Nuclear factor erythroid 2-related factor 2 antioxidant response element pathways protect bovine mammary epithelial cells against H₂O₂-induced oxidative damage in vitro

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ABSTRACT

The experiment was conducted to determine the role of nuclear factor (erythroid-derived 2)-like factor 2 (NFE2L2, formerly Nrf2) antioxidant response element (ARE) pathway in protecting bovine mammary epithelial cells (BMEC) against H₂O₂-induced oxidative stress injury. An NFE2L2 small interfering RNA (siRNA) interference or a pCMV6-XL5-NFE2L2 plasmid fragment was transfected to independently downregulate or upregulate expression of NFE2L2. Isolated BMEC in triplicate were exposed to H₂O₂ (600 μ M) for 6 h to induce oxidative stress before transient transfection with scrambled siRNA, NFE2L2-siRNA, pCMV6-XL5, and pCMV6-XL5-NFE2L2. Cell proliferation, apoptosis and necrosis rates, antioxidant enzyme activities, reactive oxygen species (ROS) and malondialdehyde (MDA) production, protein and mRNA expression of NFE2L2 and downstream target genes, and fluorescence activity of ARE were measured. The results revealed that compared with the control, BMEC transfected with NFE2L2-siRNA3 had proliferation rates that were 9 or 65% lower without or with H₂O₂, respectively. These cells also had apoptosis and necrosis rates that were 27 and 3.5 times greater with H₂O₂ compared with the control group, respectively. In contrast, transfected pCMV6-XL5-NFE2L2 had proliferation rates that were 64.3% greater or 17% lower without or with H₂O₂ compared with the control group, respectively. Apoptosis rates were 1.8 times lower with H₂O₂ compared with the control. In addition, compared with the control, production of ROS and MDA and activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and glutathi-

one-S-transferase (GST) increased markedly in cells transfected with pCMV6-XL5-NFE2L2 and without H₂O₂. However, compared with the control, production of ROS and MDA and activity of CAT and GSH-Px increased markedly, whereas activities of SOD and GST decreased in cells transfected with pCMV6-XL5-NFE2L2 and incubated with H₂O₂. Compared with the control, cells transfected with NFE2L2-siRNA3 with or without H₂O₂ had lower production of ROS and MDA and activity of SOD, CAT, GSH-Px, and GST. Cells transfected with pCMV6-XL5-NFE2L2 with or without H₂O₂ had markedly higher protein and mRNA expression of NFE2L2, heme oxygenase-1 (HMOX-1), NADH quinone oxidoreductase 1, glutamate cysteine ligase catalytic subunit, and glutamyl cystine ligase modulatory subunit compared with the control incubations. Cells transfected with NFE2L2-siRNA3 without or with H₂O₂ had markedly lower protein and mRNA expression of NFE2L2, HMOX-1, NADH quinone oxidoreductase 1, glutamyl cystine ligase modulatory subunit, and glutamate-cysteine ligase catalytic subunit compared with the control incubations. In addition, expression of HMOX-1 was 5.3-fold greater with H₂O₂ compared with the control. Overall, results indicate that NFE2L2 plays an important role in the NFE2L2-ARE pathway via the control of HMOX-1. The relevant mechanisms in vivo merit further study.

Key words: lactation, mammary gland, oxidative stress, antioxidant

INTRODUCTION

The periparturient period is the most critical period during the lactation cycle of dairy cattle (Looor et al., 2013), in large part because dairy cattle are most susceptible to infectious diseases during this time (Goff, 2006). A major contributing factor for the increased incidence of health disorders is the dysfunction of immune and oxidative stress (OS) responses (Sordillo

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et al., 2009; Sordillo and Aitken, 2009; Deng et al., 2017). In addition, the proliferation and apoptosis of bovine mammary epithelial cells (**BMEC**) under OS are modulated by cellular antioxidant status (Miranda et al., 2011).

Aside from applied research targeted at milk production, lactation, and mammary carcinogenesis, the mammary gland is an ideal experimental model system for resolving fundamental questions in areas extending from cellular and developmental biology to endocrinology and biotechnology (Ahn et al., 1995). In vitro culture systems have become increasingly important in modern research because they offer the potential to manipulate the growth and differentiation of cells through simulating the endocrine in vivo environment (Howlett and Bissell, 1993).

Apoptosis and proliferation rate control the death and survival of BMEC (Zarzyńska et al., 2007), and previous research revealed that OS can increase the rate of apoptosis leading to cell and tissue necrosis (Fernández-Checa, 2003). Although mammary cell proliferation rate during established lactation is low, it is not insignificant in relation to maintaining cell number (Fernández-Checa, 2003).

An imbalance between excessive formation of reactive oxygen species (**ROS**) and reactive nitrogen species, the reduced antioxidant capacity of cells, or both result in OS (Kruk and Duchnik, 2014). During normal cellular metabolism, the mitochondrion produces ROS including oxygen ions, free radicals, and lipid hydroperoxides as byproducts of the electron transport chain reaction (Szöcs, 2004; Sordillo and Aitken, 2009). However, in the absence of antioxidant mechanisms to counteract the excessive production of these molecules, the onset of OS can directly increase damage of proteins and DNA and induce lipid peroxide production [e.g., malondialdehyde (**MDA**); Li et al., 2016], all of which can overwhelm tissue-repair mechanisms and eventually cause irreversible cell and tissue damage (Bouwstra et al., 2010). It is now recognized that ROS generated through a variety of extracellular and intracellular actions act as signaling mediators that can affect growth, differentiation, progression, and cell death (Zhang et al., 2013; Deng et al., 2017).

