Nuclear factor erythroid 2-related factor 2-antioxidant activation through the action of ataxia telangiectasia-mutated serine/threonine kinase is essential to counteract oxidative stress in bovine mammary epithelial cells

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ABSTRACT

Nuclear factor (erythroid-derived 2)-like factor 2 (NFE2L2, formerly Nrf2) is a transcription factor that binds to the antioxidant response element (ARE) in the upstream promoter region of various antioxidant-responsive genes. Hence, at least in nonruminants, the NFE2L2-ARE signaling pathway plays an important role in the cellular antioxidant defense system. Whether oxidative stress in bovine mammary epithelial cells alters NFE2L2 or the NFE2L2-ARE pathway is unclear. Therefore, the objective of this study was to examine the response in NFE2L2- and NFE2L2-ARE-related components in bovine mammary epithelial cells (BMEC) under oxidative stress. An *in silico* analysis to identify potential phosphorylation sites on NFE2L2 and the protein kinases was performed with NetPhos 3.1 (<http://www.cbs.dtu.dk/services/NetPhos/>) and Scansite (<http://scansite.mit.edu>) software. Isolated BMEC were exposed to H₂O₂ (600 μ M) for 6 h to induce oxidative stress. *In silico* analysis revealed ataxia telangiectasia-mutated (ATM) serine/threonine kinase as a key kinase responsible for the phosphorylation of NFE2L2. Thus, after the 6 h incubation with H₂O₂, BMEC were transiently transfected with ATM-small interfering RNA (siRNA) 1, 2, or 3. Compared with the control, transfection with ATM-siRNA3 resulted in proliferation rates that were 60.7 and 36.2% lower with or without H₂O₂. In addition, production of reactive oxygen species and malondialdehyde increased markedly, but activities of superoxide dismutase, glutathione peroxidase, catalase, and glutathione-S-transferase decreased markedly in transfected cells without or with

H₂O₂ compared with the control. Transfected cells had markedly lower protein and mRNA expression of NFE2L2 without or with H₂O₂ compared with the control. In addition, fluorescent activity of the ARE in transfected BMEC indicated that NFE2L2-driven transcriptional activation decreased under oxidative stress. Overall, results indicate that ATM is a physiologically relevant NFE2L2 kinase. Furthermore, inhibition of ATM activity can cause marked alterations in oxidative stress leading to cell death as a result of diminished capacity of BMEC to cope with H₂O₂-induced cytotoxicity. The relevance of this kinase *in vivo* merits further study.

Key words: nuclear factor (erythroid-derived 2)-like factor 2-antioxidant response element pathways, ataxia telangiectasia-mutated serine/threonine kinase, oxidative stress, bovine mammary epithelial cell

INTRODUCTION

Due to the constant challenge by environmental (xenobiotics, drugs, and UV light) and endogenous [reactive oxygen species (ROS), hydroperoxides] stressors, cells are major targets of oxidative stress (Klaunig et al., 2010). These environmental and endogenous stressors or their metabolites can directly and indirectly drive the production of ROS. The ROS are produced continuously as natural by-products of the normal metabolism of oxygen and play important roles in redox balance (Lee and Griending, 2008). For example, intracellular lipid peroxidation leads to production of H₂O₂ and ROS, which in culture can decrease the number and viability of bovine mammary epithelial cells (BMEC, MAC-T; Jin et al., 2016), rodent cells (Burdon, 1995; Mori et al., 2004), and human breast adenocarcinoma cells (MCF-7; Dasari et al., 2006). Although the mechanisms associated with injury of BMEC are not well known, increasing evidence indicates that oxidative stress caused by H₂O₂ is a key factor (Jin et al., 2016).

