ABSTRACT

Ketotic dairy cows exhibit a state of negative energy balance (NEB) characterized by elevated circulating levels of β-hydroxybutyrate (BHB) and fatty acids. Impaired hepatic insulin signaling in dairy cows occurs frequently during the transition into lactation, but its role on liver function during this period is not well known. In nonruminants, endoplasmic reticulum (ER) stress is a causal factor contributing to impaired insulin signaling in the liver. Thus, the aim of this study was to investigate the status of hepatic insulin and ER stress signaling and whether ER stress contributes to impaired insulin signaling in dairy cows with ketosis. Healthy (control cows, n = 10, BHB ≤0.6 mM) and ketotic (ketotic cows, n = 10, BHB ≥1.2 mM) cows at 3 to 10 d in milk were selected for liver biopsy and blood sampling before feeding. In vitro experiments were conducted with isolated hepatocytes from 5 healthy calves (1 d old, fasted female, 30–40 kg of body weight). Treatments included BHB (0, 0.9, 1.8, 3.6 mM), tauroursodeoxycholic acid (TUDCA, a canonical inhibitor of ER stress), and different incubation times (0.5, 1, 2, 3, 5, 7, 9, or 12 h). Ketotic cows had lower daily milk yield (median: 29.50 vs. 23.00 kg), higher plasma nonesterified fatty acid (NEFA) (median: 0.33 vs. 1.17 mM), BHB (median: 0.43 vs. 3.22 mM), aspartate aminotransferase (median: 70.58 vs. 155.70 U/L), alanine aminotransferase (median: 18.31 vs. 37.90 U/L), lower plasma glucose (median: 4.32 vs. 2.37 mg/dL), and revised quantitative insulin sensitivity check index (median: 0.39 vs. 0.37) compared with healthy cows. Increased abundance of phosphorylated insulin receptor substrate-1 (IRS1) and decreased abundance of phosphorylated protein kinase B (AKT) and glycogen synthase kinase-3β (GSK3β) in ketotic cows indicated a state of insulin resistance. In addition, abundance of phosphorylated protein kinase RNA-like ER kinase (PERK) and inositol requiring protein-1α (IRE1α), and cleavage of activating transcription factor-6 (ATF6) were greater in the liver of ketotic cows. In vitro, at the early stages of incubation, treatment with BHB upregulated abundance of phosphorylated of IRE1α, PERK, and the cleavage of ATF6, as well as several unfolded protein response genes [X-box-binding protein-1 (XBP1), 78 kDa glucose-regulated protein (GRP78), and C/EBP homologous protein (CHOP)]. Furthermore, in response to increasing doses of BHB, the phosphorylation level of PERK, IRE1α, and the cleavage of ATF6, and the abundance of XBP1, GRP78, and CHOP increased. In addition, BHB treatment increased phosphorylation of IRS1 and decreased phosphorylation of AKT and GSK3β, and upregulated abundance of gluconeogenic genes (phosphoenolpyruvate carboxykinase and glucose-6-phosphatase). Importantly, these changes were reversed by inhibiting ER stress with TUDCA treatment. Overall, the present study indicated that reversing ER stress during ketosis might help alleviate hepatic insulin resistance. Targeting ER stress may represent a potential therapeutic target for controlling the negative aspects of ketosis on liver function.

Key words: dairy cow, ketosis, insulin resistance, endoplasmic reticulum stress, tauroursodeoxycholic acid

INTRODUCTION

Ketosis is a common metabolic condition in high-yielding dairy cows during the transition period during which they undergo a period of negative energy balance (NEB). Unresolved NEB initiates fat mobilization and a subsequent increase in the blood concentration of nonesterified fatty acid (NEFA; Gross et al., 2013).
Excessive NEFA absorbed by the liver can be incompletely metabolized into ketone bodies, such as BHB, acetoacetate, and acetone. Among these, BHB is considered the main ketone body and excessive accumulation contributes to oxidative stress, inflammation, and dysregulated lipid metabolism (Shi et al., 2014; Song et al., 2014; Du et al., 2018).

Insulin resistance is defined as decreased sensitivity of target tissues (skeletal muscle, liver, and adipose tissue) to the action of insulin (INS; Steppan and Lazar, 2002), for example, the inability to suppress hepatic gluconeogenesis (Brown and Goldstein, 2008). In human and mouse, there is a general agreement that INS resistance is the etiologic key component in the pathogenesis of metabolic diseases including diabetes, fatty liver, and obesity (Flamment et al., 2012; Johnson and Olefsky, 2013; Perry et al., 2014). At the molecular level, INS resistance is associated with impairment of signaling cascades involving the INS receptor, insulin receptor substrate (IRS), phosphoinositol 3-kinase, protein kinase B (AKT), and glycogen synthase kinase-3β (GSK3β; Cignarelli et al., 2019). The lower revised quantitative insulin sensitivity check index (RQUICKI; Xu et al., 2014; Youssef et al., 2017) has been used in dairy cows to assess systemic INS resistance, with data demonstrating that it is a frequent occurrence during the transition period and also in ketotic cows experiencing fatty liver (Gao et al., 2018). The state of hepatic INS signaling and underlying mechanisms in dairy cows with ketosis are not known. Given the lipotoxic property of BHB and its pathological role in dairy cow liver dysfunction, we speculated that BHB might be a causal factor in hepatic INS resistance in ketotic cows.

