Effects of supplemental chromium propionate and rumen-protected amino acids on nutrient metabolism, neutrophil activation, and adipocyte size in dairy cows during peak lactation

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ABSTRACT

The objective of this study was to evaluate effects of chromium propionate (CrPr), rumen-protected lysine and methionine (RPLM), or both on metabolism, neutrophil function, and adipocyte size in lactating dairy cows (38 ± 15 d in milk). Forty-eight individually fed Holstein cows (21 primiparous, 27 multiparous) were stratified by calving date in 12 blocks and randomly assigned to 1 of 4 treatments within block. Treatments were control, CrPr (8 mg/d of Cr, KemTRACE brand chromium propionate 0.04%, Kemin Industries Inc., Des Moines, IA), RPLM (10 g/d lysine and 5 g/d methionine intestinally available, from LysiPEARL and MetiPEARL, Kemin Industries Inc.), or CrPr plus RPLM. Treatments were fed for 35 d; blood plasma samples were collected on d 21 and 35 of treatment, and blood neutrophils were isolated from 24 cows for analysis of tumor necrosis factor α ($TNF\alpha$) and interleukin 1β ($IL-1\beta$) transcript abundance in the basal state and after 12 h of lipopolysaccharide (LPS) activation. Tailhead subcutaneous adipose tissue samples were collected on d 35 for measurement of adipocyte size. Plasma glucose, nonesterified fatty acids, and glucagon concentrations were unaffected by treatments, whereas plasma insulin concentration was increased by RPLM. Basal $TNF\alpha$ transcript abundance in neutrophils was not affected by treatment, but basal $IL-1\beta$ transcript abundance was decreased by RPLM and tended to be increased by CrPr. After LPS activation, CrPr increased neutrophil $TNF\alpha$ transcript abundance. In addition, RPLM $\times$ parity interactions were detected for both $TNF\alpha$ and $IL-1\beta$ abundance after LPS activation, reflecting enhanced responses in primiparous cows and attenuated responses in multiparous cows supplemented with RPLM. Adipocyte size was not affected by treatment. Supplemental CrPr and RPLM had minimal effects

on metabolism when fed for 35 d near peak lactation but may modulate innate immune function in lactating dairy cows.

Key words: lysine, methionine, chromium, immunity

INTRODUCTION

Chromium is a nutrient that can influence animal metabolism. Supplementation of Cr in cattle has been shown to increase milk production (Hayirli et al., 2001; McNamara and Valdez, 2005; Smith et al., 2005), improve glucose clearance and insulin sensitivity (Bunting et al., 1994; Sumner et al., 2007; Spears et al., 2012), reduce lipolysis (Besong, 1996; Bryan et al., 2004), alter adipose tissue metabolism (McNamara and Valdez, 2005), and modulate immune response (Burton et al., 1993, 1996; Burton, 1995). Although considerable research has been conducted with Cr in cattle, Cr supplementation in cattle diets has only recently been permitted by the US Food and Drug Administration. Currently, Cr-propionate (**CrPr**) is the only source of Cr allowed for supplementation to cattle in the United States, at inclusion rates up to 0.5 mg of Cr/kg of diet. By feeding CrPr to dairy cows from 21 d prepartum to 35 d postpartum, McNamara and Valdez (2005) increased DMI and milk yield in early lactation. Interestingly, they also observed that CrPr dramatically increased adipose tissue lipogenesis compared with that of controls in the postpartum period. Collectively, these results indicate that CrPr may improve production by modulating metabolism of lactating cows.

To improve the performance and productive efficiency of lactating cows, first-limiting AA such as rumen-protected lysine and methionine (**RPLM**) have been commonly supplemented (Chilliard and Doreau, 1997; Leonardi et al., 2003; Berthiaume et al., 2006). A recent meta-analysis (Patton, 2010) indicated that feeding rumen-protected methionine increased milk protein content and yield and slightly increased milk yield across 35 studies in the literature. However, little research has been conducted to determine effects of

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RPLM on glucose or lipid metabolism or immune function in dairy cows. Enhanced metabolic and immune function may contribute to improved milk production.

Currently, there are no reports regarding the interaction between CrPr and RPLM supplementation in dairy cows. We hypothesized that supplementation of both may generate effects superior to supplementing either one alone, in part because enhanced milk production in response to CrPr would increase essential AA requirements. Therefore, the objective of this study was to determine if CrPr and RPLM supplementation affects neutrophil function, adipocyte size, or intermediary nutrient metabolism in dairy cows near peak lactation.

MATERIALS AND METHODS

The Kansas State University Institutional Animal Care and Use Committee approved all experimental procedures. Production responses to these treatments are reported in a companion paper (Vargas-Rodriguez et al., 2014).

