Fatty acid-induced endoplasmic reticulum stress promoted lipid accumulation in calf hepatocytes, and endoplasmic reticulum stress existed in the liver of severe fatty liver cows

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ABSTRACT

Disruption of endoplasmic reticulum (ER) homeostasis, often termed ER stress, is intrinsically linked with perturbation of lipid metabolism in humans and mice. Whether ER homeostasis is affected in cows experiencing fatty liver is unknown. The aim of this study was to investigate the potential role of ER stress in hepatic lipid accumulation in calf hepatocytes and ER stress status in dairy cows with severe fatty liver. In vitro experiments were conducted in which hepatocytes were isolated from calves and treated with different concentrations of fatty acids, tauroursodeoxycholic acid (TUDCA; a canonical inhibitor of ER stress), or both. The increase in phosphorylation level of protein kinase RNA-like ER kinase (PERK) and inositol requiring protein-1 α (IRE1 α) proteins, and the cleavage of activating transcription factor-6 (ATF6) protein in response to increasing doses of fatty acids (which were reversed by TUDCA treatment) in primary hepatocytes underscored a mechanistic link between fatty acids and ER stress. In addition, fatty acid treatment increased the abundance of sterol regulatory element-binding protein 1c, acetyl-CoA carboxylase- α , fatty acid synthase, and diacylglycerol acyltransferase 1, and lipid accumulation in calf primary hepatocytes, whereas inhibition of ER stress by incubating with TUDCA significantly weakened these effects. Overall, results in vitro indicate that inhibition of ER stress in calf hepatocytes alleviates fatty acid-induced lipid accumulation by downregulating the expression of lipogenic genes. In vivo experiments, liver and blood samples were collected from cows diagnosed as healthy ($n = 15$) or with

severe fatty liver ($n = 15$). The phosphorylation level of PERK and IRE1 α , the cleavage of ATF6 protein, and the abundance of several unfolded protein response genes (78 kDa glucose-regulated protein, AMP-dependent transcription factor 4, and spliced X-box binding protein 1) were greater in liver of cows with severe fatty liver. The present in vivo study confirms the occurrence of ER stress in dairy cows with severe fatty liver. Considering the causative role of fatty acid-induced ER stress in hepatic lipid accumulation in calf hepatocytes, the existence of ER stress in the liver of severe fatty liver cows may presage its participation in fatty liver progression in dairy cows. However, the mechanistic relationship between ER stress and fatty liver in dairy cows remain to be determined.

Key words: fatty liver, endoplasmic reticulum stress, unfolded protein response, fatty acid

INTRODUCTION

Fatty liver is a major metabolic disorder of high-producing dairy cows during the transition period (Herdt, 1988; Grummer, 1993; Gross et al., 2013). Fatty liver during the transition period develops when the uptake of fatty acids exceeds the oxidation and secretion of lipid by the liver, which is generally secondary to negative energy balance (NEB; Fry et al., 2018). Depressed feed intake during the transition period renders cows unable to meet their energy needs for maintenance and lactation (Herdt, 1988; Rukkwamsuk et al., 1999). As a result, fatty acids are mobilized from the adipose tissue and approximately one-third of the whole-body fatty acid flux is taken up into the liver (Emery et al., 1992; Drackley et al., 2001). Over 40% of high-yielding dairy cows suffer from mild or moderate fatty liver and 5 to 10% of dairy cows have severe fatty liver (Bobe et al., 2004; Kalaitzakis et al., 2007). Fatty liver can cause huge economic losses as a result of increased veterinary

Received November 19, 2018.

Accepted April 4, 2019.

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costs, longer calving intervals, decreased milk production, and decreased average lifetime (Bobe et al., 2004). Therefore, elucidating the molecular mechanism of fatty liver and further identification of effective therapeutic strategies is of utmost importance.

It has been shown that increased plasma levels of fatty acids, the pathological basis of fatty liver in dairy cows, are leading to disruption of endoplasmic reticulum (ER) in the liver of nonruminants (Wang et al., 2006; Gentile et al., 2011; Nivala et al., 2013). The ER is a crucial site for maintenance of lipid homeostasis in hepatocytes, and also acts as a sensor for metabolic stress (Fu et al., 2012). Disruption of ER homeostasis, namely ER stress, elicits an adaptive response known as the unfolded protein response (UPR) (Baiceanu et al., 2016). The UPR has 3 canonical branches: protein kinase RNA-like ER kinase (PERK) branch, inositol requiring protein-1 α (IRE1 α) branch, and activating transcription factor-6 (ATF6) branch (Ron and Walter, 2007; Gessner et al., 2014). It is thought that activation status of PERK, IRE1, and ATF6, and mRNA abundance of several UPR downstream genes, such as 78 kDa glucose-regulated protein (GRP78), AMP-dependent transcription factor 4 (ATF4), and spliced X-box binding protein 1 (sXBP1), are reliable markers of ER stress (Baiceanu et al., 2016). The mRNA abundance of various components of the ER stress pathway, along with inflammation-related targets, was first detected in periparturient cow liver nearly 10 yr ago (Loor, 2010). More recent work with periparturient cows revealed that genes of the ER stress-induced UPR, such as *ATF4*, *GRP78*, and *XBP1*, were upregulated in dairy cows during early lactation, a time during which incidence of fatty liver is the highest (Gessner et al., 2014; Khan et al., 2015). The facts that periparturient cows have commonly strongly increased plasma concentration of fatty acids (Gessner et al., 2014) and the induction effect of fatty acids on ER stress and hepatic steatosis in nonruminants, prompted us to the hypothesis that fatty acids may be an efficacious stimulus for ER stress induction in dairy cows.

