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Study on the effect of heat stress on mammary epithelial cells and its prevention

By

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Study on the effect of heat stress on mammary epithelial cells and its prevention

（暑熱ストレスが乳腺上皮細胞に及ぼす影響とその予防に関する研究）

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Abstract

Global warming negatively influences productive parameters of dairy cattle including milk yield, milk composition, growth, and reproduction. Heat stress (HS) is a major environmental factor raising dairy cows in the Tropical and Subtropical countries of the world. It is traditionally believed that HS results in lower dry matter intake (DMI), which reduces the production of milk yield and protein contents of milk in dairy cows. However, several studies have reported that the decrease in milk production in HS accounts for 35% only by reduced feed intake. These results suggest that factors other than a reduction in energy intake may be responsible for reduced milk production, but the other factors and their mechanisms are unknown. The number of mammary epithelial cells (MECs) and their secretory activity determine the amount of milk produced during a lactation period. The secretory capacity of MECs also depends on the formation of tight junction (TJ) in mammary gland. However, the effect of HS on the number of MECs and TJ formation is still unknown. To solve this question, this study focused on unfolded protein response (UPR) activated under the endoplasmic reticulum (ER) stress condition, since accumulating evidence indicated that ER stress-mediated apoptotic cell death plays a critical role in HS-induced cellular damage. Therefore, the purpose of present study was to find out how different level of temperature affects TJ formation and viability of MEC based on the UPR signaling and its prevention.

To achieve the above objectives, MECs were culture at 39 °C temperatures (considered as mild-HS) or at ≥ 41 °C (considered as severe-HS). Severe HS (41 °C), but not 39 °C, significantly increased the transcript levels of C/EBP homologous protein (Chop), activating transcription factor 4 (Atf4), activating transcription factor 6 (Atf6) and glucose regulated protein 78 (Grp78). Moreover, severe HS significantly down regulated the TJ

protein encoding gene, *Cldn3* mRNA levels compared with the control. To measure the effect of different level of temperature on the viability of MECs, I performed 3-(4, 5-Dimethylthiazol 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Only 41 °C temperatures significantly reduced the viability of MEC. Therefore, severe HS (41 °C) induces all UPR component genes to cause severe ER stress, subsequently reduces MEC viability and decreased the TJ protein encoding gene expression. On the other hand, mild HS (39 °C) increased only Xbp1s expression among all UPR component genes. Moreover, the β-casein (a representative milk protein) and TJ protein encoding gene (Zo-1, Ocln and Cldn3) expression were significantly increased in 39 °C heat treatment cells. To explore the mechanism of TJ formation by mild HS (39 °C), I examined the expression level of temperature-sensitive transient receptor potential vanilloid 4 (Trpv4). The *Trpv4* mRNA level was significantly higher in mild HS (39 °C) treatment cells. Moreover, the mRNA levels of Zo-1, Ocln, Cldn3 and β-casein were significantly increased in response to the addition of GSK1016790A, which is a selective TRPV4 channel agonist. Therefore, mild HS (39 °C) regulates the TJ formation in MECs via cell membrane receptor TRPV4. In conclusion, the results indicate that mild heat stress induces the transcriptional level of Xbp1s via TRPV4 activity, which enhances the expression of the β-casein and TJ protein-encoding genes. On the other hand, severe heat stress induces the transcriptional level of Chop, which reduces the cell viability. It is speculated that the difference in UPR gene expression upon stimulation at 39 °C vs. 41 °C controls cell survival vs. cell death.

Based on the harmful effect of severe HS, an effective supplementation strategy is essential to maintain the cell population as well as adequate amount of milk production. 5-Aminolevulinic acid (5-ALA) is a natural non-alpha amino acid, which can reduce nephrotoxicity and apoptosis in murine tubular epithelial cells. Therefore, I investigated the possible protective effects of 5-ALA against HS-induced damage to bovine MECs.

Pretreatment with 5-ALA significantly increased the viability of MAC-T cells (bovine MECs line) and inhibited severe HS-induced ER stress-associated markers, GRP78 and CHOP expression levels. These results indicate that 5-ALA can ameliorate the HS induced ER stress and raise the cell viability.

In summary, the present study indicates that ER stress level is determined by severity of HS. Severe HS induces the ER stress with upregulation of all UPR component genes. In particular, severe HS enhances the expression of proapoptotic marker CHOP, which plays important role in reducing MEC viability. On the other hand, mild HS enhances only the expression of XBP1, which regulates the TJ formation. Moreover, it is found that 5-ALA can ameliorate the ER stress in heat stressed bovine MECs. Although detailed examination using dairy cows is necessary in the future, one of the factors causing the decrease in milk production through heat stress could be MECs death arising from ER stress. Moreover, 5-ALA is expected as a feed additive to control the HS in dairy cows.

Abbreviation

5-ALA	5-Aminolevulinic acid
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BAX	BCL2 associated X
BCL2	B-cell lymphoma 2
cDNA	Complementary DNA
CHOP	C/EBP homologous protein
Cldn3	Claudin 3
DIM	Dry matter intake
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
eIF2α	Eukaryotic translation initiation factor 2 subunit 1
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
gDNA	Genomic DNA
GRP78	Glucose-regulated protein 78
HO-1	Heme oxygenase-1
HS	Heat stress
IRE1	Inositol-requiring enzyme 1
MEC	Mammary epithelial cell
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NQO1	NAD(P)H quinone oxidoreductase 1

NRF2	Nuclear factor erythroid derived 2 like factor 2
Ocln	Occludin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
p-eIF2α	Phosphorylated-eIF2α
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
p-PERK	Phosphorylated-PERK
PVDF	Poly vinylidene difluoride
RIPA	Radio immune precipitation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative PCR
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Small interfering RNA
TJ	Tight junction
TRP	Transient receptor potential
TRPV4	Transient receptor potential vanilloid 4
UPR	Unfolded protein response
XBP1s	X-box binding protein 1 splicing form
XBP1u	X-box binding protein 1 unsplicing form
Zo-1	Zona occludens-1

Chapter I

General Introduction

The impact of global warming on dairy cattle production

Dairy farming is an important contributing sector in the economy of agricultural industries. There are several constraints to the sustainability of dairy farming, and worldwide, global warming is one of the major threats. Owing to global climate change, the yearly loss in the US dairy sector is approximately $1.2 billion (Key et al., 2014). The Intergovernmental Panel on Climate Change (IPCC) reported that the earth's temperature increases by 0.2 °C per decade and predicted that the average global surface temperature would increase to 1.4 °C–5.8 °C by 2100 (IPCC report 2007). The effects of rising temperatures on livestock vary worldwide, depending on latitude.

The thermoneutral area of the dairy animals varies from 16 °C to 25 °C, within which they maintain a physiological body temperature of 38.4 °C–39.1 °C (Youssef et al., 1985). However, air temperatures above 25 °C in tropical and subtropical countries enhance the body temperature of dairy cows (Das et al., 2016; Kumar et al., 2011). During high ambient temperatures, the body temperature increases because the heat-dissipating capacity of cows cannot disperse the heat owing to physiological limits. Therefore, the cows suffer from heat stress (HS) that negatively affects the physiology of lactation, growth, and other reproductive performance.

Lactating dairy cows are susceptible to HS compared with non-lactating cows owing to the enhanced metabolism required by increased milk production (Purwanto et al., 1990). In a heat-stressed cow, an immediate coping mechanism is initiated to reduce dry matter intake (DMI) (West, 2003; Rhoads et al., 2009). Reduction of DMI is a suitable way to

decrease heat production in hot environments because the heat increment of feeding is an important source of metabolic heat production in dairy cows (Kadzere et al., 2002). The ultimate effect of DMI reduction is the decreased milk production due to the inadequate supply of nutrients used for milk synthesis (West, 2003; Rhoads et al., 2009). Several studies have reported that only 35% of milk production responses can be explained by lower feed intake, and the remaining production losses can be attributed to changes in post-absorptive metabolism and nutrient partitioning (Rhoads et al., 2009; Wheelock et al., 2010). The above studies indicate that features other than DMI obstruct milk production under HS conditions. However, how the additional factors other than feed intake in a hot environment affect milk production and its original site of synthesis in the mammary gland remains unknown. Therefore, there is a need to determine the effect of HS at the cellular level in dairy cows for sustainable milk production.

Influence of HS on mammary epithelial cells of dairy cows

The capacity of the mammary gland to synthesize and store milk depends on the maintenance of the number and activity of secretory mammary epithelial cells (MECs) throughout lactation and the size of the alveoli (Manaman and Neville, 2003). Previously, milk yield was shown to decline after reaching a peak as the number of cells in mammary gland tissue diminishes. Cell numbers have been shown to decrease when the ratio of apoptotic cell death to epithelial proliferation is more than 1 (Knight and Peaker, 1984; Capuco et al., 2001). The secretory activity of MECs can be influenced by its extracellular structure, contributing to preserving the inner cellular components. Para-cellular barriers among the cells bear a crucial role in this regard. Loss of para-cellular barriers has been linked to reduced milk secretion function of mammary glands and increased para-cellular transport of blood components in milk, leading to disrupted cellular activity. The

maintenance of barriers between the neighboring cells is required for sufficient milk secretion (Brennan et al., 2010). Thus, cellular barriers influence the maintenance of milk production.

The temperature of the mammary gland is highly correlated with the body temperature of dairy cows and increases when exposed to HS (Bitman et al., 1984; Brown et al., 1977). When the cow is subjected to HS, cells in the mammary gland are exposed to high temperatures, showing the parallel responses of heat shock. In vitro studies indicated that bovine MECs show higher rates of programmed cell death in response to acute thermal stress (Jin et al., 2016; Tao et al. 2018), thereby reducing milk production. Also, HS decreases MEC proliferation (Tao et al., 2011). Therefore, a greater understanding of secretory activity and the number of MECs under HS conditions could be advantageous for improving milk production in the dairy industry.

Tight junction formation as a physical barrier between MECs of the mammary gland

The junctional complex between adjacent MECs plays a key role in cell–cell interaction. The tight junction (TJ) is an important member of the junctional complex. During the onset of milk secretion, TJs are formed between adjacent secretory epithelial cells in the mammary gland, consisting of two major transmembrane structural proteins, called occludin and claudin (Furuse et al., 1993; Furuse et al., 1998), which are bound to actin cytoskeletal via Zo-1 (Fanning et al., 1998). TJ is a cytoskeletal- and membrane-linked structure surrounding epithelial cells toward the apical proximity of the cell, whose basic function is to act as a barrier and fence. As a barrier, it prevents the para-cellular transport of ions and small molecules across the epithelia (Stevenson and Keon, 1998). TJ restricts the entrance of solutes between the lumen and basolateral bloodstream (Schneeberger et

al., 1992, and Itoh and Bissell, 2003). It is also the prerequisite for maintaining a small transepithelial potential difference between the basolateral and apical sides for the ideal secretory capacity of MECs. Thus, the maintenance of TJ is critical for the proper secretory activity and survival of MECs.

It is thought that HS disrupts the secretory capacity of MECs. However, a previous study indicated that mild HS at 39 °C enhances milk protein synthesis (Mizusawa et al., 2019). It is unclear whether HS affects TJ formation to increase the secretory capacity of the cell. Therefore, knowledge related to the intracellular mechanism of TJ formation under HS conditions needs to be examined.

