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Comparing oral versus intravenous calcium administration on alleviating markers of production, metabolism, and inflammation during an intravenous lipopolysaccharide challenge in mid-lactation dairy cows

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ABSTRACT

Animals, including dairy cows, develop hypocalcemia during infection. Prior independent research suggests supplementing oral Ca, but not i.v. Ca, improves multiple health metrics after immune activation. Therefore, study objectives were to directly compare the effects of administering an oral Ca bolus versus i.v. Ca on mineral and energetic metabolism variables and inflammatory parameters following an i.v. LPS challenge. Mid-lactation cows (124 ± 43 DIM) were assigned to 1 of 4 treatments: (1) saline control (CON; 4 mL of saline; n = 4), (2) LPS control (CON-LPS; 0.375 µg/kg BW; n = 6), (3) LPS with oral Ca bolus (OCa-LPS; 0.375 µg/kg BW and a 192-g bolus of Bovikalc [Boehringer Ingelheim Animal Health USA Inc., Duluth, GA] containing 43 g of Ca [71% $CaCl_2$ and 29% $CaSO_4$] supplemented at −0.5 and 6 h relative to LPS administration; n = 8), and (4) LPS with i.v. Ca (IVCa-LPS; 0.375 µg/kg BW and 500 mL of Ca-gluconate, 23% [VetOne, Boise, ID]) supplemented at −0.5 and 6 h relative to LPS infusion; n = 8). During period (P) 1 (4 d), baseline data were obtained. At the initiation of P2 (5 d), LPS and Ca supplements were administered. As anticipated, CON-LPS became hypocalcemic, but OCa-LPS and IVCa-LPS had increased ionized Ca compared with CON-LPS cows (1.11 and 1.28 vs. 0.95 ± 0.02 mmol/L, respectively). Rectal temperature increased after LPS and was additionally elevated in IVCa-LPS from 3 to 4 h (38.9 and 39.8 ± 0.1°C in CON-LPS and IVCa-LPS, respectively). Administering LPS decreased DMI and milk yield relative to CON. Circulating glucose was decreased in OCa-LPS compared with CON-LPS and IVCa-LPS during the initial hyperglycemic phase at 1 h (75.1 vs. 94.9 and 95.7 ± 3.4 mg/dL, respectively, but all LPS infused cows regardless of treatment had similar glucose concentrations thereafter, which were decreased relative to baseline during the first 12 h. Blood urea nitrogen increased after LPS but this was attenuated in OCa-LPS compared with CON-LPS and IVCa-LPS cows (8.7 vs. 10.0 and 10.4 ± 0.3 mg/dL). Glucagon increased in OCa-LPS and IVCa-LPS compared with CON-LPS cows (459 and 472 vs. 335 ± 28 pg/mL, respectively), and insulin markedly increased over time regardless of LPS treatment. Lipopolysaccharide substantially increased serum amyloid A, LPS-binding protein (LBP), and haptoglobin in all treatments, but OCa-LPS tended to have increased LBP concentrations relative to IVCa-LPS (10.7 vs. 8.6 ± 0.7 µg/mL, respectively). Several cytokines increased after LPS administration, but most temporal cytokine profiles did not differ by treatment. In summary, LPS administration intensely activated the immune system and both Ca delivery routes successfully ameliorated the hypocalcemia. The i.v. and oral Ca treatments had differential effects on multiple metabolism variables and appeared to mildly influence production responses to LPS.
Key words: hypocalcemia, immune

INTRODUCTION

During acute immune activation, dairy cows develop hypocalcemia (Thomas, 1889; Hisaeda et al., 2020), a highly conserved species response (Carlstedt et al., 2000; Toribio et al., 2005; Al-Qaisi et al., 2020). Since Ca ions stabilize LPS aggregates and LPS agglomeration is thought to elicit a hyperimmune response, reducing circulating Ca appears to be a protective strategy against excessive inflammation (Hotchkiss and Karl, 1996; Mueller et al., 2004; Harm et al., 2021). Hypocalcemia allows endotoxin sequestration in lipoproteins, a nonimmune

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system avenue of LPS clearance (Skarnes and Chedid, 1964; Munford et al., 1981). The tenet that hypocalcemia is an intentional adaptation to immune activation is supported by studies demonstrating i.v. Ca does not improve human health outcomes (Malcolm et al., 1989) or milk yield recovery (Horst et al., 2020), and actually increases human morbidity and mortality (Collage et al., 2013). However, severe or prolonged hypocalcemia is presumably not ideal because Ca is pleotropic and specifically involved with gastrointestinal motility (Huber et al., 1981), milk secretion (Burgoyne and Wilde, 1994), and gluconeogenesis (Schlumbohm and Harmeyer, 2003), functions seemingly vital to economically important dairy phenotypes.

Periparturient cows (even overtly healthy ones) experience postpartum inflammation due to a combination of parturition, nutritional changes, mastitis, endometritis, and uterine involution (Bertoni et al., 2008; Trevisi and Minuti, 2018). Incidentally, postpartum subclinical hypocalcemia afflicts a large percentage of cows (Reinhardt et al., 2011; Martinez et al., 2012). This metabolic disorder has traditionally been viewed as pathological (DeGaris and Lean, 2008; Goff, 2008) as it apparently causes immune suppression (Kimura et al., 2006; Martinez et al., 2014); a predisposition to increased disease susceptibility (Curtis et al., 1983; Chapinal et al., 2011). Subclinical hypocalcemia is thought to result from the mammary gland's acute and extensive Ca uptake, which overwhelms the homeostatic mechanisms to maintain eucalcemia (Horst et al., 1994, 2005). However, since immune activation causes hypocalcemia, some periparturient hypocalcemia may actually reflect an activated immune system (Horst et al., 2021). Administering LPS causes immune activation, a response that is repeatable and well-described (Waldron et al., 2003a; Horst et al., 2018) and this approach can thus be used (within limits) to model periparturient hypocalcemia.

Orally delivered Ca improved health, DMI, and milk production in experimentally induced immune-activated dairy cattle (Al-Qaisi et al., 2020). However, i.v. continuous infusing Ca to maintain eucalcemia following LPS administration had the opposite effect, as preventing hypocalcemia increased acute phase proteins and decreased DMI and milk yield (Horst et al., 2020). Consequently, these 2 separate experiments produced very discordant interpretations. Therefore, study objectives were to directly evaluate the effects of oral versus i.v. routes of Ca delivery on metabolism and inflammation in response to LPS administration in dairy cows, as this has implications to transition cow management. We hypothesized that oral Ca would attenuate the negative consequences of immune activation relative to both i.v. Ca and no Ca supplementation.

