Methionine supply alters mammary gland antioxidant gene networks via phosphorylation of nuclear factor erythroid 2-like 2 (NFE2L2) protein in dairy cows during the periparturient period

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ABSTRACT

The periparturient period is the most critical period during the lactation cycle of dairy cows and is characterized by increased oxidative stress status. The objective of this experiment was to evaluate the effect of supplementing rumen-protected methionine on nuclear factor erythroid 2-like 2 (NFE2L2, formerly NRF2) protein and target gene expression in the mammary gland during the early postpartal period. Multiparous Holstein cows were used in a block design experiment with 30 cows per treatment. Treatments consisting of a basal control diet (control) or the basal diet plus rumen-protected methionine (methionine) were fed from d –28 to 60 relative to parturition. Mammary tissue biopsies were harvested on d 21 postpartum from 5 cows per treatment. Compared with control, methionine increased dry matter intake, milk yield, and milk protein content. Among plasma parameters measured, methionine led to greater methionine and lower reactive oxygen metabolites. Compared with control, methionine supply resulted in greater mRNA abundance of the NFE2L2 target genes glutamate-cysteine ligase catalytic subunit (*GCLC*), glutamate-cysteine ligase modifier subunit (*GCLM*), glutathione reductase (*GSR*), glutathione peroxidase 1 (*GPX1*), malic enzyme 1 (*ME1*), ferrochelatase (*FECH*), ferritin heavy chain 1 (*FTH1*), and NAD(P) H quinone dehydrogenase 1 (*NQO1*) in the mammary tissue. In addition, methionine upregulated the mRNA abundance of *NFE2L2*, *NFKB1*, *MAPK14* and downregulated *KEAP1*. The ratio of phosphorylated NFE2L2 to total NFE2L2 protein, and total heme oxygenase 1 (HMOX1) protein

were markedly greater in response to methionine supply. In contrast, total protein abundance of Kelch-like ECH-associated protein 1 (KEAP1), which sequesters NFE2L2 in the cytosol and reduces its activity, was lower with methionine. Besides the consistent positive effect of methionine supply on systemic inflammation and oxidative stress status, the present data indicate a positive effect also on antioxidant mechanisms within the mammary gland, which are regulated, at least in part, via phosphorylation of NFE2L2 and its target genes. The exact mechanisms for these responses merit further study.

Key words: antioxidant response, lactation, oxidative stress

INTRODUCTION

Oxidative stress is associated with dairy cattle health and milk production during the periparturient period (Roche et al., 2013; Osorio et al., 2014b). During the perinatal period, the primary challenge experienced by dairy cows is a sudden state of negative energy and AA balance coupled with increased nutrient requirements for milk production (Drackley, 1999). This period of intense metabolic activity leads to a greater production of reactive oxygen metabolites (ROM) and increases the susceptibility of dairy cows to disease (Roche et al., 2013; Osorio et al., 2014b; Sordillo and Aitken, 2009).

Methionine is one of the major limiting AA in lactating dairy cows (Schwab and Broderick, 2017), and it can be used in the synthesis of glutathione (GSH) and taurine, 2 of the main intracellular antioxidants (Martinov et al., 2010). Previous research demonstrated that rumen-protected methionine (RPM) supply improved health status and production performance in dairy cows (Chen et al., 2011; Osorio et al., 2013; Zhou et al., 2016a; Batistel et al., 2017). At least in the liver, evidence indicates that RPM elicits positive effects on oxidative stress and inflammation partly through alter-

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ations in mRNA abundance (Naeem et al., 2012; Osorio et al., 2014a; Zhou et al., 2016a). Whether antioxidant mechanisms in the mammary gland also are responsive to RPM is unknown. However, the consistent increase in milk protein synthesis suggests that methionine supply has a potential biological impact within mammary tissue.

One of the key intrinsic cellular systems that protects against deleterious effects of ROM or xenobiotic compounds is regulated by nuclear factor, erythroid 2-like 2 (**NFE2L2**, formerly NRF2; Bryan et al., 2013). In cultured mammary epithelial cells, several antioxidants activate *NFE2L2*, triggering an increase in the transcription of antioxidant genes (Jin et al., 2016a,b). In an in vivo study, flax meal supplementation increased *NFE2L2* mRNA expression in mammary tissue, indicating that NFE2L2 participates in the control of the cellular antioxidant response (Schogor et al., 2013). Thus, these data suggest that NFE2L2, as a transcription factor, plays a key role in regulating mammary cell antioxidant mRNA abundance.