Role of HS on cellular stress

The endoplasmic reticulum (ER) is an important intracellular organelle covered by a continuous membrane in all eukaryotic cells. ER plays a crucial role in regulating many cellular processes, including protein synthesis, protein folding and maturation, and lipid synthesis (Baumann and Walz, 2001). Multiple physiological or environmental conditions, such as elevated protein synthesis and secretion, failure of protein folding, and transport or degradation, affect protein folding, thereby leading to ER stress. The imbalance between the demand for protein folding and the capacity of the ER for protein folding is known as ER stress (Schroder and Kaufman, 2005). The cellular system activates signal transduction pathways to respond to imbalanced ER homeostasis, which are collectively called the unfolded protein response (UPR) pathway. The functions of the UPR include the following: (1) to reduce the unfolded protein load in the ER by attenuating translation, (2) to augment the folding capacity by inducing the transcription of ER chaperones, and (3) to enhance the decomposition of abnormal proteins by inducing the transcription of ER-associated protein degradation factors. Under excessive or persistent stress, cells undergo

apoptosis and die. The canonical mammalian UPR pathway has three branches: inositol-requiring protein 1α (IRE1α), PKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) (Patil et al., 2001). These transmembrane proteins serve as proximal sensors for ER stress. As an ER chaperone, BiP (also known as GRP78), binds to those sensor proteins and renders them inactive under non-stressed conditions. However, when excessive amounts of unfolded and misfolded proteins are gathered in the ER lumen, BiP dissociates from those sensors to perform its function as a chaperone (Munro et al., 1986). The activation of PERK induces the phosphorylation of eIF2α, resulting in the overall suppression of protein translation. In the case of prolonged ER stress, eIF2α initiates the expression of activating transcription factor 4 (ATF4), which induces the expression of the proapoptotic transcription factor C/EBP homologous protein (CHOP), to induce apoptosis (Harding et. al., 1999; Zinszner et al., 1998). IRE1α is the most conserved branch of the UPR and transphosphorylates itself in the UPR, leading to the splicing of the X-box binding protein-1 (XBP-1) mRNA in the cytosol (Calfon et al., 2002).

HS influences mammalian cells in various ways depending on the level of temperature or period of heat exposure or both. Accumulating evidence explained that severe HS (> 40 °C) causes cellular damage, which initiates a series of events that may lead to organ failure and death (Becker et al., 2011). Previous studies found the induction of ER stress and expression of the UPR pathway in the cell under severe HS conditions (Xu et al, 2011, Kim et al, 2013 and Hou et al., 2014). Severe ER stress stimulates the PERK-eIF2a-ATF4 pathway to induce CHOP, which is responsible for MEC apoptosis (Sharmin et al., 2020). The increased expression of CHOP in mammary gland tissue is negatively correlated with milk production in lactating dairy cows (Yonekura et al., 2018). It has already been mentioned that milk production is reduced by the reduction of the MECs (Capuco et al.,

2001). Thus, elevated expression of CHOP may be the cause of the reduction of MEC number. However, little information is available evaluating the effects of CHOP expression on maintaining the MEC population under HS conditions.

Alternatively, mild HS (< 40 °C) increases proliferation and differentiation, thereby positively regulating the biological activity of the living cell (Han et al., 2001 and 2002). Mild HS increases the secretory capacity of MECs in terms of β-casein (a representative milk protein and differentiation marker of MECs) synthesis. The expression of β-casein increases with the upregulation of UPR-induced XBP1 under mild HS conditions (Mizusawa et al., 2019). But the detailed molecular mechanism for maintaining the secretory activity in TJ formation under HS conditions remains unknown. Therefore, this study aimed to find out the effect of different temperature levels on the MEC number and secretory activity regarding TJ formation in the context of UPR.

Prevention against severe HS-induced MEC apoptosis

Based on the harmful effects of severe HS, it was essential to determine the alternative approach to reduce severe ER stress for maintaining the cell population and adequate milk production. Several studies showed the importance of antioxidant supplementation in alleviating HS effects in in vivo and in vitro trials (Kumar et al., 2011; Pandey et al., 2012). An antioxidant protects the cell against ER and oxidative stresses. It was recommended that HS induces oxidative stress in bovine MEC, thereby acting as an oxidative agent to reduce milk yield (Jin et al., 2016 and Li et al., 2019). A previous study suggested that oxidative stress can induce ER stress, leading to apoptosis (Bhandary et al., 2012). Thus, HS is the overriding factor for lowering the milk yield by introducing ER and oxidative stress in MEC. At the cellular level, oxidative stress occurs because of altered concentrations of prooxidants and antioxidants, leading to the overproduction of free

radicals and reactive oxygen species (ROS) and a reduction in antioxidant defense (Ganaie et al., 2013). Exposure to heat enhances ROS production and induces oxidative stress, which can lead to cytotoxicity (Lord-Fontaine et al., 2002). Nuclear factor erythroid-derived 2-like factor 2 (NRF2) plays an important role in eliminating ROS by activating antioxidant genes, including hemeoxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1), which are crucial for survival in oxidative-stressed mammalian cells (Amin et al., 2014; Han et al., 2017).

This study focused on 5-Aminolevulinic acid (5-ALA), which is a natural delta amino acid and not a protein component. It is found in different foods, such as spinach, green peppers, tomatoes, shiitake mushrooms, bananas, and potatoes. Larger amounts are found in fermented products, such as wine, vinegar, sake, and soy sauce. 5-ALA is a precursor for biosynthetic tetrapyrroles, such as chlorophyll, vitamin B12, and heme (Rodriguez et al. 2012). In animals, glycine, a non-essential amino acid, combines with succinyl-CoA to form 5-ALA in the presence of 5-ALA synthase as part of the heme biosynthetic pathway (Fujino et al., 2016). 5-ALA acts as an antioxidant and protects the cell against oxidative stress (Liu et al., 2019a and 2019b). Therefore, this study examined the potential effect of 5-ALA on HS-induced harmful effects in MECs.

Considering the above information, this study seeks to unravel the following questions: (1) how different temperature levels affect the number of MECs and their secretory activity in terms of TJ formation in connection with UPR signaling and (2) how the MECs population can be preserved from the harmful effects of severe HS. Accordingly, this study was composed of the following experiments: In chapter II, an *in vitro* study conducted to elucidate the effects of different temperature levels on the viability and secretory capacity regarding TJ formation of MEC is presented. In chapter III, another *in*

vitro study performed to discover the protective effects of 5-ALA on HS-induced MEC loss is presented. Finally, chapter IV discusses the impact of different temperature levels on maintaining the viability of MEC and TJ formation along with a preventive strategy in case of detrimental effects.

Chapter II

Effects of different level of temperature on the viability and tight junction formation of mammary epithelial cells

2.1. Abstract

Severe heat stress (41–43 ºC) leads to cell damage whereas mild heat (39–40 ºC) enhances the proliferation and differentiation of cells. It has been reported that heat treatment of MEC at 39 °C activated milk production and enhanced the formation of less-permeable TJ. However, the molecular mechanisms of this response have not been delineated. In this study, I investigated that how different level of temperature affects viability and milk secretion activity in terms of TJ formation of MEC. Moreover, I examined the effect of mild or severe heat stress on the expression of UPR-related genes. Present study observed that severe heat treatment at 41 °C induced the transcription level of all UPR component genes including the *Chop* in HC11 cells. Severe HS (41 °C) negatively influences the MECs viability, but not at 39 °C temperature. The increased expression of *Chop* at 41 ºC indicates the induction of severe ER stress induced apoptosis of MEC. Besides, among the UPR related gene only *Xbp1s* was significantly increased with mild HS at 39 °C compared to control (37 °C). Mild HS enhanced the mRNA expression of β-casein and TJ protein encoding gene- *Zo-1* (Zona Occludens-1), *Ocln* (Occludin) and *Cldn3* (Claudin 3), conversely severe heat stress reduce the expression of *Cldn3* mRNA. To elucidate the mechanism behind TJ formation under mild HS, I focused on temperature-sensitive transient receptor potential vanilloid 4 (TRPV4). Result showed that the accumulation of TRPV4 mRNA was higher in mild heat stressed cell. Moreover, Transcript levels of *β-casein*, *Zo-1*, *Ocln and Cldn3* were significantly increased in response to the addition of a

selective TRPV4 channel agonist (GSK1016790A) at 37 °C. TRPV4 stimulation with GSK1016790A also caused an increase in *Xbp1s* transcript level. The increase in *Zo-1, Ocln* and *Cldn3* mRNA accumulation levels in response to 39 °C heat treatment was suppressed by *Xbp1* knockdown. These results suggest that mild heat-shock causes an increase in *Xbp1s* transcript level via *TRPV4* activity and thereby enhances the expression of TJ protein-encoding genes whereas severe heat stress negatively influence TJ formation and cell viability.

2.2. Introduction

HS reduced DMI characterizes only 35% decrease in milk yield (Rhoads et al., 2009; Wheelock et al., 2010). To uncover the other unknown features, knowledge regarding cellular physiology is indispensable. The number and secretory activity of MEC determines the milk yield of dairy cows (Capuco et al., 2001). The decrease in number and secretory activity of MECs reduces the milk yield. However, how HS affects the number and secretory activity of MECs that remains unknown.

Temperature is an important environmental factor that directly influences biological functions. Cellular damage occurs when the internal temperature increases above 40 °C, leading to a series of events that may lead to organ failure and death (Becker et al., 2011). Temperatures greater than 41 °C to 42 °C can lead to cell death from apoptosis within a few hours (Sakaguchi et al., 1995). Therefore, HS may be a cause for reduction of MEC number as well as milk yield.

It is firmly established that mild heat stress (39–40 °C) can positively regulate cell proliferation and differentiation (Park et al., 2005). Previous study also showed that mild HS (39 °C) enhanced the milk protein synthesis in MEC (Misuzawa et al., 2019), which is the indication of mild HS induced increment of secretory activity. The secretory activity of MEC depends on the extracellular structure of the cells. TJ is a junctional complex between the cells, which plays critical role for maintaining the secretory activity of MEC. In fact, a previous study showed that 39 °C heat-shock increased the milk production and enhanced the formation of less-permeable TJs (Kobayashi et al., 2018). However, detailed molecular mechanisms of mammary epithelial cell response to heat-shock have not yet been clarified.

Transient receptor potential (TRP) receptors on cellular membranes are sensitive to heat stimulation. Nine such receptors have been identified so far (Tominaga et al., 2004). TRP

receptors are expressed in many tissues including the mammary glands (Venkatachalam et al., 2007; Ouadid-Ahidouchi et al., 2013). TRP can play a role in the transport of Ca^{2+} in the epithelial cells of the mammary gland. However, information regarding TRP expression in the mammary gland is extremely limited. In the HC11 cell line, transient receptor potential vanilloid 4 (TRPV4) is selectively localized to the basolateral membrane compartment, and its activation results in calcium entry (Reiter et al., 2006). Moreover, TRPV4 can also be activated by warm temperatures (> 34 °C) (Guler et al., 2002). It is possible that TRPV4 is involved in intracellular signaling activated by temperature changes in mammary epithelial cells.

Present study considered the UPR to identify the molecular mechanism behind HS induced loss of MEC and enhancement of TJ formation. It is already established that UPR induced apoptotic factor CHOP expression is liable for the decrease in milk yield (Yonekura et al 2018). Moreover, UPR component XBP1s increases the β-casein (a representative milk protein) synthesis (Tsuchiya et al., 2017 and Mizusawa et al., 2019), thereby enhances the secretory activity of MEC. However, the understanding of UPR at different levels of temperature during milk production has not been fully elucidated. Therefore, the aim was to determine the effect of severe and mild HS on the number and TJ formation of MEC in relation to UPR.

2.3. Materials and Methods

2.3.1. Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Equitech-Bio (Cotton Gin Lane, TX, USA). Penicillin, streptomycin, hydrocortisone, and bovine insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other compounds were obtained from Nacalai Tesque (Kyoto, Japan).

2.3.2. Cell culture and heat treatment

HC11 mouse mammary epithelial cells were a kind gift from Dr. Takaharu Kozakai of Yamagata University, Yamagata, Japan. Cells were cultured in DMEM containing 10% FBS and 10 ng/mL epidermal growth factor with 1% penicillin and streptomycin. All cultures were maintained at 37 °C under 5% CO_2. For different level of heat treatment in HC11 cells, mild and severe HS are considered as 39 °C and 41 °C respectively. Cells were cultured in an incubator at 39 °C for 72 or 41 °C for 6 h.

