Phosphorylation of nuclear factor erythroid 2-like 2 (NFE2L2) in mammary tissue of Holstein cows during the periparturient period is associated with mRNA abundance of antioxidant gene networks

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ABSTRACT

Changes in the production of reactive oxygen species in the mammary gland of dairy cows during the periparturient period could lead to oxidative stress and potentially impair mammary function. Phosphorylation of the transcription factor nuclear factor erythroid 2-like 2 (*NFE2L2*), also known as nuclear factor-E2-related factor 2, controls mRNA abundance of genes encoding antioxidant proteins and enzymes. The hypothesis was that NFE2L2 phosphorylation status and target gene mRNA abundance in the mammary gland of dairy cows is altered around parturition. Total NFE2L2 protein, phosphorylated protein (p-NFE2L2), and ratio of p-NFE2L2 to NFE2L2 along with mRNA abundance of 24 genes related to the *NFE2L2* signaling pathway, apoptosis, and cell proliferation were measured in mammary tissue samples from Holstein cows at –30, 1, 15, and 30 d relative to parturition. Although total NFE2L2 protein abundance did not differ, p-NFE2L2 and p-NFE2L2-to-NFE2L2 ratio were greater after parturition. The upregulation of DNA damage inducible transcript 3 (*DDIT3*) postpartum indicated a localized oxidative stress state. Among genes evaluated, thioredoxin (*TXN*), glutathione peroxidase 1 (*GPX1*), and glutathione S-transferase mu 1 (*GSTM1*) had the highest (37.1, 15.1, and 4.8% of total mRNA measured, respectively) abundance. The mRNA abundance of various target genes with detoxifying enzymatic functions and free radical scavenging activities [glutamate-cysteine ligase catalytic subunit (*GCLC*); glutathione reductase (*GSR*); ferrochelatase (*FECH*); *TXN*; thioredoxin reductase 1 (*TXNRD1*);

and NAD(P)H quinone dehydrogenase 1 (*NQO1*)] were consistently upregulated (linear effect of time) as parturition approached and lactation began. Among the transcription regulators, *NFE2L2* had the highest mRNA abundance (7.3% of total mRNA measured). Abundance of *NFE2L2* and other transcription factors [nuclear factor kappa B subunit 1 (*NFKB1*), retinoid X receptor α (*RXRA*), and mitogen-activated protein kinase 14 (*MAPK14*)] were upregulated (linear effect of time) from –30 d to 30 d relative to parturition. Overall, NFE2L2 phosphorylation and downstream signaling leading to postpartal upregulation of genes associated with oxidative stress and inflammation in the mammary gland seem to be key components of normal cellular function to maintain proper redox homeostasis. However, if the longitudinal increases in mRNA and protein abundance of these antioxidant mechanisms are a reflection of cellular oxidative stress, then the likelihood of protein and DNA damage would be greater and might be one factor compromising cell viability and potentially lactation persistency. The actual cues coordinating these molecular responses remain to be determined.

Key words: oxidative stress, lactation, reactive oxygen metabolites

INTRODUCTION

Dairy cows experience extreme adaptations in cellular metabolism during the periparturient or transition period as the mammary gland prepares for lactation (Sordillo and Aitken, 2009; Sordillo et al., 2009). At the onset of milk synthesis, tissues such as the liver and mammary gland more than double oxidative metabolism, often leading to increased rates of reactive oxygen species (ROS) production (Aitken et al., 2009). Hence, an imbalance between ROS and antioxidant capacity (Valko et al., 2007) can lead to oxidative stress and onset of pathological conditions associated with

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reproduction, production, and welfare (Lykkesfeldt and Svendsen, 2007).

Evidence from in vitro studies indicates that in lactating mammary epithelial cells metabolic rate is associated with ROS production (Jin et al., 2014), and intracellular antioxidant systems that are controlled via transcriptional mechanisms, such as glutathione metabolism, the thioredoxin system, and heme and Fe metabolism systems, help control redox state. In fact, various types of stressors seem to trigger cellular mechanisms to prevent oxidative stress. For instance, intramammary mastitis challenge at midlactation altered abundance of nuclear factor erythroid 2-like 2 (*NFE2L2*)-mediated oxidative stress genes (Moyes et al., 2010). Nutritional management also seems to elicit a response by the *NFE2L2* pathway, as demonstrated by reductions in oxidative stress indicators in plasma and upregulation of *NFE2L2* mRNA abundance in mammary tissue when cows were fed flax meal (Schogor et al., 2013). Jin et al. (2016b) reported that upregulation of *NFE2L2* target genes attenuated heat stress-induced cell damage in bovine mammary epithelial cells. Together, these data suggest that *NFE2L2* is a key transcription factor for cellular resistance to oxidative stress in the bovine mammary gland. To our knowledge, changes in total protein abundance and phosphorylation status of *NFE2L2*, along with its target genes in bovine mammary tissue during the periparturient period, are unknown.