The Kelch-like epichlorohydrin-associated protein 1 (**Keap1**)–nuclear factor erythroid 2-related factor 2 (**NFE2L2**)–antioxidant response element (**ARE**) signaling pathway performs critical roles in maintaining cellular redox balance and metabolism. It can induce an adaptive response against OS that can otherwise lead to uncontrolled inflammation. Under higher rates of oxidative metabolism, such as after parturition, an increase of intracellular ROS can promote the dissocia-

tion of Keap1-NFE2L2-ARE (Nguyen et al., 2003; Kaspar et al., 2009; Kim et al., 2010), after which NFE2L2 transfers to the nucleus and binds to ARE on target genes, inducing a cascade of events designed to prevent OS (Jain and Jaiswal, 2007; Kensler et al., 2007). Some evidence indicates that the Keap1-NFE2L2-ARE pathway, which is functional in BMEC (Jin et al., 2016a,b), may be the most important mechanism in ruminants for protecting cells from OS (Kaspar et al., 2009; Jin et al., 2016b).

One of the few studies with BMEC recently revealed that the Keap1-NFE2L2-ARE antioxidant pathway and its downstream antioxidant enzyme heme oxygenase-1 (**HMOX-1**) have a crucial role in the ability of BMEC to cope with H₂O₂-induced OS (Jin et al., 2016a). Phase II detoxifying enzymes provide a major mechanism by which cells combat the toxicity of electrophiles and ROS, and their induction is highly effective and sufficient for protecting cells against toxic challenges (Talalay, 2000). These enzymes include HMOX-1, NADH quinone oxidoreductase 1 (**NQO1**), glutamate-cysteine ligase catalytic subunit (**GCLC**), and glutamyl cystine ligase modulatory subunit (**GCLM**). The antioxidant enzymes superoxide dismutase (**SOD**), catalase (**CAT**), glutathione-S-transferase (**GST**), and glutathione peroxidase (**GSH-Px**) are controlled by activated Keap1-NFE2L2-ARE (Kaspar et al., 2009).

Because available data indicate that BMEC might be susceptible to OS and because the Keap1-NFE2L2-ARE pathway is an important mechanism in the cellular defense against OS, we hypothesized that dysfunction in the Keap1-NFE2L2-ARE signaling pathway may result in increased sensitivity to H₂O₂-induced OS. If this hypothesis is proven correct, it could help develop means to target the pathway in vivo, specifically during the periparturient period, when cows are most susceptible to OS and mastitis. The present study sought to downregulate and upregulate NFE2L2 via silencing using small interfering RNA (**siRNA**) or transfecting an NFE2L2 construct to elucidate the effects of H₂O₂ on the Keap1-NFE2L2-ARE pathway in BMEC.

MATERIALS AND METHODS

Cell Culture and Treatment

Mammary tissue was harvested from five 4-yr-old late-lactation dairy cows from a local slaughterhouse (Hohhot, China). The midpoint area of the left rear side of the udder was clipped and surgically scrubbed. Approximately 150 mg of fresh tissue from each cow was removed and placed in sterilized tubes containing ice-cold Dulbecco's PBS (Sigma-Aldrich, St. Louis,

MO) and immediately transported to the laboratory. The samples were washed with Dulbecco's PBS 3 times and cut into 1-mm³ pieces. Tissue was transferred with tweezers onto clean, sterile plastic cell culture dishes (Corning Inc., Corning, NY). The culture dishes were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air. After 4 h, 5 mL of basal medium was added into the culture. The basal medium was replaced with fresh medium every 24 or 48 h until the cells were distributed across the bottom of the dish. Subsequently, epithelial cells were enriched by selective detachment with trypsinization using 0.25% trypsin (Gibco, Grand Island, NY). After 3 min of trypsinization, fibroblasts were removed by washing with Dulbecco's PBS. The epithelial cells attached to the dish surface were allowed to grow by addition of fresh medium. The BMEC were continuously purified using the same method. The purified BMEC were seeded at a density of 5×10^5 cells in culture flasks and continuously subcultured up to 60 passages. For cryopreservation, 1×10^6 cells/mL was suspended in freezing medium. A supraphysiologic concentration of 600 μ M H₂O₂ was applied to the BMEC to induce OS. In a preliminary unpublished study including doses of 0 to 1,000 μ M H₂O₂, we used data on cell proliferation, concentration of ROS, and activity of SOD and GSH-Px to determine an optimal dose for the study described in this paper. Basal medium comprised 85.74 mL of Dulbecco's modified Eagle medium (DMEM; 12400-024, Gibco) and 10 mL of 10% (vol/vol) fetal bovine serum (Gibco).