Design and Treatments

Forty-eight individually fed Holstein cows (21 primiparous, 27 multiparous, 38 ± 15 DIM, mean $\pm$ SD) were stratified by calving date in 12 blocks and randomly assigned to 1 of 4 treatments within block. Treatments were control, CrPr (8 mg/d of Cr in the form of 20 g/d KemTRACE chromium propionate 0.04%; Kemin Industries Inc., Des Moines, IA), RPLM (10 g/d of lysine and 5 g/d of methionine, intestinally available), or both (**CrPr+RPLM**). The RPLM supplement was composed of 48.8 g/d of LysiPEARL and 15.3 g/d of MetiPEARL (Kemin Industries Inc.). Treatments were premixed with ground corn and top-dressed at 200 g/d for 35 d. All cows were fed once daily (1600 h) for ad libitum intake of a diet formulated to meet NRC (2001) nutrient requirements (Table 1). Analysis by the Cornell Net Carbohydrate and Protein System version 6.1 (NDS version 3, Ruminant Management and Nutrition, Reggio Emilia, Italy) estimated metabolizable Met supply at 47 g/d (2.03% of MP) and metabolizable Lys supply at 148 g/d (6.38% of MP) with 22 kg/d DMI in the control diet. The RPLM supplement was predicted to result in Lys and Met supplies of 6.77% and 2.23% of MP, respectively.

Plasma Samples

On d 21 and 35 (1430 h), approximately 14 mL of blood was collected from all cows from the coccygeal vessels into 2 evacuated tubes, one containing K₃-EDTA (for analyses of NEFA, glucagon, insulin, leptin,

and adiponectin) and the other containing potassium oxalate with sodium fluoride (for analysis of glucose) as a glycolytic inhibitor (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Samples were centrifuged at $2,000 \times g$ for 15 min immediately after sample collection, and plasma was harvested and frozen at -20°C until analysis. Plasma aliquots for glucagon analysis were added to tubes containing benzamidine (50 mM final concentration; Sigma-Aldrich Chemical Co., St. Louis, MO) as a protease inhibitor. Plasma was analyzed for NEFA using an enzymatic colorimetric procedure (NEFA-HR, Wako Chemicals USA, Richmond, VA), glucose by a colorimetric kit (Autokit Glucose; Wako Chemicals USA), glucagon by a radioimmunoassay kit (#GL-32K Millipore Corp., Billerica, MA), and insulin by a bovine-specific sandwich ELISA (#10-1201-01, Mercodia AB, Uppsala, Sweden).

Plasma samples collected on d 35 were analyzed for leptin and adiponectin protein abundance by Western blot. Plasma samples (1 μL) were diluted with 19 μL of Laemmli sample buffer (Bio-Rad, Richmond, CA). The homogenate was heated at 90°C for 5 min, cooled,

Table 1. Ingredient and nutritional composition of the basal diet

Item	Value
Ingredient (% of DM)	
Corn silage	31.5
Alfalfa hay	23.4
Wet corn gluten feed ¹	6.8
Ground corn	23.1
Whole cottonseed	4.6
Mechanically extracted soybean meal ²	2.1
Solvent-extracted soybean meal	5.1
Ca salts of long-chain fatty acids ³	0.8
Micronutrient premix ⁴	2.6
Nutrient (% of DM unless otherwise noted)	
DM (% as fed)	57.9
OM	91.3
CP	16.7
NDF	31.7
ADF	20.1
fNDF ⁵	22.1
NFC	39.8
Ether extract	3.1
Model-predicted ME ⁶ (Mcal/kg)	2.50

¹SweetBran (Cargill Inc., Blair, NE).

²Soy Best (Grain States Soya, West Point, NE).

³Megalac-R (Church & Dwight Co., Princeton, NJ).

⁴Premix consisted of 45.1% limestone, 32.2% sodium bicarbonate, 6.4% magnesium oxide, 5.2% sodium chloride, 5.2% vitamin E premix (44 IU/g), 0.45% vitamin A premix (30 kIU/g), 0.19% vitamin D premix (30 kIU/g), 2.1% 4-Plex (Zinpro Corp., Eden Prairie, MN; contains 2.58% Zn, 1.48% Mn, 0.90% Cu, 0.18% Co, 8.21% Met, and 3.80% Lys), 0.96% selenium premix (600 mg/kg Se), 0.45% Zinpro 100 (Zinpro Corp.; contains 10% Zn and 20% Met), 0.03% ethylenediamine dihydriodide premix (3.65% I), 0.88% Kallsil (Kemin Industries Inc., Des Moines, IA), and 0.88% Myco CURB (Kemin Industries Inc.).

