Adaptations of hepatic lipid metabolism and mitochondria in dairy cows with mild fatty liver

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ABSTRACT

The inevitable deficiency in nutrients and energy at the onset of lactation requires an optimal adaptation of the hepatic metabolism to overcome metabolic stress. Fatty liver is one of the main health disorders after parturition. Therefore, to investigate changes in hepatic lipid metabolic status and mitochondria in dairy cows with mild fatty liver, liver and blood samples were collected from healthy cows ($n = 15$) and cows with mild fatty liver ($n = 15$). To determine the effects of palmitic acids (PA), one of the major component of fatty acids, on lipid metabolism and mitochondria in vitro, calf hepatocytes were isolated from healthy calves and treated with various concentrations of PA (0, 50, 100, and 200 μM). Dairy cows with mild fatty liver displayed hepatic lipid accumulation. The protein levels of sterol regulatory element-binding protein 1c (SREBP-1c) and peroxisome proliferator-activated receptor- α (PPAR α) and mRNA levels of acetyl CoA carboxylase 1 (*ACC1*), fatty acid synthase (*FAS*), acyl-CoA oxidase (*ACO*), and carnitine palmitoyltransferase 1A (*CPT1A*) were significantly higher in dairy cows with mild fatty liver than in control cows. The hepatic mitochondrial DNA content, mRNA levels of oxidative phosphorylation complexes I to V (*CO 1-V*), protein levels of cytochrome c oxidase subunit IV (COX IV), voltage dependent anion channel 1 (VDAC1), peroxisome proliferator activated receptor- γ coactivator-1 α (PGC-1 α) and nuclear respiratory factor 1 (NRF1), and adenosine triphosphate (ATP) content were all markedly increased in the liver of dairy cows with mild

fatty liver compared with healthy cows. The PA treatment significantly increased lipid accumulation; protein levels of SREBP-1c and PPAR α ; and mRNA levels of *ACC1*, *FAS*, *ACO*, and *CPT1A* in calf hepatocytes. Moreover, the mitochondrial DNA content, mRNA levels of *CO 1-V*, protein levels of COX IV, VDAC1, PGC-1 α , NRF1, mitochondrial transcription factor A, and ATP content were significantly increased in PA-treated hepatocytes compared with control hepatocytes. The protein level of mitofusin-2 was significantly decreased in PA-treated groups. In conclusion, lipid synthesis and oxidation, number of mitochondria, and ATP production were increased in the liver of dairy cows with mild fatty liver and PA-treated calf hepatocytes. These changes in hepatic mitochondria and lipid metabolism may be the adaptive mechanism of dairy cows with mild fatty liver.

Key words: mild fatty liver, lipid metabolism, mitochondria, adaptation, dairy cow

INTRODUCTION

The transition period in dairy cows, which spans from 3 wk before to 3 wk after calving, is defined as the changes from late pregnancy to the adaptation phase of early lactation. Voluntary DMI reduction around the time of parturition along with increases in energy requirements to meet the needs of gravidity and lactogenesis lead to cows entering a state of negative energy balance (Weber et al., 2013; Song et al., 2016). Drackley et al. (2001) reported that energy demands increased approximately 3-fold in early-lactating cows compared with pregnant and nonlactating cows. Therefore, cows mobilize fat depots to provide fatty acids as an energy fuel to meet energy requirements.

During the transition period, circulating fatty acid concentration and blood flow to the liver are both increased, and excessive amounts of fatty acids are trans-

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ported to the liver (Reynolds et al., 2003). Palmitic acid (**PA**) is an important representative of fatty acids released during lipolysis (Rukkwamsuk et al., 2000). Although many peripheral tissues remove fatty acids from the blood, up to 25% of fatty acids are removed by the liver and subsequently oxidized or re-esterified and stored as triglycerides (**TG**; Sejersen et al., 2012). High liver fat content can result in metabolic imbalances that are related to clinical diseases such as ketosis and fatty liver syndrome (Bobe et al., 2004). In the first month after calving, 5 to 10% of dairy cows have severe fatty liver, and 30 to 40% have mild and moderate fatty liver (Bobe et al., 2004). More important, fatty liver is associated with increased veterinary costs, decreased milk production, and longer calving intervals (Grummer, 2008). Therefore, the rapid adaptation of key metabolic pathways in the liver to support lactation is central to the ability of cows to make an uneventful transition.

The regulation of hepatic lipid metabolism is largely dependent on mitochondria, the primary organelle of cellular adenosine triphosphate (**ATP**) production, using the energy released from the respiratory chain mediated by oxidative phosphorylation complexes (CO I, CO II, CO III, CO IV, and CO V; **CO 1-V**; Begriche et al., 2013; Yamaguchi et al., 2016). Mitochondrial impairment can contribute to the development of fatty liver or insulin resistance in mice (Rovira-Llopis et al., 2017). More important, amelioration of mitochondrial function prevents hepatic steatosis in obese mice (Lin et al., 2014). Interestingly, Koliaki and Roden (2013) showed that obese individuals with fatty liver had increased mitochondrial respiratory rates compared with lean ones, suggesting hepatic mitochondrial flexibility at the early stages of fatty liver. Moreover, several studies reported that adaptations of mitochondrial function and lipid metabolism were observed in mice or patients with nonalcoholic fatty liver (Begriche et al., 2013; Franko et al., 2014; Sunny et al., 2017). Dairy cows during the periparturient period also displayed hepatic metabolic adaptations (Drackley et al., 2001; Ha et al., 2017), even though the cellular and molecular mechanisms have not yet been elucidated. We investigated the changes in hepatic lipid metabolism and mitochondria in cows with moderate and severe fatty liver in our previous studies (Li et al., 2015; Du et al., 2017a; Gao et al., 2018). Nevertheless, to our knowledge, few studies have evaluated the status of mitochondria in dairy cows with mild fatty liver and the adaptive mechanism of calf hepatocytes in vitro (Wathes, 2012; Laubenthal et al., 2016). In addition, the effects of PA on lipid metabolism and mitochondria of calf hepatocytes are unclear. Therefore, the aim of this study was to investigate (1) the hepatic lipid metabolic status in dairy

cows with mild fatty liver, (2) the hepatic mitochondria status in dairy cows with fatty liver, and (3) the effects of PA on lipid metabolism and mitochondria in calf hepatocytes.