Transient Transfection of NFE2L2

Small interference RNA (Beyotime Institute of Biotechnology, Shanghai, China) was used to silence the NFE2L2 gene. In brief, BMEC (2×10^6 cells/well for 6-well plates and 2×10^5 cells/well for 96-well plates) were plated into each well and cultured for 24 h. For overexpression and interference of NFE2L2, cells were transfected with pCMV6-XL5-NFE2L2 and NFE2L2-siRNA3 at 90 to 95% confluence using Lipofectamine 2000 transfection reagent (Gibco). After 12 h of transfection, cells were recovered in basal medium after removal of transfection reagents and were then incubated for an additional 36 h without (control) or with (OS) H₂O₂ treatment. The BMEC in 6-well plates were harvested 48 h after transfection for assessing mRNA and protein expression. For the cells cultured in 96-well plates, the culture medium was switched to serum-free, antibiotic-free DMEM 48 h after transfection for assessing cell proliferation rate, and cells were then subjected to 600 μ M H₂O₂ for 6 h. The BMEC proliferation rate of

control, pCMV6-XL5 transfected and scrambled siRNA transfected (minus H₂O₂), pCMV6-XL5-NFE2L2 transfected and NFE2L2-siRNA3 transfected (minus H₂O₂), pCMV6-XL5 transfected and scrambled siRNA transfected (plus 600 μ M H₂O₂), and pCMV6-XL5-NFE2L2 transfected and NFE2L2-siRNA3 transfected (600 μ M H₂O₂) was then measured.

RNA Interference

Three siRNA specific to NFE2L2 (NFE2L2-siRNA1, NFE2L2-siRNA2, and NFE2L2-siRNA3) and a scrambled sequence (scrambled siRNA) were designed and synthesized by Shanghai Genechem Co. Ltd. (Shanghai, China). The siRNA sequences are listed in Table 1. The BMEC were transfected in antibiotic-free medium following the manufacturer's instructions (Shanghai Genechem Co. Ltd.). Briefly, healthy BMEC were routinely trypsinized and resuspended to 2×10^6 cells/mL in normal growth medium without antibiotics, and cells were then set aside at 37°C. After 24 h, the cells were transfected with NFE2L2-siRNA. After incubation for 5 min at 37°C, the siRNA and Lipofectamine were mixed, incubated at room temperature for an additional 20 min, and then added to each well. The cell suspension that was previously set aside was transferred to the same wells containing the complex (2×10^6 cells per well for 6-well plates and 2×10^5 cells per well for 96-well plates), and the cell suspension was mixed by gently rocking the plates back and forth. The basal medium was replaced by the same fresh medium 12 h after transfection. The BMEC in 6-well plates were harvested 48 h after transfection to assess mRNA and protein changes. At 48 h after transfection, the culture medium for the BMEC in 96-well plates was switched to serum-free, antibiotic-free DMEM, and the cells were then subjected to 600 μ M H₂O₂ for 6 h. The cell proliferation rate of NFE2L2-silenced and control culture was measured. Scrambled transfections (using a

Table 1. Primer sequences for the nuclear factor erythroid 2-related factor 2 (NFE2L2) small interfering RNA (siRNA) tested

Primer	Primer sequence (5'→3' orientation)
NFE2L2-siRNA1	
Sense	TTGCTCAAAGAAAGAGGAGAA
Antisense	TTCTCCTCTTTCTTTGAGCAA
NFE2L2-siRNA2	
Sense	ACTGGATAAAGAAGTAGGTAA
Antisense	TTACCTACTTCTTTATCCAGT
NFE2L2-siRNA3	
Sense	CTGGAGCAAGATTTAGATCAT
Antisense	ATGATCTAAATCTTGCTCCAG

nontarget siRNA) were performed in each experiment. Three different NFE2L2-specific siRNA were tested.

BMEC Proliferation Rate Assay

To quantify BMEC proliferation rate, BMEC were plated at a density of 2×10^6 cells per well for 96-well plates overnight, and the cells were then transfected with scrambled siRNA, NFE2L2-siRNA3, pCMV6-XL5, and pCMV6-XL5-NFE2L2. At 48 h after transfection, the culture medium was switched to serum-free, antibiotic-free DMEM, and the cells were then exposed to 600 μ M H₂O₂ for 6 h. The BMEC proliferation rate was performed using the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay kit (G3582, Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, 20 μ L of MTS was added to each well and incubated for 4 h at 37°C. Subsequently, 150 μ L of dimethyl sulfoxide (Sigma-Aldrich) was added to each well and incubated for 10 min at 37°C. Last, the absorbance at 490 nm was determined with a microplate reader (Molecular Devices, Sunnyvale, CA).

Detection of OS Indicators

The activities of SOD, CAT, GSH-Px, and GST as well as the contents of MDA in BMEC were determined by using spectrophotometric diagnostic kits from Nanjing Jiancheng Biotechnology Institute (Nanjing, China) according to the manufacturer’s protocols. In brief, BMEC were washed with PBS and incubated with fresh DMEM (Gibco) at 37°C for 35 min, and then 1×10^6 cells were harvested and resuspended in PBS. Absorbance was detected at 560 nm (SOD), 420 nm (GSH-Px), 405 nm (CAT), 450 nm (GST), and 532 nm (MDA) and was recorded with a 722N spectrophotometer (Scientific Instrument Co. Ltd., Shanghai, China).