⁵Forage NDF.

⁶ME predicted by CNCPS 6.1 (NDS version 3, Ruminant Management & Nutrition, Reggio Emilia, Italy).

and loaded onto a 4 to 20% Tris-HCl gel for electrophoresis. Samples were separated by SDS-PAGE and dry-transferred onto nitrocellulose membranes (iBlot, Invitrogen, Carlsbad, CA). Membranes were blocked for 2 h in blocking buffer (5% dry milk in Tris-HCl buffer, pH 7.5, with 0.05% Tween 20). After incubation with blocking buffer, the membranes were washed 3 times for 5 min each with washing buffer (PBS, pH 7.5, containing 0.05% Tween 20). Membranes were then incubated with primary antibodies (1:500) against leptin (sc-8325, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and adiponectin (A6354, Sigma-Aldrich Co.) overnight at 4°C. After washing, a secondary antibody (sc-2313, Santa Cruz Biotechnology) diluted 1:10,000 was incubated for 1 h at room temperature. Immunodetection was performed by chemiluminescence (West-Dura; Thermo Scientific, Waltham, MA) and bands quantified by scanning densitometry (ChemiDoc-It Imaging System; UVP Inc., Upland, CA).

Neutrophil Isolation

On d 21 and 35 (0700 h), 50 mL of blood was obtained by jugular venipuncture from 6 blocks of the cows for isolation of neutrophils. Blood was collected in 50-mL tubes (Becton Dickinson) with 0.75 mL of heparin (1,000 units/mL, Acros Organics Inc., Fair Lawn, NJ). Blood was placed on ice for transport to the laboratory (<60 min) and then 20 mL of whole blood was combined with 20 mL of Ficoll-Paque PLUS (GE Healthcare, Pittsburgh, PA) and centrifuged at $800 \times g$ for 50 min at 4°C. Centrifugation separated the blood into 6 distinct bands: plasma, monocytes, isolation medium, neutrophils, more isolation medium, and the red blood cell pellet. The top 3 layers (plasma, monocytes, and isolation medium) were removed, and neutrophils were collected and washed 3 times with Ca^{2+} - and Mg^{2+} -free Hanks' balanced salt solution (Sigma-Aldrich Chemical Co.). Isolated neutrophils from each cow were split into 2 samples, one of which was immediately frozen for subsequent analysis of basal neutrophil status. The remaining neutrophils were suspended in Dulbecco's modified Eagle medium with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma-Aldrich Chemical Co.) and incubated with 1 $\mu\text{g}/\text{mL}$ LPS (from *Escherichia coli* O55:B5, Sigma-Aldrich Chemical Co.) for 12 h. Cells were frozen at -80°C until analysis of transcript abundance of tumor necrosis factor α (*TNF α*) and IL-1 β (*IL-1 β*) in the basal state or after 12 h of LPS activation.

Transcript Abundance

Total RNA was extracted from neutrophils using a commercial kit (RNeasy Lipid Tissue Mini Kit, Qiagen

Inc., Valencia, CA) according to the manufacturer's instructions. Two micrograms of total RNA was used as template for the reverse transcriptase reaction using random primers (High-Capacity cDNA RT Kit, Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in duplicate on 96-well plates with 1/20 of the cDNA product in the presence of 200 nmol/L gene-specific forward and reverse primers with real-time SYBR green fluorescent detection using SYBR Green Premix reagent (7500 Fast Real-Time PCR System, Applied Biosystems). Primers were designed (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) using the following GenBank accession numbers to generate these primer sequences (all 5'-3'): *TNF α* (NM_173966.3), forward AAGTAACAAGCCGGTAGCCCA, reverse CTTCCAGCTTCACACCGTTG; *IL-1 β* (NM_174093.1), forward CACGTGGGCTGAATAACCCCGA, reverse AGGCAGTCGGGCATG-GATCAGA; ribosomal protein subunit 9 (*RPS9*) (NM_001101152.2), forward GAACAAACGTGAGGTCTGGAGG, reverse TTACCTTCGAACAGACGCCG. All sample values were normalized against *RPS9* (Mamedova et al., 2010), and relative gene expression was quantified by using the $2^{-\Delta\text{Ct}}$ method. Efficiencies of PCR reactions, determined by serial dilution of pooled samples, were as follows: *TNF α* , 93%; *IL-1 β* , 80%; and *RPS9*, 87%.

