.4 kg of BW) were housed in adjacent individual 1.5 × 4 m pens and given free access to shade and water for the duration of the experiment. Ewes were randomly assigned to treatment combinations in a 2 × 3 factorial arrangement. Each ewe was fed either more (high CP) or slightly less (low CP) than NRC (2007) requirements for dietary CP (Table 1) at an amount equal to 3% of initial BW (as-fed basis). Ewes were fed once daily at 1800 h for 10 d. Beginning at 0900 h on d 11, each ewe was injected intravenously with a bolus of bacterial LPS (Escherichia coli O55:B55, Sigma Chemical Co., St. Louis, MO) at 0 (control), 0.75 (L75), or 1.50 (L150) μg/kg of BW. A bulk LPS solution was prepared by dissolving 2,000 μg of LPS in 100 mL of sterile physiological saline. Thus, injected volumes ranged from 1.8 to 5.6 mL of LPS solution, depending on individual BW and prescribed LPS treatment. Each control ewe was intravenously injected with 2 mL of sterile physiological saline. Blood samples, heart rate, respiratory rate, and rectal temperature were collected from each ewe 0.5 h before (−0.5 h) and immediately before (0 h) LPS injection, and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 12, and 24 h after LPS injection. Feeding on this day occurred immediately after sample collection at 12 h.

Blood Sample Analyses

Serum Cortisol, Insulin, and Glucose. Blood samples were collected via jugular venipuncture into 10-mL vacuum tubes (Corvac serum-separator, Kendall Health Care, St. Louis, MO). Blood samples were kept at room temperature for 30 to 60 min and then centrifuged (1,500 × g at 4°C for 15 min). After centrifugation, serum was stored in plastic vials at −80°C until thawed for assay. Serum cortisol concentrations were quantifyed by solid-phase RIA using components of a commercial kit (Coat-A-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA) with the modifications described by Kiyma et al. (2004). Within-assay and between-assay CV for cortisol determinations were less than 15%. Serum insulin concentrations were determined from duplicate 50-μL aliquots with a commercial ELISA kit (Ovine Insulin, ALPCO Diagnostics, Windham, NH) for samples collected at 0, 0.5, 2, 4, 12, and 24 h after the bolus. Serum glucose concentrations were quantified colorimetrically with a commercially available hexokinase reagent (Infinity TR15241, Thermo Scientific, Waltham, MA), as described previously (Waggoner et al., 2009). Within-assay and between-assay CV for insulin and glucose determinations were less than 15%.

Complete Blood Counts. Whole blood samples were collected by jugular venipuncture into EDTA-containing vacuum tubes at 0, 2, 4, 12, and 24 h after the LPS bolus. Samples were packaged on ice immediately after collection and were shipped overnight to Veterinary Diagnostics Services (Albuquerque, NM) for complete blood count analysis, which included quantification of total white blood cells, total red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelets, and neutrophil, lymphocyte, monocyte, eosinophil, and basophil fractions of total white blood cells.

Statistical Analysis

Data for respiratory rate, heart rate, rectal temperature, serum cortisol, serum glucose, serum insulin, white blood cell fractions, and hematological variables were analyzed as a split-plot design with ewe as the experimental unit. The main plot contained the 2 × 3 factorial arrangement of dietary CP (high CP or low CP), LPS (0, 0.75, or 1.50 μg/kg of BW), and the diet × LPS interaction. The subplot contained the time from the LPS bolus and all associated interactions. Data were analyzed using the MIXED procedure (SAS Inst. Inc., Cary, NC) with repeated measures (time) and a compound symmetry covariance structure. When F-tests were significant (P ≤ 0.05), linear (dose-dependent) and quadratic (dose-independent) relationships between LPS treatment means were examined with orthogonal polynomial contrasts. Area under the curve for rectal temperature over the first 6 h after the bolus was calculated by trapezoidal summation. Because

<table>
<thead>
<tr>
<th>Item</th>
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<th>High CP</th>
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<tr>
<td>Ingredient, % of DM</td>
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</tr>
<tr>
<td>Sudan hay</td>
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<td>Corn grain, cracked</td>
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<td>13.9</td>
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<td>Soybean meal</td>
<td>—</td>
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<tr>
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<tr>
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<tr>
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<td>Dicalcium phosphate</td>
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1 Analyzed by SDK Laboratories (Hutchinson, KS).
of pretreatment differences between LPS-injected and control ewes for red blood cells, hemoglobin, and hematocrit, these data were reanalyzed with the value at 0 h included in the model. Data are presented as least squares means ± SE. For comparisons between means, \( P \leq 0.05 \) was considered significant, and \( P \leq 0.10 \) was considered a tendency.

