Glutathione metabolism and nuclear factor erythroid 2-like 2 (NFE2L2)-related proteins in adipose tissue are altered by supply of ethyl-cellulose rumen-protected methionine in peripartal Holstein cows

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ABSTRACT

Enhancing the supply of rumen-protected Met (RPM) during the peripartum period alleviates inflammation and oxidative stress status in dairy cows. We tested the hypothesis that RPM could increase abundance of genes and proteins related to glutathione (GSH) metabolism and the antioxidant transcription factor nuclear factor erythroid 2-like 2 (NFE2L2) in subcutaneous adipose tissue. Multiparous Holstein cows were fed a basal diet [control prepartum diet = 1.47 Mcal/kg of dry matter (DM) and 15.3% crude protein; control postpartum diet = 1.67 Mcal/kg of DM and 17.7% crude protein] or the control plus ethyl-cellulose RPM at a rate of 0.09 and 0.10% of DM intake before expected calving and after calving, respectively. Sixty cows were assigned to treatments based on parity, previous 305-d milk yield, and body condition score at 28 d from parturition. Diets were fed from –28 to 30 d. Biopsies of subcutaneous adipose tissue collected on d –10, 10, and 30 relative to parturition from 7 cows in each group were used for measuring concentrations of GSH, reactive oxygen species, superoxide dismutase, malondialdehyde, and mRNA and protein abundance (Western blotting). A repeated-measures ANOVA was used for statistics. The statistical model included the random effect of block and fixed effects of treatment, time, and its interaction. There was a diet × time effect for reactive oxygen species due to lower concentrations in Met versus control cows specifically at d –10. Cows fed Met also had lower concentrations of malondialdehyde in subcutaneous adipose tissue. Compared with controls, overall mRNA abundance of the GSH metabolism-related genes cystathionine- β -synthase (*CBS*), glutamate-cysteine ligase modifier subunit (*GCLM*), glutathione reductase (*GSR*), and glutathione peroxi-

dase 1 (*GPX1*) was greater in cows fed Met. Furthermore, supply of Met resulted in an overall upregulation of protein abundance of glutathione peroxidase (GPX) 1, GPX3, glutathione S-transferase mu 1 (GSTM1), and glutathione S-transferase α 4 (GSTA4), all related to GSH metabolism. There was a diet × time effect for protein abundance of NFE2L2 and its repressor Kelch-like ECH associated protein 1 (KEAP1) due to lower values at 30 d in cows fed Met versus controls. The abundance of phosphorylated NFE2L2 was lower at 30 d in response to Met. Overall, the data suggest that exogenous Met may play a role in activating GSH metabolism and the antioxidant NFE2L2 pathways in subcutaneous adipose tissue.

Key words: amino acid, inflammation, lactation, oxidative stress

INTRODUCTION

During the peripartal period, dairy cows usually experience negative energy balance due to the increased nutrient requirements for fetal growth, milk synthesis, and decreased feed intake (Loor et al., 2013b; Roche et al., 2013). Therefore, mobilization of body fat and skeletal muscle helps cows satisfy the demands for energy. Reactive oxygen species (**ROS**) concentrations in plasma increase during early lactation, contributing to oxidative stress status (Bernabucci et al., 2005). Production of ROS due to the mobilization of fatty acids, lipotoxicity, and mitochondrial activity (Fernández-Sánchez et al., 2011) in the adipose tissue contributes to oxidative stress (Furukawa et al., 2004; Li et al., 2015). A pronounced state of oxidative stress could lead to inflammation and immune dysfunction, both of which contribute to metabolic disorders (Du et al., 2018; Zhu et al., 2019). For example, inadequate supply of essential vitamins and trace minerals (e.g., vitamin E or Se) due to decreased DMI around parturition could impair antioxidant synthesis and lower the functionality of circulating leukocytes (Bendich, 1993; Spears, 2000).

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Oxidative stress increases oxylipid production derived from cellular membrane omega-6 fatty acid metabolism and can contribute to inflammation (Loor et al., 2013a; Mavangira and Sordillo, 2018). Therefore, oxidative stress has a negative effect on both production performance and welfare in dairy cows (Loor et al., 2013a).