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In nonruminants, upon binding to the antioxidant response element (**ARE**) sequence, the redox sensitive transcription factor nuclear factor (erythroid-derived 2)-like factor 2 (**NFE2L2**, formerly Nrf2) regulates mRNA expression of cellular defense enzymes and certain antioxidant proteins (Kensler et al., 2007; Niture et al., 2014). Extensive work in nonruminant cells has revealed that the NFE2L2-ARE signaling pathway is one of the main defense mechanisms whereby cells can handle H₂O₂-induced oxidative damage (Kensler et al., 2007). Under physiological conditions and states of low oxidative stress, NFE2L2 is retained in the cytosol via the actin binding protein Kelch-like epichlorohydrin-associated protein (**Keap1**), which negatively regulates NFE2L2 by targeting it for ubiquitination and proteasomal degradation (Itoh et al., 1999; Hayes and McMahon, 2001). When cells are under oxidative stress, NFE2L2 is rapidly released from Keap1; it then translocates to the nucleus where it binds to ARE and induces the phase II detoxifying enzyme genes (Dinkova-Kostova et al., 2002).

Oxidative and electrophilic stresses induce alterations in the NFE2L2-Keap1 complex, preventing proteasomal degradation and enhancing NFE2L2/ARE-linked gene transcription (Tong et al., 2006). Work with nonruminant cells indicated that phase II detoxifying enzymes responsive to NFE2L2 activation include heme oxygenase-1 (**HMOX1**, formerly HO-1), NADH quinone oxidoreductase 1 (**NQO1**), glutamate-cysteine ligase catalytic subunit (**GCLC**), glutamyl cystine ligase modulatory subunit (**GCLM**), superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**), catalase (**CAT**), glutathione-S-transferase (**GST**), and antioxidative stress proteins (Itoh et al., 1999; Zhu et al., 2005). Although data in bovine cells are scarce, a recent study with BMEC indicated that the NFE2L2-ARE signaling pathway and its downstream antioxidant enzymes HMOX1, NQO1, GCLC, and GCLM have a crucial role in the ability of those cells to cope with H₂O₂-induced oxidative stress (Jin et al., 2016). However, the mechanisms that prevent BMEC from dealing with H₂O₂-induced oxidative stress and the reason for low NFE2L2 activity under oxidative conditions are largely unknown.

We hypothesized that dysfunction in the NFE2L2-ARE signaling pathway in BMEC may result in increased sensitivity to H₂O₂-induced oxidative insults. Therefore, the aim of the present study was to determine the role of protein kinases on the phosphorylation status of NFE2L2 and subsequent alterations in mRNA expression of target genes in cultured BMEC under oxidative stress.

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 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 (**DPBS**, Sigma, St. Louis, MO) and immediately transported to the laboratory. The samples were washed with DPBS 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 thoroughly 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, detached fibroblast was removed by washing with DPBS. 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. To establish oxidative stress, 600 μ M H₂O₂ was applied to the BMEC.

Basal medium was composed of 85.74 mL of DMEM/F12 (12400-024, Gibco) and 10 mL of 10% (vol/vol) fetal bovine serum (FBS, Gibco). The lactogenic medium was developed by supplementing the basal medium with 0.5 mL of 0.5% insulin and transferrin solution (Gibco), 2 mL of penicillin streptomycin (Gibco), 100 μ L of 1 μ g/mL hydrocortisone (Gibco), 100 μ L of 2.5 μ g/mL amphotericin B (Gibco), 10 μ L of 10 ng/mL epidermal growth factor (Gibco), 50 μ L of 5 μ g/mL prolactin, and 1.5 mL of glutamine (Gibco).

Transient Transfection of Ataxia Telangiectasia-Mutated Gene

Small interfering RNA (**siRNA**; Beyotime Institute of Biotechnology, Shanghai, China) was used to silence the *ATM* gene. In brief, mammary epithelial cells (2×10^6 cells per well for 6-well plates and 2×10^5 cells per

well for 96-well plates) were plated into each well and cultured for 24 h. Cells were transfected with ATM-siRNA at 90 to 95% confluence using Lipofectamine 2000 transfection reagent (Gibco). After 12 h of incubation, cells were recovered in normal medium after removal of transfection reagents and were then incubated for an additional 36 h with or without H₂O₂ treatment. Mammary epithelial cells 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 Dulbecco's modified Eagle medium (DMEM) 48 h after transfection, and the cells were then subjected to 600 μ M H₂O₂ for 6 h. This supraphysiologic dose of H₂O₂ was selected based on data from our unpublished studies including doses of 0 to 1,000 μ M in which cell proliferation, concentration of ROS, and activity of SOD and GSH-Px were evaluated. The BMEC proliferation rate in the control (no siRNA), scrambled siRNA transfected group with or without H₂O₂, ATM-siRNA3 transfected group with or without H₂O₂ was measured as described below.

