Insulin-like growth factor-1 induces IRE1-XBP1-dependent endoplasmic reticulum biogenesis in bovine mammary epithelial cells

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ABSTRACT

Insulin-like growth factor-1 (IGF-1) plays a key role in proliferation and galactopoiesis in mammary epithelial cells (MEC), but its definitive functions on endoplasmic reticulum (ER) during protein synthesis remain unknown. The present study aimed to elucidate the effects of IGF-1 on ER biogenesis in MEC in vitro and examined the expression of ER biogenesis-associated genes in the mammary gland during early lactation. We treated mammary alveolar cells–large T antigen cells (immortalized bovine MEC line established via stable transfection with simian virus-40 large T-antigen) with IGF-1 and examined ER biogenesis using the fluorescence intensity of an ER tracker and quantitative real-time PCR. We found IGF-1 significantly increased ER tracker staining and upregulated mRNA levels of ER biogenesis-related genes, such as *CHKA* (choline kinase α), *PCYT1A* (choline-phosphate cytidyltransferase A), and *SURF4* (surfeit locus protein 4). We focused on unfolded protein response to explore molecular mechanisms by which IGF-1 induces ER biogenesis. We found IGF-1 significantly increased mRNA levels of the *XBP1* splicing form (*XBP1s*). Based on western blot analysis, IGF-1 induced the expression of (inositol-requiring kinase 1 α) protein, upstream of XBP1s, and phosphorylated-IRE1 α . The inhibition of IRE1 endonuclease activity with 4-methylumbelliferone 8-carbaldehyde (4 μ 8C) significantly suppressed the increase in ER tracker fluorescence and ER biogenesis-related gene expression induced by IGF-1. Also, IGF-1-induced *XBP1s* and ER biogenesis-associated gene expression

was inhibited by rapamycin, an inhibitor of mTORC1 (mammalian target of rapamycin complex 1), indicating that IRE1-XBP1 activation by IGF-1 is mediated by mTORC1. Moreover, to clarify the expression of *XBP1s* and ER biogenesis-associated genes expression under normal physiological conditions, mammary gland tissue from biopsies of dairy cows during late gestation and lactation were analyzed. In vivo data highlighted the significant increases in the mRNA levels of *XBP1s* and ER biogenesis-related genes in mammary gland tissue immediately after calving through 6 wk of lactation. The mRNA levels of *IGF1R* (IGF-1 receptor) in mammary glands increased during 6 wk of lactation. Therefore, the present study indicated for the first time that IGF-1 induces ER biogenesis by activating the IRE1-XBP1 axis under the regulation of mTORC1 in bovine MEC line.

Key words: mammary epithelial cell, endoplasmic reticulum biogenesis, IGF-1, unfolded protein response

INTRODUCTION

The growth, development, and function of the mammary gland are regulated by several hormones, including IGF-1 (Zhao et al., 1992). The ability of IGF-1 to enhance bovine mammary epithelial cells (MEC) proliferation is known from both in vivo and in vitro studies (McGrath et al., 1991; Silva et al., 2005). The IGF-1 upregulates protein synthesis in MAC-T cells (Burgos and Cant, 2010). It also increases the milk protein (κ -CN) in bovine MEC (Wang et al., 2016). The physiological demands of proliferation and protein synthesis cause MEC to increase in size (Faridi et al., 2003; Goranov et al., 2009). Cells often reshape and resize their organelles during differentiation and adaptation to changing conditions (Reimold et al., 2001; Puhka

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et al., 2007). Gross regulation of cell proliferation and galactopoiesis by IGF-1 is unambiguously accepted, but the mode of action of IGF-1 on cellular organelles during protein synthesis remains unknown.

Synthesis and assembly of proteins and lipids occur in the endoplasmic reticulum (**ER**). The biogenesis of this specialized compartment varies depending on the demands of the external cytosolic pathway in different secretory cells. For example, during the production of copious amounts of immunoglobulin by B-lymphocytes and pancreatic juice by pancreatic acinar cells, a well-developed rough ER network is formed (Bolender, 1974; Rush et al., 1991). The ER biogenesis is also necessary to maintain the efficient secretory capacity of epithelial cells. (Bommiasamy et al., 2009). Davis et al. (2016) showed that ER development is a prerequisite for proper secretory activity in mice mammary glands. Therefore, ER biogenesis is integral to milk production by MEC.