MATERIALS AND METHODS

Animals

The Ethics Committee on the Use and Care of Animals at Jilin University (Changchun, China) approved the study protocol [2015 clinical trial (2015-121)]. To guarantee that the cows did not have any other comorbidities, all cows received a routine physical examination. Cows were fed ad libitum, and all cows received the same diet. The basal diet formulation is shown in Table 1. We chose lactating Holstein cows with similar numbers of lactations (median = 3, range = 2–4) and DIM (median = 6 d, range = 3–9 d) from a 1,000-cow dairy farm located in Changchun, Jilin Province, China. Measurement of liver TG is the gold standard for diagnosing and staging fatty liver in dairy cows (Bobe et al., 2004). According to a review by Bobe et al. (2004), fatty liver can be categorized into normal liver and mild, moderate, and severe fatty liver. In healthy cows, hepatic TG content is less than 1% (% g/g of wet weight). In cows with mild fatty liver, hepatic TG content is higher than 1% but less than 5%. Fifteen healthy Holstein cows and 15 cows with mild fatty liver were selected for the experiments. Table 2 provides a basic description of the cows with mild fatty liver and the healthy cows.

Table 1. Nutrient composition of the diets

Item	Amount
Ingredient (%)	
Corn silage	40.00
Corn	35.00
Wheat bran	8.00
Soybean meal	5.00
Sunflower	8.00
NaCl	1.00
Premix ¹	1.80
NaHCO ₃	1.20
Total	100.00
Nutrient composition (% of DM, unless noted)	
NE _L (MJ/kg)	6.70
CP	15.20
NDF	33.45
ADF	17.20
NFC	40.40
Ca	0.70
P	0.50

¹Provided the following per kilogram of diet: vitamin A 200,000 IU, vitamin D 70,000 IU, vitamin E 1,000 IU, Fe 2,000 mg, Cu 600 mg, Zn 2,400 mg, Mn 1,300 mg, I 6 mg, Co 7 mg.

The milk yield was recorded on 3 consecutive days at 0530 and 1500 h. Blood samples were collected on 3 consecutive days via jugular venipuncture between 0700 and 0800 h before feeding and immediately centrifuged at $3,500 \times g$ for 15 min at 4°C. The serum was obtained and stored at -80°C until analysis. Liver samples were collected within 3 to 9 d after calving. After blood collection, liver tissue samples were taken from the 11th or 12th right intercostal space twice from each cow by liver puncture needle (Shanghai Surgical Equipment Factory, Shanghai, China; Du et al., 2017a). 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 Co., St. Louis, MO). A scalpel blade was used to make a 3-mm stab incision in the skin. The puncture needle was then inserted through the intercostal muscle and into the liver. The liver tissues (approximately 150 mg from each collection) were immediately frozen in liquid nitrogen or fixed with 10% formaldehyde neutral buffer solution, optimal cutting temperature compound (Sakura Finetek Co., Torrance, CA), or 2.5% glutaraldehyde and 2% paraformaldehyde. During the experimental work, the cows were housed in tiestalls barn.

Determination of Blood Parameters

The glucose, BHB, and fatty acids concentrations in serum were determined using a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan) with commercially available kits (BHB: cat. no. RB1008; fatty acids: cat. no. FA115; glucose: cat. no. GL3815; Randox Laboratories, Crumlin, UK). The activities of aspartate aminotransferase and alanine aminotransferase were determined using an automatic biochemistry analyzer (Sekisui Medical Co. Ltd., Tokyo, Japan) with commercially available kits (aspartate aminotransferase: cat. no. AS3804; alanine aminotransferase: cat. no. AL3801; Randox Laboratories).

Hepatocyte Isolation and Culture

Calf hepatocytes were isolated using a modified 2-step collagenase perfusion method as previously described (Du et al., 2017b,c). Five calves (1 d old, female, 30–40 kg, healthy, fasting) were used for hepatocyte isolation in this experiment. Hepatocytes were isolated from each calf separately, and all assays were done with calf considered as random. The caudate liver lobe of Holstein calves was obtained through surgical liver excision according to the methods of a previous study (Parker and Gaughan, 1988). Subsequently, the liver was perfused with perfusion solution A (140 mM NaCl, 10 mM HEPES, 6.7 mM KCl, 0.5 mM EDTA, and 2.5 mM glucose; pH 7.2–7.4, 37°C) at a flow rate of 50 mL/min for 12 min. Then, the liver was perfused with solution B (140 mM NaCl, 30 mM HEPES, 6.7 mM KCl, 5 mM CaCl₂, and 2.5 mM glucose; pH 7.2–7.4, 37°C) at a flow rate of 50 mL/min for 3 min until the liquid became clear. Subsequently, the liver was digested with a collagenase IV solution (0.1 g of collagenase IV dissolved in 0.5 L of perfusion solution B, pH 7.2–7.4, 37°C) at a flow rate of 20 mL/min for 15 to 20 min. During the digestion step, most of the liver tissue was dissociated with a collagenase IV solution. Then, the liver was moved to a sterile flat plate after digestion, and 100 mL of fetal bovine serum (Hyclone Laboratories, Logan, UT) was added to terminate the collagenase digestion. The liver was cut open to release the hepatocytes and the liver capsule, and blood vessels and connective tissue were removed using forceps and scissors. The tissue suspension was filtered sequentially with 100-mesh (150 μm) and 200-mesh (75 μm) cell sieves. The hepatocyte suspension was washed twice in RPMI-1640 basic medium (Hyclone Laboratories) and centrifuged for 5 min at $500 \times g$ at 4°C. Cell viability was assessed with the Trypan blue dye exclusion method (Sigma-Aldrich Co.). Only when the percentage of viable hepatocytes was higher than 95% were hepatocytes used to perform further experiment. The hepatocyte suspension was

Table 2. Description of cows classified as having mild fatty liver (n = 15) or as healthy (control; n = 15)

Item	Mild fatty liver		Control		P-value
	Median	Interquartile range	Median	Interquartile range	
BW (kg)	672	623–702	643	626–662	0.2293
Milk production (kg of milk/cow per day)	29	27.8–30.5	30.6	28.7–32.7	0.017
DMI (kg/d)	19.4	18.7–20.2	20.6	19.4–20.9	0.0354
BCS	2.8	2.65–2.93	2.67	2.51–2.76	0.0256
Glucose (mM)	3.07	2.78–3.47	3.75	2.9–3.99	0.0181
Fatty acids (mM)	0.6	0.49–0.81	0.44	0.38–0.55	0.0097
BHB (mM)	1.1	0.8–1.4	0.53	0.42–1.04	0.0084
Aspartate aminotransferase (U/L)	80	74–96	73	66–77	0.0026
Alanine aminotransferase (U/L)	26	22–31	18	16–23	0.0067

seeded into a 6-well tissue culture plate (2 mL/well) at 1×10^6 cells/mL using adherent medium (RPMI-1640 basic medium supplemented with 10% fetal bovine serum, 10^{-6} mol/L of insulin, 10^{-6} mol/L of dexamethasone, 10 μ g/mL of vitamin C). The hepatocytes were incubated at 37°C in 5% CO₂. After 4 h, the medium was replaced with growth medium containing 10% fetal bovine serum. The growth medium was replaced every 24 h. Any splitting of cells was not performed in this experiment. The calf hepatocytes used in this study were harvested 72 h after isolation. Methods of adult bovine hepatocyte isolation and culture were the same as calf hepatocytes.

