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Study on the intracellular mechanism related to milk yield of mammary epithelial cells

By

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Study on the intracellular mechanism related to milk yield of mammary epithelial cells

（乳腺上皮細胞の乳量と関連する細胞内機構に関する研究）

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Contents

Abstract

Modern dairy cows are not well adapted with the higher amount of milk production. The cow starts to synthesis higher amount of milk after parturition, but suffers from physiological stresses due to negative energy balance (NEB). NEB is one of the major reasons for reduction of milk yield as well as lactation persistency during lactation. Therefore, establishment of moderated type of lactation persistency is necessary for sustainable milk production. Knowledge related to mammary gland physiology will be beneficial in this regard. The intracellular mechanisms for increase and decrease of milk yield during early lactation remains unknown. Milk yield is determined by the secretory activity and the number of mammary epithelial cells (MECs). Previous studies suggested that unfolded protein response (UPR) factor X-box binding protein 1 (XBP1) is involved in the synthesis of milk protein and that C/EBP homologous protein (CHOP) expression is negatively correlated with milk yield. However, the detailed mechanisms related to secretory activity and the loss of MECs under the context of UPR needs to be investigated. Therefore, the purpose of the study was to discover the intracellular mechanisms of enhancing the secretory activity and the loss of MECs in bovine MECs.

Firstly, I focused on endoplasmic reticulum (ER) biogenesis to search the mechanism behind the increase of milk production in early lactation. ER biogenesis in terms of ER-membrane bound phospholipid synthesis by associated genes is indispensable for the well-developed and abundant ER. Insulin like-growth factor-1 (IGF1) is the possible candidate for that, since its serum concentration increases in early lactation and is responsible for enhancing the protein synthesis of MECs. Therefore, I investigated whether XBP1s affects the ER biogenesis of bovine MECs and IGF-1 involves in this process or not. MAC-T (a cell line of bovine MECs) cells were treated with IGF-1, and the results showed that IGF-1

enhanced the expressions of *XBP1s* and ER biogenesis-associated genes, ratio of pIRE1α/IRE1α protein, staining of ER tracker dye. 4μ8C, potent inhibitor of IRE1 endonuclease activity, significantly inhibited the IGF-1-induced *XBP1s,* ER biogenesis-associated genes expression and the staining of ER tracker dye. Moreover, IGF-1-induced *XBP1s* and ER biogenesis related gene expressions were significantly reduced by rapamycin (small molecule inhibitor of mTORC1) treatment. Based on *in vitro* study, mammary gland biopsy samples of periparturient dairy cows were analyzed to know the status of ER biogenesis related genes expression under the natural physiological condition. It was found that the relatively low level of *XBP1s* expression in mammary gland tissue before parturition increased gradually from immediately after parturition through lactation. ER biogenesis-related genes and *IGF1R* expressions were also increased in mammary gland tissue during lactation. Therefore, IGF-1 increases *XBP1s* expression to induce ER biogenesis in bovine MECs to enhance the milk production. Thus, IGF-1 may play crucial role for increasing the milk yield.

Previous study found that the expression levels of *CHOP* is negatively correlated with milk yield and induces severe ER stress in MEC, but the triggering factors for *CHOP*-induced apoptosis was unknown. Fatty acids (FA) are the components of non-esterified fatty acids (NEFA), and the serum NEFA concentration also becomes higher during early lactation period in NEB condition. Therefore, the objective was to clarify the impact of FA on inducing ER stress in MECs. MAC-T cells were stimulated with various types of FA to measure the ER stress induced UPR-related genes expression and the viability of bovine MECs. Result showed that, palmitic (PA) and stearic acids induced severe ER stress and apoptosis with enhancing the CHOP expression at both mRNA and protein level. Unsaturated long-chain FA did not induce CHOP expression to reduce the cell viability, marking those FA as moderate ER stress inducer. Therefore, saturated fatty acids play

important roles in MEC viability by inducing severe ER stress. Thus, the reduction of number of MECs by saturated FA can decreases the milk yield and reduces the lactation persistency.

Based on the detrimental effect of PA, prevention was necessary to conserve number of MECs for proper milk yield. 5-Aminolevulinic acid (5-ALA) acts as antioxidant, was speculated to ameliorate the PA-induced cell death due to its anti-apoptotic properties. Therefore, I examined the efficiency of 5-ALA in inhibiting ER stress-induced apoptosis of MECs in response to PA. Result showed that pretreatment of 5-ALA reduced *CHOP* expression to decrease the PA-induced apoptosis of MECs. The apoptosis-related genes *BCL2* and *BAX* were increased and decreased respectively. Moreover, 5-ALA pretreatment elevated the expression of antioxidant gene, heme oxygenase-1. Therefore, 5-ALA has the potential to ameliorate PA-induced severe ER stress in MECs. It can be used to prevent the PA mediated severe ER stress induced cell loss to increase milk yield.

In summary, present study indicates that UPR has the significant role for increasing the ER biogenesis in terms of secretory activity and decreasing the number of MECs. IGF-1 enhances ER biogenesis via IRE-1-XBP1 axis, while excessive amount of saturated FA increases the apoptosis of MECs through PERK-ATF4-CHOP pathway. In addition, 5-ALA down regulated PA-induced *CHOP* expression to decline the bovine MECs death. These findings will be beneficial to understand the regulation of increase and decrease of milk yield at cellular level during early stage of lactation and thus will be helpful to establish moderate type of lactation persistency for sustainable milk yield.

Abbreviation

5-ALA	5-Aminolevulinic acid
ACTB	β- actin
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
BAX	BCL2 associated X
BCL2	B-cell lymphoma 2
BSA	Bovine serum albumin
CCTα	Choline cytidylyltransferase alpha
cDNA	Complementary DNA
CHKA	Choline kinase alpha
CHOP	C/EBP homologous protein
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra acetic acid
eIF2α	Eukaryotic translation initiation factor 2 subunit 1
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
gDNA	Genomic deoxyribonucleic acid
GH	Growth hormone
GRP78	Glucose-regulated protein 78
HO-1	Heme oxygenase-1
IGF-1	Insulin-like growth factor 1
IGF-1R	IGF-1 receptor
IRE1	Inositol-requiring enzyme 1
Keap1	Kelch Like ECH associated protein 1
MAC-T	Mammary alveolar cells-large T antigen.
MEC	Mammary epithelial cell
mRNA	Messenger ribonucleic acid
mTORC1	Mammalian target of rapamycin complex 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NARO	National agriculture and feed research organization
NEB	Negative energy balance
NQO1	NAD(P)H quinone oxidoreductase 1
NRF2	Nuclear factor erythroid derived 2 like factor 2
OxS	Oxidative stress
p- eIF2α	Phosphorylated-eukaryotic translation initiation factor 2 subunit 1
PA	Palmitic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCYT1A	Choline-phosphate cytidylyltransferase A
Pdtcho	Phosphatidylcholine
PERK	Protein kinase RNA(PKR)-like endoplasmic reticulum kinase
p-IRE1	Phosphorylated-inositol-requiring enzyme 1
p-PERK	Phosphorylated-protein kinase RNA(PKR)-like endoplasmic reticulum kinase
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SURF4	Surfeit locus protein 4
UPR	Unfolded protein response
XBP1s	X-box binding protein 1 splicing form
XBP1u	X-box binding protein 1 unsplicing form

Chapter I

General Introduction

1. Fluctuations in milk production during early lactation

During the past decade, global milk production has doubled. Also, high milk-producing cows experience different types of physiological stress due to transitional modification during the periparturient period. The reduction in dry matter intake before parturition (Hayirli et al., 2002) and the increase in milk production with the initiation of lactation (Doepel et al., 2002), reflecting higher nutritional requirements (Bell, 1995), are the major and common consequences of physiological stress. As a result, high-yielding cows suffer from negative energy balance (NEB) conditions, thereby leading to exposure to various metabolic disorders (Collard et al., 2000, Leblanc, 2010), which in turn reduce milk production (Drackley, 1999). The decrease in milk production causes a decline in the lactation curve, which causes a persistency reduction of the lactation curve (Capuco et al., 2003).

Therefore, it is vital for dairy physiologists to elucidate why milk production fluctuates during lactation onset, thereby economically affecting the dairy industry. The establishment of new technology, such as average lactation persistency, will be advantageous to minimize the health problems of dairy cows, thereby promoting sustainable milk production. Therefore, emphasis should be given to manipulate the average lactation persistency by understanding the reason for fluctuating milk production during early lactation. Thus, in milk production, knowledge regarding the mammary gland physiology is inevitable.

2. Physiology of milk production in the mammary gland

The mammary gland consists of a branching network of numerous ducts with terminating alveoli. Each alveolus has a lumen surrounded by a monolayer of epithelial cells. Mammary epithelial cells (MECs) can convert nutrients captured from circulating blood into milk components (McManaman and Neville, 2003).

The secretory activity and number of MECs are important for milk yield during lactation. Knight and Peaker (1984) demonstrated that milk production increases because of an increase in the number of MECs and secretory activity per cell during early lactation. In that study, DNA mass and RNA/DNA ratio were used to indicate MEC population and secretory activity per cell. Another comparative analysis based on the measurement of nucleic acid content extracted from mammary gland samples of varying stages of lactation was performed by Capuco et al., 2001. According to above study, the amount of total DNA content was highest during 14 days of lactation, reflecting the increase in the number of MECs at 14 days of lactation. Also, the increase in milk yield per epithelial cell from early to peak lactation demonstrated the continuous increase in secretory activity of MECs.

Moreover, Boutinaud et al., (2004) stated that the secretory capacity per cell and cell number are important considerations for enhancing milk production from parturition to peak lactation. After peak lactation, milk yield gradually declines because of the gradual reduction of secretory cells. A previous study revealed that a 17% decrease in DNA (as the indication of cell number) content is enough to reduce 23% of milk production from the peak to end of lactation in dairy cows (Capuco et al., 2001). In the case of goats, the reduction of milk and total DNA was 20% and 19%, respectively, from the peak to end of lactation (Knight and Peaker, 1984). Therefore, any alteration in number or secretory

activity of MECs is the principal reason for fluctuating milk production, which leads to the vulnerability of lactation persistency.

However, the augmented number and secretory activity of MECs increase milk yield and vice versa. Indeed, how the secretory activity and their population are regulated in the mammary gland remains unknown. To establish the average lactation persistency through sustainable milk production, knowledge regarding intracellular mechanisms of controlling the secretory activity and number of MECs during milk production is indispensable. Therefore, attention should be given to uncover the intracellular mechanism of determining the population and secretory activity of MECs during milk production, thereby leading to a moderate lactation persistency.

3. Association between unfolded protein response and milk synthesis in the endoplasmic reticulum (ER) of MECs

Subcellular organelles largely determine the structure and functions of cellular systems. Each cell contains a single continuous membrane-bounded organelle named the ER, which is completely unique from a structural and functional viewpoint. The most important functions of ER are synthesizing the multiple protein types, their correct folding, and maturation for the destined secretory pathway, delivery of folded proteins to their proper target sites within the secretory pathway, and the extracellular space (Paschen and Frandsen, 2001, Breckenridge et al, 2003 and Rao et al., 2004). Ideal folding and maturation of proteins can be perturbed by various causes in cellular environments, such as calcium concentration, ischemia, and production and overproduction of abnormal proteins. As a result, copious amounts of misfolded proteins are accumulated within the ER membrane. With the aggregation of excessive amounts of misfolded proteins, the

folding capacity of ER is overwhelmed. Under this condition, cells suffer from ER stress due to proteio-toxicity; thus, ER homeostasis becomes imbalanced (Ron and Walter, 2007).

A group of molecules can sense the presence of accumulated abnormal proteins within the ER lumen and transmit signals to the nucleus and cytoplasm. To restore proteio-toxic stress, the cellular system possesses a well-orchestrated process, which uses ER stress sensors, called the unfolded protein response (UPR) (Rutkowski and Hegde, 2010). The UPR has three arms: double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (Patil and Walter, 2001; Ron, 2002). The functions of UPR are the following: (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. Under non-stressed conditions, those ER transmembrane proteins remain inactive by binding with ER chaperon-like or GRP78. Upon ER stress, ER transmembrane signal transducers are released from GRP78 and become autophosphorylated to transmit signals downstream in various forms (Lee, 2005). Activated IRE1 cleaves 26 bp nucleotides through its endonuclease activity from X-box binding protein 1 (XBP1) mRNA (Calfon et al., 2002). This unconventional splicing generates XBP 1 splicing form (XBP1s), which then translocates to the nucleus and functions as a transcription factor (Yoshida et al., 2003). The PERK protein phosphorylates eukaryotic initiation factor 2α (eIF-2α) to reduce its functions, enhancing the translation of transcription factor activating transcription factor-4 (ATF4) (Harding et al., 1999, 2000). Moreover, a previous study suggested that, during ER stress, eIF-2α phosphorylation increases, which in turn increases ATF4 mRNA expression (Chan et al., 2017). ATF6α is transported to the Golgi apparatus, where it is cleaved within the membrane by sites 1 and 2 proteases, depending on the ER stress. From

this cleavage operation, N-terminal fragments migrate to the nucleus and serve as transcription factors (Ye et al., 2000). In the context of extreme or persistent ER stress, cells undergo apoptosis and die. Under this condition, ATF4 induces the transcription of C/EBP homologous protein (CHOP), a transcription factor that promotes apoptosis (Zinszner et al., 1998).

Studies suggested that UPR plays an important role in the production of milk in MECs. UPR-induced transcription factor XBP1 is involved in the increased milk protein synthesis of MECs (Davis et al. 2016). This study indicates that XBP1 has a positive influence on the secretory activity of MECs. Another study also reported that elevated expression of UPR-induced CHOP is negatively correlated with milk yield in mammary gland tissues during early lactation (Yonekura et al, 2018). The negative correlation between CHOP and milk yield suggests a reduction in the number of MECs. Thus, UPR has a significant impact on the enhancement of secretory capacity and cellular apoptosis. But the detailed molecular mechanisms regarding the increase in secretory activity and the decrease in the number of MECs in connection with UPR have not been elucidated. Therefore, this study aims to find out the intracellular mechanism of enhancement of secretory capacity and loss of MECs in terms of fluctuating milk yield during early lactation.

4. Possible stimulators to induce UPR in MEC and preventive approach in case of MEC death

To search the mechanism behind the increase in milk production during early lactation, this study first focused on ER biogenesis. It is the indispensable feature for proper development and well-abundant ER in any cell, which is mainly characterized by the biosynthesis of ER membrane-bound phospholipid through the elevation of its associated genes. ER biogenesis is necessary to maintain the efficient secretory capacity of epithelial

cells (Bommiasamy et al., 2009). The development and abundance of ER is the requirement for maintaining proper lactation capacity in mice, where XBP1 bears a significant role (Davis et al. 2016). Therefore, XBP1 may be important for ER biogenesis in bovine MEC.

For the induction of XBP1-induced ER biogenesis, insulin-like growth hormone factor (IGF-1) may be the suitable candidate since there is common evidence supporting the increase in plasma concentration of IGF-1 in early lactation (Radcliff et al., 2006; Wathes et al., 2003 and 2007). IGF-1 upregulated protein synthesis in bovine MECs (Borgos and Cant, 2010). For the synthesis of large amount of protein ER development is the requirement (Pukh et al., 2007). Therefore, I considered whether XBP1s induces ER biogenesis and IGF-1 involved in XBP1s-induced ER biogenesis in bovine MECs.

It has already been mentioned that severe ER stress induces CHOP expression level, which is negatively correlated with milk yield in the early lactation period. But what are the triggering factors for CHOP-induced bovine MEC death that remain unknown? Fatty acids (FA) were taken into consideration to uncover the mechanisms responsible for reducing the number of MECs in the context of UPR during early lactation. FAs are the components of NEFAs, produced from metabolism of adipose tissues during early lactation under NEB conditions. Also, the plasma concentration of NEFA increases within that period (Wathes et al., 2007 and Sheehy et al., 2017). Therefore, this study clarified the impact of FA in inducing ER stress in bovine MECs and, in the same way, established the mechanism for UPR-induced MEC loss during the early lactation period.

As the reduction of MECs decreases the milk yield in dairy cows, prevention is necessary to preserve the number of MECs for sustainable milk production. 5-Aminolevulinic acid (5-ALA) is a non-essential amino acid found in different natural and fermented foods. It is

a potent antioxidant and is effective against cell death induced by ER stress. *In vivo* and *in vitro* studies revealed that 5-ALA enhanced the antioxidant gene heme oxygenase-1 (HO-1) expression, which was beneficial to different stress types, 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 and Hou et al., 2013). Thus, it is speculated that 5-ALA may be effective against the loss of bovine MECs during early lactation.

Therefore, present study seeks to unravel the following questions: (1) How does IGF-1 influence the induction of ER biogenesis via the IRE1-XBP1 arm in bovine MECs, and how does IGF-1 affect the number of MECs? And (2) what are possible approaches to resolve the UPR-induced severe ER stress-mediated apoptosis of bovine MECs? Accordingly, this study was designed as follows: In chapter II, both *in vitro* and *in vivo* studies performed to uncover the molecular mechanisms of IGF-1-induced IRE1-XBP1-mediated ER biogenesis in bovine MECs are presented. In chapter III, an *in vitro* study conducted to elucidate the role of FA in inducing ER stress and apoptosis via UPR-related gene expression in bovine MECs is described. In chapter IV, another *in vitro* study conducted to discover the protective measures against palmitic acid (PA)-induced, UPR-instigated severe ER stress and apoptosis in bovine MECs via the addition of 5-ALA is arranged. Finally, chapter V discusses the role of IGF-1 and FA in the maintenance of secretory activity and the number of bovine MECs, also to establish a moderate type of lactation persistency in the dairy industry.