Glutathione (**GSH**) is a well-established antioxidant in cells and plays a critical role in the maintenance and regulation of the thiol-redox status (Aquilano et al., 2014). Methionine, which is one of the most-limiting AA for lactating dairy cattle, participates in one-carbon metabolism and synthesis of GSH, both of which occur primarily in the liver (Martinov et al., 2010; Zhou et al., 2016a, 2017). When Lys was adequate, feeding RPM to achieve a Lys:Met ratio close to 2.8:1 in the MP during the periparturient period consistently alleviated oxidative stress status and inflammation in dairy cows and improved DMI and milk yield (Osorio et al., 2014b; Batistel et al., 2017; Zhou et al., 2017). Previous data have provided some indication that adipose may play a role in the induction of oxidative stress (Bernabucci et al., 2005; Contreras et al., 2017). For instance, in non-ruminants, adipose tissue can secrete several adipokines that are associated with oxidative stress and inflammation (Shoelson et al., 2006; McGown et al., 2014; Murri et al., 2014). In nonruminants, nuclear factor erythroid 2-like 2 (**NFE2L2**; formerly Nrf2) is a key transcription factor controlling cellular oxidative stress in part by increasing the mRNA abundance of antioxidant enzymes (Kaspar et al., 2009). In dairy cows, heat stress was associated with greater protein abundance of NFE2L2 in subcutaneous adipose in late pregnancy, indicating that stressors can elicit a tissue-specific antioxidant response (Zachut et al., 2017). Thus, NFE2L2 may play an important role in the subcutaneous adipose tissue during the periparturient period. The objective of the current study was to evaluate changes in mRNA and protein abundance of various components of the GSH synthesis pathway and the NFE2L2 signaling pathway using adipose tissue samples from control and RPM-fed cows in the study of Batistel et al. (2017).

MATERIALS AND METHODS

Experiment Design and Treatments

All procedures involving animals were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee (Urbana; protocol no. 14270). Details of the experimental design have been published previously (Batistel et al., 2017). Briefly, 60 multiparous Holstein cows (average lactation number 3.22 ± 1.11) were assigned to a basal diet (control, $n = 30$) without additional Met or the basal

diet with ethyl-cellulose RPM (Met, $n = 30$; Mepron, Evonik Nutrition and Care GmbH, Hanau-Wolfgang, Germany) in a randomized complete block design. Mepron contains 85% DL-Met, and the rumen bypass coefficient of Mepron is 80% (Overton et al., 1996). The RPM was top dressed from -28 to 60 d relative to parturition at a rate of 0.09 and 0.10% of DMI of the previous day before and after calving, respectively, to achieve a Lys:Met ratio of 2.8:1. The basal diet had a Lys:Met ratio of 3.71:1 (prepartum) to 3.78:1 (postpartum). A Lys:Met ratio of 2.8:1 to 2.9:1 has proven beneficial for dairy cow production performance and health, including greater DMI and milk yield, better immune function, and lower incidence of ketosis postpartum (Osorio et al., 2013; Zhou et al., 2016b). From -45 to -29 d relative to calving date, all cows were fed the same diet without RPM. Diets were mixed daily using a TMR truck with horizontal rotors and fed once daily (1300 h). The cows were milked daily at 0600, 1400, and 2200 h. The ingredient and chemical composition of the diets was published previously (Batistel et al., 2017). Diets were formulated to meet predicted requirements for dairy cows according to NRC (2001).