At least in nonruminants, the endoplasmic reticulum (ER) orchestrates hepatic metabolic homeostasis. Disruption of ER homeostasis, often termed ER stress, elicits the unfolded protein response (UPR), which involves 3 canonical branches: protein kinase RNA-like ER kinase (PERK), inositol requiring protein-1α (IRE1α), and activating transcription factor-6 (ATF6). Upon ER stress, upregulated phosphorylation of PERK and IRE1α and cleavage of ATF6 trigger UPR pathways to cope with stressful conditions and resolve the protein-folding defects (Schröder and Kaufman, 2005; Ron and Walter, 2007). The UPR activation leads to upregulation of downstream UPR pathway components such as X-box-binding protein-1 (XBP1), 78 kDa glucose-regulated protein (GRP78), and C/EBP homologous protein (CHOP), which have been proposed as markers of ER stress (Baiceanu et al., 2016).

Studies in human, mouse, and cell lines demonstrated that excessive ER stress contributes to the development of hepatic INS resistance and progression of other liver dysfunction (Tabas and Ron, 2011; Cnop et al., 2012a; Lei et al., 2016). Early studies have shown that ER stress-related molecules are upregulated in the liver and mammary tissue in periparturient dairy cows (Invernizzi et al., 2012; Khan et al., 2015). Recently, Zhu et al. reported that ER stress is activated and contributes to liver lipid accumulation in dairy cows (Zhu et al., 2019), indicating that ER stress is associated with the progression of hepatic metabolic disorders in dairy cows. Whether ER stress plays a role in hepatic INS resistance in dairy cows with ketosis is still unknown. Thus, the objective of this study was to explore the status of INS signaling and ER stress in the liver of dairy cows with ketosis, and determine effects of BHB on INS signaling and ER stress in calf hepatocytes.

MATERIALS AND METHODS

Animals

All procedures were carried out under license approved by the Ethics Committee on the Use and Care of Animals at Jilin University (Changchun, China). Cows used in this study received humane care based on the guidelines presented in Guide for the Care and Use of Agricultural Animals in Research and Teaching (PASS, 2010). We preselected lactating Holstein cows with similar parity (median = 3, range = 2–4) and DIM (median = 6 d, range = 3–10 d) from a 10,000-cow dairy farm located in Changchun City, Jilin Province, China. All preselected cows had ad libitum access to the same diet (Sun et al., 2019). Veterinarians first classified cows as suspected ketotic if a nitroprusside powder (GL3097, Beijing Baiaolaibo Co. Ltd.) test for ketone bodies in milk was positive. To exclude the cows that had other comorbidities with ketosis, the cows then underwent a thorough clinical examination including the changes of body temperature, pulse rate, respiratory rate, feed intake, and milk yield according to previously described guidance (Divers and Peek, 2007; Rockett and Bosted, 2015). Hypocalcemia was diagnosed by detecting blood calcium concentration (<2.0 mM). Mastitis was detected by observing abnormal milk appearance and was confirmed using the California Mastitis Test according to previously described guidance (Hogan et al., 1999). Cows suspected of displaced abomasum were confirmed by auscultation and percussion of the left abdomen and rectal examination. Metritis was diagnosed as a cow having watery, purulent, or brown, and fetid vaginal discharge. The BCS was assigned based on a 1- to 5-point scale with 0.25 intervals (Edmonson et al., 1989). Accordingly, we selected 40 cows suspected of being ketotic and 40 healthy cows. Next, serum concentrations of BHB in the selected cows were measured.
and 10 ketotic cows with serum BHB concentration higher than 1.2 mM (ketotic cows) and 10 healthy cows with serum BHB concentration less than 0.6 mM (control cows) were selected for subsequent work (Itle et al., 2015; Du et al., 2018). Serum BHB concentrations and the basic description of those cows are included in Table 1.

Cows were milked twice daily, and milk yield was recorded on 3 consecutive days at 0800 and 1530 h. Blood samples were collected between 0630 and 0730 h before feeding by jugular venipuncture and centrifuged at 1,500 × g for 15 min at 4°C. Serum was obtained before feeding by jugular venipuncture and centrifuged at 1,500 × g for 15 min at 4°C. Serum was obtained and stored at −80°C until analysis. For liver biopsy, the procedure was operated by an experienced veterinarian. The liver caudate lobe was removed through surgical excision. The isolated lobe was intubated and liver samples were collected via percutaneous biopsy as described previously (Zhu et al., 2019).

Briefly, the intercostal space was shaved between the 10th and 11th ribs, sanitized with iodine scrub and 75% alcohol, and anesthetized with 2% lidocaine HCl. The intercostal space was shaved between the 10th and 11th ribs, sanitized with iodine scrub and 75% alcohol, and anesthetized with 2% lidocaine HCl. An incision (3 mm) was made in the skin using a sterile scalpel blade. A sterile puncture needle (Shanghai Surgical Equipment Factory) was used for liver puncture, and the liver tissue biopsies (approximately 200 mg and 3 cm, respectively) were snap-frozen in liquid nitrogen.

