Mitochondrial dysfunction and endoplasmic reticulum stress in calf hepatocytes are associated with fatty acid-induced ORAI calcium release-activated calcium modulator 1 signaling

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ABSTRACT

The store-operated Ca^{2+} entry (SOCE) moiety ORAI calcium release-activated calcium modulator 1 (ORAI1) located in the endoplasmic reticulum (ER) participates in key cellular functions such as protein folding, transport, and secretion, and lipid metabolism. We used an in vitro approach to test whether exogenous fatty acids alter ORAI1 signaling and to explore potential consequences on mitochondrial dysfunction and ER stress. First, hepatocytes isolated from 4 healthy female calves (1 d old, 40–50 kg) were challenged with a 1.2 mM mixture of oleic, linoleic, palmitic, stearic, and palmitoleic acids for 0.5, 1, 3, 6, 9, and 12 h to measure oxidative stress [intracellular reduced glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA), and hydrogen peroxide] and ER stress (protein abundance of PERK, IRE, ATF6, and GRP78). Concentrations of GSH and SOD decreased at 0.5 h, and MDA and hydrogen peroxide increased at 1 h; ER stress proteins increased at 6 h. To determine whether ER stress was caused by oxidative stress, primary calf hepatocytes were treated with the same 1.2 mM fatty acid mix or the reactive oxygen species (ROS) inhibitor N-acetylcysteine (NAC) for 6 h. We found that NAC prevented an increase in ER stress protein abundance. Next, the role of ORAI1 on ER stress was measured by transfecting hepatocytes with small interfering (si)ORAI1 or the ORAI1 inhibitor BTP2, followed by a challenge with 1.2 mM fatty acids for 3 h. Without inhibiting ORAI1, exogenous fatty acids upregulated *ORAI1* mRNA and protein abundance, oxidative stress, ER stress proteins, and protein abundance of marker indicators of an opened mitochondrial permeability transition pore

(mPTP). Inhibition with BTP2 or silencing via siORAI1 abrogated oxidative stress, including increased GSH concentration and SOD activity, decreased MDA, hydrogen peroxide, and ROS concentration; ER stress protein abundance was downregulated, and mitochondrial function was restored. Last, changes in markers of mPTP opening were evaluated by culturing hepatocytes for 6 h with the sarcoendoplasmic Ca^{2+} ATPase inhibitor thapsigargin or the calcium ionophore ionomycin. We detected an increase in VDAC1, CLPP, and CypD protein abundance, all of which indicated opening of the mPTP. Overall, data from these in vitro studies suggest that ORAI1 mediates ER stress induced by high concentrations of fatty acids, in part through alleviating mitochondrial dysfunction caused by oxidative stress.

Key words: lactation, nutrition, transition cow, metabolic disorder

INTRODUCTION

Fatty liver is a major metabolic disorder, with highest incidence during the periparturition period. This disorder causes substantial economic losses due to decreased milk production, longer calving intervals, or removal from the herd (Bobe et al., 2004). The increase in plasma fatty acids due to negative energy balance around parturition is one of the causes of fatty liver, which is often followed by ketosis and insulin resistance (Wang et al., 2018). Recent data from dairy cows with fatty liver also revealed an association between increased plasma fatty acids and hepatic mitochondrial dysfunction (Du et al., 2018b). In nonruminants, it is well established that oxidative stress (OXS), endoplasmic reticulum (ER) stress, and mitochondrial dysfunction are features of cells that are exposed to high levels of NEFA and, hence, constitute a cellular hallmark of fatty liver (Cao and Kaufman, 2014; Hasnain et al., 2016; Du et al., 2018a; Shen et al., 2019).