The general hypothesis was that *NFE2L2* phosphorylation status and mRNA abundance of target genes associated with responses to cellular stress in the mammary gland of dairy cows is altered around parturition. Those data would be indicative of a localized response of the mammary gland to changes in mammary function as a result of lactation, and may be related to protective mechanisms against oxidative damage. To address this hypothesis, we profiled the total protein and phosphorylated protein abundance of *NFE2L2* along with mRNA abundance of 24 target genes in mammary tissue harvested at -30, 1, 15, and 30 d relative to parturition.

MATERIALS AND METHODS

Animals and Biopsies

All procedures involving animals were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee. Eight multiparous Holstein dairy cows were used for percutaneous biopsies of the right or left rear quarter of the mammary gland at -30 (± 3 d), 1, 15, and 30 d relative to parturition according to previous procedures

and with some modifications (Bionaz and Loor, 2007). All biopsies were harvested at approximately 0700 h (after the morning milking). The midsection of the rear quarters were chosen for biopsy, with each subsequent biopsy located approximately 5 cm above or below from the original incision site. Before the procedure, cows were given a general anesthetic (0.025 mg/kg of xylazine, 20 mg/mL of solution); before performing the incision, 5 mL of lidocaine HCl (2% solution) were given subcutaneously. A 3-cm incision was made through the skin and subcutaneous tissue followed by blunt dissection of connective tissue surrounding the mammary capsule before puncture of the mammary parenchyma. Only after the pink mammary capsule was clearly visible were biopsies performed by first ensuring that the tip of the biopsy needle could go through the dissected connective tissue. Approximately 50 to 100 mg of tissue was removed after 4 punctures (per institutional guidelines) of 5 to 10 cm in depth using a biopsy needle (Bard Magnum; 12 gauge $\times$ 16 cm; C. R. Bard, Inc., Murray Hill, NJ). Tissue was blotted with gauze to remove excess blood and any connective tissue. Pressure was then applied to the wound until visual signs of bleeding were absent (~ 5 min postbiopsy). The skin incision was closed with 3 Michel clips (11 mm; Henry Schein, Melville, NY). The incision site was sprayed with topical antiseptic (10% Povidone Iodine Ointment; Taro Pharmaceuticals, Hawthorne, NY). Health was monitored after surgery by recording milk yield, rectal temperature, and feed intake daily for 7 d. Surgical clips were removed 7 d postbiopsy. Cows did not experience clinical signs of disease during the study.

RNA Isolation, cDNA Synthesis, and PCR

These procedures have been thoroughly described by Bionaz and Loor (2008, 2011). Briefly, RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). All samples had an RNA integrity number greater than 7.3. Total RNA was reverse-transcribed using the SuperScript III First Strand Synthesis kit (Thermo Fisher Scientific, Waltham MA). Real-time reverse-transcription PCR (quantitative; **qPCR**) analysis was performed using SYBR Green Fast Mix ROX (Quanta Biosciences, Gaithersburg, MD) with a 6-point standard curve. 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 with key roles in the antioxidant mechanisms evaluated (Supplemental Table S1; <https://doi.org/10.3186/jds.2017-14257>). This included the cell apoptosis regulator *BCL2*, the glutathione metabolism system (*GCLC*, *GCLM*, *GPX1*, *GSR*, *GSTM1*, *ME1* and *TALDO1*), the thioredoxin enzyme

system (*TXNRD1*, *TXN*), the heme and Fe metabolism system (*HMOX1*, *FECH*, *FTH1*, *PIR*), and NAD (P) H quinone dehydrogenase 1 gene (*NQO1*), KEAP-dependent regulators (*NFE2L2*, *KEAP1*, *CUL3*) and KEAP-independent regulators (*NFKB1*, *MAPK8*, *MAPK12*, *MAPK14*, *RXRA*). Gene symbols, primer features, and qPCR performance are reported in Supplemental Table S2 (<https://doi.org/10.3186/jds.2017-14257>).