### Determination of Blood Parameters

Serum concentrations of NEFA, BHB, and glucose (GLU) were measured with commercially available kits (NEFA: cat. no. FA115; BHB: cat. no. RB1008; GLU: cat. no. GL3815; Randox Laboratories) using a Hitachi 7170 autoanalyzer (Hitachi). The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured with commercially available kits (AST: AS3804; ALT: AL3801; Randox Laboratories) using an automatic biochemistry analyzer (Sekisui Medical Co. Ltd.). Serum concentrations of INS were determined using bovine-specific ELISA kits (cat. no. EB13RB; Invitrogen) according to the manufacturer’s guidelines (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/EB13RB.pdf). Systemic INS sensitivity was estimated using the RQUICKI (Holtenius and Holtenius, 2007), where RQUICKI = 1/[log (GLU, mg/dL) + log (INS, μU/mL) + log (NEFA, mM)].

### Primary Calf Hepatocyte Isolation and Culture

Primary calf hepatocytes were isolated using the collagenase IV perfusion method described previously (Du et al., 2018). Briefly, 5 healthy newborn fasted female Holstein calves (BW: 30–40 kg; 1 d old) were anesthetized. The liver caudate lobe was removed through surgical excision. The isolated lobe was intubated and perfused through exposed blood vessels with the prewarmed perfusion solution A (140 mM NaCl, 6.7 mM KCl, 10 mM HEPES, 2.5 mM GLU, 0.5 mM EDTA; pH 7.2–7.4) and solution B (140 mM NaCl, 6.7 mM KCl, 30 mM HEPES, 2.5 mM GLU, 5 mM CaCl₂; pH 7.2–7.4) until the returned liquid became clear. Subsequently, the liver was digested with digestion solution [solution B containing 0.02% collagenase type IV (cat. no. 17104019; Gibco); pH 7.2–7.4] for 15 to 20 min. The digested liver was then aseptically removed to a sterile cell culture dish containing 100 mL of cold RPMI-1640 basic medium (Hyclone Laboratories) with 10% fetal bovine serum (FBS; Hyclone Laboratories). Next, the
liver capsule was peeled off, and the liver was minced into small pieces. The dissociated hepatocytes were filtered through 100 (150 μm) and 200 mesh (75 μm) by aspirating with a large-bore pipette. To exclude non-parenchymal cells, the cell suspension was washed twice with RPMI-1640 basic medium by centrifugation at 50 × g for 5 min at 4°C. Cell viability was determined with Trypan Blue (Sigma-Aldrich). Only hepatocytes with viability >90% were used. Hepatocytes were resuspended in adherent medium (RPMI-1640 basic medium; 10% FBS; 10−6 mol/L INS; 10−6 mol/L dexamethasone; 10 μg/mL vitamin C; 1% penicillin/streptomycin) and plated in 6-well culture plates at a density of 2 × 106 cells per well, then cultured at 37°C in 5% CO2. After 4 h, the medium was replaced with growth medium (RPMI-1640 basic medium; 10% FBS; 1% penicillin/streptomycin). The growth medium was replaced with fresh medium every 24 h. The characterization of cultured hepatocytes was reported in Supplemental Figure S1 (https://figshare.com/articles/figure/Supplemental_Material/15138945).

**Cell Treatment**

Stock BHB solution was prepared according to a protocol described previously with modifications. In short, 1.81 g of BHB powder (cat. no. 54965; Sigma-Aldrich) was dissolved in 50 mL of distilled water, sterilized by filtration with a 0.22-μm syringe filter to obtain a concentration of 0.3 mM, and stored at −20°C. Different concentrations of BHB used in this study were chosen according to typical BHB concentrations in cow serum (Iwersen et al., 2013). Hepatocytes were subjected to the following treatments: hepatocytes were serum-free starved in RPMI-1640 basic medium for 6 h before treatment. For time-course experiments, hepatocytes were treated with BHB (1.8 mM) for 0.5, 1, 2, 3, 5, 7, 9, and 12 h, respectively. For the dose-response experiments, hepatocytes were treated with various doses of the BHB solution (0, 0.9, 1.8, or 3.6 mM) for 5 h. Hepatocytes were also treated with a single dose of BHB (1.8 mM) with the pretreatment of tauroursodeoxycholic acid (TUDCA; cat. no 700247P; Sigma-Aldrich; 100 μg/mL, for 12 h), a commonly used inhibitor of ER stress. To measure INS signaling in treated hepatocytes after treatments were imposed, hepatocytes were subsequently stimulated with 100 nM INS (Sigma-Aldrich) for 10 min.