In humans and mice with steatosis, the perturbation of hepatic lipid homeostasis is intrinsically linked to ER stress (Ozcan et al., 2004; Wang et al., 2006; Lake et al., 2014). High-fat diets induced hepatic ER stress, steatosis, and injury in rats, and evidence of ER stress preceded the development of fatty liver (Wang et al., 2006; Gentile et al., 2011). Inhibiting ER stress or depleting certain UPR pathway molecules (such as ATF4 or ATF6) ameliorates hepatic steatosis in mice or zebrafish (Kammoun et al., 2009; Xiao et al., 2013; Howarth et al., 2014). Numerous in vitro studies also detected that fatty acid-induced hepatocyte steatosis and lipoapoptosis were accompanied by ER stress, and

could be mitigated by enhancing ER folding capacity (Wei et al., 2006; Rahman et al., 2009; Akazawa et al., 2010). Hence, these studies with model organisms and cells presaged that ER stress induced by excessive fatty acids can partially induce steatosis and liver injury in nonruminants.

Mechanistically, sterol regulatory element-binding protein 1c (SREBP-1c) is a master transcriptional regulator of lipogenesis, which is bound to SREBP cleavage activating protein (SCAP) in an inactive form within the ER membranes (Porstmann et al., 2005; Lee et al., 2008; Kammoun et al., 2009). After dissociates from insulin-induced gene, SCAP escorts SREBP-1c from ER to Golgi where SREBP-1c precursor is proteolytically processed to yield the transcriptionally active form (Fang et al., 2013). The mature SREBP-1c can enter into the nucleus and activate the expression of lipogenic genes (Kammoun et al., 2009; Fang et al., 2013). Several studies have suggested that ER stress could trigger SREBP-1c activation and promotion of lipogenesis (Werstuck et al., 2001; Esfandiari et al., 2005; Ning et al., 2011). Induction of ER stress has been shown to activate both precursor SREBP-1c and nuclear SREBP-1c in HepG2 cells (Damiano et al., 2010). Of note, study in calf primary hepatocytes showed that SREBP-1c could response high concentrations of fatty acids and upregulate the expression of lipogenic genes [acetyl-CoA carboxylase- α (*ACACA*), fatty acid synthase (*FASN*), and diacylglycerol acyltransferase 1 (*DGAT1*)], thereby inducing lipid accumulation (Li et al., 2015). Also, a link between upregulation of SREBP-1c and its target genes was recently associated with fatty liver in cows (Li et al., 2015). Thus, we speculate that hepatic ER stress may contribute to fatty acid-induced upregulation of lipogenic genes, thereby increasing the risk for hepatic steatosis in dairy cows. Whether ER stress accompanies steatosis when fatty acid influx into liver increases is unknown. Thus, whether ER stress accounts for fatty liver in dairy cows is the topic of our investigation.

MATERIALS AND METHODS

In Vitro Experiments

Isolation, Culture, and Treatment of Calf Primary Hepatocytes. Primary hepatocytes from 4 Holstein calves (1 d old, female, 30–40 kg, fasting, rectal temperature: 38.7 to 39.7°C, healthy) purchased from a commercial dairy farm (Changchun, China) were isolated using a modified 2-step collagenase perfusion method as previously described (Du et al., 2018b). Briefly, the caudate liver lobe was obtained through surgical liver excision, and the hepatocytes were isolated

under sterile conditions. Blood vessels were intubated and perfused with perfusion solution A (140 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 2.5 mM glucose, and 0.5 mM EDTA, pH 7.4; 37°C, 50 mL/min for 12 min) and B (140 mM NaCl, 6.7 mM KCl, 30 mM HEPES, 2.5 mM glucose, and 5 mM CaCl₂, pH 7.4; 37°C, 50 mL/min for 3 min) until the liquid became clear. Subsequently, the liver was perfused with digestion solution (0.1 g of collagenase IV dissolved in 0.5 L of perfusion solution B, pH 7.2–7.4; 20 mL/min for 15 to 20 min) to dissociate the hepatic structure. Then, 100 mL of fetal bovine serum (**FBS**; Hyclone Laboratories, Logan, UT) was added to terminate the digestion. The liver was cut into small pieces, and the hepatic capsule, blood vessels, fat, connective tissue, and incompletely digested liver were removed. The remaining hepatic parenchyma was filtered sequentially with 100 mesh (150 μ m) and 200 mesh (75 μ m) cell sieves. The obtained hepatocytes were washed twice with RPMI-1640 basic medium (Hyclone Laboratories) and centrifuged for 5 min at 500 $\times$ *g* at 4°C. Cell viability was assessed with Trypan blue dye (Sigma-Aldrich, St. Louis, MO) exclusion method after isolation and only cells with viability >95% were used for further experiments. The hepatocyte suspension was seeded into a 6-well tissue culture plate (2 mL per well) at 1×10^6 cells/mL using adherent medium (RPMI-1640 basic medium supplemented with 10% FBS, 10^{-6} mol/L of insulin, 10^{-6} mol/L of dexamethasone, 10 μ g/mL of vitamin C) and incubated at 37°C in 5% CO₂. After 4 h, the adherent medium was replaced with growth medium (RPMI-1640 basic medium supplemented with 10% FBS). The growth medium was replaced with fresh medium every 24 h.