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In nonruminants, the secondary messenger Ca^{2+} regulates key functions such as cell proliferation, differentiation, and metabolism (Zhang et al., 2018; Varghese et al., 2019). The control of intracellular Ca^{2+} is largely regulated by the store-operated Ca^{2+} entry (SOCE) moiety ORAI calcium release-activated calcium modulator 1 (ORAI1). At least in nonruminants, the SOCE opens upon binding to its regulator stromal interaction molecule (STIM), which senses Ca^{2+} content of the ER (Fu et al., 2012; Zhu et al., 2016; Yu et al., 2018). Thus, SOCE activates Ca^{2+} entry to subsequently refill emptied Ca^{2+} stores, with depletion of Ca^{2+} leading to activation of the unfolded protein response (UPR), a cascade of events regulated by ER lumen sensors. Those include protein kinase RNA-like ER kinase (PERK; also called eukaryotic translation initiation factor 2- α kinase 3, EIF2AK3), inositol-requiring protein 1 α (IRE1 α ; also called ER-to-nucleus signaling 1, ERN1) branch, and activating transcription factor-6 (ATF6; Arruda and Hotamisligil, 2015). When inactive, all these are bound to the chaperone glucose-regulated protein 78 [GRP78; also called heat shock protein family A (Hsp70) member 5, HSPA5; Zhang et al., 2020]. In contrast, beyond a certain threshold, concentrations of Ca^{2+} can regulate formation of the mitochondrial permeability transition pore (mPTP), encompassed by mitochondrial caseinolytic protease P (ClpP), cyclophilin D (CypD), and voltage-dependent anion-selective channel 1 (VDAC-1; Addabbo et al., 2009). Clearly, intracellular Ca^{2+} is capable of controlling a myriad of cellular responses, of which lipid metabolism is quite relevant in the context of fatty liver.

Although the role of fatty acids on periparturient dairy cow liver metabolism is well known, the mechanistic events whereby high levels of fatty acids induce mitochondrial dysfunction and ER stress are not well understood. Specific objectives were to determine the effects of high fatty acid concentrations on metabolic stress and mitochondrial dysfunction, with emphasis on the role of ORAI1.

MATERIALS AND METHODS

The Ethics Committee for the Use and Care of Animals, Heilongjiang Bayi Agricultural University (Daqing, China) approved the animal use protocol.

Isolation and Culture of Primary Hepatocytes

The 2-step collagenase IV perfusion method was used (Du et al., 2017) with 4 healthy female Holstein calves (1 d old, 40–50 kg, fasting, rectal temperature 38.7–39.7°C) as donors. Calves were purchased from a commercial dairy farm (Daqing, China). Briefly, the

caudate lobe was 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 10–15 min) and solution B (140 mM NaCl, 6.7 mM KCl, 30 mM HEPES, 2.5 mM glucose, and 5 mM CaCl_2 , pH 7.4; 37°C, 50 mL/min for 5 min) through blood vessels until the liquid became clear. The liver was then perfused with collagenase IV (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 min). Fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) was added to terminate the digestion. Upon removal of the liver capsule, blood vessels, fat, and connective tissue, the liver suspension was filtered sequentially with cell sieves (100, 200, and 400 mesh). The hepatocyte suspension was washed twice with RPMI-1640 basic medium (Hyclone Laboratories) and centrifuged for 5 min at $500 \times g$ at 4°C. Primary hepatocytes (1×10^6 cells/mL) were seeded into a 6-well tissue culture plate using an adherent medium (RPMI-1640 basic medium supplemented with 10% FBS, 10 μM insulin, 1 nM dexamethasone, 10 $\mu\text{g/mL}$ vitamin C) and incubated at 37°C in 5% CO_2 and saturated humidity. After 4 h of incubation, the medium was replaced with growth medium (RPMI-1640 with 10% FBS).

The concentration of fatty acids in the mixture developed was based on previous reports from dairy cows with fatty liver (Li et al., 2013; Abdelmagid et al., 2015; Zhang et al., 2018). Stock fatty acid solution was prepared by diluting individual fatty acids in 0.1 M KOH at 60°C. The pH of the solution was adjusted to 7.4 with hydrochloric acid (1 M). The stock fatty acid (52.7 mM) solution contained oleic (22.9 mM; Sigma-Aldrich, St. Louis, MO), linoleic (2.6 mM; Sigma-Aldrich), palmitic (16.8 mM; Sigma-Aldrich), stearic (7.6 mM; Sigma-Aldrich), and palmitoleic acids (2.8 mM; Sigma-Aldrich).