**Quantitative Reverse-Transcription PCR Assay**

Quantitative reverse-transcription PCR was performed as described previously (Lei et al., 2016). Total RNA was extracted from harvested hepatocytes with TRIzol reagent (cat. no. 9108; TaKaRa Biotechnology Co. Ltd.) according to the manufacturer’s instructions (https://www.takarabio.com.cn/DownLoad/9108Q.pdf). The RNA concentration and quality were quantified using K5500 Micro-Spectrophotometer (Beijing Kaiao Technology Development Co. Ltd.) and electrophoresis with 1% agarose gels, then total RNA of 1 μg was reverse-transcribed to cDNA using PrimeScript Reverse Transcriptase Kit (cat. no. 6110B; TaKaRa Biotechnology Co. Ltd.). The mRNA abundance was measured using the SYBR green plus reagent kit (cat. no. DRR041A; TaKaRa Biotechnology Co. Ltd.) with a 7500 Real-Time PCR System (Applied Biosystems). Each cell treatment condition was performed in triplicate (n = 3). The real-time RT-PCR was conducted as follows: initial denaturation (94°C, 2 min), amplification (94°C, 10 s; 60°C, 15 s; 72°C, 30 s; 35 cycles), and extension (72°C, 5 min). Relative abundance of target genes was normalized to that of ACTB and determined by the 2−ΔΔCT method. All primers listed in Table 2 were designed using Primer Express software (Applied Biosystems) and synthesized from Sangon Biotech Co. Ltd.

**Protein Extraction and Western Blotting**

Western blotting was performed as described previously (Gao et al., 2018). Liver tissue and hepatocyte total proteins were extracted using a protein extraction kit (cat. no. C600589; Sangon Biotech Co. Ltd.) under the guidance of the instructions (https://figshare.com/articles/online_resource/C51003_EN_P/14812812). The concentration of protein was quantified using the BCA protein assay kit (cat. no. P1511; Applygen Technologies) according to the supplier’s instructions (https://figshare.com/articles/online_resource/BCA_Protein_Assay_Kit-P1511_pdf/14812800). Twenty micrograms of extracted protein was separated in 10% (p-IRS1, IRS1, p-AKT, AKT, p-GSK3β, GSK3β, p-IRE1α, IRE1α, p-PERK, PERK, and β-actin) or 12% (cleaved-ATF6) SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (cat. no. IPVH00010; Millipore) with a pore size of 0.45 μm. Membranes were blocked with 3% BSA at room temperature for 4 h. Then, membranes were hybridized with primary antibodies against phospho (p)-IRS1 (1:500; cat. no. 2381; Cell Signaling Technology), IRS1 (1:500; cat. no. 2382; Cell Signaling Technology), p-AKT (1:500; cat. no. 4060; Cell Signaling Technology), AKT (1:500; cat. no. 2920; Cell Signaling Technology), p-GSK3β (1:500; cat. no. 5558; Cell Signaling Technology), GSK3β (1:500; cat. no. 9108Q; TaKaRa Biotechnology Co. Ltd.), p-IRE1α (1:500; cat. no. Ab38; Abcam), IRE1α (1:500; cat. no. Ab77544; Abcam), p-PERK (1:500; cat. no. 4437; Cell}

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no. 3179; Cell Signaling Technology), PERK (1:500; cat. no. 3192; Cell Signaling Technology), ATF6 (1:1,000; cat. no. Ab11909; Abcam), and β-actin (1:1,000; cat. no. sc-47778; Santa Cruz) at room temperature for 45 min, respectively. Subsequently, membranes were washed 3 times with Tris-buffered saline solution with 0.01% Tween-20 for 5 min and then incubated with appropriate peroxidase-conjugated secondary antibodies (Protein technology) at room temperature for 1 h. Immunoreactive band signals were detected with an enhanced chemiluminescence solution (ECL; Millipore) and visualized using a protein imager (ProteinSimple). The gray intensity of each protein was quantified by Image-Pro Plus 6.0 (Media Cybernetics) normalized to β-actin, and the normalized value was used for calculating the ratios of p-IRS1/IRS1, p-AKT/AKT, p-GSK3β/GSK3β, p-IRE1α/IRE1α, and p-PERK/PERK to evaluate their phosphorylation level.

Immunofluorescence Staining and Image Analysis

Hepatocytes were grown and treated on glass coverslips. Cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich). The blocking solution contained 3% BSA in PBS was used for 15 min. Primary antibody against albumin (cat. no. A0353; Abclonal) was diluted (1:100) in blocking buffer and incubated overnight at 4°C. Immunoreactivity was quantified using Alexa Fluor 488-labeled secondary antibody (1:200, cat. no. A0423, Beyotime). To label ER, an ER-specific probe, ER-Tracker red dye (cat. no. C1041; Beyotime) was used according to the supplier’s instructions (https://figshare.com/articles/online_resource/C1041_ER-Tracker_Red_Endoplasmic_Reticulum_Red_Fluorescent_Probe_/14812803). Counter-staining was done with Hoechst 33258 (Beyotime) for 20 min. The coverslips were then sealed onto glass slides with glycerol, and the cells were imaged using laser confocal microscopy (Fluovie FV1200; Olympus). Fluorescence intensity and area of the ER (red) and nucleus (blue) were quantified in binary format with Image-Pro Plus software (Media Cybernetics), values were corrected for background, volume was determined by multiplying the area by intensity, and final ER volumes were divided by nucleus volumes for each cell. The computing method was performed as reported elsewhere (Hart et al., 2012).

