Glucose requirements of an activated immune system in lactating Holstein cows

S. K. Kvidera, E. A. Horst, M. Abuajamieh, E. J. Mayorga, M. V. Sanz Fernandez, and L. H. Baumgard¹

Department of Animal Sciences, Iowa State University, Ames 50011

ABSTRACT

Accurately quantifying activated immune system energy requirements in vivo is difficult, but a better understanding may advance strategies to maximize animal productivity. Study objectives were to estimate whole-body glucose utilization following an i.v. endotoxin challenge. Lactating Holstein cows were jugular catheterized and assigned 1 of 3 bolus treatments: control (CON; 5 mL of saline; $n = 6$), lipopolysaccharide (LPS)-administered (LPS-C; 1.5 $\mu\text{g}/\text{kg}$ of body weight; *Escherichia coli* 055:B5; $n = 6$), and LPS + euglycemic clamp (LPS-Eu; 1.5 $\mu\text{g}/\text{kg}$ of body weight; 50% glucose solution infusion; $n = 6$). After LPS administration, blood glucose was determined every 10 min and glucose infusion rates were adjusted in LPS-Eu cows to maintain euglycemia for 720 min. Blood samples were obtained 180, 360, 540, and 720 min postbolus for further analysis. Cows were milked 360 and 720 min postbolus. Blood glucose was increased 84% in LPS-administered cows for up to 150 min postbolus; thereafter, circulating glucose was decreased 30% in LPS-C relative to LPS-Eu and CON cows. Mild hyperthermia ($+0.5^\circ\text{C}$) occurred between 30 and 90 min postbolus in LPS-administered relative to CON cows; thereafter, rectal temperature did not differ between treatments. Milk yield and lactose percentage were decreased 80 and 11%, respectively, in LPS-administered relative to CON cows. Circulating insulin was increased 4 fold and nonesterified fatty acids, β -hydroxybutyrate, and ionized Ca were decreased $\sim 50\%$ in LPS-administered compared with CON cows. Plasma L-lactate, haptoglobin, and serum amyloid A increased ~ 160 , 260, and 75%, respectively, in LPS-administered relative to CON cows. Overall, LPS-binding protein was increased 87% in LPS-administered relative to CON cows; however, at 720 min, it was decreased 25% in LPS-Eu compared with LPS-C cows. White blood cell count decreased $\sim 90\%$ in LPS-administered cows at 180 min and progressively increased to $\sim 50\%$ of CON values by 720

min. Total glucose deficit during the 720 min following LPS administration was calculated as the decrease in the amount of glucose required to synthesize milk (due to the decrease in milk yield relative to prebolus levels) plus the amount of glucose infused to maintain euglycemia (in LPS-Eu cows only) and was 461, 1,259, and 1,553 g for CON, LPS-C, and LPS-Eu cows, respectively. Our data indicate an acutely activated immune system uses >1 kg of glucose within 720 min and maintaining euglycemia did not rescue milk synthesis.

Key words: glucose homeostasis, inflammation, insulin, lipopolysaccharide

INTRODUCTION

Maximizing nutrient and energy partitioning toward productive processes is key to economically profitable animal agriculture. Immunoactivation is characterized by decreased milk and lean tissue synthesis, inefficient feed utilization, and poor reproduction, presumably due to immune system nutrient consumption (Lochmiller and Deerenberg, 2000; Johnson, 2012). Farm animals experience frequent immune challenges, and obvious sources in dairy cattle include metritis and mastitis (Sheldon et al., 2008; Ballou, 2012). A more inconspicuous source of inflammation is increased gastrointestinal permeability, which can be a consequence of different stressors including dietary changes (e.g., grain-induced acidosis; Khafipour et al., 2009a), systemic inflammation (Hietbrink et al., 2009), or environmental factors such as heat stress (Baumgard and Rhoads, 2013). Immunoactivation begins when molecular patterns from invading pathogens (e.g., bacteria, viruses, yeast, parasites) are recognized by immune cells, eliciting the transcription and production of inflammatory cytokines, which results in an acute phase response characterized by fever, changes in circulating leukocyte numbers, and hepatic acute phase protein synthesis (Cecilian et al., 2012). Immunoactivation can be experimentally modeled by administering LPS, a cell wall component of gram-negative bacteria that elicits a well-characterized and robust immune response (van Miert and Frens, 1968; Lohuis et al., 1988b); however, the response can vary in magnitude, as repeated or continuous exposure

Received September 15, 2016.

Accepted November 6, 2016.

¹Corresponding author: baumgard@iastate.edu

to endotoxin causes hyporesponsiveness (i.e., tolerance; Beeson, 1947).

Immunoactivation markedly disrupts glucose homeostasis and is characterized by hypoglycemia and hyperlactemia (Filkins, 1978; McGuinness, 2005; Michaëli et al., 2012). In vitro experiments demonstrate a substantial increase in glucose consumption by activated immune cells, as glucose is their primary fuel and an important biosynthetic precursor (Calder et al., 2007; Palsson-McDermott and O'Neill, 2013). Despite the increase in glucose requirements, anorexia accompanies immunoactivation, which decreases diet-derived glucose or glucose precursors. Decreased milk synthesis is one of the first observable signs of infection or inflammation in dairy cattle, and this presumably represents a strategy to spare glucose for the immune system. To further ensure an adequate fuel supply for the immune system, hepatic glucose output increases via both glycogenolysis and gluconeogenesis (Filkins, 1978; McGuinness, 1994; Waldron et al., 2003a). Peripheral insulin resistance occurs synchronously, leading to decreased glucose uptake by skeletal muscle and adipose tissue (Lang et al., 1990; Song et al., 2006). These metabolic adaptations are indicative of homeorhetic partitioning toward a new dominant physiological state of immunoactivation. Despite the aforementioned efforts to spare glucose, hypoglycemia often develops following a LPS challenge, likely because the immune system's rate of glucose utilization exceeds the orchestrated capacity of the liver to export glucose and insulin sensitive tissues to reduce glucose disposal (McGuinness, 2005).