Chapter II

IGF-1 induces IRE1/XBP1-dependent endoplasmic reticulum biogenesis in bovine mammary epithelial cells

2.1. Abstract

IGF-1 plays a key role in galactopoiesis in MECs, but its definitive functions on ER during protein synthesis remain unknown. The present study aimed to elucidate the action of IGF-1 on ER biogenesis in MECs *in vitro* and examine the level of expression of ER biogenesis associated genes *in vivo*. I treated MAC-T (bovine MEC line) with IGF-1 and examined ER biogenesis using the fluorescence intensity of an ER tracker and quantitative real-time PCR. IGF-1 significantly increased ER tracker staining and upregulated mRNA levels of ER biogenesis-related genes, such as choline kinase alpha (*CHKA*), choline-phosphate cytidylyltransferase A (*PCYT1A*) and surfeit locus protein 4 (*SURF4*). I focused on UPR to explore molecular mechanisms by which IGF-1 induces ER biogenesis. IGF-1 significantly increased mRNA levels of *XBP1s*. Based on western blot analysis, IGF-1-induced the expression of IRE1α protein, upstream of XBP1s, and phosphorylated-IRE1α. The inhibition of IRE1 endoribonuclease activity with 4μ8C significantly suppressed the increase in ER tracker fluorescence and ER biogenesis-related gene expression induced by IGF-1. IGF-1-induced *XBP1s* and ER biogenesis associated genes expression was also inhibited by rapamycin (a small molecule inhibitor of mTORC1), which indicated a relationship between mTORC1 and IRE1-XBP1 axis. Moreover, to know the expression level of *XBP1s* and ER biogenesis associated genes expression under normal physiological condition, mammary gland tissue from biopsies of dairy cows during late gestation and lactation periods were analyzed. The data from *in vivo* experiment showed the significant increases in the mRNA levels of *XBP1s* and ER biogenesis-related genes in mammary gland tissue immediately after calving through 6 weeks of lactation. The mRNA levels of

IGF-1R in mammary glands increased during 6 week of lactation. Therefore, present study indicates for the first time that IGF-1 induces ER biogenesis by activating the IRE1-XBP1 axis under the regulation of mTORC1 in bovine MECs.

2.2. Introduction

Now a day's dairy industry is facing problems with high yielding dairy cows. In early lactation period, the cow initiates to secrete large amount of milk, which is stressful for cow at cellular level. Due to the physiological stress milk production of cow reduces, which in turn lowers the lactation persistency. Therefore, expected higher persistency can not be achieved with the stressful heath condition of the cow. Life time productivity of cow should be considered instead of the highest milk production per lactation. Emphasis should be given to develop the technology of moderate type of lactation persistency for sustainable milk yield. Knowledge regarding intracellular mechanism of how milk production increases in early lactation period will be advantageous in this regard.

ER plays crucial role for the biosynthesis of milk components. The biogenesis of this specialized compartment varies depending on the demands of the external cytosolic pathway in different secretory cells. For example, well-developed rough ER network was formed in different types of secretory cells (Bolender, 1974, Rush et al., 1991) including epithelial cell. (Bommiasamy et al., 2009), which is impossible without the biosynthesis of ER membrane bound phospholipid.

The membrane of ER is largely made up of phosphatidycholin (Pdtcho) from which other membrane-bound phospholipids such as sphingomyelin and phosphatidylethanolamine are synthesized (Voelker and Kennedy, 1982, Voelker, 1984). Pdtcho is necessary for the synthesis of secretory protein and lipid from different types of cells. For example, the expression of lung surfactant protein from alveolar cell type II was reduced with the reduction of pdtcho synthesis (Tian et al., 2006). The biosynthesis of pdtcho was also concomitant with the *de novo* biosynthesis of lipid in NIH-3T3 cell (Bommiasamy et al., 2009). Therefore, it was speculated that, increased biosynthesis of pdtcho is important for

the synthesis of milk proteins and lipids. The first step of Pdtcho biosynthesis is the conversion of choline to phosphocholine by choline kinase, including choline kinase alpha (CHKA) (Wu and Vance, 2010). Subsequently, choline-phosphate cytidylyltransferase A (PCYT1A), the rate-limiting enzyme for Pdtcho synthesis, yields cytidine diphosphocholine from phosphocholine (Kent, 1997). Finally, cytidine diphosphocholine and diacylglycerol are condensed to produce Pdtcho (Sundler et al., 1972).

Accumulating evidence suggested that, UPR component gene XBP1s enhanced the biosynthesis of Pdtcho (Sriburi et al., 2004). XBP1s also increased in surface area and volume of rough ER by augmenting the protein expression of phosphocholine cytidylyltransferase-α (Sriburi et al., 2007), which is encoded by the gene PCYT1A (Lykidis et al., 1999). The absence of XBP1s in mice reduced the production of immunoglobulin and pancreatic digestive enzymes from B cells and exocrine pancreas, respectively (Iwakoshi et al., 2003, Lee et al., 2005). In case of MECs, XBP1s bears the crucial role for the biosynthesis of copious amounts of milk protein and fat (Hasegawa et al., 2015 and Tsuchiya et al., 2017). Davis et al., (2016) showed that XBP1s is responsible for well-developed and abundant ER, which was already mentioned. Hence, it was hypothesized that, the IRE1-XBP1 axis may be crucial for increasing the ER biogenesis in bovine MECs.

Every cellular event happens under the regulation of physiological stimulator. Likewise, ER biogenesis also needs a triggering factor. In bovine MEC, Insulin-like growth factor-1 (IGF-1) may be considered as the as the stimulator for the induction of ER biogenesis on behalf of following reasons. Plasma IGF-1 concentration of becomes higher during early lactation, which is already mentioned. It regulates the growth, development, and functions of the mammary gland (McGrath et al., 1991, Zhao et al., 1992, Silva et al., 2005). IGF-1 upregulated the protein synthesis, including κ-casein, in bovine MECs (Burgos and Cant,

2010, Wang et al., 2016). Adaptation to change condition like increased amount of protein synthesis requires increased biosynthesis of ER membrane bound Pdtcho (Tian et al., 2006). The remodeling of ER is the fundamental characteristics during proliferation of cell (Puhka et al., 2007), which also demands for the increased amount of protein synthesis. However, the mode of action of IGF-1 on cellular organelles during protein synthesis remains obscure. Therefore, the objective of the present study was to investigate whether IRE1-XBP1 axis enhances the ER biogenesis in bovine MECs and whether IGF-1 involves in that process or not.

2.3. Materials and Methods

2.3.1. Reagents

Fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) were bought from Equitech-Bio Inc. (Kerrville, TX, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Bovine hydrocortisone, insulin, Penicillin-Streptomycin Mixed Solution, gentamycin, 4μ8C (4-methyl umbelliferone 8-carbaldehyde) and rapamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human IGF-1 was collected from Gibco (Waltham, MA, USA). Other chemicals were acquired from Nacalai Tesque (Kyoto, Japan).

2.3.2. Cell culture and treatment

MAC-T cell was generously gifted by Dr. Sangun Roh (Tohoku University, Sendai, Japan). 1.0×10^5 MAC-T cells were seeded in 35 mm dish (at least triplicates dishes for each treatment) with DMEM containing FBS (10%), bovine insulin (5 μg/mL), streptomycin and penicillin (1%), and hydrocortisone (1 μg/mL). After 48 h when the cells were reached 80%–90% confluency, then the cells were incubated in DMEM without serum for 12 h before treatment. Then cells were treated with desired concentrations of IGF-1 (10 and 100 ng/mL) for 24 h with or without 40 μM of 4μ8C, a strong inhibitor of IRE1endonuclease activity. This molecule binds with lysine 907 of IRE1 endonuclease domain and restricts the accessibility of substrate to the active site of IRE1. Thus, it inhibits the unconventional splicing of XBP1 to produce XBP1s and reduce the secretory capacity without exerting acute ER stress, Crosss et al., 2012. The cells were also treated with 100 nM of rapamycin (a potent inhibitor of mTORC1) for 24 h. Rapamycin binds with FK506-binding protein of 12 kDa (FKBP12) and interacts with the FKBP12-

rapamycin binding domain of mTOR. Thus prevents the functions of mTORC1 (Guertin and Sabatini, 2007). In all cases, cells were incubated at 37 ºC under 5% CO_2.

2.3.3. RNA extraction and quantitative real-time PCR

Total RNA was isolated from mammary glands or MAC-T cells using TRIzol (Invitrogen) following the manufacture's protocol. cDNA was synthesized from gross RNA utilizing gDNA Remover with qPCR RT Master Mix (Toyobo, Osaka, Japan). The purity of RNA was ensured based on A260/280 values ranged from 1.85-1.90 using a nanodrop analyzer. SYBR Premix Ex TaqTM ll (TaKaRa Biotechnology, Kusatsu, Japan) was used for quantitative real-time PCR. Primer sequences are shown in Table 1. Bovine β-actin (*ACTB*) and ribosomal protein S9 (*RPS9*) were used as housekeeping genes. The relative expression of bovine *XBP1s*, *ATF4*, *ATF6α*, *CHOP*, *GRP78*, *CHKA*, *PCYT1A*, *SURF4*, *IGF-1*, and *IGF-1R* mRNA are presented based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Amplification and reaction sensitivities of contaminating products, for example, the elongation of self-annealed primers, were measured by increasing the serial dilutions of cDNA. Manufacturer's instructions were strictly followed during data analysis.

2.3.4. Fluorescence image and flow cytometry

For the fluorescence image, MAC-T cells (0.10×10^5 cells/well with at least triplicates wells) were grown in 8 well chambers for 48 h to reach the confluency of 80-90% with DMEM containing 10% FBS, bovine insulin (5 µg/mL), streptomycin and penicillin (1%), and hydrocortisone (1 µg/mL). After 12 h serum free condition, the cells were treated with 100 ng/ml IGF-1 for 24 h. Finally the cells were washed with HBSS (Hank's balanced salt solution) to stain with 1 µM of (ER Tracker Green (Invitrogen) at 37 ºC for 30 minutes. At the end of incubation the ER tracker was removed and washed with HBSS. Fluorescence microphotographs were then obtained on an EVOS® FL auto imaging system (Thermo

Fisher Scientific, Rockford, USA).

For flow cytometry, cells were cultured in 35 mm dishes as previously described in cell culture and treatment section. After 100 ng/ml IGF-1 treatment cells were washed with HBSS and collected in 1.5 ml microtube using a scraper. Then ER Tracker Green at the final concentration of 1 μM was used for 30 minutes incubation at 37 ºC. Then the ER Tracker stain was removed and the cells were re-suspended in HBSS and analyzed using an SH800S cell sorter (Sony Biotechnology Inc., USA). Mean fluorescence intensity from the FITC channel was normalized to cell size as determined from forward scatter (Jia et al., 2011).

2.3.5. Western blotting analysis

Cells were washed twice and then lysed with radioimmunoprecipitation assay lysis buffer (0.05% SDS, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1% NP-40, and 0.2% sodium deoxycholate) containing protease inhibitor cocktail (Nakalai Tesque) for 30 min. Lysates were centrifuged at 20,000 × g for 10 min, and protein samples were collected. Protein concentrations were measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). SDS-PAGE was performed on 4%–20% polyacrylamide gels using 60 μg of protein. Protein bands were subsequently transferred to polyvinylidene difluoride membranes. Membranes were incubated with anti-IRE1α (Cell Signaling Technology, Danvers, USA), anti-phosphorylated-IRE1α (Thermo Fisher Scientific, Rockford, USA), or anti-α-tubulin (MBL Co., Nagoya, Japan) antibodies in blocking buffer. After incubating with horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Pittsburgh, PA, USA), labeled proteins were visualized using an ECL Prime Western Blotting Detection Reagent kit (GE Healthcare); images were captured using an Image Quant LAS 500 (GE Healthcare) and analyzed with ImageJ software from the NIH.

2.3.6. MTT assay for cell proliferation measurement

MAC-T cells were seeded at a density of 2×10^3 cells/well in 96-well plates and cultured for 48 h to reach 80-90% confluency. After 12 h of serum free condition the cells were then treated with 100 ng/ml IGF-1 and incubated for 24 h. Then the cell viability rate was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) Cell Viability Assay Kit (Biotium, Fremont, CA) according to the manufacturer's protocol. Briefly, 10 μL of the MTT solution was added to 100 μL of culture medium and incubation was done for 4 h at 37 ºC. Then 200 μL of dimethylsulfoxide was added to each well. A multimode microplate reader (iMark microplate reader, Bio-Rad Laboratories, Hercules, CA) was used to measure the absorbance at 570 nm and a reference wavelength of 630 nm.

2.3.7. Animals and sampling

The animal experiment was conducted according to the "Guideline for the Institute of Livestock and Grassland Science, NARO" and approved by the Animal Care Committee of this Institute (approval number: 14112012).

The purpose of *in vivo* experiment was to examine the level of expression of ER biogenesis related genes during lactation. Therefore, six multiparous Holstein cows (parity 2.2 ± 0.4) were used during peripartum (−1 to 6 weeks postpartum) for mammary gland biopsy. Cows were housed in free stalls except around calving time. Cows remained in a calving pen near the time of calving (about −3 to 3 d postpartum). Animals had free access to fresh water and trace mineral salt blocks (Koen ZENOAQ, Koriyama, Japan). Diet was formulated to meet or exceed the Japanese Feeding Standard for Dairy Cattle (NARO, 2006). Cows were offered diet *ad libitum* twice daily at 0930 and 1630 h during prepartum and three times daily at 0930, 1500, and 1800 h during postpartum. Diets were fed as

TMR, based on corn silage, timothy hay, alfalfa hay, beet pulp, steam-flaked corn, soybean meal, vitamin mix, and mineral mix. Animals were milked in a milking parlor twice daily at 0900 and 1730 h.

Mammary gland tissue from side to side of the rear quarters was collected by repeated biopsy at −1 week (−9 to −4 d), 0 weeks (0 d), and 6 weeks (40–45 d) (n = 6 cows × 3 time points) as previously described (Farr et al., 1996). Briefly, cows were administered an i.m. injection of 2% lidocaine (0.05 mg/kg of BW; 2% Seractal; Bayer, Leverkusen, Germany) and 5% tranexamic acid (2.5 mg/kg of BW; Vasolamin injection; Meiji-Seika Pharma, Tokyo, Japan). Quarters were clipped and scrubbed with 2% isodine solution (Meiji-Seika Pharma) and 70% ethanol. The biopsy site was selected at a midpoint of the rear quarter, the area anesthetized by application of 2% lidocaine HCl jelly (2% Xylocaine jelly; AstraZeneca, London, UK), and a subcutaneous injection of 3 mL 2% lidocaine HCl subdermal block (2% Xylocaine injection; AstraZeneca) was administered. Biopsy was performed using a Bard Magnum Biopsy Gun system with 12G, 10 cm Bard Magnum needles (Bard Biopsy Systems, Tempe, AZ, USA), avoiding any large subcutaneous blood vessels. The collected samples were rinsed with ice-cold saline solution and immediately frozen in liquid nitrogen. Samples were stored at −80 ºC until gene expression analyses. Pressure was applied to the puncture hole to reduce blood accumulation under the skin immediately after the biopsy, and the site was disinfected with 10% isodine gel (Meiji-Seika Pharma). After sampling, an i.m. dose of procaine benzylpenicillin antibiotic (Mycillin Sol; Meiji-Seika Pharma) and Vasolamin was administered, followed by application of 10% isodine gel at the biopsy site. Methyl salicylate formulations (Andres ointment; Kyoritsu Seiyaku Co., Ltd.) were applied to the udder for at least 2 d. Cows were machine-milked and hand-stripped to remove intramammary blood clots after sampling at 0 and 6 weeks postpartum. Cows were hand-stripped as required at each

milking for several days until all blood clots were removed and blood contamination was cleared.

2.3.8. Statistical analysis

Mean ± standard error was used for expressing values from a typical experiment performed, at a minimum, in triplicate dishes. All analyses used SAS (Version Add-In 7.1 for Microsoft Office; SAS Institute Japan Ltd., Tokyo, Japan). For gene expression and flow cytometry results, Tukey–Kramer tests or Paired *t*-tests were used to determine statistical differences. These tests were also considered significant at $p < 0.05$.

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

Gene	Primers (5' to 3')	R^2	PCR efficiency
XBP1s	Forward TGCTGAGTCCGCAGCAGGTG	1.00	99
	Reverse GCTGGCAGACTCTGGGGAAG		
ATF4	Forward CCGAGATGAGCTTTCTGAGC	1.00	95
	Reverse AGCATCCTCCTTGCTGTTGT		
CHOP	Forward CTGAAAGCAGAGCCTGATCC	1.00	96
	Reverse GTCCTCATACCAGGCTTCCA		
ATF6α	Forward CACTCAGCAGAACAGGGACA	1.00	101
	Reverse AGGATGGCAAGCAATGTTTC		
CHKA	Forward GGCTCTTTGGAACAATGGAA	1.00	102
	Reverse CTAGCTCCAAAGGCAGGTTG		
PCYT1A	Forward ATGAGCGCTATGATGCAGTG	1.00	98
	Reverse AAATCAATCCGGTGTTCTGC		
SURF4	Forward ACTTTGACGCCAGCTTCTTC	1.00	99
	Reverse CAAACAGCCAGACGACAAGA		
IGF-1R	Forward TGGAGTGCTGTATGCCTCTGT	1.00	97
	Reverse GGTCTCGGGCTCATCCTT		
ACTB	Forward CATCGCGGACAGGATGCAGAAA	1.00	91
	Reverse CCTGCTTGCTGATCCACATCTGCT		
RPS9	Forward CCTCGACCAAGAGCTGAAG	1.00	100
	Reverse CCTCCAGACCTCACGTTTGTTC		

2.4. Results

2.4.1. Effect of IGF-1on the proliferation of bovine MECs

To know the effect of IGF-1 on proliferation of MAC-T cells, MTT assay was conducted. The result demonstrated that there is no significant difference ($p > 0.05$) between the proliferation rates of IGF-1 treated cells and control one (Figure 1).