We hypothesized that besides benefitting the systemic inflammatory and oxidative stress status and production performance, greater methionine supply would also be associated with changes in mammary antioxidant mRNA abundance that reflect a protective mechanism against oxidative damage driven by phosphorylation of NFE2L2. Thus, the specific objectives of the present study were to evaluate the effect of methionine supply on phosphorylation of NFE2L2 and mRNA abundance of target genes in mammary tissue.

MATERIALS AND METHODS

Experimental Design and Treatments

The Institutional Animal Care and Use Committee at University of Illinois (Urbana; protocol #14270) approved all the experimental procedures. Details of the experimental design have been reported previously (Batistel et al., 2017). Briefly, multiparous Holstein cows were used in a randomized, complete, balanced block design experiment with 30 cows per treatment. Treatments consisting of a basal control (control) diet with no added methionine or the basal diet plus ethylcellulose RPM (methionine; Mepron, Evonik Nutrition and Care GmbH, Hanau-Wolfgang, Germany) and were fed from d -28 to 60 relative to parturition. Mepron was fed at a rate of 0.09% and 0.10% of DMI during the prepartum and postpartum periods, respectively, to achieve a lysine:methionine ratio of 2.8:1. This ratio has demonstrated benefits in terms of animal performance and health (Osorio et al., 2013; Zhuang et al., 2014;

Zhou et al., 2016c). During the preliminary period (from -45 to -29 d relative to the expected parturition day), all cows received the same diet with no added methionine. Treatment diets were mixed daily in a tumble-mixer and fed once daily (1300 h). The ingredients and chemical composition of the diets were reported previously (Batistel et al., 2017). Rations were formulated to meet predicted requirements for minerals and vitamins according to the NRC (2001).

Milk Samples

Milk yield was recorded daily, and consecutive morning, noon, and evening milk samples were collected weekly (Batistel et al., 2017). Milk samples were stored with preservative at 4°C before analysis of fat, protein, and lactose by mid-infrared procedures (Dairy Lab Services, Dubuque, IA).

Plasma Parameters

Blood samples were collected from the coccygeal vein before feeding using evacuated tubes containing lithium heparin (Batistel et al., 2017). Plasma was obtained by centrifugation at $2,000 \times g$ for 30 min at 4°C and frozen (-80°C) until further analysis. Methionine and taurine in plasma were analyzed by Evonik Nutrition and Care GmbH (AOAC International, 1995). Reactive oxygen metabolites in plasma were analyzed with the d-ROMs-test (cod. MC002) purchased from Diacron (Grosseto, Italy). Total antioxidant capacity of blood was assessed as ferric-reducing antioxidant power (Benzie and Strain, 1996).

Mammary Gland Samples

Mammary gland tissue was harvested on d 21 from a subset of 5 cows in each treatment via percutaneous biopsy. The procedure was conducted under mild general anesthesia with Xylazine and local anesthesia with lidocaine HCl. A 3-cm incision was made through the skin and subcutaneous tissue, and then separated from the mammary capsule at the incision site. Approximately 150 mg of tissue was removed using a biopsy needle (Bard Magnum, 12 gauge $\times$ 16 cm; C. R. Bard Inc., Murray Hill, NJ) and immediately snap-frozen in liquid N and stored at -80°C until later analysis. Pressure was applied for 10 min to the incision area with sterile gauze to prevent excessive bleeding. The skin incision was then closed with 11 mm Michel clips and antimicrobial ointment applied to the incision site. Clots resulting from the biopsies were removed by hand at each milking over the following 3 d.

Table 1. mRNA abundance of nuclear factor erythroid 2-like 2 (NFE2L2), target genes, and other regulators in mammary gland tissue at 21 d postpartum in cows fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine from –28 through 21 d around parturition