The ER membrane is largely composed of phosphatidylcholine (**PdtCho**), from which other membrane-bound phospholipids such as sphingomyelin and phosphatidylethanolamine are synthesized (Voelker and Kennedy, 1982; Voelker, 1984). Phosphatidylcholine is required for the synthesis of secretory proteins and lipids in various cell types. For example, reduced PdtCho synthesis sufficiently lowers the expression of lung surfactant protein in type II alveolar cells (Tian et al., 2007). De novo lipid biosynthesis occurs concomitantly with the biosynthesis of PdtCho in NIH-3T3 cells (Bommiasamy et al., 2009). Therefore, it is speculated that increased PdtCho expression is important for the synthesis of milk proteins and lipids. Biosynthesis of PdtCho is initiated via choline conversion to phosphocholine by choline kinase, including CHKA (Wu and Vance, 2010). Subsequently, PCYT1A, the rate-limiting enzyme for PdtCho synthesis, produces cytidine diphosphocholine from phosphocholine (Kent, 1997). Finally, cytidine diphosphocholine and diacylglycerol are condensed into PdtCho (Sundler et al., 1972).

Accumulating evidence suggests that the unfolded protein response (**UPR**)-related gene, *XBP1* spliced form (***XBP1s***), regulates PdtCho biosynthesis (Sriburi et al., 2004). Furthermore, *XBP1s* induces an increase in surface area and volume of rough ER by augmenting the protein expression of phosphocholine cytidyltransferase- α (Sriburi et al., 2007), which is encoded by the gene *PCYT1A* (Lykidis et al., 1999). In the case of MEC, *XBP1s* plays a role in the biosynthesis of copious amounts of milk protein and fat (Hasegawa et al., 2015; Davis et al., 2016; Tsuchiya et al., 2017). In a previous study, Davis et al., (2016) found that *XBP1s* ablation results in incompletely developed and

less abundant ER, thereby reducing the lactation capacity of mice MEC. Collectively, *XBP1s* is important for the synthesis of milk protein as well as development and abundance of ER. The maturation and abundance of ER is quite impossible without the biogenesis of ER membrane-bound phospholipids. Therefore, we hypothesized that *XBP1s* may be the critical factor for ER biogenesis in MEC.

The UPR is activated by 3 stress sensor transmembrane proteins: PERK, IRE1 α , and ATF6 α (Patil and Walter, 2001; Ron, 2002). Activated IRE1 triggers the production of *XBP1s* from unspliced *XBP1* mRNA (Calfon et al., 2002; Yoshida et al., 2003). Hence, we hypothesized that the IRE1-*XBP1* axis is crucial for the full secretory capacity of bovine MEC during lactation. Therefore, the purpose of the present study was to clarify the involvement of the IRE1-*XBP1* axis in ER biogenesis in bovine MEC and clarify the underlying molecular mechanisms.

MATERIALS AND METHODS

Reagents and Chemicals

Fetal bovine serum (**FBS**; cat. # SH30088.03) and Dulbecco's modified Eagle medium (**DMEM**; cat. # D7777) were purchased from Equitech-Bio Inc. (Kerrville) and Invitrogen (Carlsbad), respectively. Bovine hydrocortisone (cat. # H0396), insulin from bovine pancreas (cat. # 10516), penicillin-streptomycin mixed solution (cat. # 09367-34), gentamycin (cat. # G1397), 4-methyl umbelliferone-8-carbaldehyde (**4 μ 8C**; cat. # SML0949), and rapamycin (cat. # 553210) were purchased from Sigma-Aldrich. Recombinant human IGF-1 (cat. # PHG0071) was obtained from Gibco. Other chemicals were acquired from Nacalai Tesque.