Intracellular ROS Detection

A dichlorofluorescein staining assay was used to detect intracellular ROS. Briefly, BMEC were washed

with PBS and incubated with fresh DMEM containing 10 μ M dichlorofluorescein at 37°C for 35 min, and then 1×10^6 cells were harvested and resuspended in PBS. The optical density at 450 nm was recorded with a microplate reader (Molecular Devices).

ARE Fluorescence Activity

The ARE fluorescence activity was tested in cultured BMEC with or without H₂O₂ via an ARE reporter gene according to the manufacturer’s instructions (Promega). In brief, after the treatment of BMEC with H₂O₂ (600 μ M) for 0, 6, 12, and 24 h, 50 μ L of luciferase assay reagent II was added to the BMEC, followed by 10 μ L of cell lysis buffer. The activity of relative light unit 1 (RLU1) was detected with a biluciferase reporter gene kit (E1910; Promega). Then, 50 μ L of 50 $\times$ Stop and Glo reagent was added to the BMEC, and the activity of relative light unit 2 (RLU2) was detected again with a biluciferase reporter gene kit (E1910; Promega). The RLU1/RLU2 ratio was used to determine the transfection efficiency of ARE.

RNA Isolation, cDNA Synthesis, and Quantitative PCR

Total RNA was isolated from BMEC using the miR-Neasy kit (Qiagen, Hilden, Germany) following the manufacturer’s protocols. Samples were treated on-column with DNaseI (Qiagen, Hilden, Germany); quantification was assessed using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE), and RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). All samples had an RNA integrity number factor greater than 6.3. The quantitative PCR was performed as described previously (Osorio et al., 2014) to determine the relative mRNA abundance of NFE2L2, HMOX1, NQO1, GCLC, GCLM, and TUBB (tubulin β ; internal control). All reactions were run in triplicate. The NFE2L2-siRNA1, 2, and 3 and primer sequences are presented in Table 1. The primers of NFE2L2 and downstream target genes used for reverse-transcription PCR are listed in Table 2.

Table 2. Primers of downstream target genes in the nuclear factor erythroid 2-related factor 2 (NFE2L2) antioxidant response element (ARE) signaling pathway

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')	Accession no.
NFE2L2	CCAGCACAACACATACCA	TAGCCGAAGAAACCTCATT	001011678.2
HMOX1	GAACGCAACAAGGAGAAC	CTGGAGTCGCTGAACATAG	001014912.1
NQO1	CAACAGACCAGCCAATCA	ACCTCCCATCCTTTTCCTC	001034535.1
GCLC	ATTGGGTGGAGAGTGGA	ACAGCGGGATGAGAAAGT	001083674.1
GCLM	CCGATGAAAGAGAAGAAATG	CAACAGGAGGTGAAGCAA	001038143.1

Western Blot Analysis

After treatment, cells were washed twice with 4°C PBS (0.01 M, pH 7.2–7.3) and were then lysed with radio-immunoprecipitation assay protein lysate (Beyotime Institute of Biotechnology, Shanghai, China). Total protein was extracted from BMEC using a tissue protein extraction reagent (cat. no. 78510; Thermo Scientific, Waltham, MA) containing inhibitor cocktail (100×, cat. no. 78442; Thermo Scientific). Total protein concentration was determined using the BCA protein assay kit (Sigma-Aldrich). Protein samples were boiled for 20 min at 100°C; 50 µg of total protein per lane was resolved by 10% SDS-PAGE (Bio-Rad, Hercules, CA) and then transferred from the gel to a polyvinylidene difluoride membrane (0.45 µm; Millipore, Billerica, MA). PageRuler Plus prestained protein ladder (Fermentas, Hanover, MD) was used to confirm protein electrophoresis. Membranes were blocked in Tris-buffered saline with Tween (TBST; 50 mM Tris, pH 7.6; 150 mM NaCl; and 0.1% Tween 20) that contained 5% (wt/vol) nonfat dry milk for 2 h at room temperature with gentle agitation. The membranes were then incubated in TBST containing antibodies to NFE2L2 (cat. no. ab137550; Abcam, Cambridge, MA), HMOX-1 (cat. no. ab13248; Abcam), NQO1 (cat. no. ab28947; Abcam), GCLC (cat. no. ab80841; Abcam), and GCLM (cat. no. ab81445; Abcam) with gentle agitation at 4°C overnight. After incubating with primary antibody, the membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (HRP labeled sheep anti-mouse, 1:50,000, and HRP labeled sheep anti-rabbit, 1:50,000; cat. no. ab6721; Abcam) in TBST for 1 h at room temperature. The membranes were washed and then incubated with electro-chemi-luminescence reagent (cat. no. 170-5060; Bio-Rad). Tubulin (cat. no. ab56676; Abcam) and β -actin (cat. no. ab8226; Abcam) were used as internal controls. The images were captured using Chemi DOC MP (Bio-Rad). The intensities of the bands were measured with Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD).