Hepatocyte Treatments

Palmitic acids (P0500; Sigma-Aldrich Co.) were dissolved in 0.1 M NaOH at 70°C and then complexed with 10% bovine serum albumin at 55°C for 10 min to achieve the final palmitate concentration (100 mM; Cousin et al., 2001). Hepatocytes were maintained in RPMI-1640 basic medium containing 2% BSA and treated with 0, 50, 100, or 200 μ M PA for 12 h. Palmitic acid was applied only once, and the duration of the experiment was 12 h. Adult bovine hepatocyte treatment was the same as calf hepatocytes.

Liver Histology

Liver tissues were fixed in 10% formaldehyde neutral buffer solution, embedded in paraffin, cut into 8- μ m slices, floated on a water bath, picked up onto glass slides, and placed in slide racks. Slides were dewaxed with xylene, rehydrated through descending concentrations of alcohol, and stained with hematoxylin–eosin. For oil-red O staining, liver tissues were frozen in optimal cutting temperature compound (Sakura Finetek), sectioned into 8- μ m slices at –18°C, and fixed with 75% alcohol at room temperature for 15 min. Then, slides were stained with oil-red O (O0625; Sigma-Aldrich Co.) and counterstained with hematoxylin. Quantification of lipid droplets was performed using Image-Pro Plus software (Media Cybernetics, Rockville, MD).

Transmission Electron Microscopy

The ultrastructural characteristics of mitochondria were visualized by transmission electron microscopy (Lei et al., 2016). Liver biopsies were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde at 4°C for 12 h and then postfixed in 1% osmium tetroxide for 1 h. Subsequently, liver biopsies were dehydrated in an ethanol series and infiltrated with Spurr's resin. Ultrathin sections (50 nm) were cut and stained with 4% uranyl acetate and 0.2% lead citrate. Observations

were performed on an H-7650 electron microscope (Hitachi, Ibaraki Prefecture, Japan). To guarantee that the results of ultrastructural analysis were representative, each liver sample was used to perform ultrastructural analysis, and at least 5 random views in each sample were obtained.

ATP Content Determination

The ATP content was determined using an ATP analysis kit (A095; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, liver tissues (~20 mg) or hepatocytes were homogenized in cold PBS followed by 3 freeze–thaw cycles. The homogenate was incubated in boiling water for 10 min, vortexed for 1 min, and centrifuged for 5 min at $13,000 \times g$ at room temperature, and the supernatant was collected. The supernatant was mixed with substrate solutions and accelerant and incubated at 37°C for 30 min. Then, the mixtures were mixed with precipitant and centrifuged for 5 min at $1,500 \times g$ at room temperature, and the supernatant was collected. Subsequently, the supernatant was mixed with color developing solution and stop solution and incubated for 5 min at room temperature before being read at 636 nm. The ATP values were corrected by protein content. Total protein concentration was estimated by the bicinchoninic acid (BCA) method (P1511; Applygen Technologies Inc., Beijing, China) and performed according to the manufacturer's instructions (<http://www.applygen.com/a/danbaidingliang/94.html>). Hepatic ATP content of dairy cows with mild fatty liver was normalized to that in the control group.

MitoTracker Staining

Mitochondria were detected using MitoTracker Red CMXRos (M7512; Life Technologies, Carlsbad, CA). The cell-permeant MitoTracker Red CMXRos probes contain a mildly thiol-reactive chloromethyl moiety for labeling mitochondria. Treated calf hepatocytes were incubated with 200 nM MitoTracker Red CMXRos diluted in warmed serum free culture medium at 37°C for 30 min. The hepatocytes were washed twice with PBS and fixed with warmed 4% paraformaldehyde for 15 min at 37°C. Then, nuclei were stained with 10 μ g/mL of 4',6-diamidino-2-phenylindole (D9542; Sigma-Aldrich Co.) for 10 min at room temperature. The coverslips were then sealed with glycerol, and the samples were imaged using laser confocal microscopy (Fluoview FV1200, Olympus, Tokyo, Japan). The experiments were repeated 5 times; each time, at least 3 zones were selected for measurement. Image-Pro Plus software (Media Cybernetics) was used to quantify the signals.

Oil-Red O Staining of Hepatocytes

Oil-red O (Sigma-Aldrich Co.) working solution was prepared by diluting oil-red O stock solution (5 µg of oil-red O in 100 mL of isopropanol at 65°C for 48 h) 3:2 with double-distilled H₂O. All assay steps were performed at room temperature. Treated cells were washed once in PBS, fixed in 4% paraformaldehyde for 15 min, and then incubated for 5 min in 60% isopropanol. Subsequently, cells were incubated for 30 min with oil-red O working solution and washed for 30 s in 60% isopropanol. Cells were washed 4 times with double-distilled H₂O and counterstained with hematoxylin, and images were acquired (Olympus). Quantification of lipid droplets was performed using Image-Pro Plus software (Media Cybernetics).

Total DNA Extraction

Total DNA was extracted by Total DNA Preparation Kit (D0063; Beyotime Biotechnology, Hangzhou, China). Briefly, the homogenate of liver tissues or hepatocytes was suspended in PBS supplemented with Rnase A and proteinase K and incubated for 2 min at room temperature. Then, lysis buffer B was added and incubated for 10 min at 70°C. The samples were mixed with dehydrated alcohol and pipetted into a filter tube and centrifuged at $6,000 \times g$ for 1 min, and the flow-through was discarded. The samples were washed with washing buffer. Subsequently, elution buffer was added to the filter tube and the eluted DNA was collected by centrifugation at $18,000 \times g$ for 1 min. The DNA concentration and quality ($1.7 < \text{optical density } 260/280 < 1.9$) were measured using a K5500 microspectrophotometer (Beijing Kaiiao Technology Development Ltd., Beijing, China). The DNA samples were stored at -20°C before the next analyses.