2.4.2. IGF-1 induces ER biogenesis in bovine MECs

The total ER mass in IGF-1-treated bovine MECs was assessed using an ER tracker dye. IGF-1-treated cells induced higher levels of ER staining than did untreated control cells (Figure 2A). The flow cytometric analysis of ER tracker staining showed more than a 2.5-fold increase ($p < 0.05$) in ER fluorescence in treated cells (Figure 2B). Moreover, mRNA levels of ER biogenesis-related genes, such as *CHKA*, *PCYT1A*, and *SURF4*, were significantly upregulated ($p < 0.05$) by exposure to IGF-1 (Figure 2C).

2.4.3. IGF-1 activated the IRE1-XBP1 axis of the UPR in bovine MECs

I hypothesized that IGF-1-induced ER biogenesis in bovine MECs would be affected by UPR. Thus, I evaluated the action of IGF-1 on UPR-related gene expression. IGF-1 significantly increased ($p < 0.05$) *XBP1s* and reduced ($p < 0.01$) *ATF6α* expression levels but did not influence *ATF4*, *CHOP*, and *GRP78* expression in MECs (Figure 3A). I then analyzed the protein expression of p-IRE1α and IRE1α (upstream proteins of XBP1) to determine whether the IRE1–XBP1 arm of the UPR is activated by IGF-1. The ratio of p-IRE1α and IRE1α protein expression was significantly increased ($p < 0.05$) in bovine MECs treated with IGF-1 (Figure 3B).

2.4.4. IRE1-XBP1 axis is involved in IGF-1-induced ER biogenesis

To examine the role of the IRE1-XBP1 axis on ER biogenesis induced by IGF-1, I used the small molecule, 4μ8C, an inhibitor specific for the IRE1 RNase domain. Generally, the RNase domain of activated IRE1 cleaves a 26 bp fragment from the mRNA encoding the transcription factor X-box-binding protein 1 (XBP1) by an unconventional splicing event to generate XBP1s (Calfon et al., 2002). Then the produced XBP1s translocates to the nucleus, where it functions as a transcription factor (yoshida et al., 2003). 4μ8C inhibits the cleavage of *XBP1* via preventing the substrate entry into the endoneuclease active site of IRE1 (Crosss et al., 2012). I measured the expression of *XBP1s* mRNA to confirm the efficacy of 4μ8C (40 μM) using MECs. This treatment significantly suppressed the IGF-1-induced *XBP1s* expression. Compared with treatment with IGF-1 alone, co-treatment with IGF-1 and 4μ8C also significantly decreased ($p < 0.05$) the expression of *CHKA*, *PCYT1A*, and *SURF4* (Figure 4A). Moreover, co-treatment with IGF-1 and 4μ8C reduced ER staining compared with treatment with IGF-1 alone (Figure 4B).

2.4.5. Inhibition of IGF-1-induced XBP1s and ER biogenesis related genes expression by rapamycin

Previous study established that IGF-1 increased the protein synthesis via stimulating the mTORC1 in MAC-T cell (Burgos and Cant 2010). Therefore, I was interested to know the relationship between mTORC1 and IRE1-XBP1s axis in IGF-1 treated cell. For this reason, cells were treated with IGF-1 in the presence or absence of rapamycin (100 nM). This small molecule inhibition experiment revealed that co-treatment of IGF-1 and rapamycin significantly reduced the IGF-1-induced XBP1s expression ($p < 0.05$) than that of single IGF-1 treated cell. The expression of ER biogenesis related genes in IGF-1 and rapamycin treated cell were also down regulated ($p < 0.05$) compared to the control and single IGF-1

treated group (Figure 5). These data suggested that mTORC1 influenced the ER biogenesis of MEC through regulating the UPR factor XBP1s.

2.4.6. Changes in the gene expression in mammary gland tissue during the periparturient period

To investigate the level of expression of *XBP1s*, *IGF-1R* and ER biogenesis associated gene *in vivo*, I collected mammary gland tissues from Holstein cows from the late stages of gestation through the lactation stage and analyzed gene expression patterns using qRT-PCR. The expression levels of *XBP1s*, *CHKA*, *PCYT1A*, and *SURF4* in mammary gland tissues were gradually upregulated ($p < 0.05$) from immediately after calving through 6 week of lactation. *IGF-1R* expression was significantly increased ($p < 0.05$) during 6 week of lactation (Figure 6).

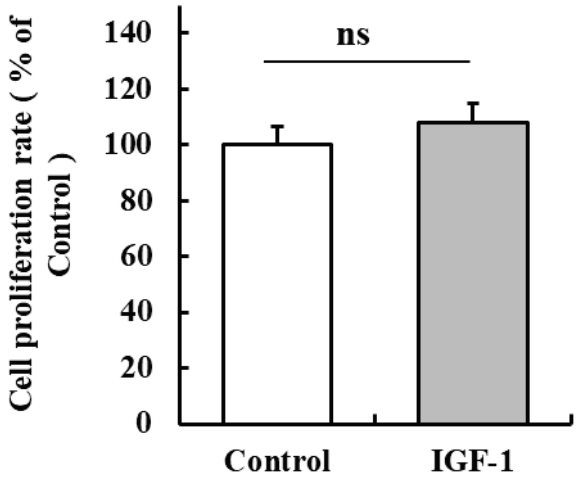

Figure 1: Assessment of proliferation rate of MAC-T cells using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 80-90% confluent cells were treated with 100 ng/ML IGF-1, for 24 h. Absorbance reading was taken at 570 and 630 nm using a multimode microplate reader to calculate the percentage of viable cells. Data are presented as mean ± SEM for 3 independent experiments. * indicates $p < 0.05$ compared with control. This picture has been taken from Journal of Dairy Science.

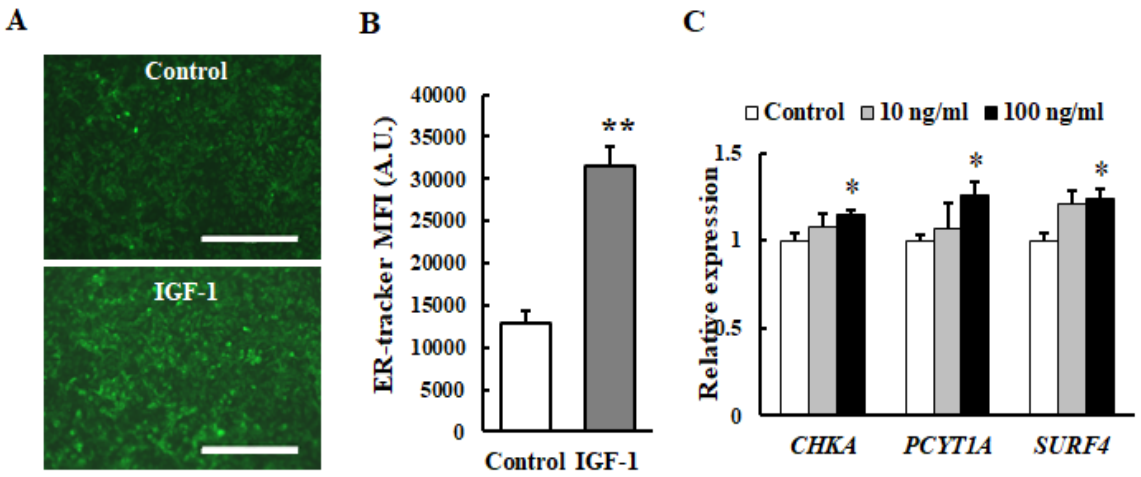

Figure 2: Effect of IGF-1 on ER biogenesis in bovine MECs. (A) Representative photomicrographs of ER tracker staining in control and IGF-1-treated bovine mammary epithelial cells (MECs). Scale bar, 400 μm. (B) Quantification of ER tracker fluorescence intensity using flow cytometry for control MECs and MECs treated with 100 ng/mL IGF-1 for 24 h. MFI, mean fluorescent intensity. (C) Relative expressions of choline kinase alpha (*CHKA*), choline-phosphate cytidylyltransferase A (*PCYT1A*), and surfeit locus protein 4 (*SURF4*) in control cells and 10 and 100 ng/mL IGF-1-treated cells for 24 h. Relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method and presented as values relative to control. Data are presented as the mean ± SEM for three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with controls. This picture has been taken from Journal of Dairy Science.

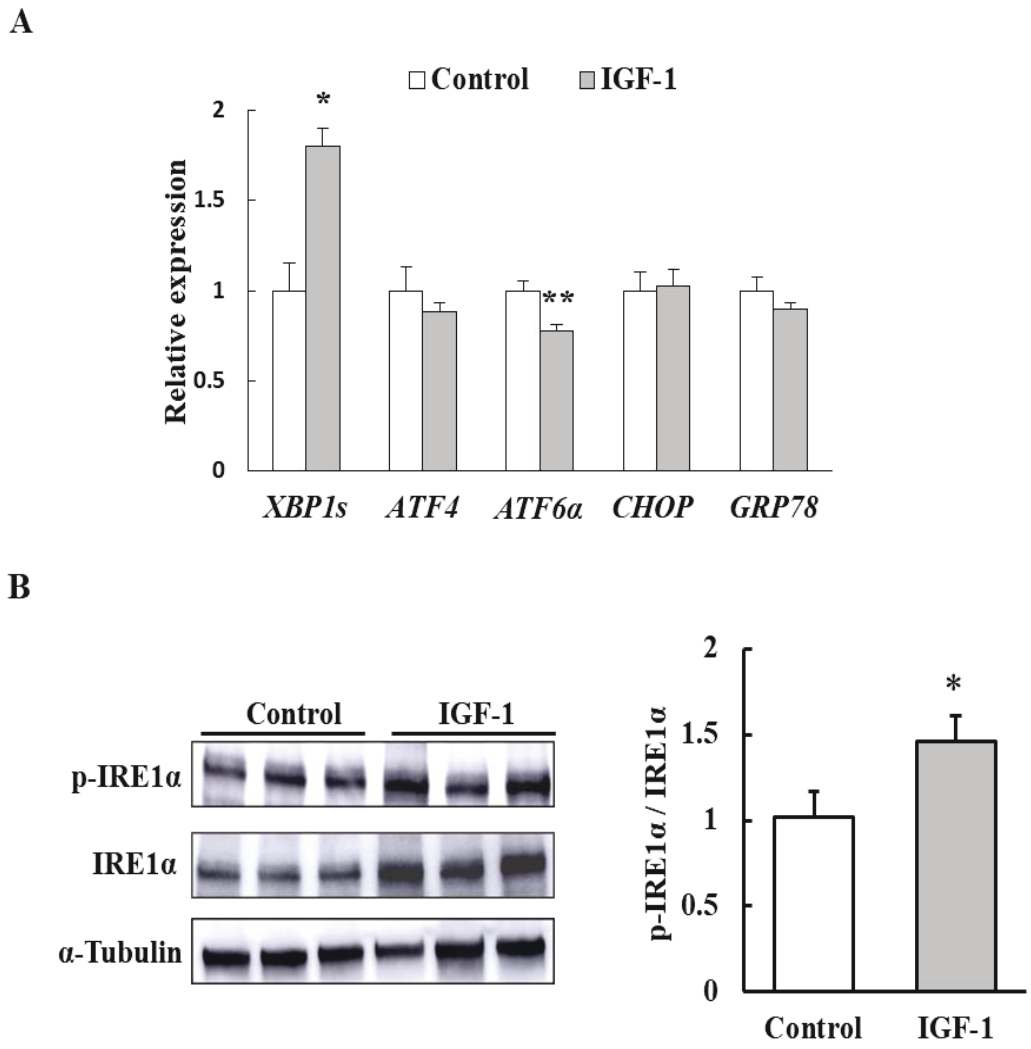

Figure 3: Effect of IGF-1 on the expression of UPR-related genes in bovine MECs. (A) Relative expression of X-box binding protein 1 splicing form (*XBP1s*), activating transcription factor 4 (*ATF4*), activating transcription factor 6 alpha (*ATF6α*), C/EBP homologous protein (*CHOP*), and glucose-regulated protein 78 (*GRP78*) in control cells and 100 ng/mL IGF-1-treated cells for 24 h. Relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method and presented as values relative to controls. (B) Bovine MECs were stimulated with 100 ng/mL IGF-1 and inositol-requiring kinase 1 alpha (IRE1α), phospho-IRE1α (p-IRE1α), and α-Tubulin (internal control) protein levels were assessed using western blotting. Representative images from at least three independent experiments are shown (Left panel). Quantification ratio of p-IRE1α/IRE1α levels is provided in the bar graph. Data are presented as mean ± SEM for three independent experiments. *$p < 0.05$ compared with controls (Right panel). This picture has been taken from Journal of Dairy Science.

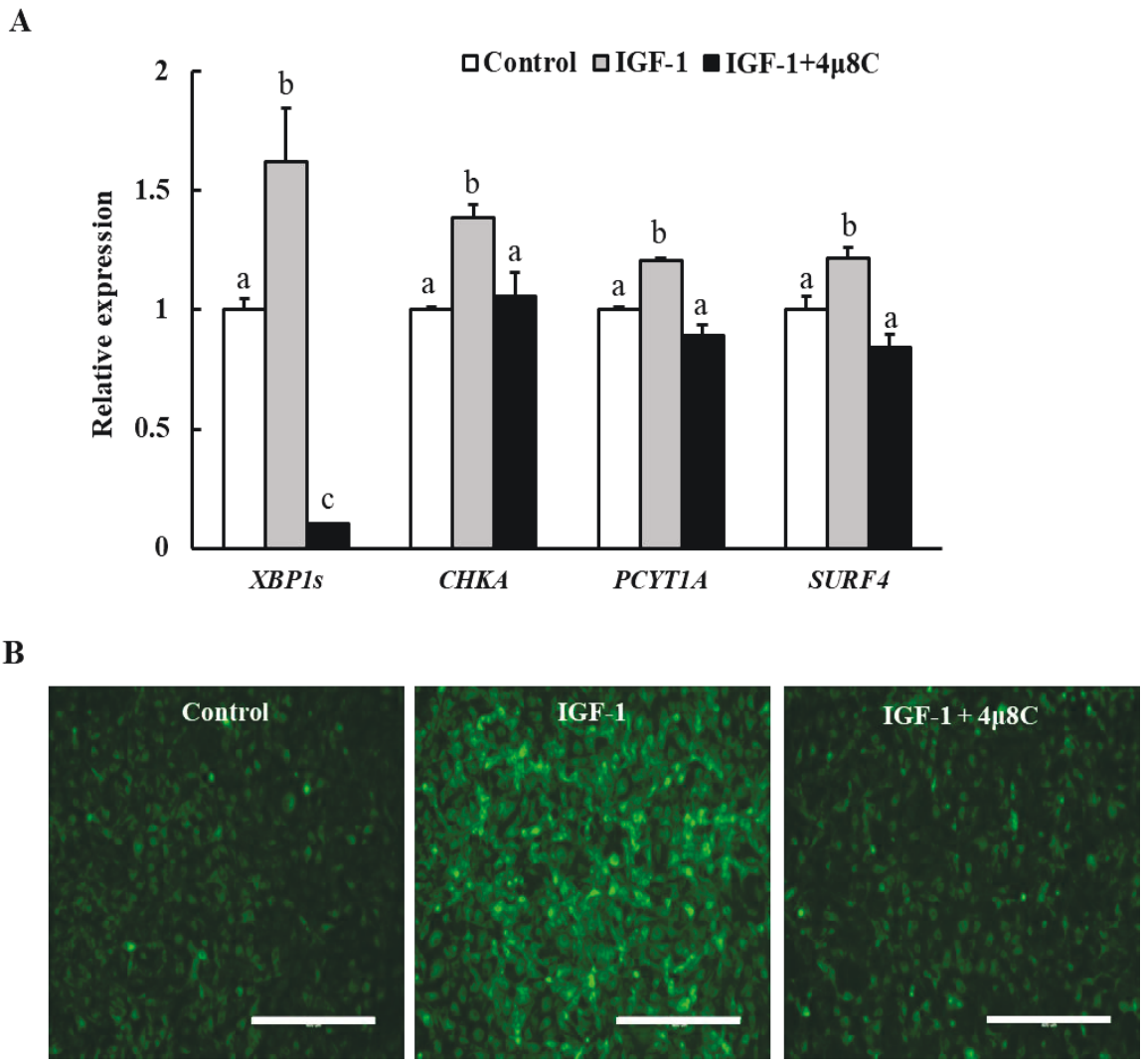

Figure 4: IRE1α inhibitor suppresses IGF-1-induced ER biogenesis in bovine MECs. (A) Bovine mammary epithelial cells (MECs) were exposed to 100 ng/mL IGF-1 in the presence of inositol-requiring kinase 1 alpha (IRE1α) inhibitor, 4μ8C (40 μM), for 24 h. Relative expression of X-box binding protein 1 splicing form (*XBP1s*), choline kinase alpha (*CHKA*), choline-phosphate cytidylyltransferase A (*PCYT1A*), and surfeit locus protein 4 (*SURF4*) were assessed via real-time PCR. Relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method and presented as values relative to control. (B) Representative photomicrographs of ER tracker staining. Scale bar, 400 μm. Data are presented as mean ± SEM for three independent experiments. Means with different letters are significantly different, $p < 0.05$; means with the same letter are not significantly different. This picture has been taken from Journal of Dairy Science.