Adipose Tissue Biopsies

A subset of 7 cows in the control or Met groups was used for adipose biopsies. These cows were selected based on actual days receiving diets prepartum (28 ± 3 d) and absence of clinical disorders, and all had a full set of biopsies. These cows did not receive any veterinary treatment. Furthermore, they averaged 21,420 to 22,062 kg of milk in the previous 305-d lactation and had a BCS of 3.5 to 3.7 at treatment assignment. Tissue was harvested from the tail head (alternating between the right and left tail head region) at -10 (± 3 d), 10, and 30 d relative to parturition according to previous procedures from our laboratory (Ji et al., 2012). Upon collection, adipose tissue was immediately placed in screw-capped microcentrifuge tubes, snap frozen in liquid nitrogen, and preserved at -80°C until further analysis. Health was monitored for 7 d after surgery. Surgical clips were removed after 7 d postbiopsy. Cows were the last to be milked and returned to their tiestalls after the 0400 h milking. They did not have access to feed before biopsy, all of which were completed at approximately 0800 h. No antibiotics were administered postbiopsy.

RNA Isolation, cDNA Synthesis, and Quantitative PCR

Total RNA was isolated from 200 mg of adipose tissue using the miRNeasy kit (Qiagen, Hilden, Germany)

according to the manufacturer's protocols. The RNA samples were digested with DNaseI, and quantification was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The quality of RNA samples was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Quantitative PCR was performed as described previously (Osorio et al., 2014b). Previously validated internal controls for adipose tissue were ribosomal protein S9 (*RPS9*), *GAPDH*, and actin β (*ACTB*; Vailati Riboni et al., 2015, 2016, 2017). A comprehensive literature search was conducted to select *NFE2L2* and GSH metabolism target genes and regulators that play crucial roles in cellular antioxidant mechanisms (Motohashi and Yamamoto, 2004; Stipanuk, 2004; Lu, 2009). Gene symbols and names, quantitative PCR performance, and primer information are presented in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-15687>).

Western Blot Analysis

Total protein was extracted from 100 mg of adipose tissue using a tissue protein extraction reagent (catalog no. 78510; Thermo Fisher Scientific) containing Halt protease and phosphatase inhibitor cocktail (100 $\times$, catalog no. 78442; Thermo Fisher Scientific). The concentration of total protein was determined using a NanoDrop ND-1000 (Thermo Fisher Scientific). Protein samples were denatured by heating at 100°C for 5 min before loading 75 μ g of protein into each lane of a 4–20% SDS-PAGE gel (catalog no. 4561096; Bio-Rad, Hercules, CA). Reactions were run for 10 min at 180 V, and then for 45 to 60 min at 110 V. After activating a polyvinylidene fluoride membrane (catalog no. 1620261; Bio-Rad) with methanol for 1 min, the protein sample was transferred to the membrane in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (catalog no. 170-3940; Bio-Rad). Membranes were then blocked in 1 $\times$ Tris-buffered saline (TBST) containing 5% nonfat milk for 2 h at room temperature. The membranes were then incubated in 1 $\times$ TBST containing primary antibodies to hydroxycarboxylic acid receptor 2 (**HCAR2**), glutathione peroxidase (**GPX**) 1, GPX3, glutathione S-transferase mu 1 (**GSTM1**), glutathione S-transferase α 4 (**GSTA4**), Kelch-like ECH associated protein 1 (**KEAP1**), heme oxygenase 1 (**HMOX1**), mitogen-activated protein kinase 3 (**ERK1**), phospho-ERK1 (Thr202), *NFE2L2*, and phospho-*NFE2L2* (Ser40); catalog number and dilution ratio are included in Supplemental Table S2 (<https://doi.org/10.3168/jds.2018-15687>) overnight at 4°C. The membranes were then washed 6 times with 1 $\times$ TBST and incubated

with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (catalog no. 7074S; dilution 1:3,000; Cell Signaling Technology, Danvers, MA) for 1 h at room temperature. Subsequently, the membranes were washed 6 times with 1 $\times$ TBST and then incubated with enhanced chemiluminescence reagent (catalog no. 170-5060; Bio-Rad) for 3 min in the dark prior to image acquisition. Actin- β (catalog no. 4967S; Cell Signaling Technology) was used as the internal control. Images were acquired using the ChemiDOC MP Imaging System (Bio-Rad). The intensities of the bands were measured with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD). Specific target protein band density values were normalized to β -actin density values. Representative blots are included in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2018-15687>).