Mitochondrial DNA Quantification

We quantified mitochondrial DNA (mtDNA) in the total DNA extracted from liver tissues (~15 mg) or hepatocytes as described before (Amaral et al., 2007; Mei et al., 2015). We used 12S rRNA and cyclophilin A to amplify mtDNA and nuclear DNA (nDNA; Bonnard et al., 2008; Laubenthal et al., 2016). The specific primers are shown in Table 3. The PCR reaction was conducted under the following conditions: initial denaturation at 95°C for 5 min, 30 cycles of amplification (denaturation at 95°C for 45 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s), and extension at 72°C for 10 min with PrimeStar Max DNA Polymerase (R045A; TaKaRa Biotechnology Co. Ltd., Dalian, China). The PCR production was purified using the

DNA Gel Extraction Kit (D0056; Beyotime Biotechnology). The DNA concentration and quality ($1.7 < \text{optical density } 260/280 < 1.9$) were measured using a K5500 microspectrophotometer (Beijing Kaiiao Technology Development Ltd.). Using the average molecular weight of the template DNA and Avogadro's constant, the number of copies of mtDNA or nDNA per microliter was calculated as

$$\text{copies}/\mu\text{L} = \frac{6.02 \times 10^{23} \times C \times 10^{-9}}{\text{Mwt}},$$

where Avogadro's constant is 6.02×10^{23} , C = concentrations (ng/µL), and Mwt = base pairs $\times 660$ (Amaral et al., 2007; Sood et al., 2015).

The purified PCR production was serially diluted to obtain a standard series from 10^8 to 10^3 copies/µL with each dilution differing by 10-fold. Quantitative reverse-transcription (qRT)-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems Inc., Foster City, CA) with the SYBR Green QuantiTect RT-PCR Kit (TaKaRa Biotechnology Co. Ltd.). The qPCR-RT was conducted under the following conditions: initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s), and extension at 72°C for 5 min. Each reaction was run in triplicate for both standards and samples as well as for negative controls.

The standard curve was established by plotting the cycle threshold (Ct) on the y-axis and the natural log of concentration (copies/µL) on the x-axis. Only reactions with high efficiencies ($95\% < \text{efficiency} < 105\%$) were considered, and only standard curves with a Pearson correlation coefficient of at least 0.99 were taken into account. The total copy number of mtDNA or nDNA was calculated by relating the Ct value to the standard curve. The relative copy number of mtDNA was normalized to that of nDNA. Then, the mtDNA/nDNA of the mild fatty liver group was normalized to that of the control group.

TG Content Determination

Liver tissues (~20 mg) or hepatocytes were homogenized in 5% Triton-X100 (T8787; Sigma-Aldrich Co.) and then heated in a water bath (85°C) for 3 min. After cooling the sample at room temperature, the sample was vortexed and centrifuged at $2,000 \times g$ for 5 min at 4°C. The supernatant was collected and stored at -80°C before TG assay. The TG content was measured using an enzymatic kit (E1013; Applygen Technologies Inc.) following the manufacturer's instructions (<http://www.applygen.com/a/chanpinmulu/>)

Table 3. Primers used during quantitative reverse-transcription PCR assay

Gene ¹	Primers ² (5'-3')	GeneBank number	Length (bp)	Annealing temperature (°C)
<i>PPARA</i>	For: TCAGATGGCTCCGTTATT Rev: CCCGCAGATCCTACACT	XM_341399	132	60
<i>ACO</i>	For: TAAGCCTTTGCCAGGTATT Rev: ATGGTCCCGTAGGTCAG	NC_015500.1	189	60
<i>CPT1A</i>	For: ACG CCG TGA AGT ATA ACC CT Rev: CCAAAAATCGCTTGTCCCTT	NC_007330.4	188	60
<i>SREBP1c</i>	For: GCAGCCCATTCATCAGCCAGACC Rev: CGACACCACCAGCATCAACCACG	NM_001113302.1	119	60
<i>ACC1</i>	For: TGTGTAATATCCTCACGGAGCT Rev: CGACGTTTTCGGACAAGATGAGT	NM_174224.2	95	60
<i>FAS</i>	For: CAGCTTTGTGTTGGCAGAGAAG Rev: AGCGAGCTGTCCAGGTTGAC	NM_001012669.1	144	60
mtDNA	For: CGCGGTCATACGATTAACCC Rev: AACCCATATTTGGTATGGTGCTT	NM_U01920.1	74	60
nDNA	For: GGTCTGGCATCTTGTCCAT Rev: TGGCAGTGCAAATGAAAACTG	NM_178320.2	73	60
β -Actin	For: GCCGTGAGGCTCTCTTCCA Rev: GCGGATGTCGACGTCACA	BC142413.1	101	60
CO I	For: TATGGACTGGAACGGGAGAG Rev: GCTTCTTTGGACACTTGAGCA	NM_205817.1	162	60
CO II	For: CAGAACCTGATGCTTTGTGC Rev: ACTCGTCAACCCTCTCCTTG	NM_174178.2	106	60
CO III	For: TCTCATTTGCTTCGTCTTGC Rev: TCTGGTGCTGTGGTGACATT	NM_174630.2	197	60
CO IV	For: ATCTCGGGTTTTGGGTTGC Rev: GGGTGGTGGTCCAGGTTCTC	NM_001077831.2	334	60
CO V	For: TCCAGCCTGCCAGAGACTAT Rev: TGCACCTCCAGGGCATTTAG	NM_175796.3	148	60
<i>GAPDH</i>	For: GTCCTCACTACCATGGAGAAGG Rev: TCATGGATGACCTTGGCCAG	NM_001034034	197	60

¹*PPARA* = peroxisome proliferator-activated receptor- α ; *ACO* = acyl-CoA oxidase; *CPT1A* = carnitine palmitoyltransferase 1A; *SREBP1c* = sterol regulatory element-binding protein-1c; *ACC1* = acetyl CoA carboxylase 1; *FAS* = fatty acid synthase; mtDNA = mitochondrial DNA; nDNA = nuclear DNA; CO I = complex I; CO II = complex II; CO III = complex III; CO IV = complex IV; CO V = complex V.