Cell Culture and Treatment

Sanggun Roh (Tohoku University, Sendai, Japan) generously provided MAC-T cells (bovine MEC line). The MAC-T cells (1.0×10^5) were seeded in 35-mm dishes (at least 3 dishes for each treatment) in DMEM containing FBS (10%), bovine insulin (5 μ g/mL), streptomycin and penicillin (1%), and hydrocortisone (1 μ g/mL). After 48 h, when the cells reached 80 to 90% confluency, 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) with or without 40 μ M 4 μ 8C, a strong inhibitor of IRE1 endonuclease activity, for 24 h. This molecule binds to lysine 907 of the IRE1 endonuclease domain and restricts the accessibility of substrates to

the IRE1 active site. Thus, 4 μ 8C inhibits the unconventional splicing of XBP1 to produce XBP1s and reduces the secretory capacity without exerting acute ER stress (Cross et al., 2012). The cells were also treated with 100 nM rapamycin (a potent inhibitor of mTORC1) for 24 h. Rapamycin complexes with the FK506-binding protein of 12 kDa (FKBP12) and specifically acts as an allosteric inhibitor of mTORC1 (Guertin and Sabatini, 2007). In case of 4 μ 8C (40 μ M) or rapamycin (100 nM) treatment, the cells were treated with 100 ng/mL IGF-1. In all cases, cells were incubated at 37°C under 5% CO₂.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was isolated from MAC-T cells or mammary glands (see animal and sampling) using TRIzol (Invitrogen) following the manufacturer's protocol. The absence of RNA degradation was confirmed by electrophoresis in 2% agarose gel plates. The concentration of total isolated RNA was determined by optical density measurements at 260 nm, and its purity was measured at a wavelength ratio of 260:280 nm (1.85–2.0 was the acceptable value range) using a spectrophotometer (NanoDrop One Spectrophotometer, Thermo Fisher Scientific). We synthesized cDNA from gross RNA using gDNA Remover with qPCR RT Master Mix (Toyobo). We used SYBR Premix Ex Taq™ II (TaKaRa Biotechnology) for quantitative real-time PCR. All primer sequences are shown in Supplemental Table S1 (<https://doi.org/10.6084/m9.figshare.15049083.v1>). Primer3 software, freely available online (<https://bioinfo.ut.ee/primer3-0.4.0/>), was used to design the primers for *CHKA*, *PCYT1A*, and *SURF4*. Agarose gel electrophoresis was performed using the PCR products. The clear PCR bands were then removed from the gel using a NucleoSpin Gel and PCR Clean-Up (TaKaRa Biotechnology). The amplicons were ligated using the pCR4-TOPO vector (Thermo Fisher Scientific) for DNA sequencing. Sequencing results were analyzed against annotated bovine genomes using the basic local alignment search tool (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>; Supplemental Table S2, <https://doi.org/10.6084/m9.figshare.15049083.v1>). We used *ACTB* (bovine β -actin) and *RPS9* (ribosomal protein S9) as housekeeping genes. The housekeeping genes were tested across all samples using NormFinder software (Andersen et al., 2004). The relative expression of bovine *XBP1s*, *ATF4*, *ATF6A*, *CHOP*, *GRP78*, *CHKA*, *PCYT1A*, *SURF4*, *IGF1*, and *IGF1R* mRNA are presented based on the 2^{− $\Delta\Delta C_t$} method (Pfaffl, 2001). Amplification and reaction sensitivities of contaminating products (e.g., the elongation of self-annealed primers)

were measured by increasing the serial dilutions of cDNA. Manufacturer's instructions were strictly followed during data analysis.

Fluorescence Image and Flow Cytometry

For the fluorescence image, MAC-T cells (0.1×10^5 cells/well with at least 3 wells) were grown in 8-well chambers for 48 h (80–90% confluency) in DMEM containing 10% FBS, bovine insulin (5 μ g/mL), streptomycin and penicillin (1%), and hydrocortisone (1 μ g/mL). After 12 h of incubation in serum-free medium, the cells were treated with 100 ng/mL IGF-1 for 24 h. Finally, the cells were washed with Hanks' balanced salt solution (HBSS) and then stained with 1 μ M ER Tracker Green (Invitrogen) at 37°C for 30 min. At the end of incubation, the ER tracker was removed, and the cells were washed with HBSS. Fluorescence microphotographs were then obtained using an EVOS FL auto imaging system (Thermo Fisher Scientific).