RNA Interference

Three siRNA specific for ATM (ATM-siRNA1, ATM-siRNA2, and ATM-siRNA3) and a scrambled sequence (scrambled-siRNA) were designed and synthesized (Table 1). Mammary epithelial cells were transfected in antibiotic-free medium following the manufacturer's instructions. 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 ATM-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 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 medium was replaced by fresh medium 12 h after transfection. The BMEC in 6-well plates were harvested 48 h after transfection to assess mRNA and protein changes, respectively. 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 ATM-silenced and control culture was measured. Scrambled transfections (using a nontargeting siRNA) were performed in each experi-

BMEC Proliferation Rate Assay

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 according to the manufacturer's instruction. Briefly, MTS (20 μ L/well, Sigma-Aldrich) was added to each well and incubated for 4 h at 37°C. Subsequently, 150 μ L of dimethyl sulfoxide (DMSO, 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 Oxidative Stress Indicators

The total superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and glutathione-S-transferase (GST) activities, and the malondialdehyde (MDA) production in BMEC were determined using the xanthine oxidase, 5'-dithio-bis-nitrobenzoic acid display color, ammonium molybdate, and thiobarbituric acid, respectively. In brief, BMEC were washed with PBS and incubated with fresh DMEM containing 50 μ L of xanthine oxidase, 5'-dithio-bis-nitrobenzoic acid display color, ammonium molybdate, and thiobarbituric acid, at 37°C for 35 min, respectively, then 1×10^6 cells were harvested and resuspended in PBS. Absorbance was detected at 450 nm and 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, then 1×10^6 cells were harvested and resuspended in PBS.

Table 1. Primer sequence for the ataxia telangiectasia-mutated (ATM)-small interfering RNA (siRNA) tested

Name	Primer sequence (5'→3' orientation)
LV-ATM-siRNA1	
Sense	TGAGGAAATACTGGTGTGAAA
Antisense	TTTCACACCAGTATTTCTCTCA
LV-ATM-siRNA2	
Sense	AATCAAGAAAGTGGAGAAGTA
Antisense	TACTTCTCCACTTTCTTGATT
LV-ATM-siRNA3	
Sense	TTCAGAAGATGCTGTGAGAAA
Antisense	TTTCTCACAGCATCTTCTGAA

The optical density at 450 nm was recorded with a microplate reader (Molecular Devices, Sunnyvale, CA).

ARE Fluorescence Activity

The ARE fluorescence activity was tested in cultured BMEC without or with H₂O₂ via an ARE reporter gene according to the manufacturer's instruction (Beyotime Biotechnology). 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 (LAR II) was added to the BMEC and 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 (Beyotime Biotechnology); 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 (Beyotime Biotechnology). The RLU1/RLU2 ratio was used to determine the transfection efficiency of ARE.

Real-Time Reverse-Transcription PCR Analysis

The reverse-transcription (**RT**) PCR was performed with SYBR Premix Ex Taq II (TaKaRa, Otsu, Japan) on a Chromo 4 continuous fluorescence detector with a PTC-200 DNA Engine Cycler (Bio-Rad, Hercules, CA) to determine the mRNA expression of *NFE2L2*, *HMOX-1*, *NQO-1*, *GCLC*, *GCLM*, and β -actin (internal control). The cycling conditions were as follows: pre-denaturation at 95°C for 30 s followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 20 s, at 72°C for 15 s, and extension at 72°C for 30 s. The specificity of the PCR amplification was verified by a dissociation curve analysis. All reactions were run in triplicate. The primers used for RT-PCR are listed in Table 2. Analyses of gene expression data were done using the $2^{-\Delta\Delta C_t}$ (Livak) method (Livak and Schmittgen, 2001).