The extent of in vivo glucose consumption by the activated immune system is difficult to assess due to the ubiquitous and fluctuating distribution of immune cells and organ-specific changes in insulin sensitivity; however, better understanding its impact on bioenergetics is a prerequisite to developing strategies aimed at minimizing production losses in immunoactivated animals. Therefore, our experimental objective was to estimate the amount of glucose needed to maintain euglycemia following an LPS challenge as a proxy for the amount of glucose required to fuel an acute immune response.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures were approved by the Iowa State University Institutional Animal Care and Use Committee. Eighteen Holstein cows (718 ± 16 kg; 169 ± 7 DIM; parity 2 or 3) were used and housed in individual boxstalls (4.57×4.57 m) at the Iowa State University Dairy Farm. Due to space constraints, the study was conducted in 2 replications occurring 14 d apart

with 9 different cows in each replication. Treatment was balanced within replicate. Cows were allowed 5 d to acclimate, during which they were implanted with bilateral jugular catheters. Cows were fed ad libitum once daily (0600 h) with a diet formulated to meet or exceed the predicted requirements (NRC, 2001; Table 1) of energy, protein, minerals, and vitamins. Cows were milked 4 times daily (0000, 0600, 1200, 1800 h) throughout the experiment. During the final 3 d of acclimation, milk yields were recorded and a sample for composition analysis was obtained at each milking. To estimate the glucose requirements of an activated immune system, we employed an LPS-euglycemic clamp technique, as we have recently described (Kvidera et al., 2016a). Cows were randomly assigned to 1 of 3 i.v. bolus treatments administered immediately after the 0600-h milking: (1) control (**CON**; 3 mL of sterile saline; $n = 6$), (2) LPS-administered controls in which hypoglycemia was allowed to develop (**LPS-C**; $1.5 \mu\text{g/kg}$ of BW of LPS; $n = 6$), and (3) LPS-administered in which euglycemia was maintained (**LPS-Eu**; $1.5 \mu\text{g/kg}$ of BW of LPS; $n = 6$). Selection of the LPS dose was influenced by the magnitude of hypoglycemia observed in earlier reports (Giri et al., 1990; Waldron et al., 2003a; Waggoner et al., 2009). Lipopolysaccharide (*Escherichia coli* O55:B5; Sigma Aldrich, St. Louis, MO) was dissolved in sterile saline at a concentration of $200 \mu\text{g/mL}$ and passed through a $0.2\text{-}\mu\text{m}$ sterile syringe filter (Thermo Scientific, Waltham, MA). The total volume of LPS solution administered was approximately 5 mL. In the LPS-Eu treatment, we performed a euglycemic clamp, where a 50% glucose solution (as dextrose; VetOne, Boise, ID) was i.v. infused at a known and adjustable rate utilizing a modular pump (Deltec 3000, Deltec Inc., St. Paul, MN) to maintain the pre-LPS administration blood glucose concentrations.

Cows were tethered during the 12-h challenge (but allowed to stand up and lay down) to allow for frequent sampling. Water was provided ad libitum and feed was removed ~ 1 h before treatment administration and animals remained fasted during the 720-min data collection period to eliminate the confounding effect of dissimilar nutrient intake. Cows were milked immediately before administration (0600 h) and continued to be milked every 360 min (6 h) with yields and samples for composition analysis obtained at each milking. Samples were stored at 4°C with a preservative (bronopol tablet; D & F Control System, San Ramon, CA) until analysis by Dairy Lab Services (Dubuque, IA) using AOAC-approved (AOAC International, 1995) infrared analysis equipment and procedures.

Blood samples were obtained at -30 , -20 , and 0 min relative to LPS administration to establish baseline glucose levels. Each respective treatment bolus was

Table 1. Ingredients and composition of diet¹

Item	Measurement ²
Ingredient (% of DM)	
Corn silage	20.5
Alfalfa hay	10.2
Lactation grain	8.9
Ground corn	4.7
Whole cotton	4.6
Soy Plus ³	1.8
Soy 48 ⁴	2.3
Chemical analysis (% of DM)	
Starch	25.3
CP	16.6
NDF	31.4
ADF	22.2
NE _L (Mcal/kg of DM)	1.62

¹Diet moisture averaged 42.32%.

²Average nutrient levels: 4.72% fat, 0.81% Ca, 0.47% P, 0.36% Mg, 0.19% S, 1.20% K, 0.45% Na, 0.47% Cl, 56.05 mg/kg of Zn, 62.79 mg/kg of Mn, 129.60 mg/kg of Fe, 17.54 mg/kg of Cu, 0.22 mg/kg of Co, 0.28 mg/kg of Se, 0.84 mg/kg of I, 4,453.3 IU/kg of vitamin A, 1,190.5 IU/kg of vitamin D, and 26.5 IU/kg of vitamin E.

³Soy Plus: Landus Cooperative, Ames, IA.