A schematic diagram of the cell treatment is shown in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2018-16015>). Briefly, after 44 h cultured, hepatocytes were serum-free starved in RPMI-1640 basic medium for 6 h, and then treated with 0 or 500 μ g/mL of TUDCA for 12 h. After that, hepatocytes were maintained in RPMI-1640 basic medium containing 2% BSA and treated with 0, 0.4, 0.8, or 1.2 mM fatty acids for 6 h. Hepatocytes were harvested 72 h after isolation. The components and concentrations of fatty acids used in this study were chosen based on the hematology standards of dairy cows with severe fatty liver (Bertics et al., 1992; Li et al., 2015). Stock fatty acid solution was prepared by diluting the fatty acid components in 0.1 M KOH at 60°C, then the pH of the solution was adjusted to 7.4 with hydrochloric acid (1 M). The stock fatty acid (52.7 mM; Sigma-Aldrich) solution included oleic acid (22.9 mM; Sigma-Aldrich), linoleic acid (2.6 mM; Sigma-Aldrich), palmitic acid (16.8 mM; Sigma-Aldrich), stearic acid (7.6 mM; Sigma-Aldrich), and palmitoleic acid (2.8 mM; Sigma-Aldrich).

Measurement of Triglyceride Content. Triglyceride (**TG**) content was measured using commercially available TG assay kit (E1013, Applygen Technologies Inc., Beijing, China) following the manufacturer's instructions. For cell samples, every 1×10^6 hepatocytes were lysed with 0.1 mL of lysis buffer. Then, a sample of supernatant was used to determine the total protein concentration using the bicinchoninic acid assay (P1511, Applygen Technologies Inc.). The remaining supernatant was heated in a water bath (70°C) for 10 min and then centrifuged at $800 \times g$ for 5 min at room temperature. Then, a sample of 10 μ L of supernatant was mixed with 190 μ L of chromogenic liquid for TG assay. The mixture absorbance at 550 nm was proportional to the concentration of TG in each sample (run in triplicate). The TG values of cell samples were normalized to protein concentrations and expressed as micromoles per milligram of protein.

Oil Red O Staining in Calf Primary Hepatocytes. Oil Red O working solution was prepared by diluting Oil Red O stock solution (3.5 g of Oil Red O diluted in 1 L of isopropanol) 3:2 with double-distilled H₂O. All assay steps were performed at room temperature. Hepatocytes were washed twice in PBS and fixed with 4% formaldehyde for 30 min. After washing with PBS twice, hepatocytes were incubated in 60% isopropanol for 10 s, dried, and then stained with 1 mL of Oil Red O working solution for 10 min. After washing with PBS twice, hepatocytes were counterstained with hematoxylin for 5 min. The features were observed and captured at 400 $\times$ under a light microscope (Olympus, Tokyo, Japan).

Protein Extraction and Western Blotting. Protein abundance of phospho-PERK (Thr980), total PERK, phospho-IRE1 α (S724), total IRE1 α , cleaved-ATF6, total SREBP-1c, and β -actin were determined by semiquantitative Western blotting. Total proteins were extracted from cultured primary hepatocytes, and prepared for Western blotting according to manufacturer's instructions (P0013B, Beyotime Institute of Biotechnology, Jiangsu, China). The bicinchoninic acid assay (P1511, Applygen Technologies Inc.) was performed to determine protein concentration, which was used to standardize the quantity of total protein loaded onto each gel. Proteins (30 μ g per lane) were separated in 10% (phospho-PERK, PERK, phospho-IRE1 α , IRE1 α , and β -actin) or 12% (cleaved-ATF6, SREBP-1c, and β -actin) SDS-polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride membranes. Following transfer, membranes were blocked in 3% BSA/Tris-buffered saline/Tween at room temperature for 4 h. Blots were incubated with phospho-PERK antibody (1:500; catalog no. 3179, Cell Signaling Technology, Beverly, MA), PERK antibody

(1:500; catalog no. 3192, Cell Signaling Technology), phospho-IRE1 α antibody (1:500; catalog Ab104157, Abcam, Cambridge, MA), IRE1 α antibody (1:500; catalog Ab77544, Abcam), cleaved-ATF6 antibody (1:1000; catalog Ab11909, Abcam), SREBP-1c antibody (1:500; catalog NB100–2215, Novus Biologicals, Littleton, CO), or β -actin antibody (1:1,000; sc-47778; Santa Cruz) overnight at 4°C, followed by incubation with an appropriate secondary antibody (phospho-PERK: anti-rabbit; PERK: anti-rabbit; phospho-IRE1 α : anti-rabbit; IRE1 α : anti-goat; cleaved-ATF6: anti-mouse; SREBP-1c: anti-mouse; β -actin: anti-mouse) at room temperature for 45 min, respectively. Immunoreactive bands were visualized by enhanced chemiluminescence solution (Beyotime Biotechnology Inc.) and imaged using a simple protein imager (ProteinSimple, Santa Clara, CA). Each sample was run in triplicate. Protein gray intensity was quantified by Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD) normalized to β -actin (Morey et al., 2011).

