Low abundance of mitophagy markers is associated with reactive oxygen species overproduction in cows with fatty liver and causes reactive oxygen species overproduction and lipid accumulation in calf hepatocytes

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ABSTRACT

Mitochondria are the main site of fatty acid oxidation and reactive oxygen species (ROS) formation. Damaged or dysfunctional mitochondria induce oxidative stress and increase the risk of lipid accumulation. During the process of mitophagy, PTEN induced kinase 1 (PINK1) accumulates on damaged mitochondria and recruits cytoplasmic Parkin to mitochondria. As an autophagy receptor protein, sequestosome-1 (p62) binds Parkin-ubiquitinated outer mitochondrial membrane proteins and microtubule-associated protein 1 light chain 3 (LC3) to facilitate degradation of damaged mitochondria. In nonruminants, clearance of dysfunctional mitochondria through the PINK1/Parkin-mediated mitophagy pathway contributes to reducing ROS production and maintaining metabolic homeostasis. Whether PINK1/Parkin-mediated mitophagy plays a similar role in dairy cow liver is not well known. Thus, the objective of this study was to investigate mitophagy status in dairy cows with fatty liver and its role in free fatty acid (FFA)-induced oxidative stress and lipid accumulation. Liver and blood samples were collected from healthy dairy cows (n = 10) and cows with fatty liver (n = 10) that had a similar number of lactations (median = 3, range = 2 to 4) and days in milk (median = 6 d, range = 3 to 9 d). Calf hepatocytes were isolated from 5 healthy newborn female Holstein calves (1 d of age, 30–40 kg). Hepatocytes were transfected with small interfering RNA targeted against PRKN for 48 h, followed by treatment with FFA (0.3 or 1.2 mM) for 12 h. Mitochondria were isolated from fresh liver tissue or calf hepatocytes. Serum concentrations of β-hydroxybutyrate were higher in dairy cows with fatty liver. Hepatic malondialdehyde (MDA) and hydrogen peroxide (H2O2) were greater in cows with fatty liver. The lower protein abundance of PINK1, Parkin, p62, and LC3-II in hepatic mitochondrial fraction of dairy cows with fatty liver indicated the mitophagy was impaired. In hepatocytes, knockdown of PRKN decreased protein abundance of p62 and LC3-II in the mitochondrial fraction, and increased contents of triacylglycerol (TG), MDA, and H2O2. In addition, protein abundances of PINK1, Parkin, p62, and LC3-II were lower in the mitochondrial fraction from hepatocytes treated with 1.2 mM FFA than the hepatocytes treated with 0.3 mM FFA, whereas the content of TG, MDA, and H2O2 increased. In 1.2 mM FFA-treated hepatocytes, PRKN overexpression increased protein abundance of p62 and LC3-II in the mitochondrial fraction and decreased contents of TG, MDA, and H2O2. Together, our data demonstrate that low abundance of mitophagy markers is associated with ROS overproduction in dairy cows with fatty liver and impaired mitophagy induced by a high concentration of FFA promotes ROS production and lipid accumulation in female calf hepatocytes.

Key words: autophagy, hepatocyte, mitochondria, transition period, ROS

INTRODUCTION

Fatty liver occurs frequently in high-yielding dairy cows (Starke et al., 2011; Ringseis et al., 2015), and its pathological basis rests primarily on lipolysis dysregulation of adipose tissue that leads to increased circulating free fatty acids (FFA) for extended periods of time (Janovick et al., 2011). However, a high influx of FFA into the liver results in excessive accumulation
of triacylglycerol (TG) and causes fatty liver (Grummer, 1993), which decreases milk yield and increases the risk of removal and economic losses (Bobe et al., 2004). Thus, exploring mechanisms underlying the pathogenesis of fatty liver is important for prevention and treatment of fatty liver in dairy cows during the peripartal period.