2.3.3. Cell transfection

For the transient transfection, HC11 cells were transfected using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. *Xbp1* small interfering RNA (siRNA) was purchased from Sigma-Aldrich and non-targeting control siRNA was purchased from Santa Cruz (Santa Cruz, CA, USA).

2.3.4. RNA extraction and RT-qPCR

Total RNA was extracted from the HC11 cells using TRIzol reagent (Life Technologies) following the manufacturer's protocol. A qPCR RT Master Mix with gDNA Remover

(Toyobo, Osaka, Japan) was utilized for reverse transcription. Quantitative real-time PCR was carried out using the SYBR Premix Ex TaqTM ll (TaKaRa Biotechnology, Kusatsu, Japan), to quantify the relative expression level of mRNA. Relative mRNA expression was estimated by the double delta delta Ct method and represented as the relative values to the control. GAPDH was used as the housekeeping gene. The primers used for the quantitative PCR are shown in Table 1. The reaction sensitivity and amplification of the contaminating products were examined by amplifying serial dilutions of cDNA.

2.3.5. Cell viability test

MTT Cell Viability Assay Kit (Biotium, Fremont, CA, USA) was used to assess the cell viability according to the manufacturer's protocol. Briefly, mammary epithelial cells (HC11) were cultured at a density of 2×10^3 cells/well on 96-well plates. After 48 h, cells were placed in an incubator at 39 °C or 41 °C for 72 h or 6 h, respectively. Then 10 μL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to 100 μL of culture medium. After 4 h of incubation at 37 °C, 200 μL of dimethylsulfoxide (DMSO) was added to each well. The absorbance was measured using a multimode microplate reader (iMark microplate reader, Bio-Rad) at 570 nm and a reference wavelength of 630 nm.

2.3.6. Statistical analysis

In all experiments, values are expressed as means ± standard error of the mean, with at least 3 replicates in each experimental group. Tukey-Kramer test or Student's *t*-test were applied to determine statistical differences between groups. Differences were considered to be significant at $P < 0.05$.

Table 1. Sequences of primers used for real-time PCR amplification

Gene	Primers (5' to 3')
Zo-1	Forward GGGAGGGTCAAATGAAGACA
	Reverse GGCATTCCTGCTGGTTACAT
Cldn3	Forward AGCCAGTCTCCAAAGCCACA
	Reverse CTGGGAATCAACTGCCCTTC
Ocln	Forward CGGACCCTGACCACTATGAAA
	Reverse CCTGCAGACCTGCATCAAAA
Trpv4	Forward ATGGCAGATCCTGGTGATGG
	Reverse GGAACTTCATACGCAGGTTTGG
β-casein	Forward GATGCCCCTCCTTAACTCTGAA
	Reverse TTAGCAAGACTGGCAAGGCTG
Xbp1s	Forward TGAGAACCAGGAGTTAAGAACACGC
	Reverse CCTGCACCTGCTGCGGAC
Ire1α	Forward ACGAAGGCCTGACGAAACTT
	Reverse ATCTGAACTTCGGCATGGGG
Atf4	Forward GAGCTTCCTGAACAGCGAAGTG
	Reverse TGGCCACCTCCAGATAGTCATC
Chop	Forward CCTAGCTTGGCTGACAGAGG
	Reverse CTGCTCCTTCTCCTTCATGC
Atf6α	Forward CTTCCTCCAGTTGCTCCATC
	Reverse CAACTCCTCAGGAACGTGCT
Grp78	Forward GAAAGGATGGTTAATGATGCTGAG
	Reverse GTCTTCAATGTCCGCATCCTG
Gapdh	Forward TTGTGATGGGTGTGAACCACGAG
	Reverse CATGAGCCCTTCCACAATGCCAA

2.4. Results

2.4.1. Effects of different temperatures on cell viability and expression level of UPR-related genes

MTT assay was performed to examine whether heat treatment affects HC11 cell viability. Only heat treatment at 41 °C significantly decreased cell viability, but no significant difference found in case of 39 °C heat treated group (Figure 1A). Quantitative RT-PCR was used to analyze the mRNA levels of the UPR-related genes in HC11 cells cultured at mild HS (39 °C) or severe HS (41 °C). The result showed that transcription levels of *Atf4*, *Chop*, *Atf6α,* and *Grp78* increased significantly and expression of *Ire1α* decreased with heat treatment at 41 °C. However, the expression level of *Xbp1s* was significantly increased with both mild and severe heat treatment (Figure 1B). Besides, mild heat stress did not enhance the transcription level of *Atf4, Chop, Atf6α,* and *Grp78* expression.

2.4.2. Effect of different temperatures on TJ Protein encoding genes expression

I investigated the effect of severe and mild heat treatment on the TJ protein-encoding gene and β-casein transcription levels in mammary epithelial cells using RT-qPCR. Heat stress at 41 °C significantly downregulated the TJ protein-encoding gene- *Cldn3* mRNA levels compared with the control (Figure 2B). Conversely, the *Zo-1*, *Ocln*, *Cldn3*, and *β-casein* mRNA levels were significantly higher in heat-shocked MECs at 39 °C (Figure 2C).

2.4.3. Mild heat treatment at 39ºC increases *Trpv4* mRNA levels in HC11 cells

To explore the mechanism how mild heat stress enhances the TJ formation, it was investigated the transcript level of cellular membrane heat sensitive receptor- *Trpv4* expression. The *Trpv4* mRNA level was significantly higher in mild heat treated HC11 cells (Figure 3).

2.4.4. TRPV4 agonist increases TJ protein-encoding gene and *β-casein* expression levels

I further investigated the *Zo-1, Ocln, Cldn3* and *β-casein* transcript levels in mammary epithelial cells cultured with a selective TRPV4 channel agonist (GSK1016790A) at 37 °C. The mRNA levels of *Zo-1, Ocln, Cldn3,* and *β-casein* were significantly increased in response to the addition of GSK1016790A (Figure 4).

2.4.5. Mild heat shock at 39 °C increases TJ protein-encoding gene transcript levels via XBP1

The transcript levels of *Xbp1s* in the HC11 cells cultured with GSK1016790A at 37 °C were investigated to determine whether *TRPV4* activity can induce *Xbp1s* transcript accumulation. TRPV4 activation with GSK1016790A increased the *Xbp1s* transcript level (Figure 5A).

Next, it was examined if *Xbp1* is involved in upregulation of the TJ protein-encoding gene upon mild heat treatment. I generated *Xbp1* knockdown of HC11 cells using *Xbp1* small interfering RNA (siRNA). After mild heat treatment, the *Zo-1, Ocln,* and *Cldn3* mRNA levels were analyzed using RT-qPCR. Results reveal that the increased mRNA expressions of *Zo-1, Ocln,* and *Cldn3* by mild heat treatment were inhibited by *Xbp1* silencing (Figure 5B).

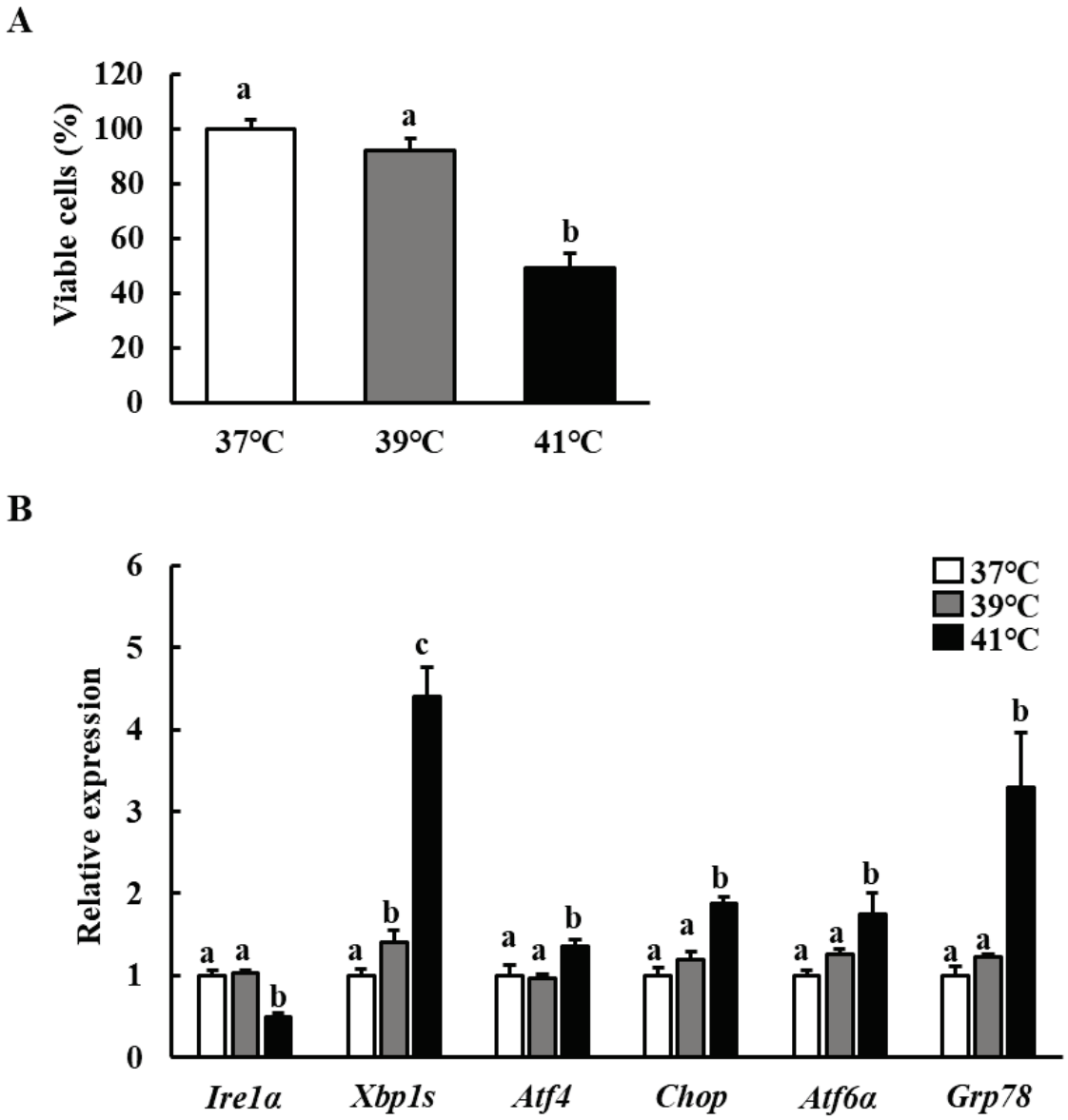

Figure 1. Effect of different temperatures on the cell viability and mRNA expression levels of the UPR-related genes. After reaching 90%–100% confluence, HC11 cells were incubated at 37 °C (control), 39 °C for 72 h, or 41 °C for 6 h. (A) Cell viability was measured using an MTT assay. (B) The mRNA level of *Ire1α, Xbp1s, Atf4, Chop, Atf6α*, and *Grp78* were determined using RT-qPCR and normalized to that of GAPDH. The results are expressed as the mean values ± SEM for four independent experiments. Means with different letters are significantly different, $p < 0.05$. This figure is taken from "Animals" journal.