RNA Extraction and Quantitative Real-Time PCR Assay. Total RNA was extracted from cultured primary hepatocytes using RNAiso Plus (D9108A, TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The purity of RNA was measured by calculating the ratio of UV activity at 260/280 nm using a K5500 Micro-Spectrophotometer (Beijing Kaiiao Technology Development Co. Ltd., Beijing, China). The A_{260}/A_{280} and A_{260}/A_{230} ratios ranged from 1.96 to 2.05 and from 2.06 to 2.16, respectively. Therefore, all total RNA were pure and free from protein and organic pollutants derived from RNA extraction. The RNA integrity was assessed by native agarose gel electrophoresis using the Gel Loading Solution (All-Purpose, Native Agarose, AM8556; Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The 18S and 28S ribosomal RNA bands were clearly visible and the 28S rRNA band was approximately twice as intense as the 18S rRNA band, which indicated that the RNA was intact. The concentration of purified RNA was determined by UV spectrum at 260 nm. The mean total RNA concentration was 1.98 $\mu\text{g}/\mu\text{L}$. The cDNA was reverse transcribed from 1 μg of purified total RNA using a reverse transcription kit (RR047A, TaKaRa Biotechnology Co. Ltd.) and subsequently stored at -80°C . The relative mRNA expression of target genes was detected using the SYBR green plus reagent kit (Roche, Norwalk, CT) in the 7500 Real-Time PCR System (Applied Biosystems Inc., Waltham, MA). All primer sequences are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-16015>). The reaction conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each

sample was run in triplicate. The relative quantitation values normalized to the geometric mean of each reference gene cycle threshold (Cq) value was extrapolated using the $2^{-\Delta\Delta\text{Cq}}$ method. *ACTB* and *GAPDH* were the chosen, stably expressed, reference genes used for normalization (Supplemental Figure S2, <https://doi.org/10.3168/jds.2018-16015>; Morey et al., 2011).

In Vivo Experiments

Animals. All animal protocols were approved by the Ethics Committee for Animal Care and Use of Jilin University (2017 clinical trial 201709031, Changchun, China). The animals received humane care according to the principles and specific guidelines presented in *Guide for the Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010).

Dairy cows were selected from a 1,000-cow dairy farm located in Changchun City, Jilin Province, China. The TMR was formulated to meet lactating cow nutritional requirements, and cows had ad libitum access throughout the day. The basal diet formulation is shown in Supplemental Table S2 (<https://doi.org/10.3168/jds.2018-16015>). All selected cows were subjected to routine physical examination to guarantee absence of co-morbidities. First, 134 lactating cows with similar number of lactation (mean $\pm$ SD = 2.73 ± 0.70) and DIM (mean $\pm$ SD = 6.33 ± 1.59) were sampled to arrive at the 2 experimental groups. Then, a total of 30 cows were selected into a healthy ($n = 15$) or severe fatty liver group ($n = 15$) according to their hepatic TG content (% wet liver). Cows with hepatic TG content below 1% were classified as healthy, whereas cows with hepatic TG content higher than 10% were classified as having severe fatty liver. Body condition score was assigned based on a 1- to 5-point scale according to a previous report (Edmonson et al., 1989). The basic description of healthy and severe fatty liver cows is provided in Table 1.

Sample Acquisition. Blood samples were collected on 3 consecutive days via coccygeal venipuncture (no anticoagulant) between 0700 and 0800 h before feeding. Separation of serum took place within an hour by centrifugation at $3,500 \times g$ for 15 min at 4°C . Collected serum was stored at -80°C until analysis.

Liver samples were taken from the 11th or 12th right intercostal space twice by an experienced veterinarian using a liver puncture needle (14 gauge $\times$ 16 cm, Shanghai Surgical Equipment Factory, Shanghai, China) as previously described (Zhu et al., 2019). Before liver biopsy, the intercostal space was shaved, sanitized with iodine scrub and 75% alcohol, and anesthetized with a subcutaneous injection of 2% lidocaine HCl (Sigma-Aldrich). The #22 scalpel blade was used to make a

Table 1. A basic description of the healthy cows and cows with severe fatty liver¹

Item	Healthy (n = 15)		Severe fatty liver (n = 15)		P-value
	Median	IQR	Median	IQR	
BW (kg)	624	34	681	27	<0.001
BCS	2.91	0.12	3.39	0.18	<0.001
Milk production (kg of milk/cow per d)	31.2	1.6	27.2	1.5	<0.001
DMI (kg/d)	21.3	0.8	18.8	1.1	<0.001

¹The data were analyzed using the Wilcoxon test. The data are presented as the median and interquartile range (IQR).

3-mm stab incision in the skin. The puncture needle was then inserted through the intercostal muscle and into the liver. The liver tissue biopsies (approximately 200 mg and 3 cm) were immediately frozen in liquid nitrogen or fixed with 4% paraformaldehyde or OCT compound (Sakura Finetek Co., Torrance, CA).