Oxidative Stress Analysis in Subcutaneous Adipose Tissue

Superoxide dismutase (catalog no. 706002; Cayman Chemical, Ann Arbor, MI), malondialdehyde (**MDA**; catalog no. 10009055; Cayman Chemical), GSH (catalog no. NWK-GSH01; Northwest Life Science Specialties, Vancouver, WA), and ROS (catalog no. STA-347, Cell Biolabs, San Diego, CA) were analyzed in subcutaneous adipose tissue using commercial kits. Concentration of total protein of the adipose samples was measured using the BCA assay kit (catalog no. 23227; Thermo Fisher Scientific).

Statistical Analysis

The data were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC) according to the following model with repeated measures:

$$Y_{ji} = \mu + M_j + T_1 + MT_{ji} + e_{ji},$$

where Y_{ji} = dependent continuous variable, μ = overall mean, M_j = fixed effect of diet (j = control vs. Met), T_1 = fixed effect of day (−10, 10, and 30 d), MT_{ji} = interaction between diet and day, and e_{ji} = residual error. Cow nested within treatment was the random effect. The Kenward-Roger statement was used for computing the denominator degrees of freedom. The covariance structure of the repeated measurements was spatial power. When the interaction was significant, least squares means separation between and within time points was performed using the PDIFF statement with Tukey adjustment. Normality of the residuals was checked with normal probability and box plots, and homogeneity of variances was checked with plots

of residuals versus predicted values. Significance was determined at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

NFE2L2 Target Gene Abundance

Main effects of diet, day, and interactions are reported in Figures 1, 2, and 3. The diet $\times$ day effect was not significant ($P > 0.05$) for any gene measured. Abundance of *NFE2L2* ($P = 0.06$; Figure 1) and *CBS* ($P = 0.06$; Figure 2) tended to be greater in response to enhanced Met supply. In addition, enhanced Met supply led to greater overall mRNA abundance (Figure 3) of *GCLM* ($P = 0.03$), *GSR* ($P = 0.02$), *TALDO1* ($P = 0.04$), and *ME1* ($P = 0.04$).

Protein Abundance

Main effects of diet, day, and interactions are reported in Figures 4, 5, and 6. The diet $\times$ day interaction was significant for protein abundance of *NFE2L2* ($P = 0.05$) and *KEAP1* ($P < 0.01$), namely due to lower abundance of *NFE2L2* ($P < 0.01$) at 10 d and greater abundance of *KEAP1* at 30 d in Met compared with control cows (Figure 4). Although abundance of phosphorylated (p)-*NFE2L2* could not be detected at -10 and 10 d in both control and Met cows, at 30 d postpartum abundance of p-*NFE2L2* ($P < 0.05$) was lower in Met cows compared with controls (Figure 4). However, there was no difference in p-*NFE2L2*:*NFE2L2* ratio between Met cows and the controls. Compared with controls, cows fed Met had lower abundance of the *NFE2L2* target *HMOX1* ($P < 0.05$).

Among the enzymes associated with GSH metabolism, enhancing the supply of Met resulted in an overall upregulation ($P < 0.05$) of *GPX1*, *GPX3*, *GSTM1*, and *GSTA4* ($P < 0.05$; Figure 5). The diet $\times$ day interaction tended to be significant ($P = 0.06$) for p-*ERK1* due to Met cows having greater ($P < 0.01$) abundance at 10 d compared with controls (Figure 6). In addition, p-*ERK1* tended to be greater ($P = 0.06$) in Met compared with control cows at 30 d. Overall abundance of *HCAR2* and *ERK1* in response to enhancing Met supply was greater ($P < 0.05$; Figure 6). The ratio of p-*ERK1* to total *ERK1* was greater ($P < 0.01$) in cows fed Met compared with controls due to greater ($P < 0.05$) values at d 10.