BMEC Apoptosis and Necrosis Rate

To quantify apoptotic and necrotic death, BMEC were plated at a density of 2×10^6 cells per well for 96-well plates overnight, and the cells were then transfected with scrambled siRNA, NFE2L2-siRNA3, pCMV6-XL5, and pCMV6-XL5-NFE2L2. At 48 h after transfection, the culture medium was switched to serum-free, antibiotic-free DMEM, and the cells were then exposed to

600 µM H₂O₂ for 6 h. The apoptosis rate and necrosis rate of cells were detected with the dead cell apoptosis kit (V13241, Thermo Fisher). Briefly, 5 µL of Alexa Fluor 488 Annexin V (V13241, Thermo Fisher) and 1 µL of 100 µg/mL propidium iodide working solution was added to each 100 µL of cell suspension, and the cells were incubated at room temperature for 15 min. Subsequently, 400 µL of 1× annexin-binding buffer was added to each well and mixed gently, and the samples were kept on ice. The stained cells were subsequently analyzed by flow cytometry (FACSCantoII, BD Biosciences, San Jose, CA), and fluorescence emission was measured at 530 nm.

Statistical Analysis

All statistical tests were carried out using SAS 9.0 (SAS Institute Inc., Cary, NC). One-way ANOVA followed by a Student–Newman–Keuls multiple-comparison test was used to determine statistical significance for multiple comparisons, and a Student's *t*-test was used for 2 group comparisons. All data are expressed as means $\pm$ standard error of the mean for the indicated number of independent experiments performed. Differences were considered significant at $P < 0.05$.

RESULTS

Verification and Screening of NFE2L2-siRNA Interference Fragment

Compared with the control group, the BMEC transfected with NFE2L2-siRNA1, NFE2L2-siRNA2, and NFE2L2-siRNA3 had reduced protein and mRNA expression of NFE2L2; the best interference efficiency was achieved with NFE2L2-siRNA3 (>50%; $P < 0.01$; Figure 1). Therefore, NFE2L2-siRNA3 was chosen as the interference fragment for subsequent experiments.

Effect of Downregulation and Upregulation of NFE2L2 on the Proliferation Rate of BMEC

The transfection with NFE2L2-siRNA3 decreased the proliferation rate by 65% ($P < 0.05$) under OS compared with the control group (Figure 2). This result suggested that downregulation of NFE2L2 decreased the ability of BMEC to cope with H₂O₂-induced cytotoxicity. Transfection with pCMV6-XL5 and pCMV6-XL5-NFE2L2 without H₂O₂ increased the proliferation rate by 50% ($P < 0.01$) and 180% ($P < 0.01$), respectively, compared with the control group (Figure 2).

Figure 1. Screening for efficacy of nuclear factor erythroid 2-related factor 2 (NFE2L2) small interfering RNA (siRNA) fragments. The mRNA and protein expression of NFE2L2 after siRNA treatment was detected by reverse-transcription PCR and Western blot. * $P < 0.05$, ** $P < 0.01$ compared with the control group. Error bars indicate SEM.

Effect of Downregulation and Upregulation of NFE2L2 on Apoptosis and Necrosis Rate in BMEC Under OS

The results revealed that transfection with NFE2L2-siRNA3 with H_2O_2 sharply increased ($P < 0.01$) the apoptosis and necrosis rate by 4.5- and 5-fold, respectively, compared with the control group. However, transfection with pCMV6-XL5-NFE2L2 plus H_2O_2 sharply decreased ($P < 0.05$) the apoptosis rate by 70% compared with the control group (Figure 3). These results suggested that downregulation of NFE2L2 sharply decreased the ability of BMEC to cope with H_2O_2 -induced apoptosis, whereas upregulation of NFE2L2 sharply enhanced the ability of BMEC to cope with H_2O_2 -induced apoptosis.

Effect of Downregulation and Upregulation of NFE2L2 on Antioxidant Enzyme Activities in BMEC

Transfection of NFE2L2-siRNA3 without H_2O_2 sharply decreased SOD ($P < 0.05$), CAT ($P < 0.01$), GSH-Px ($P < 0.01$), and GST ($P < 0.01$) activity and

Figure 2. Effect of regulating nuclear factor erythroid 2-related factor 2 (NFE2L2) expression on bovine mammary epithelial cell (BMEC) proliferation rate. (A) The BMEC were transiently transfected with control fragment scrambled small interfering RNA (siRNA) and the interference fragment NFE2L2 siRNA3 for 48 h under normal conditions, followed by transfection with scrambled siRNA and NFE2L2 siRNA3 for 48 h after treatment with H_2O_2 (600 μM) for 6 h. (B) The BMEC were transiently transfected with the empty plasmid pCMV6-XL5 and the overexpression vector pCMV6-XL5 NFE2L2 for 48 h under normal conditions followed by transfection with pCMV6-XL5 and pCMV6-XL5 NFE2L2 for 48 h after treatment with H_2O_2 (600 μM) for 6 h. * $P < 0.05$, ** $P < 0.01$ compared with the control group. Error bars indicate SEM.

ROS ($P < 0.01$) and MDA ($P < 0.05$) production compared with the control group, respectively (Figure 4). However, transfecting with NFE2L2-siRNA3 plus H_2O_2 sharply decreased SOD ($P < 0.01$), CAT ($P < 0.001$), GSH-Px ($P < 0.001$), and GST ($P < 0.001$) activity as

well as concentration of ROS ($P < 0.01$) and MDA ($P < 0.01$) compared with the control group.