MATERIALS AND METHODS

Experimental Design

All procedures were approved by the Iowa State University Animal Care and Use Committee, and the experiment was conducted in the fall of 2022. Twenty-six multiparous mid-lactation cows (124 ± 43 DIM; 660 ± 57 kg; 2.3 ± 0.5 lactations) were assigned to (1) saline control (**CON**; 4 mL of saline; n = 4), (2) LPS control (**CON-LPS**; 0.375 µg/kg BW; n = 6), (3) LPS with oral Ca bolus (**OCa-LPS**; 0.375 µg/kg BW and one 192-g bolus of Bovikalc [Boehringer Ingelheim Animal Health USA Inc., Duluth, GA] containing 43 g of Ca [71% $CaCl_2$ and 29% $CaSO_4$] supplemented at −0.5 and 6 h relative to LPS administration; n = 8), and (4) LPS with i.v. Ca administration (**IVCa-LPS**; 0.375 µg/kg BW and 500 mL of Ca-gluconate, 23% [VetOne; Boise, ID] provided at −0.5 and 6 h relative to LPS administration; n = 8). Calcium doses were chosen for their commercial practicality; therefore, the administration routes knowingly did not provide an equivalent amount of Ca at the same rate. Oral Ca sourced from $CaCl_2$ allows for rapid absorption, whereas $CaSO_4$ is absorbed at a slower rate. Although the experiment has implications to the postpartum cow, mid-lactation cows were used as a model to reduce the physiologic, metabolic, and inflammatory variability that often accompany early-lactation animals (a common practice in LPS models; Vernay et al., 2012; Zarrin et al., 2014). Due to logistical and facility limitations, fewer cows were assigned to the CON-LPS and CON treatments. Sample size was determined by considering logistical constraints, previous research (Al-Qaisi et al., 2020; Horst et al., 2020), and power analysis (PROC POWER; SAS Institute Inc., Cary, NC) based on our central objective (effect of Ca treatments on ionized Ca [**iCa**]), which indicated a statistical power of 80% (α = 0.05) to detect meaningful group mean differences of 1.1 and 1.4 ± 0.2 mmol/L between oral and i.v. Ca supplements with a sample size of 16 (n = 8 per Ca treatment).

The experiment was completed in 2 replicates where treatments were balanced equally. Cows resided in individual box-stalls (4.57 × 4.57 m) at the Iowa State University Dairy Farm (Ames, IA) throughout the experiment. Cows were given 3 d to acclimate during which an indwelling jugular catheter was implanted. Briefly, cows were restrained in stanchions where the neck was washed with alternating povidone-iodine, 7.5% and 70% ethanol 3 times. After occluding the jugular vein, a 14-gauge, 3.75-cm introducer (MILA International, Erlanger, KY) was inserted to pass a sterilized Tygon tube (Saint-Gobain Performance Plastics, Akron, OH) in the vein. The tubing was sutured with a butterfly tape to the skin,

and a tubing adapter (BD, Franklin Lakes, NJ) closed the external opening.

The trial was conducted with 2 experimental periods (**P1** and **P2**). Period 1 lasted 4 d during which cows were fed ad libitum and baseline production data were obtained. Period 2 began after the last milking of P1 and lasted 5 d during which the response to saline or LPS administration were recorded. Approximately 30 min following the last milking of P1, cows were administered a single i.v. infusion of LPS (0.375 µg/kg BW in ~4 mL of sterile saline; *Escherichia coli* O55:B5; Sigma-Aldrich, St. Louis, MO). Briefly, LPS was dissolved in sterile saline and passed through a 0.2-µm sterile syringe filter (Thermo Scientific, Waltham, MA). Cows receiving Ca were administered the first treatment (either oral or i.v.) immediately after the last milking of P1 (30 min before LPS administration), and the second treatment (either oral or i.v.) at 6 h following LPS administration, which closely followed a Ca dosing regimen we previously used (Al-Qaisi et al., 2020).

Throughout the experiment, cows were milked twice daily (0500 and 1700 h). After each milking during P1 and P2, individual milk yields were recorded and composite milk samples were collected to evaluate milk components and SCC. Samples were stored at 4°C with a preservative (bronopol tablet; D & F Control System, San Ramon, CA) until analysis (CentralStar, Kaukauna, WI) using mid-infrared spectrometry. Energy-corrected milk was calculated with the following formula: ECM = 0.327 × milk yield + 12.95 × fat yield + 7.2 × protein yield. Water and feed were provided ad libitum, and the diet was formulated to meet or exceed the predicted requirements (NRC, 2001; Supplemental Table S1, see Notes) of energy, protein, minerals, and vitamins. Samples of the TMR were collected at the beginning of P1 and P2 in both replicates and were composited into 1 sample for nutrient analysis (Dairyland Laboratories, Arcadia, WI).

Blood Analysis

Blood samples were collected via jugular catheter. Serum and plasma (from tubes containing serum clot activating factor and K_2EDTA, respectively; BD, Franklin Lakes, NJ) were harvested after centrifugation at 1,500 × *g* for 15 min at 4°C before storing at −20°C until analysis. Another set of whole blood samples in K_2EDTA were submitted to the Iowa State University's Department of Veterinary Pathology (Ames, IA) for complete blood cell count analysis. Whole blood in heparin were analyzed immediately following sampling for blood gas, hematology, and circulating chemistry and electrolyte markers via an iSTAT handheld machine and cartridge (CG8+; Abbott Point of Care, Princeton, NJ).

Blood samples were collected from the jugular catheter at −0.5, 0, 1, 2, 3, 4, 5, 6, 6.5, 7, 8, 9, 10, 11, 12, 24, 48, and 72 h relative to LPS administration. Plasma nonesterified fatty acids (**NEFA**), BHB, BUN, insulin, glucagon, LPS-binding protein (LBP), haptoglobin (**Hp**), and serum amyloid A (**SAA**) concentrations were determined with commercially available kits (NEFA, Wako Chemicals USA Inc., Richmond, VA; BHB, Pointe-Scientific Inc., Canton, MI; BUN, Teco Diagnostics, Anaheim, CA; insulin, Mercodia AB, Uppsala, Sweden; glucagon, R&D Systems, Minneapolis, MN; LBP, Hycult Biotech, Uden, the Netherlands; Hp, Life Diagnostics Inc., West Chester, PA; SAA, Tridelta Development Ltd., Kildare, Ireland). The inter- and intra-assay CV were the following for NEFA, BHB, BUN, insulin, glucagon, LBP, Hp, and SAA: 8.9% and 4.5%, 1.7% and 6.4%, 2.4% and 2.7%, 1.8% and 5.1%, 12.5% and 6.5%, 4.0% and 5.9%, 2.8% and 4.7%, and 0.3% and 7.6%, respectively.

Cytokine concentration analysis was conducted at the Iowa State University College of Veterinary Medicine. Briefly, a MILLIPLEX Bovine Cytokine/Chemokine 15-plex kit (BCYT1–33K-PXBK15; EMD Millipore Corporation, Billerica, MA) utilizing antibodies to bovine IL-6, IL-8, IL-10, macrophage inflammatory protein (MIP)-1α, IL-36 receptor antagonist (RA), macrophage chemo-attractant protein (MCP)-1, MIP-1β, and tumor necrosis factor (TNF)-α was used to evaluate serum samples. The assay was performed according to the manufacturer's instructions. Concentrations of markers were measured on a BioPlex 200 system (Bio-Rad, Hercules, CA) with Bio-Plex Manager Software (v 6.1.1). Quality control values for each marker were consistently within the range indicated by the manufacturer.

Rectal temperatures were measured using a digital thermometer (GLA M900 Digital Thermometer, San Luis Obispo, CA) at the same times as blood sampling for the first 12 h of P2 and immediately following each milking thereafter.