Oxidative Stress in Subcutaneous Adipose Tissue

The main effects of diet, time, and interactions are reported in Figure 7. The diet $\times$ day interaction was

Figure 1. Messenger RNA abundance (log-10 scale) of the antioxidant transcription factor *NFE2L2* (A) and the *NFE2L2* repressors *KEAP1* (B) and *CUL3* (C) in subcutaneous adipose tissue harvested at -10 , 10, and 30 d relative to parturition. Holstein cows were fed a basal diet (control prepartum diet: 1.47 Mcal/kg of DM and 15.3% CP; control postpartum diet: 1.67 Mcal/kg of DM and 17.7% CP) or the control plus ethyl-cellulose rumen-protected Met at a rate of 0.09 and 0.10% of DMI from -28 d to calving and from 1 through 30 d postcalving, respectively. Data are LSM $\pm$ pooled SEM, $n = 7$ cows/diet. *NFE2L2* = nuclear factor erythroid 2-like 2; *KEAP1* = Kelch-like ECH associated protein 1; *CUL3* = cullin 3.

Figure 2. Messenger RNA abundance (log-10 scale) of the one-carbon metabolism-related genes *MTR* (A), *BHMT* (B), *CBS* (C), and *CDO* (D) in subcutaneous adipose tissue harvested at -10, 10, and 30 d relative to parturition. Holstein cows were fed a basal diet (control prepartum diet: 1.47 Mcal/kg of DM and 15.3% CP; control postpartum diet: 1.67 Mcal/kg of DM and 17.7% CP) or the control plus ethyl-cellulose rumen-protected Met at a rate of 0.09 and 0.10% of DMI from -28 d to calving and from 1 through 30 d postcalving, respectively. Data are LSM $\pm$ pooled SEM, $n = 7$ cows/diet. *MTR* = methionine synthase; *BHMT* = betaine homocysteine methyltransferase; *CBS* = cystathionine β -synthase; *CDO* = cysteine dioxygenase.

significant for ROS ($P < 0.05$), namely due to a lower concentration ($P < 0.05$) at -10 d in Met compared with control cows (Figure 7). The concentration of MDA was lower ($P < 0.05$) overall in Met cows.

DISCUSSION

At a mechanistic level, changes in mRNA abundance associated with GSH metabolism and the NFE2L2 pathway in the liver and mammary gland tissue contribute to the reduction in oxidative stress in dairy cows receiving enhanced postruminal supply of Met (Osorio et al., 2014a; Han et al., 2018a). Furthermore, enhanced supply of Met increased the phosphorylation status of NFE2L2 in the mammary gland (Han et al., 2018a). In the current study, the potential benefit of Met in the subcutaneous adipose tissue antioxidant response is likely part of the overall benefits observed in alleviation of inflammation and oxidative stress in the same group of cows.

Oxidative Stress Biomarkers and mRNA Abundance of Genes in the One-Carbon and GSH Metabolism Pathways in Adipose Tissue

The concentration of ROS is a well-established biomarker for oxidative stress. The lower plasma ROS concentration detected in the entire cohort of cows (Batistel et al., 2017, 2018) used in this study agreed with previous data indicating a lower oxidative stress status (Osorio et al., 2014a; Zhou et al., 2016b, 2017). Although we did not attempt to quantify Met content in subcutaneous adipose, the lower concentration of MDA (measure of peroxides derived from PUFA) in cows fed Met suggests a less pronounced state of oxidative stress, which is consistent with plasma data from the bigger cohort of cows in this study (Batistel et al., 2018). Because nonspecific products of lipid peroxidation could interfere with the MDA assay, additional research is needed to confirm whether nutrients or stressors alter oxidative status in bovine adipose tissue.

Glutathione, one of the major antioxidants in the cell, helps prevent damage caused by ROS (Yu, 1994). At least in nonruminants, Cys availability limits GSH biosynthesis (Griffith, 1999). Methionine can be converted to Cys by the transsulfuration pathway, with the enzyme CBS being rate limiting (Kane et al., 1990). The greater overall mRNA abundance of *CBS* coupled with greater DMI (Batistel et al., 2017) in Met-fed cows could be taken as an indication that adipocytes had greater biosynthesis of cystathionine, Cys, and eventually GSH. Besides greater availability of Met in response to greater DMI, cows fed Met also would have had increased intakes of trace minerals

