

# Estradiol plus progesterone treatment modulates select elements of the proinflammatory cytokine cascade in steers: Attenuated nitric oxide and thromboxane B<sub>2</sub> production in endotoxemia<sup>1</sup>

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**ABSTRACT:** Estradiol plus progesterone (EP) implants have been shown to favorably alter the time course or decrease the severity of many of the clinical manifestations associated with coccidiosis and endotoxemia in calves. This study evaluated the effect of EP treatment on plasma tumor necrosis factor- $\alpha$  (TNF), thromboxane (TXB), prostacyclin (PRC), nitrite and nitrate (NO<sub>[x]</sub>), and cortisol. Holstein steer calves were divided into four groups: control, EP, endotoxin (LPS), and EP+LPS (n = five/group). Estradiol/progesterone pellets (Synovex-S) were implanted subcutaneously when calves reached 20 wk of age. One week after implantation, calves were injected IV with endotoxin (i.e., lipopolysaccharide; LPS, 0.6  $\mu$ g/kg of BW) or nonpyrogenic saline placebo. Body temperature was measured and blood was collected before injection and at 1, 2, 3, 4, 6, and 8 h thereafter. Plasma concentrations of TNF,

cortisol, TXB, PRC, NO<sub>[x]</sub>, were measured. Body temperature increased in both LPS and LPS-EP calves, but had returned to normal by 6 h in the LPS-EP group ( $P < 0.05$ ). Plasma TNF and cortisol increased after LPS ( $P < 0.01$ ), but were not differentially affected by EP treatment. Likewise, EP did not affect the magnitude of increase in LPS-induced PRC, but EP decreased the magnitude of increase in TXB ( $P < 0.05$ ). Plasma NO<sub>[x]</sub> levels were increased ( $P < 0.01$ ) in calves after LPS; treatment with EP attenuated the LPS-associated increase in plasma NO<sub>[x]</sub> levels. These results suggest that EP exerts specific effects on different components of the proinflammatory cytokine cascade. Although the initiation of responses mediated by TNF, cortisol, and PRC do not seem to be differentially affected by EP, components of the nitric oxide- and TXB-axis responses to LPS are decreased in calves pretreated with EP.

Key Words: Cattle, Cortisol, Endotoxins, Growth Promoters, Prostacyclin, Tumor Necrosis Factor

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## Introduction

Diseases produce a gradient of catabolic processes in animals, which are characterized by poor growth of young infected animals, reduced appetite (McMahon et al., 1999), and a series of metabolic perturbations that may persist beyond the point where clinical signs have resolved (Elsasser et al., 2000b). Anabolic agents have been studied as a means of assisting animals and humans through catabolic disease with variable success (Zeigler et al., 1994; Sartin et al., 2000). For example,

GH treatment of calves resulted in a beneficial impact on the response to endotoxin (Elsasser et al., 1994; 1997b). Conversely, in models of cachectic wasting, there were reductions in BW that persisted in spite of treatment with GH (Elsasser et al., 1997a; 1998; Sartin et al., 1998).

Recent studies (Heath et al., 1997; McMahon et al., 1998) utilizing an estradiol and progesterone (EP) preparation with known anabolic effects in cattle (Rumsey et al., 1992) have demonstrated improvement in an animal's recovery from a disease process. Calves treated with EP and infected with *Eimeria bovis* had fewer days of fever and diarrhea and recovered food intake more quickly. Moreover, infected calves that were EP treated had a positive weight gain during the infection (Heath et al., 1997). There were also improvements in responses of EP-treated calves injected with *Escherichia coli* endotoxin or lipopolysaccharide (LPS) (McMahon et al., 1998), which include effects on plasma IGF-I concentrations and appetite.

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At present, the mechanism underlying these protective effects of EP in cattle is unknown. The endotoxin model was selected based on a previous experiment (McMahon et al., 1998) and because there is a wavelike pattern of release of cytokines, prostaglandins, neurohormones, glucocorticoids, and nitric oxide that will allow study of specific sites for the mechanism of action of EP. Therefore, this study was designed to determine where in this cascade EP treatment might act to improve an animal's response to LPS.