⁴Soy 48: Key Cooperative, Gilbert, IA.

administered immediately following the 0-min blood sample collection. For LPS-Eu cows, postbolus blood samples (1 mL) were collected every 10 min and immediately analyzed for glucose concentration (TRUEbalance glucometer; McKesson, San Francisco, CA). Glucose infusion began when blood glucose concentration declined below baseline levels, and its rate of infusion was adjusted as necessary to maintain blood glucose concentration at baseline levels ($\pm 10\%$). The rate of 50% glucose solution infusion (mL/h) was transformed to rate of glucose infusion (ROGI; g/h). The total glucose infused for each cow was calculated using the ROGI for each 10-min interval (72 intervals in total) according to the following equation:

$$\sum_{i=0}^{72} \text{ROGI (g/h)}_i \times \frac{1 \text{ h}}{60 \text{ min}} \times 10 \text{ min.}$$

Blood glucose was measured every 30 min in CON and LPS-C cows for the first 300 min, and every 60 min thereafter. Additional plasma and serum samples (~10 mL each) were collected from all treatments at -30, 0, 180, 360, 540, and 720 min relative to LPS administration. Plasma and serum were harvested following centrifugation at $1,500 \times g$ for 15 min at 4°C and were subsequently frozen at -20°C until analysis. Urine was collected at 0, 180, 360, 540, and 720 min relative to LPS administration and was frozen at -20°C until analysis. Rectal temperatures were measured at -30 and 0 min relative to LPS administration, every 30 min for the first 300 min postbolus, and every 60 min

thereafter using a digital thermometer (GLA M700, San Luis Obispo, CA).

Plasma insulin, nonesterified fatty acids (NEFA), BHB, LPS-binding protein (LBP), L-lactate, serum amyloid A (SAA), haptoglobin, and BUN as well as urine glucose concentrations were determined using commercially available kits according to manufacturers' instructions (insulin, Mercodia AB, Uppsala, Sweden; NEFA, Wako Chemicals USA, Richmond, VA; BHB, Pointe Scientific Inc., Canton, MI; LBP, Hycult Biotech, Uden, the Netherlands; L-lactate, Biomedical Research Service Center, Buffalo, NY; SAA, Tridelta Development Ltd., Kildare, Ireland; haptoglobin, Immunology Consultants Laboratory Inc., Portland, OR; BUN, Teco Diagnostics, Anaheim, CA; glucose, Wako Chemicals USA Inc.). The inter- and intra-assay coefficients of variation for haptoglobin, insulin, NEFA, BHB, L-lactate, SAA, LBP, and BUN assays were 1.2 and 26.1%, 8.3 and 13.8%, 6.7 and 2.0%, 10.5 and 5.3%, 18.3 and 10.2%, 43.0 and 20.8%, 12.1 and 7.4%, and 2.8 and 3.7%, respectively. The intra-assay coefficient of variation for urine glucose was 4.9%. Blood ionized calcium was measured using an i-STAT hand-held machine and cartridge (CG8+; Abbott Point of Care, Princeton, NJ). For white blood cell (WBC) count, a 3-mL blood sample was collected (K₂EDTA; BD Franklin Lakes, NJ) and stored at 4°C for ~12 h before submission to the Iowa State University's Department of Veterinary Pathology for complete blood count analysis.

Calculations and Statistical Analyses

Administration of LPS decreased milk yield and therefore decreased glucose used for milk synthesis. The decrease in milk yield allows us to estimate the amount of glucose conserved (milk glucose deficit) and channeled toward the activated immune system in LPS-administered cows. As described in the results section, the CON cows also reduced their milk yield due to fasting, allowing us to account for the milk glucose deficit due to reduced feed intake alone. The amount of glucose used for milk synthesis was calculated for each milking based on Kronfeld's (1982) estimation of 72 g of glucose required to synthesize 1 kg of milk. Milk glucose utilization values obtained before the challenge were averaged to establish a baseline value. Milk glucose utilization at both 360 and 720 min was subtracted from the baseline to calculate the milk glucose deficit. For CON and LPS-C cows, milk glucose deficit was solely used to calculate total glucose deficit. For LPS-Eu cows, milk glucose deficit plus the amount of glucose infused to maintain euglycemia were combined to obtain total glucose deficit.

The temporal pattern of postbolus blood glucose was divided into 2 phases, a hyperglycemic (0–150 min) and hypoglycemic (180–720 min) phase, which were statistically analyzed separately. Rectal temperature and ROGI were analyzed for the entire postbolus period. Remaining parameters were analyzed for the hypoglycemic phase (180–720 min, during which blood samples, milk yield, and milk composition were obtained). Each animal's respective parameter was analyzed using repeated measures with an autoregressive covariance structure for blood and milk parameters and spatial power law for rectal temperature and blood glucose. The repeated effect was minute after LPS administration. Each specific variable's prebolus value (when available) served as a covariate. Effects of treatment, time, treatment by time interaction, and replicate (except for ROGI, where only the effect of time within the LPS-Eu treatment was analyzed) were assessed as a completely randomized design using PROC MIXED (SAS Institute Inc., Cary, NC). Preformed contrasts were used to estimate differences between CON and LPS-administered cows (LPS-C and LPS-Eu) as well as between the 2 LPS-administered treatments (LPS-C vs. LPS-Eu). Data are reported as least squares means and considered significant if $P \leq 0.05$ and a tendency if $0.05 < P \leq 0.10$.