Western Blot Analysis

After treatment, cells were washed twice with 4°C PBS (0.01 M, pH 7.2–7.3) and 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 (catalog no. 78510; Thermo Fisher Scientific, Waltham, MA) containing inhibitor cocktail (100 $\times$, catalog no. 78442; Thermo Fisher Scientific). Total protein concentration was determined using the BCA Protein Assay Kit (Sigma, St. Louis, MO). Protein samples were boiled for 20 min at 100°C. Fifty micrograms of total protein per lane was resolved by 10% SDS-PAGE (Bio-Rad) and then transferred from the gel to a polyvinylidene difluoride membrane (0.45 μ m, 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), which 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 ATM (catalog no. ab103970, Abcam, Cambridge, MA), NFE2L2 (catalog no. ab137550, Abcam), HMOX-1 (catalog no. ab13248, Abcam), NQO1 (catalog no. ab 28947, Abcam), GCLC (catalog no. ab 80841, Abcam), and GCLM (catalog no. ab81445, Abcam) with gentle agitation at 4°C overnight. After incubating with the primary antibody, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (horseradish peroxidase-labeled sheep anti-mouse, 1:50,000; horseradish peroxidase-labeled sheep anti-rabbit, 1:50,000; catalog no. ab6721, Abcam) in TBST for 1 h at room temperature. The membranes were washed and then incubated with electro-chemi-luminescence reagent (catalog no. 170–5060; Bio-Rad). β -Actin (catalog no. ab8226; Abcam) were used as internal control. 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).

Statistical Analysis

All data are expressed as the means $\pm$ standard deviation of the mean from the indicated number of independent experiments performed in triplicate. One-way ANOVA followed by a Student–Newman–Keuls multi-

Table 2. Primers of downstream genes in the Nrf2-antioxidant response element (ARE) signaling pathway

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')	Accession number
<i>NFE2L2</i>	CCAGCACAACACATACCA	TAGCCGAAGAAACCTCATT	001011678.2
<i>HMOX-1</i>	GAACGCAACAAGGAGAAC	CTGGAGTCGCTGAACATAG	001014912.1
<i>NQO1</i>	CAACAGACCAGCCAATCA	ACCTCCCATCCTTTCCTC	001034535.1
<i>GCLC</i>	ATTGGGTGGAGAGTGGAA	ACAGCGGATGAGAAAGT	001083674.1
<i>GCLM</i>	CCGATGAAAGAGAAGAAATG	CAACAGGAGGTGAAGCAA	001038143.1

ple-comparison test was used to determine statistical significance for multiple comparisons, and Student's *t*-test was used for 2 group comparisons. Differences were considered significant at $P < 0.05$ and very significant at $P < 0.01$. All statistical tests were carried out using SAS 9.0 (SAS Institute Inc., Cary, NC).

RESULTS

NFE2L2 Phosphorylation

The NetPhos 3.1 software can predict phosphorylated sites and the identity of the protein kinase (Blom et al., 1999, 2004). Thus, it was used to predict the possible phosphorylation positions on NFE2L2 (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-13954>). Results revealed that NFE2L2 is mainly phosphorylated at serine, threonine, and tyrosine sites, and it was also predicted that phosphorylation of NFE2L2 would occur through the kinases ATM serine/threonine kinase (ATM), cyclin-dependent kinase inhibitor, casein kinase II, calmodulin kinase II, DNA-dependent protein kinase, epidermal growth factor receptor, glycogen synthase kinase, insulin receptor, protein kinase A, protein kinase B, protein kinase C, protein kinase G, recombinant streptokinase, tyrosine kinase C, cell division cycle kinase 2, cyclin-dependent kinase 5, and p38 mitogen-activated protein kinase. Subsequently, we used the protein phosphorylation analysis software Scansite (<http://scansite.mit.edu>) to predict protein kinases that could phosphorylate NFE2L2 (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-13954>). The highest score was for ATM with threonine 457 (T457) on NFE2L2 as the target for ATM. Thus, this analysis further suggested that ATM may be the physiologically relevant kinase that phosphorylates NFE2L2.