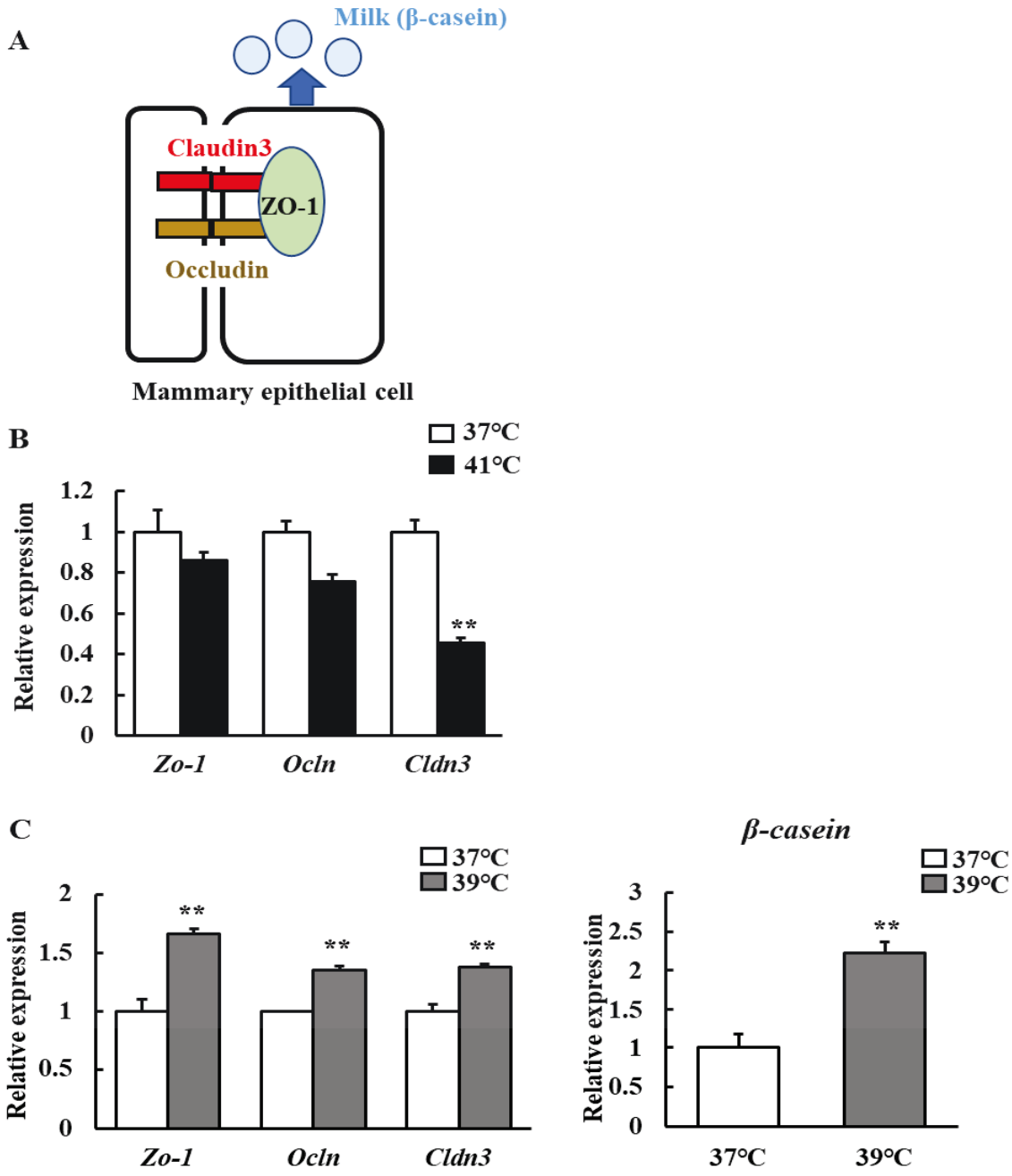

Figure 2. Effects of different temperatures on TJ protein-encoding gene and β-casein expression (A) Schematic diagram of Cloudin3, Occludin, ZO-1, and β-casein in mammary epithelial cells. (B) HC11 cells were maintained at 37 °C or exposed to 41 °C without differentiation medium for 6 h or (C) HC11 cells were maintained at 37 °C or exposed to 39 °C without differentiation medium for 72 h. The mRNA levels of Zo-1, Ocln, Cldn3 and β-casein were detected by RT-qPCR. GAPDH was used as the reference gene. The results are expressed as the mean values ± standard error of the mean from four independent experiments. ** $p < 0.01$ compared to the control. This figure is taken from "Animals" journal.

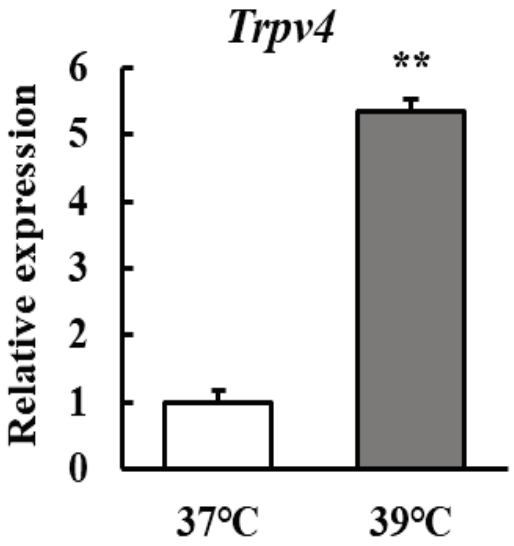

Figure 3. Heat treatment at 39 °C increases the Trpv4 mRNA level in HC11 cells. HC11 cells were maintained at 37 °C or exposed to 39 °C for 72 h. The Trpv4 mRNA level was detected by RT-qPCR. GAPDH was used as the reference gene. The results are expressed as the mean values ± standard error of the mean from four independent experiments. ** $p < 0.01$ compared to the control. This figure is taken from "Animals" journal.

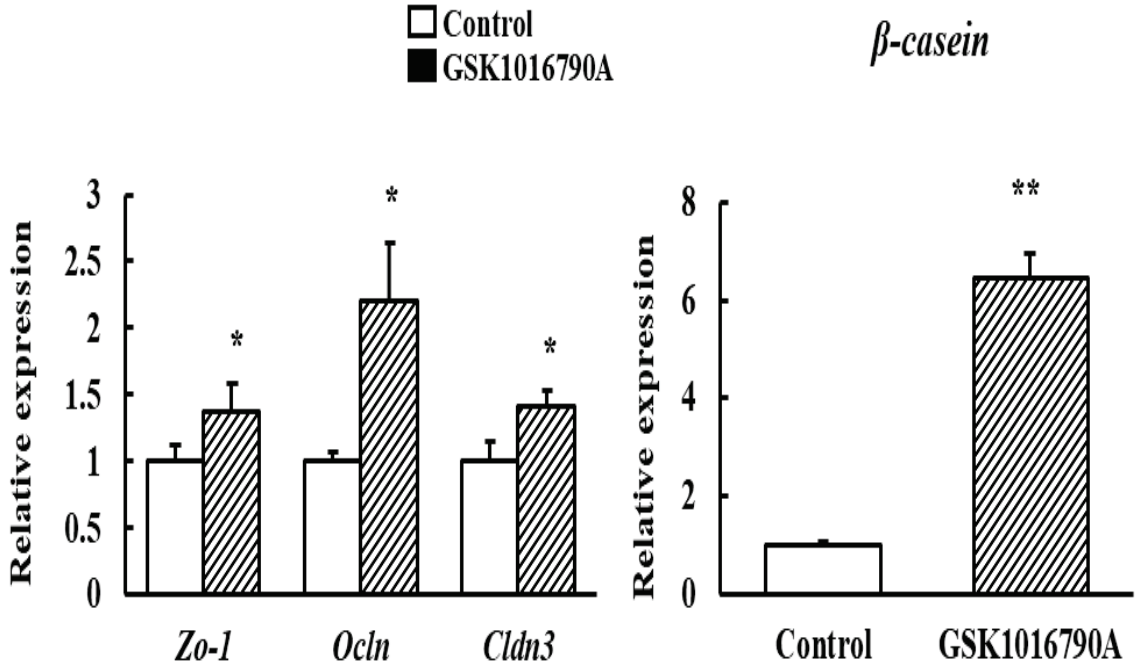

Figure 4. TRPV4 agonist increases the expression level of TJ protein encoding gene and β-casein transcripts. HC11 cells were incubated at 37 °C without (control) or with 10 μM GSK1016790A for 24 h. The mRNA level of *Zo-1, Ocln, Cldn3* and *β-casein* were detected by RT-qPCR. GAPDH was used as the reference gene. The results are expressed as the mean values ± SEM for four independent experiments. * $p < 0.05$ and ** $p < 0.01$ compared to the control. This figure is taken from "Animals" journal.

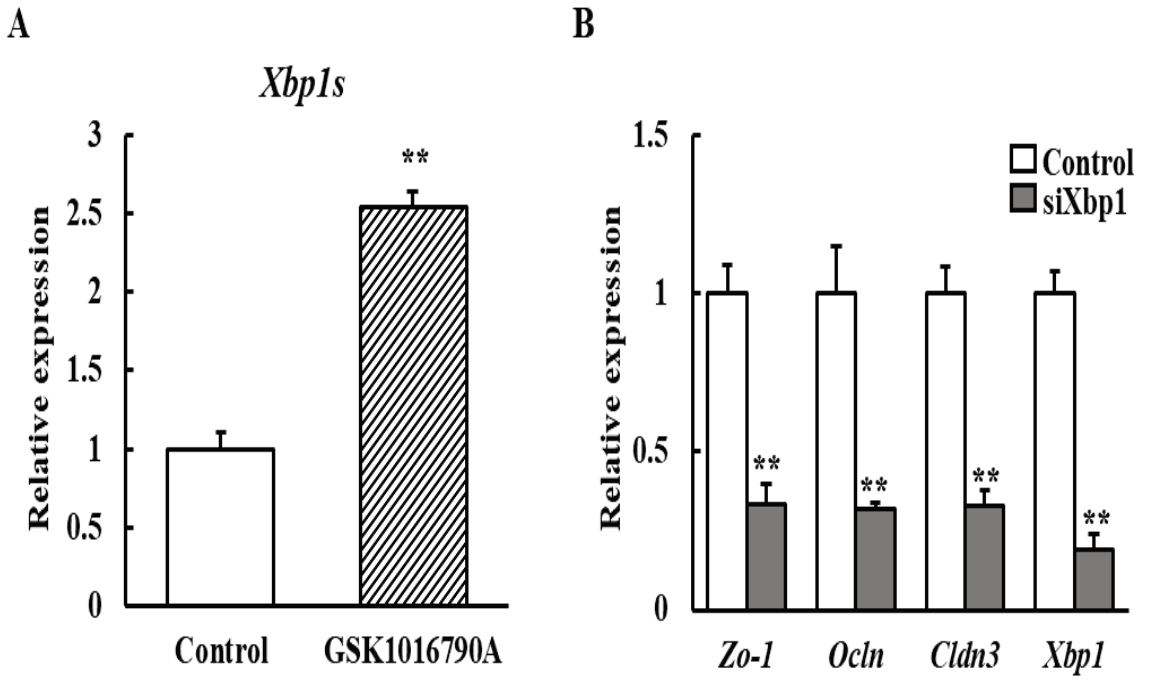

Figure 5. Effects of *XBP1* knockdown on the mRNA expression of TJ protein-encoding gene after heat stimulation at 39 °C. (A) HC11 cells were incubated at 37 °C without (control) or with 10 μM GSK1016790A for 24 h. The mRNA level of *Xbp1s* was detected by RT-qPCR. GAPDH was used as the reference gene. (B) HC11 cells were transfected with *XBP1* siRNA or control scramble siRNA for 24 h. Then the cells were maintained at 37 °C or exposed to 39 °C without differentiation medium for 72 h. The mRNA level of *Zo-1, Ocln and Cldn3* were detected by RT-qPCR. GAPDH was used as the reference gene. The results are expressed as the mean values ± SEM for four independent experiments. $^{**}p < 0.01$ compared to the control. This figure is taken from "Animals" journal.