For flow cytometry, cells were cultured on 35-mm dishes as previously described in the Cell Culture and Treatment section. After treatment with 100 ng/mL IGF-1, cells were washed with HBSS and collected in 1.5-mL microtubes using a scraper. Then, cells were incubated with ER Tracker Green at a final concentration of 1 μ M for 30 min incubation at 37°C. After removing the stain, the cells were resuspended in HBSS and analyzed using an SH800S cell sorter (Sony Biotechnology Inc.). The mean fluorescence intensity from the FITC channel was normalized to cell size as determined from forward scatter (Jia et al., 2011).

Western Blotting Analysis

Cells were washed twice with PBS 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 $\times g$ for 10 min at 4°C, and protein samples were collected. Protein concentrations were measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories). We performed SDS-PAGE on 4 to 20% polyacrylamide gels using 60 μ g of protein. Protein bands were subsequently transferred to polyvinylidene difluoride membranes. Membranes were incubated with anti-IRE1 α (Cell Signaling Technology), antiphosphorylated-IRE1 α (Thermo Fisher Scientific), or anti- α -tubulin (MBL Co.) antibodies in blocking buffer. After incubating with horseradish peroxidase-conjugated secondary antibody (GE Healthcare), 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 National Institutes of Health. Intensity of the bands was quantified by ImageJ software from the National Institutes of Health (<https://imagej.nih.gov/ij/>).

MTT Assay for Cell Proliferation Measurement

Cell proliferation was measured using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Cell Viability Assay Kit (Biotium) following the manufacturer's protocol. Briefly, MAC-T cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured for 24 h to reach 80 to 90% confluency. Then, cells were incubated in DMEM without serum for 12 h before treatment. Cells were then treated with or without IGF-1 (100 ng/mL) for 24 h. After incubation, 10 μ L of the MTT solution were added to 100 μ L of culture medium. After 4 h of incubation at 37°C, 200 μ L of dimethyl sulfoxide were added to each well. Absorbance was measured at 570 nm and a reference wavelength of 630 nm using a multimode microplate reader (iMark microplate reader, Bio-Rad).

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 the *in vivo* experiment was to examine the expression levels of ER biogenesis-related genes at parturition and during lactation. Therefore, 6 multiparous Holstein cows (parity 2.2 ± 0.4 , SD) were used during peripartum (−1 to 6 wk postpartum) for mammary gland biopsy. Cows were housed in freestalls 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 (containing per 1 kg: Fe, 1,232 mg; Cu, 150 mg; Co, 25 mg; Zn, 500 mg; Mn, 500 mg; I, 50 mg; Se, 15 mg; and Na, 382 g; ZENOAG). Diet was formulated to meet or exceed the Japanese Feeding Standard for Dairy Cattle (NARO, 2006). The diets were fed as fermented TMR from the same batch (stock preparation) of roll-bale storage as Supplemental Table S3 (<https://doi.org/10.6084/m9.figshare.15049083.v1>). In addition, we also used unfermented TMR during the postpartum period, which was based on 26.7% corn silage, 3.2% timothy hay, 15.7% alfalfa hay, 9.8% beet

pulp, 32.7% steam-flaked corn, 10.0% soybean meal, 0.4% vitamin mix, and 1.5% mineral mix (DM basis). We added the unfermented TMR (about 9.5% of the total amount of feeding, DM basis) to the fermented TMR in each trough and mixed by hand or shovel just before each feeding. Cows were offered diet *ad libitum* twice daily at 0930 and 1630 h during prepartum and 3 times daily at 0930, 1500, and 1800 h during postpartum. 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 (e.g., left, right, left) was collected by repeated biopsy at −1 wk (−9 to −4 d), 0 wk (0 d), and 6 wk (40–45 d; $n = 6$ cows $\times$ 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) and 5% tranexamic acid (2.5 mg/kg of BW; Vasolamin injection; Meiji-Seika Pharma). Quarters were clipped and scrubbed with 2% iodine 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), and a subcutaneous injection of 3 mL of 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), 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°C 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% iodine 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% iodine 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 wk postpartum. Cows were hand-stripped as required at each milking for several days until all blood clots were removed and blood contamination was cleared.