## Materials and Methods

This study was reviewed and approved by the Auburn University Institutional Animal Care and Use Committee. Castrated male Holstein calves (mean BW = 147 kg) were obtained from the Auburn University dairy and maintained from approximately 20 wk of age in the experimental facility with controlled temperature and photoperiod (12 h of light). All calves were fed daily a concentrate feed (14% CP) at 4% of BW. Hay and water were provided ad libitum. Seven days before the administration of test challenges with endotoxin, calves were fitted with EP ear implants (Synovex S, courtesy of F. Prouty, Ft. Dodge Animal Health, Overland Park, KS), containing a slow-release formulation of 20 mg of 17  $\beta$ -estradiol benzoate and 200 mg of progesterone. The calves were divided into four groups in a 2  $\times$  2 factorial arrangement: control, EP, endotoxin, EP + endotoxin (n = five calves per group). The day before an experiment, heparin-filled cannulas were placed into an external jugular vein. The following morning, cannulas were opened, heparin was removed, and cannulas were flushed with saline; calves were allowed to stand undisturbed for 1 h. A 10-mL baseline blood sample was collected, followed by the intravenous injection of 0.6  $\mu$ g/kg of BW of *E. coli* endotoxin (O55 B5; Sigma Chemical Company, St. Louis, MO). This dose was determined for this batch of endotoxin based on a consistent fever response and plasma tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) response to several doses of LPS. Subsequent blood samples were collected at 1, 2, 3, 4, 6, and 8 h after endotoxin challenge. Blood was centrifuged, and the plasma was aliquoted to vials and stored at  $-20^{\circ}\text{C}$  until assay.

Plasma was assayed for TNF- $\alpha$  (Kenison et al., 1991), cortisol (Thompson et al., 1995; McMahon et al., 1998), and prostacyclin (PRC; Shafer and Malik, 1982) using previously validated radioimmunoassays. The intraassay coefficients of variation were 8.4% for TNF- $\alpha$ , 4.7% for cortisol, and 8.8% for PRC. Thromboxane-B<sub>2</sub> (TXB) was assayed using a commercial ELISA kit (Oxford Biomedical Research Inc., Oxford, MI). The TXB analyte was extracted from plasma (1 mL) into a supernatant by adding 0.2 mL of methanol and then vortexing and centrifuging the sample. The supernatant was applied to a 400-mg Sep Pak C<sub>18</sub> column (Waters Corp., Milford, MA) preconditioned with sequential washes with chloroform and methanol. The column was washed

with 2 mL of 15% methanol followed by 2 mL of petroleum ether. The TXB was eluted with 2 mL of methyl formate that was evaporated under a stream of nitrogen gas. Samples were frozen until assayed using the kit instructions. Increasing volume equivalents of bovine plasma extracts displaced tracer parallel to that of the standard curve; extraction efficiency was determined to be >90% for three samples representing different concentration points (0.01, 0.04, 0.2 ng/mL) on the standard curve. The intraassay CV was 6.4% and the assay sensitivity was 0.004 ng/mL.

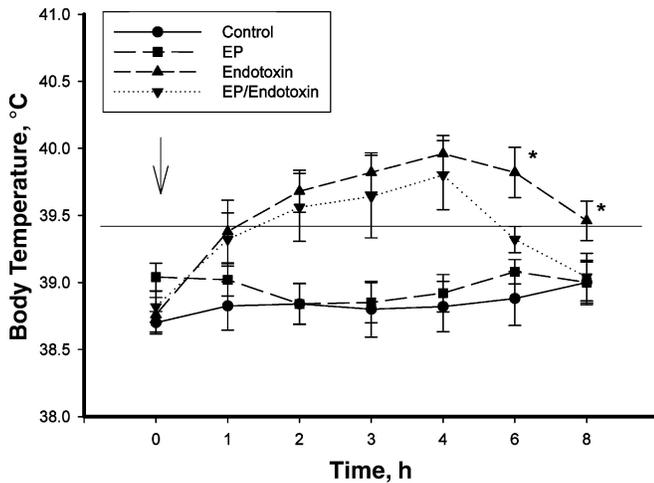
The effects of endotoxin and EP on nitric oxide production were assessed and inferred (Hibbs et al., 1992; Santak et al., 1997) in terms of changes in plasma nitrate levels after subjecting plasma samples to a treatment with bacterial nitrate reductase to form stoichiometric concentrations of nitrite, which is the NO<sub>(x)</sub> form measured in the Griess reaction, as previously described and validated for bovine plasma (Kahl et al., 1997). Data on the nitric oxide response to endotoxin challenge were assessed in terms of the NO<sub>(x)</sub> response above baseline with an individual animal's plasma concentration responses at each time point summated to a single indexed variable calculated as the area under the concentration  $\times$  time curve. The response area under the curve (AUC) for the NO<sub>(x)</sub> plasma response was calculated between 0 and 8 h of blood sampling after endotoxin challenge with baseline concentration area (at time 0 h) was subtracted. In addition, body temperature was recorded as rectal temperature using an electronic digital thermometer at 0, 1, 2, 4, 6, and 8 h.