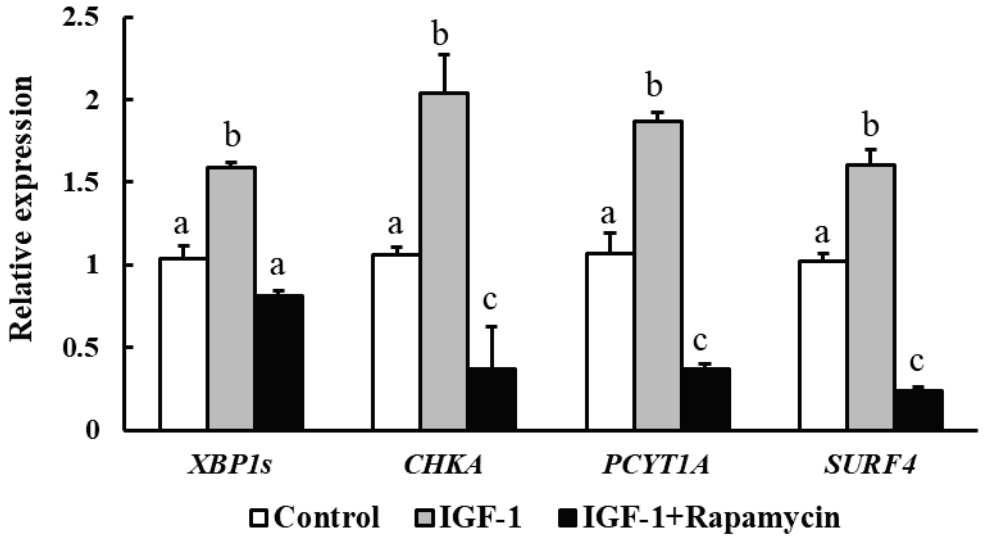

Figure 5: Rapamycin, an inhibitor of mammalian target of rapamycin (mTOR) reduces the IGF-1-induced ER biogenesis in bovine MECs. Bovine mammary epithelial cells (MECs) were exposed to 100 ng/mL IGF-1 in the presence or absence of rapamycin (100 nM), for 24 h. Relative expression of X-box binding protein 1 splicing form (*XBP1s*), choline kinase alpha (*CHKA*), choline-phosphate cytidylyltransferase A (*PCYT1A*), and surfeit locus protein 4 (*SURF4*) were assessed via real-time PCR. Relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method and presented as values relative to control. Data are presented as mean ± SEM for three independent experiments. Means with different letters are significantly different, $p < 0.05$; means with the same letter are not significantly different. This picture has been taken from Journal of Dairy Science.

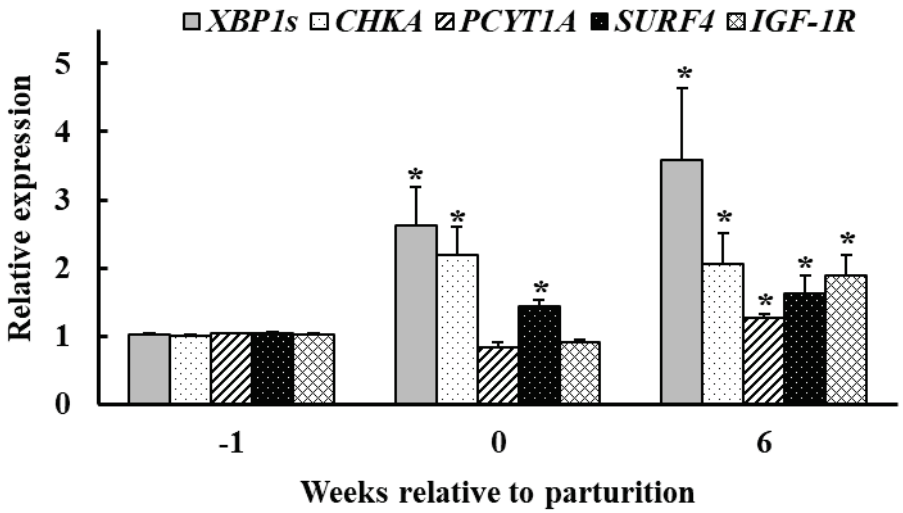

Figure 6: Changes in gene expression in mammary gland tissue of dairy cows at parturition and during lactation. Relative expression of *XBP1s*, choline kinase alpha (*CHKA*), choline-phosphate cytidylyltransferase A (*PCYT1A*), surfeit locus protein 4 (*SURF4*) and IGF-1 receptor (*IGF-1R*) in bovine mammary glands at indicated points were determined by real-time PCR. Relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method and represented as relative values to −1 week relative to parturition. Data are presented as the mean ± SEM for three independent experiments. *$p < 0.05$, compared with −1 week. This picture has been taken from Journal of Dairy Science.

Figure 7: Potential binding sites of XBP1 in the -500 to +1 region of the *PCYT1A* promoter predicted by using the Contra v3 bioinformatics tool and shown in red and over lined. This picture has been taken from Journal of Dairy Science.

2.5. Discussion

The important function of IGF-1 is galactopoiesis, which occurs via IGF-1-induced protein synthesis in MECs (Burgos and Cant, 2010, Wang et al., 2016). This activity is in addition to the fundamental functions of IGF-1 related to mammary gland growth and development (McGrath et al., 1991, Zhao et al., 1992). During increased amount of protein synthesis, bovine MECs are expected to exhibit ER biogenesis, as do other secretory cells (Rush et al., 1991, Kirk et al., 2010, Davis et al., 2016); however, whether IGF-1 mediated protein synthesis induces ER biogenesis is not understood. Present study found that IGF-1 significantly increases staining by an ER tracker dye and increases the mRNA levels of ER biogenesis-related genes (Figure 2). Therefore, the data predominantly show that IGF-1 induces ER biogenesis in bovine MECs through upregulating genes responsible for this process.

I first evaluated the rate of proliferation of bovine MECs due to IGF-1 treatment. The result revealed that IGF-1 did not increase ($p > 0.05$) the proliferation of MECs (Figure 1). Previous study showed that IGF-1 increased the protein synthesis of bovine MECs (Burgos and Cant, 2010). Moreover, organelle remodeling is the fundamental characteristics during differentiation or adaptation to change condition (Reimold et al., 2001, Puhka et al., 2007) like synthesis of increased amount of protein synthesis in MEC. Therefore, ER biogenesis is needed by the bovine MECs.

To search the molecular mechanism of how IGF-1-induced ER biogenesis, I then focused on UPR. IGF-1 increased the mRNA expression of *XBP1s*. A previous study found similar results in myotubes (Acosta-Alvear et al., 2007). Furthermore, the protein expression of p-IRE1α and IRE1α (upstream of *XBP1s*) were upregulated by IGF-1 treatment (Figure 3). IRE1α, which exhibits endonuclease activity, cuts the definite intron from unspliced *XBP1*

and thus generates transcription factor *XBP1s* (Calfon et al., 2002). Hence, the elevated ratio of p-IRE1α/ IRE1α protein expression provides mechanistic support for IGF-1-stimulated *XBP1s* mRNA expression. Then combined treatment with IGF-1 and 4μ8C was performed; co-treatment significantly masked IGF-1-induced *XBP1s* expression. Consistent with *XBP1s* expression, 4μ8C exposure decreased the mRNA expression of *CHKA*, *PCYT1A* and *SURF4* in IGF-1-treated cells. Furthermore, the enhanced fluorescence intensity of IGF-1-treated cells was inhibited by 4μ8C (Figure 4), which indicated the presence of increased number of ER in IGF-1 treated cells and successful inhibition of endonuclease activity of IRE1α to suppress the ER biogenesis in IGF-1 and 4μ8C co-treated cell. Therefore, IGF-1 induces the IRE1-XBP1s axis to regulate ER biogenesis in bovine MECs.

XBP1s is a transcriptional factor, but how it regulates the ER biogenesis-related genes is unknown. Previous studies established that XBP1s expression is indispensable for ER remodeling in various secretory cells (Iwakoshi et al., 2003, Lee et al., 2005). But, during ER biogenesis in mouse NIH-3T3 fibroblast cells, the expression of *PCYT1A* or *CCTα* mRNA was unchanged by *XBP1s* overexpression (Sriburi et al., 2004). Rather, enhanced rough ER formation and Pdtcho synthesis occurred because of an increase in CCT enzyme activity. Here, the enhanced the Pdtcho synthesis in XBP1s overexpressing cell was largely due to the post-transcriptional regulation of CCTα enzyme (Sriburi et al., 2007). However, surprisingly in the present study, IGF-1-induced *XBP1s* expression elevated the *PCYT1A* gene expression in MAC-T cell. Indeed, the bioinformatics study of *PCYT1A* using the Contra v3 tool indicated four potential binding sites in the −500 to +1 region of the bovine *PCYT1A* promoter (figure 7). XBP1s could thus directly modulate *PCYT1A* gene expression. Species variation in the experimental unit may be a major reason for this difference. Although, there is lack of information regarding the effect of *XBP1s* on *CHKA*

expression during ER biogenesis, but present study was the pioneer that, *XBP1s* increased the *CHKA* expression during ER biogenesis in bovine MECs. *CHKA* plays significant role as the initiator of Pdtcho synthesis pathway (Wu and Vance, 2010), which pathway is indispensable for ER biogenesis (Sriburi et al., 2007). SURF4 is known as a secretory cargo receptor (Lin et al., 2020). A previous microarray study found that XBP1s overexpression increased the expression of the *SURF4* gene in NIH-3T3 cells (Sriburi et al., 2007). However, whether IGF-1-induced *XBP1s* expression directly or indirectly affects *CHKA* and *SURF4* genes during ER elaboration needs further investigation. Collectively, in bovine MECs, ER biogenesis occurs under IGF-1-stimulated *XBP1s* upregulation via increasing *CHKA*, *PCYT1A*, and *SURF4* gene expression.

Previous study found that protein synthesis was increased in IGF-1 treated bovine MEC stimulating the mTORC1 (Burgos and Cant, 2010). Furthermore, a gene specific knock out study in mice established that, mTORC1 requires the UPR component XBP1 for the immunoglobulin production from plasma cell (Benhamron et al., 2015). Therefore, I speculated that a linkage might be existed between mTORC1 signaling and XBP1s in IGF-1 treated cell. To proof the hypothesis I had used rapamycin in IGF-1 treated cell which is a potent inhibitor of mTORC1 (Guertin and Sabatini, 2007). I was able to see that IGF-1-induced *XBP1s* expression was significantly down regulated in IGF-1 and rapamycin co-treated cell. In addition, the expression of ER biogenesis related genes were significantly decreased in that combine treated cell (Figure 5). These data suggested that, mTORC1 was involved in the XBP1s-induced ER biogenesis due to IGF-1 treatment in bovine MEC. In addition there was a relationship between mTORC1 activation and IRE1-XBP1 branch of UPR.

Since the *in vitro* study confirmed that IGF-1-induced XBP1s expression increased the ER biogenesis of bovine MECs, I therefore was queries about the status of ER biogenesis

related genes under the natural physiological condition during periparturient period. *In vivo* study, the relatively low XBP1s expression levels in mammary gland tissue before parturition increased gradually from immediately after parturition through lactation. I previously found that *XBP1s* mRNA expression in mammary glands also increased immediately after calving (Yonekura et al., 2018). The mRNA expression of ER biogenesis-related genes was upregulated during lactation compared with before parturition. Furthermore, the mRNA expression of *IGF-1R* increased during lactation in the present study (Figure 6). *IGF-1R* was expressed in dairy cows during pregnancy and lactation stages (Hadsell et al., 1990), and the expression of *IGF-1R* in the present study was consistent with this finding (Plath-Gabler et al., 2001). Hence, the increased XBP1s, ER biogenesis related genes and *IGF-1R* mRNA expression in mammary glands, focused on possible occurrence of ER biogenesis in bovine MECs during lactation.

2.6. Conclusion

The crucial effect of IGF-1 on mammary tissue is galactopoiesis, including protein synthesis, during lactation. However, how IGF-1 affects the physiology of ER during periods of greatly increased protein synthesis remains unknown. *In vitro* results show that IGF-1 induces ER biogenesis activating IRE1-XBP1 arm of UPR under the regulation of mTORC1. In addition, the enhanced expression of *XBP1s*, *IGF-1R*, and ER biogenesis-related genes in bovine mammary gland tissue indicates the possible occurrence of ER biogenesis during lactation. Thus, present study summarizes that, IGF-1 induces ER biogenesis in bovine MECs establishing a linkage between mTORC1 and IRE1-XBP1 axis of UPR, of which a working model has been described in figure 8. Therefore, milk production is largely affected by ER biogenesis since previous study reasons the undeveloped ER for decreasing the milk yield. However, future research related to molecular mechanisms of IGF-1-induced ER biogenesis and milk yield will increase the knowledge of how lactation curves are defined at the cellular level.

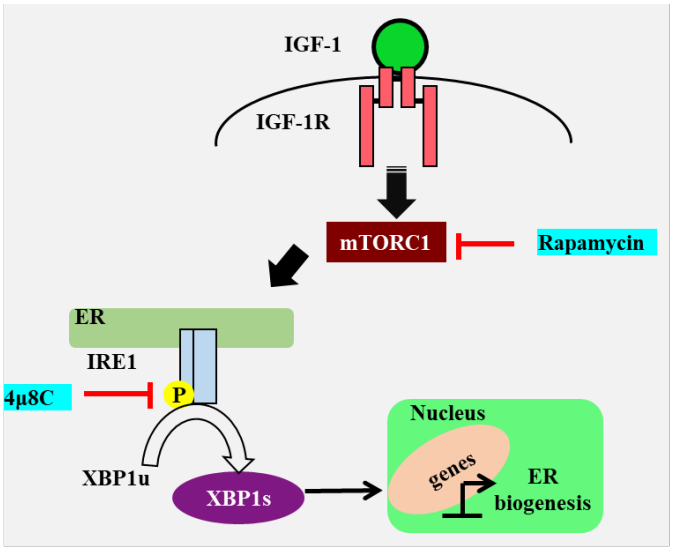

Figure 8: Working model of IGF-1 induced ER biogenesis in bovine MECs. This picture has been taken from Journal of Dairy Science.

Chapter III

Effects of fatty acids on inducing endoplasmic reticulum stress in bovine mammary epithelial cells

3.1. Abstract

FA plays important roles in the regulation of ER stress-induced apoptosis in different cells. Currently, the effects of FA on bovine MEC remain unknown. Present study examined bovine MEC viability and measured UPR-related gene and protein expressions following fatty acid treatments. To evaluate the role of FA, I treated MAC-T cells with 100 to 400 μM of saturated (palmitic and stearic acid) and unsaturated (palmitoleic, oleic, linoleic, and linolenic acid) FA and 1 to 5 mM of short- and medium-chain FA (acetic, propionic, butyric, and octanoic acid). Thereafter, I determined UPR-related gene expression using quantitative real-time PCR. Palmitic acid stimulated expression of *XBP1s*, *ATF4*, *ATF6A*, and *CHOP*. Stearic acid increased expression of *XBP1s* and *CHOP* and decreased expression of *ATF4* and *ATF6A*. Results of Western blot analysis and MTT assay revealed that palmitic and stearic acid reduced MAC-T cell viability and induced extreme ER stress by increasing the protein expression of ER stress markers, such as phospho-PKR-like endoplasmic reticulum kinase, phospho-eIF2α, cleaved CASP-3, and CHOP. Among unsaturated long-chain FA, palmitoleic acid increased expression of *ATF4* and *ATF6A*. Oleic acid increased expression of *XBP1s*, *ATF4*, and *ATF6A*. Linoleic and linolenic acids increased expression of *XBP1s*, *ATF4*, and *ATF6A* but decreased expression of *XBP1s* and *ATF6A* at the highest dose. Although palmitoleic, oleic, and linoleic acid decreased CHOP expression, only palmitoleic acid increased MAC-T cell viability. Therefore, unsaturated long-chain FA did not induce severe ER stress. Acetic, propionic, and butyric acids

decreased expression of *ATF4*, *ATF6A*, and *CHOP* and increased *XBP1s* expression. Although only octanoic acid increased *ATF4* and *ATF6A* expressions, it lowered expression of *XBP1s* and *CHOP*. Although fatty acid treatment did not increase the levels of ER stress proteins, butyric and octanoic acids reduced cell viability, possibly because of early differentiation. These results suggest that saturated FA play important roles in MEC viability by inducing severe ER stress compared with unsaturated FA. In addition, acetic and propionic acids (short- and medium-chain FA) reduced ER stress. Therefore, the present study reflects the new insight that serum fatty acid concentration plays an important role in maintaining the lactation physiology of dairy cows.

3.2. Introduction

The reduction of milk yield in early lactation is strongly associated with NEB condition. NEB is the result of physiological stress due to the transitional modification. In this condition, there is inadequate supply of energy required by the increased amount of milk synthesis. For maintaining the energy requirements, dairy cows utilize body fat and produce NEFA, mainly C16:0, C18:0, and *cis*-9 C18:1 and C14:0, *cis*-9 C16:1, C17:0, *trans*-11 C18:1, and minor FA, albeit to a much lesser extent (Bas et al., 1987). The MEC utilize FA as energy substrates as well as for milk lipid synthesis; however, FA exerts innumerable effects on various tissues. Several studies have demonstrated that saturated FA (palmitic and stearic acids) are proapoptotic, whereas unsaturated FA are antiapoptotic factors in β-cells (Eitel et al., 2002), coronary artery endothelial cells (Staiger et al., 2006), and human breast cancer cells (Hardy et al., 2000, 2003). In addition, palmitic and stearic acids cause ER stress in various types of nonruminant cells (Maedler et al., 2001; Wei et al., 2006; Sieber et al., 2010; Haywood and Yammani, 2016). However, the mode of action of increased amounts of free FA on bovine MEC remains unclear.