Computational Analysis

The sequence of *PCTY1A* promoter (500 b upstream from the transcription start site) was retrieved from *Bos taurus* genome (Ensemble; ENSBTAG00000016979) for

analyzing binding sites for XBP1. ContraV3 bioinformatics tool (Kreft et al., 2017) was used for identification of potential binding sites of XBP1 (TRANFAC database, <https://genexplain.com/transfac/>; JASPAR database, <http://jaspar.genereg.net/>) in *PCTY1a* promoter region.

Statistical Analysis

Mean $\pm$ standard error was used for expressing values from a typical experiment performed, at a minimum, in triplicate dishes. All analyses used SAS version 9.4 (SAS Institute Inc.). Gene expressions of mammary gland were analyzed statistically using repeated measures with a MIXED model procedure; time (sampling weeks) was the fixed effect, and animal was a random effect. When $P < 0.05$ for time, differences among least squares means were analyzed using Tukey-Kramer tests, again using $P < 0.05$ as a threshold for significance. For gene expression, flow cytometry and MTT assay results of in vitro experiments, one-way ANOVA with Tukey-Kramer tests or Student's *t*-tests were used to determine statistical differences. These tests were also considered significant at $P < 0.05$.

RESULTS

IGF-1 Induced ER Biogenesis in Bovine MEC

We initially assessed the total ER in IGF-1-treated bovine MEC using an ER tracker dye. The IGF-1-treated cells induced higher levels of ER staining than did untreated control cells (Figure 1A). The flow cytometric analysis of ER tracker revealed a greater than 2.5-fold increase in ER fluorescence in treated cells ($P < 0.05$, Figure 1B). Moreover, the mRNA expressions of ER biogenesis-related genes, such as *CHKA*, *PCYT1A*, and *SURF4*, were significantly increased by exposure to IGF-1 (all $P < 0.05$, Figure 1C).

IGF-1 Activated the IRE1-XBP1 Axis of the UPR in Bovine MEC

We hypothesized that IGF-1-induced ER biogenesis in bovine MEC would be affected by UPR. Thus, we evaluated the action of IGF-1 on UPR-related gene expression. The IGF-1 significantly increased *XBP1s* and reduced *ATF6A* expression levels (both $P < 0.05$) but did not influence *ATF4*, *CHOP*, and *GRP78* expression in MEC (Figure 2A). We then analyzed the protein expression of p-IRE1 α and IRE1 α (upstream proteins of XBP1) to determine whether the IRE1-XBP1 arm of the UPR is activated by IGF-1. The protein expression ratio of p-IRE1 α to IRE1 α was significantly increased

Figure 1. Effect of IGF-1 on endoplasmic reticulum (ER) biogenesis in bovine mammary epithelial cells (MEC). (A) Representative photomicrographs of ER tracker staining in control and IGF-1-treated bovine MEC. Scale bar: 400 μ m. (B) Quantification of ER tracker fluorescence intensity using flow cytometry for control mammary epithelial cells (MEC) and MEC treated with 100 ng/mL IGF-1 for 24 h. MFI = mean fluorescent intensity. (A.U. = arbitrary units.) (C) Relative expressions of *CHKA*, *PCYT1A*, and *SURF4* in control cells and 10 and 100 ng/mL IGF-1-treated cells for 24 h. Relative transcript expression was calculated by the $2^{-\Delta\Delta C_t}$ method and presented as values relative to control. Data are presented as the mean $\pm$ SEM for 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with controls.