After 44-h culture, hepatocytes were serum-free starved in RPMI-1640 basic medium for 12 h. Subsequently, hepatocytes were maintained in RPMI-1640 basic medium supplemented with 2% BSA and treated with vehicle only or 1.2 mM fatty acids for 0.5, 1, 3, 6, 9, and 12 h.

Cellular Manipulations of Oxidative Stress and ORAI1 Abundance

Hepatocytes were cultured in 6-well plates (1×10^6) for 48 h with growth medium before inhibition of ORAI1. Cells were pretreated with 20 μM CRAC channel inhibitor BTP2 (Sigma-Aldrich), 10 mM OXS inhibitor N-acetylcysteine (NAC, Beyotime Biotechnology, Jiangsu, China), or vehicle (dimethyl sulfoxide; DMSO) only. A dose of 1.2 mM fatty acids was added

to cells after 2-h exposure to the pretreatments. This incubation lasted for another 6 h.

To induce downregulation of ORAI1, hepatocytes were transfected with 40 pM cattle ORAI1 small interfering (si)RNA (GenePharma, Shanghai, China) and a non-target siRNA (siRNA-mate, Shanghai GenePharma Co. Ltd., Shanghai, China) according to manufacturer's protocols (Zhang et al., 2019). Hepatocytes were cultured in 6-well plates for 48 h with growth medium.

Cellular Manipulation with the Sarcoendoplasmic Ca^{2+} ATPase Inhibitor Thapsigargin and the Ca^{2+} Ionophore Ionomycin

As previously described, hepatocytes were treated with 1 μM thapsigargin to block uptake of Ca^{2+} or 100 nM ionomycin to raise intracellular levels of Ca^{2+} for 6 h. These treatments would have prolonged or enhanced the response to changes in ORAI1 abundance.

Cellular Measures of Oxidative Stress Indices

Intracellular concentrations of glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), and hydrogen peroxide were determined using commercial kits following the manufacturer's instructions [Micro-Reduced GSH Test Kit (A006-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), MDA Assay Kit (S0131S, Beyotime), Total Superoxide Dismutase Assay Kit with WST-8 (S0101, Beyotime), and Hydrogen Peroxide Assay Kit (S0038, Beyotime)].

Measurement of Reactive Oxygen Species

A reactive oxygen species (ROS) assay kit (S0033S, Beyotime) was used to determine intracellular changes in ROS. A total of 1×10^5 cells were incubated with 10 μM carboxy-2',7'-dichloro-dihydro-fluorescein diacetate probe in PBS for 15 min at 37°C. Fluorescence was measured at 488 nm (excitation) and 525 nm (emission) using a Beckman CytoFLEX flow cytometer (Beckman Coulter, Brea, CA).

Reverse-Transcription PCR

Total RNA was extracted from hepatocytes using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols, and reverse-transcribed using a PrimeScript RT reagent kit with Gdnase Eraser (Takara Bio, Dalian, China). Calculated mRNA abundance in each sample was normalized to *ACTB*. Abundance was quantified with the $2^{-\Delta\Delta\text{CT}}$ method. Reactions were performed in a Bio-Rad iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules,

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

Gene	Primer (5' to 3')
Bovine <i>ORAI1</i>	Forward: TTTGCCGTCCACTTCTAC Reverse: CCTCTTTCCTCCACTTCT
Bovine <i>ACTB</i>	Forward: GCTAACAGTCCGCTAGAAGCA Reverse: GTCATCACCATCGGCAATGAG

les, CA). Primers used for bovine *ORAI1* and *ACTB* are shown in Table 1.