Blood Biochemical Assays. Concentrations of fatty acids, BHB, and glucose in serum were determined using a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan) with commercially available kits (BHB: RB1008; fatty acids: FA115; glucose: GL3815; Randox Laboratories, Crumlin, UK). The activities of aspartate aminotransferase (**AST**), alanine aminotransferase (**ALT**), glutamate dehydrogenase (**GLDH**), and gamma-glutamyl transferase (**GGT**) were determined using an automatic biochemistry analyzer (Sekisui Medical Co. Ltd., Tokyo, Japan) with commercially available kits (AST: AS3804; ALT: AL3801; GLDH: GL442; GGT: GT3817; Randox Laboratories).

Histomorphology Analysis. Tissue samples from each cow (n = 15 cows per group for each staining) were used to evaluate histological features captured at 200× under a light microscope (Olympus). The frozen liver sections that were prepared using OCT compound were subjected to Oil Red O staining to visualize lipid droplets. Briefly, liver tissue was frozen in OCT compound (Sakura Finetek Co.), sectioned to 8 μm at −18°C, stained with Oil Red O working solution (Sigma-Aldrich), and counterstained with hematoxylin. Oil Red O working solution was prepared as described above.

Liver sections embedded in paraffin were subjected to hematoxylin and eosin (**H&E**) staining to observe the distribution of accumulated lipid. Briefly, samples were fixed in 4% paraformaldehyde overnight, dehydrated, cleared, and embedded in paraffin. Sections 6 to 8 μm in thickness were cut and stained with H&E.

Measurement of TG Content. The TG content was measured using commercially available TG assay kit (E1013, Applygen Technologies Inc., Beijing, China) as previously described (Zhu et al., 2019). Approximately 50 mg of liver tissue was chopped and homogenized in 1 mL of lysis buffer. The next protocol was the same as the in vitro experiments described above. The TG

values of tissue samples were expressed as a percentage of wet liver weight.

Protein Extraction and Western Blotting. Protein abundance of phospho-PERK (Thr980), total PERK, phospho-IRE1α (S724), total IRE1α, cleaved-ATF6, total SREBP-1c, and β-actin were determined by semiquantitative Western blotting. Total proteins were extracted from liver samples and prepared for Western blotting according to the manufacturer's instructions (P0013B, Beyotime Institute of Biotechnology). The protocol of Western blotting was the same as the in vitro experiments described before.

RNA Extraction and Quantitative Real-Time PCR Assay. Total RNA was extracted from liver samples using RNAiso Plus (D9108A, TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The protocol of the quantitative real-time PCR assay was the same as the in vitro experiments described before.

Statistical Analysis

Each experiment was repeated at least 3 times. All data were analyzed using the appropriate statistical analysis methods with the Statistical Package for the Social Sciences (SPSS) 19.0 software (SPSS Inc., Chicago, IL). All data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene tests, respectively. For data with Gaussian distribution, parametric statistical analysis was performed using the independent-samples *t*-test for 2 groups; 1-way ANOVA was performed for multiple comparisons with Bonferroni correction for data meeting homogeneity of variance or with Tamhane's T2 analysis for data of heteroscedasticity. For data with skewed distribution, nonparametric statistical analysis was performed using the Wilcoxon test for 2 groups. The present statistical analyses included all the observations including outliers. The statistical analysis was also repeated after the outliers were removed according to the interquartile range (**IQR**) criteria, for which values less than $Q1 - 1.5 \times \text{IQR}$ or greater than $Q3 + 1.5 \times \text{IQR}$ were labeled as outliers. $Q1$ and $Q3$ are defined to be the first and third quartiles, and the value of $Q3 - Q1$ is

Figure 1. Effects of fatty acids, tauroursodeoxycholic acid (TUDCA), or both, on endoplasmic reticulum (ER) stress induction and lipogenic gene expression in calf primary hepatocytes. Calf primary hepatocytes were treated with different concentrations of fatty acids (0, 0.4, 0.8, or 1.2 mM), or 500 μg/mL of TUDCA, or co-treated with 500 μg/mL of TUDCA and 1.2 mM fatty acids (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-16015>). (A) Representative Western blots (i) of hepatic phospho-PERK (Thr980), protein kinase RNA-like ER kinase (PERK), phospho-IRE1α (S724), inositol requiring protein-1α (IRE1α), cleaved activating transcription factor-6 (ATF6), and sterol regulatory element-binding protein 1c (SREBP-1c), and quantification of phospho-PERK/PERK (ii), phospho-IRE1α/IRE1α (iii), cleaved-ATF6 (iv), and SREBP-1c (v) in calf primary hepatocytes. (B) Relative mRNA expression levels of *SREBP1* in calf primary hepatocytes. (C) Relative mRNA expression levels of *ACACA* in calf primary hepatocytes. (D) Relative mRNA expression levels of *FASN* in calf primary hepatocytes. (E) Relative mRNA expression levels of *DGAT1* in calf primary hepatocytes. For all bar plots shown, data are expressed as the mean ± SEM. * $P < 0.05$, ** $P < 0.01$, by 1-way ANOVA with subsequent Bonferroni correction (B, C, E) or Tamhane's T2 analysis (D). Results are representative of at least 3 independent measurements. n.s. = not significant.

the IQR. The significance levels for all of the analyses were the same with or without these outliers. Data are presented as the mean ± SEM or the median and IQR. A $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Fatty Acids and TUDCA on ER Stress Induction, Lipogenic Gene Expression, and Lipid Accumulation in Calf Primary Hepatocytes