²For = forward; Rev = reverse.

meixueyushenghuaceding/ganyousanzhic/2012/0905/276.html). Total protein concentration was estimated by the BCA method (P1511; Applygen Technologies Inc.).

qRT-PCR Assay

The total RNA of hepatocytes and liver tissues (~15 mg) was extracted using RNAiso Plus (TaKaRa Biotechnology Co. Ltd.) according to the manufacturer's instructions (<http://www.takarabiomed.com.cn/Download/9108-9109.pdf>). The RNA concentration and quality were measured using a K5500 microspectrophotometer (Beijing Kaiiao Technology Development Ltd.) and electrophoresis (1% agarose gels). Then, 1 μ g of total RNA in each sample was reverse-transcribed to cDNA in 20- μ L reactions using a reverse transcription kit (TaKaRa Biotechnology Co. Ltd.) according to the supplier's protocol (<http://www.takarabiomed.com.cn/Download/RR036A.pdf>). We evaluated mRNA expression level using qRT-PCR technology with the SYBR Green QuantiTect RT-PCR Kit (Ta-

KaRa Biotechnology Co. Ltd.) and a 7500 Real-Time PCR System (Applied Biosystems Inc.). The qRT-PCR was conducted as described in "Mitochondrial DNA Quantification." The relative expression of each target gene was normalized to 2 reference genes, β -actin and *GAPDH*, and calculated using the $2^{-\Delta\Delta C_t}$ method. For in vivo qRT-PCR experiments, the qRT-PCR reaction was performed in triplicate for each cow and 15 cows were included per group. For in vitro qRT-PCR experiments, the qRT-PCR reaction was performed 15 times in each group. The primers used for peroxisome proliferator-activated receptor- α (*PPARA*), acyl-CoA oxidase (*ACO*), carnitine palmitoyltransferase 1A (*CPT1A*), sterol regulatory element-binding protein-1c (*SREBP1c*), acetyl CoA carboxylase1 (*ACC1*), fatty acid synthase (*FAS*), β -actin, *GAPDH*, and *CO 1-V* are shown in Table 3. The cycles-to-threshold values of β -actin and *GAPDH* were not affected by liver fat content and PA treatment, findings that validated their usefulness as a control gene (Morey et al., 2011; Du et al., 2017a, 2018; Supplemental Figure S1, <https://doi.org/10.3168/jds.2018-14546>).

Western Blotting

Western blotting was performed as previously described (Du et al., 2017b; Sun et al., 2017). The liver (~30 mg) and hepatocyte total proteins were extracted using a protein extraction kit (C510003; Sangon Biotech Co. Ltd., Shanghai, China; http://www.sangon.com/productImage/DOC/C510003/C510003_ZH_P.pdf). Total protein concentration was estimated by the BCA method (P1511; Applygen Technologies Inc.). A total of 30 µg of protein from each sample was separated by 12% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 3% BSA/Tris-buffered saline/Tween (TBS-T) buffer for 4 h. The blocked membranes were incubated overnight at 4°C with primary antibodies against PPARα (1:1,000; ab8934), cytochrome c oxidase subunit IV (COX IV; 1:1,000; ab14744), voltage-dependent anion channel 1 (VDAC1; 1:1,000; ab15895), peroxisome proliferator activated receptor-γ coactivator-1α (PGC-1α; 1:1,000; ab54481), mitofusin-2 (MFN2; 1:1,000; ab56889), nuclear respiratory factor 1 (NRF1; 1:1,000; ab154269), β-actin (1:2,000; ab8226; Abcam, Cambridge, MA), mitochondrial transcription factor A (TFAM; 1:200; sc166965; Santa Cruz Biotechnology, Santa Cruz, CA), and SREBP-1c (1:500; NB100-2215; Novus Biologicals, Littleton, CO). The membranes were then washed with TBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig at room temperature for 45 min. Immunoreactive bands were visualized by enhanced chemiluminescence solution (Millipore, Bedford, MA). All bands were analyzed using Image-Pro Plus (Media Cybernetics). β-Actin was used as a reference protein in this study, and each band was related to β-actin.

Statistical Analysis

All experiments were repeated at least 3 times. In the in vivo studies, data from oil-red O staining quantification, hepatic TG content, Western blotting, and qRT-PCR were normally distributed and analyzed with paired *t*-tests; other data were not normally distributed and analyzed with Wilcoxon signed-rank test. In the in vitro studies, comparisons among groups were calculated using a 1-way ANOVA with subsequent Bonferroni correction. All analyses were performed using GraphPad Prism 5.0 (Graph Pad Software, San Diego, CA) or SPSS (Statistical Package for the Social Sciences) 19.0 software (IBM, Chicago, IL). Baseline characteristics are expressed as the median and interquartile range, and other data are expressed as the mean ± standard error of the mean; *P* < 0.05 was considered statistically significant.

RESULTS

Changes of Hepatic Lipid Synthesis and Oxidation Status in Healthy Cows and Dairy Cows with Mild Fatty Liver

As shown in Table 2, the milk production and blood concentration of glucose were lower (*P* < 0.05) in dairy cows with mild fatty liver than in healthy cows. Conversely, the BCS (*P* < 0.05), blood concentrations of fatty acids and BHB, and serum activities of aspartate aminotransferase and alanine aminotransferase were higher (*P* < 0.01) in dairy cows with mild fatty liver than in healthy cows (Table 2).

The results of hematoxylin-eosin and oil-red O showed that the liver of dairy cows with mild fatty liver displayed significant lipid accumulation (Figure 1A and B). Furthermore, increased lipid droplets were observed in the liver of dairy cows with mild fatty liver. The hepatic TG content of dairy cows with mild fatty liver (mean = 3.01% g/g of wet weight) was much higher (*P* < 0.01) than that of control cows (mean = 0.58% g/g of wet weight; Figure 1C). The protein (*P* < 0.05) and mRNA (*P* < 0.01) expression levels of hepatic SREBP-1c, a master regulator of lipogenic pathway, and the mRNA expression levels of *ACC1* (*P* < 0.01) and *FAS* (*P* < 0.05) were higher in dairy cows with mild fatty liver than in control cows (Figure 2A, B, and D; Supplemental Figure S2, <https://doi.org/10.3168/jds.2018-14546>). These data indicate that dairy cows with mild fatty liver display increased hepatic lipid synthesis compared with healthy cows.

Moreover, the protein (*P* < 0.05) and mRNA (*P* < 0.01) expression levels of PPARα and the mRNA expression levels of lipid oxidation genes, *ACO* and *CPT1A* (*P* < 0.05), were also significantly increased in the liver of dairy cows with mild fatty liver (Figure 2A, B, and C). These data indicate that dairy cows with mild fatty liver display increased lipid oxidation in the liver.