Verification and Screening of ATM-siRNA Interference Fragments

The BMEC were transfected with different ATM-siRNA interference fragments (ATM-siRNA1, ATM-siRNA2, and ATM-siRNA3) and cultured for 48 h without (control) or with H_2O_2 to induce oxidative stress. Changes in protein and mRNA expression were detected with RT-PCR and Western blot (Figure 1). The results showed that compared with the control group, transfected ATM-siRNA1, ATM-siRNA2, and ATM-siRNA3 reduced protein and mRNA expression of ATM with the best interference efficiency achieved with ATM-siRNA3 (>94.1%; $P < 0.01$). Thus, ATM-siRNA3 was chosen as the interference fragment for subsequent experiments because it was the most efficient and least cytotoxic among the siRNA (Figure 1).

Effect of ATM Expression on the Proliferation Rate of BMEC

The BMEC were transiently transfected with ATM-siRNA3 and the control scrambled-siRNA with or without exposure to H_2O_2 . The MTS assay was used to investigate cell proliferation rate after transfection for 48 h (Figure 2). The results revealed that transfection with scrambled-siRNA and ATM-siRNA3 without H_2O_2 decreased ($P < 0.05$) the proliferation rate by 25.8 and 36.2% compared with the control group. However, under oxidative stress, transfection with scrambled-siRNA and ATM-siRNA3 decreased ($P < 0.01$) the proliferation rate by 52.9 and 60.7% compared with the control group (Figure 2). No apparent difference in cell proliferation rate was detected between the scrambled-siRNA and ATM-siRNA3 without H_2O_2 , between the scrambled-siRNA and ATM-siRNA3 with H_2O_2 , but all of these were significantly lower than the control BMEC. In contrast, 600 μM H_2O_2 caused a significant ($P < 0.01$) reduction in BMEC proliferation rate compared with control BMEC and BMEC without H_2O_2 . These data further suggested that ATM silencing decreased the ability of BMEC to cope with H_2O_2 -induced cytotoxicity.

Effect of Regulation of ATM on Antioxidant Enzyme Activities in BMEC

Activities of SOD, GSH-Px, CAT, and GST and production of ROS and MDA were analyzed after treatment of BMEC with or without H_2O_2 (600 μM) for 6 h, followed by scrambled-siRNA and ATM-siRNA3 transfection for 48 h. As shown in Figure 3, the transfection

Figure 1. Screening of the efficacy of ataxia telangiectasia-mutated (ATM)-small interfering RNA (siRNA) fragments. The mRNA and protein expression of ATM after siRNA treatment was detected with real-time reverse transcription PCR and Western Blot. * $P < 0.05$, ** $P < 0.01$, differ from untreated cells. Error bars indicate SEM.

of ATM-siRNA3 without H_2O_2 sharply decreased SOD ($P < 0.05$), CAT ($P < 0.05$), GSH-Px ($P < 0.05$), and GST ($P < 0.05$) activity compared with the control. It also increased ROS ($P < 0.01$) and MDA ($P < 0.01$) production compared with the control. A similar effect was observed in cells transfected with ATM-siRNA3 and incubated with H_2O_2 . Compared with all groups under normal conditions, the transfection of ATM-siRNA3 and scrambled-siRNA plus addition of H_2O_2 decreased SOD ($P < 0.05$), CAT ($P < 0.05$), GSH-Px ($P < 0.05$), and GST ($P < 0.01$) activity. Concentration of ROS ($P < 0.05$) and MDA ($P < 0.05$) also decreased (Figure 3). These results further support the hypothesis that ATM serine/threonine kinase is essential for induction of several antioxidant defense enzymes in BMEC under oxidative stress. Results also suggest that the antioxidant enzyme system is impaired in BMEC exposed to supraphysiologic levels of H_2O_2 .