Statistical Analysis

Data were analyzed with PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, NC). Data from P1 and P2 were analyzed separately. Each animal's respective response was analyzed using repeated measures (represented as d or h) with an autoregressive covariance structure for production responses, Hp, and cytokines, and a spatial power covariance structure for rectal temperature and all other blood responses. Both models included cow nested within treatment as the subject. Effects of treatment, time, their interaction, and replicate were assessed as fixed effects. Statistical analysis included baseline data from either P1 or −0.5 h (before Ca and LPS administration)

as a covariate. Orthogonal contrast statements comparing (1) CON relative to all 3 LPS treatments, (2) LPS relative to Ca supplements (OCa-LPS and IVCa-LPS), and (3) OCa-LPS relative to IVCa-LPS were included in the analysis. Cows in the CON treatment were included in production responses and rectal temperature analysis but were omitted from blood response analyses due to cost. A logarithmic transformation was performed for MIP-1β. Data were reported as LSM ± SEM (pooled SEM were used when reporting LSM of multiple treatment groups) and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$. Within the Results section, all *P*-values independent of time point are reported with orthogonal contrasts when applicable, and meaningful ($P \leq 0.10$) effects of treatment and treatment by time are reported with multiple comparisons *P*-values adjusted with Tukey's honestly significant difference test.

RESULTS

As anticipated, iCa concentrations were decreased post-LPS in CON-LPS cows (0.83 ± 0.04 mmol/L at 8 h nadir; $P < 0.01$; Figure 1). Overall, cows supplemented with OCa-LPS and IVCa-LPS had increased iCa relative to CON-LPS (1.11 and 1.24 vs. 0.95 ± 0.02 mmol/L, respectively; $P < 0.01$). Further, iCa concentrations were increased in IVCa-LPS relative to OCa-LPS ($P < 0.01$). After each IVCa-LPS infusion, cows became temporally hypercalcemic (1.79 ± 0.03 mmol/L).

Production and Vital Signs

During P2, CON-LPS, OCa-LPS, and IVCa-LPS cows had decreased DMI compared with CON cows (23.5 vs. 28.3 ± 1.2 kg, respectively; $P < 0.01$; Figure 2A), and the pattern and extent of reduced DMI did not differ between LPS groups. Milk yield was decreased in LPS cows relative to CON during P2 (18.1 and 21.9 ± 0.6 kg, respectively; $P < 0.01$; Figure 2B). The pattern and extent of reduced milk yield did not differ overall between CON-LPS and OCa-LPS cows. However, IVCa-LPS was increased relative to both CON-LPS and OCa-LPS throughout P2 (19.6 vs. 17.1 and 17.7 ± 0.5 kg; $P = 0.01$ and $P < 0.01$, respectively). Cows administered LPS had increased rectal temperature compared with CON (38.8 vs. 38.5 ± 0.04°C, respectively; $P < 0.01$; Figure 2C). Further, IVCa-LPS had increased rectal temperature relative to CON-LPS from 3 to 4 h (39.8 vs. 38.9 ± 0.1°C, respectively; $P < 0.01$).

Milk components, MUN, and SCS are reported in Supplemental Table S2 (see Notes). Briefly, in response to LPS, ECM decreased relative to CON (20.3 vs. 24.3

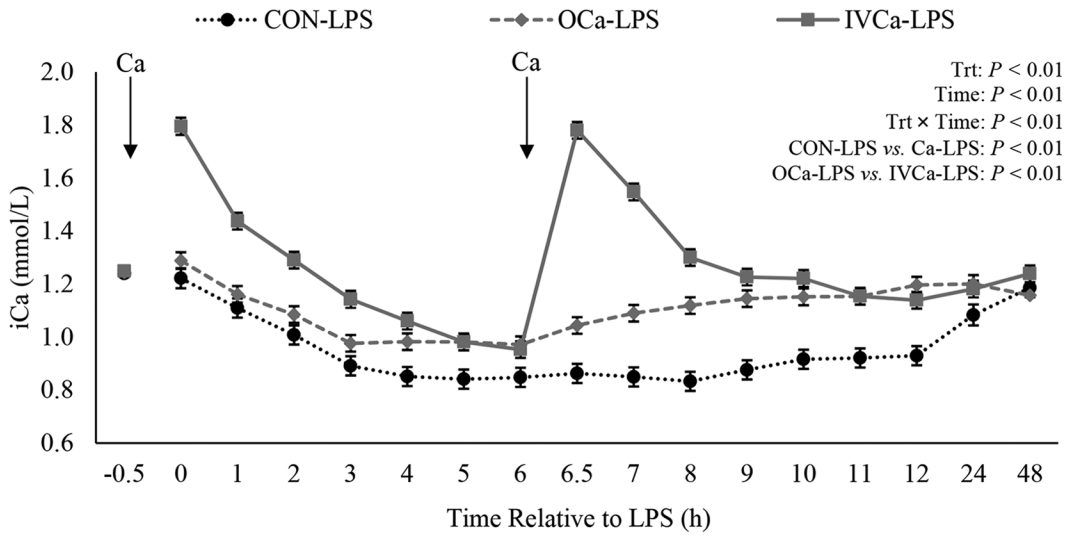

Figure 1. Effects of LPS and Ca on circulating ionized Ca (iCa). Treatments (Trt) included LPS control (CON-LPS; 0.375 µg/kg BW; n = 6), LPS with oral Ca bolus (OCa-LPS; 0.375 µg/kg BW and one 192-g bolus of Bovikalc [Boehringer Ingelheim Animal Health USA Inc., Duluth, GA] containing 43 g of Ca [71% $CaCl_2$ and 29% $CaSO_4$] supplemented at −0.5 and 6 h relative to LPS administration; n = 8), and LPS with i.v. Ca infusion (IVCa-LPS; 0.375 µg/kg BW and 500 mL of Ca-gluconate, 23% [VetOne; Boise, ID] administered at −0.5 and 6 h relative to LPS administration; n = 8). Arrows (↓) depict when Ca treatments were administered (immediately after blood samples were collected at −0.5 and 6 h relative to LPS). Contrasts compare effects of CON-LPS relative to Ca treatments (OCa-LPS and IVCa-LPS [Ca-LPS]; CON-LPS vs. Ca-LPS) and OCa-LPS relative to IVCa-LPS (OCa-LPS vs. IVCa-LPS). Results are expressed as LSM ± SEM. Mean baseline values collected before LPS are reported to the left of the dataset.