Hepatic Mitochondria Status in Healthy Cows and Dairy Cows with Mild Fatty Liver

The hepatic mtDNA content was increased (*P* < 0.05) in dairy cows with mild fatty liver (Figure 3A). Our results showed that the hepatic protein levels of COX IV (the inner mitochondrial membrane marker; *P* < 0.05) and VDAC1 (the outer mitochondrial membrane marker; *P* < 0.01) were increased in dairy cows with mild fatty liver compared with healthy cows (Figure 3B and C; Supplemental Figure S3, <https://doi.org/10.3168/jds.2018-14546>). The protein levels of mitochondrial function regulators, PGC-1α and NRF1,

were higher ($P < 0.05$) in the liver of dairy cows with mild fatty liver than in control cows (Figure 3B and C). However, the protein levels of MFN2 and TFAM were not significantly different between dairy cows with mild fatty liver and healthy cows (Figure 3B and C). In addition, we found that the mRNA levels of *CO 1-V* ($P < 0.05$) and ATP content ($P < 0.01$) were all increased in the livers of dairy cows with mild fatty liver compared with healthy cows (Figure 3D and E). More important, ultrastructural analysis revealed an increased number and volume of mitochondria in the liver of dairy cows with mild fatty liver (Figure 4). Taken together, these data demonstrate that number of mitochondria and

ATP production were increased in the liver of dairy cows with mild fatty liver.

Effects of PA on Lipid Synthesis and Oxidation in Calf Hepatocytes

We found that PA treatment increased the intracellular TG content gradually and was significantly higher in the 100 and 200 μM PA groups (Figure 5A). The oil-red O staining also showed similar results (Figure 5B and C). The mRNA and protein expression levels of SREBP-1c and PPAR α and mRNA expression levels of *ACO* and *CPT1A* were increased gradually and

Figure 1. Liver histology and triglyceride (TG) content in control cows and dairy cows with mild fatty liver. (A) Representative images of hematoxylin-eosin (H&E) and oil-red O staining of liver sections. (B) Quantification of oil-red O staining. (C) Hepatic TG content in control cows ($n = 15$) and dairy cows with mild fatty liver ($n = 15$). Data were analyzed with paired t -tests and expressed as mean $\pm$ SEM. Color version available online.

Figure 2. Hepatic lipid metabolic status in control cows and dairy cows with mild fatty liver. (A) Hepatic protein levels of peroxisome proliferator-activated receptor- α (PPAR α) and sterol regulatory element-binding protein 1c (SREBP-1c) in control cows ($n = 15$) and dairy cows with mild fatty liver ($n = 15$). Representative blots are shown. (B) Quantification of hepatic protein levels of PPAR α and SREBP-1c. (C) Relative hepatic mRNA levels of PPAR α , acyl-CoA oxidase (ACO), and carnitine palmitoyltransferase 1A (CPT1A) in control cows ($n = 15$) and dairy cows with mild fatty liver ($n = 15$). (D) Relative hepatic mRNA levels of SREBP1c, acetyl CoA carboxylase 1 (ACC1), and fatty acid synthase (FAS) in control cows ($n = 15$) and dairy cows with mild fatty liver ($n = 15$). Data were analyzed with paired t -tests and expressed as mean $\pm$ SEM.

significantly higher in the PA-treated groups than in the control group (Figure 5D, E, F, and G; Supplemental Figure S4, <https://doi.org/10.3168/jds.2018-14546>). Furthermore, the mRNA expression levels of ACC1 and FAS were significantly increased in 100 and 200 μ M PA-treated groups compared with the control group (Figure 5G). The expression changes of PPAR α and SREBP1c were similar between PA-treated adult bovine hepatocytes and calf hepatocytes (Supplemental Figure S5, <https://doi.org/10.3168/jds.2018-14546>). These results demonstrate that PA increases lipid synthesis and oxidation in calf hepatocytes.

Effects of PA on Mitochondria Status in Calf Hepatocytes

To investigate the effects of PA on mitochondria, calf and adult bovine hepatocytes were treated with different concentrations of PA. Following treatment with PA, the mtDNA content increased gradually in a PA dose-dependent manner, and it was significantly higher in the PA treatment groups than in the control group (Figure 6A; Supplemental Figure S6A, <https://doi.org/10.3168/jds.2018-14546>). The protein levels of COX IV and VDAC1 all increased significantly in the PA treatment groups (Figure 6B and C). Hepatocytes stained with MitoTracker Red CMXRos also showed

that mitochondria content was increased in the group treated with 200 μ M PA compared with the control group (Figure 6F and G). The protein levels of PGC-1 α , NRF1, and TFAM were significantly increased in the group treated with 200 μ M PA compared with the control group (Figure 6B and C). However, the protein level of MFN2 was significantly decreased in PA-treated groups (Figure 6B and C). Furthermore, our results showed that 100 and 200 μ M PA treatment increased the mRNA levels of CO 1-V (Figure 6D). More important, the intracellular ATP content was increased gradually and markedly higher in the 100 and 200 μ M PA treatment groups than in the control group (Figure 6E; Supplemental Figure S6B, <https://doi.org/10.3168/jds.2018-14546>). These data indicate that PA increase mitochondria content and ATP production in calf hepatocytes.

DISCUSSION

Major physiological, nutritional, metabolic, and immunological changes occur within the transition period, and tremendous metabolic and endocrine adjustments must be made as dairy cows move from late gestation to early lactation (Sundrum, 2015; McCarthy et al., 2016). Lipid mobilization of adipose tissue is an efficient physiological adaptation in dairy cows to support en-

Figure 3. Hepatic mitochondria status in control cows and dairy cows with mild fatty liver. (A) Relative hepatic mitochondrial DNA (mtDNA) content in control cows ($n = 15$) and dairy cows with mild fatty liver ($n = 15$). (B) Hepatic protein levels of peroxisome proliferator activated receptor- γ coactivator-1 α (PGC-1 α), mitofusin-2 (MFN2), mitochondrial transcription factor A (TFAM), nuclear respiratory factor 1 (NRF1), cytochrome c oxidase subunit IV (COX IV), voltage-dependent anion channel 1 (VDAC1), and β -actin in control cows ($n = 15$) and dairy cows with mild fatty liver ($n = 15$). Representative blots are shown. (C) Quantification of hepatic protein levels of PGC-1 α , MFN2, TFAM, NRF1, COX IV, and VDAC1. (D) Relative hepatic mRNA levels of complexes I to V (CO 1-V) in control cows ($n = 15$) and dairy cows with mild fatty liver ($n = 15$). (E) Hepatic ATP content in control cows ($n = 15$) and dairy cows with mild fatty liver ($n = 15$). Panels A and E were analyzed with Wilcoxon signed-rank test and expressed as mean $\pm$ SEM. Panels B, C, and D were analyzed with paired t -tests and expressed as mean $\pm$ SEM. nDNA = nuclear DNA.