Adipose Tissue Biopsy

On d 35, tailhead subcutaneous adipose tissue samples were collected from all cows. At the time of sampling, the site was shaved and aseptically prepared. The tailhead was then anesthetized with 2 mL of 2% lidocaine hydrochloride. An incision of 2 to 3 cm in length was made between the tailhead and the tuber ischii, and adipose tissue was collected as described previously (Smith and McNamara, 1990). Incisions were closed by a skin stapler (Oasis Disposable Skin Stapler, Med-Vet International, Mettawa, IL). Tissue samples were trimmed to eliminate contaminating tissue and were cut into small pieces (approximately 1 cm^3). One sample from each cow was embedded in Optimal Cutting Temperature compound (OCT, Tissue-Tek, Sakura Finetek, Torrance, CA) and frozen at -80°C for subsequent analysis of adipocyte size.

Adipocyte Morphology

Adipose tissue cryosections (14 μm) were prepared on a cryostat (Microm HM550, Thermo Fisher Scientific Inc., Kalamazoo, MI) and mounted on SuperFrost slides (Thermo Fisher Scientific Inc.). Tissue sections were fixed in 4% paraformaldehyde for 10 min, rinsed

twice with PBS, and covered with a coverslip. Representative images were captured using a Nikon Eclipse TI-U inverted microscope with 10 $\times$ working distance magnification (Nikon Instruments Inc., Melville, NY). Five representative photomicrographs per animal were captured using a Nikon DS-QiMc digital camera (Nikon Instruments Inc.). All the adipocytes in each photomicrograph and at least 150 adipocytes per sample were analyzed for adipocyte cross-sectional area using NIS-Elements Imaging Software (Basic Research, 3.3; Nikon Instruments Inc.).

Calculations and Statistical Analysis

An insulin sensitivity measure, revised quantitative insulin sensitivity check index (**RQUICKI**) was calculated as (Holtenius and Holtenius, 2007): $RQUICKI = 1/[\log(G_b) + \log(I_b) + \log(NEFA_b)]$, where G_b = glucose (mg/dL), I_b = insulin (μ U/mL), and $NEFA_b$ = NEFA (mmol/L), such that a lower RQUICKI indicates greater insulin resistance. Insulin, determined in nanograms per milliliter, was converted to micro-units per milliliter by assuming an activity of 25.1 μ U/ng (JAMA, 2010). The distribution of adipocyte area values within each cow (from 5 randomly selected cows) was plotted to assess the normality of the population. In contrast to some previous reports demonstrating bimodal adipocyte populations in relatively obese animals (Cruz et al., 2012), adipocyte populations within animal were unimodal in this study. However, distributions were right-skewed because of small numbers of very large adipocytes. Therefore, median adipocyte area from each cow was used instead of the mean value for further analysis.

One cow receiving CrPr+RPLM developed severe mastitis on d 20 of treatment and was subsequently removed from the study; no data were collected or analyzed for this cow. Data were analyzed using Proc MIXED of SAS (version 9.2; SAS Institute Inc., Cary, NC) to assess the fixed effects of day, CrPr, RPLM, parity, and all interactions, and the random effect of block. With the exception of CrPr $\times$ RPLM, interactions were removed from models when $P > 0.30$. Data for *TNF α* and *IL-1 β* transcript abundance in neutrophils were log-transformed for analysis and reported values are back-transformed. Repeated measures over time within cow were modeled with an autoregressive [AR(1)] covariance structure. Denominator degrees of freedom were estimated using the Kenward-Rogers method. Values were deemed outliers and omitted from analysis when Studentized residuals were >3.5 or <-3.5 . Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. Treatment means were separated with pair-wise *t*-tests when interactions were significant.

RESULTS

Plasma Data

Plasma metabolite and hormone concentrations are shown in Table 2. Glucose and NEFA concentrations were not affected ($P > 0.10$) by RPLM or CrPr. Compared with values on d 21, glucose tended to be decreased (50.5 vs. 53.1 ± 1.5 mg/dL, $P = 0.10$) and NEFA was decreased (223 vs. 342 ± 22 μ M, $P < 0.001$) on d 35. Insulin was unaffected ($P > 0.10$) by CrPr, but was increased ($P = 0.03$) by RPLM and was greater on d 35 than on d 21 (311 vs. 259 ± 23 pg/mL, $P = 0.04$). Although glucagon was not affected ($P > 0.10$) by CrPr or RPLM, we detected an interaction of RPLM $\times$ day ($P = 0.04$), reflecting increased ($P = 0.05$) glucagon concentration (173 v. 159 ± 5.6 pg/mL) by RPLM on d 35; no difference ($P = 0.53$) was detected on d 21. The RQUICKI measure of insulin sensitivity did not show treatment effects ($P > 0.10$) but tended to be increased on d 35 compared with d 21 (0.569 vs. 0.536 ± 0.014 , $P = 0.08$). Primiparous cows tended to have lesser RQUICKI values than multiparous cows (0.536 vs. 0.570 ± 0.014 , $P = 0.07$).