Data were analyzed using GLM procedures for repeated measures of SAS version 8 (SAS Inst., Inc., Cary, NC). Plasma concentrations of NO<sub>(x)</sub>, cortisol, PRC, and TNF- $\alpha$  were tested for effects of treatment (endotoxin or saline), implant, time, and interactions. For effects of treatment, animal within treatment  $\times$  implant was used as the error term. The AUC was calculated for TXB using the trapezoidal method. The AUC for TXB was tested for effects of treatment, implant, and interactions. For significant effects ( $P < 0.05$ ), means separation procedures were performed using preplanned comparisons and the least squares means option and differences option (PDIF) of SAS.

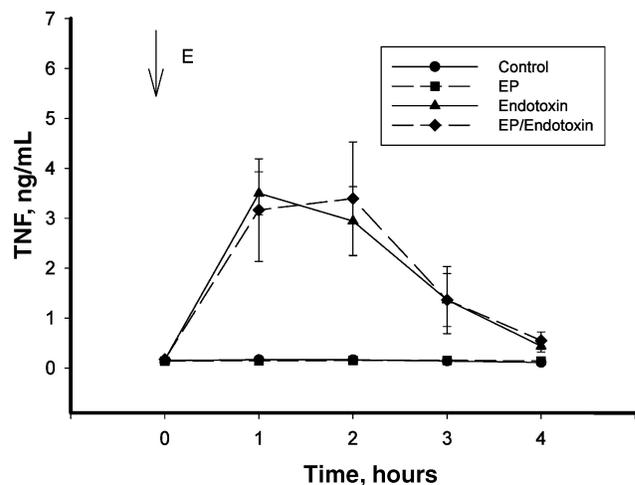
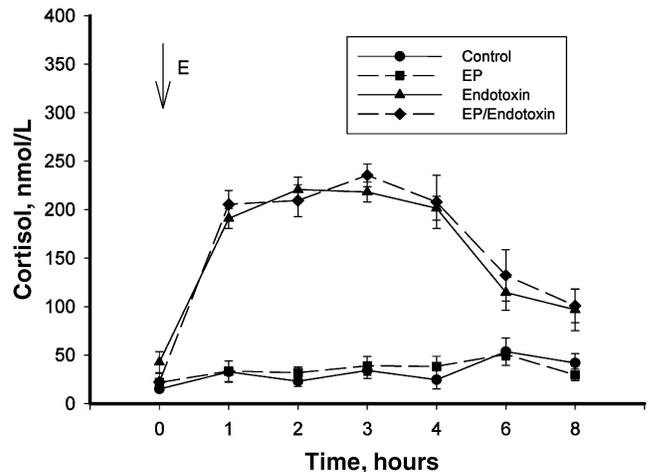
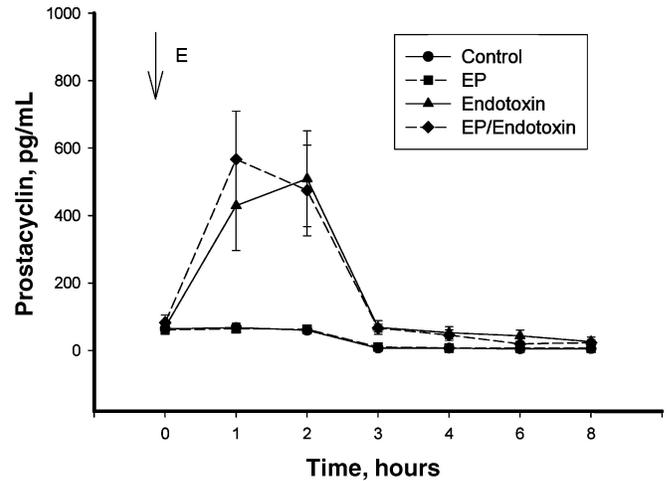
## Results

There was a significant effect of time ( $P < 0.0001$ ) and endotoxin treatment ( $P < 0.001$ ) on body temperature (Figure 1). There was also a significant implant  $\times$  endotoxin treatment interaction ( $P < 0.0002$ ) and a time  $\times$  implant  $\times$  endotoxin interaction ( $P < 0.0001$ ), where EP/endotoxin-treated calves returned to normal temperature more quickly than endotoxin-treated calves.