Figure 2. Effect of IGF-1 on the expression of unfolded protein response-related genes in bovine mammary epithelial cells (MEC). (A) Relative expression of *XBP1* splicing form (*XBP1s*), *ATF4*, *ATF6α*, *CHOP*, and *GRP78* in control cells and 100 ng/mL IGF-1-treated cells for 24 h. Relative transcript expression was calculated by the $2^{-\Delta\Delta C_t}$ method and presented as values relative to controls. (B) Bovine MEC were stimulated with 100 ng/mL IGF-1, and IRE1α, phospho-IRE1α (p-IRE1α), and α-tubulin (internal control) protein levels were assessed using western blotting. Representative images from at least 3 independent experiments are shown (left panel). Quantification ratio of p-IRE1α:IRE1α levels is provided in the bar graph. Data are presented as mean $\pm$ SEM for 3 independent experiments. * $P < 0.05$ compared with controls (right panel).

in bovine MEC treated with IGF-1 ($P < 0.05$, Figure 2B).

IRE1-XBP1 Axis in IGF-1-induced ER Biogenesis

To examine the role of the IRE1-XBP1 axis on ER biogenesis induced by IGF-1, we 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 mRNA, encoding the transcription factor XBP1 through an unconventional splicing event to generate XBP1s. (Calton et al., 2002). Then, the produced XBP1s translocates to the nucleus, where it functions as a transcription factor (Yoshida et al., 2003). The 4μ8C inhibits the cleavage of *XBP1* by preventing substrate entry into the endonuclease active site of IRE1 (Cross et al., 2012). We measured the mRNA expression of *XBP1s* to confirm the efficacy of 4μ8C (40 μM) using MEC. This treatment significantly suppressed the IGF-1-induced *XBP1s* expression. Compared with treatment with IGF-1 alone, cotreat-

ment with IGF-1 and 4μ8C also significantly decreased the expression of *CHKA*, *PCYT1A*, and *SURF4* (all $P < 0.05$, Figure 3A). Moreover, cotreatment with IGF-1 and 4μ8C reduced ER staining compared with the finding for treatment with IGF-1 alone (Figure 3B).

Inhibition of IGF-1-induced XBP1s and ER Biogenesis-related Genes Expression by Rapamycin

A previous study established that IGF-1 increased protein synthesis by stimulating mTORC1 in MAC-T cells (Burgos and Cant, 2010). Therefore, we examined the relationship between mTORC1 and the IRE1-XBP1s axis in IGF-1-treated cells. 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 cotreatment with IGF-1 and rapamycin more strongly reduced IGF-1-induced XBP1s expression than IGF-1 alone ($P < 0.05$). The expression of ER biogenesis-related genes in cells treated with IGF-1 and rapamycin was also downregulated compared with the findings in control and IGF-1-treated cells (all $P < 0.05$, Figure 4). Thus, rapamycin abolished the IGF-1-mediated *XBP1s*-induced ER biogenesis. These data suggested that mTORC1 influenced ER biogenesis in MEC through regulating the UPR factor XBP1s.

Changes in the Gene Expression in Mammary Gland Tissue During the Periparturient Period

To investigate the expression of *XBP1s*, *IGF1R*, and ER biogenesis-related genes in vivo, we 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 from immediately after calving through 6 wk of lactation (all $P < 0.05$). Expression of *IGF1R* was significantly increased during 6 wk of lactation ($P < 0.05$, Figure 5).

DISCUSSION

It is known that IGF-1 stimulates protein synthesis in MEC (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 the periods of extensive protein synthesis, bovine MEC 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 pro-

tein synthesis induces ER biogenesis is not understood. We found that IGF-1 significantly increased staining by an ER tracker dye and upregulated the mRNA levels of ER biogenesis-related genes (Figure 1). Therefore, for the first time, our data showed that IGF-1 induced ER biogenesis in bovine MEC through upregulating genes responsible for this process.