Transmission Electron Microscopy

Transmission electron microscopy analysis was performed as described previously (Lei et al., 2016). Hepatocytes for electron microscopy grown on plates were washed with sterile PBS in situ and scraped off gently with a cell scraper, and then centrifuged at 150 × g for 10 min at 4°C to collect cell pellets. Cell pellets were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde at 4°C for 2 h, post-fixed in 1% osmium tetroxide for 1 h. Cell pellets were dehydrated in an ethanol series (70, 80, 90, and 100%) and infiltrated with Spurr’s resin. Ultrathin sections (50 nm) were cut and stained with 4% uranyl acetate and 0.2% lead citrate. Sections with the cells were visualized with an H-7650 electron microscope at 80 kV (Hitachi) at 6,800 × magnification. At least 5 random views of cells in each sample were obtained.

Statistical Analysis

All experiments were repeated at least 3 times, and data were analyzed using GraphPad Prism 5 (GraphPad InStat Software, https://www.graphpad.com/scientific-software/instat/) or SPSS 20.0 software package (IBM Inc.). All data were tested for normality and homogeneity of variance. For data with Gaussian distribution, unpaired 2-sided Student t-test and one-way ANOVA with a Bonferroni post-test were used to compare differences between 2 groups and more than 2

Table 2. Primers used for quantitative real-time PCR (F, forward; R, reverse)

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Sequence number</th>
<th>Primer sequence (5’-3’)</th>
<th>PCR product size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XBP1</td>
<td>NM_001271737.1</td>
<td>F: GCCGGGTCTGCTGAGTC R: TTTCTGGGTCACTTCTGGGA</td>
<td>207</td>
<td>60</td>
</tr>
<tr>
<td>GRP78</td>
<td>NM_001075148.1</td>
<td>F: GCCCTGTTTCCCAACATCA R: CAGGTCACTATTAGTCTTCAA</td>
<td>111</td>
<td>60</td>
</tr>
<tr>
<td>CHOP</td>
<td>NM_001078163.1</td>
<td>F: GAACCTGGAGGAGGCTCTTCCA R: AGTGACCTAGGCTGCATCTCTGT</td>
<td>101</td>
<td>60</td>
</tr>
<tr>
<td>PEPCK</td>
<td>NM_174737.2</td>
<td>F: AAGTACCTTGAGGAGCAAGTTGA R: GGTGCGTTGATGGATTTGGA</td>
<td>133</td>
<td>60</td>
</tr>
<tr>
<td>G6PC1</td>
<td>NM_001076124.2</td>
<td>F: AGCAAGTGTTTCCCGTTTC R: ACCCAGGGGAGGCGTA</td>
<td>179</td>
<td>60</td>
</tr>
<tr>
<td>ACTB</td>
<td>NM_173979.3</td>
<td>F: GCCCTGAGGCTCTTCTCCA R: GCGGATGCTGACGTCACA</td>
<td>101</td>
<td>60</td>
</tr>
</tbody>
</table>
groups, respectively. For data with skewed distribution, nonparametric statistical analysis was performed using the Mann Whitney U test and Kruskal-Wallis test with Dunn's post-hoc test for 2 groups and more than 2 groups, respectively. Linear and quadratic contrasts were conducted to evaluated time- and dose-dependent effects. Data are expressed as means ± standard error (unless otherwise specified). A significant difference was defined as $P < 0.05$, and $P < 0.01$ was considered highly significant.

RESULTS

Baseline Characteristics and Analyzed Blood Parameters

As shown in Table 1, BW in healthy and ketotic cows did not differ ($P = 0.15$). The DMI ($P = 0.01$), milk yield ($P = 0.03$), blood concentration of GLU ($P < 0.01$), and INS ($P = 0.08$) of ketotic cows was lower. Additionally, the BCS ($P = 0.04$), blood concentrations of NEFA and BHB, and serum activities of AST and ALT were greater ($P < 0.01$) in cows with ketosis. Indeces of INS sensitivity revealed a decrease in RQUICKI ($P = 0.03$) in ketotic compared with control cows.

Ketotic Cows Display Hepatic Insulin Resistance and ER Stress

As shown in Figure 1A, compared with healthy cows, the phosphorylation level of IRS (S307) was greater ($P < 0.01$) and was coupled with lower ($P < 0.01$) phosphorylation of AKT (S473) and GSK 3β (Ser9) in the liver of ketotic cows. The key ER stress activation indicators, including the phosphorylation of IRE1α and PERK, as well as cleavage of ATF6, were greater ($P < 0.05$) in the liver of ketotic cows (Figure 1B).

Effects of BHB Treatment Duration on ER Stress Signaling in Calf Hepatocytes

Calf hepatocytes were treated with BHB (1.8 mM) for 0.5, 1, 2, 3, 5, 7, 9, and 12 h, respectively. Compared with the 0.5-h group, BHB treatment caused a quadratic ($P < 0.01$) time-dependent increasing effect on phosphorylation of IRE1α, peaking at 5 h (Figure 2A; Supplemental Table S1, https://figshare.com/articles/figure/Supplemental_Material/15138945). Upon BHB treatment, phosphorylation of PERK and the cleavage of ATF6 increased with treatment and time, being strongest at 3 h ($P < 0.01$) and 5 h ($P < 0.01$), respectively, and starting to fade away at later time points, resulting in both linear ($P < 0.01$) and quadratic ($P < 0.01$) effects (Figure 2A; Supplemental Table S1). In addition, the mRNA abundance of ER stress markers, including XBP1, GRP78, and CHOP, had both linear and quadratic trends with BHB treatment (Supplemental Table S1). The mRNA abundance of XBP1 (Figure 2B) after BHB treatment was similar to the phosphorylation levels of IRE1α, peaking at 5 h ($P < 0.05$). The mRNA abundance of GRP78 (Figure 2C) increased gradually and was significantly higher ($P < 0.01$) from 3 to 9 h of BHB treatment. The BHB treatment caused a rapid upregulation of CHOP (Figure 2D) abundance at 1 h ($P < 0.05$) after BHB treatment.