Plasma leptin and adiponectin protein abundance on d 35 is shown in Figure 1. Plasma leptin protein abundance (Figure 1A) tended to be increased ($P = 0.09$) by RPLM, but was unaffected ($P > 0.10$) by CrPr. In addition, we observed a tendency for a CrPr $\times$ parity interaction ($P = 0.08$), reflecting decreased ($P = 0.04$) leptin abundance by CrPr in multiparous cows but not ($P = 0.53$) in primiparous cows (Figure 1B). Plasma adiponectin protein abundance (Figure 1C) was not altered ($P > 0.10$) by treatments.

Neutrophil Transcript Abundance

Basal *TNF α* transcript abundance (Figure 2A) in neutrophils was not affected ($P > 0.10$) by treatment or day. After LPS activation, *TNF α* transcript abundance increased compared with nonstimulated controls and tended ($P = 0.07$) to be greater in cells collected on d 21 compared with those collected on d 35. Dietary CrPr increased ($P = 0.02$) *TNF α* abundance in LPS-activated neutrophils (Figure 2B). We observed an RPLM $\times$ parity interaction ($P < 0.01$), reflecting increased *TNF α* transcript abundance in LPS-stimulated neutrophils from primiparous cows ($P < 0.01$) and a tendency for decreased *TNF α* in multiparous cows ($P = 0.07$) in response to RPLM (Figure 2C).

Basal *IL-1 β* transcript abundance in neutrophils was decreased ($P = 0.05$) by RPLM, and tended to be increased by CrPr ($P = 0.08$, Figure 3A). Basal *IL-1 β* transcript abundance was increased between d

Table 2. Effects of chromium propionate (CrPr) and rumen-protected lysine and methionine (RPLM) on plasma metabolites and hormones in lactating dairy cows on d 21 and 35 of dietary treatment¹

Item	Treatment				SEM	P-value ²		
	Control	CrPr	RPLM	CrPr+RPLM		CrPr	RPLM	Day
Glucose (mg/dL)	51.4	51.9	52.2	51.8	2.0	0.96	0.83	0.10
Insulin (pg/mL)	259	247	273	357	32	0.20	0.03	0.04
NEFA (μ M)	314	292	287	238	38	0.34	0.28	<0.001
Glucagon ³ (pg/mL)	163	173	169	173	6.0	0.30	0.38	0.27
RQUIKI ⁴	0.522	0.533	0.534	0.533	0.015	0.68	0.64	0.08

¹Values are least squares means across d 21 and 35.

²No interactions were significant except as noted for glucagon.

³RPLM $\times$ day interaction ($P = 0.04$). Glucagon concentration was increased by RPLM on d 35 (0.173 vs. 0.159 ± 0.006 ng/mL, $P = 0.05$), but not on d 21 (0.169 vs. 0.173 ± 0.006 ng/mL, $P = 0.53$).

⁴Revised quantitative insulin sensitivity check index (Holtenius and Holtenius, 2007).

21 and d 35 ($P = 0.01$) and was greater ($P = 0.04$) in primiparous cows than multiparous cows (12.2 ± 6.3 vs. 2.7 ± 1.2 arbitrary units). After LPS activation, *IL-1 β* transcript abundance increased dramatically, but no treatment or day effects ($P > 0.10$) were observed (Figure 3B). However, we detected a RPLM $\times$ parity interaction ($P < 0.01$), reflecting increased *IL-1 β* transcript abundance in primiparous cows ($P = 0.03$) and decreased *IL-1 β* in multiparous cows ($P = 0.01$) in response to RPLM (Figure 3C).

Adipocyte Morphology

Figure 4A depicts a representative photomicrograph of an adipose tissue cross-section. Tailhead subcutaneous adipocyte cross-sectional area was not affected ($P > 0.10$) by RPLM or CrPr (Figure 4B), but was greater ($P = 0.02$) in primiparous compared with multiparous cows ($4,942$ vs. $3,577 \pm 427$ μ m²). Median adipocyte cross-sectional area was positively correlated with d 35 BCS ($P < 0.001$, Figure 5), but not BW ($P > 0.10$).