2.5. Discussion

It has been reported that heat treatment at 41–43 °C induced UPR-mediated apoptosis in human umbilical vein endothelial cells or bovine MECs (Li et al., 2014; Jin et al., 2016). Mild heat stress (39–40 ºC) can positively regulate cell proliferation and differentiation (Park et al., 2005). These results suggested that the UPR was involved in both cellular survival under mild heat stress (39 °C) and cell death under severe heat stress (41–43 °C). In recent years, it has been reported that responses vary depending on cell type, even with the same degree heat-stress. Further, the cellular response to stress differs depending stress type and stress intensity (Markmiller et al., 2018). However, it is unknown how mild heat-stress at 39 °C and severe heat stress at 41 °C differentially affect the UPR in MECs. Results from present study revealed that the transcript levels of *Xbp1*, *Atf4, Chop, Atf6α* and *Grp78* were significantly increased and *Ire1α* expression decreased with heat treatment at 41 °C. Consistent with a previous report Homma et al., 2016, the expression level of *Ire1α* transcript was significantly decreased by heat treatment at 41 °C. It is speculated that the downregulation of *Ire1α* might result from a negative feedback system. Besides, among the UPR related gene, only *Xbp1s* expression level was significantly increased with mild heat treatment (Figure 1B). I further examined whether heat treatment affected HC11 cell viability using the MTT assay. Only heat treatment at 41 °C significantly decreased cell viability (Figure 1A). When excessive ER stress is initiated or continued for a long period of time, cells undergo apoptosis. The UPR pathway protects cells from apoptotic death in response to stress stimulus, but can also lead to apoptosis when it is difficult to adapt to the stress (Urra et al., 2013). The UPR is facilitated by three ER receptors: PERK, IRE1 and ATF6. Among these proteins, PERK is essential for the induction expression of the proapoptotic transcriptional factor CHOP under ER stress conditions and is dominant over the ATF6 and IRE1/XBP-1 signaling pathways (Harding

et al., 2000). Current data already revealed that heat treatment at 41 °C, but not 39 °C, significantly increased the transcript levels of Atf4 and Chop. It is thought that these changes are associated with a decrease in cell viability.

Next, I examined the effect of severe and mild heat treatment on β-casein and TJ protein-encoding transcript levels in HC11 cells. Severe HS at 41 °C significantly downregulated the expression of Cldn3 mRNA (Figure 2B). Conversely, transcript levels of *Zo-1*, *Ocln*, *Cldn3* and *β-casein* were significantly higher in HC11 cells cultured at 39 °C compared to those in cells cultured at 37 °C (Figure 2C), which corroborated previous findings (Kobayashi et al., 2018; Mizusawa et al., 2019). Therefore, results indicate that mild heat stress enhances the formation of TJ and β-casein whereas severe HS negatively affects the TJ formation. To find out the mechanism of TJ formation under mild HS, current study examined either the temperature-sensitive TRPV4 transcript level in HC11 cells was affected by mild heat stimulation at 39 °C. The transcript level of *Trpv4* was significantly higher in cells cultured at 39 °C compared to that in cells cultured at 37 °C (Figure 3). Research revealed that TRPV4 is involved in the differentiation of osteoblastic cells (Suzuki et al., 2013). A previous study suggested that TRPV4 maintains a substantial degree of temperature-dependent activity over a range of temperatures (between 30 and 40 °C) under many different experimental conditions (Watanabe et al., 2002; Chung et al., 2003). Therefore, it is thought that mild heat treatment at 39 °C induces TRPV4 activity.

Again, I was interested to investigate the relationship between TRPV4 and TJ protein encoding genes. Thus, present study further examined the transcript levels of *β-casein*, *Zo-1*, *Ocln* and *Cldn3* in HC11 cells cultured with a selective TRPV4 channel *agonist* (GSK1016790A) at 37 °C. The transcript levels of *β-casein*, *Zo-1*, *Ocln* and *Cldn3* were significantly increased in response to the addition of GSK1016790A (Figure 4). Previous studies indicated that activation of TRPV4 strengthens the TJ-associated barrier through

up-regulation of OCLN in skin keratinocytes and cornel epithelial cells (Akazawa et al., 2013; Martinez-Rendon et al., 2017). On the other hand, the TRPV4 activator (4α-PDD) decreased the expression level of Cldn3 in HC11 cells (Reiter et al., 2006), which differs from current results. It is reported that GSK1016790A is a potent and selective agonist of TRPV4 in different cell types (Darby et al., 2016) and that 4α-PDD is 300-fold less potent than GSK1016790A in activating TRPV4 current (Thorneloe et al., 2008). 4αPDD can also act through other pathways (Alexander et al., 2013; White et al., 2016). Although further investigation is necessary, the present results suggest that TRPV4 is involved in the increase in β-casein and TJ protein-encoding transcript levels upon mild heat-shock at 39 °C.

It is known that the UPR is activated under heat-stress conditions (Xu et al., 2011; Kim et al., 2013). Previous research also demonstrated that the expression of β-casein is regulated by UPR-related factors including XBP1 (Tsuchiya et al., 2017). When IRE1α is activated, a spliceosome-independent frame switch is induced. As a consequence, the active transcription factor XBP1s is expressed (Calfon et al., 2002). To investigate whether TRPV4 activity can induce *Xbp1s* transcript accumulation, I investigated the transcript levels of *Xbp1s* in HC11 cells cultured with GSK1016790A at 37 °C. I found that TRPV4 activation with GSK1016790A caused an increase in *Xbp1s* transcript level (Figure 5A). TRPV4 is regulated by progesterone and activated by heat stimulation, hypotonic pressure, chemical and mechanical stress, and its activation leads to Ca^{2+} influx (Garcia-Elias et al., 2014; Jung et al., 2009). The endoplasmic reticulum (ER) is an organelle that is responsible for the storage of Ca^{2+}. Moreover, it is reported that ER Ca^{2+} depletion activates XBP1 (Celli et al., 2011). Though further investigation is necessary, it is speculated that TRPV4-mediated Ca^{2+} signaling regulates *Xbp1s* expression. Furthermore, present study next examined whether XBP1 mediates an increase in TJ protein-encoding

mRNA levels upon mild heat-shock at 39 °C. Cells with siRNA (small interfering RNA) knockdown of *Xbp1* and control cells using control siRNA were created and treated at 39 °C. I found that the increase in *β-casein*, *Zo-1*, *Ocln* and *Cldn3* mRNA expression levels induced by mild heat treatment were suppressed in *Xbp1* knockdown cells (Figure 5B). It has been revealed that IRE1-XBP1 pathway regulates retinal pigment epithelium tight junctions (Ma et al., 2016). These results suggest that XBP1 regulates the expression of TJ protein-encoding genes. Because XBP1 is a transcription factor, it will be necessary to clarify whether it directly regulates the expression of these genes.

2.6. Conclusion

In summary, results indicate that mild heat-stress causes an increase in the transcriptional level of XBP1s via TRPV4 activity, which enhances the expression of β-casein and TJ protein-encoding genes. Besides, severe HS activates severe ER stress with upregulation of all UPR component genes. In particular, severe HS enhances the expression of proapoptosis marker CHOP which plays important role in reducing MEC viability and milk production. Moreover, the UPR expression pattern is different after heat stimulation at 39 °C versus that at 41 °C. it is speculated that the difference in UPR gene expression upon stimulation at 39 °C versus 41 °C controls cell survival versus cell death. These findings have implications on controlling positive outcomes of heat stress in tissues including mammary epithelium.

Chapter III

Effects of 5-aminolevulinic acid on severe heat stress induced ER stress and viability loss of bovine mammary epithelial cells

3.1. Abstract

Cells have increased susceptibility to activation of apoptosis when suffering severe heat stress (HS). An effective supplementation strategy to mimic heat-induced apoptosis of bovine mammary epithelial cells (MECs) is necessary to maintain optimal milk production. This study aimed to investigate possible protective effects of the anti-apoptotic activity of 5-aminolevulinic acid (5-ALA) against HS-induced damage of bovine MECs. Bovine MECs were pretreated with or without 5-ALA at concentrations of 10, 100, and 500 μM for 24 h followed by HS (42.5 °C for 24 h and 48 h). Cell viability was measured with MTT assays. Real-time qPCR and Western blotting were used to explore the regulation of genes associated with apoptosis, oxidative stress, and endoplasmic reticulum (ER) stress marker genes. I found that 5-ALA induces cytoprotection via inhibition of apoptosis markers after HS-induced damage. Pretreatment of bovine MECs with 5-ALA resulted in dramatic upregulation of mRNA for nuclear factor erythroid-derived 2-like factor 2 (*NRF2*), heme oxygenase-1 (*HO-1*), and NAD(P)H quinone oxidoreductase 1 (*NQO1*), all of which are antioxidant stress genes. Moreover, 5-ALA pretreatment significantly suppressed HS-induced ER stress-associated markers, *GRP78* and *CHOP* expression levels. Therefore, 5-ALA has the ability to ameliorate the ER stress in heat stressed bovine MEC via enhancing the expression of antioxidant gene.

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3.2. Introduction

In chapter II, results showed that severe heat stress in mouse mammary epithelial cell (HC11) activated severe ER stress induced proapoptotic marker CHOP and reduced the viability. In addition, HS is responsible for inducing oxidative stress and subsequent cellular damage. Heat induced excessive ROS (marker of oxidative stress) production in bovine MECs provides direct evidence of a redox imbalance status which impairs the cellular antioxidant capacity (Jin et al., 2016). Disruption of cellular antioxidant capacity as well as overproduction of ROS can upset ER homeostasis and impair protein folding leading to induce ER stress (Cao et al., 2014). Therefore, ER stress induced MEC death under severe HS condition has been identified as harmful factor for reduction of milk yield. An effective supplementation strategy to counter heat load-induced apoptosis in MECs is necessary for maintaining sustainable milk production of dairy cow.

Previous study reported that antioxidant is the potent reducer of ER stress induced apoptosis (Malhotra et al., 2008). 5-ALA is a natural non-alpha amino acid found in different food and fermented products. It has the property of antioxidant. 5-ALA can reduce nephrotoxicity and apoptosis in murine tubular epithelial cells and can protect kidneys from acute injury (Liu et al., 2019a; Uchida et al., 2019). Moreover, both in vivo and in vitro studies show that 5-ALA can induce HO-1 expression via Nrf2 in kidney cells (Liu et al., 2019b; Zhao et al., 2016). Nrf2 is a transcription factor that binds to the antioxidant respond elements (ARE) in the upstream promoter region of many antioxidant genes such as HO-1 and NQO1 which are involved in the elimination of oxidative stress. Enhanced expression of HO-1 protects against oxidative and other stresses, such as cisplatin-induced nephrotoxicity, hydrogen peroxide-induced cardiomyocyte hypertrophy, and ischemia-reperfusion-induced renal injury (Uchida et al., 2019; Zhao et al., 2016;

Terada et al., 2013; Hou et al., 2013). Therefore, this study aimed to investigate the possible protective effects of 5-ALA against HS-induced damage to bovine MECs. In this current research, I assessed the MAC-T cell (a MEC line) viability after pretreatment with 5-ALA followed by HS. I also evaluate ER and oxidative stress marker gene expression in heat stressed MAC-T cell with and without 5-ALA treatment. Furthermore, I investigate the expression of ER stress marker protein.

3.3. Materials and methods

3.3.1. Chemicals and reagents

5-ALA was provided by Neopharma Japan Co. Ltd. (Tokyo, Japan). All other chemical and reagents were purchased and maintained as explained in section 2.3.1.

3.3.2. Cell culture and HS treatment

Immortalized bovine MECs (MAC-T cells) were generously provided by Dr. Sangun Roh of Tohoku University, Sendai, Japan. Bovine MECs of passage 35 were cultured in DMEM containing 10% FBS, 1% penicillin and streptomycin, 5 μg/mL bovine insulin, and 1 μg/mL hydrocortisone. Cells were maintained in a humidified incubator at 37 °C in an atmosphere of 5% CO_2. I applied modified methods of heat treatment; 42.5 °C temperature for 24 h or 48 h for heat stress in MAC-T cell which was adopted from previous study (Jin et al., 2016; Li et al., 2019) . Cells cultured at 37 °C were used as controls. 5-ALA was added to bovine MECs for 24 h before heat treatment to examine its activity in preventing HS-related damage.