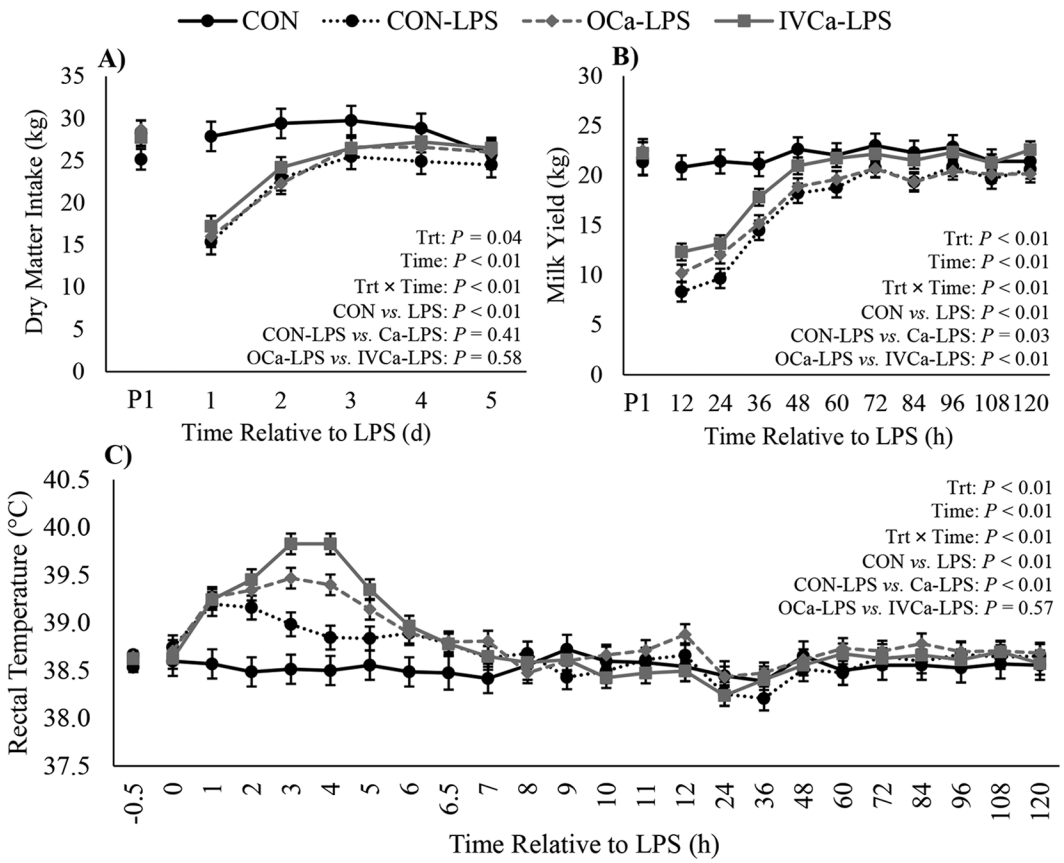

Figure 2. Effects of LPS and Ca on production parameters and rectal temperature. Treatments (Trt) included saline control (CON; 4 mL of saline; n = 4), LPS control (CON-LPS; 0.375 µg/kg BW; n = 6), LPS with oral Ca bolus (OCa-LPS; 0.375 µg/kg BW and one 192-g bolus of Bovikalc [Boehringer Ingelheim Animal Health USA Inc., Duluth, GA] containing 43 g of Ca [71% $CaCl_2$ and 29% $CaSO_4$] supplemented at −0.5 and 6 h relative to LPS administration; n = 8), and LPS with i.v. Ca infusion (IVCa-LPS; 0.375 µg/kg BW and 500 mL of Ca-gluconate, 23% [VetOne; Boise, ID] administered at −0.5 and 6 h relative to LPS administration; n = 8). Figures depict (A) DMI, (B) milk yield, and (C) rectal temperature. Contrasts compare effects of CON relative to LPS treatments (CON vs. LPS), CON-LPS relative to Ca treatments (OCa-LPS and IVCa-LPS [Ca-LPS]; CON-LPS vs. Ca-LPS), and OCa-LPS relative to IVCa-LPS (OCa-LPS vs. IVCa-LPS). Results are expressed as LSM ± SEM. Least squares means ± SEM of P1 and mean baseline values collected before LPS are reported to the left of the dataset.

± 0.5 kg, respectively; $P < 0.01$). Cows assigned to OCa-LPS had reduced ECM compared with IVCa-LPS (19.4 vs. 20.9 ± 0.4 kg, respectively; $P = 0.02$) but did not differ from CON-LPS. Milk concentrations of fat and protein were largely unaltered by LPS relative to CON cows, but lactose concentrations were reduced in LPS cows compared with CON (4.65 vs. 4.81 ± 0.04%, respectively; $P < 0.01$). Yield of milk fat, protein, and lactose decreased in LPS groups relative to CON ($P < 0.01$). Overall milk fat and protein yield were decreased in OCa-LPS relative to IVCa-LPS (0.75 vs. 0.80 ± 0.02 kg and 0.56 and 0.61 ± 0.02 kg, respectively; $P = 0.05$). Milk urea nitrogen increased on P2 d 1 in all LPS treatments compared with CON, but overall MUN concentrations during P2 did not differ. Milk SCS was increased in LPS cows compared with CON ($P = 0.04$) but did not differ between LPS groups.

Metabolism

Cows assigned to CON-LPS and IVCa-LPS treatments had increased glucose concentrations relative to OCa-LPS cows at 1 h (94.9 and 95.7 vs. 75.1 ± 3.4 mg/dL, respectively; $P = 0.05$; Figure 3A). All LPS-infused groups had similarly reduced glucose concentrations by 2 h. Insulin concentrations increased in all LPS groups but did not differ by treatment (Figure 3B). Circulating glucagon increased in OCa-LPS and IVCa-LPS, but not CON-LPS (459 and 472 vs. 335 ± 28 pg/mL, respectively; $P < 0.01$; Figure 3C). Concentrations of BUN increased post-LPS, peaked at 12 h and gradually decreased thereafter; however, OCa-LPS had attenuated BUN compared with CON-LPS and IVCa-LPS (8.7 vs. 10.0 and 10.4 ± 0.3 mg/dL, respectively; $P < 0.01$; Figure 3D). Circulating BHB decreased at 6 h post-LPS,