Relative mRNA Abundance of Transcripts

These procedures have been described in detail elsewhere (Bionaz and Loor, 2007, 2008, 2011). Briefly, RT-PCR was performed using 4 μ L of diluted cDNA combined with 6 μ L of a mixture composed of 5 μ L of 1 SYBR Green master mix (Quanta Biosciences), 0.4 μ L each of 10 μ M forward and reverse primers, and 0.2 μ L of DNase/RNase free water in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Foster City, CA). Each sample was run in triplicate and a 6-point standard curve (a pool from cDNA of all samples) plus the nontemplate control were used. The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems). The mRNA abundance for each gene was calculated relative to the 6-point standard curve with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems). The overall percentage mRNA abundance for each gene among all those measured was calculated using the inverse of PCR efficiency (E) raised to Δ Ct [gene abundance 1/E Median Δ Ct, where the median of Δ Ct is calculated as (cycle threshold of the target gene – geometrical mean cycle threshold of 3 internal controls) for each time point]. The efficiency of PCR amplification for each gene was calculated using the standard curve method [$E = 10^{(-1/\text{standard curve slope})}$] as described in various manuscripts from our laboratory (Bionaz and Loor, 2007, 2008, 2011). The final data were normalized using the geometric mean of *UXT*, *RPS9*, and *RPS15* (Bionaz and Loor, 2007).

Western Blot Analysis

Total protein was extracted from mammary gland tissue using T-PER tissue protein extraction reagent (catalog number: 78510; Thermo Fisher Scientific) including a protease and phosphatase inhibitor cocktail (catalog number 78440; Thermo Fisher Scientific). The concentrations of total protein was determined with the Pierce BCA protein assay kit (catalog number 23227; Thermo Fisher Scientific). After protein samples were boiled at 100°C for 10 min, 50 μ g of protein was loaded into wells of a 10% SDS-PAGE gel and run at 120 V for 120 min. After activating a polyvinylidene fluoride

membrane (catalog number 1620261; Bio-Rad, Hercules, CA) with methanol for 1 min, the protein sample was transferred to the membrane using a semidry transfer assembly (Bio-Rad). Membranes were blocked for 2 h at room temperature using TBST (50 mM Tris, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) containing 5% (wt/vol) nonfat milk. The membranes were then washed 5 times and incubated with TBST containing antibodies to NFE2L2 (catalog number 137550; Abcam, Cambridge, UK) and Phospho-NFE2L2 (catalog number PA5-67520; Invitrogen, Carlsbad, CA) with gentle agitation at 4°C overnight. Subsequently, the membranes were washed 5 times with TBST, 5 min each time at room temperature. After incubated with horseradish peroxidase-conjugated secondary antibodies (catalog number ab6721; Abcam) at room temperature for 1 h, anti-GAPDH antibody (catalog number ab22555; Abcam) was used as internal control. The membranes were incubated with Clarity ECL Western blotting substrate (catalog number 170-5060; Bio-Rad) and images were captured using a ChemiDOC MP system (Bio-Rad). The intensity of the bands were measured using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD).

Statistical Analysis

Normalized qPCR data for *DDIT3*, *GCLC*, and *KEAP1* had to be log-2 transformed before statistical analysis because the Shapiro-Wilk test *P*-value was <0.05. Subsequently, statistical analysis on all mRNA data was performed using repeated measures ANOVA with the MIXED procedure of SAS 9.3 (SAS Institute Inc., Cary, NC) to evaluate the effect of time relative to parturition. The model included the fixed effect of time (–30, 1, 15, and 30 d) and the random effect of cow. Orthogonal polynomial contrasts were used to evaluate linear and quadratic effects of time on protein and mRNA abundance. Significance was declared at a corrected $P \leq 0.05$. Data for *DDIT3*, *GCLC*, and *KEAP1* in Figures are the back-transformed least squares means.

RESULTS

Total NFE2L2 Protein Abundance and Phosphorylation Status

Although total protein abundance did not differ ($P = 0.18$) at 30 d postpartum relative to –30 d (Figure 1), the abundance of phosphorylated (p) NFE2L2 tended ($P = 0.09$) to increase and the p-NFE2L2-to-total NFE2L2 ratio increased ($P = 0.05$) after calving.

