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The unfolded protein response is involved in both differentiation and apoptosis of bovine mammary epithelial cells

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ABSTRACT

The unfolded protein response (UPR) describes a process involved in the homeostasis of endoplasmic reticulum (ER) and the differentiation of secretory cells. At present, the roles of UPR in the mammary gland tissue of dairy cattle are unknown. In the current study, we investigated the expression of UPR-related genes in Holstein cows during the developmental and lactating stages of the mammary gland tissue. To investigate the roles of UPR during the differentiation of mammary epithelial cells (MEC), we used MAC-T cells, a line of MEC. We collected samples of mammary gland tissue in dairy cows by biopsy during the late gestation and lactation periods and examined the expression of UPR-related genes by quantitative real-time PCR. Expression levels of the spliced X-box binding protein 1 (*XBP1*) and activating transcription factor 4 (*ATF4*) were found to be significantly higher in the mammary gland tissue 10 d before delivery compared with 40 d before delivery. An investigation before and after differentiation in MAC-T cells showed that the expression of *ATF4* increased after differentiation of MEC, whereas that of the spliced *XBP1* did not significantly change. Western blot analysis revealed that the differentiation-inducing stimulus induced phosphorylation of eukaryotic initiation factor 2α (eIF2α) but reduced that of protein kinase RNA-like endoplasmic reticulum kinase (PERK). Additionally, in ATF4-knockdown bovine MEC, differentiation was significantly suppressed; ATF4 knockdown also significantly suppressed the expression of glucocorticoid and insulin receptors. These results revealed that ER stress–independent ATF4 is involved in the cell differentiation mechanism, either directly or indirectly, via the control of the expression of lactogenic hormone receptors in bovine MEC. Immediately after parturition, gene expression levels of the spliced *XBP1*, *ATF4*, and C/EBP homologous protein (*CHOP*) markedly increased in mammary gland tissue, with a strong negative correlation between expression of *CHOP* and initial milk yield; CHOP is an apoptosis-related protein induced by ER stress. The above findings indicate that UPR is intrinsically associated with apoptosis of MEC, thus affecting the differentiation of these cells, as well as milk yield.
Key words: dairy cow, unfolded protein response (UPR), mammary epithelial cell

INTRODUCTION

The mammary gland is an exocrine tissue that synthesizes proteins in large quantities during lactation. For a proper lactation, the mammary gland needs to develop its functions over the period from gestation to lactation. Specifically, mammary epithelial cells (**MEC**) constituting the mammary alveoli need to differentiate into secretory cells that are capable of synthesizing milk. From gestation to lactation, the development of mammary glands is regulated by interactions among multiple hormones, including progesterone, prolactin, growth hormone, glucocorticoid, and epidermal growth factor (Neville et al., 2002). However, the detailed molecular mechanism for this process remains unknown. Once differentiated, MEC need to maintain their ability to synthesize proteins for an extended period; however, the mechanism to support this process remains unclear.

Endoplasmic reticulum (**ER**) is a subcellular organelle essential for the biosynthesis of secretory proteins. To exert its innate physiological functions, a newly

