Nutritional Regulation of Plasma Tumor Necrosis Factor–α and Plasma and Urinary Nitrite/Nitrate Responses to Endotoxin in Cattle (44146)

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Abstract. **Effects of dietary protein level with and without L-arginine (Arg) infusion on plasma tumor necrosis factor–α (TNF-α) response to endotoxin (lipopolysaccharide [LPS]) as well as plasma concentration and urine output of nitrite and nitrate (NO_x), the stable end products of nitric oxide radical (NO), were studied in beef heifers (275–310 kg body wt). The animals were fed low- (LP; 7.96%) or high- (HP; 13.94%) protein diets for 10 days before LPS administration (*Escherichia coli;* 0.2 μg/kg, iv). L-Arginine in saline (0.5 g/kg body wt) or saline was infused for 8 hr with one-third of total Arg infused before LPS administration. Plasma TNF-α concentrations increased in all heifers after LPS injection (peak at 1 hr and return to baseline at 4 hr); however, concentrations were lower in HP- than in LP-fed heifers at 1, 2, and 3 hr. Infusion of Arg did not affect plasma TNF-α response to LPS. Plasma NO_x concentrations increased in all heifers after LPS challenge; compared with saline, Arg infusion increased the total response (integrated area under concentration curve) in LP- but not in HP-fed heifers. Relative to pretreatment period, the rate of NO_x output in urine collected 2–6 hr after LPS administration increased in all heifers regardless of dietary protein level and was further amplified by Arg infusion. The rate of NO_x output in urine collected 6–24 hr after LPS challenge was even higher in LP-fed heifers infused with Arg but returned to the basal values in other groups. Activity of hepatic inducible NO synthase was not affected by LPS, Arg, or dietary protein level at the time points studied. The data suggest that dietary protein levels can modulate both TNF-α and NO responses to LPS in cattle; high dietary protein intake decreases TNF-α response and attenuates the conversion of supplemental Arg to NO.** [P.S.E.B.M. 1997, Vol 215]

Many physiological effects of septicemia and endotoxemia are mediated by the acute release of cytokines from circulating and resident populations of immune cells, monocytes, macrophages, and neutrophils in particular (1). Cellular immune responses as well as a variety of physiological processes, including regulation of blood flow, hormone release, reproductive function, neurotransmission, and tumoricidal and microbicidal activities, are linked to the production of nitric oxide (NO), a short-lived gaseous effector molecule synthesized from L-arginine (Arg) by several isoforms of nitric oxide synthase (2, 3). Two principal categories of nitric oxide synthase (NOS) exist, a Ca^{2+}-calmodulin–regulated, constitutively produced NOS (cNOS) mainly present in vascular endothelium (NOS III) and neurons (NOS I) and associated with generalized maintenance functions in the body, and a Ca^{2+}-independent, inducible NOS (iNOS, NOS II) present in macrophages, neutrophils, hepatocytes, and other cells (2, 4, 5). The iNOS isoform is induced mainly by endotoxin, (lipopolysaccharide [LPS]) and cytokines (6, 7). During disease stress,

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pathological consequences of both over- and underproduction of the cytokine tumor necrosis factor-α (TNF-α) and NO have been documented (8, 9).

Powanda (10) suggested that the state of nutrition at the time of immune stress encounter profoundly impacts the intensity of the acute-phase response and recovery from disease insult. It is clear that plane of nutrition affects immune response in general and may affect the release and sensitivity of the body to cytokines (11). Modulation of disease responses in cattle could have significant impact on the economics of food production. Presently, few studies have characterized cytokine responses to systemic disease stress and the impact of changes in NOS activity in cattle *in vivo*. Currently available cell-based cytotoxicity or proliferation assays for cytokines in plasma of ruminants are confounded by the presence of endogenous undefined compounds that are themselves toxic to cytokine-specific target cells (12). Because of the short *in vivo* half-life of NO, traditional implications of change in NOS activity have been made in association with measurements of nitrate and nitrite in plasma and urine (13). The origin of cytokine-mediated conversion of Arg to nitrite and nitrate was also validated in studies wherein ^{15}N-arginine was infused in humans and the measured increase in excreted $^{15}NO_3^-$ was highly correlated with substrate origin (14). Böger *et al.* (15) demonstrated that long-term oral administration of Arg increased NO production in rats, as assessed by the urinary excretion of nitrate and cyclic guanosine monophosphate.