Table 1. Relative percentage mRNA abundance of target genes measured

Gene name	Symbol ¹	RNA ² (%)
DNA damage inducible transcript 3	<i>DDIT3</i>	<0.01
BCL2 apoptosis regulator	<i>BCL2</i>	1.09
Glutathione reductase	<i>GSR</i>	0.91
Glutamate-cysteine ligase catalytic subunit	<i>GCLC</i>	1.38
Glutathione peroxidase 1	<i>GPX1</i>	15.1
Glutamate-cysteine ligase modifier subunit	<i>GCLM</i>	1.43
Glutathione S-transferase M1	<i>GSTM1</i>	4.78
Malic enzyme 1	<i>ME1</i>	0.73
Transaldolase 1	<i>TALDO1</i>	0.38
Thioredoxin	<i>TXN</i>	37.0
Thioredoxin reductase 1	<i>TXNRD1</i>	3.36
Ferrochelatase	<i>FECH</i>	0.72
Heme oxygenase 1	<i>HMOX1</i>	3.99
Ferritin heavy chain 1	<i>FTH1</i>	4.01
Pirin (iron-binding nuclear protein)	<i>PIR</i>	1.23
NAD(P)H quinone dehydrogenase 1	<i>NQO1</i>	0.47
Nuclear factor, erythroid 2-like 2	<i>NFE2L2</i>	7.32
Kelch-like ECH-associated protein 1	<i>KEAP1</i>	3.06
Cullin 3	<i>CUL3</i>	4.26
Nuclear factor kappa B subunit 1	<i>NFKB1</i>	3.25
Mitogen-activated protein kinase 12	<i>MAPK12</i>	0.72
Mitogen-activated protein kinase 14	<i>MAPK14</i>	1.34
Mitogen-activated protein kinase 8	<i>MAPK8</i>	1.10
Retinoid X receptor α	<i>RXRA</i>	2.19

¹From National Center for Biotechnology Information (NCBI; <https://www.ncbi.nlm.nih.gov/>).

²Calculated as $[(1/E\Delta Ct) \text{ specific gene} / \sum (1/E\Delta Ct) \text{ all genes}] \times 100$, where E = efficiency and ΔCt = median of Δ cycle threshold (calculated as cycle threshold of the target gene minus geometrical mean cycle threshold of 3 internal controls).

Oxidative Stress and NFE2L2 Target Gene Abundance

Among the measured *NFE2L2* target genes, *TXN*, *GPX1*, and *GSTM1* had the highest percentage mRNA abundance (Table 1). Compared with -30 d, abundance of *GPX1* and *GSTM1* increased at 30 d (linear and quadratic effect, $P < 0.05$; Figure 2). Abundance of the thioredoxin enzyme system (*TXN* and *TXNRD1*) increased gradually during early lactation with a linear pattern ($P < 0.05$, Figure 2). The antioxidant enzyme genes measured in the glutathione metabolism system (*GSR*, *GCLC*, *ME1*, and *TALDO1*) decreased at 1 d and then peaked in mRNA abundance at 15 or 30 d postpartum (linear effect, $P < 0.05$, Figure 2).

In the heme and Fe metabolism system, percentage mRNA abundance of *FECH* and *PIR* was low ($<1.5\%$ of total mRNA; Table 1), but both genes experienced a linear increase in abundance after parturition compared with -30 d ($P < 0.05$, Figure 3). The abundance of *HMOX1* and *FTH1* was not affected over time ($P > 0.09$; Figure 3).

Although not among the most abundant genes on a percentage basis, the abundance over time of *DDIT3* increased linearly from -30 to 30 d relative to parturition ($P < 0.01$, Figure 4). Compared with -30 d, abundance of *BCL2* increased at 1 d and peaked at 30 d postpartum (linear and quadratic effect, $P < 0.05$);

this gene accounted for $\sim 1\%$ of total mRNA abundance measured (Figure 4). Compared with -30 d, *NQO1* abundance was downregulated (linear effect, $P < 0.05$)

Figure 1. Abundance of total NFE2L2, phosphorylated NFE2L2 (p-NFE2L2), and the ratio of p-NFE2L2 to total NFE2L2 protein in mammary gland tissue of multiparous Holstein dairy cows during the periparturient period. Different lowercase letters (a,b) indicate means differ ($P \leq 0.05$); different uppercase letters (A,B) indicate means tend to differ ($P = 0.09$). Error bars represent the SEM.

Figure 2. Abundance of mRNA for genes associated with glutathione metabolism (*GSR*, *GCLC*, *GPX1*, *GCLM*, *GSTM1*, *ME1*, and *TALDO1*) and the thioredoxin pathway (*TXN*, *TXNRD1*). *GSR* = glutathione reductase; *GCLC* = glutamate-cysteine ligase catalytic subunit; *GPX1* = glutathione peroxidase; *GCLM* = glutamate-cysteine ligase modifier subunit; *GSTM1* = glutathione S-transferase M1; *ME1* = malic enzyme 1; *TALDO1* = transaldolase 1; *TXN* = thioredoxin; *TXNRD1* = thioredoxin reductase 1. Different letters (a–c) indicate means differ ($P \leq 0.05$). Error bars represent the SEM.

at 1 d and then upregulated at 15 d before reaching peak expression at 30 d (Figure 4).