Calf primary hepatocytes were treated with different concentrations of fatty acids (0, 0.4, 0.8, or 1.2 mM), 500 μg/mL of TUDCA, or co-treated with 1.2 mM fatty acids and 500 μg/mL of TUDCA, an agent commonly used to inhibit ER stress (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-16015>). Following treatment with fatty acids, the phosphorylation levels of PERK and IRE1α, and the cleaved-ATF6 increased gradually in a fatty acid dose-dependent manner; it was highest in the 1.2 mM fatty acid treatment group (Figure 1A). The protein expression of SREBP-1c and mRNA expression of *SREBP1*, *ACACA*, *FASN*, and *DGAT1* had a similar trend (Figure 1A, B, C, D, E). Furthermore, hepatic TG content was greater ($P < 0.01$) with the 0.8 and 1.2 mM fatty acid treatment

compared with the control (Figure 2A). The result of Oil Red O staining showing that lipid droplets excessive infiltrated in the 1.2 mM fatty acid treatment group, which further demonstrated a link between fatty acid supply, ER stress, and lipid accumulation in calf primary hepatocytes (Figure 2B).

Compared with 1.2 mM fatty acids alone, the phosphorylation level of PERK and IRE1α protein, the protein expression of cleaved-ATF6 and SREBP-1c, and the mRNA expression of *SREBP1*, *ACACA*, *FASN*, and *DGAT1*, were lower ($P < 0.01$ or $P < 0.05$) in response to co-treatment with TUDCA plus 1.2 mM fatty acids (Figure 1A, B, C, D, E). In addition, treatment with TUDCA significantly alleviated the fatty acid-induced lipid accumulation (Figure 2A and B).

Hepatic TG Content, Baseline Characteristics, and Blood Parameters in Healthy Cows and Dairy Cows with Severe Fatty Liver

Hepatic TG content (% wet weight) of dairy cows with severe fatty liver was greater ($P < 0.01$) than healthy cows (Figure 3A). Furthermore, Oil Red O and H&E staining revealed a clear state of steatosis in the liver of cows with severe fatty liver (Figure 3B).

Figure 2. Effects of fatty acids, tauroursodeoxycholic acid (TUDCA), or both, on lipid accumulation in calf primary hepatocytes. Calf primary hepatocytes were treated with different concentrations of fatty acids (0, 0.4, 0.8, or 1.2 mM), or co-treated with 500 μg/mL of TUDCA and 1.2 mM fatty acids (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-16015>). (A) Triglyceride (TG) content in calf primary hepatocytes. (B) Representative Oil Red O stained images of calf primary hepatocytes; scale bar = 20 μm. For all bar plots shown, data are expressed as the mean ± SEM. ** $P < 0.01$, n.s. = not significant, by 1-way ANOVA with subsequent Bonferroni correction. Results are representative of at least 3 independent measurements.

Figure 3. Hepatic triglyceride (TG) content and histomorphology analysis. (A) Hepatic TG content in healthy ($n = 15$) and severe fatty liver cows ($n = 15$). (B) Representative hepatic Oil Red O (top) and hematoxylin and eosin (bottom) stained images of liver sections. Scale bar = 50 μm. For all scatter plots, data are presented as the median and interquartile range (IQR); boundary lines represent the 25th and 75th percentiles, respectively; center lines are medians; and every point represents an individual sample. ** $P < 0.01$ by Wilcoxon test. Results are representative of at least 3 independent measurements.

As shown in Table 1, BW and BCS of dairy cows with severe fatty liver were greater ($P < 0.01$) than healthy cows. However, milk production ($P < 0.01$) and DMI ($P < 0.01$) of cows with severe fatty liver were lower than healthy cows (Table 1).

Concentrations of fatty acids and BHB were greater ($P < 0.01$) in cows with severe fatty liver than healthy cows (Figure 4A and B). In contrast, the blood concentration of glucose was lower ($P < 0.01$) in dairy cows with severe fatty liver than healthy cows (Figure 4C).

Activities of AST, ALT, GLDH, and GGT, blood biomarkers of liver function, were all greater ($P < 0.01$) in serum of dairy cows with severe fatty liver than healthy cows (Figure 4D, E, F, and G).

Hepatic ER Stress Status in Healthy Cows and Dairy Cows with Severe Fatty Liver

Western blotting results showed that phosphorylation of UPR indicators, including PERK phosphorylation, IRE1 α phosphorylation, and cleaved-ATF6, was greater ($P < 0.01$) in liver of cows with severe fatty liver than healthy cows (Figure 5A). In addition, several downstream genes of UPR, including *GRP78*, *ATF4*, and *sXBP1*, were upregulated ($P < 0.01$ or $P < 0.05$) in the liver of cows with severe fatty liver (Figure 5B). The protein expression of SREBP-1c and mRNA expression of *SREBP1*, *ACACA*, *FASN*, and *DGAT1* were greater ($P < 0.01$) in the liver of cows with severe fatty liver than healthy cows (Figure 5A, B).