Protein Extraction and Western Blotting

Hepatocytes with radioimmunoprecipitation assay (RIPA) buffer (Beyotime) containing protease inhibitors were lysed, incubated on ice for 30 min, then centrifuged at $14,000 \times g$ for 5 min. Total protein was collected and measured using a bicinchoninic acid (BCA) Protein Assay Kit (Beyotime). Subsequently, equal amounts of protein (30 $\mu\text{g}/\text{lane}$) were separated on 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Billerica, MA). Then, the membrane was blocked for 1 h at room temperature in blocking buffer (0.1% Triton-X/PBS containing 5% skim milk powder). The blocked membranes were incubated overnight at 4°C with specific antibodies for GRP78 (1:250, sc-376768; Santa Cruz Biotechnology, Dallas, TX), PERK (1:1,000, ab229912; Abcam, Cambridge, UK), IRE1 (1:1,000, sc-376768; Abcam), ATF6 (1:1,000, sc-376768; Abcam), ORAI1 (1:1,000, 66223-1-Ig; ProteinTech Group Inc., Rosemont, IL), VDAC1 (1:1,000, ab15895; Abcam), CLPP (1:1,000, ab124822; Abcam), CypD (1:1,000, ab110324; Abcam), and ACTB (internal control; 1:1,000, sc-47778; Santa Cruz Biotechnology), respectively. Subsequently, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (3:5,000; Beyotime) for 30 min at room temperature. Protein abundance was visualized via enhanced chemiluminescence solution (Beyotime) using a ProteinSimple imager (ProteinSimple, San Jose, CA). Band intensity was quantified using Image Lab software (Bio-Rad, Hercules, CA).

Ca^{2+} Release Assay (mPTP)

Hepatocytes were treated with fatty acids, BTP2, or small interfering (si)ORAI1, respectively, and a Transition Pore Assay Kit (I35103, Thermo Fisher Scientific, Waltham, MA) used according to manufacturer's instructions. Hepatocytes were washed twice with modified Hank's balanced salt solution (HBSS) buffer (14175095, Thermo Fisher Scientific) including

HEPES (10 mM), L-glutamine (2 mM), and succinate (100 μ M), and incubated with labeling solution (composed of 1 mM calcein and 1 mM CoCl_2) for 15 min protected from light at 37°C. Cells were then washed with warm modified HBSS buffer to remove residual dye. The Beckman CytoFLEX flow cytometer was used for analysis (Beckman Coulter). Opening of mPTP was assessed via fluorescence using a laser confocal microscope (LSM 5 PASCAL, Zeiss, Oberkochen, Germany).

JC-1 Staining

The fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Beyotime, C2006) was used to stain hepatocytes ac-

cording to manufacturer's instructions. Hepatocytes were loaded with 10 μ g/mL of JC-1 for 10 min at 37°C. The labeling solution was then removed by aspiration, and cells were rinsed 3 times in cell culture medium without JC-1. Fluorescence images were acquired via the Beckman CytoFLEX flow cytometer (Beckman Coulter).

Statistical Analysis

All treatments were run in triplicate, and results are reported as mean $\pm$ standard error of the mean. The data were analyzed using SPSS 22.0 (IBM Corp., Armonk, NY). Statistical significance was evaluated by one-way ANOVA with a Duncan test for post hoc

Figure 1. Effects of exogenous fatty acids (FA) on oxidative and endoplasmic reticulum stress in calf hepatocytes. Concentrations [means $\pm$ SEM ($n = 12$)] of glutathione (GSH; gprot = g protein; Ctrl = control; A), activity of superoxide dismutase (SOD; B), and concentrations of malondialdehyde (MDA; C) and hydrogen peroxide (D) in calf hepatocytes incubated without (white bar) or with (black bars) a mixture of fatty acids (1.2 mM). Western blot analysis of PERK, IRE1, GRP78, ATF6, and ACTB (E). Abundance of PERK (F), IRE1 (G), ATF6 (H), and GRP78 (I) relative to ACTB protein in calf hepatocytes incubated without (white bar) or with (black bars) a mixture of fatty acids (1.2 mM). * $P < 0.05$ and ** $P < 0.01$ indicate significant differences (one-way ANOVA).