ergy demands during the transition period (Ingvarsten and Moyes, 2013). Increased lipid mobilization results in a large increase in blood fatty acids concentration, and excessive blood fatty acids are taken up by the liver (Akbar et al., 2015; Sundrum, 2015). Therefore, the liver must adapt quickly to provide the increased energy demand and to process the flood of fatty acids taken up from extensive mobilization of adipose TG. Our data demonstrated that hepatic lipid synthesis and oxidation, number of mitochondria, and ATP production were enhanced in dairy cows with mild fatty liver, indicating that the hepatic adaptive capacity was increased in dairy cows with fatty liver.

Fatty liver develops when the hepatic synthesis of lipid exceeds the oxidation and secretion of lipids by the liver (Bobe et al., 2004). In this study, we observed that dairy cows with mild fatty liver displayed hepatic lipid accumulation. More important, SREBP-1c and its target genes *ACC1* and *FAS* were markedly enhanced in cows with mild fatty liver. The transcription factor SREBP-1c plays a crucial role in the regulation of lipid

synthesis by stimulating the expression of lipogenic genes, such as *ACC1* and *FAS* (Shimomura et al., 1999; Liu et al., 2014; Li et al., 2015). Our previous study also showed that the hepatic protein and mRNA levels of SREBP-1c, *ACC1*, and *FAS* were significantly higher in cows with hepatic steatosis (mild, moderate, and severe fatty liver) than in healthy cows (Li et al., 2015). Several studies indicated that mRNA abundances of hepatic *FAS* and *ACC1* were higher in early lactation than in late gestation (Graber et al., 2010; Gross et al., 2013). Consequently, increased SREBP-1c and its target gene expression reflect the increased lipogenesis and participate in the lipid accumulation in the liver of cows with mild fatty liver.

Energy demands increase approximately 2- to 3-fold in early-lactating cows (Drackley et al., 2001). Fatty acids can be used as energy substrate for β -oxidation in the mitochondria (Contreras and Sordillo, 2011). Peroxisome proliferator-activated receptor- α is a ligand-activated transcription factor that plays a key role in the regulation of the expression of fatty acid

Figure 4. Ultrastructural analysis of liver sections. (A) Hepatic ultrastructural analysis of control cows. (B, C) Hepatic ultrastructural analysis of dairy cows with mild fatty liver.

oxidation genes, including *CPT1A* and *ACO* (Ntambi et al., 2002; Lee et al., 2006; König et al., 2009). Our experiment found that the expression levels of *PPARα* and its target genes *ACO* and *CPT1A* were markedly increased in the liver of cows with mild fatty liver. Weber et al. (2013) observed increased hepatic mRNA expression of *PPARA* and *CPT1A* in dairy cows with high liver fat content, which was in agreement with our study. Our study also showed that dairy cows with mild fatty liver displayed higher blood concentration of BHB than healthy cows. Interestingly, the study from van Dorland et al. (2009) indicated that dairy cows with high blood concentration of BHB displayed increased

hepatic mRNA expression of *PPARA* and *CPT1A* compared with dairy cows with low blood concentration of BHB in early lactation, which further supported our results. Taken together, previous studies and our data demonstrated that dairy cows with mild fatty liver displayed increased hepatic lipid oxidation and adaptive performance.

Mitochondria produce about 90% of the cellular ATP, and many metabolic processes occur in mitochondria, including β -oxidation and ketogenesis (Sunny et al., 2017). Our previous study demonstrated that dairy cows with severe fatty liver displayed mitochondria dysfunction (Gao et al., 2018). However, few studies in-

Figure 5. Effects of palmitic acids (PA) on lipid metabolism in calf hepatocytes. Calf hepatocytes were treated with various concentrations of PA (0, 50, 100, and 200 μ M) for 12 h. (A) Triglyceride (TG) content in calf hepatocytes. (B) Oil-red O staining of calf hepatocytes. (C) Quantification of oil-red O staining. (D) Protein levels of peroxisome proliferator-activated receptor- α (PPAR α), sterol regulatory element-binding protein 1c (SREBP-1c), and β -actin in calf hepatocytes. Representative blots are shown. (E) Quantification of protein levels of PPAR α and SREBP-1c. (F) Relative mRNA levels of *PPARA*, acyl-CoA oxidase (*ACO*), and carnitine palmitoyltransferase 1A (*CPT1A*) in calf hepatocytes. (G) Relative mRNA levels of *SREBP1c*, acetyl CoA carboxylase 1 (*ACC1*), and fatty acid synthase (*FAS*) in calf hepatocytes. Comparisons among groups were calculated using a 1-way ANOVA with subsequent Bonferroni correction. Data were expressed as mean $\pm$ SEM. * P < 0.05; ** P < 0.01. Color version available online.

investigated hepatic mitochondria function in dairy cows with mild fatty liver. The copy number of the mtDNA and the expression of COX IV and VDAC1 reflect the

abundance of mitochondria (Laubenthal et al., 2016). In our study, we first found that hepatic mtDNA and protein levels of COX IV and VDAC1 were increased in

Figure 6. Effects of palmitic acids (PA) on mitochondria in calf hepatocytes. Calf hepatocytes were treated with various concentrations of PA (0, 50, 100, and 200 μ M) for 12 h. (A) Relative mitochondrial DNA (mtDNA) content in calf hepatocytes. (B) Protein levels of peroxisome proliferator activated receptor- γ coactivator-1 α (PGC-1 α), mitofusin-2 (MFN2), mitochondrial transcription factor A (TFAM), nuclear respiratory factor 1 (NRF1), cytochrome c oxidase subunit IV (COX IV), voltage-dependent anion channel 1 (VDAC1), and β -actin in calf hepatocytes. Representative blots are shown. (C) Quantification of protein levels of PGC-1 α , MFN2, TFAM, NRF1, COX IV, and VDAC1. (D) Relative mRNA levels of complexes I to V (CO I-V) in calf hepatocytes. (E) Adenosine triphosphate (ATP) content in calf hepatocytes. (F) MitoTracker (Life Technologies, Carlsbad, CA) staining of calf hepatocytes. DAPI was used to stain nuclei and indicated nuclei in (F). Merge indicated that nuclei and MitoTracker staining were combined into one figure. Comparisons among groups were calculated using a 1-way ANOVA with subsequent Bonferroni correction. Data were expressed as mean $\pm$ SEM. * P < 0.05; ** P < 0.01. nDNA = nuclear DNA. Color version available online.