Bovine MEC sensed ER stress due to increases in milk synthesis and elevated the UPR factor *CHOP* expression during the early lactation period (Invernizzi et al. 2012 and Yonekura et al., 2018). It was previously stated that *CHOP* expression was negatively correlated with initial milk yield in bovine mammary gland tissue (Yonekura et al., 2018), strongly affirming the loss of numerous MEC because of ER stress affecting milk yield. Therefore, the UPR plays a significant role in the survival of MEC. Loss of MECs reduces the milk yield, which is stated by the previous study (Capuco et al., 2001). For the sustainable milk production maintenance of MEC number is necessary. However, the mechanism of MEC death due to induction of CHOP and the triggering factors for

determination of the cell fate (in terms of apoptosis and survival) needs to be investigated. Accordingly, in the present study, I determined whether long- and short-chain FA alters the ER stress induced UPR related gene expression, and consequently cell viability, in bovine MEC. Thus, I focused on the effects of FA on the mammary gland environment of dairy cows.

3.3. Materials and Methods

3.3.1. Reagents

Reagents details were given in section 2.3.1 of chapter II. In addition, sodium stearate and sodium octanoate were bought from Sigma-Aldrich (St. Louis, MO).

3.3.2. Preparation of FA solutions

I prepared the palmitic and stearic acid solutions, following the procedure described by Qi et al. (2014) with minor modifications. The stock solution of above saturated FA was prepared by dissolving the required amount of a specific acid in dimethyl sulfoxide warmed at 60 ºC with continuous shaking. Then, various concentrations of the prepared solutions were heated for 10 to 15 min at 55 ºC with continuous shaking after mixing with 10% (wt/vol) BSA/PBS (fatty acid-free) and. The resulting solutions were added to the cell culture medium before treatment to obtain the desired final concentrations (100, 200, 300, 400, and 500 μM) and 0.1% BSA. For the preparation of unsaturated long-chain fatty acid solutions, the optimum amounts of specific FA were dissolved in fatty acid-free 10% BSA/PBS warmed at 37 ºC for 30 min and mixed by vortexing. The required amounts of specific short- and medium-chain FA were taken to dissolve in growth medium containing 10% FBS heated at 37 ºC for 30 min, to obtain the stock solution, which was further mixed by vortexing. After sterilization, all the long-chain fatty acid solutions were stored at −20 ºC under shade, except for stearic acid. Steric acid and all of the short- and medium-chain fatty acid solutions were stored at 4 ºC and protected from light until further use.

3.3.3. Cell culture and FA treatments

MAC-T cells were cultured according to section 2.3.2 of chapter II. Additionally, 100% confluent cells were treated with saturated and unsaturated long-chain FA (100, 200, 300, and 400 μM) and short- and medium-chain FA (1, 3, and 5 mM) in growth medium containing 10% FBS for 12 h.

3.3.4. RNA extraction and quantitative real-time PCR

The procedure for RNA extraction and cDNA synthesis was mentioned in section 2.3.3 of chapter II. The level of mRNA expression of *XBP1s*, *ATF4*, *ATF6A*, and *CHOP* were measured via the $2^{-\Delta\Delta Ct}$ method. The *GAPDH* and *VPS4A* genes were used as house-keeping gene. The primer sequences were used according to Yonekura et al. (2018). The primer sequence of *GAPDH* was AATGGAAAGGCCATCA (forward) and GTGGTTCACGCCCATC (reverse). The primer sequence of *VPS4A* was CAAAGCCAAGGAGAGCATTC (forward) and ATGTTGGGCTTCTCCATCAC (reverse). Data analysis was performed according to the manufacturer's instructions.

3.3.5. Cell Viability Test

MTT assay was followed according to section 2.3.6 of previous chapter. Further, the cells were treated with 200 and 400 μM saturated and unsaturated FA and 1, 3, and 5 mM short- and medium-chain FA.

3.3.6. Western blot analysis

For western blot analysis, the detailed procedure explained in section 2.3.5 of the previous chapter. In this section membranes were probed using antibodies against phosphorylated PERK (Santa Cruz Biotechnology, Santa Cruz, CA), total PERK (Santa Cruz

Biotechnology), phosphorylated eIF2α (Invitrogen), total eIF2α (Invitrogen), CHOP (Life Span Bioscience Inc., Seattle, WA), cleaved CASP-3 (Cell Signaling Technology, Danvers, MA), and α-tubulin (MBL Co., Nagoya, Japan)

3.3.7. Statistical analysis

The values were expressed as the mean ± standard error of the mean from a typical experiment that was run in triplicates dishes. Statistical difference was determined using one-way ANOVA followed by Dunnett's test. The test was considered significant at $p < 0.05$.

3.4. Results

3.3.1. Effect of saturated long-chain FA (palmitic and stearic acid) on MAC-T cells

The mRNA expression of proapoptotic factor *CHOP* was significantly upregulated by 200, 300, and 400 μM palmitic and stearic acids treatment. Both palmitic and stearic acids significantly increased the level of *XBP1s* mRNA expression. The gene expressions of *ATF4* and *ATF6A* were elevated by palmitic acid but downregulated by stearic acid treatment (Figure 1A).

I measured the phospho-PERK, phospho-eIF2α, CHOP, and cleaved CASP-3 protein expressions via Western blot analysis, to confirm the detrimental effect of palmitic and stearic acids on bovine MEC. Palmitic and stearic acid treated cells increased the expression of phospho-PERK, phospho-eIF2α CHOP, and cleaved CASP-3 protein (Figure 1B).

As both palmitic and stearic acids treatment upregulated the expression of proapoptotic marker CHOP and cleaved caspase-3, I examined whether saturated long-chain FA affected MAC-T cell viability using the MTT assay. Compared with the control, both palmitic and stearic acids at 200 and 400 μM significantly decreased the bovine MEC viability (Figure 1C).

3.3.2. Effect of unsaturated long-chain FA (palmitoleic, oleic, linoleic, and linolenic acids) treatment in MAC-T cells

I hypothesized that unsaturated long-chain FA (100 to 400 μM) treatment will affect the ER stress-induced UPR-related gene expression in bovine MEC. Result showed that, with the exception of linolenic acid, the highest dose of palmitoleic, oleic, and linoleic acids

significantly reduced *CHOP* gene expression level compared with the control (Figure 2A). The expression of *XBP1s* mRNA was increased by oleic, linoleic, and linolenic acids at different concentrations. However, only the highest doses of linoleic and linolenic acids decreased the *XBP1s* expression. The expression level of *ATF4* mRNA was upregulated by all of the unsaturated FA. Moreover, the expression of *ATF6A* was increased by all of the unsaturated FA, except for the highest doses of linoleic and linolenic acids, compared with the controls.

I considered that unsaturated long-chain FA would not negatively affect bovine MEC, because those FA did not increase the *CHOP* gene expression. Thereafter, I measured the phospho-PERK, phospho-eIF2α, CHOP, and cleaved CASP-3 protein expressions using Western blot analysis. The results of Western blot analysis suggested that unsaturated long-chain FA did not enhance the expression of phospho-PERK, phospho-eIF2α, cleaved CASP-3, or CHOP proteins compared with the palmitic acid-treated group (Figure 2B).

I also examined MAC-T cell viability using the MTT assay. None of the unsaturated FA affected bovine MEC viability, and 400 μM palmitoleic acid significantly increased the proliferation of MEC (Figure 2C).

3.3.3. Effect of short- and medium-chain FA (acetic, propionic, butyric, and octanoic acids) in MAC-T cells

The result from Quantitative real-time PCR analysis showed that all short- and medium-chain FA significantly downregulated *CHOP* mRNA expression compared with the controls. Acetic, propionic, and butyric acids increased the *XBP1s* expression but reduced *ATF4* and *ATF6A* expression level. Only 5 mM of octanoic acid increased the *ATF4* and *ATF6A* expression and also downregulated the *XBP1s* expression (Figure 3A).

The mRNA expression of *ATF4*, *ATF6A*, and *CHOP* was downregulated by most short- and medium-chain FA. Therefore, I hypothesized that short- and medium-chain FA might reduce ER stress. The Western blot analysis results showed that none of the short- and medium-chain FA increased the p-PERK, p-eIF2α, or CHOP protein levels compared with the palmitic acid-treated cells. In addition, cleaved CASP-3 protein expression was not visible via Western blot for any short- or medium-chain fatty acid-treated cells (Figure 3B).

To confirm the ER stress-reducing characteristics of short- and medium-chain FA, I performed the MTT assay to analyze cell viability. Only 3 and 5 mM butyric and 5 mM octanoic acid significantly decreased the cell viability (Figure 3C).

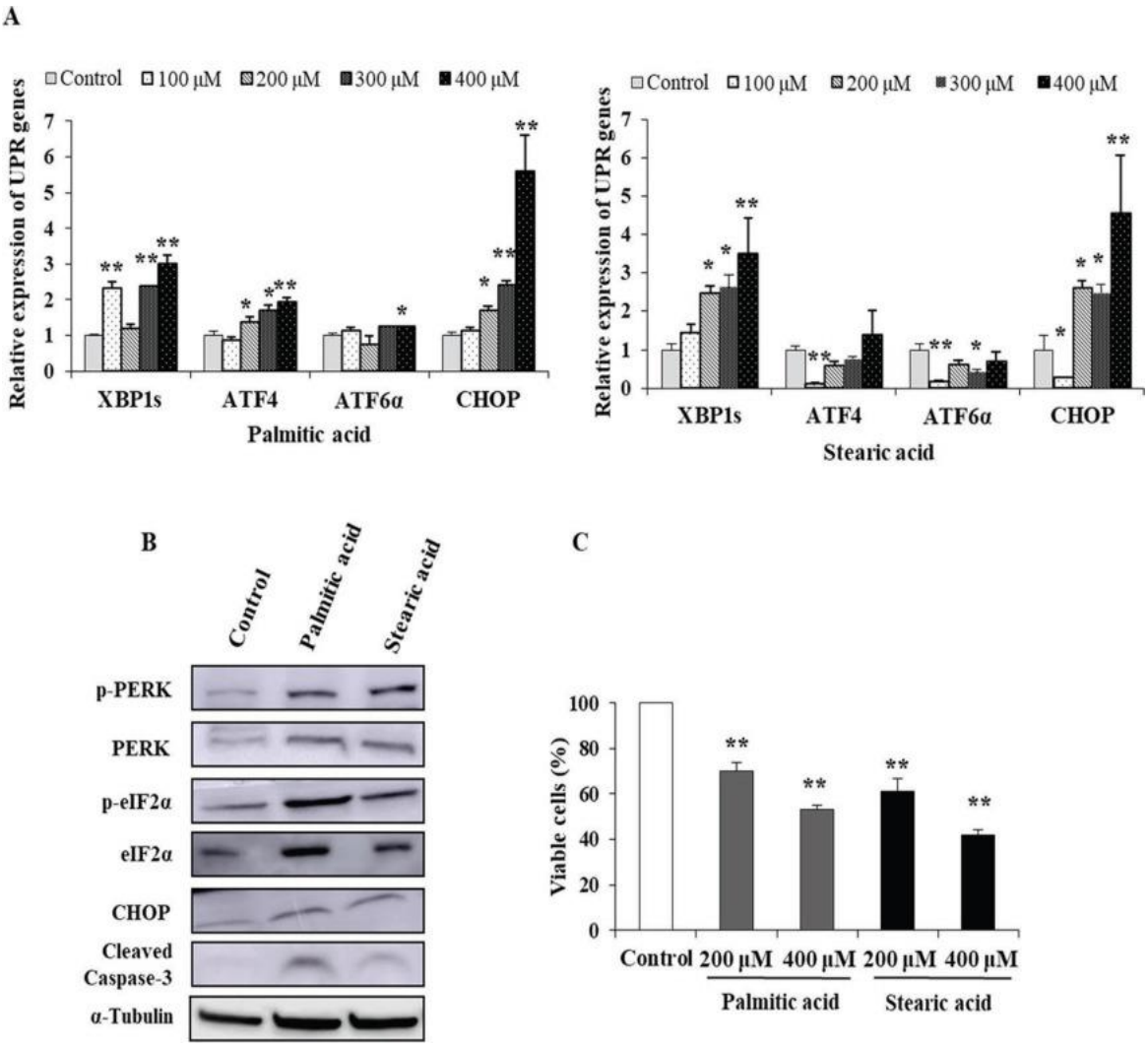

Figure 1. Effects of saturated long-chain FA on endoplasmic reticulum stress-induced unfolded protein response (UPR)-related gene and protein expression and bovine mammary epithelial MAC-T cell viability. (A) The expression pattern of UPR-related genes in control and 100, 200, 300, and 400 μM palmitic and stearic acid-treated MAC-T cells for 12 h, determined using real-time PCR of *XBP1s*, *ATF4*, *ATF6A*, and *CHOP* mRNA. Relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method and values are presented as relative to control. (B) MAC-T cells were stimulated with 400 μM palmitic acid and stearic acids for 12 h, and phosphorylated (P) The protein expression levels of phosphorylated (P) PERK, PERK, P-eIF2α, eIF2α, CHOP, cleaved CASP-3, and α-tubulin (internal control) were detected using the Western blot method. Representative images from at least 3 independent experiments with at least 4 replicates in each experiment are shown. (C) The viability of MAC-T cells was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. 90 to 100% confluent cells were treated with 200 and 400 μM palmitic and stearic acids for 48 h. Absorbance was measured at 570 and

630 nm using a multimode microplate reader to calculate survival rates (%) of cells. Data are presented as mean ± SEM for 3 independent experiments. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with control, determined using 1-way ANOVA followed by Dunnett's test. This figure has been taken from Journal of Dairy Science.

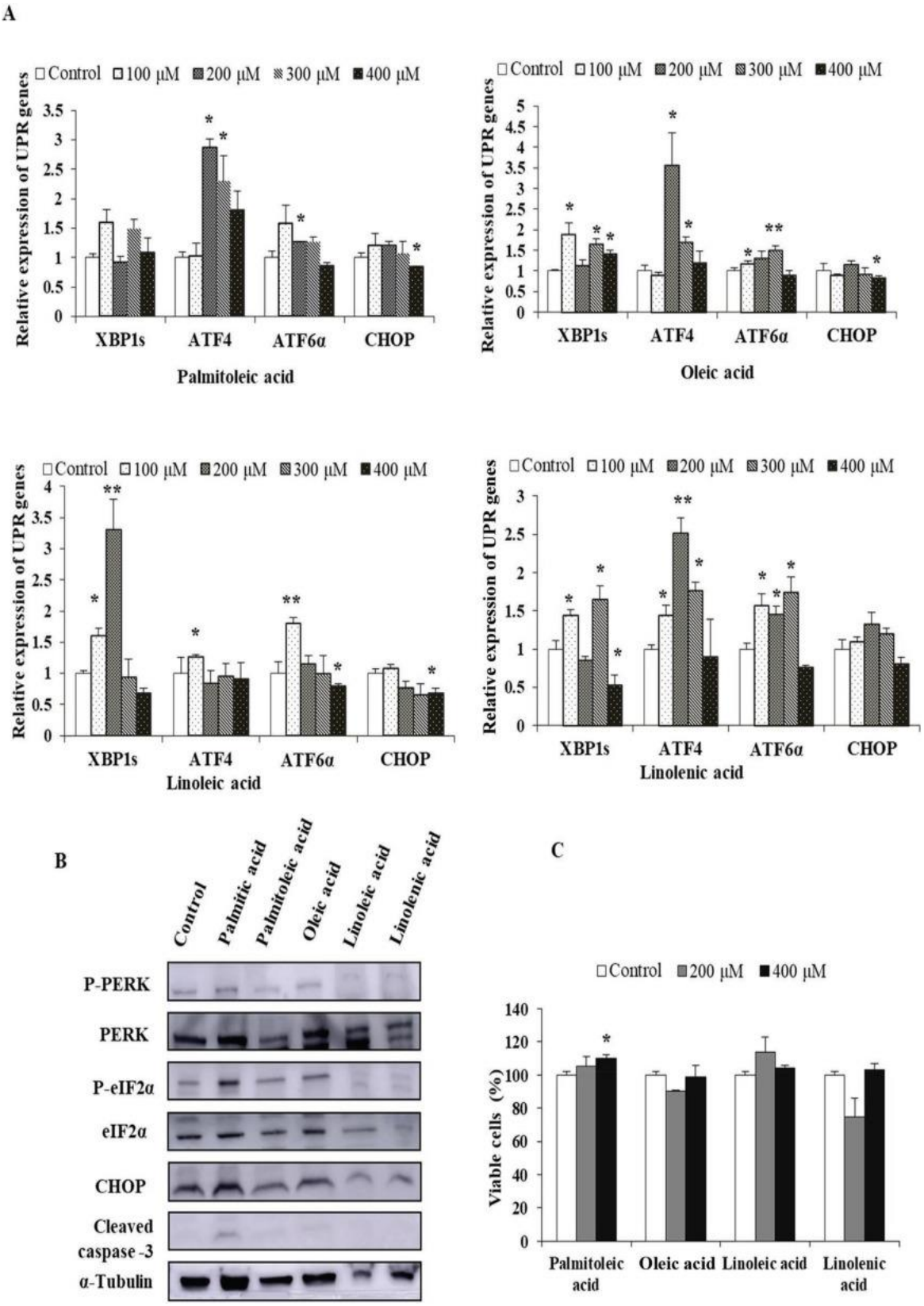

Figure 2. Effects of unsaturated long-chain FA on endoplasmic reticulum stress-induced unfolded protein response (UPR)-related gene and protein expression and bovine mammary epithelial MAC-T cell viability. (A) The expression of UPR-related gene in

control and 100, 200, 300 and 400 μM palmitoleic, oleic, linoleic, and linolenic acid-treated MAC-T cells for 12 h, examined using real-time PCR of *XBP1s*, *ATF4*, *ATF6A*, and *CHOP* mRNA. Relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method and presented as values relative to control. (B) The cells were stimulated with 400 μM palmitic acid (as a positive control), palmitoleic acid, oleic acid, linoleic acid, and linolenic acid for 12 h, and the phosphorylated (P) PERK, PERK, P-eIF2α, eIF2α, cleaved CASP-3, CHOP, and α-tubulin (internal control) protein levels were detected using Western blotting. Representative images from at least 3 independent experiments with at least 4 replicates in each experiment are shown. (C) Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells that were 90 to 100% confluent were treated with 200 and 400 μM palmitoleic, oleic, linoleic, and linolenic acids for 48 h. Absorbance was measured at 570 and 630 nm using a multimode microplate reader to calculate survival rates (%) of cells. Data are presented as mean ± SEM for 3 independent experiments. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with control, determined using 1-way ANOVA followed by Dunnett's test. This figure has been taken from Journal of Dairy Science.