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

To further determine whether ATM has a significant effect on the NFE2L2-ARE pathway in control or ATM-silenced BMEC, and which downstream genes in the NFE2L2-ARE pathway are most affected, we examined

the protein and mRNA abundance of NFE2L2 and 4 key phase II detoxifying enzymes of the NFE2L2-ARE pathway: HMOX-1, NQO-1, GCLC, and GCLM. Figures 4 and 5 show the expression of the 4 main phase II detoxifying genes in BMEC after the treatment with and without 600 μM H_2O_2 for 6 h. The RT-PCR and Western blot results in Figures 4 and 5 demonstrate that transfection of ATM-siRNA3 sharply decreased the protein and mRNA expression of NFE2L2 ($P < 0.05$), HMOX-1 ($P < 0.05$), NQO1 ($P < 0.05$), GCLM ($P < 0.05$), and GCLC ($P < 0.01$) under normal conditions compared with the control group, and was lower compared with the transfected scrambled-siRNA ($P < 0.05$). Under oxidative stress, compared with the control group, the transfection of ATM-siRNA3 sharply decreased the protein and mRNA expression of NFE2L2 ($P < 0.01$), HMOX-1 ($P < 0.05$), NQO1 ($P < 0.05$), GCLM ($P < 0.05$), and GCLC ($P < 0.01$), and the protein and mRNA expression of HMOX-1 ($P < 0.05$) was significantly lower than NQO1, GCLM, and GCLC. In all cases, expression was lower compared with the transfection of scrambled-siRNA ($P < 0.05$).

These results suggest that ATM silencing decreased the expression levels of NFE2L2 and all 4 genes in BMEC with or without H_2O_2 , and revealed that HMOX-1 is the main antioxidant target gene protect-

Figure 2. Effects of ataxia telangiectasia-mutated (ATM)-small interfering RNA (siRNA) on the proliferation rate of cultured bovine mammary epithelial cells (BMEC). Cells were cultured with H_2O_2 (600 μM) for 6 h, followed by scrambled-siRNA and ATM-siRNA3 transfection for 48 h before measuring proliferation rate in oxidative stress-damaged and normal BMEC. * $P < 0.05$, ** $P < 0.01$ compared with the control group. Error bars indicate SEM. SCR-siRNA = scrambled-siRNA.

Figure 3. Effect of ataxia telangiectasia-mutated (ATM) on antioxidative enzyme activity in cultured bovine mammary epithelial cells (BMEC). Cells were cultured with H₂O₂ (600 μ M) for 6 h, followed by scrambled-small interfering RNA (siRNA) and ATM-siRNA3 transfection for 48 h before measuring superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and glutathione-S-transferase (GST) activity, and reactive oxygen species (ROS) and malondialdehyde (MDA) production in oxidative stress-damaged and normal BMEC. * P < 0.05, ** P < 0.01 compared with the control group. Error bars indicate SEM. SCR-siRNA = scrambled-siRNA.

ing BMEC from H_2O_2 -induced oxidative stress in the NFE2L2-ARE pathway.

Impaired NFE2L2 Transcriptional Activity in the NFE2L2-ARE Pathway

The normal BMEC and the oxidative-damaged BMEC were transiently transfected with an ARE-luciferase reporter plasmid (pGL3-ARE) and a Renilla luciferase reporter plasmid (pRLtk) followed by treatment with

600 μM H_2O_2 for 0, 6, 12, and 24 h before the luciferase activity was measured (Figure 6). The results showed that treatment with H_2O_2 increased ($P < 0.05$) the transcriptional activity of NFE2L2 in a time-dependent manner in both normal and oxidative-damaged groups. In contrast, in the presence of H_2O_2 , ARE luciferase activity of oxidative-damaged BMEC was much lower ($P < 0.05$) than normal BMEC. These data clearly indicate that NFE2L2-driven transcriptional activation of ARE is aberrant in oxidative-damaged BMEC.