DISCUSSION

In this study, CrPr supplementation did not affect plasma glucose, NEFA, insulin, or glucagon concentrations in lactating cows. Most studies evaluating the effects of Cr supplementation on dairy cattle metabolism have been conducted in the periparturient period, and effects on circulating hormones and metabolites in lactating animals have been limited. Hayirli et al. (2001) reported that supplementing periparturient cows with 0, 0.03, 0.06, and 0.12 mg/kg of BW^{0.75} of Cr as chromium-L-methionine (**Cr-Met**) decreased plasma insulin but did not affect plasma glucose, glucagon, or NEFA concentrations in early lactation. Smith et al. (2008) reported that supplementation of 0.03 or 0.06 mg/kg BW^{0.75} of Cr as Cr-Met had no effects on these plasma concentrations in early lactation despite some

alterations in late gestation. Interestingly, we found that RPLM supplementation increased plasma insulin concentration. Essential AA have long been considered to be physiologic stimuli for insulin secretion in humans (Floyd et al., 1966), but few studies have focused on the influence of dietary AA supplementation on insulin concentrations in dairy cows. By feeding a rumen-protected methionine product, Blum et al. (1999) observed increased plasma insulin concentrations in lactating cows within 5 d of supplementation. In the present study, although insulin was significantly increased by RPLM, this increase was not likely of great consequence for the metabolism and performance of animals because other metabolites were unaffected.

One important characteristic of Cr is its modulatory effect on insulin sensitivity. It has been established that Cr supplementation alleviates insulin resistance, enhances glucose tolerance, and improves metabolism in obese or type 2 diabetic subjects (Cefalu et al., 2010; Wang and Cefalu, 2010; Kim et al., 2011). Although the mode of action is not precisely known, Cr has been shown to enhance insulin binding, insulin receptor abundance, and pancreatic β -cell sensitivity (Wang and Cefalu, 2010). A recent paper also proposed that Cr improves insulin action by activating glucose transporter-4 trafficking and enhancing insulin-stimulated glucose transport in adipocytes (Chen et al., 2006). In the bovine, Sumner et al. (2007) reported that CrPr supplementation improved glucose tolerance in growing Holstein heifers. There has also been interest in the effects of Cr supplementation on insulin sensitivity in the periparturient period (Sano et al., 1993), when insulin resistance typically occurs (Bell and Bauman, 1997). Subiyatno et al. (1996) found that supplementation of 0.5 mg of Cr/kg of DM beginning at 6 wk prepartum increased insulin sensitivity in primiparous but not multiparous dairy cows at 2 wk before parturition. Hayirli et al. (2001) reported that Cr-Met supplementation from 21 d prepartum to 28 d postpartum improved glu-

Figure 1. Plasma leptin and adiponectin protein abundance in lactating dairy cows supplemented with control (Con), chromium propionate (CrPr), rumen-protected lysine and methionine (RPLM), or both (CrPr+RPLM). Plasma samples were collected on d 35 of dietary treatment, and protein abundance was measured by Western blot. (A) We observed a tendency for increased leptin abundance in response to RPLM ($P = 0.09$) but no effects of CrPr ($P = 0.33$) or RPLM $\times$ CrPr ($P = 0.56$). (B) We observed a tendency for a CrPr $\times$ parity interaction ($P = 0.08$), reflecting decreased ($P = 0.04$) leptin abundance by CrPr in multiparous cows, but not in primiparous cows ($P = 0.53$). (C) Relative abundance of adiponectin was not affected by RPLM ($P = 0.18$), CrPr ($P = 0.66$), or RPLM $\times$ CrPr ($P = 0.19$). Error bars are SEM; $n = 12$. Color version available in the online PDF.

cose tolerance and insulin responsiveness measured at 28 d postpartum in multiparous dairy cows. Therefore, by modulating insulin action, Cr may have significant implications for health and performance of lactating dairy cows. In the current study, we used RQUICKI to estimate insulin sensitivity (Holtenius and Holtenius, 2007). We found that RQUICKI tended to be lesser on d 21 compared with d 35, which is consistent with insulin resistance in early lactation. Furthermore, RQUICKI tended to be lesser in primiparous compared with multiparous cows, indicating that, in this study, first-lactation heifers were relatively more insulin resistant. However, RQUICKI was not affected by treatments, suggesting that CrPr and RPLM did not alter insulin sensitivity.

Neutrophils are the first line of immune defense in dairy cows. Upon maturation, neutrophils are released into circulation and play key roles for immune surveillance and defense against pathogens (Burton and Erskine, 2003). Impaired neutrophil trafficking, phagocytosis, and killing directly contribute to immunosuppression and increased disease incidence in early-lactation cows (Kehrli et al., 1989). Among other functions, neutrophils serve as a significant source of proinflammatory cytokines, such as $TNF\alpha$ and $IL-1\beta$ (Sohn et al., 2007). These cytokines are critical in activating and coordinating immune responses. As a cell wall component of gram-negative bacteria, LPS has frequently been used to stimulate immune cells; the capacity of these activated cells to produce proinflammatory cytokines can be used to assess the competence of these immune cells (Mani et al., 2012).