Gene	Gene name	Diet		% Difference ¹	SEM	P-value
		Control	Methionine			
Cell apoptosis						
<i>BCL2</i>	Apoptosis regulator BCL2	0.75	0.85	–33	0.11	0.39
Glutathione metabolism						
<i>GSR</i>	Glutathione reductase	0.99	1.20	21	0.04	0.02
<i>GCLC</i>	Glutamate-cysteine ligase catalytic subunit	0.66	0.98	48	0.11	0.04
<i>GPX1</i>	Glutathione peroxidase 1	0.92	1.04	–12	0.03	0.04
<i>GCLM</i>	Glutamate-cysteine ligase modifier subunit	0.45	1.04	131	0.17	0.01
<i>GSTM1</i>	Glutathione S-transferase M1	1.05	0.99	–6	0.03	0.28
<i>ME1</i>	Malic enzyme 1	0.65	0.92	42	0.11	0.03
<i>TALDO1</i>	Transaldolase 1	1.00	1.00	0	0.02	0.98
Thioredoxin metabolism						
<i>TXN</i>	Thioredoxin	0.94	0.97	3	0.02	0.35
<i>TXNRD1</i>	Thioredoxin reductase 1	0.88	1.03	17	0.06	0.07
Heme and Fe metabolism						
<i>FECH</i>	Ferrochelatase	0.63	0.99	57	0.09	0.01
<i>HMOX1</i>	Heme oxygenase 1	0.92	0.95	3	0.08	0.76
<i>FTH1</i>	Ferritin heavy chain 1	0.76	0.98	29	0.06	0.02
<i>PIR</i>	Pirin	0.66	0.82	24	0.15	0.33
Quinone metabolism						
<i>NQO1</i>	NAD(P)H quinine dehydrogenase 1	0.51	1.07	110	0.21	0.04
KEAP-dependent regulators						
<i>NFE2L2</i>	Nuclear factor, erythroid 2-like 2	0.57	1.01	77	0.13	0.02
<i>KEAP1</i>	Kelch-like ECH-associated protein 1	1.15	0.92	–20	0.05	0.01
<i>CUL3</i>	Cullin 3	0.82	1.01	23	0.06	0.05
KEAP-independent regulators						
<i>NFKB1</i>	Nuclear factor kappa B subunit 1	0.75	0.99	32	0.07	0.03
<i>MAPK12</i>	Mitogen-activated protein kinase 12	1.09	1.04	–5	0.15	0.84
<i>MAPK14</i>	Mitogen-activated protein kinase 14	0.76	1.03	36	0.07	0.01
<i>MAPK8</i>	Mitogen-activated protein kinase 8	0.72	0.90	25	0.15	0.32
<i>RXRA</i>	Retinoid X receptor α	0.97	1.01	4	0.08	0.77

¹Difference in mRNA abundance = (methionine – control)/control $\times$ 100.

RNA Isolation, cDNA Synthesis, and Quantitative PCR

Total RNA was isolated from 50 mg of mammary gland tissue using the miRNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The RNA samples were digested with DNaseI and quantification was accessed 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), and all samples had an RNA integrity number factor greater than 7.3. The quantitative PCR was performed as described previously (Osorio et al., 2014a). The *RPS9*, *RPS15*, and *UXT* were selected as the internal control genes to normalize mRNA abundance data (Bionaz and Looor, 2007). A comprehensive literature search (Chorley et al., 2012; Niture and Jaiswal, 2012; Jin et al., 2016a) was conducted to select *NFE2L2* target genes and regulators that play key roles in cellular antioxidant mechanisms. Gene symbols and names are presented

in Table 1. Quantitative PCR performance and primer information are included in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-14206>).

Western Blot Analysis

Protein was extracted from mammary gland tissue (50 mg) using a tissue protein extraction reagent (catalog no. 78510, Thermo Fisher Scientific) containing inhibitor cocktail (100 $\times$, catalog no. 78442; Thermo Fisher Scientific; Xu et al., 2017). Protein concentration was measured using the BCA Assay Kit (catalog no. 23227; Thermo Fisher Scientific). Fifty micrograms of total protein per lane was loaded on a 10% SDS-PAGE and ran at 120 V for 120 min. Electrotransfer to polyvinylidene difluoride membranes (#162–0261, Bio-Rad, Hercules, CA) was performed in the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (#170–3940, Bio-Rad). Membranes were blocked in Tris-buffered saline (TBST) containing 5% nonfat milk for 2 h at room temperature. The membranes were then incubated in TBST containing primary antibodies

to NFE2L2 (catalog no.137550, Abcam, Cambridge, UK), Phospho-NFE2L2 (Ser40, catalog no. PA5-67520, Invitrogen, Carlsbad, CA), KEAP1 (catalog no. 8047, Cell Signaling Technology, Danvers, MA), HMOX1 (catalog no. 70081, Cell Signaling Technology), and GPX1 (catalog no. ARP41491, Aviva Systems Biology, San Diego, CA) overnight at 4°C. Subsequently, the membranes were washed 5 times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (catalog no. ab6721, Abcam) for 1 h at room temperature. The membranes were washed with TBST and then incubated with Clarity Western ECL Substrate (catalog no. 170-5060, Bio-Rad), and GAPDH (catalog no. ab22555, Abcam) was used as the internal control. Images were obtained with the ChemiDOC MP instrument (Bio-Rad). The intensities of the bands were measured with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD; Supplemental Figure S1, <https://doi.org/10.3168/jds.2017-14206>).