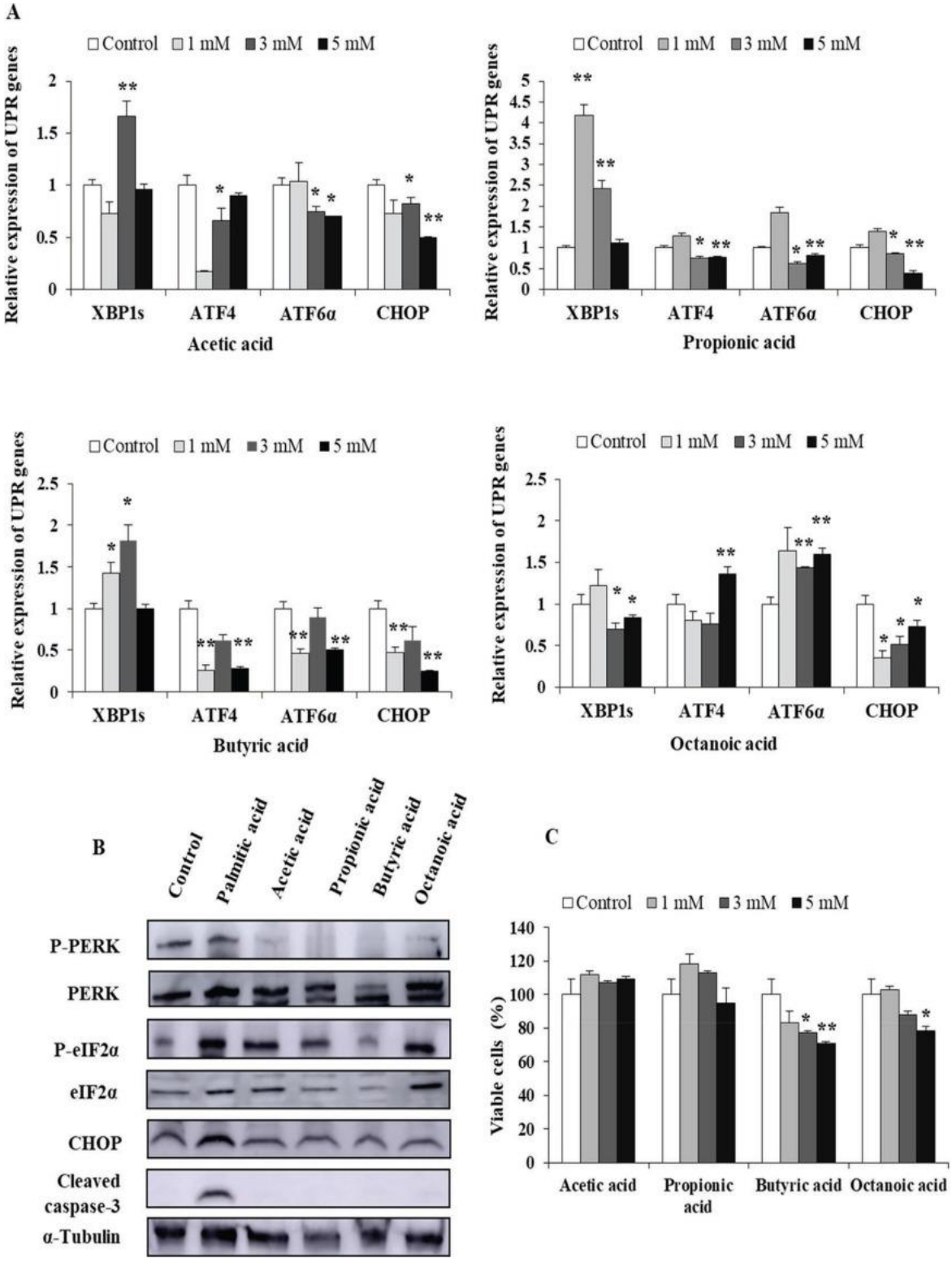

Figure 3. Effect of short- and medium-chain FA on endoplasmic reticulum stress-induced unfolded protein response (UPR)-related gene and protein expression and bovine mammary epithelial MAC-T cell viability. (A) Expression pattern of UPR-related genes in control, 1, 3, and 5 mM acetic, propionic, butyric, and octanoic acid-treated MAC-T cells

for 12 h, determined using real-time PCR of *XBP1s*, *ATF4*, *ATF6A*, and *CHOP* mRNA. (B) Relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method and represented as values relative to control. MAC-T cells were supplemented with 400 μM palmitic acid (as a positive control group) or 5 mM acetic, propionic, butyric, or octanoic acid for 12 h, and the phosphorylated (P) PERK, PERK, P-eIF2α, eIF2α, cleaved CASP-3, CHOP and α-tubulin (internal control) protein levels were detected using Western blotting. Representative images from 3 independent experiments with at least 4 replicates in each experiment are shown. (C) Cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Cells that were 90 to 100% confluent were treated with 1, 3, and 5 mM acetic, propionic, butyric, and octanoic acid for 48 h. Absorbance was measured at 570 and 630 nm using a multimode microplate reader to calculate survival rates (%) of cells. Data are presented as mean ± SEM for 3 independent experiments. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with the control, determined using 1-way ANOVA followed by Dunnett's test. This figure has been taken from Journal of Dairy Science.

3.5. Discussion

The accumulation of free FA in excessive amount in any non-adipose tissue may cause the cells to dysfunction and undergo apoptosis. The cell signaling pathways and molecular mechanisms involved in cell death induced by the accumulation of FA have not yet been completely elucidated. As far I know, this was the first study making a relationship between fatty acid treatment and ER stress-induced UPR-related gene expression in bovine MEC.

The results explained that both palmitic and stearic acids induced CHOP overexpression at both the mRNA and protein levels in dose-dependent manner in bovine MEC (Figure 1 A and B). The 100 μM solutions of palmitic and stearic acids did not increase the *CHOP* expression in bovine MEC. During the periparturient period, the concentration of nonesterified FA mainly changed according to the degree of lipomobilization in dairy cows. The saturated and unsaturated long-chain fatty acid treatment (100 to 400 μM) used in this study may be higher compared with the *in vivo* concentration of bovine serum free fatty acid; however, knowledge from the previous *in vitro* experiment helped us to fix the dosage of fatty acid treatment that was applied to MAC-T cells. Qi et al., (2014) used the 0 to 600 μM palmitic and stearic acids to stimulate the bovine MEC, to discover the effects of saturated FA on milk fat and protein biosynthesis related gene expressions. Yonezawa et al. (2004a, 2008) treated the bovine MEC with 50 to 400 μM saturated long-chain FA for measuring cytosolic triacylglycerol accumulation, lipid droplet formation, and uncoupling protein-2 expression. Furthermore, MAC-T cells were provided with 100 to 400 μM saturated long-chain FA to determine cytosolic triacylglycerol content (Vargas-Bello-Pérez et al. 2019). It was noted that, I followed previous studies (Yonezawa et al.,

2004b; Sun et al., 2016) for fixing the doses of short- and medium-chain fatty acid treatments in the present study.

It was observed that, ER membrane integrity was impaired by the permeabilization and leakage of ER contents into the cytosol and also by ER stress induced by palmitic acid treatment (Hitomi et al., 2004; Kanekura et al., 2015). When cells were cultured with palmitic acid, the saturation of the ER membrane resident lipid species was increased, and thus the membrane became less flexible (Weijers, 2012; Kanekura et al., 2015). Kanekura et. al. (2015) also found that the ER stress-induced UPR-related gene *CHOP* was upregulated, in palmitic acid-treated ER membrane disrupted cells, which was consistent with the results of the 200 to 400 μM saturated long-chain fatty acid treatment in the present study. Therefore, palmitic and stearic acids are considered as the ligands for disrupting ER membrane homeostasis and inducing ER stress. However, in fatty acid treatment of MAC-T cells where growth medium containing FBS was used, a percentage of FA was detected, although I also supplied the same FBS-containing growth medium for the control cells. Therefore, I thought that the cells of both control and fatty acid-treated groups may have received an indistinguishable amount of FA from FBS.

In a typical condition, CHOP is ubiquitously expressed at extremely low levels in non-stress cell; however, in most cells, CHOP expression increases when the cells are subjected to severe stress (Chikka et al., 2013). The death of cell mediated by ER stress, requires CHOP overexpression (Matsumoto et al., 1996; Maytin et al., 2001; Oyadomari et al., 2001), which was found following high doses of saturated long-chain fatty acid treatments in MAC-T cells. The UPR is sensed by three ER transmembrane protein: PERK, IRE1, and ATF6. Among these proteins, the PERK/eIF2α-signaling pathway is essential for induction 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; Scheuner et al., 2001). Both palmitic and stearic acids significantly increased expression of phospho-PERK and phospho-eIF2α compared with control cells (Figure 1B), leading to the activation of the PERK arm of the UPR under ER stress conditions in MAC-T cells. Wei et al. (2006) postulated that palmitic and stearic acids caused rat liver cell death by upregulating expression of the *CHOP* gene. Consistent with the results of present study, that study found that palmitic and stearic acids disrupted ER homeostasis, increased phosphorylation of *eIF2α* and *ATF4* mRNA, and induced *CHOP* expression.

Both of the saturated FA treated-cells showed higher cleaved CASP-3 protein expression compared with control cells (Figure 1C). CASP-3 is a major executioner caspase that undergoes cleavage and is activated for the degradation of multiple cellular proteins, and it is responsible for morphological changes and DNA fragmentation in cells during apoptosis (McIlwain et al., 2013). Previous studies have demonstrated saturated fatty acid-induced CASP-3-dependent apoptosis in various cell types (Hardy et al., 2003; Wang et al., 2006; Wei et al., 2006). Considering the findings of Hardy et al. (2003), Wang et al. (2006), and Wei et al. (2006), palmitic and stearic acids induced apoptosis of bovine MEC by increasing the expression of cleaved CASP-3. Therefore, for the first time, current study suggested that bovine MEC experienced extreme ER stress due to treatment with 200 to 400 μM palmitic and stearic acids. This study indicated that excessive saturated long-chain FA in the mammary glands of dairy cows may be harmful, as it negatively affects optimum milk production.

Palmitoleic, oleic, linoleic, and linolenic acids reduced expression of the *CHOP* gene as well as phospho-PERK, phospho-eIF2α, and CHOP protein expression in bovine MEC (Figure 2 A and B). Previous studies have shown that palmitoleic, oleic, and linoleic acid-

treated cells did not upregulate CHOP expression. Further, those FA rescued the palmitic acid-induced CHOP expression under coculture conditions (Listenberger et al., 2003; Wei et al., 2006; Sieber et al., 2010). In addition to decreasing the capacity of CHOP, palmitoleic and α-linolenic acids exhibited the ability to reduce ER stress and facilitated downstream signaling of phospho-eIF2α induced by either palmitic acid, salubrinal (phosphatase inhibitor), or tunicamycin (noble ER stress inducer) in the rat renal cell line NRK-52E (Diakogiannaki et al., 2008; Katsoulieris et al., 2009). Therefore, the cytoprotective effect of unsaturated long-chain FA provides mechanistic support for the reduction of ER stress-associated markers, such as phospho-PERK, phospho-eIF2α, and CHOP, when MAC-T cells were cultured with palmitoleic, oleic, linoleic, and linolenic acids. Finally, the present study concluded that unsaturated FA did not cause severe ER stress in bovine MEC, considering that the principal arm of the UPR, involving PERK, was not upregulated following unsaturated fatty acid treatment. Moreover, the transcription factor CHOP may interact with *ATF4* to bind the promoter regions of genes encoding protein synthesis, thereby enhancing ER stress-induced cell death in wild-type mouse embryo fibroblasts (Han et al., 2013). I speculated that in the unsaturated fatty acid treatment, the upregulated *ATF4* mRNA could not become a dimer with a low amount of *CHOP*. Unlike saturated FA, unsaturated FA did not affect the cell viability of bovine MEC, possibly because of the reduced expression of the *CHOP* gene and ER stress proteins in the present study. Previous studies have observed that palmitoleic and oleic acids did not negatively affect cardiac myocytes (de Vries et al., 1997; Van Bilsen et al., 1997; Welters et al., 2004); this finding is similar to the findings that oleic, linoleic, and linolenic acids did not affect bovine MEC viability. Furthermore, palmitoleic acid treatment increased bovine MEC viability (Figure 2C). This finding is consistent with the explanation provided by Maedler et al. (2001), who stated that palmitoleic acid treatment

increased β-cell viability by providing more energy at a normoglycemic glucose concentration. Finally, the present study primarily concluded that high doses of unsaturated FA induce moderate ER stress and partly contribute to the cytoprotective effect in bovine MEC. Therefore, unsaturated FA, especially palmitoleic acid, have potential to maintain bovine udder physiology through cytoprotection.

This is the first study to introduce ER stress-related molecular mechanisms of short- and medium-chain fatty acid treatment to bovine MEC. Acetic, propionic, and butyric acids accounts for 45 to 70%, 15 to 40%, and 5 to 20%, respectively, of 60 to 160 mM total short-chain FA produced in the rumen and the attached reticulum (Bergman, 1990; Aschenbach et al., 2009). These 3 short-chain FA are quantitatively the most important for providing 70% of the energy required by ruminant animals (Kristensen et al., 1998). The study showed that all short- and medium-chain FA reduced the proapoptotic marker *CHOP* expression (Figure 3A). Reductions of the ER stress-associated proteins phospho-PERK, phospho-eIF2α, and CHOP were observed following by acetic, propionic, butyric, and octanoic acid treatment in MAC-T cells (Figure 3B). Furthermore, expression of the cleaved CASP-3 protein was completely absent in these short- and medium-chain fatty acid-treated cells. Diao et al. (2019) identified antiapoptotic properties of acetic, propionic, butyric, and octanoic acids in the intestinal cells of weaned piglets. That study reported that the gastric infusion of the earlier-mentioned FA decreased the percentage of apoptotic cells via reduction of gene and protein abundances of CASP-3; this finding is strongly consistent with the result. In accordance with present findings, another study found that feeding lactating goats with sodium butyrate reduces high-concentrate diet-induced apoptosis via reduction of the CASP-3 protein in mammary cells (Chang et al., 2018). Therefore, present study demonstrated that acetic, propionic, butyric, and octanoic acids may provide beneficial effects by reducing ER stress proteins.

Although all short- and medium-chain FA significantly downregulated CHOP without increasing cleaved CASP-3 protein expression, only butyric and octanoic acids affected bovine MEC viability (Figure 3C). Previous studies have shown that acetic and propionic acid did not affect cell viability (Yonezawa et al., 2009; Wei et al., 2017). I observed that acetic and propionic acids did not reduce bovine MEC viability. The antiproliferative effect of butyric acid for bovine MEC, previously established by Yonezawa et al. (2009), was also demonstrated by the present study. Furthermore, the proliferation of HT29 and DLD-1 cells was inhibited by butyric acid via the inhibition of the expression of genes encoding proteins involved in DNA replication, cell cycle, and cell proliferation (Gamet et al., 1992; Ohara and Mori, 2019) and those that induced differentiation (Toscani et al., 1988). Alva-Murillo et al. (2013) observed that sodium octanoate did not reduce bovine MEC viability. By contrast, only the highest dose of octanoic acid affected bovine MEC viability in my study. The decreased viability observed with butyric and octanoic acids in the present study can be attributed to early differentiation. However, the present study suggests that without affecting cell viability, the reduction of ER stress proteins by short- and medium-chain FA, particularly acetic and propionic acids, renders them a favorable option for ameliorating ER stress in bovine MEC. Therefore, of the short- and medium-chain FA, at least acetic and propionic acids are favorable for conserving MEC for dairy cows to maintain optimal milk production.

3.6. Conclusion

The findings of the present study discovers the antagonistic effects of saturated and unsaturated and short- and medium-chain FA on the UPR, ER stress, and bovine MEC viability. Excessive amounts of saturated FA are detrimental in regulating bovine MEC cell death via ER stress-induced *CHOP* gene expression. Since *CHOP* was negatively correlated with milk production (Yonekura et al., 2018), it has been postulated that saturated FA might reduce milk production during the transition period via lowering the number of bovine MEC. Unsaturated FA do not induce severe ER stress in bovine MEC. On the other hand, short- and medium-chain FA, particularly acetic and propionic acids, reduce ER stress via downregulation of UPR genes. In summary, the present study reflects the new insight that serum fatty acid concentration plays an important role in maintaining the lactation physiology of dairy cows.