Morphological Characteristics of ER with BHB Treatment in Calf Hepatocytes

Calf hepatocytes were treated with or without BHB (1.8 mM) treatment for 5 h. Compared with the control group, morphologically, the ER with BHB treatment was enlarged, as indicated by the greater ($P < 0.01$) ratio of ER/nucleus in ER-Tracker red immunostaining (Figure 3A). Ultrastructural analysis by transmission electron microscopy showed that BHB exposure led to ER dilation and degranulation (Figure 3B).

TUDCA Attenuated BHB-Induced ER Stress in Calf Hepatocytes

Calf hepatocytes were treated with various doses of BHB (0, 0.9, 1.8, or 3.6 mM) for 5 h, or TUDCA (100 μg/mL, for 12 h), or pretreated with TUDCA (100 μg/mL, for 12 h) and followed by BHB (1.8 mM, for 5 h). Following treatment with BHB, compared with the control group (0 mM), the phosphorylation levels of IRE1α and PERK and the cleaved-ATF6 were significantly greater ($P < 0.01$) and displayed both linear ($P < 0.01$) and quadratic ($P < 0.01$) trends (Figure 4A; Supplemental Table S2, https://figshare.com/articles/figure/Supplemental_Material/15138945). Compared with the control group, we detected a linear ($P < 0.01$) dose-dependent effect on the mRNA abundance of XBP1, GRP78, and CHOP (Figure 4B-D; Supplemental Table S2). Importantly, compared with 1.8 mM BHB group, pretreatment with TUDCA significantly ($P < 0.01$) decreased the phosphorylation levels of IRE1α and PERK, and cleaved-ATF6 (Figure 4A), as well as the mRNA abundance of XBP1, GRP78, and CHOP (Figure 4B-D).

BHB-Induced Insulin Resistance, and Blocking ER Stress by TUDCA Reversed BHB-induced Insulin Resistance in Calf Hepatocytes

Calf hepatocytes were treated with various doses of BHB (0, 0.9, 1.8, or 3.6 mM) for 5 h, or TUDCA (100 μg/mL, for 12 h), or pretreated with TUDCA (100 μg/mL, for 12 h) and followed by BHB (1.8 mM, for 5 h). Following treatment with BHB, compared with the control group (0 mM), the phosphorylation levels of IRE1α and PERK and the cleaved-ATF6 were significantly greater ($P < 0.01$) and displayed both linear ($P < 0.01$) and quadratic ($P < 0.01$) trends (Figure 4A; Supplemental Table S2, https://figshare.com/articles/figure/Supplemental_Material/15138945). Compared with the control group, we detected a linear ($P < 0.01$) dose-dependent effect on the mRNA abundance of XBP1, GRP78, and CHOP (Figure 4B-D; Supplemental Table S2). Importantly, compared with 1.8 mM BHB group, pretreatment with TUDCA significantly ($P < 0.01$) decreased the phosphorylation levels of IRE1α and PERK, and cleaved-ATF6 (Figure 4A), as well as the mRNA abundance of XBP1, GRP78, and CHOP (Figure 4B-D).
μg/mL, for 12 h), or pretreated with TUDCA (100 μg/mL, for 12 h) and followed by BHB (1.8 mM, for 5 h). After treatments, calf hepatocytes were subsequently stimulated with 100 nM INS (Sigma-Aldrich) for 10 min to measure INS signaling. Compared with the control group, we observed a linear (P < 0.01) dose-dependent effect with BHB treatment on all INS signaling detected molecules (Supplemental Table S3, https://figshare.com/articles/figure/Supplemental_Material/15138945). As shown in Figure 5A, compared with the control group, the phosphorylation level of IRS (S307) was significantly increased (P < 0.01), and that of GSK3β (Ser9) was significantly decreased (P < 0.01) in both 1.8 and 3.6 mM BHB treatment groups. Furthermore, the phosphorylation level of AKT (S473) was significantly suppressed (P < 0.01) in all BHB (0.9,