The transfection of pCMV6-XL5-NFE2L2 without H_2O_2 sharply increased SOD ($P < 0.05$), CAT ($P < 0.001$), and GSH-Px ($P < 0.01$) activity compared with the control group (Figure 5). However, transfection with pCMV6-XL5-NFE2L2 plus H_2O_2 increased CAT ($P < 0.01$) activity as well as ROS ($P < 0.05$) and MDA ($P < 0.05$) production and sharply decreased SOD ($P < 0.01$) and GST ($P < 0.01$) activity compared with the control group. These results further support the hypothesis that NFE2L2 is essential for induction of several antioxidant defense enzymes in BMEC under OS. Results also suggest that the antioxidant enzyme system is impaired in BMEC exposed to supraphysiologic levels of H_2O_2 .

Effect of Regulating NFE2L2 Expression on Target Genes in the NFE2L2-ARE Pathway

Figures 6 and 7 depict the expression of NFE2L2 and 4 main phase II detoxifying genes in BMEC after treatment with or without 600 μM H_2O_2 for 6 h. The western blot and reverse-transcription PCR results demonstrated that transfection of NFE2L2-siRNA3 without H_2O_2 sharply decreased the protein and mRNA expression of NFE2L2 ($P < 0.05$), HMOX-1 ($P < 0.01$), NQO1 ($P < 0.05$), GCLM ($P < 0.05$), and GCLC ($P < 0.05$) compared with the control group. Furthermore, those results were lower compared with the transfection

of scrambled siRNA ($P < 0.05$). The transfection of NFE2L2-siRNA3 with H_2O_2 sharply decreased the protein and mRNA expression of NFE2L2 ($P < 0.01$), HMOX-1 ($P < 0.05$), NQO1 ($P < 0.01$), GCLM ($P < 0.05$), and GCLC ($P < 0.05$) compared with the control group. Furthermore, those results were significantly lower compared with the transfection of scrambled siRNA ($P < 0.05$). Overall, these results suggested that downregulation of NFE2L2 decreased the expression of NFE2L2 and all 4 genes regardless of H_2O_2 .

The transfection of pCMV6-XL5-NFE2L2 without H_2O_2 sharply increased the protein and mRNA expression of HMOX-1 ($P < 0.05$), NQO1 ($P < 0.05$), and GCLM ($P < 0.05$) compared with the control group, respectively, and the response was significantly higher compared with the transfection of pCMV6-XL5 ($P < 0.05$; Figures 8 and 9). However, transfection with pCMV6-XL5-NFE2L2 plus H_2O_2 increased the protein and mRNA expression of NFE2L2 ($P < 0.01$), NQO1 ($P < 0.05$), GCLM ($P < 0.01$), and GCLC ($P < 0.05$) compared with the control group, respectively. Overall, these results suggested that upregulation of NFE2L2 enhanced the expression levels of NFE2L2 and all 4 genes regardless of H_2O_2 . The most significant change was detected for HMOX-1, with a 5.3-fold increase when compared with the control.

HMOX-1 Is a Main Target Gene of the NFE2L2-ARE Pathway

Incubation with H_2O_2 led to a greater than 5.3-fold increase in *HMOX-1* mRNA expression in oxidative-damaged BMEC, whereas the increases in *NQO1*, *GCLM*, and *GCLC* mRNA expression were of smaller magnitude, with only a 2- to 2.8-fold increase compared with the H_2O_2 -untreated group (control group; Figure 10).

Impaired NFE2L2 Transcriptional Activity in the NFE2L2-ARE Pathway

To compare the NFE2L2-ARE transcriptional activity between control and oxidative damage, we used a dual-luciferase reporter assay to measure ARE luciferase activity in the presence and absence of H_2O_2 . The results revealed that treatment with H_2O_2 increased ($P < 0.05$) the transcriptional activity of NFE2L2 in terms of ARE luciferase activity in a time-dependent manner in both control and oxidative-damaged groups. In contrast, ARE luciferase activity of oxidative-damaged BMEC was much lower ($P < 0.05$) than control BMEC (Figure 11). These data clearly indicate that NFE2L2-driven transcriptional activation of ARE is aberrant in oxidative-damaged BMEC.

Figure 3. Effect of regulating nuclear factor erythroid 2-related factor 2 (NFE2L2) expression on apoptosis and necrosis rate of bovine mammary epithelial cells (BMEC) under oxidative stress. Apoptosis and necrosis rate of BMEC were detected following treatment with H_2O_2 (600 μM) for 6 h followed by transfection with pCMV6-XL5 and pCMV6-XL5 NFE2L2, scrambled small interfering RNA (siRNA), and NFE2L2 siRNA3 for 48 h. * $P < 0.05$, ** $P < 0.01$ compared with the control group. Error bars indicate SEM.