We evaluated the rate of bovine MEC proliferation following IGF-1 exposure. The result revealed that IGF-1 did not increase ($P > 0.05$) the proliferation of MEC (Supplemental Figure S1, <https://doi.org/10.6084/m9.figshare.15049083.v1>). Previous research indicated that IGF-1 increased protein synthesis in bovine MEC (Burgos and Cant, 2010). Organelle remodeling is a fundamental characteristic of differentiation or adaptation to changing conditions (Reimold et al., 2001; Puhka et al., 2007) such as increased protein synthesis

in MEC. Therefore, ER biogenesis induced by IGF-1 in bovine MEC occurs independently of cell proliferation.

To uncover the molecular mechanism by which IGF-1 induces ER biogenesis, we then focused on UPR. The 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-IRE1 α and IRE1 α (upstream of *XBP1s*) was upregulated by IGF-1 treatment (Figure 2). The IRE1 α , which exhibits endonuclease activity, cuts the definite intron from unspliced *XBP1* and thus generates transcription factor *XBP1s* (Calfon et al., 2002). Hence, an elevated protein expression ratio of p-IRE1 α :IRE1 α provides mechanistic support for IGF-1-stimulated *XBP1s* mRNA expression. We then used combined treatment with IGF-1 and 4 μ 8C, a potent inhibitor of IRE1 RNase activity (Stewart et al.,

Figure 3. IRE1 α inhibitor suppresses IGF-1-induced endoplasmic reticulum (ER) biogenesis in bovine mammary epithelial cells (MEC). (A) Bovine MEC were exposed to 100 ng/mL IGF-1 in the presence of IRE1 α inhibitor, 4 μ 8C (40 μ M), for 24 h. Relative expression of *XBP1* splicing form (*XBP1s*), *CHKA*, *PCYT1A*, and *SURF4* were assessed via real-time PCR. Relative transcript expression was calculated by the $2^{-\Delta\Delta C_t}$ method and presented as values relative to control. (B) Representative photomicrographs of ER tracker staining. Scale bar: 400 μ m. Data are presented as mean $\pm$ SEM for 3 independent experiments. Means with different letters (a,b) are significantly different, $P < 0.05$; means with the same letter are not significantly different.

Figure 4. Rapamycin, an inhibitor of mTOR, suppresses IGF-1-induced endoplasmic reticulum biogenesis in bovine mammary epithelial cells (MEC). Bovine MEC were exposed to 100 ng/mL IGF-1 in the presence or absence of rapamycin (100 nM) for 24 h. Relative expression of *XBP1* splicing form (*XBP1s*), *CHKA*, *PCYT1A*, and *SURF4* was assessed via real-time PCR. Relative transcript expression was calculated by the $2^{-\Delta\Delta C_t}$ method and presented as values relative to the control. Data are presented as the mean $\pm$ SEM for 3 independent experiments. Means with different letters (a–c) are significantly different ($P < 0.05$), and those with the same letter are not significantly different.

2017); cotreatment significantly masked IGF-1-induced *XBP1s* expression. Consistent with *XBP1s* expression, 4 μ 8C 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 4 μ 8C (Figure 3C), which suggested the inhibition of the endonuclease activity of IRE1 α and thus suppressed IGF-1-induced ER biogenesis. Therefore, IGF-1 induces the IRE1-XBP1s axis to regulate ER biogenesis in bovine MEC.

Figure 5. Changes in gene expression in mammary gland tissue of dairy cows at parturition and during lactation. Relative expression of *XBP1* splicing form (*XBP1s*), *CHKA*, *PCYT1A*, *SURF4*, and *IGF1R* in bovine mammary glands at indicated points was determined by real-time PCR. Relative transcript expression was calculated by the $2^{-\Delta\Delta C_t}$ method and represented as relative values to -1 wk relative to parturition. Data show mean $\pm$ SEM of values from 6 dairy cows. * $P < 0.05$, compared with -1 wk.