In this study we evaluated the effect of different levels of dietary protein on the plasma TNF-α and plasma and urine nitrite and nitrate (NO_x) responses to LPS challenge in cattle. In addition, we determined whether coadministration of substrate L-Arg could amplify underlying NOS-mediated NO_x production in response to LPS. Knowing that the levels of protein fed were capable of altering liver urea metabolism enzymes, arginase in particular (16), and that Arg is utilized in both the NOS and urea cycle pathways (17), we hypothesized that these specific components of nutrition might alter the TNF-α and NOS aspects of immune response to LPS.

Materials and Methods

Chemicals. All chemicals were from Sigma Chemical Co. (St. Louis, MO) unless specified.

Animals and Experimental Design. This experiment was performed in accordance with approval of the Animal Care and Use Committee at the USDA Agricultural Research Service (Beltsville, MD). The experiment was conducted as an unbalanced 2 × 2 factorial in two replications with dietary protein level and Arg infusion as main treatments. In the first replication, eight heifers (275–310 kg body wt) were used, with two heifers assigned to each treatment. In the second replication, the same heifers were used, and two additional animals were added. The heifers were fed low- (LP: 7.96% crude protein; 6.5 kg/day) or high- (HP: 13.94% crude protein; 7.2 kg/day) protein isocaloric diets (1.96 Mcal metabolizable energy/kg dry matter) for 10 days before LPS administration. L-Arginine (0.5 g/kg) in 0.9% saline or 0.9% saline alone was infused *via* a jugular cannula for 8 hr starting at 0600, with one-third of total Arg infused before LPS administration at 0800 (0.2 μg/kg, iv bolus, *Escherichia coli,* 055:B5; Sigma). Treatment assignments for heifers were switched between the first and second replication such that animals on HP became LP, animals on LP became HP; additionally, the assignments to Arg or saline infusion were reversed. The two extra heifers in Replication 2 were assigned to LP + saline and HP + Arg, respectively. In a given replication, heifers were divided into two equal groups, which were sampled and challenged on successive weeks. At least 3 weeks elapsed between LPS challenges in heifers used in both replicates. Blood samples were obtained immediately before (0 hr) and then 1, 2, 3, 4, 6, and 8 hr after LPS injection. Total urine output was collected *via* urinary catheter during five collection periods starting 20 hr before and ending 24 hr after LPS challenge (−20 to −12, −12 to −2, −2 to 2, 2 to 6, and 6 to 24 hr). For liver biopsy, heifers were sedated with xylazine (Rompun) and biopsy samples (~20 mg/sample, four samples) were obtained transcutaneously with external and internal local anesthesia using lidocaine with epinephrine-HCl. Puncture sites were infiltrated with procaine penicillin at the end of the procedure. Biopsy samples were collected 20 hr before, and then 6 and 24 hr after LPS injection using a 2-in. by 12-gauge guide trochar to reach the inner abdomen and a commercial biopsy instrument (True-Cut; Baxter, Columbia, MD) inserted to a depth of approximately 10 cm. Immediately after removal, liver samples were frozen in liquid N_2. Blood plasma and urine samples were stored at −20°C and biopsy samples at −80°C until assayed.

Plasma TNF-α Determination. Immunoreactive TNF-α was measured by specific double antibody RIA as previously described (18), using antisera generated in our laboratory to recombinant bovine TNF-α (Ciba-Geigy, Basel, Switzerland).

Nitrite + Nitrate Determination in Plasma and Urine. The stable end products of the NO pathway, $NO_2^- + NO_3^-$, were measured according to a modified method of Schmidt *et al.* (19). The method is based on the diazotization reaction after initial enzymatic reduction of NO_3^- to NO_2^- by nitrate reductase (*Aspergillus* species; EC 1.6.6.2; Boehringer-Mannheim, Indianapolis, IN). In brief, samples were diluted in water (urine 1:4, plasma 1:2) and incubated with nitrate reductase in the presence of NADPH and FAD followed by incubation with lactate dehydrogenase (EC 1.1.1.27; Boehringer-Mannheim) and sodium pyruvate to oxidize excess NADPH. After the incubation, sulfanilamide and HCl were added to the samples and protein was precipitated with trichloroacetic acid. Samples were then centrifuged and supernatant was transferred to a microtiter plate. After the addition of *N*-[1-naphthyl]ethylene diamine and incubation at room temperature, the absorbance was measured at 546 nm and compared with a stan-

dard of $NaNO_2$. Results obtained by this assay will be referred to as NO_x.