3.3.3. Cell viability and 5-ALA toxicity determination

Cell viability and 5-ALA toxicity evaluations were performed using an MTT Cell Viability Assay Kit (Biotium, Fremont, CA, USA) following the manufacturer's protocol. Briefly, bovine MECs were seeded in 96-well plates at a density of 1×10^4 cells/well and were cultured for 24h to reach an optimal density. Various concentrations of 5-ALA (0, 50, 100, 250, and 500 μM) were added to cells, and incubation continued for 48 h to test for 5-ALA toxicity. Different doses of 5-ALA were determined based on the knowledge of previous study (Zhao et al., 2016). Bovine MECs were pretreated with 5-ALA (10, 100, and 500 μM) followed by HS challenged for 48 h to examine 5-ALA effects on cellular

damage caused by HS. After incubation, 10 µL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to 100 µL of culture medium. After 4 h of incubation at 37 °C, 200 µL of dimethylsulfoxide (DMSO) was added to each well. Absorbance reading was measured as described in section 2.3.5.

3.3.4. RNA extraction and quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from bovine MECs using TRIzol (Invitrogen), according to the manufacturer's protocol. For quantitative real-time (qRT) PCR, the protocol was followed as mention in section 2.3.4. β-actin (*ACTB*) was used as the reference gene. Primers used for quantitative PCR are shown in Table 2.

3.3.5. Western blot analysis

Cell were treated described above, washed with PBS twice, harvested and then lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (50 m*M* Tris-HCl, 150 m*M* NaCl pH 7.4, 0.05% SDS, 0.2% sodium deoxycholate, 1 m*M* EDTA, 1% Tergitol-type NP-40) containing protease inhibitor cocktail (Nacalai Tesque). Following centrifugation (10 min at 20000 ×g), protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Cell extracts (60 µg) were subjected to SDS-PAGE on 4–20% polyacrylamide gels and were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated for 1 h in 1% PBT and 0.01% Tween 20 solution containing 4% skim milk powder (blocking buffer). Previous study established that ER stress induced apoptosis enhance the p-PERK, CHOP and cleaved caspase-3 protein expression in bovine MECs (Sharmin et al., 2020). Therefore, I measured PERK, p-PERK, CHOP and cleaved caspase-3 protein expression level using western blot analysis. Thus, membranes were then incubated with anti-phosphorylated PERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PERK (Santa Cruz), anti-CHOP (Life

Span Bioscience, Inc., Seattle, USA), anti-cleaved caspase-3 (Cell Signaling, MA, USA), and anti-α-tubulin (MBL Co., Nagoya, Japan) antibodies diluted in blocking buffer at room temperature. Next, the membranes were incubated with anti-rabbit IgG secondary antibody (GE Healthcare, Pittsburgh, PA, USA), and enhanced chemiluminescent (ECL) membranes were visualized using an ECL Prime Western Blotting Detection Reagent Kit (GE Healthcare) and images obtained using an Image Quant LAS 500 (GE Healthcare).

3.3.6. Statistical Analysis

Data are presented as the mean ± SEM with at least three replicates in each experimental group. Tukey-Kramer test or Student's *t*-test were used to assess statistical differences between groups. Differences were considered significant at $p < 0.05$.

Table 2. Sequences of primers used for real-time PCR amplification

Gene	Primers (5' to 3')
XBP1s	Forward TGCTGAGTCCGCAGCAGGTG
	Reverse GCTGGCAGACTCTGGGGAAG
GRP78	Forward GATTGAAGTCACCTTTGAGATAGATGTG
	Reverse GATCTTATTTTTGTTGCCTGTACCTTT
CHOP	Forward CTGAAAGCAGAGCCTGATCC
	Reverse GTCCTCATACCAGGCTTCCA
BAX	Forward TGGACATTGGACTTCCTTCG
	Reverse CCAGCCACAAAGATGGTCAC
BCL2	Forward GGATGACCGAGTACCTGAACC
	Reverse GCCCAGATAGGCACCCAG
NRF2	Forward CCAGCACAACACATACCA
	Reverse TAGCCGAAGAAACCTCATT
HO-1	Forward GGCAGCAAGGTGCAAGA
	Reverse GAAGGAAGCCAGCCAAGAG
NQO1	Forward CAACAGACCAGCCAATCA
	Reverse ACCTCCCATCCTTTCCTC
ACTB	Forward CATCGCGGACAGGATGCAGAAA
	Reverse CCTGCTTGCTGATCCACATCTGCT

3.4. Results

3.4.1. 5-ALA prevents HS reduced cell viability in Bovine MECs

Cytotoxicity of 5-ALA was evaluated following 48 h treatment of bovine MECs with 5-ALA concentrations of 50, 100, 250, and 500 μM, and no cytotoxic effect was found (Figure 6). HS significantly reduced bovine MEC viability (Figure 7A).

I investigated the effects of 5-ALA treatment on bovine MEC viability after HS. Cell viability markedly increased when cells were pretreated with 5-ALA at concentrations of 100 and 500 μM. Real-time qPCR analysis showed that expression of anti-apoptosis gene B-cell lymphoma 2 (*BCL2*) was downregulated and expression of pro-apoptosis gene BCL2-associated X (*BAX*) was upregulated in heat-stressed bovine MECs. Pretreatment with 5-ALA (10, 100, and 500 μM) resulted in a significant reduction of *BAX* expression and an increase in *BCL2* expression (Figure 7B).

3.4.2. Effect of 5-ALA on the expression of oxidative stress-related gene during HS

I examined the effect of pretreatment with 5-ALA on the expression of nuclear factor erythroid-derived 2-like factor 2 (*NRF2*) and downstream signaling antioxidant genes *HO-1* and *NQO1* under HS. Pretreatment of bovine MECs with 5-ALA induced dramatic mRNA upregulation of *NRF2, HO-1,* and *NQO1* (Figure 8). 5-ALA likely provides a cytoprotective effect via upregulating *NRF2* along with downstream genes *HO-1* and *NQO-1* expression.

3.4.3. 5-ALA reduces HS-induced ER stress

Finally, the expression of *GRP78*, a central regulator of ER stress, and *CHOP,* a key signaling component involved in ER stress-induced apoptosis, and *XBP1s*, a key

transcriptional gene involved in UPR, were examined as ER stress markers in bovine MECs after pretreatment with 5-ALA. 5-ALA pretreatment significantly suppressed HS-induced *GRP78* and *CHOP* expression levels and decreased *XBP1s* expression (Figure 9A).

I performed Western blot analysis to measure phospho-PERK, CHOP, and cleaved caspase-3 protein expressions to confirm the beneficial effects of 5-ALA on bovine MECs. 5-ALA pretreatment suppressed HS-induced phospho-PERK, CHOP, and cleaved caspase-3 protein expressions (Figure 9B).

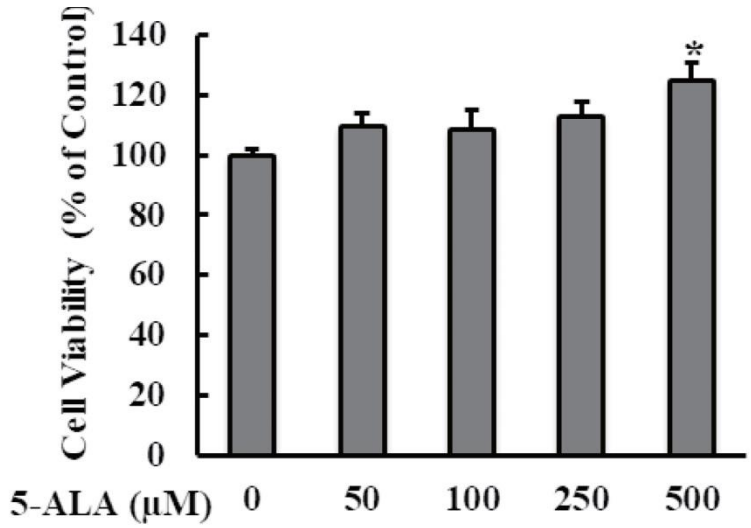

Figure 6. Effect of different doses of 5-ALA on bovine MEC viability. Confluent bovine MECs were treated with 50, 100, 250, and 500 μM of 5-ALA for 48 h. Cell viability was measured using MTT assays. Absorbance was measured at 570 and 630 nm using a multimode microplate reader to calculate survival rates expressed as the percentage of the control cells without 5-ALA treatment. Data are presented as mean ± SEM for three independent experiments. * indicates $P< 0.05$ compared with the control. This figure is taken from "Animal Bioscience" journal.

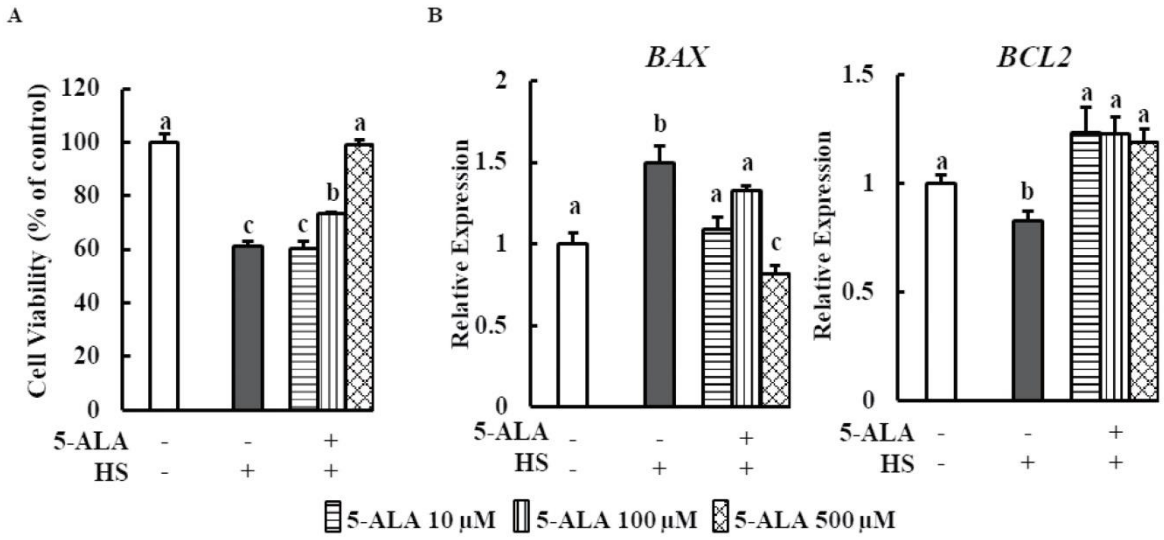

Figure 7. 5-ALA counters HS reduced cell viability in bovine MECs. (A) Bovine MECs were pretreated with or without 5-ALA at concentrations of 10, 100, and 500 µM for 24 h followed by HS (42.5 °C for 48 h). Cell viability was measured by MTT assay and expressed the percentage of control cells continuously cultured at 37 °C and received no 5-ALA treatment. (B) Bovine MECs were pretreated with or without 5-ALA at concentrations of 10, 100, and 500 µM for 24 h followed by HS (42.5 °C for 24 h). mRNA levels of *BAX* and *BCL2* were determined by RT-qPCR and normalized to *ACTB* levels. Data are presented as mean ± SEM for three independent experiments. Means with different letters are significantly different, $p < 0.05$. This figure is taken from "Animal Bioscience" journal.