dairy cows with mild fatty liver. In addition, ultrastructural analysis revealed an increased number and volume of mitochondria in the liver of dairy cows with mild fatty liver. Chiche et al. (2010) reported that enlarged mitochondria were functional as they conserved their ATP production. Our results also demonstrated that dairy cows with mild fatty liver displayed increased hepatic ATP content. These results demonstrate that the number of mitochondria and ATP production are increased in the liver of dairy cows with mild fatty liver. More important, these changes in mitochondria indicate that hepatic mitochondria of dairy cows with mild fatty liver can metabolize more fatty acids and provide more energy to rescue the energy deficit and adapt to metabolic changes in the transition period.

In this study, the hepatic lipid synthesis, TG content, lipid oxidation, and mitochondrial function were all higher in cows with mild fatty liver than in healthy cows. This contradictory phenomenon results from high concentrations of fatty acids in cows with mild fatty liver. Dairy cows in the transition period generally have negative energy balance and high concentrations of fatty acids and display lipotoxicity, which is defined as lipid- and lipid metabolite-induced lean tissue dysfunction (Weber et al., 2013; Ertunc and Hotamisligil, 2016). Therefore, to provide energy and defeat fatty acid-induced lipotoxicity, the liver must enhance mitochondrial function and lipid oxidation. Nevertheless, when enhanced mitochondrial function and lipid oxidation could not metabolize overloaded fatty acids, fatty acids would re-esterify and be stored as TG in the liver. This may explain the discrepancy that dairy cows with mild fatty liver had increased hepatic lipid oxidation and enhanced mitochondrial function and lipid synthesis.

Increased number of mitochondria and ATP production reflect enhanced adaptive capacity of dairy cows with mild fatty liver; however, the detail molecular mechanisms underlying this adaptation are not clearly understood. Peroxisome proliferator activated receptor- γ coactivator-1 α coordinates numerous genes needed for mitochondrial function and biogenesis by regulating the activity of several nuclear transcription factors, such as NRF1 and TFAM (Laubenthal et al., 2016, 2017). In our study, the protein expression of hepatic PGC-1 α , NRF1, and TFAM was increased in dairy cows with mild fatty liver. We speculated that upregulation of PGC-1 α , NRF1, and TFAM might promote the increased number of mitochondria and ATP production. However, our previous study indicated that protein level of PGC-1 α was reduced in the liver of dairy cows with moderate or severe fatty liver compared with healthy cows (Du et al., 2017c). The discrepancies between these studies could be due to differences in the

extent of fatty liver, and the adaptive capacity of hepatic mitochondrial function may have decreased in dairy cows with moderate or severe fatty liver. The oxidative phosphorylation complexes system forms the basis for mitochondrial ATP production (Boekema and Braun, 2007). In this study, we first found that mRNA expression of oxidative phosphorylation complexes (*CO I-V*) was increased in the liver of dairy cows with mild fatty liver, which is consistent with ATP content. Increased gene expression of *CO I-V* also reflects enhanced ATP production. Several studies reported that the expression and activities of oxidative phosphorylation complexes were increased in the liver of patients and mice with simple fatty liver or type 2 diabetes (Szendroedi et al., 2011; Begriche et al., 2013). These studies and our study also indicate that mitochondrial adaptation may exist in different species. Taken together, increased expression of mitochondrial function regulator and oxidative phosphorylation complexes contributes to the enhanced number of mitochondria and ATP production, which further increase the adaptive capacity of dairy cows with mild fatty liver.

Fatty acids increase sharply in the serum and liver of dairy cows after parturition and are metabolized to provide energy (Rukkwamsuk et al., 2000). However, fatty acids also displayed lipotoxicity in liver tissues (Bobe et al., 2004). Increased lipid oxidation and mitochondrial function may be an adaptive mechanism to defeat increased fatty acids concentration. Therefore, to further investigate the adaptive mechanism of mitochondria, the status of lipid metabolism and mitochondria in PA-treated calf hepatocytes was measured. We found that PA treatment could increase the expression of SREBP-1c and its target genes *ACC1* and *FAS* and increase TG accumulation in calf hepatocytes. Furthermore, PA treatment induced the augmented expression of PPAR α , *ACO*, and *CPT1A* in calf hepatocytes. More important, the number of mitochondria and ATP production; the protein expression of PGC-1 α , NRF1, and TFAM; and the mRNA expression of *CO I-V* all increased in calf hepatocytes treated with PA. Mitofusin-2 is a mitochondrial membrane protein that participates in mitochondria fusion, and decreased of MFN2 expression was accompanied by reduced mitochondria fusion events and increased mitochondria content (Zorzano et al., 2015). Our results showed that PA treatment significantly decreased protein expression of MFN2 and increased mitochondria content in calf hepatocytes, which is consistent with a previous study (Zorzano et al., 2015). In summary, these data indicate that hepatocytes treated with PA trigger adaptive changes, such as increased lipid synthesis and oxidation, and enhanced number of mitochondria and ATP production, which is consistent with hepatic adaptive

performance in dairy cows with mild fatty liver. In addition, increased hepatic mitochondria function is an effective mechanism to metabolize excessive fatty acids and provide energy in dairy cows with mild fatty liver.

CONCLUSIONS

The mRNA expression of lipid synthesis genes *SREBP1c*, *ACC1*, and *FAS* and lipid oxidation genes *PPARA*, *ACO*, and *CPT1A* was higher in the liver of dairy cows with mild fatty liver than in healthy cows, indicating increased hepatic lipid synthesis and oxidation. More important, the hepatic mitochondria content, mRNA levels of oxidative phosphorylation complexes, and ATP content increased in the liver of dairy cows with mild fatty liver compared with healthy cows, indicating enhanced hepatic mitochondria function. The same results were also observed in PA-treated calf hepatocytes. These changes in hepatic mitochondria and lipid metabolism may be the adaptive mechanism of dairy cows with mild fatty liver. Our study contributes to the understanding of hepatic energy metabolism around parturition, which may help develop strategies for enhancing hepatic metabolic adaptation during the transition period.

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