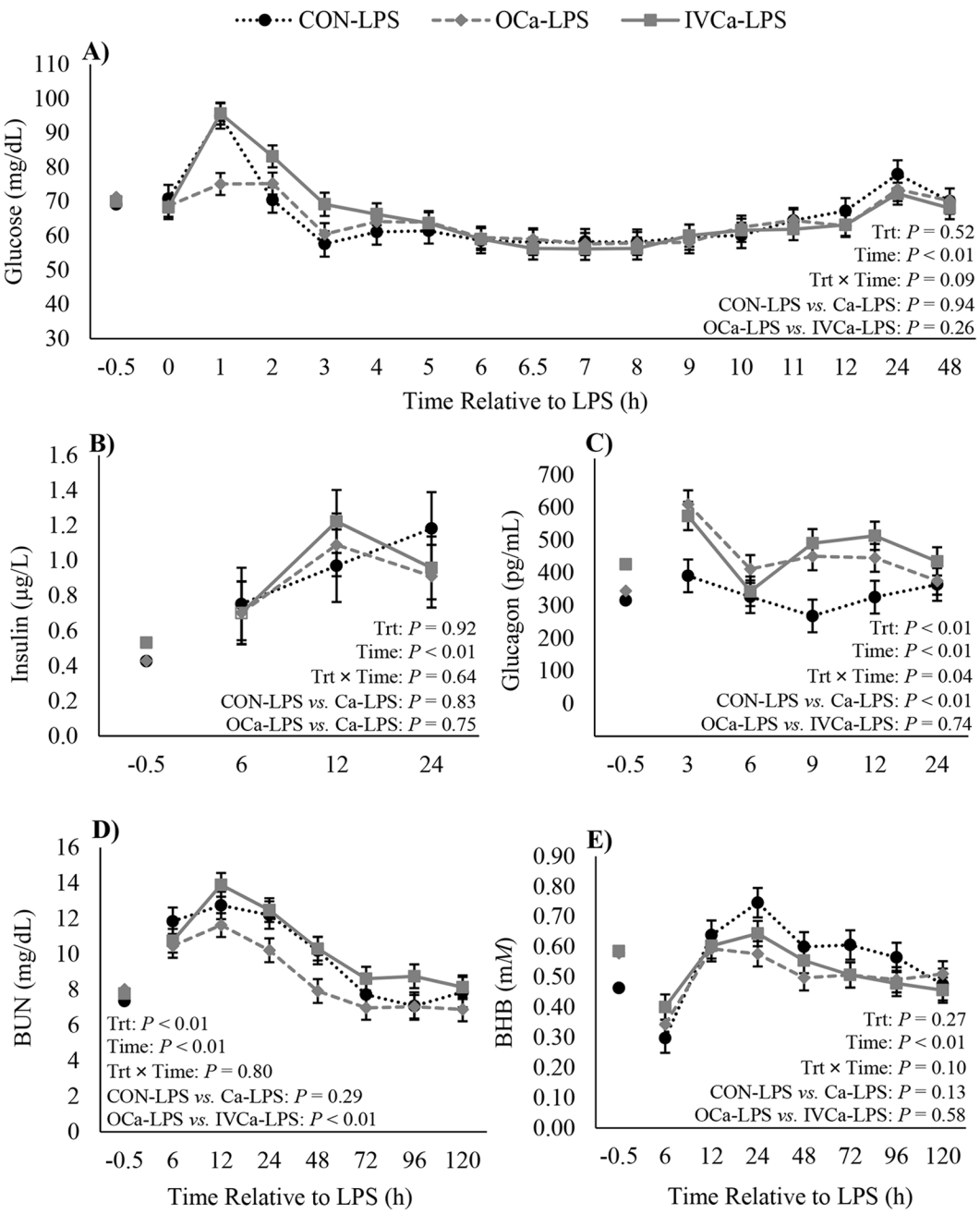

Figure 3. Effects of LPS and Ca on metabolic parameters. Treatments (Trt) included LPS control (CON-LPS; 0.375 µg/kg BW; n = 6), LPS with oral Ca bolus (OCa-LPS; 0.375 µg/kg BW and one 192-g bolus of Bovikalc [Boehringer Ingelheim Animal Health USA Inc., Duluth, GA] containing 43 g of Ca [71% $CaCl_2$ and 29% $CaSO_4$] supplemented at −0.5 and 6 h relative to LPS administration; n = 8), and LPS with i.v. Ca infusion (IVCa-LPS; 0.375 µg/kg BW and 500 mL of Ca-gluconate, 23% [VetOne; Boise, ID] administered at −0.5 and 6 h relative to LPS administration; n = 8). Figures depict circulating (A) glucose, (B) insulin, (C) glucagon, (D) BUN, and (E) BHB. Contrasts compare effects of CON-LPS relative to Ca treatments (OCa-LPS and IVCa-LPS [Ca-LPS]; CON-LPS vs. Ca-LPS) and OCa-LPS relative to IVCa-LPS (OCa-LPS vs. IVCa-LPS). Results are expressed as LSM ± SEM. Mean baseline values collected before LPS are reported to the left of the dataset.

then increased and returned to baseline thereafter, but was undifferentiated by treatment group overall (Figure 3E). Circulating NEFA slightly increased post-LPS over the course of P2 but did not differ by treatment (P = 0.75; data not shown).

Hematological parameters are provided in Supplemental Table S3 (see Notes). Following LPS administration, blood pH, base excess, and HCO_3 concentrations in cows decreased with time, and all 3 parameters were increased in IVCa-LPS relative to other LPS groups (P < 0.01).

Inflammation

Alterations in leukocyte counts followed typical patterns of LPS-induced immune activation, but Ca treatments did not affect blood cell counts during P2 differently than CON-LPS (Supplemental Table S4, see Notes). Administering LPS increased LBP, SAA, and Hp concentrations in all treatments (Figure 4A–C). However, while LBP did not differ between CON-LPS and OCa-LPS, OCa-LPS tended to have increased LBP compared with IVCa-LPS (10.7 vs. 8.6 ± 0.7 µg/mL, respectively; $P = 0.06$).

Circulating IL-6 peaked at 3 h in all cows after LPS and decreased thereafter (9,557 to 3,778 ± 832 pg/mL from 3 to 6 h; Figure 5A) but did not differ by treatment. Concentrations of IL-8 decreased over time post-LPS in all treatments ($P < 0.01$; Figure 5B); however, OCa-LPS tended to have increased IL-8 concentrations relative to IVCa-LPS (2,301 vs. 1,410 ± 340 pg/mL, respectively; $P = 0.08$) but neither differed from CON-LPS. Post-LPS, circulating IL-10 increased at 3 h and decreased thereafter but was undifferentiated by treatment (Figure 5C). Circulating IL-36RA was reduced post-LPS in CON-LPS and IVCa-LPS, but OCa-LPS cows had increased IL-36RA compared with CON-LPS and IVCa-LPS (535 vs. 333 and 285 ± 59 pg/mL; $P = 0.08$ and $P < 0.01$, respectively; Figure 5D). Further, all cows had a similar increase in TNF-α, MCP-1, and MIP-1α concentrations post-LPS (Figure 5E–G). Lastly, MIP-1β concentrations increased after LPS but were attenuated in OCa-LPS compared with CON-LPS (7.88 vs. 8.56 ± 0.18 ln [pg/mL], respectively; $P = 0.05$; Figure 2H), and IVCa-LPS did not differ from other LPS treatments.

DISCUSSION

Hypocalcemia is a conserved species response to an immune insult (Carlstedt et al., 2000; Waldron et al., 2003b), and a common supportive therapy during infection in dairy cattle includes Ca supplementation (Green et al., 1997; Oliveira and Ruegg, 2014). However, numerous studies suggest i.v. Ca therapy is not beneficial or even detrimental to survival during endotoxemia in a variety of species (Malcolm et al., 1989; Steinhorn et al., 1990; Zaloga et al., 1992; Collage et al., 2013; Dotson et al., 2016), does not improve milk yield recovery in dairy cows (Horst et al., 2020; Chandler et al., 2023a), and disrupts normal Ca homeostasis (Wilms et al., 2019).