Figure 3. Messenger RNA abundance (log-10 scale) of the glutathione metabolism-associated genes *GCLM* (A), *GPX1* (B), *GCLC* (C), *GSR* (D), *GSTM1* (E), *ME1* (F), and *TALDO1* (G) in subcutaneous adipose tissue harvested at -10, 10, and 30 d relative to parturition. Holstein cows were fed a basal diet (control prepartum diet: 1.47 Mcal/kg of DM and 15.3% CP; control postpartum diet: 1.67 Mcal/kg of DM and 17.7% CP) or the control plus ethyl-cellulose rumen-protected Met at a rate of 0.09 and 0.10% of DMI from -28 d to calving and from 1 through 30 d postcalving, respectively. Data are LSM $\pm$ pooled SEM, $n = 7$ cows/diet. *GCLM* = glutamate-cysteine ligase modifier subunit; *GPX1* = glutathione peroxidase 1; *GCLC* = glutamate-cysteine ligase catalytic subunit; *GSR* = glutathione reductase; *GSTM1* = glutathione S-transferase M1; *ME1* = malic enzyme 1; *TALDO1* = transaldolase 1.

Figure 4. Protein abundance (relative to β -actin) of the antioxidant transcription factor phosphorylated (p)-NFE2L2 (active; A), NFE2L2 repressor KEAP1 (B), NFE2L2 (inactive; C), NFE2L2 target HMOX1 (D), and the p-NFE2L2/NFE2L2 ratio (E) in subcutaneous adipose tissue harvested at -10, 10, and 30 d relative to parturition. Holstein cows were fed a basal diet (control prepartum diet: 1.47 Mcal/kg of DM and 15.3% CP; control postpartum diet: 1.67 Mcal/kg of DM and 17.7% CP) or the control plus ethyl-cellulose rumen-protected Met at a rate of 0.09 and 0.10% of DMI from -28 d to calving and from 1 through 30 d postcalving, respectively. Data are LSM $\pm$ pooled SEM, $n = 7$ cows/diet. Abundance of p-NFE2L2 was detectable only at 30 d postpartum. NFE2L2 = nuclear factor erythroid 2-like 2; KEAP1 = Kelch-like ECH associated protein 1; HMOX1 = heme oxygenase 1. Means with different letters (a, b) differ (diet $\times$ day, $P \leq 0.05$). *Means between control and Met differ ($P \leq 0.05$).

(e.g., Se) and vitamins with roles as antioxidants; for example, concentrations of β -carotene and tocopherol were greater in plasma from Met-fed cows (Batistel et al., 2018). Further in vitro studies with bovine primary adipocytes, for example, can be designed to measure mRNA and protein abundance, concentrations of ROS, and other intermediates related to GSH metabolism in relation to supply of Met or antioxidants such as Se,

β -carotene, and tocopherol. Such studies would be helpful for better understanding the role of nutrients in the antioxidant response of adipose tissue.

Glutathione reductase is an essential enzyme that converts oxidized GSH to the reduced form (Couto et al., 2016). The enzymes glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modifier subunit (GCLM) are rate limiting for GSH

metabolism. Malic enzyme 1, a multifunctional protein linking the glycolytic and citric acid cycles, is important for NADPH production (Wen et al., 2015; Nakashima et al., 2018). The enzyme transaldolase 1 (TALDO1) participates in the pentose phosphate pathway and helps provide cells with NADPH, an important redox cofactor (Samland and Sprenger, 2009) known for its role in mitigating oxidative stress. Thus, the upregulation of GSH metabolism genes (*GCLM*, *GSR*, *ME1*, and *TALDO1*) in response to enhanced postpartum supply of Met indicates that this AA, either directly or via its positive effect on DMI, plays a role in helping adipose tissue alleviate oxidative stress during the transition period.