The XBP1s is a transcription factor; however, how it regulates the ER biogenesis-related genes is unclear. Previous studies established that XBP1s expression is indispensable for ER remodeling in various secretory cells (Iwakoshi et al., 2003; Lee et al., 2005). However, during ER biogenesis in mouse NIH-3T3 fibroblast cells, the expression of *PCYT1A* or *CCTA* 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. In this study, the enhancement of PdtCho synthesis in XBP1s-overexpressing cells was largely attributable to the posttranscriptional regulation of CCT α (Sriburi et al., 2007). Surprisingly, IGF-1-induced *XBP1s* expression in our present study elevated *PCYT1A* expression in MAC-T cells. Indeed, the bioinformatics study of *PCYT1A* using the Contra v3 tool indicated 4 potential binding sites in the -500 to +1 region of the bovine *PCYT1A* promoter (Supplemental Figure S2, <https://doi.org/10.6084/m9.figshare.15049083.v1>). Therefore, XBP1s could directly modulate *PCYT1A* gene expression. Species variation in the experimental unit may be a major reason for this difference. Although there is a lack of information regarding the effect of *XBP1s* on *CHKA* expression during ER biogenesis, the present study was the first to report that *XBP1s* increased *CHKA* expression during ER biogenesis in bovine MEC. Gene *CHKA* plays a significant role as the initiator of the PdtCho synthesis pathway (Wu and Vance, 2010), which is indispensable for ER biogenesis (Sriburi et al., 2007). Protein 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. Overall, in bovine MEC, ER remodeling occurs under IGF-1-stimulated *XBP1s* upregulation via increasing *CHKA*, *PCYT1A*, and *SURF4* gene expression.

Burgos and Cant (2010) found that protein synthesis was increased in IGF-1-treated bovine MEC through upregulation of mTORC1. Furthermore, a gene-specific knock out study in mice established that mTORC1 requires the UPR component XBP1 for immunoglobulin production from plasma cells (Benhamron et al., 2015). Therefore, we hypothesized mTORC1 signaling and XBP1s might be linked in IGF-1-treated cells. To test our hypothesis, we cotreated cells with IGF-1 and the mTORC1 inhibitor rapamycin. (Guertin and Sabatini, 2007). We revealed that IGF-1-induced *XBP1s* expression was significantly downregulated in cells

cotreated with rapamycin. Moreover, the expression of ER biogenesis-related genes was significantly decreased in cotreated cells. These data suggested that mTORC1 participates in XBP1s-induced ER biogenesis following IGF-1 treatment in bovine MEC. In addition, there was a relationship between mTORC1 activation and the IRE1-XBP1 branch of the UPR, and a model of this process is presented in Figure 6.

As our in vitro study confirmed that IGF-1-induced *XBP1s* expression increased ER biogenesis in bovine MEC, we examined the status of ER biogenesis-related genes under natural physiological conditions during the periparturient period. In this in vivo experiment, the relatively low *XBP1s* expression levels in mammary gland tissue before parturition increased gradually from immediately after parturition through lactation. We 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 *IGF1R* increased during lactation in the present study (Figure 5). The *IGF1R* was expressed in dairy cows during pregnancy and lactation (Hadsell et al., 1990), and the expression of *IGF1R* in our present study was consistent with this finding (Plath-Gabler et al., 2001). Hence, the increased mRNA expression of *XBP1s*, ER biogenesis-related genes, and *IGF1R* in mammary glands supported the

possible occurrence of ER biogenesis in bovine MEC during lactation.

CONCLUSIONS

Our in vitro results showed that IGF-1 induced ER biogenesis by activating the IRE1-XBP1 arm of UPR under the regulation of mTORC1. In addition, the enhanced expression of *XBP1s*, *IGF1R*, and ER biogenesis-related genes in bovine mammary gland tissue indicated the possible occurrence of ER biogenesis during lactation. Thus, we found that IGF-1 induces ER biogenesis in bovine MEC by establishing a linkage between mTORC1 and the IRE1-XBP1 axis of the UPR.

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Figure 6. A model of IGF-1-induced endoplasmic reticulum (ER) biogenesis in bovine mammary epithelial cells. *XBP1s* = spliced *XBP1*; *XBP1u* = unspliced *XBP1*.

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