Chapter IV

5-ALA attenuates the palmitic acid-induced ER stress and apoptosis in bovine mammary epithelial cells

4.1. Abstract

Conservation of mammary gland physiology by maintaining the maximum number of MECs is of the utmost importance for optimum amount of milk production. In a state of negative energy balance, palmitic acid (PA) reduces the number of bovine MECs. However, there is no effective strategy against PA-induced apoptosis of MECs. In the present study, 5-ALA was established as a remedial agent against PA-induced apoptosis of MAC-T cells. In PA-treated cells, the apoptosis-related genes *BCL2* and *BAX* were down- and upregulated, respectively. Elevated expression of major genes of the UPR, such as *CHOP* reduced the viability of PA-treated MAC-T cells. In contrast, 5-ALA pretreatment increased and decreased *BCL2* and *BAX* expression, respectively. Moreover, cleaved caspase-3 protein expression was significantly reduced in 5-ALA pretreated group in comparison with PA group. Downregulation of major UPR-related genes, including *CHOP*, extended the viability of MAC-T cells pretreated with 5-ALA and also reduced the enhanced intensity of PA-induced expression of phospho-PERK. Moreover, the enhanced expression of antioxidant gene *HO-1* by 5-ALA reduced PA-induced oxidative stress (OxS). *HO-1* is not only protective against OxS, but also effective against ER stress. Collectively, these findings offer new insights into the protective effects of 5-ALA against PA-induced apoptosis of bovine MECs.

4.2. Introduction

The excessive amount of palmitic acid (PA) was invented as a causal agent for the MEC death, which had been well illustrated in chapter III. It had upregulated the expression level of CHOP transcription factor via the PERK arm of the UPR, which resulted in severe ER stress-induced apoptosis of bovine MECs (Sharmin et al., 2020). PA had also been reported to induce oxidative stress (OxS) through the production of intracellular reactive oxygen species, which subsequently stimulate apoptosis of hepatocytes (Lambertucci et al., 2008). Therefore, it was an important factor for the reduction of MECs number as well as milk yield in the early lactation period. Hence, a successful approach to inhibit PA-induced apoptosis of MECs is needed as an efficient management tool to improve the lactation performance of dairy cows.

5-ALA is an endogenous amino acid of both animals and plants found in foods, such as spinach, green peppers, tomatoes, shiitake mushrooms, bananas, and potatoes. Greater amounts are found in fermented products, such as wine, vinegar, sake, and soy sauce. It is a precursor for biosynthetic tetrapyrroles, such as heme, vitamin B_{12}, and chlorophyll (Rodriguez et al., 2012). In animals, it can be synthesized from glycine and succinyl-CoA in the presence of 5-ALA synthase as part of heme biosynthetic pathway (Ishizuka et al., 2011). Previous study showed that 5-ALA can induce heme oxygenase-1 (HO-1) thereby provides protection against oxidative and other stresses, such as cisplatin-induced nephrotoxicity, hydrogen peroxide-induced cardiomyocyte hypertrophy, and ischemia-reperfusion-induced renal injury (Hou et al., 2013, Terada et al., 2013 and Zaho et al., 2016). A recent study found that 5-ALA can reduce heat stress-mediated ER stress-induced apoptosis of bovine MECs (Islam et al., 2021). Therefore, the aim of current study was to examine the efficiency of 5-ALA to inhibit ER stress-induced apoptosis of MECs in response to palmitic acid.

4.3. Materials and Methods

4.3.1. Reagents

Chapter II section 2.3.1 described the reagents information. Moreover, Neopharma Japan Co. Ltd. (Tokyo, Japan) has provided the 5-ALA.

4.3.2. Cell culture and treatment

Cell culture technique was mentioned in section 2.3.2 of chapter II. In addition, 5-ALA was added to MAC-T cells for 48 h before PA treatment to examine its activity in preventing PA-related damage. PA solution was prepared as previously described by Sharmin et al (2020). The preserved PA stock solution in 0.1% BSA was added to the cell culture medium before treatment to obtain the desired final concentration of 300 μM.

4.3.3. Cell viability test

For MTT assay section 2.3.6 of chapter II had been followed. Here, the cells were pretreated with 5-ALA (100, 250, and 500 μM) followed by PA challenged for 48 h.

4.3.4. RNA extraction and quantitative real-time PCR

The RNA extraction and quantitative real time PCR procedure was followed according to section 2.3.3 of chapter II. Primer sequences were used according to Islam et al., (2021) and Sharmin et al (2020).

4.3.5. Western blot analysis

The procedure for western blot analysis was written in section 2.3.5 of chapter II. Membranes were incubated with anti-phosphorylated PERK (Santa Cruz Biotechnology (sc-32577), Santa Cruz, CA, USA), anti-PERK (Santa Cruz Biotechnology (sc-13073)),

anti-cleaved caspase-3 (Cell Signaling Technology (9661), Danvers, MA, USA), and anti-α-tubulin (MBL Co., Nagoya, Japan) antibodies.

4.3.6. Apoptosis rate analysis by flow cytometer

Apoptosis rate was determined using tunnel assay with an *in situ* apoptosis detection kit (TaKaRa Bio Inc.) according to manufacturer's instruction. Briefly, Cells incubated with 4% paraformaldehyde at room temperature for 15-30 minutes for fixing up. After buffer permeabilization cells were stained with TdT (Terminal deoxynucleotidyl Transferase) for 60-90 minutes at 37 ºC. Finally TdT positive cells were analyzed using the SH800S cell sorter (Sony Biotechnology Inc. USA).

4.3.6. Statistical analysis

Paired *t*-test was utilized to measure the statistical significance to make a comparison between two samples. For multiple comparisons ANOVA with post hoc Tukey–Kramer's honestly significant difference test was performed. Statistically significant was considered at $p < 0.05$.

4.4. Results

4.4.1. PA reduced but 5-ALA enhanced the MAC-T cells viability

It was previously reported that PA-induced severe ER stress and reduced the viability of bovine MECs (Sharmin et al., 2020). Consistent with the result of that previous study, PA also decreased the viability of MAC-T cells in the current study. The reduction of cell viability was 20% in PA treated group compared to the control one. Pretreatment with all doses of 5-ALA (100, 250, and 500 μM) significantly enhanced the viability of MAC-T cells, as compared with PA-treated cells (Figure 1). Therefore, 5-ALA has the potentiality to enhance the viability of MAC-T cells which was decreased by PA.

4.4.2. 5-ALA reduced the apoptosis produced by PA in MAC-T cells

The rate of apoptosis was measured by flow cytometry analysis using the cells pretreated with 5-ALA and treated with PA. The apoptosis rate was 13.71% for cells treated with PA and 7.25% for those co-treated with PA and 5-ALA (Figure 2A). The results of qRT-PCR analysis determined that *BCL2* and *BAX* were related to apoptosis. Expression of the anti-apoptosis gene *BCL2* was downregulated, while that of the pro-apoptosis gene *BAX* was upregulated, respectively, in PA-treated cells, whereas *BCL2* expression was significantly upregulated and that of *BAX* was downregulated in cells pretreated with 5-ALA (Figure 2B). Moreover cleaved caspase-3 protein expression was higher in PA treated group compared to control and it was significantly reduced in 5-ALA pretreated group in comparison with PA group (Figure 2C). These data indicated that 5-ALA rescued MAC-T cells from PA-induced apoptosis.

4.4.3. 5-ALA ameliorated the PA-induced ER stress

The UPR-component genes *GRP78*, *CHOP*, *ATF4*, and *XBP1s* expressions were examined as markers of ER stress in 5-ALA pre-treated MAC-T cells following treatment with PA. According to Figure 3A, PA treatment upregulated the expression levels of all of the above ER stress marker genes, as compared with the control group, while pretreatment with 5-ALA downregulated PA-induced upregulation of *GRP78*, *CHOP*, *ATF4*, and *XBP1s*.

Western blot analysis of phospho-PERK expression was performed to confirm the advantageous effect of 5-ALA. Figure 3B showed that, PA application increased phospho-PERK expression, but was reduced in cells pretreated with 5-ALA, indicating that 5-ALA can decrease PA-induced ER stress in MAC-T cells. Therefore, these data suggested that 5-ALA reduces PA-induced ER stress in MAC-T cells.

4.4.4. 5-ALA culminated the PA-induced OxS

PA is a potent inducer of OxS in a variety of cell types (Barlow et al., 2013 and Sato et al., 2014). The upregulation of NRF2, a master regulator of OxS and its target antioxidant gene HO-1 (heme oxygenase 1), by PA treatment was a marker of OxS. The stronger upregulation of NRF2 and HO-1 by 5-ALA pretreatment, as compared with PA treatment, was indicative of OxS (Figure 4). As a result, enhanced expression of *NRF2* and *HO-1* in 5-ALA pretreated cell showed the protective antioxidant effect of 5-ALA for MACT-cells.

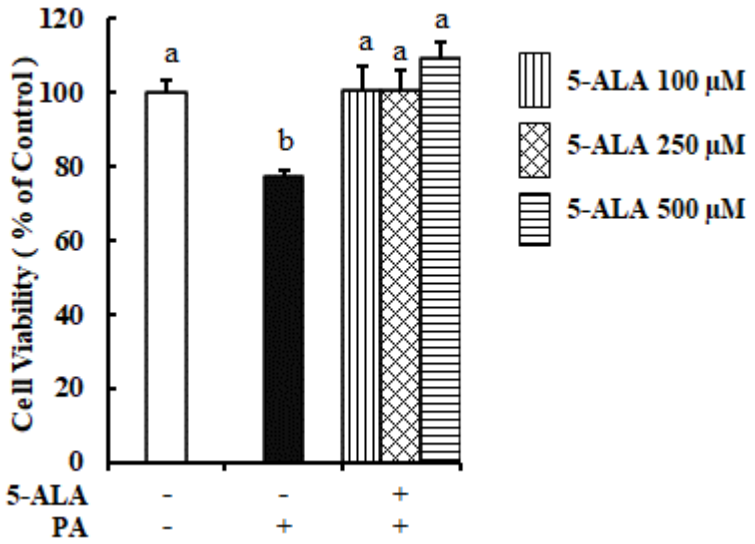

Figure 1. Measurement of the viability of MAC-T cells using 5-ALA pretreatment. 300 μM PA with or without 5-ALA pretreatment at 100, 250, and 500 μM for 48 h were used to treat the confluent cells. The MTT assay was applied to determine the cell viability. Survival rates are expressed as percentages of the control cells. Three standard experiments were performed independently to show the data as mean ± SEM (standard error of the mean) with different letters indicating significant differences at $p < 0.05$. This figure has been taken from Molecules.

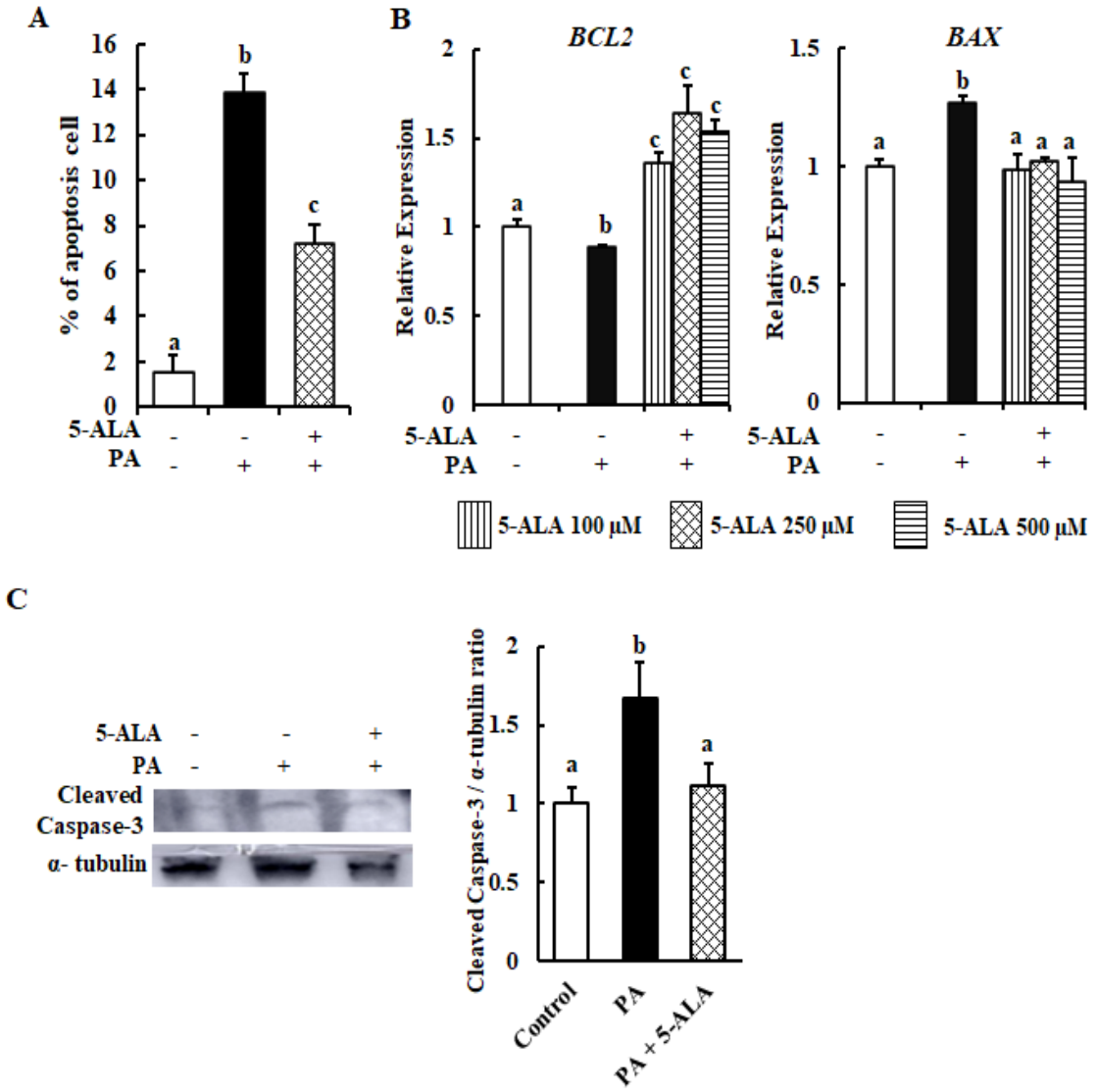

Figure 2. Assessment of the apoptosis rate and the expression levels of apoptosis-related genes from 5-ALA pretreated MAC-T cells. (A) FACS analysis was performed to assess the apoptotic rates of bovine MECs treated with 300 µM PA with or without 5-ALA pretreatment at 250 µM for 24 h. (B) Bovine MECs were treated with 300 µM PA with or without 5-ALA pretreatment at 100, 250, and 500 µM for 24 h. BCL2 and BAX mRNA expression levels were determined by RT-qPCR and normalized to ACTB levels. Three standard experiments were performed independently to show the data as mean ± SEM (standard error of the mean) with different letters indicating significant differences at $p < 0.05$. (C) MAC-T cells were served with 300 µM PA with or without 5-ALA pretreatment at 250 µM for 24 h and the expression levels of cleaved caspase-3, and α-tubulin protein (internal control) were determined by western blot analysis. Left: Data of three independent experiments of at least four replicates are shown. Right: Quantification of the cleaved caspase-3/α-tubulin ratio obtained by densitometric analysis. Three standard

experiments were performed independently to show the data as mean ± SEM (standard error of the mean) with different letters indicating significant differences at $p < 0.05$. This figure has been taken from Molecules.

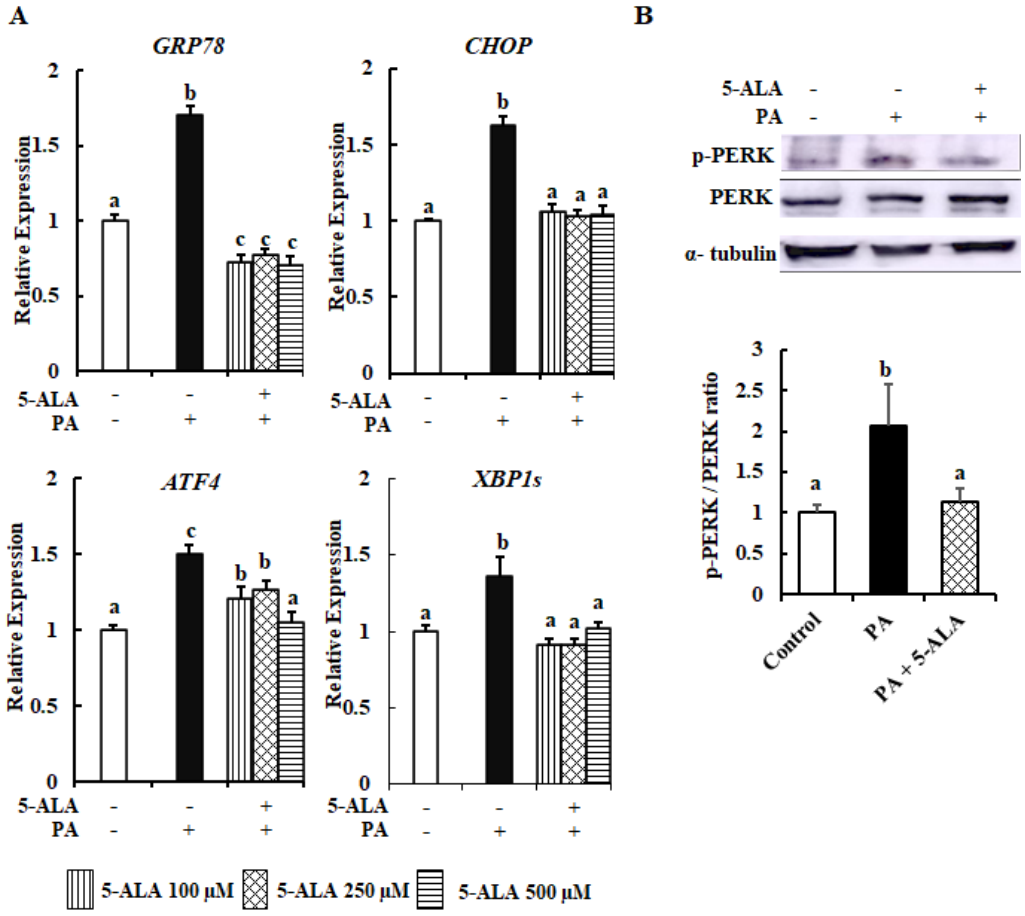

Figure 3. 5-ALA reduced the expression levels of marker genes and a protein of PA-induced ER stress. Confluent MAC-T cells were treated with 300 μM PA with or without 5-ALA pretreatment at 100, 250, and 500 μM for 24 h. (A). The mRNA expression levels of *GRP78*, *CHOP*, *ATF4*, and *XBP1s* were measured by RT-qPCR and normalized to *ACTB* levels. Three standard experiments were performed independently to show the data as mean ± SEM (standard error of the mean). (B) Confluent MAC-T cells were stimulated with 300 μM PA with or without 5-ALA pretreatment at 250 μM for 24 h. Expression levels of phosphor-PERK, PERK, and α-tubulin (internal control) were determined by western blot analysis. Upper: Data of three independent experiments of at least four replicates are shown. Lower: Quantification of the phospho-PERK/PERK ratio obtained by densitometric analysis. The data presented as mean ± SEM (standard error of the mean) with different letters indicating significant differences at $p < 0.05$. This figure has been taken from Molecules.