Inducible NO Synthase Determination. Activity of iNOS was determined by analyzing the conversion of [^{3}H]arginine to [^{3}H]citrulline according to a modified method of Salter *et al.* (4). In brief, liver biopsy samples were homogenized (1:5, w/v) using a microcentrifuge pestle (Kontes, Vineland, NJ) in 50 m*M* HEPES (pH 7.4) containing 320 m*M* sucrose, 1 m*M* dithiothreitol, 1 m*M* EDTA, and protease inhibitors (1 μg/ml antipain, 1 μg/ml aprotinin, 1 μg/ml leupeptine, 1 μg/ml pepstatin, 10 μg/ml soy trypsin inhibitor, and 100 μg/ml phenylmethylsulphonyl fluoride). After centrifugation (30 min at 100,000g) the cytosol fraction was incubated for 30 min at 37°C with 18 n*M* L-[2,3-^{3}H]-arginine (36.8 Ci/mmole; DuPont-New England Nuclear, Boston, MA) in 50 m*M* HEPES (pH 7.4) containing 1 m*M* dithiothreitol, 4 μ*M* FAD, 4 μ*M* FMN, 0.2 m*M* NADPH, 50 μ*M* tetrahydrobiopterine (ICN Biomedicals, Inc., Aurora, OH), and 25 μ*M* L-arginine. Valine (50 m*M*), citrulline (1 m*M*), and ornithine (1 m*M*) were included in the reaction mixture to inhibit activity of arginase and other enzymes of the urea cycle. The reaction was terminated by the addition of Dowex AG50WX-8, 200-400 Mesh Na^+ resin (Bio-Rad Laboratories, Hercules, CA) and L-[2,3-^{3}H]-citrulline was quantified in the supernatant by liquid-scintillation counting. Enzyme activity was expressed as citrulline formation (pmol/min/mg protein) that could be inhibited by *S*-methylisothiourea sulfate (1 m*M*), the selective inhibitor of iNOS (20). Protein concentration in cytosol fractions was determined with bicinchoninic acid reagent and bovine serum albumin as a standard (Pierce Chemical Co., Rockford, IL).

Plasma Urea Nitrogen Determination. Plasma urea nitrogen (PUN) concentration was determined using a commercially available kit based on endpoint ultraviolet method (Sigma Diagnostics, St. Louis, MO).

Statistical Analysis. Data are expressed as mean ± SEM. Response area for plasma TNF-α and nitrite/nitrate was calculated as an area under the concentration time-response curve using the trapezoidal rule. Data were analyzed using the General Linear Model procedure of SAS (21). The response area data for plasma concentrations of nitrite + nitrate and TNF-α were analyzed as a 2 × 2 factorial with dietary protein level (LH versus HP), infusion (Arg versus saline) and their interaction as main effects. Plasma concentrations of nitrite + nitrate and TNF-α and activity of iNOS in the liver were analyzed using a 2 × 2 factorial model split-plot-in-time. Urinary output data of NO_x were analyzed within each collection period as a 2 × 2 factorial with diet, infusion and their interaction as main effects and analyzed across collection periods as a split-plot with diet, infusion and their interaction as main plot effects and period as the subplot. When a significant *F* test result was found ($P < 0.05$), the least significant difference was used to separate appropriate group means.