In this study, we evaluated the effect of CrPr and RPLM supplementation on neutrophil function by determining $TNF\alpha$ and $IL-1\beta$ transcript abundance in neutrophils in the basal state and after 12 h of LPS activation. We found that CrPr did not affect basal $TNF\alpha$ transcript abundance but significantly increased its abundance after LPS activation. These results suggest that CrPr supplementation may improve innate immune response upon activation. Several studies have highlighted the role of Cr in bovine immunity. Burton et al. (1993) reported that Cr supplementation at 0.5 mg of Cr/kg of DM from 6 wk prepartum through 16 wk postpartum increased antibody responses to ovalbumin administration and mitogen-stimulated blastogenesis of blood monocytes in lactating cows. In addition, $TNF\alpha$ and $IL-2$ production from mitogen-stimulated monocytes was significantly decreased for cows supplemented with Cr compared with controls, particularly around peak lactation (Burton et al., 1996).

Interestingly, RPLM increased LPS responsiveness of neutrophils from primiparous cows but decreased responsiveness of neutrophils from multiparous cows,

based on both *TNF α* and *IL-1 β* results. The reasons underlying the discrepant responses to RPLM supplementation in primiparous and multiparous cows are unclear. We also observed that basal *IL-1 β* transcript abundance in neutrophils was decreased by RPLM and tended to be increased by CrPr. Recent research has suggested that AA play an important role in immune modulation by regulating immune cell activation, proliferation, antibody production, and cytokine production, whereas dietary AA deficiency directly impairs immune function of animals (Li et al., 2007). For example, lysine deficiency limits cytokine synthesis and lymphocyte proliferation, and impairs immune responses in chickens (Konashi et al., 2000). Methionine supplementation markedly enhanced several key aspects of innate and humoral immune responses in chickens infected with virus (Li et al., 2007). Although little research has directly assessed the effects of these AA on bovine inflammation and immune function, the interaction between these nutrients and immunity warrants further research.

Several studies have been conducted in an attempt to modulate lipolysis in lactating dairy cows by dietary manipulations (Baumgard et al., 2002; McNamara and Valdez, 2005; Yuan et al., 2012). For example, CLA (*trans*-10,*cis*-12 isomer) decreased milk fat synthesis in lactating cows and reduced body fat accretion in growing animals by decreasing *de novo* lipogenesis (Bauman et al., 2000). Recently, Akter et al. (2011) reported that supplementation of dairy cows with 100 g of CLA/d (about 6% each of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers) for the first 100 d of lactation considerably decreased adipocyte sizes in different fat depots. This finding supports the concept that adipocyte size is sensitive to altered rates of lipogenesis or lipolysis in dairy cows (McNamara, 1991). By feeding 10 mg/d of Cr as CrPr to dairy cows from 21 d prepartum to 35 d postpartum, McNamara and Valdez (2005) dramatically increased adipose tissue lipogenesis postpartum but did not affect rates of lipolysis. In the present study, however, we did not detect effects of CrPr or RPLM on subcutaneous adipocyte size. Interestingly, adipocyte size was positively correlated with BCS, supporting the observation that fatter cows typically have greater adipocyte size (McNamara, 1991). In addition, we found that adipocyte size was greater in primiparous than multiparous cows, which is consistent with the observation that primiparous cows tended to have greater BCS than multiparous cows (2.94 vs. 2.70 ± 0.085 , $P = 0.07$).

We also measured the plasma protein abundance of several key hormones secreted from adipocytes to assess adipose tissue endocrine status. Leptin is primarily secreted by adipocytes and plays a central role in the

Figure 2. Neutrophil transcript abundance of tumor necrosis factor α (*TNF α*) in the basal state or after 12 h of LPS (1 μ g/mL) activation. Cows were supplemented with control (Con), chromium propionate (CrPr), rumen-protected lysine and methionine (RPLM), or both (CrPr+RPLM). Neutrophils were isolated from blood samples collected on d 21 and 35 of dietary treatments. (A) Basal *TNF α* abundance was not affected by RPLM ($P = 0.59$), CrPr ($P = 0.82$), or RPLM $\times$ CrPr ($P = 0.53$). We observed a tendency for a CrPr $\times$ day interaction ($P = 0.09$), but CrPr did not affect ($P > 0.10$) *TNF α* transcript abundance on either d 21 or 35 (data not shown); (B) LPS-activated *TNF α* transcript abundance was increased ($P = 0.02$) by CrPr supplementation but no effects were detected for RPLM ($P = 0.14$) or RPLM $\times$ CrPr ($P = 0.76$); *TNF α* transcript abundance tended to be greater ($P = 0.07$) on d 21 compared with d 35 (data not shown); (C) LPS-activated *TNF α* transcript abundance was affected by an RPLM $\times$ parity interaction ($P < 0.01$), reflecting increased *TNF α* transcript abundance in primiparous cows ($P < 0.01$) and a tendency for decreased *TNF α* transcript abundance in multiparous cows ($P = 0.07$) in response to RPLM. Error bars are SEM; $n = 6$. Color version available in the online PDF.