NFE2L2 Regulator Genes

Among the measured regulators of NFE2L2, KEAP-dependent regulators (*NFE2L2*, *CUL3*, and *KEAP1*) had the highest mRNA abundance (Table 1), accounting for 4 to 7% of total mRNA abundance measured. The mRNA abundance of *NFE2L2* was lowest at 1 d and then reached highest abundance at 30 d (linear effect, $P < 0.05$; Figure 5). The abundance of *KEAP1* decreased at 1 d postpartum, and then had a linear increase at 15 and 30 d, where it peaked relative to –30 d (Figure 5). The abundance of *NFKB1*, *MAPK14*, and *RXRA* was greater at 30 compared with –30 d (linear effect of time, $P < 0.05$; Figure 5). No changes in abun-

dance of *MAPK8* and *CUL3* around parturition were observed ($P > 0.10$, Figure 5).

DISCUSSION

Although multiple biopsies of the same tissue could potentially confound the interpretation of mRNA and protein abundance data, it is unlikely that the procedure used in the present study had an effect on the biologic processes of interest. First, biopsies alternated between the rear quarters such that the same quarter was not biopsied for a period of weeks. Second, a different location was biopsied each time, ensuring that scar tissue was avoided. Lastly, in previous work from our group performing similar experiments (Bionaz and Loo, 2012), at least from the metabolic standpoint, we have been able to capture the expected temporal

Figure 3. Abundance of mRNA for genes associated with heme and iron metabolism. *FECH* = ferrochelatase; *HMOX1* = heme oxygenase 1; *FTH1* = ferritin heavy chain 1; *PIR* = pirin (iron-binding nuclear protein). Different letters (a,b) indicate means differ ($P \leq 0.05$). Error bars represent the SEM.

Figure 4. Abundance of mRNA for genes associated with apoptosis (*DDIT3*), cell proliferation (*BCL2*), and electron reduction (*NQO1*). *DDIT3* = DNA damage inducible transcript 3; *BCL2* = BCL2 apoptosis regulator; *NQO1* = NAD(P)H quinone dehydrogenase 1. Different letters (a,b) indicate means differ ($P \leq 0.05$). Error bars represent the SEM.

biologic response of the mammary gland (e.g., increase in lipogenesis; Mellenberger et al., 1973). The fact that repeated biopsies of liver during a 48-h period in lactating Holstein cows had minimal effects on mRNA abundance and systemic indicators of inflammation and stress also support the applicability of this type of procedure (Vels et al., 2009).

The onset of lactation is characterized not only by metabolic and endocrine changes, but also by changes in immune and oxidative stress status (Everts et al., 2005; Loor et al., 2013). These multifaceted adaptations are partly driven by the increased demands for nutrients in the mammary gland that are needed to support milk component synthesis. The sudden increase in metabolic activity of tissues at the onset of lactation is a well-coordinated process that ensures proper nutrient supply to mammary gland, but indirectly results in the production of ROS (Sordillo and Aitken,

2009; Sordillo et al., 2009). In fact, systemic biomarker analysis has demonstrated that dairy cows experience oxidative stress throughout the transition period, and particularly after parturition (Berton et al., 2008). When produced in excess or in a chronic fashion and without proper antioxidant mechanisms, ROS can lead to oxidative stress and harm cells that could result in loss of function and tissue damage (e.g., cell apoptosis; Young and Woodside, 2001). Because the abundance of *DDIT3* mRNA and cellular apoptosis is increased by hydrogen peroxide (Ariyama et al., 2008) and ROS-induced oxidative stress (Feng et al., 2014), the gradual increase in *DDIT3* expression after calving in the present study suggests some degree of oxidative stress in mammary gland. Alternatively, the profile of *DDIT3* may represent a normal response in cellular apoptosis, which in a previous study was reported to be highest at 14 d postpartum (Capuco et al., 2001).

Figure 5. Abundance of mRNA for genes associated with redox-sensitive regulation of nuclear factor, erythroid 2-like 2 (*NFE2L2*) action. Kelch-like ECH associated protein 1 (*KEAP1*)-dependent regulators: *NFE2L2*, *KEAP1*; *CUL3* = cullin 3. *KEAP1*-independent regulators: *MAPK8* = mitogen-activated protein kinase 8; *MAPK12* = mitogen-activated protein kinase 12; *MAPK14* = mitogen-activated protein kinase 14; *RXRA* = retinoid X receptor α . Different letters (a-c) indicate means differ ($P \leq 0.05$). Error bars represent the SEM.