**Figure 1.** Ketotic cows exhibit hepatic insulin resistance (IR) and endoplasmic reticulum (ER) stress. (A) Representative western blots (left) of phosphorylated insulin receptor substrate-1 (p-IRS1, S307), insulin receptor substrate-1 (IRS1), phosphorylated protein kinase B (p-AKT, S473), protein kinase B (AKT), phosphorylated glycogen synthase kinase-3β (p-GSK3β, Ser9), and β-actin. Box plots showing the ratios of p-IRS1/IRS1, p-AKT/AKT, and p-GSK3β/GSK3β were quantified (right) in healthy (n = 10) and ketotic cows (n = 10). (B) Representative western blots (left) of phosphorylated inositol requiring protein-1α (p-IRE1α), inositol requiring protein-1α (IRE1α), phosphorylated protein kinase RNA-like ER kinase (p-PERK), protein kinase RNA-like ER kinase (PERK), cleaved activating transcription factor-6 (cleaved-ATF6), and β-actin. Box plots showing the ratios of p-IRE1α/IRE1α, p-PERK/PERK, and cleaved-ATF6 (right) in healthy (control; n = 10) and ketotic cows (n = 10). Data are expressed as box-and-whisker plots with median (middle line), 25th–75th percentiles (box), and minimum–maximum values (whiskers). **P < 0.01 compared with the control group; statistical differences were assessed by Mann Whitney U test.
1.8, or 3.6 mM) treatment groups. As expected, we also observed that mRNA abundance of gluconeogenic genes, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6PC1) increased ($P < 0.05$ or $P < 0.01$) in a BHB dose-dependent manner (Figure 5B, C).

As shown in Figure 5A, pretreatment with TUDCA significantly ($P < 0.01$) decreased the phosphorylation level of IRS (S307) and increased the phosphorylation levels of AKT (S473) and GSK3β (Ser9) induced by 1.8 mM BHB treatment. Furthermore, TUDCA markedly alleviated ($P < 0.01$) the increase in mRNA abundance of PEPCK and G6PC1 induced by BHB (Figure 5B, C).

**DISCUSSION**

Dairy cows are exposed to drastic physiological changes and metabolic stress in the transition period. Ketotic cows often display high serum concentrations of BHB (Deng et al., 2015; Song et al., 2021), which can induce lipotoxicity and oxidative stress in the liver, consequently, to some extent, resulting in liver damage (Deng et al., 2015; Du et al., 2018; Gao et al., 2018). We confirmed that ketotic cows with high serum concentrations of BHB also had greater serum concentrations of AST and ALT, indicators of liver damage, suggesting the existence of liver injury. The need for GLU by the mammary gland after calving triggers adaptations to
ensure a constant supply, including lower INS concentrations (Drackley et al., 2001) and also increased fat mobilization through lipolysis that enhances serum levels of NEFA (Sordillo and Raphael, 2013). Excessive NEFA are incompletely oxidized into ketone bodies in the liver (Bobe et al., 2004). When these physiological adaptations occur, the dairy cows display the characteristics of hypoglycemia and hypoinsulinemia, which are consistent with our observations in ketotic cows. Indeed, another etiological form of ketosis, characterized by hyperglycemia and hyperinsulinemia, can occur earlier in lactation as a consequence of overfeeding the animals in the dry period (Holtenius and Holtenius, 1996; Mair et al., 2016). The lower milk production of ketotic cows also agreed with the expected responses. Together, these results imply that ketotic cows are not able to adapt to metabolic alterations that are “beneficial” for milk production, but tend to decrease milk production and develop important metabolic derangements.

Insulin resistance has been defined as the decreased sensitivity of target tissues (skeletal muscle, liver, and
adipose tissue) to the action of INS (Steppan and Lazar, 2002), which is associated with impairment of signaling cascade involving the IRS1, AKT, and GSK3β (Cignarelli et al., 2019). In nonruminants, it is well known that INS resistance is a major metabolic abnormality in a great majority of patients with T2DM, fatty liver, or obesity (Bugianesi et al., 2005; Wilcox, 2005). As indicated by a lower RQUICKI value, previous work indicated that systemic INS resistance develops frequently in dairy cows during the transition period and ketosis, and is associated with liver dysfunction and oxidative stress (Xu et al., 2014; Abuelo et al., 2016; Youssef et al., 2017). This was confirmed in the present study through the use of RQUICKI, demonstrating lower values in ketotic cows.

In addition to the obvious benefits of INS resistance in terms of milk synthesis after calving, this state also may contribute to the etiology of abomasal displacement (Pravettoni et al., 2004; Doll et al., 2009) and enhanced fat lipolysis, which could lead to hepatic triacylglycerol accumulation, consequently compromising the health, fertility, and longevity of the cow (Davis et al., 2017). In spite of the liver playing a crucial role in responding to the challenges of metabolic stress during the transition period, few studies have addressed hepatic INS resistance in dairy cows (De Koster and Opsomer, 2013; Gao et al., 2018; Angeli et al., 2021). Based on the decrease in phosphorylation levels of IRS2, AKT, GSK3β in the liver, Gao et al. concluded that dairy cows with fatty liver exhibited hepatic INS