Figure 4. Effect of downregulating nuclear factor erythroid 2-related factor 2 (NFE2L2) expression on antioxidative enzyme activity, reactive oxygen species (ROS), and malondialdehyde (MDA) production in bovine mammary epithelial cells (BMEC). Activity of (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) glutathione peroxidase (GSH-Px), and (D) glutathione transferase (GST), and production of (E) ROS and (F) MDA in oxidative-damaged and control BMEC. Cells were treated with H_2O_2 (600 μM) for 6 h followed by transfection with scrambled small interfering RNA (siRNA) and NFE2L2 siRNA3 for 48 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control group. Error bars indicate SEM.

Figure 5. Effect of upregulating nuclear factor erythroid 2-related factor 2 (NFE2L2) expression on antioxidative enzyme activity, reactive oxygen species (ROS), and malondialdehyde (MDA) production in bovine mammary epithelial cells (BMEC). Activity of (A) superoxide dismutase (SOD), (B) catalase (CAT), (C) glutathione peroxidase (GSH-Px), and (D) glutathione transferase (GST) and production of (E) ROS and (F) MDA in oxidative-damaged and control BMEC. Cells were treated with H₂O₂ (600 μ M) for 6 h followed by transfection with pCMV6-XL5 NFE2L2 and pCMV6-XL5 for 48 h. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with the control group. Error bars indicate SEM.

Figure 6. Effects of nuclear factor erythroid 2-related factor 2 (NFE2L2) small interfering RNA (siRNA)-3 interference fragment on downstream protein expression of NFE2L2 antioxidant response element signal pathway in bovine mammary epithelial cells (BMEC). Protein expression of (A) glutamate-cysteine ligase catalytic subunit (GCLC), (B) heme oxygenase-1 (HMOX-1), (C) NADH quinone oxidoreductase 1 (NQO1), and (D) glutamyl cystine ligase modulatory subunit (GCLM) was measured in oxidative-damaged and control BMEC by Western blot. Cells were treated with H_2O_2 (600 μM) for 6 h followed by transfection with scrambled small interfering RNA (siRNA) and NFE2L2 siRNA3 for 48 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control group. Error bars indicate SEM.

DISCUSSION

Taken together, the changes in BMEC proliferation rate, ROS and MDA production, and antioxidant enzyme activity in response to up or downregulation of NFE2L2 indicated that BMEC are susceptible to oxidative damage induced by H_2O_2 . Thus, along with data from a previous study (Jin et al., 2016a), the data support the assertion that the NFE2L2-ARE signaling pathway is one of the main defense mechanisms of resistance against OS. Oxidative stress induced by H_2O_2 can increase ROS and MDA production, leading to decreased antioxidant ability, partly due to lower

NFE2L2 and HMOX-1 abundance. These responses in vitro could have bearing in vivo, particularly during times when the mammary gland experiences changes in metabolism or the number of potential challenges to normal function (e.g., mastitis).

Recent evidence, including our findings, indicated that dairy cows during the perinatal and transition into lactation periods are highly susceptible to altered redox (oxidation reduction) balance, resulting in OS (Abuelo et al., 2015; Hanschke et al., 2016). A large portion of this response is due to high metabolic rates and physiological adaptations to the onset of lactation. The period of negative energy balance during calving

Figure 7. Effects of nuclear factor erythroid 2-related factor 2 (NFE2L2) small interfering RNA (siRNA)-3 interference fragment on downstream expression of target genes in the NFE2L2 antioxidant response element signaling pathway in bovine mammary epithelial cells (BMEC). The mRNA expression of (A) *NFE2L2*, (B) heme oxygenase-1 (*HMOX-1*), (C) NADH quinone oxidoreductase 1 (*NQO1*), (D) glutamyl cystine ligase modulatory subunit (*GCLM*), and (E) glutamate-cysteine ligase catalytic subunit (*GCLC*) was measured in oxidative-damaged and control BMEC by reverse-transcription PCR. Cells were treated with H_2O_2 (600 μM) for 6 h followed by transfection with scrambled small interfering RNA (siRNA) or NFE2L2 siRNA3 for 48 h. * $P < 0.05$, ** $P < 0.01$ compared with the control group. Error bars indicate SEM.

Figure 8. Effects of pCMV6-XL5 nuclear factor erythroid 2-related factor 2 (NFE2L2) overexpression vector on downstream protein expression of NFE2L2 antioxidant response element signaling pathway in bovine mammary epithelial cells (BMEC). Protein expression of (A) NFE2L2, (B) heme oxygenase-1 (HMOX-1), (C) NADH quinone oxidoreductase 1 (NQO1), (D) glutamyl cystine ligase modulatory subunit (GCLM), and (E) glutamate-cysteine ligase catalytic subunit (GCLC) in oxidative-damaged and control BMEC was measured by Western blot. Cells were treated with H_2O_2 (600 μM) for 6 h followed by transfection with pCMV6-XL5 and PCMV6-XL5 NFE2L2 for 48 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control group. Error bars indicate SEM.