Results

Plasma TNF-α Concentrations. Infusion of Arg did not affect plasma TNF-α response to LPS challenge regardless of dietary protein level. Therefore, the data were pooled for each dietary protein level and presented in Figure 1. Plasma concentrations of immunoreactive TNF-α were increased in all heifers after exposure to LPS. Peak concentrations were attained 1 hr after LPS and returned to baseline by 4 hr. Plasma TNF-α concentrations were significantly lower in HP than in LP fed heifers 1 ($P < 0.01$), 2 ($P < 0.01$), and 3 hr ($P < 0.05$) after LPS injection. Also, calculated response area was blunted in heifers fed the HP diet (2.81 versus 4.32 units, SEM = 0.59; $P < 0.1$).

Plasma Nitrite + Nitrate Concentrations. After LPS challenge, plasma NO_x concentrations increased in all heifers; however, the pattern of response was different among treatments (Fig. 2). As compared to the basal concentration at time 0, the highest level within the 12-hr period after LPS administration was attained in the LP + Arg group (170% increase at 6 hr), followed by the LP (137% at 8 hr), HP (127% at 3 hr), and HP + Arg (126% at 8 hr) groups. The calculated response area of plasma NO_x (Fig. 3) was influenced by dietary protein × Arg interaction ($P < 0.05$). Infusion of Arg increased the response in LP-fed heifers ($P < 0.05$) but did not affect the response in HP-fed heifers.

Infusion of Arg alone for 8 hr without LPS challenge did not affect plasma concentrations of NO_x within 24 hr (data not shown).

Urinary Output of Nitrite + Nitrate. Because of different volumes of urine collected and different durations of each collection period, the urinary output of NO_x within collection period was expressed as μmol/heifer/hr (Fig. 4.) Compared with the same treatment before LPS challenge (–20 to –2 hr), the rate of NO_x output in urine collected 2–6

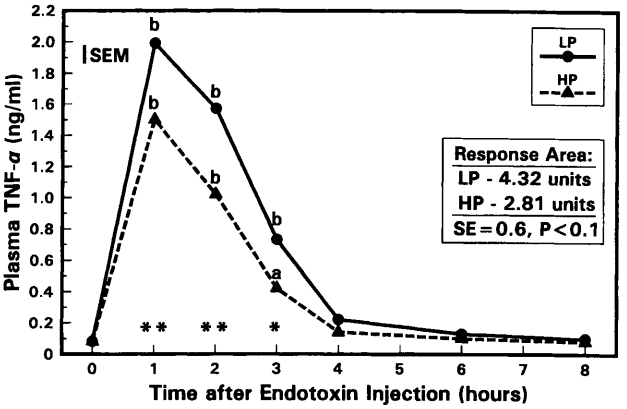

Figure 1. Profile of mean plasma concentrations of immunoreactive TNF-α after LPS challenge (0.2 μg/kg, iv bolus at time 0) in heifers fed a low- (LP) or high- (HP) protein diet and infused with Arg (0.5 g/kg) or 0.9% saline. Because infusion of Arg did not affect plasma TNF-α concentration, the data were pooled for each protein diet (n = 8–9 observations/diet). Response (insert) was calculated as area under the concentration time-response curve. $^aP < 0.05$; $^bP < 0.01$, versus time 0 within the same diet. $^*P < 0.05$; $^{**}P < 0.01$, LP versus HP within the same time. SEM, the common standard error of the mean taken from analysis of variance.

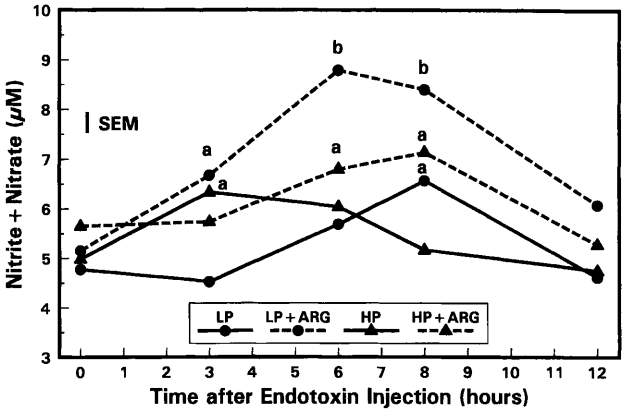

Figure 2. Plasma concentrations of nitrite + nitrate (NO_x) after LPS challenge (0.2 μg/kg, iv bolus at time 0) in heifers fed a low- (LP) or high- (HP) protein diet and infused with Arg (0.5 g/kg) or 0.9% saline (n = 4–5 observations/treatment). Compared with the basal NO_x concentrations at 0 time, plasma NO_x concentrations increased in all treatment groups within 12 hr after LPS injection ($^aP < 0.05$; $^bP < 0.01$) although the pattern of response was different among treatments (Fig. 3). SEM, the common standard error of the mean taken from analysis of variance.