Figure 2. Effects of fatty acids (FA) on ORAI1 and endoplasmic reticulum stress proteins in calf hepatocytes with or without supplemental N-acetylcysteine (NAC; representative Western blot; A). Abundance [means $\pm$ SEM ($n = 9$)] of PERK (B), IRE1 (C), GRP78 (D), ATF6 (E), CHOP (F), and ORAI1 (G) relative to ACTB in calf hepatocytes incubated without (left bar) or with (middle and right bar) fatty acids (1.2 mM) in the presence (right bars) of the oxidative stress inhibitor NAC (10 mM). * $P < 0.05$, ** $P < 0.01$ indicate significant difference from control; # $P < 0.05$, ## $P < 0.01$ indicate significant difference from cells incubated with fatty acids alone (one-way ANOVA).

Figure 3. Effect of ORAI1 inhibition on mitochondrial dysfunction protein levels in calf hepatocytes. Abundance of VDAC1, CLPP, CypD, ORAI1, and ACTB in calf hepatocytes incubated without or with fatty acids (FA; 1.2 mM, 6 h) in the absence or presence of ORAI1 inhibitor BTP2 (200 nM; representative Western blot; A). Abundance [means $\pm$ SEM ($n = 9$)] of VDAC1 (B), CLPP (C), CypD (D), and ORAI1 (E) in calf hepatocytes incubated without or with fatty acids (1.2 mM, 6 h) in the absence (left bars) or presence (right bars) of ORAI1 inhibitor BTP2 (200 nM). ** $P < 0.01$ indicates significant difference from control; ## $P < 0.01$ indicates significant differences from cells incubated with fatty acids alone (one-way ANOVA).

analysis. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Time Sequence of Oxidative Stress and ER Stress

As shown in Figure 1A–D, GSH and SOD levels significantly decreased ($P < 0.05$) from 0.5 h, and MDA and hydrogen peroxide levels increased markedly from 1 h after the challenge. In contrast, abundances of GRP78, PERK, IRE, and ATF6 were greater by 6 h (Figure 1E–I). Furthermore, compared with the 1.2 mM fatty acid treatment, calf hepatocytes treated with the oxidative stress inhibitor NAC had markedly lower abundance of the ER stress-associated proteins PERK, IRE1, ATF6, and CHOP (Figure 2).

Effects of Inhibition or Silencing of ORAI1

Compared with the control group, the transcription level of ORAI1 was markedly greater in the 1.2 mM

fatty acid treatment. Protein abundances of ORAI1, VDAC1, CLPP, and CypD were greater in cells challenged with fatty acids (Figure 3). Silencing of ORAI1 via transient transfection or inhibition of ORAI1 via ORAI1 inhibitor BTP2 decreased mRNA (Supplemental Figure S1, <https://doi.org/10.3168/jds.2020-18684>) and protein abundance of ORAI1 (Figure 3A, E; Supplemental Figure S2A, E, <https://doi.org/10.3168/jds.2020-18684>). Furthermore, inhibition and silencing of ORAI1 abrogated the effects of fatty acids on the cytosolic content of GSH, MDA, SOD, hydrogen peroxide (Figure 4), and VDAC1, CLPP, and CypD protein abundance (Figure 3 A–D; Supplemental Figure S2A–D, <https://doi.org/10.3168/jds.2020-18684>). In addition, they markedly alleviated the increase in mRNA and protein abundance of PERK, IRE1, ATF6, CHOP, and GRP78 induced by 1.2 mM fatty acid (Figure 5, Supplemental Figure S3, <https://doi.org/10.3168/jds.2020-18684>). Although ROS concentrations increased with fatty acids, inhibition and silencing of ORAI1 reduced ROS (Figure 6).