Figure 4. Effects of ataxia telangiectasia-mutated (ATM)-small interfering RNA (siRNA) on downstream expression of genes associated with the nuclear factor erythroid 2-related factor 2-antioxidant response element (ARE) signaling pathway in cultured bovine mammary epithelial cells (BMEC). Cells were cultured with H_2O_2 (600 μM) for 6 h, followed by scrambled-siRNA and ATM-siRNA3 transfection for 48 h before measuring the mRNA expression of nuclear factor erythroid 2-related factor 2 (NFE2L2), heme oxygenase-1 (HMOX-1), NADH quinone oxidoreductase 1 (NQO1), glutamyl cystine ligase modulatory subunit (GCLM), and glutamate-cysteine ligase catalytic subunit (GCLC) in oxidative stress-damaged and normal BMEC. CON = control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control group. Error bars indicate SEM. SCR-siRNA = scrambled-siRNA.

DISCUSSION

Several studies in nonruminant cells indicate that excess production of ROS when endogenously or exogenously produced ROS exceed cellular antioxidant defenses may cause oxidative damage (Jena, 2012). Oxidative stress leads to induction of lipid peroxidation and protein modifications followed by cellular dysfunction and disease (Deng et al., 2017). It is well established that oxidative stress is related to the health status of the udder and mammary secretory cell number (Rainard et al., 2013; Thompson-Crispi et al., 2014). A recent study revealed that oxidative stress

causes impaired function of BMEC (Jin et al., 2016), with the NFE2L2-ARE pathway apparently playing an important role in the cell's defense mechanisms against oxidative stress. Therefore, the NFE2L2-ARE pathway and its downstream genes may protect BMEC from H₂O₂-induced oxidative damage.

The protein NFE2L2 is a redox-sensitive transcription factor that can regulate the expression of several cytoprotective genes (Tang et al., 2011). At least in nonruminants, the ARE pathway controlled by NFE2L2 is one of the main regulators of redox signaling. Activation of ARE stimulates the production of phase II detoxifying enzymes with antioxidant properties that

Figure 5. Effects of ataxia telangiectasia-mutated (ATM)-small interfering RNA (siRNA) on downstream expression of genes associated with the nuclear factor erythroid 2-related factor 2-antioxidant response element (ARE) signaling pathway in cultured bovine mammary epithelial cells (BMEC). Cells were cultured with H₂O₂ (600 μ M) for 6 h, followed by scrambled-siRNA and ATM-siRNA3 transfection for 48 h before measuring the protein expression of nuclear factor erythroid 2-related factor 2 (NFE2L2), heme oxygenase-1 (HMOX-1), NADH quinone oxidoreductase 1 (NQO1), glutamyl cystine ligase modulatory subunit (GCLM), and glutamate-cysteine ligase catalytic subunit (GCLC) in oxidative stress-damaged and normal BMEC. CON = control. * $P < 0.05$, ** $P < 0.01$ compared with the control group. Error bars indicate SEM. SCR-siRNA = scrambled-siRNA.

Figure 6. Fluorescence activity of the antioxidant response element (ARE) in control and oxidative stress bovine mammary epithelial cells. Fluorescence activity of ARE in the control and oxidative stress cells was detected with the Biluciferase gene reporter assay after treatment of cells with H_2O_2 (600 μM) for 0, 6, 12, or 24 h. # $P < 0.05$, ## $P < 0.01$ compared with the control; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with 0 h. Error bars indicate SEM.

protect cells from oxidative damage (Johnson et al., 2008). The NFE2L2-ARE signaling pathway is a vital regulator of oxidative damage and inflammation, both of which are involved in various types of pathologies in various organs including the mammary gland (Chen et al., 2011; Jian et al., 2011).