Statistical Analysis

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

$$Y_{ij} = \mu + b_i + M_j + e_{ij},$$

where Y_{ij} = dependent, continuous variable, μ = overall mean, b_i = random effect of block, M_j = fixed effect of treatment (j = control vs. methionine), and e_{ij} = residual error. Normality of the residuals was checked with normal probability and box plots and homogeneity of variances with plots of residuals versus predicted values. Significance was determined at $P < 0.05$.

RESULTS

NFE2L2 and Target Gene mRNA Abundance

Compared with control, methionine supplementation resulted in greater mRNA abundance of *NFE2L2* target genes including *GCLC* ($P = 0.04$), *GCLM* ($P = 0.01$), *GPX1* ($P = 0.04$), *GSR* ($P = 0.02$), *ME1* ($P = 0.03$), *FECH* ($P = 0.01$), *FTH1* ($P = 0.02$), and *NQO1* ($P = 0.04$). Other *NFE2L2* target genes (*HMOX1*, *TXN*, *GSTM1*, *TALDO1*, *BCL2*, and *PIR*) were not affected by methionine (Table 1).

Compared with control, expression of *NFE2L2* ($P = 0.02$), *CUL3* ($P = 0.05$), *NFKB1* ($P = 0.03$), and *MAPK14* ($P = 0.01$) was upregulated in response to methionine supply, whereas *KEAP1* abundance was downregulated ($P = 0.01$). Other regulators (*MAPK8*, *MAPK12*, and *RXRA*) were not affected by methionine supply (Table 1).

Lactation Performance

Methionine supplementation led to greater DMI (+1.7 kg/d; $P = 0.01$), milk yield (+3.5 kg/d; $P < 0.01$), and milk protein content (+0.12%; $P = 0.02$) during the first 21 d postpartum (Table 2). No differences ($P > 0.10$) between treatments were observed for milk fat and lactose, or SCC (Table 2). A time effect ($P < 0.01$) during the first 21 d postpartum was observed for milk yield, % milk protein, % milk fat, % milk lactose, SCC, and DMI.

Plasma Biomarkers

Compared with control, methionine cows tended to have lower ($P = 0.08$) concentration of ROM and

Table 2. Effects of ethyl-cellulose rumen-protected methionine supplementation to multiparous Holstein dairy cows from -28 through 21 d around parturition on lactation performance and selected blood biomarkers

Item	Diet		SEM	P-value		
	Control	Methionine		Treatment ¹	Time	Treatment × time
Milk yield, kg/d	33.7	37.2	0.78	<0.01	<0.01	0.81
Milk protein, %	3.33	3.45	0.04	0.02	<0.01	0.85
Milk fat, %	4.09	3.97	0.11	0.35	<0.01	0.79
Milk lactose, %	4.71	4.75	0.05	0.41	<0.01	0.85
SCC ²	1.81	1.83	0.07	0.54	<0.01	0.38
DMI, kg/d	14.8	16.5	0.30	0.01	<0.01	0.79
Plasma ³						
Taurine, mg/dL	0.39	0.32	0.03	0.17	0.01	0.58
Methionine, mg/dL	0.15	0.18	0.01	0.09	0.05	0.78
ROM, ⁴ mg of H ₂ O ₂ /100 mL	18.2	17.2	0.31	0.08	<0.01	0.86

¹Methionine effect.

²Data were log-transformed.

³Data from d 7 and 21.

⁴Reactive oxygen metabolites.

Table 3. Expression of proteins (relative units) involved in the nuclear factor erythroid 2-like 2 (NFE2L2) pathway in mammary gland tissue from cows fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine from –28 through 21 d around parturition

Protein ¹	Diet		SEM	P-value
	Control	Methionine		
NFE2L2	0.92	0.45	0.08	<0.01
p-NFE2L2	1.35	1.30	0.15	0.32
Ratio NFE2L2/p-NFE2L2	1.46	2.88	0.35	<0.01
KEAP1	0.98	0.79	0.11	0.02
HMOX1	0.35	0.55	0.09	<0.01
GPX1	0.29	0.30	0.10	0.27

¹p-NFE2L2 = phosphorylated NFE2L2; KEAP1 = total Kelch-like ECH-associated protein 1; HMOX1 = total heme oxygenase 1; and GPX1 = total glutathione peroxidase 1.

higher ($P = 0.09$) concentration of methionine in the plasma. No differences ($P > 0.10$) between groups were observed for taurine concentrations (Table 2). A time effect ($P < 0.05$) was observed for the concentration of taurine, methionine, and ROM.