Figure 4. Effect of ORAI1 inhibitor BTP2 and silencing siORAI1 on oxidative stress in calf hepatocytes. Concentrations [means $\pm$ SEM ($n = 12$)] of glutathione (GSH; gprot = g protein), activity of superoxide dismutase (SOD), and concentrations of malondialdehyde (MDA) and hydrogen peroxide in calf hepatocytes incubated without (white bar) or with (black bar) fatty acids (FA; 1.2 mM, 6 h) in the absence (left bars) or presence (right bars) of ORAI1 inhibitor BTP2 (200 nM). Concentrations [means $\pm$ SEM ($n = 12$)] of GSH, activity of SOD, and concentrations of MDA and hydrogen peroxide in calf hepatocytes incubated without (white bar) or with (black bar) fatty acids (1.2 mM, 6 h) in the absence (left bars) or presence (right bars) of ORAI1 silencing siORAI1 (40 pM). * $P < 0.05$, ** $P < 0.01$ indicate significant differences from control; # $P < 0.05$, ## $P < 0.01$ indicate significant differences from cells incubated with fatty acids alone (one-way ANOVA).

Effect of Sarcoendoplasmatic Ca^{2+} ATPase Inhibitor Thapsigargin and Ca^{2+} Ionophore Ionomycin on Mitochondrial Dysfunction

Thapsigargin and ionomycin upregulated the protein abundance of ORAI1 (Supplemental Figure S4E and J, <https://doi.org/10.3168/jds.2020-18684>) and markedly increased protein abundances of VDAC1, CLPP, and CypD (Supplemental Figure S4A–I, <https://doi.org/10.3168/jds.2020-18684>). Furthermore, we also observed a larger reduction in the JC-1 fluorescence ratio upon challenge with thapsigargin and ionomycin, which increased ORAI1 abundance (Figure 7A, B).

Status of mPTP

To determine the status of mPTP during the early stage of lactation in dairy cows with hepatic lipid de-

position, we monitored the mPTP opening via calcein release assay in calf hepatocytes during fatty acid-mediated mitochondrial dysfunction. Challenge with fatty acids induced mPTP opening (Figure 8), but the inhibitor BTP2 and silencing of ORAI1 abrogated mPTP opening (Figure 8). Use of JC-1, a mitochondrial membrane potential ($\Delta\Psi_m$) dye, is another method to detect mPTP opening. After cells were permeabilized and treated with CaCl_2 , we observed a greater decrease in the JC-1 fluorescence ratio in cells challenged with fatty acids, confirming that mPTP opening was greater (Figure 7C–F).

DISCUSSION

Fatty liver is the most important metabolic disease afflicting dairy cows early postpartum. It is usually characterized by increased plasma fatty acid concen-

Figure 5. Effect of ORAI1 inhibitor BTP2 on endoplasmic reticulum stress in calf hepatocytes. Abundance of PERK, IRE1, ATF6, and GRP78 relative to ACTB in calf hepatocytes incubated without or with fatty acids (FA; 1.2 mM, 6 h) in the absence or presence of ORAI1 inhibitor BTP2 (200 nM; representative Western blot; A). Abundance [means $\pm$ SEM ($n = 12$)] of PERK, IRE1, GRP78, ATF6, and CHOP relative to ACTB in calf hepatocytes incubated without (white bar) or with (black bar) fatty acids (1.2 mM, 6 h) in the absence (left bars) or presence (right bars) of ORAI1 inhibitor BTP2 (200 nM). * $P < 0.05$, ** $P < 0.01$ indicate significant difference from control; # $P < 0.05$, ## $P < 0.01$ indicate significant differences from cells incubated with fatty acids alone (one-way ANOVA).

trations, accumulation of lipid droplets in hepatocytes, OXS (Song et al., 2014), and mitochondrial dysfunction in hepatocytes (Zhu et al., 2019). Lipid peroxidation of polyunsaturated fatty acids leads to formation of ROS and MDA, both of which are markers of OXS. The toxic stress in cells arising from exposure to MDA

is due to the character of reactive electrophile species (Moore and Roberts, 1998; Del Rio et al., 2005) that lead to formation of lipoxidation end products (Farmer and Davoine, 2007). The endogenous antioxidant GSH protects cellular membranes from free radical oxidative damage because it can reduce concentrations of hydro-