RESULTS

Overall rectal temperature did not differ between treatments ($P = 0.94$); however, mild hyperthermia ($+0.5^{\circ}\text{C}$) was observed between 30 to 90 min postbolus in LPS-administered relative to CON cows ($P = 0.04$; Figure 1). Cows treated with LPS (LPS-C and LPS-Eu) anecdotally developed visible symptoms of immunoactivation within 120 min postbolus, including lethargy, drooping ears, and diarrhea. Cows administered LPS had increased (84%) circulating glucose from 0 to 150 min postbolus relative to CON cows ($P < 0.01$; Figure 2A). From 180 to 720 min, LPS-C cows had 30% decreased blood glucose compared with CON and LPS-Eu cows. There were no differences in circulating glucose between CON and LPS-Eu cows from 180 to 720 min ($P = 0.26$), indicating successful maintenance of euglycemia in LPS-Eu cows. No treatment differences were detected in urine glucose (<1.5 mg/dL; $P = 0.29$; data not shown). Glucose infusion began 143 ± 18 min post-LPS administration (range 60–170) and increased with time ($P < 0.01$; Figure 2B). An average of 265 ± 98 g of glucose were infused to maintain euglycemia during the entire 720-min clamp.

Milk yield decreased $\sim 80\%$ for both LPS treatments relative to CON cows at both 360 and 720 min post-

bolus ($P < 0.01$; Figure 3A). Relative to the baseline value, milk yield of CON cows was decreased at both 360 and 720 min (21%; $P = 0.07$ and 39%; $P < 0.01$, respectively), which can be attributed to fasting. Milk lactose percentage decreased 11% in LPS-treated cows relative to CON cows ($P = 0.01$; Table 2). Milk fat percentage increased in all treatments with time (36%; $P = 0.02$; Table 2), and there were no effects on milk protein percentage ($P > 0.10$; Table 2). There was a progressive increase in MUN ($\sim 20\%$) for all treatments ($P < 0.01$; Table 2). Overall, SCC increased 100% in LPS-C relative to CON cows, whereas LPS-Eu cows did not differ from either treatment ($P = 0.03$; Table 2). The milk glucose deficit accumulated over 720 min was increased $\sim 175\%$ in LPS-administered cows relative to controls ($P < 0.01$; Figure 3B). Total glucose deficit accumulated over 720 min differed between all treatments, with CON, LPS-C, and LPS-Eu cows having 461-, 1,259-, and 1,553-g glucose deficits, respectively ($P < 0.01$; Figure 3B).

Insulin increased 4-fold in LPS relative to CON cows ($P = 0.01$; Figure 4A). Circulating NEFA and BHB were decreased 46 and 53%, respectively, in LPS versus CON cows ($P < 0.01$; Figure 4B and 4C). Blood urea nitrogen increased $\sim 30\%$ in all treatments over time ($P = 0.01$; data not shown). Ionized calcium decreased 46% in both LPS treatments relative to CON cows ($P < 0.01$; Figure 4D). Compared with CON cows, L-lactate, haptoglobin, and SAA increased ~ 160 , 260, and 75%, respectively, in LPS-treated cows ($P \leq 0.01$; Figure 5A, 5B, and 5C). Overall, an 87% increase in LBP was observed in LPS compared with CON cows, and by 720 min LBP was reduced 25% in LPS-Eu relative to LPS-C cows ($P < 0.01$; Figure 5D).

There was a treatment by time interaction ($P < 0.01$; Figure 6A) for WBC counts, as LPS-administered cows experienced an initial decrease in cell number (180 min; 90%) followed by a progressive increase with time, and WBC counts were 54% of the CON values by 720 min. Lymphocytes, neutrophils, and monocytes primarily contributed to the WBC temporal changes, as they were initially reduced (86, 98, and 85%, respectively; $P < 0.01$) at 180 min post-LPS administration and gradually increased to 50, 53, and 47% of CON values, respectively, by 720 min (Figure 6B and 6C; Table 3). The neutrophil-to-lymphocyte ratio decreased 80% at 180 min in LPS-administered cows relative to controls and returned to baseline levels by 540 min ($P = 0.02$; Table 3). Red blood cells, hemoglobin, and hematocrit increased in LPS-administered cows relative to CON (6, 8, and 10%; $P \leq 0.01$; Table 3). Platelets were decreased 63% in LPS-administered cows relative to controls, whereas mean platelet volume was increased 105% ($P < 0.01$; Table 3). There were no differences

Figure 1. Rectal temperature in cows administered a bolus of saline (CON), lipopolysaccharide (LPS-C), or lipopolysaccharide accompanied with a euglycemic clamp (LPS-Eu). Results are expressed as LSM $\pm$ SEM.

between LPS-C and LPS-Eu cows for any complete blood count parameter.

DISCUSSION

Accurately determining nutrient requirements of the immune system is difficult due to its ubiquitous and fluctuating distribution throughout tissues. From a metabolic perspective, both whole-body energy expenditure and glucose utilization markedly increase during infection (Lang and Dobrescu, 1991; Lang et al., 1993; Plank et al., 1998), ostensibly because immune cells become substantial and obligate glucose consumers to support the energetic and substrate needs of activation (Calder et al., 2007; Maratou et al., 2007; Maciver et al., 2008; Palsson-McDermott and O'Neill, 2013; Kelly and O'Neill, 2015). For instance, in tissues with a large immune compartment (spleen, liver, lung, and ileum), LPS increases glucose utilization, which is enhanced by maintaining euglycemia (Lang et al., 1993). Herein, we propose using a euglycemic clamp after LPS administration to estimate the amount of glucose utilized during an intense immune response.

Immunoactivation was successfully induced in the current study, as indicated by increased circulating levels of acute phase proteins haptoglobin, SAA, and LBP.