RESULTS

**Respiratory Rate and Heart Rate**

An LPS dose \( \times \) time interaction (\( P \leq 0.005 \)) was observed for respiratory rate and heart rate. Respiratory rate was greater (\( P \leq 0.056 \)) in control ewes than in LPS-treated ewes at 1 and 2.5 h after the bolus, and heart rate was increased (\( P \leq 0.031 \)) in L150 ewes at 5 and 12 h compared with controls. Respiratory rates in L75 and L150 ewes increased (\( P \leq 0.001 \)) from 3 to 4 h after the bolus compared with rates at 0 h. In control and L75 ewes, heart rates were constant across the 24-h sampling period, whereas the rate transiently increased (\( P = 0.051 \)) in L150 ewes at 1.5 h compared with the rate at 0 h (data not shown).

**Rectal Temperature**

A LPS \( \times \) dietary CP concentration \( \times \) time interaction (\( P = 0.020 \)) was observed for rectal temperature (Figure 1). In general, rectal temperatures were greater (\( P \leq 0.05 \)) in control ewes fed high CP compared with low CP, but LPS increased (\( P \leq 0.05 \)) rectal temperature in a dose-dependent manner, regardless of diet. The peak rectal temperature occurred 4 h after the bolus in both L75 and L150. Area under the curve for rectal temperature during the first 6 h after the bolus did not differ (\( P = 0.580 \)) between ewes fed high and low CP (108,545 ± 286 and 108,772 ± 286 units, respectively), but was increased (\( P < 0.001 \)) by LPS independent of dose (106,039 ± 350, 110,061 ± 350, and 109,876 ± 350 units for controls, L75, and L150, respectively).

**Serum Cortisol Concentration**

An LPS \( \times \) time interaction (\( P \leq 0.001 \)) was observed for serum cortisol concentrations. In control ewes, serum cortisol concentrations (Figure 2) were constant (\( P \geq 0.10 \)) across the 24-h sampling period, but the LPS injection increased (\( P \leq 0.023 \)) cortisol in a dose-

![Figure 1](image-url)
independent manner at 0.5 h after the bolus and at all subsequent times. Peak serum cortisol in L75 and L150 ewes occurred 4 h after the bolus. Serum cortisol concentrations did not differ ($P = 0.293$) between ewes fed high- or low-CP diets.

**Serum Glucose and Insulin Concentrations**

A dietary CP concentration × time interaction ($P = 0.002$) was observed for serum glucose concentrations. Serum glucose (Figure 3) was greater ($P \leq 0.033$) in ewes fed high-CP diets compared with those fed low-CP diets at 1 and 2 h, and tended ($P \leq 0.092$) to be greater at 0, 0.5, and 2.5 h after the bolus. Serum glucose was reduced ($P \leq 0.059$) in a dose-independent manner by LPS at 1.5 h and from 2.5 to 24 h after the bolus. An LPS × time interaction ($P = 0.066$) was observed for serum insulin concentrations. Additionally, a tendency ($P = 0.083$) for an LPS × CP interaction was observed. Insulin (Figure 4) was constant ($P \geq 0.10$) across the 24-h sampling period. Neutrophil fractions of total white blood cells, and a dietary CP concentration × time interaction ($P = 0.050$) was observed for the eosinophil fraction. The number of total white blood cells per microliter of blood was not affected ($P \geq 0.431$) by LPS dose or CP concentration, but was less ($P \leq 0.014$) at 2 h (7.7 ± 1.1 cells × 10$^3$) and 4 h (8.6 ± 1.2 cells × 10$^3$) than at 0 h (13.7 ± 1.0 cells × 10$^3$), 12 h (14.0 ± 1.4 cells × 10$^3$), and 24 h (16.5 ± 1.5 cells × 10$^3$) in all ewes, independent of the diet or LPS dose. In control ewes, the neutrophil, lymphocyte, and monocyte fractions of total white blood cells (Figure 5) were constant ($P \geq 0.10$) across the 24-h sampling period. Neutrophil fractions were increased ($P \leq 0.010$) by LPS in a dose-independent manner at 12 and 24 h. Lymphocyte fractions were increased ($P \leq 0.051$) by LPS in a dose-independent manner at 2 h, were not changed ($P = 0.558$) at 4 h, and decreased ($P \leq 0.006$) in a dose-independent manner at 14 and 24 h after the bolus. Monocyte fractions were increased by LPS ($P = 0.026$) in a dose-independent manner 24 h after the bolus but were not different ($P \geq 0.05$) before 24 h. Eosinophil fractions did not differ ($P = 0.158$) among LPS treatments but were less ($P = 0.043$) in ewes fed high CP at 2 and 24 h after the bolus. We did not observe any effects ($P \geq 0.10$) of dietary CP, LPS, or time from the bolus on the basophil fraction of total white blood cells (data not shown).