DISCUSSION

Severe fatty liver is characterized by high concentration of fatty acids in blood and hepatic lipid accumulation (Li et al., 2015). An overload of fatty acids directly induces ER stress in hepatocytes (Pagliassotti et al., 2007; Gentile et al., 2011). The ER is an organelle where several enzymes involved in lipid metabolism reside, hence rendering it essential in the control of lipid homeostasis in tissues like the liver (Fu et al., 2012). Recently, mounting evidence has shown that ER stress occurs in the liver of dairy cows during early lactation (as evidenced by increased expression of 13 UPR genes, including *GRP78* and *XBP1*), a time during which the blood concentration of fatty acids is higher than other periods (Gessner et al., 2014; Khan et al., 2015). Although ER stress can be induced by different stimuli (Pagliassotti et al., 2007; Gentile et al., 2011; Nivala et al., 2013), the most likely and vital candidate for ER stress induction in the liver of early-lactating cows is fatty acids. However, there were few pieces of research on fatty acid-induced ER stress in the hepatocytes of dairy cows. Therefore, hepatocytes were isolated from

calves and treated with different concentration of fatty acids. The results that fatty acids dose-dependently activated ER stress transducers (PERK, IRE1 α , and ATF6) in calf primary hepatocytes indicated that fatty acids can induce hepatic ER stress in dairy cows in vitro.

Mounting evidence suggests that induction of ER stress has deleterious consequences and precipitates hepatic lipogenesis (Kammoun et al., 2009; Pagliassotti, 2012; Baiceanu et al., 2016). The inhibition of ER stress ameliorates fatty acid-induced lipid accumulation in Huh-7 cells (Kammoun et al., 2009; Akazawa et al., 2010). Intriguingly, fatty acids can induce lipid accumulation in calf hepatocytes (Li et al., 2015), which was in agreement with our result. These studies underscored a causative role between ER stress and lipid accumulation in hepatocytes, an association we sought to study in calf primary hepatocytes by inhibiting ER stress with TUDCA (a canonical ER stress inhibitor). In accordance with our prediction, fatty acid-induced activation of ER stress transducers (PERK, IRE1 α , and ATF6) was decreased by TUDCA treatment. Incidentally, TUDCA is a hydrophilic bile acid with chaperone properties (Berger and Haller, 2011; Cortez and Sim, 2014), which can inhibit the thermal aggregation of different proteins and promote calcium balance (Gleeson et al., 1990). Thus, the inhibitory effects of TUDCA on ER stress in our study may be secondary to the direct stabilization of the protein or calcium homeostasis. More importantly, the accumulation of lipid was alleviated following the mitigation of ER stress induced by fatty acids in response to TUDCA. These results suggest that high concentrations of fatty acids exacerbate hepatic lipogenesis partly by inducing ER stress in calf primary hepatocytes. By the way, the capacity to metabolize fatty acids may be different in the preruminant liver and the ruminant liver. Although previous studies have confirmed the fatty acid catabolism and anabolism ability of calf hepatocytes (Li et al., 2014; Liu et al., 2014; Du et al., 2018a), the differences of metabolic profiles between hepatocytes isolated from calf liver and adult bovine liver still need to be further studied.

A growing body of evidence in vitro has revealed that induction of ER stress promotes proteolytic cleavage of SREBP-1c and subsequent lipogenic gene expression (Kammoun et al., 2009). Activity of XBP1, a downstream target of UPR, also reinforces the action of SREBP-1c and participates in the regulation of enzymes involved in fatty acid synthesis (Lee et al., 2008; Ning et al., 2011). The SREBP-1c is a master transcriptional regulator of lipogenesis (Li et al., 2014, 2015), and our previous study revealed that treatment of calf primary hepatocytes with high concentrations

Figure 4. Blood parameters. The serum concentrations of (A) fatty acids, (B) BHB, (C) glucose, (D) aspartate aminotransferase (AST), (E) alanine aminotransferase (ALT), (F) glutamate dehydrogenase (GLDH), and (G) gamma-glutamyl transferase (GGT) in healthy (n = 15) and severe fatty liver (n = 15) cows. For all scatter plots, data are presented as the median and interquartile range (IQR); boundary lines represent the 25th and 75th percentiles, respectively; center lines are medians; and every point represents an individual sample. ** $P < 0.01$ by Wilcoxon test. Results are representative of at least 3 independent measurements.

of fatty acids upregulated the expression of lipogenic genes (*ACACA*, *FASN*, and *DGAT1*) through increasing the expression and activity of SREBP-1c. The present study confirmed those effects. On the basis of these facts, we deduce that ER stress may participate in the fatty acid-induced lipid accumulation process in calf hepatocytes by regulating lipogenic genes. To confirm the link between ER stress and lipogenesis, we measured the abundance of SREBP-1c and its target genes in fatty acid- or TUDCA-treated hepatocytes (or both). Results revealed that the increased expression of SREBP-1c and its target genes (*ACACA*, *FASN*, and *DGAT1*) induced by fatty acids was significantly attenuated by TUDCA treatment in calf primary hepatocytes. Taken together, the present study indicates that fatty acid-induced ER stress favors the expression of

lipogenic genes, thereby exacerbating lipid accumulation in calf hepatocytes.