Figure 6. Production of reactive oxygen species (ROS) in calf hepatocytes. Histogram overlays of ROS production in calf hepatocytes incubated without or with fatty acids (FA; 1.2 mM, 6 h) in the absence (left bars) or presence (right bars) of ORAI1 inhibitor BTP2 (200 nM; A). P1 = percentage of fluorescence. Production of ROS [means $\pm$ SEM (n = 20)] in calf hepatocytes incubated without or with fatty acids (1.2 mM, 6 h) in the absence (left bars) or presence (right bars) of BTP2 (200 nM; B). Original histogram overlays of ROS production in calf hepatocytes incubated without or with fatty acids (1.2 mM, 6 h) in the absence (left bars) or presence (right bars) of ORAI1 silencing siORAI1 (10 pM; C). Production of ROS [means $\pm$ SEM (n = 20)] in calf hepatocytes incubated without or with fatty acids (1.2 mM, 6 h) in the absence (left bars) or presence (right bars) of ORAI1 silencing siORAI1 (10 pM; D). * $P < 0.05$, ** $P < 0.01$ indicate significant differences from control; ## $P < 0.01$ indicates significant differences from cells incubated with fatty acids alone (one-way ANOVA).

Figure 7. (A) Original histogram overlays of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) ratio in calf hepatocytes incubated without or with ionomycin (100 nM, 6 h) or thapsigargin (1 μ M, 6 h). (B) Means $\pm$ SEM (n = 20) of JC-1 ratio in calf hepatocytes incubated without or with ionomycin (100 nM, 6 h) or thapsigargin (1 μ M, 6 h). (C) Original histogram overlays of JC-1 ratio in calf hepatocytes incubated without or with fatty acids (FA; 1.2 mM, 6 h) in the absence (left) or presence (right) of ORAI1 inhibitor BTP₂ (200 nM). NEFA = nonesterified fatty acids. (D) Means $\pm$ SEM (n = 20) of JC-1 ratio in calf hepatocytes incubated without or with fatty acids (1.2 mM, 6 h) in the absence (siNeg; left) or presence (right) of BTP₂ (200 nM). (E) Original histogram overlays of JC-1 ratio in calf hepatocytes incubated without or with fatty acids (1.2 mM, 6 h) in the absence (siNeg; left) or presence (right) of ORAI1 silencing siORAI1 (10 pM). (F) Means $\pm$ SEM (n = 20) of JC-1 ratio in calf hepatocytes incubated without or with fatty acids (1.2 mM, 6 h) in the absence (left bars) or presence (right bars) of ORAI1 silencing siORAI1 (10 pM). ** P < 0.01 indicates significant differences from control; ## P < 0.01 indicates significant differences from fatty acids alone (one-way ANOVA).

peroxides and organic peroxides, including lipid peroxides (Zitka et al., 2012). Therefore, GSH is considered a free radical scavenger of ROS and a potent inhibitor of lipid peroxidation (Owen and Butterfield, 2010).

Superoxide dismutase catalyzes the dismutation (or partitioning) of the superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or H_2O_2 to defend against OXS (Younus, 2018). Hydrogen peroxide, a ROS with unique chemical properties, is produced endogenously in living systems as a destructive oxidant to ward off pathogens or as a finely-tuned secondary messenger in cellular signaling pathways (Lin et al., 2013). Marked increases in mitochondrial β -oxidation can increase permeability of the inner mitochondrial membrane, leading to dissipation of membrane potential and loss of ATP synthesis capacity, all of which impairs mitochondrial function and enhances ROS production (Masarone et al., 2018; Yang et al., 2020). Thus, the lower GSH and SOD activity along with greater MDA and hydrogen peroxide after cells were challenged with fatty acids were indicative of increased OXS. Therefore, influx of fatty acids induced OXS partly through decreasing the ability of GSH to reduce lipid peroxides and impairment of the ability of SOD to remove O_2^- radicals.