**White Blood Cells**

An LPS × time interaction ($P \leq 0.049$) was observed for the neutrophil, lymphocyte, monocyte, and eosinophil fractions of total white blood cells, and a dietary CP concentration × time interaction ($P = 0.050$) was observed for the eosinophil fraction. The number of total white blood cells per microliter of blood was not affected ($P \geq 0.431$) by LPS dose or CP concentration, but was less ($P \leq 0.014$) at 2 h (7.7 ± 1.1 cells × 10$^3$) and 4 h (8.6 ± 1.2 cells × 10$^3$) than at 0 h (13.7 ± 1.0 cells × 10$^3$), 12 h (14.0 ± 1.4 cells × 10$^3$), and 24 h (16.5 ± 1.5 cells × 10$^3$) in all ewes, independent of the diet or LPS dose. In control ewes, the neutrophil, lymphocyte, and monocyte fractions of total white blood cells (Figure 5) were constant ($P \geq 0.10$) across the 24-h sampling period. Neutrophil fractions were increased ($P \leq 0.010$) by LPS in a dose-independent manner at 12 and 24 h. Lymphocyte fractions were increased ($P \leq 0.051$) by LPS in a dose-independent manner at 2 h, were not changed ($P = 0.558$) at 4 h, and decreased ($P \leq 0.006$) in a dose-independent manner at 14 and 24 h after the bolus. Monocyte fractions were increased by LPS ($P = 0.026$) in a dose-independent manner 24 h after the bolus but were not different ($P \geq 0.05$) before 24 h. Eosinophil fractions did not differ ($P = 0.158$) among LPS treatments but were less ($P = 0.043$) in ewes fed high CP at 2 and 24 h after the bolus. We did not observe any effects ($P \geq 0.10$) of dietary CP, LPS, or time from the bolus on the basophil fraction of total white blood cells (data not shown).

**Hematological Variables**

An LPS × time interaction ($P \leq 0.039$) was observed for red blood cells, hemoglobin, and hematocrit. Red
blood cells, hemoglobin, and hematocrit (Figure 6) did not differ ($P \geq 0.10$) between dietary CP concentrations, but were increased ($P \leq 0.022$) in a dose-independent manner by LPS at 2 and 4 h after the bolus. We did not observe any effects ($P \geq 0.10$) of dietary CP, LPS, or time from the bolus on mean corpuscular volume, mean corpuscular hemoglobin concentrations, or platelets (data not shown).

**DISCUSSION**

Our results showed that feeding CP above NRC (2007) requirements for 10 d did not reduce inflammation during the first 24 h after exposure to bacterial endotoxins. Rectal temperatures were greater in control ewes fed high CP compared with low CP for most of the first 6 h after the saline bolus. Furthermore, rectal

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**Figure 3.** Serum glucose concentrations of ewes administered intravenous boluses of lipopolysaccharides (LPS) or saline (top; n = 5 per LPS × CP) after being fed high- or low-CP diets for 10 d (bottom). An asterisk (*) indicates times at which a dose-independent effect of LPS was observed (top; $P \leq 0.059$) or times at which means for ewes fed high or low dietary CP differed (bottom; $P \leq 0.033$). A pound sign (#) indicates times at which means for ewes fed high or low CP tended to differ ($P \leq 0.092$). Effects on serum glucose concentration were LPS × CP × time ($P = 0.841$), LPS × time ($P = 0.197$), CP × time ($P = 0.002$), CP × LPS ($P = 0.806$), time within CP ($P \leq 0.001$), and LPS ($P = 0.022$). Parentheses reflect gaps in time of 6 and 12 h.
temperatures in all ewes fed high CP were generally greater than in those fed low CP before the LPS bolus, indicating the greater CP concentration in the diet increased metabolic heat production, as previously shown in rats (Yamaoka et al., 2009). Ahmed and Abdellatif (1995) found similar effects of dietary CP on body temperature in unchallenged desert sheep. However, effects of CP were not observed in ewes injected with LPS because rectal temperatures were increased in a dose-dependent manner to magnitudes that were similar between ewes fed high and low CP. This finding indicates that the increased metabolic heat production attributable to greater CP was masked by the febrile response to LPS or that metabolic dysregulation occurred with endotoxicity.