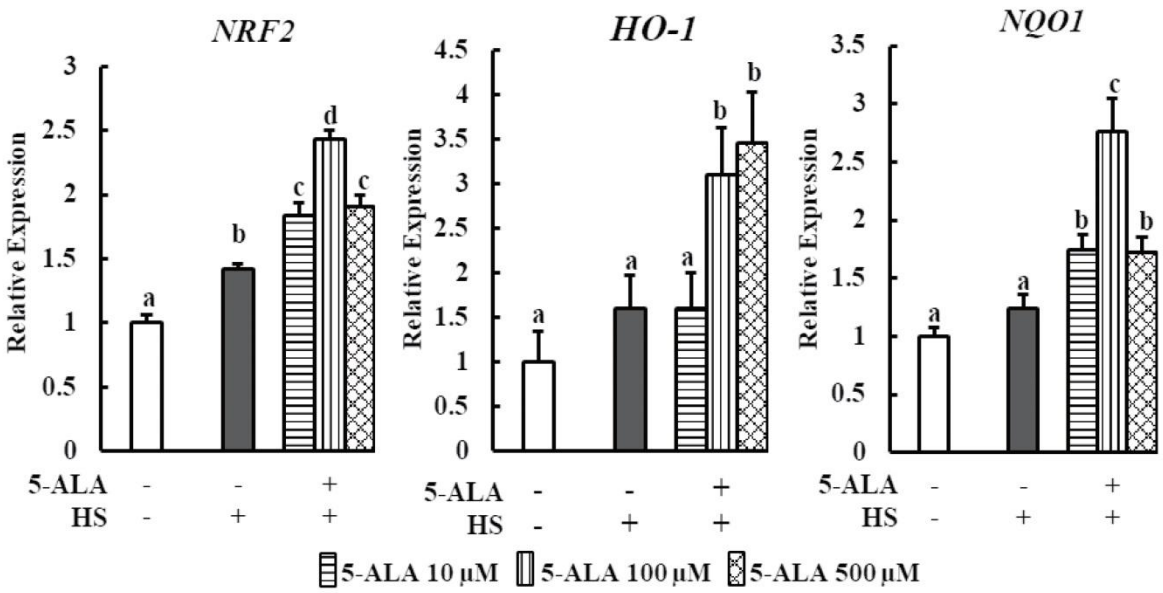

Figure 8. Effect of 5-ALA on the expression of oxidative stress-related genes during HS. Bovine MECs were pretreated with or without 5-ALA at concentrations of 10, 100, and 500 μM for 24 h followed by HS (42.5 °C for 24 h). Cells that were consistently cultured at 37 °C and received no 5-ALA treatment were used as the control group. mRNA levels of *NRF2*, *HO-1*, and *NQO1* were determined by RT-qPCR and normalized to *ACTB* levels. Data are presented as mean ± SEM for three independent experiments. Means with different letters are significantly different, $p < 0.05$. This figure is taken from "Animal Bioscience" journal.

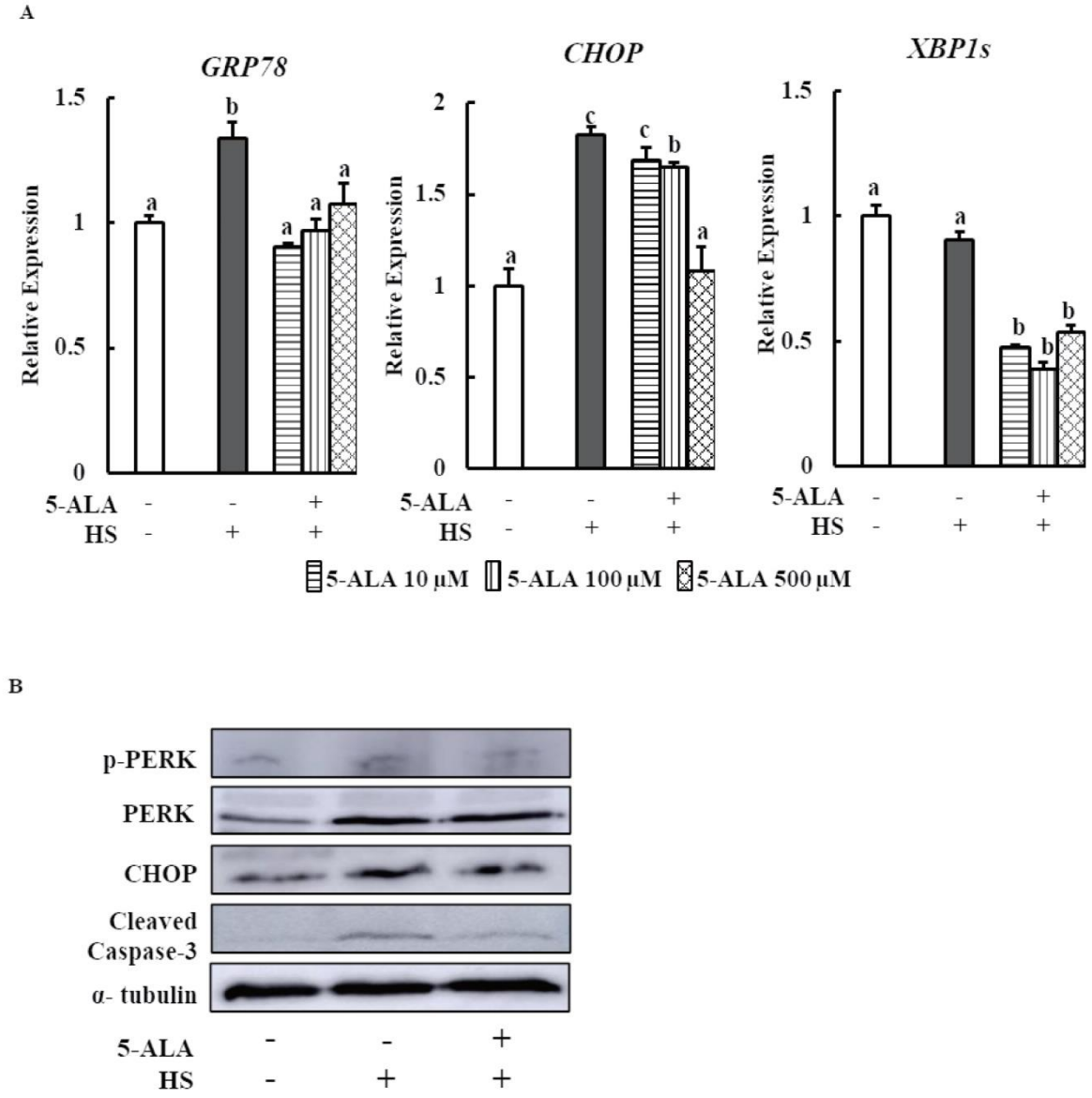

Figure 9. 5-ALA reduces HS induced ER stress. Bovine MECs were pretreated with or without 5-ALA at concentrations of 10, 100, and 500 μM for 24 h followed by HS (42.5 °C for 24 h). Cells that were consistently cultured at 37 °C and received no 5-ALA treatment were used as the control group. (A) mRNA levels of *GRP78*, *CHOP*, and *XBP1s* were determined by RT-qPCR and normalized to *ACTB* levels. Data are presented as mean ± SEM for three independent experiments. Means with different letters are significantly different, $p < 0.05$. (B) The phosphorylated PERK, PERK, CHOP, cleaved caspase3, and α-tubulin (internal control) protein levels were detected using Western blotting. Representative images from three independent experiments with at least four replicates in each experiment are shown. This figure is taken from "Animal Bioscience" journal.

3.5. Discussion

A number of studies have been suggested that the major cause of HS-induced cellular impairment is the ER stress mediated cellular apoptosis (Jin et al., 2016; Xu et al., 2011). But the cellular and molecular events underlying the effect of HS on bovine mammary epithelial cells remain poorly uncovered. This study again showed that severe HS increased the mRNA expression of *GRP78* and *CHOP* and protein expression of CHOP, p-PERK, and cleaved caspase-3 in bovine MECs (Figure 9). The major ER stress chaperon *GRP78*, plays a vital role in maintaining the ER homeostasis. In non-stressed cells, three UPR transducers remain as inactive form through binding with *GRP78*. Upon ER stress, all three sensors are released from *GRP78*. In this case, PERK is activated (Bertolotti et al., 2000), subsequent transcription factor CHOP and major apoptosis executioner caspase-3 is upregulated (Zinszner et al., 1998). Previous study indicated that hyperthermia increases the *GRP78* and caspase-3 protein expression in human osteosarcoma and bovine granulosa cells (Hou et al., 2014; Alemu et al., 2018). It has been demonstrated that HS activates PERK-eIF2α-ATF4-CHOP pathway that enhances ER stress-mediated apoptosis in different types of cell including bovine MECs (Jin et al., 2016).

I also found that anti- and pro-apoptosis gene *BAX* and *BCL2* expressions were enhanced and suppressed respectively by HS in MAC-T cells, which is consistent with the results of Jin et al., 2016. It is well established that during ER stress condition, *CHOP* upregulates the expression of *BAX* and downregulates the expression of *BCL2* expression (Iurlao et al., 2016). Thus, *CHOP* is responsible for heat treatment mediated ER stress induced cell death. Surprisingly, *XBP1s* expression did not differ between control and HS-treated bovine MECs in the present study (Figure 9A). Hence, HS activated the PERK arm of

UPR, which leads to apoptosis. In brief, current data postulated that HS induced UPR signaling and ER stress mediated apoptosis in bMECs. Therefore, HS has the adverse effect on mammary gland physiology of dairy cows.

Findings from this research showed that *Nrf2* mRNA upregulation was higher in bMECs at all pretreatment concentrations of 5-ALA followed by HS. *Nrf2* acts as a master regulator of cellular antioxidant response, which protects against oxidative stress-induced DNA damage and apoptosis (Gan et al., 2014). Previous study demonstrated that 5-ALA is an enhancer of heme synthesis, which reduces cardiomyocyte hypertrophy via *Nrf2* activation (Zhao et al., 2016). In response to oxidative stress, *Nrf2* induces cellular antioxidant defense enzymes, such as *HO-1* and *NQO1* (Stefanson et al., 2014). Further, data from this study showed that bovine MECs pretreated with 5-ALA significantly increased the mRNA expression of *HO-1* and *NQO1* (Figure 8). 5-ALA showed a renoprotective effect via inducing *HO-1* expression in response to renal ischemia-reperfusion injury and cisplatin nephropathy in the mouse kidney (Terada et al., 2013; Huo et al., 2013). Another research showed that enhanced expression of *Nrf2* mediated *NQO1*, a cytosolic flavoprotein, facilitates cytoprotection against oxidative stress induced DNA damage and the apoptosis of muscle cells by suppressing intracellular reactive oxygen species (Han et al., 2017). 5-ALA thus provides a cytoprotective effect via upregulating *Nrf2* that in turn upregulates downstream antioxidant genes, *HO-1* and *NQO-1* expression, and increases the viability of heat stressed bovine MECs. Interestingly, this study also indicates that HS slightly activates *Nrf2/HO-1* signaling in bMECs even without 5-ALA treatment, suggesting a self-defense mechanism in bMECs during HS. These findings are consistent with the study of Jin et al., who showed that Nrf2-ARE signaling is also slightly activated in heat-stressed bMECs (Jin et al., 2016). However, 5-

ALA refrain the cell from HS induced apoptosis via increasing the *Nrf2* and antioxidant genes expression.

Moreover, the present study showed that 5-ALA treatment may prevent HS-induced apoptosis of mammary epithelial cells because 5-ALA pretreatment significantly suppressed HS-induced *GRP78*, *CHOP, caspase-3 and BAX* expression and enhanced *BCL2 expression* (Figure 9 and 7). The key regulator of ER stress transducers, *GRP78* reduction by 5-ALA may restore the basal level of p-PERK. Thereby, 5-ALA may enhance the cell viability by decreasing the CHOP expression of heat stressed cell and also simultaneously regulating the anti- and pro-apoptotic gene expression in this study. Therefore, 5-ALA can ameliorate the HS induced ER stress and raise the cell viability as well as shows the beneficial effect for udder environment of dairy cows.