Whereas the increase in acute phase proteins is consistent with the literature, substantial variation exists in baseline, temporal pattern, and severity of increase (Jacobsen et al., 2004; Carroll et al., 2009; Graugnard et al., 2013; Moyes et al., 2014). This may be the result of different experimental models, route of LPS administration, animal variation, or laboratory procedures, but the variation (even from the same laboratory; Khafipour et al., 2009a,b) highlights why repeated sampling from the same animal pre- and postimmunoactivation is necessary to put the magnitude of change into perspective and context. Ionized calcium was severely decreased (46%) following LPS administration, which agrees with others (Griel et al., 1975; Elsasser et al., 1996; Waldron et al., 2003b), and is presumably due to calcium's involvement with immune system activation (Hendy and Canaff, 2016).

Severe hypoglycemia (30% decrease; Figure 2A) and hyperlactemia (160% increase; Figure 5A) developed $\sim$ 180 min postbolus and continued through 720 min. These indications of glucose dyshomeostasis are similar to other models of endotoxemia in ruminants (Giri et al., 1990; Gerros et al., 1995; Elsasser et al., 1996; Yates et al., 2011; Burdick Sanchez et al., 2013; Zarrin et al., 2014) and other species (Wolfe et al., 1977; Bruins et al., 2003; Michaeli et al., 2012). Glucose infusion initia-

tion, coinciding with development of hypoglycemia, was fairly consistent between cows (143 ± 18 min post-LPS administration; range 60–170). Interestingly, ROGI was maximum at 210 min postbolus and decreased to ~ 20 g/h between 360 and 540 min before increasing steadily to a plateau of ~ 40 g/h at 650 min (Figure 2B). Urine was not a source of glucose disposal, as it was almost similarly immeasurable (<1.5 mg/dL) from cows in all

treatments. Milk yield was acutely decreased $\sim 80\%$ in both LPS-administered treatments, which ostensibly represents a strategy to spare glucose for immune cell utilization. Admittedly, this is an extreme decrease, but similar magnitudes of milk synthesis cessation are frequent during *Escherichia coli* mastitis (Gröhn et al., 2004) and the acute model was necessary to achieve appreciable hypoglycemia to employ the euglycemic

Figure 2. (A) Blood glucose levels in cows administered a bolus of saline (CON), lipopolysaccharide (LPS-C), or lipopolysaccharide accompanied with a euglycemic clamp (LPS-Eu) during both hyperglycemic (0–150 min) and hypoglycemic (180–720 min) phases and (B) the average rate of glucose infusion in LPS-Eu cows. Trt = treatment. Results are expressed as LSM $\pm$ SEM.

Figure 3. (A) Milk yield at 360 and 720 min postbolus and (B) milk or total glucose deficit from 0 to 360, 360 to 720, and accumulated over 720 min in cows administered a bolus of saline (CON), lipopolysaccharide (LPS-C), or lipopolysaccharide accompanied with a euglycemic clamp (LPS-Eu). Different letters (x,y) represent differences between milk glucose deficits ($P \leq 0.05$). Different letters (a-c) represent differences between total glucose deficits ($P \leq 0.05$; total glucose deficit = milk glucose deficit in CON and LPS-C cows; total deficit = milk glucose deficit + infused glucose in LPS-Eu cows). Trt = treatment. Results are expressed as LSM $\pm$ SEM.

clamp. In CON cows, the ~30% decrease in milk yield can be solely attributed to fasting. The decrease in milk yield allows us to estimate the amount of glucose conserved (milk glucose deficit) due to both reduced feed intake and glucose utilization by the immune system.

The milk glucose deficit was 461, 1,259, and 1,288 g for CON, LPS-C, and LPS-Eu cows, respectively. In CON and LPS-C cows, milk glucose deficit makes up the entirety of the total glucose deficit calculation. In LPS-Eu cows, the accumulated ROGI was ~265 g, which

Table 2. Milk composition parameters in cows given a bolus of saline (CON), lipopolysaccharide (LPS-C), or lipopolysaccharide accompanied with a euglycemic clamp (LPS-Eu) at 360 and 720 min post-LPS administration

Parameter ¹	Treatment ²				P-value			Contrast	
	CON	LPS-C	LPS-Eu	SEM	Treatment	Time	Treatment × time	CON vs. LPS ²	LPS vs. LPS-Eu
Milk solids (%)									
Fat	3.4	3.4	3.2	0.3	0.91	0.02	0.86	0.81	0.71
Protein	3.1	2.9	3.1	0.1	0.13	0.36	0.75	0.14	0.15
Lactose	4.7 ^a	4.1 ^b	4.3 ^b	0.1	0.01	<0.01	0.08	0.01	0.29
TS	12.2	11.2	11.3	0.3	0.14	0.02	0.78	0.05	0.80
MUN (mg/dL)	18.1	16.8	16.4	0.6	0.26	<0.01	0.68	0.12	0.67
Milk SCC	54 ^a	108 ^b	81 ^{ab}	13	0.03	0.01	0.23	0.02	0.15

^{a,b}Means with different letters differ ($P < 0.05$).

¹Data are presented as LSM from milk samples collected 360 and 720 min postbolus.

²LPS-C and LPS-Eu treatments.

provides a total glucose deficit of 1,553 g in LPS-Eu cows. Taking into account the reduction of feed intake by subtracting the 461-g deficit observed in CON cows,

we estimated the activated immune system used approximately 1,092 g of glucose in a 720-min period. This calculation is likely underestimated, as it does

Figure 4. Circulating (A) insulin, (B) nonesterified fatty acids (NEFA), (C) BHB, and (D) ionized calcium during the hypoglycemic phase (180–720 min) in cows administered a bolus of saline (CON), lipopolysaccharide (LPS-C), or lipopolysaccharide accompanied with a euglycemic clamp (LPS-Eu). Trt = treatment. Results are expressed as LSM $\pm$ SEM.

not incorporate glucose use by immune cells during the hyperglycemic phase. On a metabolic BW basis, the glucose requirement is approximately 0.66 g/kg of $BW^{0.75}/h$, which is comparable with data we generated in other LPS-euglycemic clamp experiments in growing steers and pigs (1.0 and 1.1 g/kg of $BW^{0.75}/h$, respectively; Kvidera et al., 2016a; S. K. Kvidera and L. H. Baumgard, unpublished data). The immune system uses a large quantity of glucose following immunosactivation, and the rate is seemingly conserved across species and life stages.