Abundance of Proteins Associated with GSH Metabolism and NFE2L2

The enzymes GSTM1, GSTA4, GPX1, and GPX3 play key roles in inactivating pro-oxidant products

such as H_2O_2 and oxygen radicals in various tissues, including adipose (Lee et al., 2008; Curtis et al., 2010). For instance, GPX3 (a selenium-dependent enzyme; Yu et al., 2007) and GSTA4 in nonruminant adipose tissue are essential for preventing oxidative stress in conditions associated with excessive fat deposition (Lee et al., 2008). Downregulation of GSTA4 in murine adipocytes resulted in increased ROS production along with lipolysis and reduced glucose uptake (Curtis et al., 2010). The GPX enzymes are oxidized by H_2O_2 and reduced by GSH (Sena and Chandel, 2012). Both GSTM1 and GSTA4 belong to the glutathione-S-transferase enzymes that are generally considered to be important for detoxification of electrophiles via GSH conjugation (Strange et al., 2001). Overall, protein abundance of GSH-related protein targets was consistent with mRNA abundance data. The upregulation of protein abundance of GPX1, GPX3, GSTM1, and GSTA4 in response to enhanced Met supply underscores not only that adipose tissue has antioxidant mechanisms related

Figure 5. Protein abundance (relative to β -actin) of the glutathione metabolism-related enzymes GSTM1 (A), GPX1 (B), GSTA4 (C), and GPX3 (D) in subcutaneous adipose tissue harvested at -10, 10, and 30 d relative to parturition. Holstein cows were fed a basal diet (control prepartum diet: 1.47 Mcal/kg of DM and 15.3% CP; control postpartum diet: 1.67 Mcal/kg of DM and 17.7% CP) or the control plus ethylcellulose rumen-protected Met at a rate of 0.09 and 0.10% of DMI from -28 d to calving and from 1 through 30 d postcalving, respectively. Data are LSM $\pm$ pooled SEM, $n = 7$ cows/diet. GSTM1 = glutathione S-transferase mu 1; GPX1 = glutathione peroxidase 1; GPX3 = glutathione peroxidase 3; GSTA4 = glutathione S-transferase α 4.

Figure 6. Protein abundance (relative to β -actin) of the oxidative stress-sensitive protein ERK1 (inactive; A), phosphorylated (p)-ERK1 (active; B), p-ERK1:ERK1 ratio (C), and the inflammation-related G-coupled protein receptor HCAR2 (D) in subcutaneous adipose tissue harvested at -10, 10, and 30 d relative to parturition. Holstein cows were fed a basal diet (control prepartum diet: 1.47 Mcal/kg of DM and 15.3% CP; control postpartum diet: 1.67 Mcal/kg of DM and 17.7% CP) or the control plus ethyl-cellulose rumen-protected Met at a rate of 0.09 and 0.10% of DMI from -28 d to calving and from 1 through 30 d postcalving, respectively. Data are LSM $\pm$ pooled SEM, $n = 7$ cows/diet. ERK1 = mitogen-activated protein kinase 3; HCAR2 = hydroxycarboxylic acid receptor 2. Means with different letters (a, b) differ (diet $\times$ day, $P \leq 0.05$).

Figure 7. Activity of superoxide dismutase (SOD; A) and concentrations of reactive oxygen species (ROS; B), glutathione (GSH; C), and malondialdehyde (MDA; D) in subcutaneous adipose tissue harvested at -10, 10, and 30 d relative to parturition. Holstein cows were fed a basal diet (control prepartum diet: 1.47 Mcal/kg of DM and 15.3% CP; control postpartum diet: 1.67 Mcal/kg of DM and 17.7% CP) or the control plus ethyl-cellulose rumen-protected Met at a rate of 0.09 and 0.10% of DMI from -28 d to calving and from 1 through 30 d postcalving, respectively. Data are LSM $\pm$ pooled SEM, $n = 7$ cows/diet. Means with different letters (a, b) differ (diet $\times$ day, $P \leq 0.05$).

to GSH but also that these are responsive to greater Met supply or DMI.