Protein Expression

Western blot analysis revealed that total NFE2L2 protein expression was lower ($P < 0.01$), whereas the p-NRF2/NRF2 ratio was greater ($P < 0.01$) in response to increasing the methionine supply (Table 3). Methionine supply led to lower ($P = 0.02$) KEAP1 and greater ($P < 0.01$) HMOX1. Abundance of GPX1 was not affected ($P = 0.27$) by methionine supply.

DISCUSSION

In vitro studies with bovine mammary cells revealed that enhancing methionine supply protects against thermal damage by increasing intracellular antioxidant (Mu et al., 2014; Han et al., 2015). Whether methionine elicits similar effects in vivo is unknown. The present study revealed for the first time that enhancing methionine supply via RPM feeding can upregulate the mRNA abundance of antioxidant genes in part through the phosphorylation of NFE2L2 protein. The potential benefit of methionine on the mammary antioxidant response is likely part of the overall benefits observed on lactation performance and alleviation of inflammation and oxidative stress status in the same group of cows.

Lactation Performance and Plasma Biomarkers

The beneficial effects of enhancing the supply of methionine during the periparturient period on production performance have been associated with a reduction in inflammation and oxidative stress status (Osorio et al., 2013; Zhou et al., 2016d). The better performance

(DMI, milk yield, and milk protein content) and lower concentration of ROM detected in the subset of cows used in the present study confirm previous data (Batistel et al., 2017, 2018) indicating a reduced oxidative stress status. The greater plasma methionine concentration also agrees with previous research supplementing RPM (Ordway et al., 2009; Zhou et al., 2016b). Therefore, the greater plasma circulating levels of methionine in the methionine cows coupled with greater milk protein content, GSH, and taurine concentrations suggest that a portion of this AA was metabolized to generate these potent antioxidants (Martinov et al., 2010).

NFE2L2-Target Gene Abundance

Glutathione metabolism via the disulfide reductase systems in the cell involves the transfer of electrons from reduced to oxidized molecules (Berndt et al., 2007). The enzymes GCLC and GCLM are rate-limiting of GSH biosynthesis. The enzyme GSR uses NADPH to reduce GSSG into GSH, and GPX1 uses GSH to neutralize harmful peroxides and ROM (Lu, 2013). The cytosolic enzyme ME1 catalyzes the oxidative decarboxylation of malate to convert NADP^+ to NADPH (Ratledge, 2014). Concentration of GSH in periparturient liver is increased in response to enhanced methionine supply (Osorio et al., 2014b; Zhou et al., 2017; Batistel et al., 2018). In bovine mammary cells under thermal stress, methionine supply significantly increased GPX enzymatic activity (Han et al., 2015). Thus, the upregulation of GSH metabolism genes (*GCLC*, *GCLM*, *GSR*, *GPX1*, and *ME1*) in mammary tissue of methionine cows suggests that this AA plays a role in attenuating oxidative stress by regulating GSH metabolism in mammary cells.

The reaction of hydrogen peroxide (Fenton reaction) to produce ROM can lead to damage of cellular molecules with important biologic roles (proteins, lipids, and DNA). Considering the potential harmful effects

of high concentrations of free iron, the cells transport, acquire, and store it by using a dedicated group of proteins (Jansova et al., 2014) including ferritin (FTH1; an iron storage protein) and ferrochelatase (FECH; converts Fe^{2+} to heme; Gozzelino and Soares, 2014). In human endothelial cells, methionine induces an increase in FTH and suppresses the formation of free radicals (Erdmann et al., 2005). Thus, we speculate that the observed upregulation of *FTH1* and *FECH* abundance are indicative of a protective effect of methionine supply from iron-catalyzed oxidative damage through the increase of free iron binding and storage.

The cytoplasmic enzyme NQO1 is important for the reduction of reactive quinones and scavenging of superoxide (Dietz et al., 2013). In bovine mammary epithelial cells, the antioxidant effect of resveratrol was due to its free radical scavenging activity assessed though an increase in the mRNA abundance of *NQO1* (Jin et al., 2016a). Thus, the observed upregulation of NQO1 abundance in response to enhanced methionine supply is suggestive that the antioxidant potential of methionine is partly related to an increase in the cellular capacity to reduce oxidative substances and scavenge free radicals.