An additional reason for increased fuel oxidation (glucose in particular) following LPS administration could be to facilitate a febrile response. However, increased glucose utilization continues independently of rectal temperature during sepsis in experimental animal models (Lang et al., 1987). In support of this, we observed only mild and transient pyrexia in LPS-administered cows (between 30 and 90 min postbolus)

despite marked metabolic changes that existed for the entire experiment. This agrees with Giri et al. (1990), who also observed short-lived fever post-LPS despite large and prolonged changes in glucose homeostasis. Moreover, the fever response to LPS in ruminants is inconsistent and not dose-dependent, as sometimes higher doses attenuate the febrile response (Lohuis et al., 1988a; Gerros et al., 1995; Waldron et al., 2003a; Jacobsen et al., 2005), whereas high LPS doses consistently cause hypoglycemia. Regardless, increased glucose utilization during our experiment did not appear to be an attempt to mount a fever.

The current experimental design had some limitations. First, the extent of peripheral tissue glucose consumption limited our capacity to accurately estimate immune system glucose utilization. However, insulin sensitivity and glucose utilization were reduced in both muscle and adipose tissue (typically large glucose consumers) during endotoxemia, both in vivo (Raymond et

Figure 5. Circulating (A) L-lactate, (B) haptoglobin, (C) serum amyloid A (SAA), and (D) lipopolysaccharide-binding protein (LBP) during the hypoglycemic phase (180–720 min) in cows administered a bolus of saline (CON), lipopolysaccharide (LPS-C), or lipopolysaccharide accompanied with a euglycemic clamp (LPS-Eu). Trt = treatment. Results are expressed as LSM $\pm$ SEM.

Figure 6. Circulating (A) white blood cell (WBC), (B) lymphocyte, and (C) neutrophil counts during the hypoglycemic phase (180–720 min) in cows administered a bolus of saline (CON), lipopolysaccharide (LPS-C), or lipopolysaccharide accompanied with a euglycemic clamp (LPS-Eu). Trt = treatment. Results are expressed as LSM $\pm$ SEM.

al., 1981; Ling et al., 1994; Poggi et al., 2007; Mulligan et al., 2012) and in vitro (Song et al., 2006; Liang et al., 2013). As mentioned before, tissues with a large immune compartment (spleen, liver, lung, and ileum) actually increase glucose utilization following LPS administration (Lang et al., 1993). Further, Mészáros

et al. (1991) examined different cell fractions within the liver after an i.v. LPS challenge and demonstrated glucose uptake did not change in parenchymal cells but markedly increased in Kupffer cells (i.e., resident hepatic macrophages) and neutrophils. Overall, these results highlight the relative importance of activated

Table 3. Complete blood count parameters in cows given a bolus of saline (CON), lipopolysaccharide (LPS-C), or lipopolysaccharide accompanied with a euglycemic clamp (LPS-Eu) during the hypoglycemic phase 180–720 min post LPS-administration

Parameter	Treatment				P-value			Contrasts	
	CON	LPS-C	LPS-Eu	SEM	Treatment	Time	Treatment × time	CON vs. LPS ¹	LPS-C vs. LPS-Eu
Monocytes ($\times 10^3/\mu\text{L}$)	0.24 ^a	0.04 ^b	0.05 ^b	0.03	<0.01	0.02	0.07	<0.01	0.80
Eosinophils ($\times 10^3/\mu\text{L}$)	0.35	0.36	0.23	0.05	0.25	0.03	<0.01	0.43	0.13
Basophils ($\times 10^3/\mu\text{L}$)	0.05 ^a	0.02 ^b	0.02 ^b	0.01	0.01	<0.01	0.24	<0.01	0.67
Red blood cells ($\times 10^6/\mu\text{L}$)	6.2 ^a	6.6 ^b	6.5 ^b	0.1	0.04	0.08	0.58	0.02	0.65
Hemoglobin (mg/dL)	10.8 ^a	11.9 ^b	11.5 ^b	0.2	0.02	0.04	0.36	0.01	0.23
Hematocrit (%)	28.9 ^a	32.3 ^b	31.3 ^b	0.7	0.02	0.60	0.24	0.01	0.39
Platelets ($\times 10^3/\mu\text{L}$)	323 ^a	136 ^b	102 ^b	15	<0.01	0.02	0.62	<0.01	0.12
Mean platelet volume (fL)	6.6 ^a	13.4 ^b	13.7 ^b	1.0	<0.01	0.22	0.37	<0.01	0.84
NLR ²	1.05 ^a	0.65 ^b	0.56 ^b	0.13	0.05	<0.01	0.02	0.02	0.63

^{a,b}Means with different letters differ ($P < 0.05$).

¹LPS-C and LPS-Eu treatments.

²Neutrophil-to-lymphocyte ratio.

immune cells to tissue-specific glucose consumption. In addition, immune cell distribution radically changes after LPS administration, as demonstrated by leukopenia observed in the current and other studies (Griel et al., 1975; Bieniek et al., 1998), which is likely due to tissue leukocyte sequestration, especially tissues with important immune functions (Mészáros et al., 1991; Lang and Dobrescu, 1991). Endotoxemia also likely causes macrophage infiltration into adipose and muscle (Caesar et al., 2012; Pilon et al., 2013). The aforementioned studies have demonstrated glucose incorporation into immune cells increases, whereas extraimmune cells decrease glucose utilization following LPS administration, which supports our assumption glucose was primarily used by immune cells rather than peripheral tissue.