Endotoxin-stimulated increases in plasma TNF- $\alpha$  concentrations ( $P < 0.01$ ) that peaked in the blood-sampling scheme at 1 h remained elevated through 3 h ( $P < 0.008$ ) and returned to baseline by 4 h after endotoxin injection (Figure 2). There were no differences in TNF-



**Figure 1.** Effects of an estradiol + progesterone (EP) implant on mean rectal temperature in calves following challenge with intravenous endotoxin ( $0.6 \mu\text{g}/\text{kg}$  of BW, *Escherichia coli* 055-B5). Data are mean  $\pm$  SEM,  $n =$  five calves/group. There was a significant effect of time ( $P < 0.0001$ ) and endotoxin treatment ( $P = 0.001$ ) on body temperature. There was also a significant implant  $\times$  endotoxin treatment interaction ( $P = 0.0002$ ) and time  $\times$  implant  $\times$  endotoxin interaction ( $P < 0.0001$ ). The horizontal line indicates the point for a fever ( $39.4^\circ\text{C}$ ). The asterisk indicates differences between endotoxin- and EP/endotoxin-treated calves,  $P < 0.009$ .

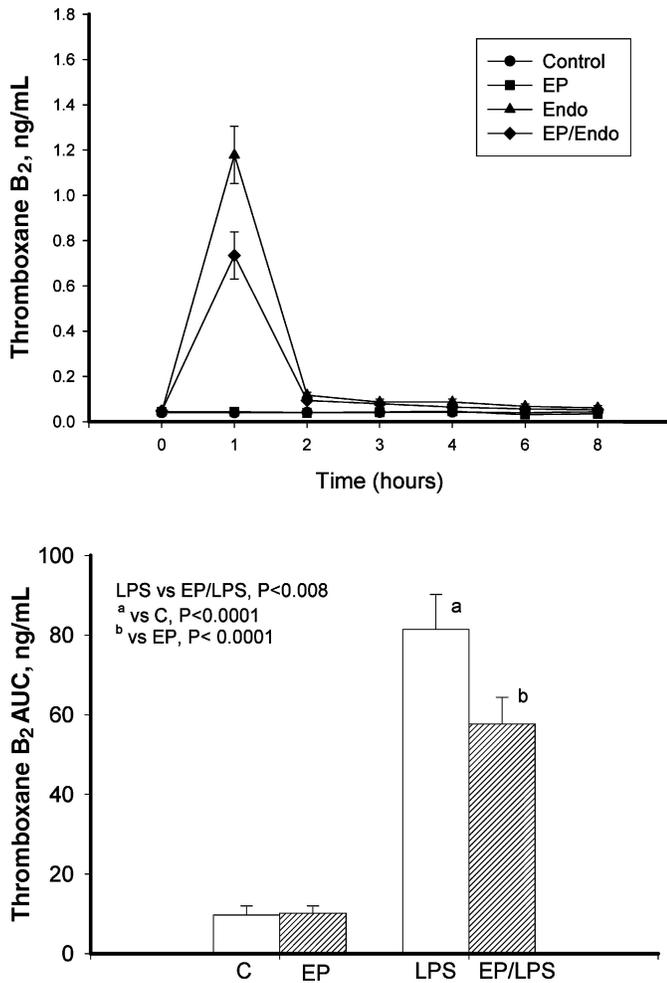


**Figure 2.** Mean plasma concentrations of prostacyclin, cortisol, and tumor necrosis factor- $\alpha$  (TNF) in calves following intravenous challenge with endotoxin ( $0.6 \mu\text{g}/\text{kg}$  of BW, *Escherichia coli* 055-B5). Data are mean  $\pm$  SEM,  $n =$  five calves/group. Arrows indicate the time of treatment (saline or endotoxin). For each variable, the mean concentration changes of animals receiving endotoxin (endotoxin, estradiol + progesterone [EP]/endotoxin) were significant (prostacyclin,  $P < 0.005$ ; cortisol,  $P < 0.0001$ ; TNF,  $P < 0.01$ ) compared with those receiving only saline (control, EP).