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synthesized secretory protein first needs to be folded into its correct steric conformation within the ER. Normal maturation of proteins can be disrupted by changes in the cellular environment, such as ischemia, and in calcium concentrations and by the production and overproduction of abnormal proteins translated from mutated genes. This causes the unfolded proteins to accumulate within the ER, thereby damaging the cells. Endoplasmic reticulum stress is defined as this type of loss of homeostasis within the ER (Ron and Walter, 2007). Cells possess a defense mechanism against functional abnormalities in the ER, which is referred to as unfolded protein response (**UPR**), which is widely conserved in eukaryotes from yeasts to mammals (Mori, 2000). Endoplasmic reticulum stress sensors are a group of molecules that detect abnormal proteins accumulated within the ER lumen and transmit signals to the nucleus and cytoplasm. Mammals possess 3 ER stress sensors: protein kinase RNA-like endoplasmic reticulum kinase (**PERK**), activating transcription factor 6 (**ATF6**), and inositol-requiring enzyme 1 (**IRE1**; Patil and Walter, 2001; Ron, 2002). The UPR comprises the following functions: (1) to augment the folding capacity by inducing the transcription of ER chaperones, (2) to suppress protein translation, and (3) to promote the decomposition of abnormal proteins by inducing the transcription of ER-associated protein degradation factors. All 3 stress sensors are ER transmembrane proteins, and each forms a characteristic effecter domain in the cytoplasm and transmitting signals downstream in different forms. When IRE1 is activated, unconventional spliceosome-independent splicing of X-box binding protein 1 (***XBP1***) mRNA was elicited; XBP 1 splicing form (the spliced *XBP1*) was produced from the spliced substrate *XBP1* mRNA (Calfon et al., 2002). After migrating into the nucleus, the spliced XBP1 functions as a transcription factor (Yoshida et al., 2003). The PERK protein phosphorylates eukaryotic initiation factor 2α (**eIF-2α**) to reduce its functions, thereby promoting the translation of the transcription factor ATF4 (Harding et al., 1999, 2000). A recent study suggested that ER stress through eIF-2α phosphorylation also promotes an increase in *ATF4* mRNA expression (Chan et al., 2017). Dependent on the ER stress, ATF6α is transported to the Golgi apparatus, where it is cleaved within the membrane by site 1 and 2 proteases. The N-terminal fragments from the cleavage reaction then migrate into the nucleus and serve as transcription factors (Ye et al., 2000). Under excessive or persistent stress, cells undergo apoptosis and die. In such cases, ATF4 induces the transcription of C/EBP homologous protein (**CHOP**), a transcription factor that promotes apoptosis (Zinszner et al., 1998).

The UPR has also been reported to be a requisite for the differentiation of pancreatic B cells that secrete insulin as well as for the differentiation of osteoblasts that secrete proteins constituting bone matrices, such as collagen (Lee et al., 2005; Saito et al., 2011). Furthermore, we revealed that UPR-related transcription factors XBP1 and ATF4 are involved in the differentiation of mammary gland epithelial cells in mice (Tsuchiya et al., 2017). Thus, UPR plays a critical role in the development of mammary glands, which are exocrine tissues that synthesize large quantities of proteins.

Years of selective breeding and improvement have made it possible to maintain high lactation volumes in dairy cows, including Holsteins. Invernizzi et al. (2012) observed transcriptional changes in UPR-related genes at different stages of the lactation cycle in Holstein cows. A recent study has indicated that exogenous essential amino acids regulate the expression of UPR-related gene expression in bovine mammary gland (Nichols et al., 2017). However, roles of UPR in the mammary gland tissue of cows remain unknown. In this study, we examined the expression levels of UPR-related genes in the mammary gland tissue of Holstein cows during the developmental and lactation stages. Using MAC-T cells (a MEC line), we investigated the roles of UPR in the differentiation of MEC. Additionally, we assessed the correlation between initial milk yield and expression levels of UPR-related genes.

MATERIALS AND METHODS

All experiments were conducted in accordance with the "Guideline for the Institute of Livestock and Grassland Science, NARO" and approved by the Animal Care Committee of Institute of Livestock and Grassland Science, NARO (Tochigi, Japan).

Animals and Sample Collection

Ten multiparous Holstein cows (parity 2.5 ± 0.4) were used during the peripartum period (–50 to 40 d postpartum) to carry out the mammary gland tissue biopsy sampling. During the experiment, except around calving, the cows were housed in individual tiestalls or freestalls. Cows remained in the calving pen around the time of calving (3 d prepartum to 3 d postpartum). They had free access to fresh water and trace mineral salt blocks. The diet was formulated to meet or exceed the Japanese Feeding Standard for Dairy Cattle (NARO, 2006). The cows were offered diets for ad libitum intake twice daily (0930 and 1630 h) prepartum and 3 times (0930, 1500, and 1800 h) postpartum. The diets were fed as fermented TMR or unfermented TMR, which was based on corn silage, timothy hay, alfalfa hay,