A second drawback to our experimental model was the lack of hepatic glucose output measurements, which prevented us from estimating the liver's contribution to the circulating glucose pool. However, endotoxemia-induced increased hepatic glycogenolysis and gluconeogenesis has been reported in multiple species (Wolfe et al., 1977; Filkins, 1978; Spitzer et al., 1985; Lang et al., 1985; McGuinness, 1994; McGuinness, 2005) including ruminants (Waldron et al., 2003a). Hepatic glycogenolysis is likely the primary contributor to the acute hyperglycemia observed following LPS administration in the current study (Figure 2A), but we were unable to calculate the amount of endogenous glucose used by the immune system before the initiation of glucose infusion (~180 min). The hyperglycemic phase results from orchestrated peripheral insulin insensitivity coupled with enhanced hepatic glucose production, which provides glucose in surplus of immune cell utilization capacity. The hypoglycemic phase represents the inability of such glucose-sparing mechanisms to keep pace with the activated immune system's glucose consumption. If increased rates of hepatic glycogenolysis and gluconeogenesis

described in ruminants and nonruminants apply to the current model, then we are further underestimating the quantity of glucose entering the circulating pool and, subsequently, the total amount of glucose used by the activated immune system.

Little or no overall metabolic, production, or immune differences were detected between LPS-C and LPS-Eu cows, indicating a similar response following LPS regardless of glycemic status. Most notably, maintaining euglycemia did not rescue milk yield or lactose content, indicating that decreased milk synthesis is not due to LPS-induced hypoglycemia. It is likely LPS induces a direct effect on mammary tissue, as mammary epithelial cells express toll-like receptor 4, an LPS receptor (Ibeagha-Awemu et al., 2008). Alternatively, endotoxin-induced increases in proinflammatory cytokines and hormonal changes (e.g., cortisol) may indirectly inhibit milk synthesis (Verheijden et al., 1983). Interestingly, LPS reduces the integrity of the mammary epithelial barrier (Wellnitz et al., 2016), and cows from the LPS-C treatment had increased milk SCC, which agrees with a previous i.v. LPS report (Shuster et al., 1991). Further, euglycemia appeared to attenuate the LPS-induced mammary permeability, as milk SCC from LPS-Eu cows was not different from either treatment. Markers of inflammation in the mammary gland post-LPS administration are increased when euglycemia is maintained compared with hypoglycemic cows (Vernay et al., 2012), suggesting glucose may be a limiting factor in mammary immune cell activation and subsequent pathogen clearance. Likewise, the reduction in circulating LBP at 540 and 720 min coupled with the increase in circulating neutrophils at 720 min in LPS-Eu relative to LPS-C cows indicates that glucose availability may be a bottleneck to mounting a full immune response.

Despite fasting, administering LPS induced acute and sustained hyperinsulinemia, which has been previ-

ously observed in cattle (Waldron et al., 2003a; Burdick Sanchez et al., 2013; Zarrin et al., 2014; Kvidera et al., 2016a) and pigs (Stoakes et al., 2015). Contrary to our previous results in growing steers (Kvidera et al., 2016a), infusing glucose did not exacerbate hyperinsulinemia to levels above LPS-C. An interaction between LPS and glucose infusion has been recently proposed (Baumgard et al., 2016) and may be due to either direct action of LPS on the pancreas (Vives-Pi et al., 2003; Bhat et al., 2014), LPS-induced hyperglycemia (Figure 2A), or LPS-stimulated secretion of the insulin secretagogue glucagon-like peptide 1 (Nguyen et al., 2014). Circulating insulin remained increased (~4 fold) during the hypoglycemic phase (180–720 min postbolus), so acute hepatic glycogenolysis is not likely the cause. Rationale for why circulating insulin increases are not clear, but insulin is important for immune cell glucose uptake and development during activation (Shimizu et al., 1983; Helderman, 1984; Calder et al., 2007; Maratou et al., 2007). Severe hyperinsulinemia likely contributes to decreased plasma NEFA observed in LPS-administered cows relative to controls, as insulin is a potent antilipolytic hormone (Vernon, 1992). Interestingly, despite the gradual increase in NEFA over time in all treatments (more pronounced in the controls), BHB was markedly decreased (>50%) throughout the experiment. This agrees with other ruminant models (Waldron et al., 2003a; Graugnard et al., 2013; Kvidera et al., 2016a) yet contradicts our pig results, where BHB was increased following LPS administration (Stoakes et al., 2015). Reasons for decreased circulating ketones are not clear; most circulating BHB in ruminants in positive energy balance (akin to the current study) originate from the rumen epithelium, as opposed to the liver (Pennington, 1952). Administering LPS decreases rumen motility (Jing et al., 2014), and whether or not this gastrointestinal stasis affects ketone synthesis is not known. An additional reason for decreased circulating BHB could be reduced liver ketone production, as data from rodent models have demonstrated a decreased capacity for hepatic ketone synthesis during endotoxemia (Takeyama et al., 1990; Gitomer et al., 1995); however, Waldron et al., (2003a) observed no LPS effects on ketone synthesis in incubated liver slices obtained from lactating cows. Additionally, an increased rate of BHB infusion was required to maintain hyperketonemia in lactating cows challenged with LPS (Zarrin et al., 2014), indicating increased circulating BHB clearance and peripheral tissue utilization could help explain the decrease; this may be a strategy used to spare glucose for immune cells. Nevertheless, having a better understanding of how endotoxemia influences NEFA and ketone metabolism likely has practical implications to a variety of dairy cow pathological conditions (e.g., ketosis).