3.6. Conclusion

Current study indicates that 5-ALA provides cytoprotection via inhibition of apoptosis markers in HS-induced damage of bovine MECs. Moreover, this study demonstrates that antioxidant actions of 5-ALA are caused by the upregulation of *NRF2* and its downstream antioxidant gene such as *HO-1, NQO1*. Overall, results showed that the upregulation of antioxidant gene and reduction of ER stress by 5-ALA could attenuate HS-induced cell damage.

Chapter IV

General Discussion

Despite global attention to intensify research to better understand the biology of HS, dairy cow productivity remains severely depressed during the summer. This is particularly true in countries that do not have resources to alleviate environmental stress. Globally, the effects of HS cause a yearly production loss worth millions of dollars. Dairy cows suffer from heat stress (HS) in air temperatures above 25 °C (Das et al., 2016). The negative impact of HS is a reduction in dry matter intake (DMI), which in turn declines milk yield. A study reported that the 35% reduction in milk yield can be explained by the decrease in DMI under HS conditions (Rhoads et al., 2009), indicating the involvement of other factors in addition to DMI. Therefore, cellular physiology in the mammary gland has been considered to uncover the mechanism of decreasing milk yield under HS.

The mammary gland is an important economical organ in dairy animals because of its special function in milk synthesis and secretion, which is affected by HS. The decrease in the number and secretory activity of the mammary epithelial cells (MECs) reduces milk yield (Capuco et al., 2001). Severe HS also declines the proliferation (Tao et al., 2011) and enhances the loss of MEC (Jin et al., 2016), thereby reducing milk yield. Similarly, mammary glands face challenges such as lower milking frequency and decreased milk yield of lactating dairy cows, which are accompanied by disrupted mammary epithelial integrity (Stelwagen and Sing 2014). Thus, intact mammary epithelial integrity and optimum population of MECs are the prerequisite in maintaining maximum milk production. Extracellular structures such as tight junction (TJ) formation influence the secretory activity of MECs. Also, TJ prevents para-cellular transport, thereby maintaining

a small transepithelial potential difference between the basolateral and apical sides needed for the ideal secretory capacity of MECs (Stevenson and Keon, 1998, and Itoh and Bissell, 2003). It is therefore speculated that HS disrupts the secretory capacity of MECs. However, an earlier study indicated that mild HS at 39 °C enhanced milk protein synthesis (Mizusawa et al., 2019), although it is unknown how HS affects TJ formation to alter the secretory capacity of MECs.

Temperature directly affects the biological function of mammary glands to induce cellular stress. To know the effect on MEC loss and TJ formation under severe and mild HS conditions, this study focused on endoplasmic reticulum (ER) stress response, which plays a crucial role in milk production. The Unfolded protein response (UPR) induced the proapoptotic factor *CHOP* (Yonekura et al., 2018), which is responsible for decreasing milk production, thereby proposing a reason for the reduction in the number of MECs in the mammary gland. Previous studies indicate that severe HS activates the UPR pathway in cells (Xu et al, 2011, and Hou et al., 2014). However, how CHOP affects the number of MECs under HS conditions remains unclear. Furthermore, the UPR transcription factor *XBP1* involves an increase in β-casein synthesis under mild HS conditions (Mizusawa et al., 2019), thereby proposing an impact of mild HS on the secretory activity of MECs. It is therefore uncovered how mild HS affects the secretory activity in terms of TJ formation. Thus, this study elucidated the effect of different levels of temperature on MEC number and TJ formation in the context of UPR.

Survival on cellular and organismal levels relies on the appropriate response to changes in temperature. When the core temperature rises above 40°C, cellular damage occurs, thereby initiating a series of events that lead to organ failure and death (Becker et al., 2011). Temperatures greater than 41 °C–42 °C leads to cell death from apoptosis within a few

hours (Sakaguchi et al., 1995). However, the study in chapter II revealed that severe heat treatment at 41°C significantly decreased cell viability and TJ protein-encoding gene-Cldn3 expression. Moreover, UPR-related transcript levels of *Atf4* and *Chop* were increased than mild HS (39 °C) and control (37 °C) conditions. These results also indicate that severe HS stimulates the PERK arm of UPR to induce ER stress-mediated apoptosis. Therefore, PERK is essential for the induced expression of the proapoptotic transcriptional factor *CHOP* under ER stress conditions (Harding et al., 2000). Temperatures greater than 41 °C–42 °C also leads to apoptosis-induced cell death within a few hours (Sakaguchi et al., 1995).

Conversely, hormesis is a phenomenon defined operationally as lower-dose stimulation, followed by higher-dose inhibition. Hormesis induces adaptive responses in cells when exposed to various stressors, including mild HS (Chapman et al., 2005). Mild HS (39 °C–40 °C) positively regulates cell proliferation and differentiation (Park et al., 2005). Indeed, a previous study has shown that heat shock at 39 °C activated milk production and enhanced the formation of less permeable TJs (Kobayashi et al., 2018; Misuzawa et al., 2019). However, detailed molecular mechanisms on the MEC response to mild heat shock have not yet been elucidated. Furthermore, this study showed that the TJ protein-encoding genes *Zo-1, Ocln*, and *Cldn3* transcript levels were significantly higher in cells cultured at 39 °C. Moreover, a 39 °C heat treatment increased *Xbp1s* and *β-casein* (a representative milk protein) expression compared with that of control, which is consistent with previous studies (Mizusawa et al., 2019). Metabolic heat is produced during the milk production process in a lactating mammary gland (Berman et al., 1985). This condition exposes MECs to temperatures above body temperatures. Therefore, mild HS is proposed to enhance or maintain milk production in vivo.

Therefore, to explore the mechanism of TJ formation under mild HS conditions, the current research investigated the effect of mild HS on the expression of temperature-sensitive cell membrane receptors. A membrane surrounds every living cell, keeping the cells' interior separated and protected from the outside world. Many factors affect how this membrane behaves and temperature is among the most important. Results of mild HS at 39 °C increased the expression of temperature-sensitive transient receptor potential vanilloid 4 (TRPV4). It was also reported that under different experimental conditions, TRPV4 maintained a substantial degree of temperature-dependent activity over a range of 30 °C and 40 °C temperatures (Watanabe et al., 2002; Chung et al., 2003). Therefore, TRPV4 was activated under mild HS conditions in MECs.

The activation of TRPV4 strengthens the TJ-associated barrier, thus enhancing the expression of *Ocln* in skin keratinocytes and corneal epithelial cells (Akazawa et al., 2013; Martinez-Rendon et al., 2017). *TRPV4* activation with GSK1016790A also increased *Xbp1s* transcript levels in the study. This study also revealed that *Xbp1* knockdown reduced the mRNA expression of *β-casein*, *Zo-1, Ocln, and Cldn3*, which was increased by mild heat treatment. Thus, *XBP1* regulates the expression of the TJ protein-encoding genes under mild HS conditions. This result is consistent with previous reports that the IRE1-XBP1 pathway regulates retinal pigment epithelium TJs (Ma et al., 2016). Also, although *TRPV4* is activated at 39°C, different TRPs are proposed to be activated at 41°C since TRP channels cover the range of temperatures sensed. However, further mechanistic examinations are necessary, as it is speculated that a difference in UPR gene expression occurs upon stimulation at 39 °C vs. 41 °C in controls' cell survival vs. cell death, respectively (Figure 10). Overall, severe HS lowers cell number by elevating the *CHOP*, whereas mild HS enhances TJ integrity by up-regulating *XBP1s* expression.

It is unambiguously identified that severe HS induces MEC death by increasing the expression of the ER stress proapoptotic marker CHOP, thereby reducing milk production and hamper the sustainability of the dairy industry. However, reduction strategies for HS in the dairy industry are limited by the fact that most dairy cows are exposed to elements as they graze the pasture. Therefore, techniques such as providing shade, sprinklers, and fans for dairy cows have become challenging and expensive for the producers. Additionally, a more practical amelioration strategy is the development of feeding supplementation to maintain the number of MECs, since a reduction in the number of MECs declines milk yield (Capuco et al., 2001). Accordingly, chapter III is well arranged to assess the effective prevention strategy by 5-Aminolevulinic acid (5-ALA) against severe HS-induced apoptosis of MEC. Results showed that pretreatment of bovine MEC with 5-ALA increased the viability and decreased mRNA and protein expression of ER stress markers *GRP78*, CHOP, p-PERK, and cleaved caspase-3 induced by severe HS at 42.5 °C. However, previous studies found that HS activated the PERK–eIF2α–ATF4–CHOP pathway to enhance ER stress-mediated apoptosis in different types of cells, including bovine MECs. Suppressions of those markers by 5-ALA also facilitated more viable bovine MECs than did severe HS conditions alone. Furthermore, 5-ALA enhanced *Nrf2* and its antioxidant genes such as *HO-1* and *NQO1*, which helped in reducing HS-induced oxidative damage in this study. It has also been established that 5-ALA showed its protective effect against apoptosis via *Nrf2* and *HO-1* in different types of injuries (Terada et al., 2013; Huo et al., 2013 and Han et al., 2017). Therefore, 5-ALA enhances the antioxidant gene and provides cytoprotection to bovine MEC against severe ER stress (Figure 10).

In summary, this study revealed that severe ER stress induced apoptosis and loss of TJ formation of MECs, thereby causing reduced milk production besides reduced DMI of

dairy during severe HS conditions. ER stress-induced apoptosis of MECs can therefore be reduced by supplementing with 5-ALA. From a practical viewpoint, further studies are needed to discover the specific level of temperature and duration of HS, which determines the ER stress status in MECs in vivo. Additionally, as a feed additive, a suitable dose of 5-ALA should be determined by assessing the efficacy and response of the UPR component in MECs of the dairy cows during HS conditions.

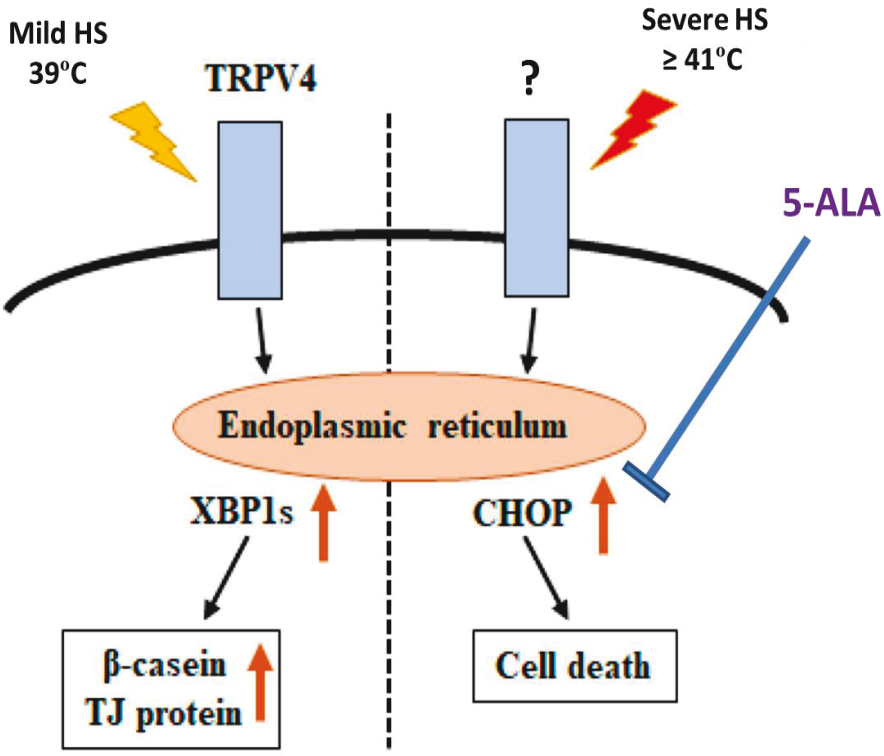

Figure 10: Schematic model showing unfolded protein response (UPR)-mediated crosstalk between temperature and cellular response.

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