Eosinophils were the only class of leukocytes affected by CP concentration and, along with basophils, were not affected by the LPS injection. All other leukocytes responded to LPS at some point within 24 h of exposure, and all these responses were independent of
dietary CP. Dietary CP concentration previously had no effect on leukocyte profiles in healthy, unchallenged ewes (Gentry et al., 1999), and neutrophil and monocyte responses to endotoxin exposure were consistent with observations in pigs (Williams et al., 2009). Although leukocytes are known to produce pyrogenic cytokines, LPS-induced febrile responses in the present study preceded the observed changes in blood leukocyte profiles and were likely initiated by a source other than white blood cells. Furthermore, we observed this increase in rectal temperature to be monophasic, whereas Whyte et al. (1989) observed a biphasic febrile response that bracketed changes in leukocyte profiles; they attributed only the second peak in rectal temperature

Figure 5. Neutrophil (top), lymphocyte (middle), and monocyte (bottom) fractions of total white blood cells (WBC) of ewes fed high- or low-CP diets and administered intravenous boluses of 0 (♦), 0.75 (■), or 1.50 (Δ) μg of lipopolysaccharides (LPS)/kg of BW (n = 5 per LPS × CP). The asterisk (*) dose-dependent and pound sign (#; dose-dependent) indicate times at which effects of LPS were observed (P ≤ 0.016). Effects on these fractions were LPS × dietary CP × time (P ≥ 0.217), LPS × time (P ≤ 0.004), CP × time (P ≥ 0.075), CP × LPS (P ≥ 0.300), time (P ≤ 0.004), and CP (P ≥ 0.224). Parentheses reflect gaps in time of 6 and 12 h.

Figure 6. Red blood cells (top), hemoglobin (middle), and hematocrit (bottom) of ewes fed high- or low-CP diets and administered intravenous boluses of 0 (♦), 0.75 (■), or 1.50 (Δ) μg of lipopolysaccharides (LPS)/kg of BW (n = 5 per LPS × CP). An asterisk (*) indicates times at which a dose-independent effect of LPS was observed (P ≤ 0.022). Effects on these blood components were LPS × dietary CP × time (P ≥ 0.388), LPS × time (P ≤ 0.034), CP × time (P ≥ 0.181), CP × LPS (P ≥ 0.217), time (P ≤ 0.002), CP (P ≥ 0.316), and time 0 (P ≤ 0.001). Parentheses reflect gaps in time of 6 and 12 h.
to leukocyte-derived cytokines. Morimoto et al. (1987) further explained that in rabbits, the initial increase in temperature was due to nonleukocyte-derived pyrogenic compounds and that any leukocyte-induced pyrogenesis might be delayed by several hours. Regardless of the influence of leukocytes on rectal temperature, leukocyte responses to LPS exposure were not affected by dietary CP concentration. Red blood cells, hemoglobin, and hematocrit all increased after the LPS injection. Thorgersen et al. (2010) reported a similar increase in hemoglobin in pigs, although the mechanism by which this occurs is unclear.

Unlike other components of inflammation, the insulin response to LPS was affected by dietary CP concentrations. In ewes fed low CP, insulin concentrations after the injection of either LPS dose increased similarly when compared with controls. However, in ewes fed high CP, the smaller LPS dose did not affect insulin, whereas the larger dose increased it to an even greater extent than in ewes fed low CP. Additionally, increased insulin concentrations were not preceded by hyperglycemia but instead coincided with increased cortisol concentrations, indicating that serum insulin was increased as a result of insulin insensitivity. Andrews and Walker (1999) reported that increased glucocorticoid action can oppose insulin signaling and reduce sensitivity in tissues, whereas cytokines may also contribute to transient insulin resistance (Kushibiki et al., 2000). Our data indicate that a larger dose of LPS was needed to elicit increased insulin in ewes fed high-CP diets, but when elicited, the increase was more robust than in ewes fed low CP. Increased insulin concentrations may also have resulted from stimulation by non-glucose substrates, such as propionate, butyrate, or AA (McAtee and Trenkle, 1971; Brockman, 1982), which may increase during inflammation.

In summary, feeding CP above NRC (2007) requirements for 10 d did little to alter the inflammatory response to injection of bacterial endotoxins but did appear to increase the dose needed to elicit acute insulin resistance. Furthermore, the effects of high CP on serum glucose and rectal temperatures that were observed in the absence of LPS were lost after LPS exposure. These findings indicate that increasing the CP concentration in the diet for a short time does not reduce inflammation during the first 24 h after endotoxin exposure but may benefit livestock by tempering insulin resistance when endotoxin exposure is mild.

**LITERATURE CITED**