$\alpha$  mean concentrations between endotoxin- and EP/endotoxin-treated calves at measurement time points. Cortisol ( $P < 0.0001$ ) and PRC ( $P < 0.005$ ) concentrations likewise were increased following endotoxin administration, but there was no effect of EP to modify the endotoxin effect on either of these marker hormones (Figure 2). Whereas plasma PRC had returned to baseline by 3 h after endotoxin, cortisol concentrations in endotoxin- and EP/endotoxin-treated calves had not returned to baseline by 8 h ( $P < 0.001$ ) after endotoxin. Plasma TBX concentrations (Figure 3) were also elevated by endotoxin ( $P < 0.001$ ) with EP pretreatment associated with a measured attenuation in the endotoxin-induced concentration of TBX in plasma ( $P < 0.008$ ). Levels of  $\text{NO}_{(x)}$  (Figure 4) increased following endotoxin injection ( $P < 0.01$ ), with a significant differentiation relative to time 0 beginning at 4 h and persisting beyond the 8-h sampling interval. There was no significant increase in mean plasma  $\text{NO}_{(x)}$  concentrations in calves treated with EP + endotoxin.

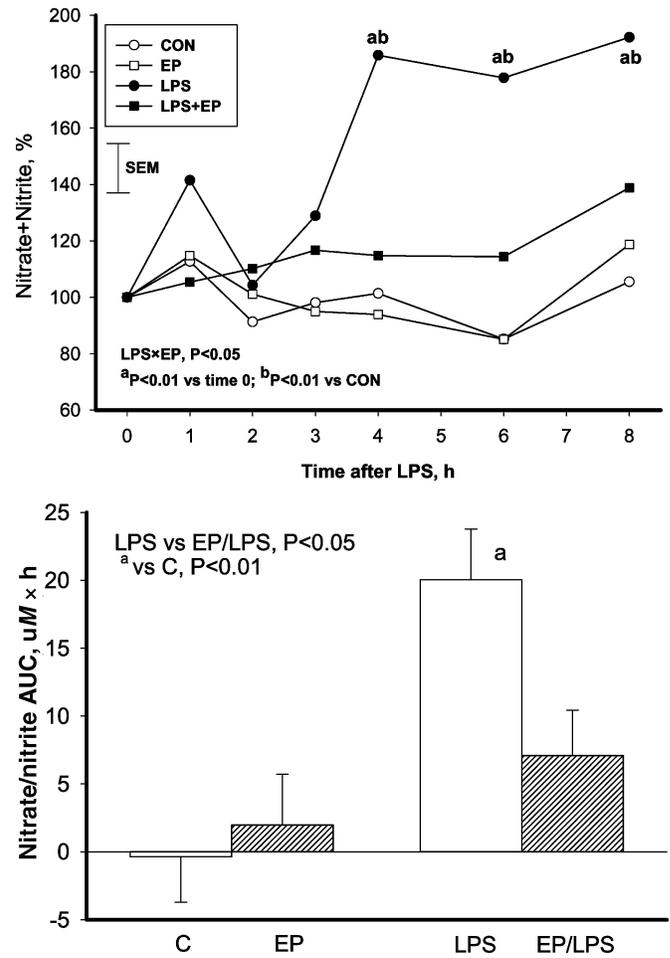
## Discussion

Initial studies with EP and endotoxin examined metabolic regulation as a site for EP action to improve endotoxin responses in calves. There was a 60% reduction in 24-h food intake in endotoxin-treated calves, whereas calves first treated with EP had a 30% reduction in food intake (McMahon et al., 1998). There was



**Figure 3.** Thromboxane responses to intravenous endotoxin (0.6  $\mu\text{g}/\text{kg}$  of BW, *Escherichia coli* 055-B5) challenge in calves receiving saline without endotoxin (C), an estradiol + progesterone implant without endotoxin (E/P), endotoxin only (LPS), and endotoxin and an implant (EP/LPS). Data are mean  $\pm$  SEM, n = five calves/group. The upper panel represents actual values. The lower panel represents means of the area-under-curve (AUC) responses.

also less of a catabolic effect in EP-treated calves, as evidenced by the attenuation in the elevation in blood urea nitrogen following the endotoxin injection (McMahon et al., 1998). Interestingly, free fatty acids and glucose were more elevated after endotoxin injection in EP-treated calves, and there was a greater evidence for insulin resistance in EP-treated calves than in calves not given EP. This latter effect may provide energy for noninsulin-dependent tissues, such as the brain and cells of the immune system. Interestingly, plasma IGF-I concentrations were recovered more quickly in the endotoxin animals also receiving EP (McMahon et al., 1998). However, whereas these results provide interesting explanations for some of the physiological changes, they do not point to a cellular mechanism for EP actions in this catabolic model.