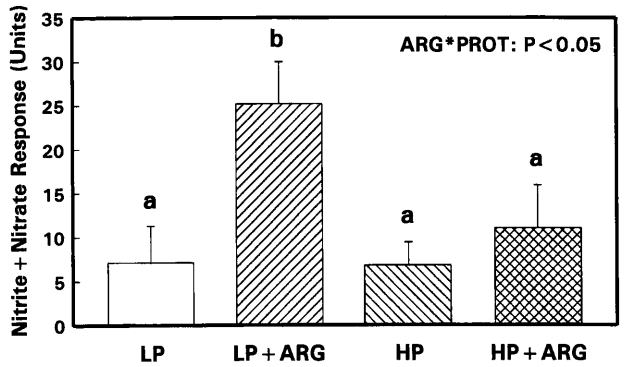

Figure 3. Effect of LPS challenge (0.2 μg/kg, iv bolus) on plasma nitrite + nitrate (NO_x) response in heifers fed a low- (LP) or high- (HP) protein diet and infused with Arg (0.5 g/kg) or 0.9% saline. Response was calculated as area under the concentration time-response curve (Fig. 2) by trapezoidal rule. Data are presented as mean ± SEM (n = 4–5 observations/treatment). Bars with different letters are significantly different ($P < 0.05$).

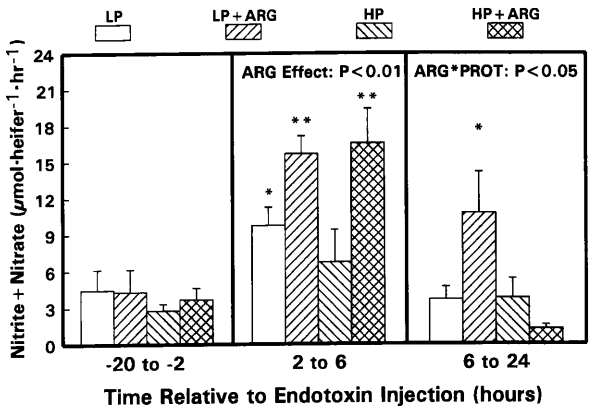

Figure 4. Effect of LPS challenge (0.2 μg/kg, iv bolus) on urinary output of nitrite + nitrate (NO_x) in heifers fed a low- (LP) or high- (HP) protein diet and infused with Arg (0.5 g/kg) or 0.9% saline. The output (μmole/heifer/hr) was calculated from urine NO_x concentration and volume of urine collected during specified time relative to LPS injection. Compared with the same treatment before LPS challenge (–20 to –2 hr) the NO_x output was different at $P < 0.05$ (*) or $P < 0.01$ (**). Nitrite + nitrate output in urine collected 2–6 hr after LPS was higher in Arg infused heifers ($P < 0.01$). Data are presented as mean ± SEM (n = 4–5 observations/treatment).

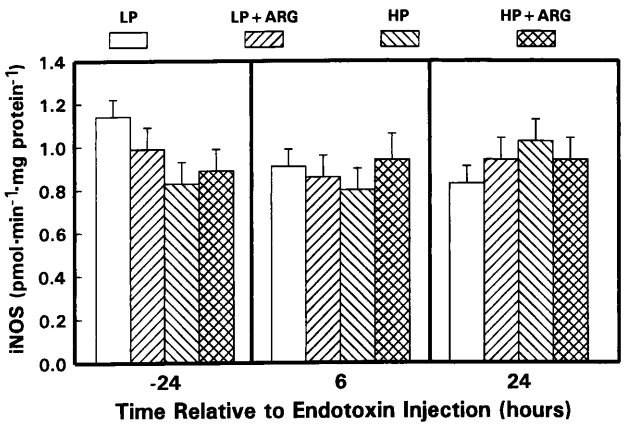

Figure 5. Effects of LPS challenge on hepatic iNOS activity in heifers fed a low- (LP) or high- (HP) protein diet and infused with Arg (0.5 g/kg) or 0.9% saline. Liver biopsy samples were obtained 24 hr before, and then 6 and 24 hr after LPS injection. Data are presented as mean ± SEM (n = 4–5 observations/treatment).

