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Nuclear factor-like 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 H_2O_2 (600 μM) 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 H_2O_2, 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 H_2O_2. 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 H_2O_2 compared with the control. Transfected cells had markedly lower protein and mRNA expression of NFE2L2 without or with H_2O_2 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 H_2O_2-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 Griendling, 2008). For example, intracellular lipid peroxidation leads to production of H_2O_2 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 H_2O_2 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 H_2O_2-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 H_2O_2-induced oxidative stress (Jin et al., 2016). However, the mechanisms that prevent BMEC from dealing with H_2O_2-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 H_2O_2-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 mm^3 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% CO_2 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 μM H_2O_2 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 μL of 1 μg/mL hydrocortisone (Gibco), 100 μL of 2.5 μg/mL amphotericin B (Gibco), 10 μL of 10 ng/mL epidermal growth factor (Gibco), 50 μL of 5 μg/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