Figure 9. Effects of pCMV6-XL5 nuclear factor erythroid 2-related factor 2 (NFE2L2) overexpression vector on expression of target genes in the NFE2L2 antioxidant response element signaling pathway in bovine mammary epithelial cells (BMEC). The mRNA expression of (A) *NFE2L2*, (B) heme oxygenase-1 (*HMOX-1*), (C) NADH quinone oxidoreductase 1 (*NQO1*), (D) glutamyl cystine ligase modulatory subunit (*GCLM*), and (E) glutamate-cysteine ligase catalytic subunit (*GCLC*) in oxidative-damaged and control BMEC was measured by reverse-transcription PCR. Cells were treated with H_2O_2 (600 μM) for 6 h followed by transfection with pCMV6-XL5 and PCMV6-XL5 NFE2L2 for 48 h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control group. Error bars indicate SEM.

Figure 10. Downstream expression of gene targets in the nuclear factor erythroid 2-related factor 2 (NFE2L2) antioxidant response element pathway in bovine mammary epithelial cells under oxidative stress. The mRNA expression of heme oxygenase-1 (*HMOX-1*), NADH quinone oxidoreductase 1 (*NQO1*), glutamyl cystine ligase modulatory subunit (*GCLM*), and glutamate-cysteine ligase catalytic subunit (*GCLC*) was measured by reverse-transcription PCR after treatment of cells with H_2O_2 (600 μM) for 6 h. * $P < 0.05$ compared with the control group. Error bars indicate SEM.

and early lactation also can further cause disorders associated with OS (Bernabucci et al., 2005; Castillo et al., 2005; Patton et al., 2006; van Knegsel et al., 2007).

Figure 11. Fluorescence activity of antioxidant response element (ARE) in control and oxidative stress groups. The fluorescence activity of ARE was detected with the biluciferase gene reporter assay (E1910; Promega, Madison, WI) after treatment of cells with H_2O_2 (600 μM) for 0, 6, 12, and 24 h. # $P < 0.05$, ## $P < 0.01$ compared with the control group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with 0 h. Error bars indicate SEM.

An imbalance between ROS production and antioxidant defenses can alter normal cell function and decrease immune function in dairy cows (Sordillo et al., 2009). Thus, maintaining intracellular redox balance to decrease the damage induced by OS is important. Excess production of ROS and MDA or insufficient antioxidant enzymes may cause oxidative damage in BMEC that could compromise cell viability. The focus of the present study was on enzymatic antioxidants, and future research would have to assess the role of other molecules with antioxidative properties that are not enzymes.

In the present study, the lower activity of SOD, GSH-Px, CAT, and GSH coupled with the higher content of ROS and MDA induced by an acute supraphysiologic concentration of H_2O_2 overpowered the ability of BMEC to scavenge ROS to prevent cell damage. The data underscore that these antioxidant systems were insufficient to counteract the increase in ROS and MDA in response to H_2O_2 , causing cellular apoptosis and necrosis also. Whether such an acute oxidative attack mirrors the in vivo condition that periparturient cows may experience is unknown. Thus, results from the present study should be carefully translated to the in vivo condition. It is more likely that at the onset of lactation cows are exposed to a chronic increase in both ROS and reactive nitrogen species; hence, in vivo studies focusing on the NFE2L2-ARE signaling pathway should be performed to better address the relevance of in vitro findings from the present study.

At least in ruminant cells, NFE2L2 is one of the most important transcription factors regulating expression of several antioxidant or detoxifying enzymes through its interaction with ARE (Jin et al., 2016a). Recent studies have demonstrated a crucial function for the NFE2L2-ARE signaling pathway in regulating BMEC homeostasis under OS (Jin et al., 2016a,b). In fact, this pathway is an early sensor of OS, acting to dampen oxidative damage by upregulating the expression of phase II detoxifying enzymes (Lee and Johnson, 2004). Thus, the finding that up- or downregulating the expression of NFE2L2 could alter the degree of apoptosis induced by H_2O_2 underscores its importance for BMEC. Hence, we speculate that the vulnerability to oxidative damage and death in the mammary gland might depend heavily on NFE2L2.

In this study, we demonstrated that HMOX-1 is a key antioxidant gene that helps protect BMEC against OS, and it is under the control of NFE2L2 through the ARE (Bae et al., 2017). Previous studies confirmed that the expression of HMOX-1 is mediated by the ARE and depends on NFE2L2 activation (Jian et al., 2011). Thus, the present data confirm that NFE2L2 itself does not elicit an antioxidant effect but rather helps

launch a series of transcriptional changes upregulating the expression of antioxidant detoxifying enzymes. The specific mechanisms whereby activated NFE2L2 travels to the nucleus and activates the transcriptional program remain to be elucidated.

Regardless of addition of H₂O₂, the dual-luciferase data revealing that treatment with H₂O₂ decreased the fluorescence intensity of ARE in a time-dependent manner along with the decrease in protein and mRNA expression of NFE2L2 under oxidative-damaged conditions suggested the existence of an “activation barrier” preventing NFE2L2-ARE signaling under oxidative damage conditions. Such an activation barrier of NFE2L2 can further aggravate the toxicity and damage of cells, resulting in physiological dysfunction leading to apoptosis or necrosis.

CONCLUSIONS

Our study provides evidence that OS can increase the oxidative damage of BMEC by reducing nuclear translocation and transcriptional activity of NFE2L2. As a result, several molecular and antioxidant enzymatic mechanisms are impaired, leading to an unbalanced redox state.

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