The detrimental effects of i.v. Ca infusion during LPS exposure might be due to excessive cellular Ca accumulation and the ensuing necrosis (Song et al., 1993; Malcolm et al., 1989). Thus, i.v. Ca administration during infection is generally not recommended as a critical care strategy in humans (Zaloga, 2000; Rhodes et al., 2017; Melchers

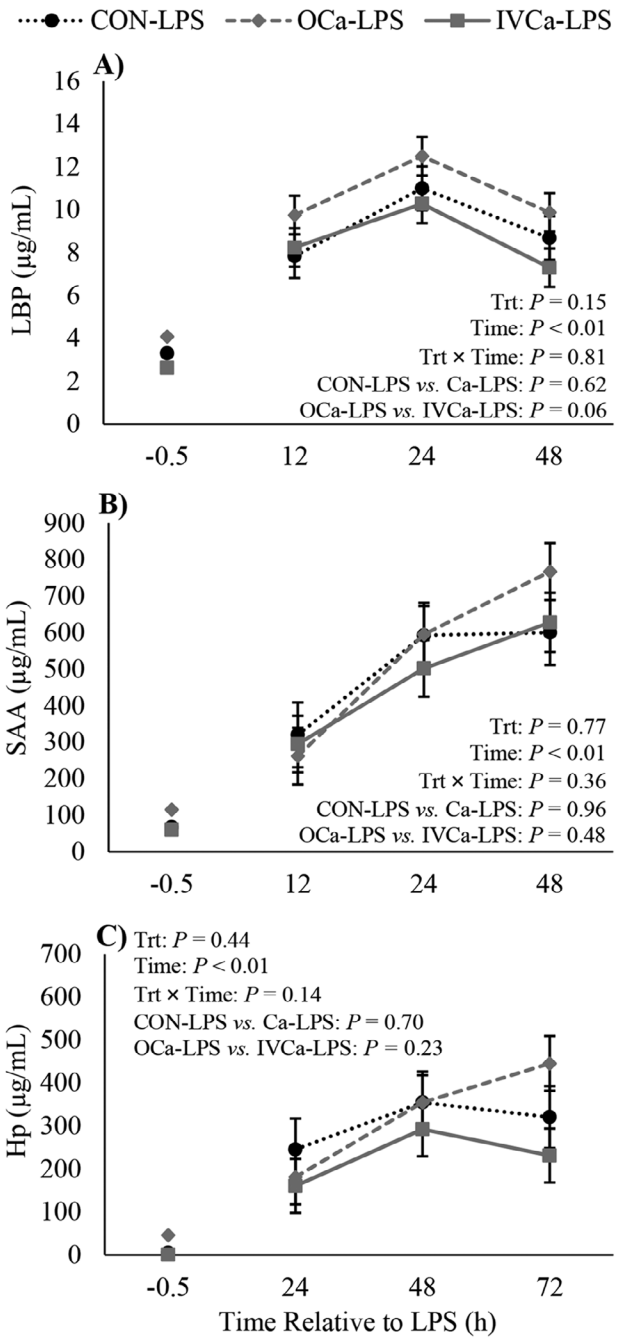

Figure 4. Effects of LPS and Ca on acute phase proteins. Treatments (Trt) included LPS control (CON-LPS; 0.375 µg/kg BW; n = 6), LPS with oral Ca bolus (OCa-LPS; 0.375 µg/kg BW and one 192-g bolus of Bovikalc [Boehringer Ingelheim Animal Health USA Inc., Duluth, GA] containing 43 g of Ca [71% $CaCl_2$ and 29% $CaSO_4$] supplemented at −0.5 and 6 h relative to LPS administration; n = 8), and LPS with i.v. Ca infusion (IVCa-LPS; 0.375 µg/kg BW and 500 mL of Ca-gluconate, 23% [VetOne; Boise, ID] administered at −0.5 and 6 h relative to LPS administration; n = 8). Figures depict (A) LPS-binding protein (LBP), (B) serum amyloid A (SAA), and (C) haptoglobin (Hp). Contrasts compare effects of CON-LPS relative to Ca treatments (OCa-LPS and IVCa-LPS [Ca-LPS]; CON-LPS vs. Ca-LPS) and OCa-LPS relative to IVCa-LPS (OCa-LPS vs. IVCa-LPS). Results are expressed as LSM ± SEM. Mean baseline values collected before LPS are reported to the left of the dataset.