KEAP-Dependent NFE2L2 Regulators

Under oxidative stress conditions, at least in nonruminants, the active form of NFE2L2 translocates to the nucleus, where it can upregulate its target genes by binding to the antioxidant response element, ultimately triggering molecular events that help reduce ROS levels (Menegon et al., 2016). The stability of NFE2L2 protein is primarily regulated by Kelch-like ECH-associated protein 1 (**KEAP1**), which forms a homodimer to inactivate NFE2L2 in the cytosol. In addition, KEAP1 facilitates CUL3-dependent NFE2L2 ubiquitination, leading to its proteasomal degradation (Hine and Mitchell, 2012). Although, to our knowledge, *in vivo* data in bovine mammary tissue are not available, research with bovine embryos underscored the importance of *KEAP1* and *NFE2L2* interactions in the context of controlling endogenous antioxidant mechanisms during oxidative stress conditions (Amin et al., 2014). If such a mechanism also functions in mammary tissue, it would partly explain the similar pattern of increase in postpartum abundance of *NFE2L2* and *KEAP1*.

Previous research with bovine mammary tissue or mammary cells has reported changes in mRNA abundance of NFE2L2 in response to LPS or has associated its expression with oxidative status. For instance, greater *NFE2L2* mRNA abundance in mammary tissue was accompanied with improved oxidative status in midlactation Holstein cows (Schogor et al., 2013). In bovine mammary cells, LPS downregulated the abundance of *NFE2L2* mRNA (Yu et al., 2017). A cytoprotective effect of *NFE2L2* upregulation in MAC-T cells during heat shock when incubating with the antioxidant tert-butylhydroquinone was due in part to upregulation of *NFE2L2* target genes (Jin et al., 2016b). Therefore, the finding that KEAP-dependent regulators had the third highest mRNA abundance among the genes evaluated underscores their importance in bovine mammary tissue around parturition. This idea is further substantiated by the increase in the ratio of p-NFE2L2 to total NFE2L2 after calving and the fact that the profile of *NFE2L2* mRNA abundance was similar to that of genes related to antioxidant enzymes.

In nonruminants, several studies have pointed toward stress-induced phosphorylation of NFE2L2 and its nuclear translocation followed by activation of gene expression (Bloom and Jaiswal, 2003; Jaiswal, 2004; Huang et al., 2015). In the present study, the increased ratio of p-NFE2L2 to total NFE2L2 and target gene abundance (e.g., *GSR*, *GCLC*, *NQO1*) after calving suggest that NFE2L2, as a transcription factor, plays a key role in the antioxidant mechanisms of the mammary cell during a period where the animal may experience

a chronic state of excessive ROS production. These mechanisms might be one of the physiologic means to prevent tissue damage as a result of ROS production.

KEAP-Independent Regulators

Although the regulation of *NFE2L2* is mainly via KEAP, a body of evidence exists suggesting that NFE2L2 can be positively or negatively regulated by other proteins (Bryan et al., 2013), such as the inflammation-related transcription factor nuclear factor- κ B (**NFKB**) and retinoic X receptor α (**RXRA**). For instance, an antagonistic relationship exists between the NFE2L2 and NFKB pathway in nonruminant cells (Cuadrado et al., 2014), leading to changes in mRNA abundance of their respective target genes (Wakabayashi et al., 2010). Although no data are available for mammary tissue, midlactation cows undergoing inflammation as a result of feeding a high-grain diet had lower *NFE2L2* mRNA abundance and greater *NFKB* abundance in liver tissue (Abaker et al., 2017). Retinoic X receptor α , a nuclear retinoic acid receptor, regulates nuclear hormone signaling through forming a heterodimer with other nuclear receptors (Xu et al., 1999) and was recently shown to inhibit NFE2L2 (Wu et al., 2014) activity through a protein-protein interaction (Wang et al., 2013). Therefore, considering the upregulation in postpartal abundance of *NFKB1* and *RXRA* along with their relatively high percentage mRNA in the present study, it seems plausible that these transcription regulators exert some degree of control (i.e., as co-repressors) on *NFE2L2* and its target genes. However, the marked increase in p-NFE2L2 abundance after parturition seems to underscore its activation as a dominant mechanism during a time when mammary metabolism is at its peak.