hr after LPS increased in LP ($P < 0.05$), LP + Arg ($P < 0.01$), and HP + Arg ($P < 0.01$) and was significantly amplified by Arg infusion regardless of dietary protein level (Arg effect: $P < 0.01$). The rate of NO_x output in urine collected 6–24 hr after LPS was affected by Arg × dietary protein interaction ($P < 0.05$): the output remained higher only in LP-fed heifers infused with Arg ($P < 0.05$) but returned to the basal values in other treatment groups.

Hepatic iNOS Activity. Although very low, activities of hepatic iNOS were detectable in all heifers before LPS administration (Fig. 5) and they were not affected by LPS challenge, Arg infusion or dietary protein level.

PUN Concentrations. Concentrations of PUN were higher ($P < 0.001$) in heifers on the HP diet than on the LP diet. Average PUN concentrations in samples collected before Arg infusion and LPS challenge (–2 and 0 hr relative to LPS administration) were 10.26 and 3.46 mg/dl (SEM = 0.22 mg/dl, n = 9) for HP- and LP-fed heifers, respectively.

Discussion

The experiment described herein was a replicated design. It was essential to the validity of the conclusions that none of the measured responses to the second LPS challenge were affected by residual tolerance to the first challenge. It is well documented that repeated exposure to LPS results in the development of tolerance as we have also described previously in cattle with two LPS challenges administered 5 days apart (22). In this experiment, parameter responses to LPS challenge were not statistically different between the two experimental replications as separated by the 3-week interval. This indicates that, in the heifers used

in this replicated experiment, the responses to the second LPS challenge were not blunted due to prior exposure to LPS.

The data presented here indicate that bolus administration of LPS to cattle induces the activity of the NO pathway and increases the accumulation of stable end products of NO metabolism, nitrite and nitrate, as previously reported for other species (7, 23–25). This is the first report in cattle on *in vivo* modulation of the NO pathway by LPS administration. Previous *in vitro* studies demonstrated enhanced production of NO by bovine bone marrow–derived macrophages stimulated with heat-killed bacteria (26) and by bovine blood-derived monocytes stimulated by γ-interferon and LPS (27). The magnitude of response in plasma NO_x in the present experiment (26% to 70% increase) was lower than previously reported by others in nonruminant species (20- to 30-fold increase: 7, 23, 24, 25). This difference could be related to the much lower dose of LPS used in our heifers (0.2 μg/kg) than in laboratory rodents (2–10 mg/kg) or to a different potential for NOS stimulation in cattle and rodents. Based on our previous studies (22), we chose the dose of LPS sufficiently low in cattle as to cause transient signs of response (e.g., increased rectal temperature for 3–6 hr, a short period of labored breathing, decreased plasma glucose, and increased plasma cortisol and TNF-α for 4 hr) but without prolonged or severe response or mortality. Although the dose of LPS used in this study was approximately 10^4 times lower than doses used in most *in vivo* studies in rodents, the response in plasma NO_x in this study was only 40–60 times lower. This may suggest the higher potential for iNOS stimulation by LPS *in vivo* in cattle than in mice and rats. In our preliminary experiment on mice (unpublished data), we have compared the effect of low (0.2 μg/kg) and high (2 mg/kg) doses of LPS injected ip. We found that plasma concentrations of NO_x were not affected by a low LPS dose but were significantly increased after a high LPS dose (5.4 ± 1.0 μ*M* at 0 hr; 3.7 ± 0.3 and 150.0 ± 4.3 μ*M* at 8 hr after LPS injection for low and high LPS dose, respectively; $n = 3$). On the other hand, the dose of LPS used in the present study in bovine was still 50 times greater than that producing significant cardiovascular changes and increasing plasma TNF concentrations in humans (28).