Fatty acids, which were demonstrated as the potent ER stress inducer in calf hepatocytes by our in vitro study, are also the pathological basis of fatty liver development in dairy cows. Pathophysiologically, DMI reduction during early lactation, coupled with increases in energy requirements to meet the C supply of maintenance and lactation, results in cows entering a state of NEB (Bertics et al., 1992; Bobe et al., 2004; Gross et al., 2011). Fat mobilization due to NEB causes high blood concentrations of fatty acids (Roche et al., 2013). The great amount of fatty acids taken up into the liver exceeds its oxidation capacity, and thus, is re-esterified into TG or incompletely oxidized to ketone bodies (Bobe et al., 2004), leading to the onset of fatty liver

syndrome or ketosis (White, 2015). Obese cows characterized by higher BW and BCS experience greater decreases in DMI and more pronounced NEB, and consequently, experience higher blood fatty acid concentration and are more susceptible to fatty liver than lighter cows (McNamara, 2000; Stockdale, 2001). The present results of BW, BCS, and blood concentration of fatty acids agree with this statement. In addition, the content of TG and staining with Oil Red O and H&E revealed a clear state of hepatic steatosis in cows with severe fatty liver. Considering the high blood fatty acid concentration in cows with fatty liver, and the causative effect of fatty acid-induced ER stress on hepatic lipid accumulation in calf hepatocytes, we hypothesized that ER stress may participate in the occurrence and development of fatty liver in dairy cows. In the present study, we observed the activation of the ER stress

transducers (PERK, IRE1 α , and ATF6) and upregulation of UPR downstream genes (*GRP78*, *ATF4*, and *sXBP1*) in the liver of severe fatty liver cows. These results strongly indicate ER stress was induced in the liver of dairy cows with severe fatty liver. As ER stress was convincingly demonstrated to reinforce the expression of lipogenesis genes and thus contribute to hepatic lipid accumulation in calf hepatocytes, the occurrence of ER stress and increased expression lipogenic genes in the liver of severe fatty liver cows may presage the participation of ER stress in fatty liver progression. However, the exact role and underlying mechanisms of ER stress in fatty liver development still need further research.

It is well established that ER stress induces cell death and apoptosis (Szegezdi et al., 2006). An overload of fatty acids and accumulation of intrahepatic

Figure 5. Hepatic endoplasmic reticulum (ER) stress and lipogenesis state in dairy cows with severe fatty liver. (A) Representative Western blots (left) of hepatic phospho-PERK (Thr980), protein kinase RNA-like ER kinase (PERK), phospho-IRE1 α (S724), inositol requiring protein-1 α (IRE1 α), cleaved activating transcription factor-6 (ATF6), and sterol regulatory element-binding protein 1c (SREBP-1c), and quantification (right) of phospho-PERK/PERK, phospho-IRE1 α /IRE1 α , cleaved-ATF6, and SREBP-1c in healthy (n = 15) and severe fatty liver (n = 15) cows. (B) Relative mRNA expression levels of *GRP78*, *ATF4*, *sXBP1*, *SREBP1*, *ACACA*, *FASN*, and *DGAT1* in healthy (n = 15) and severe fatty liver (n = 15) cows. For all bar plots shown, data are expressed as the mean $\pm$ SEM. * P < 0.05, ** P < 0.01 by independent-samples t -test. Results are representative of at least 3 independent measurements.

lipid induces ER stress, oxidative stress, and inflammation (Grauagnard et al., 2013; Li et al., 2015). These mechanisms interact and collectively promote the development of fatty liver, ultimately resulting in liver injury (Li et al., 2015). Data from mice and humans also revealed that ER stress is involved in the induction of liver injury (Ji, 2008). Based on these fundamental views, the greater concentrations of AST, ALT, GLDH, and GGT in blood of severe fatty liver cows may presage the potential role of ER stress on the induction of liver injury in dairy cows with severe fatty liver. However, the mechanistic relationship among ER stress, inflammation, apoptosis, and liver injury in dairy cows with fatty liver has not been elucidated in this study.

CONCLUSIONS

In vitro results show that a high concentration of fatty acids can induce ER stress, upregulate the expression of lipogenic genes, and promote lipid accumulation in calf hepatocytes. In addition, inhibition of ER stress in calf hepatocytes alleviates fatty acid-induced lipid accumulation by downregulating the expression of lipogenic genes. These results indicate that fatty acid-induced ER stress favors the expression of lipogenic genes, thereby exacerbating lipid accumulation in calf hepatocytes. The present in vivo study confirms the occurrence of ER stress in dairy cows with severe fatty liver. Considering the causative role of fatty acid-induced ER stress in hepatic lipid accumulation in calf hepatocytes, we deduced that the induction of ER stress may represent the molecular basis for fatty liver progression and account for the frequent occurrence of fatty liver in high-yielding dairy cows. These findings provide new insights regarding the role of ER stress in the fatty liver progression in dairy cows. However, the mechanistic relationship between ER stress and fatty liver in dairy cows remain to be determined.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Program (Beijing, China; grant no. 2016YFD0501206), the National Natural Science Foundation of China (Beijing, China; grant no. 31772810, 31672621, and 31572581), the Natural Science Foundation of Jilin Province (Changchun, China; grant no. 20170101148JC), the Natural Science Scientific Research Project Jointly Established by Jilin University and Jilin Province (Changchun, China; grant no. SXGJSF2017-6), and the Talents Cultivation Program of Jilin University. The authors declare no competing financial interests.

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