J. Dairy Sci. 99:1–10
http://dx.doi.org/10.3168/jds.2016-11031

Nuclear factor-like factor 2-antioxidant response element signaling activation by tert-butylhydroquinone attenuates acute heat stress in bovine mammary epithelial cells

X. L. Jin,*† K. Wang,† L. Liu,*† H. Y. Liu,*†[1] F.-Q. Zhao,*‡ and J. X. Liu*†[1]
*Institute of Dairy Science, Key Laboratory of Molecular Animal Nutrition, Ministry of Education, and
†College of Animal Sciences, Zhejiang University, Hangzhou 310058, P.R. China
‡Laboratory of Lactation and Metabolic Physiology, Department of Animal and Veterinary Sciences, University of Vermont, Burlington 05405

ABSTRACT

Nuclear factor (erythroid-derived 2)-like factor 2 (Nrf2) is a transcription factor that binds to the antioxidant response element (ARE) in the upstream promoter region of many antioxidative genes. The Nrf2-ARE signaling plays a key role in the cellular antioxidant-defense system, but whether Nrf2 activation has protective effects against heat shock (HS) stress in mammary epithelial cells (MEC) remains unclear. The objective of this study was to determine whether tert-butylhydroquinone (tBHQ), a well-known Nrf2 activator, could attenuate heat stress-induced cell damage in MAC-T cells of the bovine MEC line. The MAC-T cells were exposed to HS (42.5°C for 1 h) followed by recovery at 37°C to mimic HS. Compared with cells that were consistently cultured at normothermia (37°C), the cell viability levels significantly decreased after HS stress. In parallel, heat stress increased the reactive oxygen species levels and induced cellular apoptosis and endoplasmic reticulum stress. The MAC-T cells that were pretreated with tBHQ (10 μM) for 2 h followed by HS had a reduction in the loss of cell viability. The tBHQ pretreatment significantly decreased cellular reactive oxygen species levels and stress-related marker gene expression. The tBHQ-treated MAC-T cells showed strong Nrf2-ARE signaling activation and a nuclear accumulation of Nrf2 and upregulated expression of Nrf2-ARE downstream genes. Small interfering RNA silencing of Nrf2 in HS-treated MAC-T cells almost completely abolished the cytoprotective effects by tBHQ. Overall, our results demonstrated that HS could cause cell damage in cultured bovine MEC, and that activation of Nrf2 by tBHQ could attenuate HS-induced cell damage.
Key words: antioxidation, bovine mammary epithelial cell, heat shock, tBHQ

INTRODUCTION

Dairy cows are sensitive to high temperature and easily suffer stress when the temperature-humidity index exceeds 72, resulting in reduced milk productivity and economic losses (Rhoads et al., 2009; Bernabucci et al., 2014). Heat-stressed cows are mainly characterized by high body temperatures accompanied with increased respiration rates and water consumption and reduced feed intake and milk yield. Meanwhile, physiological disturbances, increased susceptibility to parasites, and disease occurrences are often observed in heat-stressed cows (Hahn, 1999). Under heat stress, cows have reduced antioxidant capacity and over-production of free radicals at systemic and cellular levels, suggesting that free radicals may play important roles in heat stress (Liu et al., 2010; Chauhan et al., 2014). Therefore, restoring the redox balance and strengthening the antioxidative capacity of the cows by supplying sufficient antioxidants or enhancing expression of antioxidant enzymes can be efficient management tools to protect dairy cow from heat stress.

Nuclear factor (erythroid-derived 2)-like factor 2 (**Nrf2**) is a transcription factor that binds to the antioxidant response element (**ARE**) in the upstream promoter region of many antioxidative genes. Signaling of Nrf2-ARE regulates expression of several different antioxidant enzymes involved in the elimination of reactive oxygen species (**ROS**), which are widely acknowledged as essential chemical agents that cause oxidative stress (Ma, 2013). We previously validated that the activation of Nrf2-ARE signaling promotes MAC-T cells, a bovine mammary epithelial cell (**bMEC**) line, survival following hydrogen peroxide treatment, which induces

Received February 15, 2016.
Accepted July 16, 2016.
[1]Corresponding authors: hyliu@zju.edu.cn; liujx@zju.edu.cn

cellular oxidative damage as well as endoplasmic reticulum (**ER**) and mitochondria stress-induced cell death (Jin et al., 2016). For some drugs, Nrf2-ARE signaling activation contributes to their critical therapeutic or preventative effects in alleviating inflammation and combating other diseases in humans and in animals, such as various cancers, cardiovascular diseases, and Alzheimer diseases (Liby et al., 2005; Zhu et al., 2005). Activation of Nrf2 has been proven to be a molecular target candidate, and its promising effects have been shown to promote human and animal health (Ma, 2013).