The ability of nutrition to impact and modulate immune function has been recognized for decades (10, 11). In general, nutritional extremes in terms of both energy and protein insufficiencies as well as excesses are associated with perturbed immune response to disease stress (10, 11, 29). Data on the relationship between more subtle changes in nutrition and immune function are considerably fewer, particularly where the overall plane of nutrition is sufficient for adequate rates of live weight gain in a target species. The diets used and protein intake levels fed in this study, fundamentally bracketed dietary ranges typically used in the cattle feedlot industry. As such, the present study has served to define observations made on experimental animals free of nutritional stress and within physiological dietary norms. Protein nutrition and metabolism are somewhat less straight forward in ruminant species than in monogastrics since the metabolic fate of ingested crude protein by ruminants is determined by the extent of microbial conversion of ingested dietary crude protein and energy into the characteristic components of volatile fatty acids (principal form of energy in ruminants), microbial-derived protein, and amino acids (30). In addition, urea metabolism and urea recycling are especially important for ruminant nutrition because urea serves as a nitrogen donor to ruminal microbes (31). Arginine, derived indirectly from dietary protein or synthesized in the urea cycle, is catabolized mainly by liver arginase, releasing ornithine and urea, and—to much lesser degree—by NOS, releasing citrulline and NO (17). Hibbs *et al.* (14) reported that in humans, 0.1% and 17% of infused L-[^{15}N]-arginine were excreted in the urine as [^{15}N]nitrate and [^{15}N]urea, respectively. Thus the potential for interplay between the urea cycle and the NOS pathway suggests a mechanism through which protein nutrition can modulate immune response to disease challenge.

In the present paper we demonstrated that plasma concentrations of TNF-α after low-level bolus LPS administration were lower in heifers fed a high-protein diet than in similar heifers fed a low-protein diet. Furthermore, intravenous administration of Arg was shown to be effective in amplifying underlying NOS-mediated NO_x production and the high-protein diet significantly blunted NO_x production from Arg after LPS challenge. The diets fed in this study were previously validated in cattle to promote significant differences in rate of weight gain, N-retention (32), PUN concentration, and liver arginase activity (16). It was the impact of the different protein content of the diets on arginase activity that prompted the consideration that changes in the more dominant urea cycle pathway might alter either the relative availability of Arg substrate for NOS or that diet itself might change NOS activity. Liver arginase activity was previously demonstrated to be increased almost 100% by a high-protein diet when the same diets as in the present study were fed at similar intake levels to equal sized steers (16). It was also shown that arginase administration to rats during endotoxic shock decreased NO production due to Arg depletion (25). In the present study, PUN concentrations before Arg infusion and LPS administration were higher in heifers fed the high-protein diet than in heifers fed the low-protein diet indicating that liver arginase activity was influenced by dietary protein intake. Using the same dose of LPS as in the present study, we have previously shown elevated PUN concentrations after LPS challenge in calves (22), the finding consistent with a diminished capacity for nitrogen retention, decreased protein synthesis, and a mobilization of amino acids from muscle (33).

Tumor necrosis factor–α affects protein metabolism during trauma or septic shock and, conversely, has its regulation modulated by certain aspects of dietary nutrition. During endotoxemia or infection, mobilization of tissue

proteins and amino acids is ascribed to the quantity and timing of release patterns of cytokines elicited from circulatory and resident populations of macrophages. The effects of TNF-α to mobilize muscle proteins and redirect protein metabolism in other tissues were suggested by Zamir *et al.* (33) as well as others (8). Similarly the tissue wasting associated with administration of recombinant TNF-α to sheep was blunted during the coadministration of insulin-like growth factor-I (34). Glycine and cysteine supplementation have been purported to modify the biological response to administered TNF-α (35). Few, if any, studies have examined whether supplementation with Arg affects the circulating TNF-α response to LPS challenge. We used the infusion of Arg because of its overall significance in urea and protein metabolism, its function as substrate for NOS, and impact to improve immune function and recovery from stress (17, 36). Also, when rapidly infused intravenously, Arg has been shown to stimulate the release of several hormones including growth hormone (17, 36). Recently, we demonstrated that administration of recombinant bovine growth hormone to cattle blunted the magnitude of the release of TNF-α into the circulation and decreased the severity of some physiological responses to endotoxemia (22). However, in the present experiment the rate of Arg infusion (0.5 g/kg in 8 hr) was much slower than that needed to affect growth hormone secretion (0.5 g/kg in 10 min). Mean plasma concentrations of growth hormone were not affected by Arg infusion or LPS administration (data not shown). It seems unlikely, therefore, that endogenous growth hormone status was a factor in the observed effects of nutrients on TNF-α responses to LPS.