Figure 3. Neutrophil transcript abundance of IL-1 β (*IL-1 β*) in the basal state or after 12 h of LPS (1 μ g/mL) activation. Cows were supplemented with control (Con), chromium propionate (CrPr), rumen-protected lysine and methionine (RPLM), or both (CrPr+RPLM). Neutrophils were isolated from blood samples collected on d 21 and 35 of dietary treatments. (A) Basal *IL-1 β* transcript abundance was decreased ($P = 0.05$) by RPLM, and tended to be increased ($P = 0.08$) by CrPr, but we detected no RPLM $\times$ CrPr interaction ($P = 0.13$); (B) LPS-activated *IL-1 β* transcript abundance was not affected by RPLM ($P = 0.91$), CrPr ($P = 0.60$), or RPLM $\times$ CrPr ($P = 0.54$); (C) LPS-activated *IL-1 β* transcript abundance was affected by a RPLM $\times$ parity interaction ($P < 0.01$), reflecting increased *IL-1 β* transcript abundance in primiparous cows ($P = 0.03$) and decreased *IL-1 β* transcript abundance in multiparous cows ($P = 0.01$) in response to RPLM. Error bars are SEM; $n = 6$. Color version available in the online PDF.

Figure 4. Subcutaneous adipocyte size in lactating dairy cows. Cows were supplemented with control (Con), chromium propionate (CrPr), rumen-protected lysine and methionine (RPLM), or both (CrPr+RPLM). Adipose tissue samples were collected from the tail-head on d 35 of dietary treatment for assessment of median (≥ 150 cells/cow) cross-sectional area of adipocytes. (A) Representative photomicrograph (original magnification 10 $\times$) of adipose tissue section. Scale bar = 100 μ m. (B) There were no effects of RPLM ($P = 0.14$), CrPr ($P = 0.58$), or RPLM $\times$ CrPr ($P = 0.46$) on adipocyte size. Color version available in the online PDF.

regulation of feed intake, energy metabolism, and body condition (Ingvarsen and Boisclair, 2001; Block et al., 2003). In dairy cows, plasma leptin concentrations have been shown to be positively related to body fatness (Reist et al., 2003; Kokkonen et al., 2005). In this study, however, plasma leptin protein abundance was not correlated with BCS ($P = 0.53$, $R^2 = 0.009$, data not shown). Leptin abundance tended to be increased by RPLM, and was decreased by CrPr in multiparous cows. Adiponectin, another adipokine secreted by adi-

Figure 5. Correlation between median cross-sectional area of subcutaneous adipocytes and BCS (on d 35) in lactating dairy cows. Adipose tissue samples were collected on d 35 of dietary treatment. Adipocyte sizes were positively correlated with BCS ($P < 0.001$, $R^2 = 0.38$). Color version available in the online PDF.

pose tissue, plays a key role in glucose and lipid metabolism (Kadowaki and Yamauchi, 2005). Paradoxically, despite being produced in adipose tissue, adiponectin is negatively correlated with body fatness in humans (Kadowaki and Yamauchi, 2005; Ahima, 2006). In dairy cows, however, a previous study did not detect a relationship between plasma adiponectin concentrations and BCS (Raddatz et al., 2008). Interestingly, we found a negative correlation ($P = 0.02$, $R^2 = 0.16$, data not shown) between plasma adiponectin protein abundance and BCS, which agrees with the human data mentioned above. Here, we did not find effects of CrPr or RPLM on adiponectin protein abundance.

Taken together, our results indicate that supplemental CrPr (8 mg/d of Cr), RPLM (10 g/d of Lys and 5 g/d of Met), or both provided for 35 d had minimal effects on metabolism and adipocyte size but may modulate neutrophil function in lactating dairy cows. Neutrophil inflammatory responses to pathogen-associated molecules may be enhanced by CrPr supplementation, and RPLM can also influence these responses in a parity-dependent manner.

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