**Figure 4.** Nitric oxide production responses to intravenous endotoxin (0.6  $\mu\text{g}/\text{kg}$  of BW, *Escherichia coli* 055-B5) challenge in calves receiving saline without endotoxin (C), an estradiol + progesterone implant without endotoxin (E/P), endotoxin only (LPS), and endotoxin and an implant (EP/LPS). Data are mean  $\pm$  SEM, n = five calves/group. Values in the upper panel represent change from control (%). In the lower panel, values represent means of the area-under-curve (AUC) plasma nitrate concentration responses.

A protective effect for GH, similar to EP, has been described in cattle exposed to lower doses of endotoxin (0.2 vs. 0.6 mg/kg of BW; Elsasser et al., 1994). Therefore, comparison of a probable mechanism for the positive effects of GH in cattle to those of EP may provide clues to the mechanism of action of EP. Examination of the effects of GH in cattle administered endotoxin indicates that GH reduced cortisol production and release, reduced TNF production and release, and reduced the generation of thromboxane B<sub>2</sub> production. Comparison of the data of Elsasser et al. (1994) in cattle to these data with EP suggests differences in the effects of EP and GH. Indeed, the only point of similarity is the ability of EP to reduce the plasma concentrations of thromboxane B<sub>2</sub>. If this effect were a nonspecific effect of anabolic hormones, GH and EP administration would

be expected to produce the same response profile in an animal. Therefore we conclude that the actions of these two hormonal strategies operate on distinct mechanisms not dependent solely on anabolic effects in the animal.

Endotoxin will increase plasma TXB levels in ruminants (Demling et al., 1981). Thromboxane B<sub>2</sub> is a stable endproduct of the metabolism of thromboxane A<sub>2</sub>, which promotes vasoconstriction and platelet aggregation. An imbalance in the ratio of TXB:PRC (a vasodilator that decreases platelet aggregation) can produce thrombus formation in the coronary and cerebral vessels (Cotran et al., 1999). In addition, the primary cause of pulmonary hypertension, in septic shock, is associated with pathological imbalance in the steady-state production of these vasohormones (Demling et al., 1986). In the present experiment, EP reduced the effects of endotoxin to increase TXB, whereas EP had no effect on PRC. This could provide some measure of protection for the animals against severe vasoconstriction, thrombus formation, and other compromises, such as increased vascular permeability. However, the small effects seen in this study are unlikely to account for the types of effects observed in either the *E. bovis* infection model or the transient endotoxin injection model. Interestingly, a more robust attenuation in TXB concentration responses was observed by Elsasser et al. (1994) in GH + endotoxin-treated cattle, although the GH treatment decreased the PRC response as well.

Excess NO generation in animals has been demonstrated to link endotoxemia to adaptive responses of the cell, as well as more severe disruptions in cellular function and even apoptosis (Gross and Wolin, 1995; Le et al., 1995; Adams et al., 1996). Indeed, excess NO generation may be responsible for many of the deleterious changes observed with endotoxemia in cattle (Elsasser et al., 1994; 2000a; Kahl et al., 1996). Therefore, an ability to reduce the generation of NO in cattle could produce favorable effects on the outcome of a disease process. The current data demonstrate an action of EP to reduce the effects of endotoxemia on nitrate/nitrite accumulation in plasma. Since nitrate and nitrite are the stable end products of NO metabolism, these results suggest that EP reduces NO production in cattle. This is consistent with our knowledge of EP actions in physiological control systems. For example, E and P can independently block NO production in lymphocytes in rodent models (McCrudden and Stimson, 1994; Miller et al., 1996). In addition, NO production in the hypothalamus associated with GnRH regulation is also inhibited by E (Ceccatelli et al., 1996). This inhibition is thought to be due to a reduction in activity of the inducible NO synthase. The ability of EP to block NO production would provide an explanation for the major effects of EP to protect against the effects of endotoxemia in cattle.

### Implications

Estradiol plus progesterone treatments aid animals in body weight maintenance in the presence of infec-

tions by *Eimeria bovis* and protect against the adaptive responses of the animal to coccidiosis and endotoxemia. However, additional research on specific and separate effects of estrogen and progesterone still warrant investigation. Results indicate a possible mechanism for estradiol plus progesterone actions that might be exploited as a means of preventing reductions in animal productivity associated with some parasitic and bacterial infections.

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