In the present study, the infusion of Arg was without effect on the plasma concentrations of TNF-α within dietary protein treatment group. Arginine did increase the plasma concentration as well as urinary output of NO_x in heifers fed low-protein diets. In heifers fed the higher–protein level diet, where in previous studies the arginase content of the liver was higher (16), the NO_x output was also augmented by supplemental Arg but to a significantly lower magnitude: output was increased only in urine collected within 6 hr but not in urine collected 6–24 hr after LPS challenge. In both plasma and urinary NO_x responses, there was evidence of increased NO_x concentration or output earlier (at 3 hr in plasma and within 6 hr in urine) than expected. Induction of iNOS in various organs (4, 7, 23) and macrophages (19, 37) is purported to begin 4–6 hr after LPS treatment. The measurement of increased concentrations and output of NO_x in the present study may be related to forms of NOS other than iNOS and simply reflect alterations in the membrane flux of Arg into cells as stimulated by the γ^+-mediated L-arginine transport system. Pacitti *et al.* (38) demonstrated in rats that TNF-α stimulated a time- and dose-dependent increase in hepatocyte Arg transport. Also in rats, LPS treatment was reported to induce an *in vivo* L-arginine transport in hepatocytes (5). In the present experiment, activities of hepatic iNOS were not affected by LPS challenge in any treatment group (Fig. 5). However, low activities of hepatic iNOS were detected in all heifers before LPS administration. These basic activities combined with increased Arg flux into cells could be involved in the early NO_x response to LPS challenge in ruminants. The origin of the basic activity of hepatic iNOS in our heifers is not clear but could be related to the continuous exposure to endotoxin produced by the microbial population of the rumen (39). In our study, plasma concentrations of TNF-α peaked approximately 1 hr after LPS. The temporal relationship between the peak TNF-α concentrations and the initial change in plasma and urinary NO_x, and the larger increase, especially in urinary output, in the sampling windows beyond 3 hr is consistent with an early overall response due to increased membrane transport of Arg and a later increased production of NO and ultimately nitrite and nitrate *via* iNOS at sites other than liver, perhaps peripheral macrophages, neutrophils and mast cells. This suggestion is also supported by the observation that in Arg-infused heifers, the high-protein diet, which was previously shown to increase liver arginase activity (16), decreased NO_x output in urine collected between 6 and 24 hr but not between 2 and 6 hr after LPS challenge.

Nitric oxide, as a mediator of many of the purported pathological consequences of endotoxemia or infection, is generated in association with the acute induction of the iNOS isoform of the NOS family (4, 7, 23) following gene induction by various cytokines (37, 40) but TNF-α in particular (7, 40). A close relationship between TNF-α and NO production during endotoxemia has been previously suggested. Harbrecht *et al.* (24) have shown that TNF-α plays a significant role in the *in vivo* induction of NO synthesis in rats. Conversely, NO synthesized by the constitutive isoform of NOS positively modulates TNF-α production in endotoxemic rats (41). In the present study, there was relatively little difference in the integrated plasma NO_x response area over time in the absence of supplemental Arg. However, in the presence of supplemental Arg, there appeared to be a positive relationship between the magnitude of the TNF-α response and the subsequent NO_x response ($r = 0.89$; $p < 0.01$), the greater TNF-α concentrations being associated with higher plasma concentrations of the nitrogen metabolites. Supplemental dietary Arg has been previously associated with increased urinary excretion of nitrate (15).

The overall implication of the present data is that changes in dietary protein within levels that modulate growth are capable of altering the magnitude of response to LPS challenge in both TNF-α and nitrite/nitrate, the stable metabolites of NO. The data underscore the capability of normal dietary protein ranges to modulate immune response to a simulated low-level disease challenge in ruminants.

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