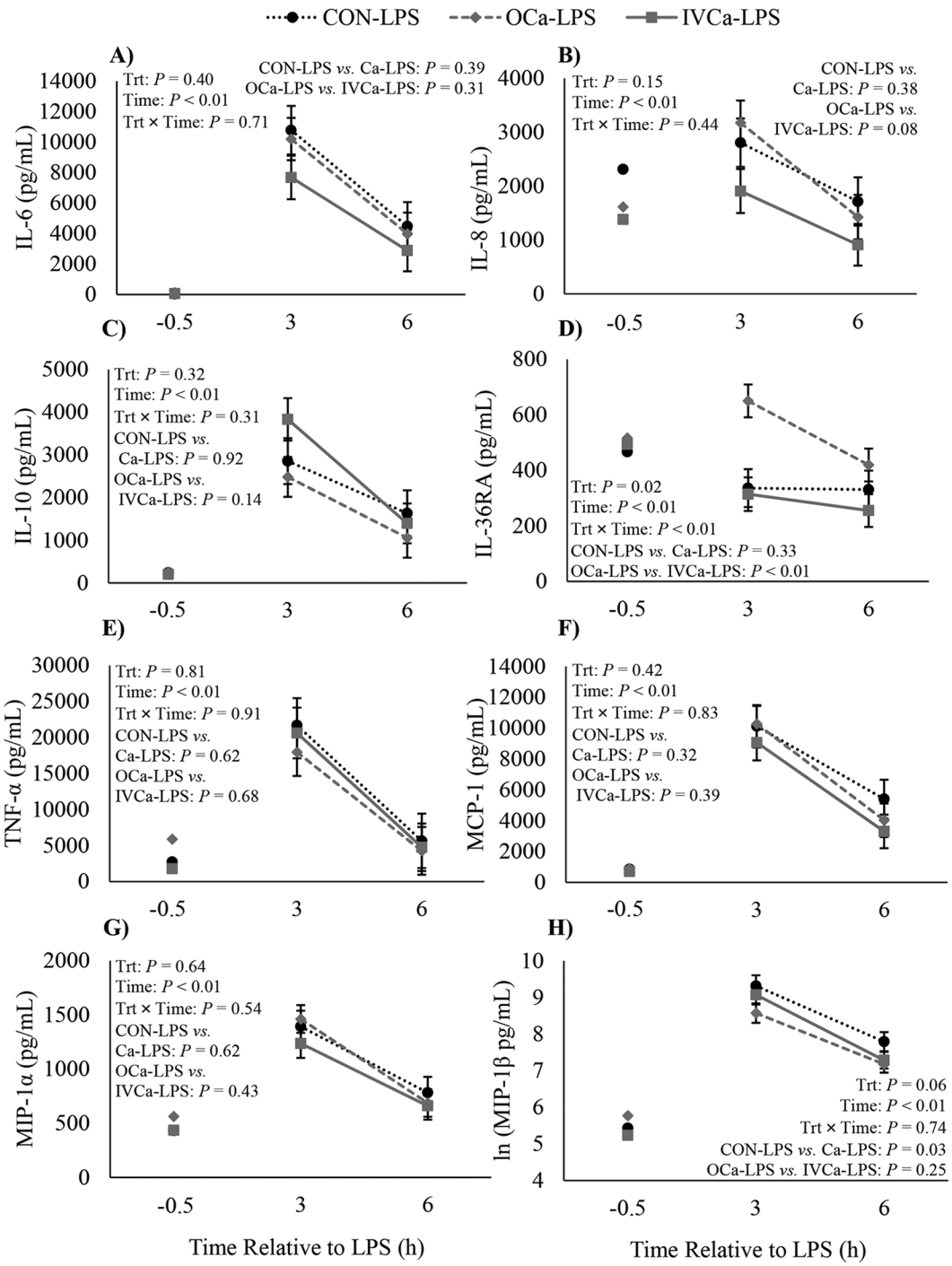

Figure 5. Effects of LPS and Ca on cytokines. Treatments (Trt) included LPS control (CON-LPS; 0.375 µg/kg BW; n = 6), LPS with oral Ca bolus (OCa-LPS; 0.375 µg/kg BW and one 192-g bolus of Bovikalc [Boehringer Ingelheim Animal Health USA Inc., Duluth, GA] containing 43 g of Ca [71% $CaCl_2$ and 29% $CaSO_4$] supplemented at −0.5 and 6 h relative to LPS administration; n = 8), and LPS with i.v. Ca infusion (IVCa-LPS; 0.375 µg/kg BW and 500 mL of Ca-gluconate, 23% [VetOne; Boise, ID] administered at −0.5 and 6 h relative to LPS administration; n = 8) on circulating (A) IL-6, (B) IL-8, (C) IL-10, (D) IL-36 receptor antagonist (IL-36RA), (E) tumor necrosis factor (TNF)-α, (F) monocyte chemo-attractant protein (MCP)-1, (G) macrophage inflammatory protein (MIP)-1α, and (H) macrophage inflammatory protein (MIP)-1β. Contrasts compare effects of CON-LPS relative to Ca treatments (OCa-LPS and IVCa-LPS [Ca-LPS]; CON-LPS vs. Ca-LPS) and OCa-LPS relative to IVCa-LPS (OCa-LPS vs. IVCa-LPS). Results are expressed as LSM ± SEM. Mean baseline values collected before LPS are reported to the left of the dataset.

and van Zanten, 2023). However, Ca presumably remains crucial for host survival. During immune activation, immune cells use Ca to exert effector functions (Lewis, 2001; Varga-Szabo et al., 2009). Further, Ca is thought to increase stability and reduce proteolytic degradation of acyloxyacyl hydrolase, an important LPS detoxifying enzyme (Gorelik et al., 2018). From a dairy production perspective, Ca plays important roles in gastrointestinal tract motility, energetic metabolism, and milk synthesis (Burgoyne and Wilde, 1994; Klec et al., 2019; Goff et al., 2020). In contrast to the apparent adverse effects of i.v. Ca infusion, oral Ca seems to benefit milk yield recovery after LPS administration (Al-Qaisi et al., 2020). Further, oral Ca does not appear to meaningfully augment inflammation (Couto Serrenho et al., 2024). Therefore, we hypothesized supplementing oral Ca would improve the response following immune activation when compared with i.v. Ca, and this would be accompanied with attenuated markers of immune activation and less severe changes in metabolism.

As expected, LPS successfully induced hypocalcemia in CON-LPS cows, which was predictably ameliorated in OCa-LPS and IVCa-LPS cows. Relieving hypocalcemia with oral Ca supplementation herein confirms our previous experiment (Al-Qaisi et al., 2020). Further, IVCa-LPS cows had abrupt increases in Ca after each i.v. Ca infusion and this pattern agrees with others (Blanc et al., 2014; Wilms et al., 2019). Taken together, the temporal alterations in iCa following LPS and Ca administration were as anticipated and indicated successful implementation of the experimental design.

Cows decreased DMI and milk yield following LPS, and this corroborates other models (Waldron et al., 2006; Horst et al., 2018). However, the DMI response did not differ between treatments, which disagreed with our hypothesis that OCa-LPS cows would have increased appetite relative to CON-LPS and IVCa-LPS. Al-Qaisi et al. (2020) observed a tendency for improved DMI post-LPS with oral Ca supplementation, which was foundational to our tenet. An important difference between trials was that the oral Ca supplement that benefited DMI included live yeast, and the experiment was unable to isolate differential effects of the Ca and yeast (Al-Qaisi et al., 2020). Additionally, differences in Ca sources and quantity between boluses may also explain inconsistencies between experiments (Al-Qaisi et al., 2020). Nevertheless, no differences in DMI were detected between LPS treatment groups herein, but this demonstrates that the metabolic effects of Ca treatments (discussed below) were not mediated by differences in nutritional plane.

Immune activation markedly decreased milk yield, which is consistent with prior LPS reports (Waldron et al., 2003a; Horst et al., 2019; Opgenorth et al., 2024). Milk production did not differ between OCa-LPS and CON-LPS cows, which contrasted from Al-Qaisi et al. (2020) and may be due to differences in supplement components as described previously. Some investigations observe improved milk yield following oral Ca boluses in transition cows, but this appears dependent upon specific interactions of parity, previous lactation milk yield, BCS, days carried calf, and lameness (Oetzel and Miller, 2012; Martinez et al., 2016; Leno et al., 2018). Further, sometimes oral Ca does not affect transition cow milk production (Domino et al., 2017; Menta et al., 2021) and sometimes it actually decreases postpartum milk production (Martinez et al., 2016). Reasons for the inconsistencies are unclear, but overall, oral Ca boluses do not appear to consistently influence milk synthesis in transition (Valldecabres et al., 2023) or immune-activated dairy cows.

Additionally, IVCa-LPS slightly increased milk yield compared with CON-LPS and OCa-LPS groups. This contrasted from our hypothesis because we previously observed that a 12-h continuous i.v. Ca infusion detrimentally affected milk production (Horst et al., 2020), and others utilizing early-lactation cows under a similar experimental design did not observe any difference between i.v. Ca infusion and placebo (Chandler et al., 2023a). The abrupt hypercalcemic state achieved following each i.v. Ca infusion was unique compared with the previous studies and might influence physiology differently than eucalcemic conditions. Although we analyzed a wide range of variables, there is not an obvious reason milk yield appeared to improve with IVCa-LPS. Regardless, the primary areas of interest were inflammation and metabolism, as the experiment was knowingly underpowered to detect differences in milk yield.

Rectal temperature increased post-LPS, which agrees with temporal pyrexia observed in other LPS models (Gross et al., 2018; Horst et al., 2019; Chandler et al., 2023b). Interestingly, the febrile response at 3 to 4 h was elevated by IVCa-LPS relative to CON-LPS, but was no different from OCa-LPS cows. The heightened post-LPS rectal temperature with i.v. Ca supplementation corroborates previous observations in mid-lactation cows with eucalcemic i.v. Ca infusion (Horst et al., 2020). Augmented pyrexia is generally suggestive of a magnified inflammatory response, though acute fever also amplifies leukocyte functions (Johansen et al., 1983), enhances cellular response to LPS, and improves survival during infection (Rosenspire et al., 2002; Lee et al., 2012). Reasons as to why i.v. Ca supplementation exaggerated the post-LPS febrile response compared with CON-LPS herein may be due in part to the postinfusion hypercalcemia as increased leukocyte Ca uptake can exacerbate fever-inducing cytokine secretion (Rossol et al., 2012). Though we did not detect differences in the pryogenic compounds measured (discussed below), it is possible that other nonanalyzed immune originating

compounds (i.e., prostaglandins) were affected by i.v. Ca supplementation. Regardless, health providers should be aware of the febrile enhancing effects that may accompany supplemental i.v. Ca when evaluating therapeutic strategies.