NFE2L2 Regulated Pathways

The transcription factor NFE2L2 belongs to the leucine zipper-type family and binds to antioxidant response elements located in the regulatory regions of many antioxidant genes (Suzuki and Yamamoto, 2015). The current model of NFE2L2 activation takes into account the negative repressor complex formed by KEAP1 and CUL3 (Canning et al., 2015). Alternate mechanisms of NFE2L2 regulation include phosphorylation of NFE2L2 by kinases and interaction with other proteins such as NFKB1 and RXRA. The NFE2L2 is activated under several conditions including oxidative stress and specific oxidant compounds. For instance, NFE2L2 activity is stimulated by inflammation (Bryan et al., 2013) and the production of ROM stimulates the release of NFE2L2 from KEAP1, leading to nuclear translocation and activation of the antioxidant response elements in antioxidant target genes (Ho et al., 2005). In addition, antioxidants such as carotenoids and taurine chloramine also can stimulate the activation of the NFE2L2 pathway (Kim et al., 2010; Inoue et al., 2017). A recent study in nonruminants reported that methionine supplementation downregulated *KEAP1* mRNA abundance and increased *NFE2L2*, resulting in improved immune function (Pan et al., 2016). Thus, the upregulation of *NFE2L2* and downregulation of *KEAP1* abundance in cows with greater methionine

supply suggests that it may regulate transcription of the *KEAP1/NFE2L2* pathway in mammary tissue.

Mitogen-activated protein kinases (MAPK) are a family of highly related kinases that are composed of extracellular signal-regulated protein kinases, c-jun N-terminal kinases, and p38 kinases. Although, to our knowledge, no data are available on the effect of methionine on the phosphorylation of NFE2L2 protein, the markedly greater ratio of p-NFE2L2/NFE2L2 and upregulation of *MAPK14* abundance with methionine suggests a potential role for methionine in the control of NFE2L2 phosphorylation status in mammary tissue. Thus, methionine could potentially exert a posttranslational level of control on phosphorylation of total NFE2L2 protein. Serine phosphorylation of NFE2L2 can occur via MAPK (Sun et al., 2009), PKC (Bloom and Jaiswal, 2003), and ATM serine/threonine kinase (Ma et al., 2017). It is unclear if these kinases regulate NFE2L2 through direct or indirect phosphorylation, but phosphorylation of NFE2L2 in response to oxidative stress promotes its translocation into the nucleus and upregulates transcription of target genes (Huang et al., 2000, 2015). Thus, a central mechanism for the antioxidant potential of methionine in mammary tissue during the periparturient period could occur via phosphorylated NFE2L2 and the upregulation of downstream antioxidant gene abundance.

The regulation of transcription of *NFKB* and *NFE2L2* is affected by redox balance, including protein-protein interactions that control the transcription of downstream target genes (Wakabayashi et al., 2010). In a rat hepatocarcinogenesis model, greater concentration of S-adenosylmethionine abolished the activation of NFKB1 (Garcia-Roman et al., 2008). Thus, the reason for the upregulation of *NFKB1* mRNA abundance with enhanced methionine supply is not readily apparent. It could be possible that differences in the flux through the 1-carbon cycle between organs and mammalian species dictates how much S-adenosylmethionine might be available for methylation reactions versus S-adenosylhomocysteine and homocysteine synthesis followed by transsulfuration. In addition, considering that the interaction between NFE2L2 and the NFKB1 pathway operate mainly through posttranscriptional mechanisms (Wardyn et al., 2015), it is possible that changes in *NFKB1* abundance do not elicit functional outcomes.

CONCLUSIONS

Enhancing the supply of methionine during the periparturient period led to greater phosphorylation of the antioxidant transcription regulator NFE2L2 along

with a reduction in protein abundance of its negative regulator KEAP1 in mammary tissue. As a result, the abundance of the antioxidant protein HMOX1 and various target genes (*GPX1*, *GCLC*, *GCLM*, *GSR*, *ME1*, *FTH1*, and *FECH*) under the control of NFE2L2 was upregulated. Although further studies with greater number of cows and additional measurements of lipid peroxidation products at other time points are needed for a better mechanistic understanding, the data suggest the existence of posttranslational and transcriptional mechanisms in mammary tissue during early lactation that likely modulate the antioxidant effect of methionine.

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