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Nuclear factor erythroid 2-related factor 2 antioxidant response element pathways protect bovine mammary epithelial cells against H_2O_2-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 H_2O_2-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 H_2O_2 (600 μM) 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 H_2O_2, respectively. These cells also had apoptosis and necrosis rates that were 27 and 3.5 times greater with H_2O_2 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 H_2O_2 compared with the control group, respectively. Apoptosis rates were 1.8 times lower with H_2O_2 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 glutathione-S-transferase (GST) increased markedly in cells transfected with pCMV6-XL5-NFE2L2 and without H_2O_2. 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 H_2O_2. Compared with the control, cells transfected with NFE2L2-siRNA3 with or without H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 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 (Loor 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 (Szőcs, 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 dissociation 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 H_2O_2-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 H_2O_2-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 H_2O_2 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,