Figure 4. Effects of BHB, tauroursodeoxycholic acid (TUDCA), or both on endoplasmic reticulum (ER) stress signaling in calf hepatocytes. Calf hepatocytes were treated with various doses of BHB (0, 0.9, 1.8, or 3.6 mM) for 5 h, or TUDCA (100 μg/mL, for 12 h), or pretreated with TUDCA (100 μg/mL, for 12 h) and followed by BHB (1.8 mM, for 5 h). (A) Representative western blots (left) of phosphorylated inositol requiring protein-1α (p-IRE1α), inositol requiring protein-1α (IRE1α), phosphorylated protein kinase RNA-like ER kinase (p-PERK), protein kinase RNA-like ER kinase (PERK), cleaved activating transcription factor-6 (cleaved-ATF6), and β-actin. The ratios of p-IRE1α/IRE1α, p-PERK/PERK, and cleaved-ATF6 were quantified (right); mRNA expression of (B) XBP1, (C) GRP78, and (D) CHOP in calf hepatocytes. All data are expressed as mean ± SEM based on 3 independent experiments. * P < 0.05, ** P < 0.01 compared with control (0 mM) group; ##P < 0.01 compared with the BHB (1.8 mM) group; statistical differences were assessed by one-way ANOVA with subsequent Bonferroni correction.
resistance (Gao et al., 2018). In a more recent study, the cows with high BCS exhibit hepatic INS resistance, as demonstrated by their lower phosphorylation level of AKT (Angeli et al., 2021). In the present study, the greater IRS1 serine phosphorylation and lower AKT and GSK3β phosphorylation levels in ketotic cows confirmed the impairment in hepatic INS signaling.

The etiology of INS resistance has been intensely studied in nonruminants (Petersen and Shulman, 2006; Glass and Olefsky, 2012), and it is now recognized that ER stress is an essential cause of INS resistance in liver and adipose tissue (Ozcan et al., 2004; Hotamisligil, 2010). In dairy cows, increasing evidence has demonstrated activation of ER stress in tissues of cows after parturition (Invernizzi et al., 2012; Gessner et al., 2014; Khan et al., 2015). Of note, a recent study reported that ER stress was activated and contributed to liver lipid accumulation in dairy cows with fatty liver (Zhu et al., 2019). Indeed, studies in human and mouse showed that ER stress itself could be induced by dif-
different stimuli such as reactive oxygen species (ROS), NEFA, proinflammatory cytokines, and microbial components (Cnop et al., 2012b), all of which are frequently increased in the systemic circulation of dairy cows in the transition period (Bradford et al., 2015). Thus, the greater IRE1α and PERK phosphorylation levels and ATF6 cleavage in ketotic cows confirmed that ER stress was activated. Taken together, our results confirm that the liver of ketotic cows displays INS resistance along with activation of ER stress. We speculated that ER stress might be a causal factor of INS resistance in the liver of dairy cows with ketosis. However, one limitation is that we cannot ensure that these signaling changes were contributed by hepatocytes, or other liver cell types, such as cholangiocytes and endothelial cells.

Previous data have provided evidence that NEFA could induce ER stress, and NEFA-induced ER stress promotes lipid accumulation in calf hepatocytes, which might contribute to the progression of fatty liver (Zhu et al., 2019). However, the factors that could induce ER stress in the liver during ketosis remain unknown. β-Hydroxybutyrate, the main ketone body, can be lipotoxic and contribute to inflammation, oxidative stress, apoptosis, and hormone dysregulation in hepatocytes (Shi et al., 2014; Song et al., 2016; Du et al., 2018). Furthermore, it induces programmed cell death in bovine smooth muscle cells (Tian et al., 2014). Thus, the greater levels of phosphorylation of IRE1α, PERK, and cleavage of ATF6, as well as upregulation of downstream ER stress-related genes (XBPI, GRP78, and CHOP) in hepatocytes treated with BHB suggested a direct link between BHB and ER stress. These results indicated that hepatocytes could contribute to ER stress in ketotic cows in response to the high level of BHB. Interestingly, apoptosis was shown to occur as a result of long-term (24 h) BHB treatment (Shi et al., 2014). However, in the present study, we found no significant pyknosis, chromatin condensation, or DNA fragmentation but did find activation of ER stress under short-term (5 h) BHB treatment. It might be suggested that apoptosis was triggered by prolonged ER stress (Szegedy et al., 2006).

It is noteworthy that CHOP, a downstream molecular of the PERK branch, increased rapidly with BHB treatment, which suggested that it could be used as a stress-related predictor during NEB and ketosis. Further investigation would help ascertain the usefulness of this protein as a biomarker. The TUDCA, a canonical ER stress inhibitor, alleviates ER stress by acting as chemical chaperones to prevent protein misfolding and aggregation in the cell. A considerable amount of research has provided evidence that TUDCA displays hepatoprotection targeting both hepatocytes and biliary epithelium (Alpini et al., 2002; Alpini et al., 2004; Li et al., 2018). Moreover, TUDCA has been approved by the Food and Drug Administration as a safe compound to treat primary biliary cirrhosis in humans, hence underscoring its potential for application in farm animals (Cao and Kaufman, 2012). The attenuated BHB-induced ER stress in calf hepatocytes treated with TUDCA support this idea.

### CONCLUSIONS

In vivo data indicated that hepatic INS signaling is impaired and ER stress pathways are overactivated in ketotic cows. Our in vitro data indicate that BHB, as a causal factor, induces INS resistance and ER stress in calf hepatocytes. In addition, blocking of ER stress by TUDCA in calf hepatocytes ameliorates BHB-induced INS resistance. Our study gives insights into potential strategies by targeting ER stress for improving hepatic INS resistance and other liver disorders in dairy cows with ketosis.

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