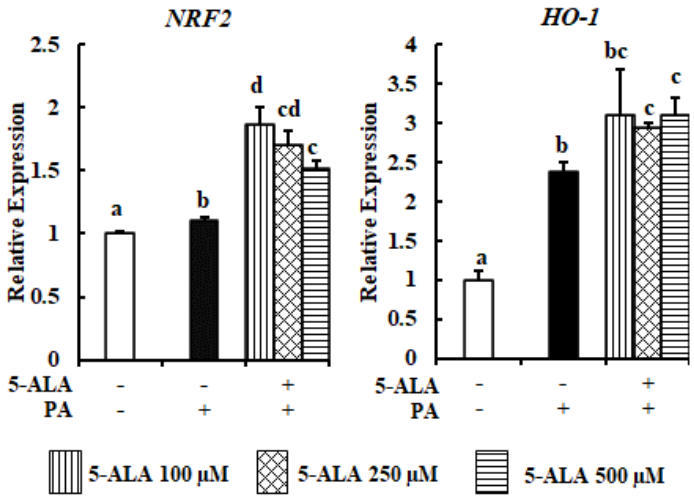

Figure 4. Effect of 5-ALA on the expression levels of OxS-related genes following PA treatment. Confluent MAC-T cells were treated with 300 µM PA with or without 5-ALA pretreatment at 100, 250, and 500 µM for 24 h. *NRF2* and *HO-1* mRNA expressions were quantified by RT-qPCR and normalized to *ACTB* levels. Three standard experiments were performed independently to show the data as mean ± SEM (standard error of the mean) with different letters indicating significant differences at $p < 0.05$. This figure has been taken from Molecules.

4.5. Discussion

Present study indicated that PA reduced 20% viability and caused 13.71% apoptosis of MAC-T cells, while pretreatment with different doses of 5-ALA enhanced viability, consistent with the findings of previous studies (Sharmin et al., 2020). I believe that the rest of the cell death is due to PA induced necrosis. Unfortunately, I did not check the PA mediated necrosis related cell death. But previous studies explained that PA induced necrosis and caused different types of cell death (Khan et al., 2012 and Zhou et al., 2013). Therefore, I speculated that the remaining 6.29% dead cell was due to necrosis in PA treated group. PA treatment decreased the expression of *BCL2* and increased that of *BAX*, indicating that PA augmented the proportion of apoptotic cells, while 5-ALA pre-treatment had an opposite effect. Moreover, cleaved caspase-3 protein expression was significantly decreased by 5-ALA pretreatment. Overall, the proportion of apoptotic cells was decreased by co-treatment with 5-ALA and PA. Previous study also reported that PA downregulated expression of BCL2 and upregulated that of BAX and cleaved caspase-3, as markers of the induction of apoptosis (Peterson et al., 2008 and Yuan et al., 2013). However, 5-ALA blocked apoptosis and promoted the viability of MAC-T cells by increasing the expression levels of anti-apoptotic genes.

In the present study, mRNA expression of *GRP78* and *CHOP*, *ATF4*, and *XBP1s*, and phospho-PERK protein expressions were aggravated in PA-treated bovine MECs. GRP78, a major ER stress chaperon, acts to conserve ER homeostasis. Under physiological conditions, GRP78 binds to and inactivates all three UPR arms within the ER membrane. But, in response to ER stress, GRP78 disassociated from and activated all three sensors. Thereafter, phospho-PERK activated the transcription factors ATF4 and CHOP (Zinszner et al., 1998). This CHOP expression has a negative correlation with milk yield, identified by an *in vivo* study (Yonekura et al., 2018). Previous findings explained that PA treatment

enhanced *GRP78* expression (Zhang et al., 2018 and Chen et al., 2019), which led to an imbalance in ER homeostasis. A recent study found that PA induced severe ER stress-mediated apoptosis of MAC-T cells by increasing the *XBP1s*, *ATF4* and *CHOP* mRNA expression, and protein expression of phospho-PERK (Sharmin et al., 2020). In contrast to PA treatment, pretreatment with 5-ALA decreased the expression levels of *GRP78*, *CHOP*, *ATF4*, and *XBP1s*, as well as phospho-PERK. Therefore, 5-ALA has the potential to ameliorate PA-induced severe ER stress in MAC-T cells.

The results of the present study also revealed that *NRF2* and *HO-1* expressions by 5-ALA treatment were higher as compared with PA treatment only. Notably, *NRF2* and *HO-1* expression levels were significantly upregulated in PA-treated cells as compared with control cells, which is compatible with previous study (Shi et al., 2018). Under non-stress conditions, in the cytoplasm, NRF2 binds with Kelch-like ECH-associated protein 1 (Keap1) to maintain NRF2 at a constant level of ubiquitination and degradation. Thus, a lower level of NRF2 is maintained (Wardyn et al., 2015). In case of OxS, due to modification of the cysteine residue, Keap1 releases NRF2. The released NRF2 becomes activated and translocates into the nucleus. In the nucleus, NRF2 binds with an antioxidant response element and promotes transcriptional activation of antioxidant genes, such as HO-1 (Furfaro et al., 2015). So, the enhanced expression of *NRF2* and *HO-1* by PA treatment is a self-defense mechanism, but yet insufficient to diminish PA-induced OxS. A previous study also demonstrated that prolonged upregulation of *HO-1* with the use of an NRF2-specific activator compensates PA-induced OxS (Shi et al., 2018).

A working model of 5-ALA-mediated enhancement of *HO-1* in response to PA-induced ER stress is illustrated in the diagram presented in Figure 5. Here, 5-ALA-instigated elevation of *HO-1* inhibited PA-induced activation of phospho-PERK, which subsequently downregulated expression of *ATF4* and *CHOP*. Therefore, the proportion of viable cells

was increased, while that of apoptotic cells was decreased in the 5-ALA pretreatment group as compared with the PA treatment group. Accumulating evidence suggests that HO-1 upregulation is necessary to decrease ER stress-induced apoptosis of hepatic (Jung et al., 2017), endothelial (Maamoun et al., 2017), and myocardial (Yu et al., 2017) cells. The enhanced expression of HO-1 suppressed PA-mediated expression of phospho-eIF2α (downstream molecule of phospho-PERK) and CHOP to reduce apoptosis of HepG2 cells. Meanwhile, HO-1 silencing was unable to abrogate the upregulated expression of phospho-eIF2α and CHOP or to ameliorate apoptosis of hepatocytes (Jung et al., 2017). Furthermore, several studies have reported the anti-apoptotic effect of increased HO-1 expression by suppression of the PERK arm of the UPR (Yu et al., 2015, 2017 and Chen et al., 2018). Therefore, increased mRNA expression of *NRF2* and *HO-1* by 5-ALA suggests that 5-ALA may act as an antioxidant response element to reduce OxS and ER stress and can effectively protect MAC-T cells from the detrimental effects of PA.

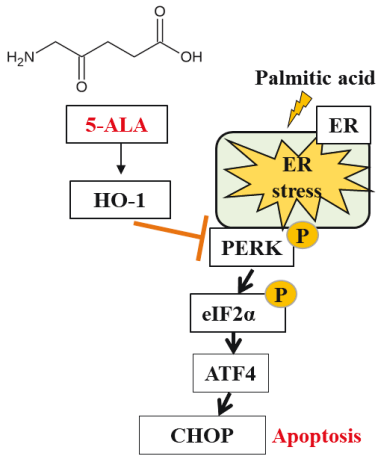

Figure 5. Schematic model of 5-ALA for the reduction of PA induced apoptosis in bovine MEC. This figure has been taken from Molecules.

4.6. Conclusion

In conclusion, present study indicates that, as compared with PA, 5-ALA promoted the viability of bovine MECs by reducing the apoptosis rate via altering the expression levels of apoptosis-related genes. PA exerts severe ER stress and OxS in bovine MECs. In contrast, 5-ALA reduces the expression levels of UPR component genes and proteins to circumvent PA-mediated ER stress. In addition, prolonged *HO-1* expression by 5-ALA is not only operative against OxS, but also suppressed signaling of the PERK pathway of the UPR. Therefore, 5-ALA pretreatment is beneficial to bovine MECs as well as udder physiology.

Chapter V

General Discussion

Modern dairy cows are not well habituated with increased milk yield. In early lactation, the NEB condition creates physiological stress to the cow. In consequence, milk yield decreases, thereby reducing lactation persistency (Capuco et al., 2003). The invention of a new strategy for establishing moderate lactation persistency will be advantageous for sustainable milk production. Possible mechanisms regarding the fluctuation in milk yield in early lactation should be identified to regulate the lactation curve at cellular levels to develop moderate-type lactation persistency.

Previous studies have identified secretory activities and number of MECs that play a crucial role in regulating the milk production during lactation (Capuco et al., 2001 and Boutinaud et al., 2004). Thus, an alteration in the secretory activity and number of MECs greatly influences milk yield. However, it is unknown how the secretory activity and number of MECs is controlled in the mammary gland.

Knowledge regarding the intracellular mechanisms of controlling the secretory activity and number of MECs will be helpful for sustainable milk yield. Previous studies also showed that UPR-induced XBP1 increased protein synthesis of MECs and augmented its secretory activity as well (Davis et al., 2016). Therefore, this study leaves a research gap that uncovers the detailed molecular mechanisms of increasing secretory activities in connection with XBP1. Furthermore, UPR-induced CHOP expression is responsible for a decrease in milk yield during early lactation (Yonekura et al., 2018), which proposes the loss of MECs due to CHOP elevation. However, the detailed mechanism of CHOP-induced MEC loss remains unknown. Therefore, this study reveals the intracellular

mechanism of increasing the secretory activity and loss of MECs under the context of UPR.

Also, this study considered ER biogenesis on the basis of increased secretory activities since it is a prerequisite for enhancing ER development and abundance. Similarly, well-developed and abundant ER is needed for the maintenance of an ideal secretory capacity in MECs, where XBP1 plays a critical role (Davis et al., 2016). Therefore, I propose that XBP1 plays important functions during ER biogenesis. For increasing the XBP1-induced ER biogenesis, IGF-1 was chosen as a stimulator since it increases protein synthesis in bovine MECs (Burgos and Cant, 2010) in addition to broad term mammary gland functions. Moreover, its serum concentration was elevated during early lactation (Radcliff et al., 2006; Wathes et al., 2003 and 2007).

Chapter II has been fully furnished with mechanisms in which IGF-1-induced XBP1s mediates ER biogenesis in bovine MECs. Results showed that IGF-1 increased the fluorescence intensity of the ER tracker dye and ER biogenesis-related gene expression.

Also, an increased ratio of p-IRE1α/IRE1α protein expression mechanistically supported the stimulation of XBP1s by IGF-1 since IRE1α slices the 26 bp intron from XBP1 mRNA to yield XBP1s via endonuclease activity (Calfon et al., 2002). The enhanced IRE1 RNAse activity in IGF-1 treated cell was also ensured by 4μ8C (potent inhibitor of IRE1 RNAse activity, Stewart et al., 2017) treatment. Result revealed that IGF-1-induced *XBP1s* expression was suppressed due to 4μ8C exposure. Keeping consistency with *XBP1s* expression, IGF-1 induced fluorescence intensity and ER biogenesis related gene expressions were also inhibited by 4μ8C treatment. Therefore, IGF-1 stimulates IRE1-XBP1s to regulate the ER biogenesis in bovine MECs.

The bioinformatics study explained that the role of *XBP1s* as a transcriptional factor also induced ER biogenesis. On the basis of the results, four potential binding sites were present in the −500 to +1 region of the bovine PCYT1A promoter in *XBP1s*, which is consistent with *XBP1s*-induced *PCYT1A* gene expression *in vitro*. *XBP1s* expression was therefore proposed to be necessary for ER remodeling in various secretory cells (Iwakoshi et al., 2003). Although there is a scarcity of information regarding XBP1-induced *CHKA* expression, this study explained that *XBP1s* mediated *CHKA* expression during ER biogenesis. IGF-1-induced *XBP1s* also increased *SURF4* expression that accounted for ER biogenesis in MECs. A micro-array study was also carried out to confirm this result (Sriburi et al., 2007). Overall, ER biogenesis occurs through the stimulation of IGF-1-induced *XBP1s* by elevating its associated gene in MECs.

Furthermore, this study also stated that IGF-1 regulated *XBP1s* via mTORC1, thereby increasing ER biogenesis. This observation was based on the fact that rapamycin (a small molecule inhibitor of mTORC1) treatment suppressed IGF-1-induced *XBP1s* and ER biogenesis-related genes. Previously, it was also discovered that IGF-1 enhanced protein synthesis stimulating mTORC1 in MECs (Burgos and Cant, 2010), while *XBP1s* expression was required by mTORC1 during immunoglobulin production (Benhamron et al., 2015). Therefore, it is established that IGF-1 regulates *XBP1s* through mTORC1during ER biogenesis in MEC.

The results of the *in vivo* study further confirmed the upregulation of *XBP1s*, ER biogenesis-related genes, and *IGF-1R* expression in bovine mammary gland tissue during lactation, therefore indicating the possible occurrence of ER biogenesis. The findings of *XBP1s* and *IGF-1R* expression were also similar to the previous results (Yonekura et al., 2018 and Plath-Gabler et al., 2001). Additionally, *in vitro* and *in vivo* study results

summarize that the IRE1-XBP1 axis enhances ER biogenesis in bovine MECs and that IGF-1 is involved in this process.

Chapter III focuses on FA to know if the mechanism behind MEC loss was due to CHOP expression. The serum concentration of FA increased as a component of NEFA during early lactation, which was taken by MECs as ingredients of milk synthesis and source of energy. However, the impact of FA on MEC was unknown. Therefore, MECs were treated with different concentrations of long-chain saturated, unsaturated, and short- and middle-chain FA. Results demonstrated that PA and stearic acids reduced the viability of cells. Alternatively, the molecular mechanism revealed that those FA stimulated the PERK arm to increase CHOP at both the mRNA and protein levels, thus inducing severe ER stress. Previous studies have also postulated that those acids responsible for the upregulation of CHOP expression induced severe ER stress-mediated apoptosis in different non-ruminant cells (Wei et al. 2006 and Kanekura et. al. 2015). Therefore, PA and stearic acids are considered ligands for severe ER stress-induced apoptosis in bovine MECs.

Furthermore, neither of the unsaturated FA induced severe ER stress or reduced viability of MECs since those acids did not instigate the PERK arm to increase the CHOP expression. Rather, palmitoleic acid increased the viability of MECs by providing more energy, which is reported in a previous study (Maedler et al. 2001).

This study also examined the effect of short- and medium-chain FA in MECs, which are important in ruminant animals, since those compounds cover 70% of their energy requirement, especially short-chain FA (Kristensen et al., 1998). Therefore, I characterized those FA, particularly acetic and propionic acids as antiapoptotic owing to the observed reduction in CHOP expression. These results were also consistent with previous studies (Chang et al., 2018, Diao et al. 2019).

In chapter III as well, it was identified that copious amount of PA increased apoptosis of MECs. In this manner, an efficient supplementary strategy is necessary to inhibit PA-induced MEC loss. It is also speculated that 5-ALA reduced the effect of PA-induced MEC death. Previous study showed that it inhibited apoptosis by elevating *HO-1* expression in various types of cells (Liu et al., 2019 and Zaho et al., 2016). This study, however, showed that pretreatment of 5-ALA reduced major UPR-related genes, including *CHOP* expression to augment viability and decrease the PA-induced severe ER stress-mediated apoptosis. A recent study also found that 5-ALA protected MECs from heat stress-induced severe ER stress and apoptosis (Islam et al., 2021). Moreover, 5-ALA pretreatment elevated the expression of the antioxidant gene *HO-1*, thus offering new insights into the protective effects of 5-ALA on PA-induced MEC loss. These results prove that 5-ALA reduced PA-induced CHOP-mediated apoptosis of MECs through its antioxidant and antiapoptotic properties. It can therefore be used to prevent the PA-mediated severe ER stress-induced cell loss to increase milk yield.

In summary, this study discovered that the UPR-induced increase in ER biogenesis and MEC loss as the mechanism for the increase and decrease in milk yield, respectively. This study partly covers the knowledge required for developing a moderate type of lactation persistency. Therefore, an understanding of the role of ER biogenesis in enhancing secretory activities and MEC death to reduce milk yield in the context of UPR at early lactation, will be helpful for the final setup of an average lactation persistency.

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