The transcription factor NFE2L2 represents a major mechanism for cells to defend against oxidative stress. Expression of the gene was first reported in the transition cow liver (Loor, 2010), and more recent research provided some evidence that it can play an important role during the transition period and late pregnancy (Gessner et al., 2013; Zachut et al., 2017). Therefore, it seems that NFE2L2 plays an important role in the adipose tissue adaptations to the onset of lactation in dairy cows. Most dairy cows mobilize body fat to aid in meeting energy demands for milk synthesis during the transition period, and in that process oxidation of fatty acids also generates ROS (Loor et al., 2013a,b; Roche et al., 2013). Both nutrients and ROS can activate the NFE2L2 pathway (Nguyen et al., 2009; Cardozo et al., 2013), which triggers adaptations in the abundance of KEAP1 (Ade et al., 2009). In the cytosol, KEAP1 binds to NFE2L2, which inhibits its release and promotes proteasomal degradation (Kensler et al., 2007).

The fact that p-NFE2L2 was detectable only at 30 d postpartum agrees with other data indicating that oxidative stress status increases gradually after calving (Abuelo et al., 2013). In the entire cohort of cows (Batistel et al., 2018), we detected not only a temporal increase in concentration of ROS in plasma but also greater overall values in control cows compared with Met-fed cows. This may partly explain the fact that p-NFE2L2 was not detectable at -10 and 10 d relative to parturition. Along with mammary tissue data demonstrating a marked upregulation in abundance of p-NFE2L2:total NFE2L2 at 30 d versus -30 d around parturition (Han et al., 2018b), these temporal responses underscore the potential role of NFE2L2 signaling in the defense mechanisms of mammary gland and adipose tissue against oxidative stress.

Taking into account the lower p-NFE2L2 at 30 d in cows fed Met and the similar p-NFE2L2:total NFE2L2 ratio in the present study along with previous data in which enhanced Met supply upregulated the phosphorylation status of NFE2L2 in the bovine mammary gland (Han et al., 2018a), it seems plausible that this transcription factor is less responsive to Met or less susceptible to ROS in adipose than mammary tissue. Although the greater abundance of KEAP1 at 30 d in cows fed Met may partly explain the lower p-NFE2L2, the lack of difference in ROS concentration between groups coupled with greater overall abundance of GSTM1, GPX1, GSTA4, and GPX3 in response to Met suggests that this AA helps control tissue ROS independent of NFE2L2.

Protein Abundance of Targets Associated with Response to Inflammation

It has been proposed that uncontrolled adipose tissue inflammation could intensify lipolysis (Contreras et al., 2018). Hydroxycarboxylic acid receptor 2 belongs to the G protein-coupled receptor family, and in nonruminants it can serve both an antilipolytic (Offermanns et al., 2011) and anti-inflammatory (Graff et al., 2016) role. Previous work demonstrated wide expression of HCAR2 in bovine tissues, including adipose (Titgemeyer et al., 2011). In addition, there is an indication that enhancing the postruminal supply of nicotinic acid (an agonist of HCAR2) can signal through this receptor and reduce plasma free fatty acid concentrations (Morey et al., 2011; Yuan et al., 2012). Thus, the greater abundance of HCAR2 in response to enhanced Met supply might have served a dual role as in nonruminants (i.e., help control lipolysis as well as inflammation).

Previous research has clearly demonstrated that bovine adipose tissue undergoes an inflammatory response around parturition, which likely affects the antioxidant defense system of the tissue (Vailati Riboni et al., 2015, 2016, 2017). Oxidative stress is known to cause apoptosis in various cell types, and oxidative damage-induced apoptosis is partly mediated by ERK1/2 phosphorylation (Lee et al., 2003; Du et al., 2017). Thus, the greater phosphorylation status of ERK1 in response to enhanced Met supply was indicative that adipocyte survival might have been sustained in those cows.

CONCLUSIONS

Several components of the GSH metabolism and NFE2L2 antioxidant signaling pathways are expressed in subcutaneous adipose tissue and respond to the change in physiological state from late pregnancy to early lactation. The gradual increase in protein abundance of key components of these pathways reflects the state of oxidative stress experienced by cows. Supplementation of Met was associated with lower MDA and higher activity of the GSH antioxidant enzymes. Further in vitro studies using adipocytes may help us better understand the role and mechanisms for these responses.

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