The first signaling pathway known to influence NFE2L2 activity was the mitogen-activated protein kinase (**MAPK**) cascade (Kwak et al., 2004). In fact, the NFE2L2 protein contains several threonine, tyrosine, and serine residues, which can be phosphorylated by various kinases. The MAPK pathway, including extracellular signal-regulated kinase (**ERK**), c-Jun N-terminal kinase (**JNK**), and p38 kinase, could all regulate NFE2L2 (Sun et al., 2009). In general, at least in nonruminants, the activation of c-Jun N-terminal kinase and ERK activates NFE2L2, whereas p38 is inhibitory (Huang et al., 2015). Using cultured bovine mammary cells (MAC-T), Jin et al. (2016a) reported that inhibiting p38 kinase enhanced the expression of *NFE2L2* and several antioxidant genes. In addition, ERK activation was associated with *NFE2L2*-mediated activation of antioxidant enzymes (Zipper and Mulcahy, 2003). Similar to *NFKB1* and *RXRA*, the gradual

increase in mRNA abundance of *MAPK12* (ERK) and *MAPK14* (p38) likely underscores their participation in a regulatory mechanism that helps control NFE2L2 activity.

NFE2L2 Target Genes

Regulation of cell death by inducing or inhibiting apoptosis is one of the main roles of *BCL2* (Siddiqui et al., 2015), and it has been demonstrated that its promoter region contains an antioxidant response element to which *NFE2L2* can bind and activate (Niture and Jaiswal, 2012). In fact, NFE2L2-mediated upregulation of *BCL2* enhanced cell survival and drug resistance in cancer cells (Niture and Jaiswal, 2012). Work in murine mammary gland also indicated that *BCL2* can inhibit apoptosis (Metcalf et al., 1999) and extend lactation by promoting survival of mammary epithelial cells (Schorr et al., 1999). In bovine mammary gland tissue, an increase in the proliferation-to-apoptotic cell ratio was associated with a sustained expression of anti-apoptotic *BCL2* (Colitti et al., 2004). Therefore, the gradual upregulation of *BCL2* postpartum that was detected suggests a role for *BCL2* in modulating mammary epithelial cell survival during the transition period.

The first enzyme of GSH biosynthesis that catalyzes the reaction between glutamate and cysteine to form γ -glutamyl-L-cysteine is glutamate cysteine ligase (*GCL*), which is composed of catalytic (*GCLC*) and modifier (*GCLM*) subunits (Lu, 2013). Glutathione exists in its reduced or oxidized form (i.e., GSH or GSSG, respectively), with reduced GSH playing important functions including antioxidant defense, detoxification, and maintenance of thiol status, which could scavenge ROS (Lu, 2009). Our previous work demonstrated a reduction in liver and plasma GSH concentration from prepartum to early postpartum, followed by a gradual increase through 30 d postpartum (Zhou et al., 2017; Batistel et al., 2018). Pathological conditions are known to affect GSH concentration in milk, and this compound is considered an oxidative stress biomarker (Talukder et al., 2015; Sadek et al., 2017).

At least in nonruminants, oxidative stress is associated with increased concentration of cellular GSH and upregulation of *GCL* mRNA abundance (Yamane et al., 1998; Huang et al., 2001). Therefore, the postpartal upregulation of *GCLC* detected in the present study underscores the importance of this subunit during early lactation and within the context of GSH biosynthesis. Alternately, the nadir in mRNA abundance of *GCLM* at 1 d postpartum could be related to an acute oxidative stress status shortly after calving.

In the present study, *GPX1* and *GSTM1* were the second and fourth most abundant transcripts (15 and 4.7%, respectively) among all genes evaluated, suggesting a major role in glutathione metabolism. The up-regulation of *GPX1* mRNA abundance after parturition is consistent with a previous study (Aitken et al., 2009) and underscores the importance of the GPX enzyme system and its use of GSH to neutralize harmful peroxides and ROS. Another important component of the GSH system is the enzyme *GSTM1*, which catalyzes the conjugation of GSH to xenobiotic substrates for the purpose of detoxification (Lu, 2013). In bovine mammary cells, CLA isomers increased GPX and *GSTM1* activity, enhancing cell resistance against H₂O₂-induced oxidative stress (Basiricò et al., 2015). Therefore, the upregulated abundance of *GPX1* and *GSTM1* after parturition suggests a gradual increase of free radical scavenging and detoxification capacity in the mammary gland postpartum and seems to underscore the importance of NFE2L2 phosphorylation status in the control of this process.