Administrating LPS often results in a biphasic glucose response when hyperglycemia develops in the first 2 h followed by prolonged hypoglycemia (Giri et al., 1990; Yates et al., 2011; Burdick Sanchez et al., 2013). Hyperglycemia is a consequence of increased hepatic glucose output and LPS-induced peripheral insulin resistance, but hypoglycemia eventually develops when the activated immune system's glucose consumption exceeds the individual's glucose-producing and glucose-sparing mechanisms (Lang and Dobrescu, 1991; Lang, 1993). Herein, hyperglycemia developed in CON-LPS and IVCa-LPS, but not OCa-LPS cows, though all treatment groups had similarly reduced glucose concentrations thereafter. Reasons why OCa-LPS did not acutely increase glucose are not clear, especially because oral Ca did in our previous experiment (Al-Qaisi et al., 2020). Cortisol sharply increases post-LPS in dairy cows (Horst et al., 2019), and this glucocorticoid response presumably contributes to the increased hepatic glucose output. Thus, it is of interest to determine if intestinally absorbed Ca affects cortisol homeostasis.

Other metabolic parameters, including insulin, glucagon, BUN, BHB, and NEFA concentrations were altered post-LPS; temporal changes that closely agree with prior reports (Waldron et al., 2003a; Kvidera et al., 2017; Horst et al., 2018, 2019). Although the insulin response did not differ by treatment, glucagon only increased in OCa-LPS and IVCa-LPS cows. Calcium is critically involved with the secretion of both pancreatic insulin and glucagon (Iversen and Hermansen, 1977; Rorsman and Trube, 1986). While there are species differences (specifically ruminants) in glucagon's role in intermediary metabolism (Bassett, 1978; Brockman, 1978), it is unclear why there were differences in the magnitude of hyperglycemia at 1 h between Ca treatments despite having a similar glucagon response.

Circulating BUN increased post-LPS in all treatments, and while endotoxin can cause kidney dysfunction and interfere with BUN filtration (Peng et al., 2020), elevated BUN could also be indicative of increased skeletal muscle proteolysis and amino acid deamination as gluconeogenic precursors (Beisel and Wannemacher, 1980). Oral Ca blunted the BUN response whereas i.v. Ca and CON-LPS had similarly elevated BUN. Decreased BUN with oral Ca agrees with the decreased post-LPS MUN previously described (Al-Qaisi et al., 2020). This may imply that oral Ca reduced the extent of post-LPS skeletal muscle mobilization or decreased the gastrointestinal tract ammonia absorption. Regardless of whether this is a kidney or a skeletal muscle effect, the route of Ca delivery seems to influence nitrogen metabolism during immune activation and having a better understanding of this would likely have practical implications to nutrition and management decisions.

Lipopolysaccharide increased acute phase proteins and cytokines and altered circulating leukocytes, changes that closely corroborate other LPS models (Kvidera et al., 2017; Horst et al., 2019; Chandler et al., 2023b) and are indicative of intense immune activation. The concentrations of LBP and IL-8 tended to be reduced in IVCa-LPS compared with OCa-LPS, but neither Ca treatment differed from CON-LPS. A primary function of IL-8 is neutrophil recruitment and activation (Harada et al., 1994). Interestingly, the increase in MIP-1β (a pro-inflammatory chemokine that recruits monocytes and lymphocytes; Rollins, 1997) was blunted in OCa-LPS compared with CON-LPS cows. Further, IL-36RA (an anti-inflammatory compound) concentrations were increased in OCa-LPS relative to CON-LPS and IVCa-LPS. This cytokine binds to the IL-36 receptor and prevents IL-36 from eliciting proinflammatory activity (Bassoy et al., 2018). Incidentally, IL-36 plays important roles in mediating gastrointestinal tract inflammation (Ngo et al., 2021), which may be more relevant to oral Ca supplementation due to the potential involvement of the gut during Ca absorption (Goff, 2018). Collectively, alterations in LBP, IL-8, MIP-1β, and enhanced IL-36A suggests oral Ca may modify the inflammatory milieu following intense immune activation.

Collectively, neither Ca delivery route had much influence on inflammation, and this agrees with previous oral Ca investigations (Al-Qaisi et al., 2020; Couto Serrenho et al., 2024), but disagrees with an i.v. eucalcemic model (Horst et al., 2020). Calcium infusion may cause a hyperinflammatory response through several mechanisms: (1) iCa stabilizes circulating LPS aggregates, which interferes with the ability of lipoproteins to sequester LPS (Skarnes and Chedid, 1964; Munford et al., 1981); a scenario that forces TLR-4 presenting cells to detoxify LPS (Rossol et al., 2012; Eckel and Ametaj, 2016; Klein et al., 2016), (2) calcium is a chemo-attractant for macrophages and may enhance cytokine production (Shi et al., 1996; Iamartino and Brandi, 2022), and (3) supplemented Ca can cause cellular Ca overload and provoke cell injury and necrosis (Malcolm et al., 1989; Nicotera et al., 1992; Song et al., 1993). Regardless of the aforementioned speculation, both routes of Ca supplementation were administered as acute boluses instead of constant infusion like in previous models (Zaloga et al., 1992; Horst et al., 2020), which may have provided cows the ability to regulate circulating Ca and thus contributed to the lack of deleterious effects of Ca on inflammation.

Inflammation is often experimentally modeled by administering a single or repeated LPS bolus, which elicits a well-described and robust immune response (van Miert and Frens, 1968; Lohuis et al., 1988). This approach has provided an enormous amount of insight and information about how immune activation coordinates metabolic adjustments to homeorhetically prioritize survival. However, natural infection (e.g., mastitis, metritis, pneumonia, and intestinal hyperpermeability) likely comprises a persistent, dynamic exposure to multiple (maybe hundreds of) different endotoxins with varying virulences (Bonnett et al., 1991; Oliveira et al., 2020). Consequently, extrapolating the intense and acute response to LPS administration (i.v., intramammary, and so on) to normal on-farm ailments and transition cow inflammation needs appropriate context and restraint. Thus, the field of immunometabolism needs a more comprehensive and pragmatic approach to advance our understanding of Ca dyshomeostasis during disease and evaluate strategies that improve animal health.

CONCLUSIONS

Lipopolysaccharide administration decreased production performance, altered metabolism, increased inflammation, generated an intense febrile response, and caused hypocalcemia. Supplementing i.v. and oral Ca during an LPS challenge ameliorated the hypocalcemia and both Ca delivery routes increased circulating glucagon. Inflammatory markers increased as expected post-LPS in all treatments. Some inflammatory markers differed or tended to differ with Ca supplementation, including LBP, IL-8, MIP-1β, and IL-36RA, but altogether, it does not indicate substantial detriment or benefit to the immune response with oral Ca delivery. Collectively, these data suggest that the route of Ca delivery has modest impacts on the immune response and its coordination of the accompanying metabolic adjustments. Whether or not supplemental Ca should be used therapeutically during an infection requires further exploration.

NOTES

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Nonstandard abbreviations used: Ca-LPS = Ca treatments; CON = saline control; CON-LPS = LPS control; Hp = haptoglobin; iCa = ionized Ca; IVCa-LPS = LPS with i.v. Ca administration; NEFA = nonesterified fatty acids; OCa-LPS = LPS with oral Ca bolus; P1 = period 1; P2 = period 2; SAA = serum amyloid A; Trt = treatment.

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