Tert-Butylhydroquinone (**tBHQ**), a widely used food additive, is one of the most potent inducers of Nrf2 activity by increasing Nrf2 protein stability (Kraft et al., 2004). The stabilization of Nrf2 by tBHQ mainly occurs by blocking the ubiquitination and degradation of Nrf2 and by promoting its nuclear translocalization, which leads to increased ARE-mediated gene expression levels (Ma, 2013). These effects have been partly explained as a result of a direct reaction between the thiol group of Keap1 and tBHQ (Kraft et al., 2004; Gharavi et al., 2007; Wang et al., 2008). In vivo and in vitro studies have shown that the tBHQ-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against various cellular or body dysfunctions (Eftekharzadeh et al., 2010). However, it is not known whether any protective effects exists on heat shock (**HS**) stress by the tBHQ activation of Nrf2-ARE in bMEC. Therefore, the aims of our study were to determine whether tBHQ can provide protection against heat stress-induced cell damage in cultured bMEC and to elucidate the roles of Nrf2 during this protection.

MATERIALS AND METHODS

Chemical Reagents

Tert-Butylhydroquinone (112941), 2′,7′-Dichlorofluorescin diacetate (**DCHF-DA**, D6883), and alkaline phosphatase-conjugated secondary antibody (anti-rabbit IgG) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco′s Modified Eagle Medium (**DMEM**), fetal bovine serum, and penicillin-streptomycin were obtained from Life Technologies (Thermo Fisher Scientific, Boston, MA). Primary antibodies against β-tublin, Nrf2, heme oxygenase 1 (**HO-1**), thioredoxin reductase 1 (**Txnrd-1**), Bax, and Bcl2 were purchased from Abcam (Cambridge, MA), and primary antibodies that recognize total and phosphorylated eukaryotic initiation factor 2α (**eIF2α**) were obtained from Cell Signaling Technology (Danvers, MA). The commercial kits used in this study included a CCK-8 kit (Dojido, Kumamoto, Japan), RNApure kit (Aidlab Biotechnologies, Beijing, China), PrimeScript RT reagent (Takara, Dalian, China), SYBR premix EX Taq (Takara), RIPA lysis buffer (Beyotime, Haimen, China), and a BCA protein quantification kit (Beyotime). Other analytical grade chemicals were purchased from Sangon Biotechnology (Shanghai, China).

Cell Culture and Acute HS Treatment

The MAC-T cells were cultured in DMEM that was supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin (Sigma-Aldrich; Huynh et al., 1991). The cells were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air. The HS procedure was performed as described in our previous study (Liu et al., 2010). Briefly, the dispersed cells were seeded at a density of 1×10^5 cells/mL and cultured for 24 h under normal conditions (37°C) before the cells were challenged under a HS condition. The medium was replaced by 42.5°C prewarmed normal culture medium, then the cells were continuously maintained at this temperature for 1 h. The cells were then returned to the normal culture condition, and the time point when the cells were returned was recorded as 0 h. Cells consistently cultured in 37°C conditions were used as corresponding controls.

Cell Viability and tBHQ Toxicity Determination

Cell viability and the tBHQ toxicity evaluations were performed using a CCK-8 kit according to the manufacturer's instruction. For tBHQ toxicity determination, various concentrations of tBHQ were added to MAC-T cells for 24 h. After optimal tBHQ concentration was defined, MAC-T cells were pretreated with tBHQ for 2 h before HS. At the end of the experiments, 10 μL/well of CCK-8 reagent was added to the cells cultured in triplicate wells in the 96-well plates. After incubation for 2 h, the optical density at 450 nm was recorded with a microplate reader (Molecular Devices, Sunnyvale, CA).

Intracellular ROS Detection

A DCHF-DA staining assay was used to detect intracellular ROS as described in our previous study (Wang et al., 2015). Briefly, MAC-T cells were washed with PBS and incubated with fresh DMEM containing 10 μM DCHF-DA at 37°C for 30 min, then 1×10^6 cells were harvested and resuspended in PBS. The percentage of fluorescence-positive cells was determined with