In the current study, we identified the potential phosphorylation sites on NFE2L2 through in silico analysis, and found that ATM serine/threonine kinase is a key kinase responsible for the phosphorylation of NFE2L2. Furthermore, we demonstrated that inhibition of ATM activity or ATM silencing can cause marked alterations in oxidative stress leading to decreased BMEC proliferation rate and cell death. Thus, H_2O_2 -induced cytotoxicity diminishes the capacity of BMEC to cope with this type of stress. This evidence seems to support ATM serine/threonine kinase as a physiologically relevant NFE2L2 kinase.

The ATM serine/threonine kinase protein is activated by breaks in DNA or chromatin induced during meiosis or oxidation (Lavin, 2008). In nonruminants under physiological conditions, ATM mediates the activation of multiple signal transduction pathways (Brown et al., 1999). The protein is primarily localized to the nucleus as a catalytically inactive homodimer (Elson et al., 1996). Once activated, ATM phosphorylates serine/threonine-glutamine (S/T-Q) motifs on more than 700 protein substrates (Brown et al., 1999). Indeed, a recent large-scale phosphoproteomics screen identified cytoplasmic ATM activation in response to

oxidative stress with over 2,500 unique protein targets (Kozlov et al., 2016). The lower ARE luciferase activity in response to H_2O_2 stimulus strongly suggested that the NFE2L2-ARE pathway is dysfunctional when a supraphysiological concentration of H_2O_2 is produced in BMEC.

The antioxidant enzymes SOD, GSH-Px, CAT, and GST can scavenge ROS to prevent cell damage, and numerous reports from research with nonruminant systems have confirmed that NFE2L2 plays a crucial role in eliciting adaptive responses to diverse stresses by upregulating these enzymes (Miranda et al., 2011), all of which helps restore cellular redox status (Son et al., 2010; Magesh et al., 2012; Tsou et al., 2015). Therefore, activation of the NFE2L2-ARE pathway might potentially be a new target in the treatment of oxidative stress in the dairy cow mammary gland (Loor et al., 2013). In this study, we confirmed that silencing ATM increased ROS and MDA concentrations when challenging with a supraphysiological dose of H_2O_2 because of a decrease in antioxidant enzyme activity. Thus, the data support the role of ATM in the antioxidant response of BMEC, which is in accordance with previous reports in nonruminant cells (Kamsler et al., 2001; Perry and Tainer, 2011).

Among the known antioxidant enzymes, HMOX-1 is an inducible antioxidant enzyme and protects cells against oxidative stress. Expression of HMOX-1 is triggered by diverse stressors including UV radiation, hypoxia, inflammation, H_2O_2 , and nitric oxide (Moterlini, 2000; Naughton et al., 2002). The decrease in antioxidative enzyme activity, protein, and mRNA expression level of NFE2L2, HMOX-1, NOQ1, GCLC, and GCLM, along with the increased concentration of ROS and MDA in response to supraphysiological treatment with H_2O_2 when ATM was silenced, provided a strong indication that ATM kinase plays an important role in the NFE2L2-ARE signaling pathway. Thus, as demonstrated in vitro (Matsuda, 2014; Biagiotti et al., 2016) and in vivo (Matsuda, 2014) in nonruminant cells, manipulating ATM kinase activity might be a useful strategy for controlling oxidative stress.

Clearly, our data provide evidence that oxidative stress in response to a supraphysiological dose of H_2O_2 decreases NFE2L2-driven transcriptional activation of ARE in BMEC. The abnormal expression of ATM kinase is one key factor preventing NFE2L2 phosphorylation and nuclear activity. The exact mechanisms that control ATM expression remain unclear, but the present data offer support for ATM kinase as a novel target to help regulate or control activity of the NFE2L2-ARE pathway under oxidative stress.

CONCLUSIONS

Abnormal expression and activation of ATM serine/threonine kinase during oxidative stress compromises NFE2L2 activation, leading to lower antioxidative enzyme activity and expression of antioxidant genes. As such, cellular proliferation rates are decreased. Further studies should focus on the regulation of ATM serine/threonine kinase.

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