A previous *in vivo* study confirmed not only the occurrence of ER stress in dairy cows with severe fatty liver, but also that high concentrations of fatty acids

induced OXS (Song et al., 2014), ER stress, and lipid accumulation in calf hepatocytes (Zhu et al., 2019). In the present study, the greater abundance of PERK, IRE1, ATF6, and GRP78, along with the abrogation of these effects by NAC (an antioxidant), underscored a potent effect of fatty acids on ER stress in hepatocytes in part through OXS. The greater abundance of major ER stress proteins PERK, IRE1, ATF6, and GRP78, along with the abrogation of these effects by NAC (an antioxidant), indicated a potent effect of fatty acids on OXS status within hepatocytes. Store-operated Ca^{2+} entry leads to emptying of ER Ca^{2+} stores, which in turn triggers influx of Ca^{2+} across the plasma membrane (Soboloff et al., 2012). Thus, the ER acts as a reservoir for Ca^{2+} to help maintain intracellular homeostasis, and upon Ca^{2+} depletion in the ER, ORAI1 channels are activated and promote SOCE activity (Brandman et al., 2007). Thus, although we could not discern the exact mechanisms, the increase in ORAI1 abundance in response to challenge with fatty acids and the abrogation of this effect by inhibition or silencing of ORAI1 highlighted an important role of intracellular Ca^{2+} in the normal functioning of ruminant hepatocytes.

An increase in mitochondrial Ca^{2+} enhances metabolic rate and ROS generation (Gorlach et al., 2006), leading to mitochondrial dysfunction and OXS, both of which can trigger mPTP opening. VDAC1, ClpP, and CypD are essential components of the mPTP and work

Figure 8. (A) Original immunofluorescence images demonstrating nuclear staining (blue; leftmost column of images), calcein (green; second column from left), Mito Tracker (Thermo Fisher Scientific, Waltham, MA; red; third column from left), and an overlaying of all (right column) in calf hepatocytes incubated without (uppermost row of images) or with fatty acids (FA; 1.2 mM, 6 h; second row from top), siORAI1 (third row from top), or siORAI1 with fatty acids (bottom row). Scale bar: 25 μ m. (B) Original immunofluorescence images demonstrating nuclear staining (blue; leftmost column of images), calcein (green; second column from left), Mito Tracker (red; third column from left), and an overlaying of all (right column) in calf hepatocytes incubated without (uppermost row of images) or with fatty acids (1.2 mM, 6 h; second row from top), BTP2 (third row from top), or BTP2 with FA (bottom row). Scale bar: 25 μ m.

in concert to protect against mitochondrial dysfunction. VDAC1 is the gatekeeper for the passage of metabolites, nucleotides, and ions, and reflects the abundance of mitochondria (Camara et al., 2017). ClpP maintains the integrity of oxidative phosphorylation (Cole et al., 2015). In the present study, high concentrations of fatty acids decreased GSH and increased ROS production, likely via enhanced oxidative phosphorylation, leading to mPTP opening. In contrast, inhibition or silencing of ORAI1 alleviated the effect of fatty acids on mitochondrial dysfunction. The increase in intracellular Ca^{2+} concentration and ORAI1 abundance in response to ionomycin or thapsigargin suggests that derangements in intracellular Ca^{2+} signaling induced by excessive concentrations of fatty acids could contribute to mitochondrial dysfunction.

Although several published studies addressing mechanistic aspects of adult ruminant liver metabolism have relied on calf hepatocytes (Mashek and Grummer, 2003; Chandler and White, 2017; Zhou et al., 2018; Chandler and White, 2019), caution should be taken when trying to extrapolate findings using hepatocytes from 1-d-old calves to whole-animal liver metabolism in periparturient cows. Although in vitro data suggest that ORAI1 signaling could affect mitochondrial and ER function, the relevance of these data to the transition cow remains to be determined.

CONCLUSIONS

Excessive fatty acid influx into hepatocytes activates oxidative stress in part through its effects on intracellular Ca^{2+} signaling and ORAI1 abundance. These adaptations induce mitochondrial dysfunction and ER stress. Because of its central role in cellular Ca^{2+} homeostasis, ORAI1 may serve as a therapeutic target for prevention of fatty liver and hepatic inflammation.

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