Oxidative stress depends not only on the production of ROS, but also on the level of NADPH and GSH during electron transport in organelles (Perl et al., 2011). The conversion of NADP⁺ to NADPH during the oxidative decarboxylation of (S)-malate to pyruvate via malic enzyme 1 (*ME1*) and the activity of transaldolase 1 (*TALDO1*) link glycolysis, pentose phosphate, and citric acid pathways in mammary cells (Gumaa et al., 1973). In *NFE2L2*-KO mice, *ME1* was significantly downregulated and was identified as an *NFE2L2*-regulated gene (Chartoumpekis et al., 2013); *TALDO1*-deficient liver is characterized by NADPH and GSH depletion along with oxidative stress (Hanczko et al., 2009). Although in ruminant mammary glands the major source of NADPH is the isocitrate dehydrogenase pathway, in nonruminants *ME1* is a major source of NADPH (Gumaa et al., 1973). In addition to *ME1*, *TALDO1* activity as part of the pentose phosphate pathway and helps generate NADPH and ribose-5-phosphate for nucleic acid synthesis in mammary cells (Gumaa et al., 1973). Therefore, *ME1* and *TALDO1* may not be directly involved in the removal of ROS, but rather through the metabolism of NADPH and GSH through their active participation in the antioxidant mechanism of glutathione. Overall, the up-regulation in abundance of the glutathione metabolism enzymes suggest this system may play a key role in the control of oxidative stress in mammary cells.

The activity of thioredoxin reductase1 (*TXNRD1*) and availability of thioredoxin (*TXN*) constitute a powerful oxidoreductase system with important antioxidant effects (Nordberg and Arner, 2001). The thio-

redoxin system reduces disulfide bridges of proteins and removes peroxides or H_2O_2 by using NADPH as an electron donor (Lu and Holmgren, 2014). The TXNRD1 is the only enzyme that catalyzes the transfer of electrons to the active site of TXN, which continues to reduce protein disulfide bridges or other substrates (Du et al., 2013). The importance of this gene or protein in bovine mammary tissue clearly is underscored by the fact it had the greatest mRNA abundance (37% of all mRNA measured) among all genes evaluated. The gradual increase in *TXNRD1* abundance over time suggested this was a sustained mechanism potentially dedicated to removing ROS via TXN. Our data are in agreement with a previous study reporting that TXNRD1 activity in blood is an important antioxidant defense mechanism during the transition period in dairy cows (Gong and Xiao, 2016).

Hemoxygenase-1 catalyzes the breakdown of heme to carbon monoxide, biliverdin, and iron (Wegiel et al., 2014). When Fe is rapidly lost from the hemoglobin porphyrin ring, free heme can cause oxidative damage and contribute to the ferrous state producing ROS. Free iron is stored by ferritin1, whereas ferrochelatase catalyzes the biosynthesis of heme (Kim et al., 2014). Pirin is an Fe(II)-binding nuclear protein that belongs to the redox-sensitive cupin superfamily. The upregulation in abundance of *FECH* after calving is in agreement with previous data in dairy cows (Wang et al., 2015). Free iron is toxic to cells, as it acts as a catalyst and leads to tissue damage derived from free radical toxicity via the Fenton reaction (Jomova and Valko, 2011). Therefore, the increase in *FECH* and *PIR* after calving suggest a response of mammary cells to stress conditions potentially caused by Fe^{2+} .

By using NADPH as the hydrogen donor, NQO1 catalyzes the reduction of a broad array of quinones to their corresponding hydroquinones, hence this protein prevents the 1-electron reduction of quinones that results in ROS production (Dinkova-Kostova et al., 2004; Dinkova-Kostova and Talalay, 2010). In addition to its catalytic role as an antioxidant enzyme in the reduction of quinines (Glorieux et al., 2016), NQO1 has been reported to protect against estrogen quinone-mediated DNA and protein damage in human mammary epithelial cells (Yang et al., 2013). Similar to the observed postpartal upregulation of *NQO1* in mammary tissue, it was reported that *NQO1* was upregulated in the liver of dairy cows during the transition period (Gessner et al., 2013). Because both liver and mammary tissue undergo a significant degree of oxidative stress postpartum (Wang et al., 2012), it is conceivable that *NQO1* plays a protective role against oxidative stress.

CONCLUSIONS

The increase in phosphorylation status of the NFE2L2 transcription regulator during the postpartum period appears to be one mechanism that helps coordinate the upregulation of mRNA abundance of various antioxidant target genes. Although the present study did not specifically examine oxidative stress status, ROS production, or antioxidant capacity of mammary tissue, the data suggest that signaling through the NFE2L2 pathway is a key component of normal cellular function to maintain proper redox homeostasis. However, if the longitudinal increases in mRNA and protein abundance of these antioxidant mechanisms are a reflection of cellular oxidative stress, then the likelihood of protein and DNA damage would be greater and might be one factor compromising cell viability and potentially lactation persistency.

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