Hyperlactemia is another characteristic of endotoxemia (Wolfe et al., 1977; Michaeli et al., 2012), which is likely due to enhanced glucose utilization via aerobic glycolysis in activated immune cells (Palsson-McDermott and O'Neill, 2013). In the current study, LPS-administered cows had >150% increases in circulating L-lactate. Other potential sources include skeletal muscle, which may export L-lactate as an oxidative fuel for nonimmune cells in an attempt to spare glucose for the immune system, a process akin to the Warburg effect (Tannahill and O'Neill, 2011). Muscle proteolysis is another adaptation employed during immunoactivation to support the demand for gluconeogenic precursors and AA for acute phase protein production (Klasing and Austic, 1984; Doyle et al., 2011; Michaeli et al., 2012; Johnson, 2012). Similar to results of Zarrin et al. (2014) in lactating cows, we detected no treatment differences in circulating BUN concentrations but did observe a similar progressive increase over time. This is in contrast to findings in LPS-administered nonlactating ruminants (Elsasser et al., 1996; Ballou et al., 2008; Kvidera et al., 2016a) and other species (Myers et al., 1997; Bruins et al., 2003; Rose et al., 2007), where LPS administration markedly increases BUN. Relatively little data exists on circulating BUN following endotoxin challenge in lactating animals; the source of differences between growing and lactating physiological states is not clear.

Regarding immune parameters, leukopenia was evident at 180 min post-LPS and WBC count gradually increased to ~50% of CON levels by 720 min. Lymphocytes, neutrophils, and monocytes were the main contributors, as they all followed the same temporal pattern. Leukopenia has been observed in other ruminant endotoxemia studies (Griel et al., 1975; Bieniek et al., 1998; Jacobsen et al., 2005) and is likely due to accelerated leukocyte extravasation into tissues. Samples obtained beyond 720 min have observed leukocytosis following an initial period of leukopenia (Griel et al., 1975; Jacobsen et al., 2005; E. A. Horst and L. H. Baumgard, unpublished data), indicating that circulating immune cells eventually exceed baseline levels. In summary, concentrations of circulating immune cells are highly dependent on time relative to immune activation. Leukopenia in the current study indicated immune cell mobilization into tissue, and the current experimental duration was likely not long enough to observe the characteristic leukocytosis in later stages of immunoactivation.

Glucose is obviously a critically important metabolite in all species, but it is especially pertinent to lactating dairy cows, as ruminants rely almost exclusively on hepatic gluconeogenesis instead of dietary carbohydrate digestion and absorption to meet their

glucose requirements. Further, glucose is the substrate for lactose synthesis and lactose production is the primary osmoregulator of milk yield (Chaiyabutr et al., 1980); thus, extra-mammary processes that increase glucose utilization have the potential to decrease glucose supply to the mammary gland and decrease productivity. Herein we describe the immune system as a substantial glucose user, and this has implications to several practical scenarios. Metritis and mastitis are common infections, especially in early lactation, which prevent optimal productivity. There are 2 less overt situations where immune system glucose utilization limits maximum milk yield. The first is heat stress, as heat-stressed animals have a compromised intestinal barrier function and, thus, intestinal-derived LPS infiltration (Baumgard and Rhoads, 2013). Immune system glucose consumption during heat stress likely explains why decreased feed intake is only responsible for about 50% of reduced milk yield. The second is maladaptation to lactation, as the transition period is associated with increased inflammatory markers (Bertoni and Trevisi, 2013; Bradford et al., 2015), although a source of blatant infection is often unclear. We have extended this and recently demonstrated increased circulating inflammatory biomarkers in periparturient cows diagnosed with ketosis and no other obvious disorder (i.e., mastitis or metritis; Abuajamieh et al., 2016). We hypothesize dietary shifts or inadequate feed intake associated with the transition period cause leaky gut and, thus, endotoxin infiltration (Abuajamieh et al., 2016), a scenario similar to heat stress (Kvidera et al., 2016b). Having a better understanding of how the immune system influences nutrient partitioning may allow for developing strategies to ameliorate production losses and reduce morbidity.

CONCLUSIONS

Our experiment demonstrates that the induction of acute endotoxemia induces hypoglycemia resulting from the immune system's rate of glucose utilization exceeding whole-body glucose-sparing mechanisms. From the combination of reduced milk carbohydrate output and ROGI, we estimate the activated immune system uses >1 kg of glucose within 720 min. This is ostensibly underestimated, because we are unable to account for immune system glucose utilization during the acute hyperglycemic phase and the liver's increased contribution to the circulating pool. The consistency between glucose utilization in the current experiment and other LPS-Eu clamps we have performed, despite differences in animal ages, physiological status, and species, suggests the reprioritization and extent of fuel utilization by immune cells upon activation is a con-

served response. Whether glucose can become a limiting factor in the immune response is not clear; thus, the benefits of supplemental glucose to sick animals remains unknown. However, from an animal production perspective, infection and inflammation noticeably redirect resources toward the immune system and away from utilization and synthesis of economically relevant products. Having a better understanding of the energetic and nutrient requirements of the immune response is critical to developing strategies to minimize productivity losses when physiological states or environmental conditions activate the immune system.

ACKNOWLEDGMENTS

The authors thank Andy Mueller and recognize the Norman Jacobson Endowment for funding.

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