Regulation of hepatic FFA β-oxidation is largely dependent on mitochondria, the primary organelle responsible for cellular adenosine triphosphate production (Bongard et al., 2015). Despite its high capacity for handling FFA, at high levels these molecules can cause lipotoxicity and impair hepatic mitochondrial function (Gao et al., 2018; Qin et al., 2019; Zhang et al., 2020). High levels of FFA in cells render the mitochondria prone to overproduction of reactive oxygen species (ROS; Zorov et al., 2014), which can lead to mitochondrial damage (Sanz et al., 2006) and aggrivate hepatic lipidosis (Rector et al., 2010). Thus, timely elimination of damaged mitochondria contributes to reduction in ROS production and helps maintain lipid metabolism homeostasis (Rector et al., 2010; Zhao et al., 2021; Cioffi et al., 2022).

In nonruminants, damaged mitochondria are mainly removed via the PTEN induced kinase 1 (PINK1)/Parkin-mediated mitophagy pathway, a specialized form of autophagy (Williams et al., 2015; Yamada et al., 2019; Zhou et al., 2019). The protein PINK1 accumulates on the outer membrane of damaged mitochondria, and activates and recruits the E3 ubiquitin ligase Parkin to dysfunctional mitochondria. Subsequently, Parkin ubiquitinates outer mitochondrial membrane (OMM) proteins to trigger mitophagy (Matsuda et al., 2010; Vives-Bauza et al., 2010). During this process, sequesosomes-1 (p62), acting as a bridge between the autophagic cargo and autophagosome, is recruited to the mitochondria by ubiquitinated OMM proteins (Geisler et al., 2010). In addition, p62 contains a microtubule-associated protein light chain 3 (LC3)-interacting region motif that is recognized by autophagosome marker LC3 to recruit the tagged mitochondria to the autophagosome (Lazarou et al., 2015). Autophagosomes then fuse with lysosomes forming an autolysosomal unit in which the damaged mitochondrial cargo is degraded (Figure 1).

Dairy cows with fatty liver are characterized by hepatic steatosis (Jorritsma et al., 2001; Bobe et al., 2004). Studies have reported that oxidative stress contributes to development of fatty liver in murine and human (Gao et al., 2020, 2022; Pereira et al., 2022).
Studies with bovine hepatocytes have revealed that treatment with FFA induces lipid accumulation and oxidative stress (Gao et al., 2018; Wang et al., 2022). Furthermore, high concentrations of FFA reduced the colocalization of PINK1, LC3, and mitochondria in alpha mouse liver 12 and human hepatocellular carcinoma cells and suppressed autophagy activity in calf hepatocytes (Li et al., 2020; Gao et al., 2021; Shen et al., 2021a). More importantly, studies have revealed that defective mitophagy renders mice more susceptible to diet-induced hepatic oxidative stress and steatosis (Williams et al., 2015; Edmunds et al., 2020). Based on those studies, we hypothesized that high concentrations of FFA impair PINK1/Parkin-mediated mitophagy, which contributes to hepatic oxidative stress and steatosis in dairy cows with fatty liver. Thus, the objective of this study was to investigate (1) the hepatic status of mitophagy in dairy cows with fatty liver and (2) the role of mitophagy in the process of FFA-induced oxidative stress and lipid accumulation using calf hepatocytes as a model.

**MATERIALS AND METHODS**

**Animals**

The protocol for the current study was approved by the Jilin University Ethics Committee on the Use and Care of Animals (Changchun, China). Cows for the present study were selected from a dairy farm located in Changchun, Jilin Province, China, and underwent a thorough routine physical examination to ensure the chosen cohort was only afflicted with fatty liver. All cows were housed in a tiestall barn and offered a TMR at 0730 and 1300 h daily for ad libitum intake with free access to tap water. The basal diet formulation is described in a previous study (Shen et al., 2021b). Dairy cows had a similar number of lactations (median = 3, range = 2–4) and DIM (median = 6 d, range = 3–9 d). Considering that dairy cows with fatty liver have high circulating concentrations of FFA (Rukkwamsuk et al., 2000; Contreras et al., 2010), we performed a preliminary screen based on serum FFA using a Hitachi 3110 autoanalyzer (Hitachi) with commercially available kits (cat. no. FA115; Randox Laboratories). The limit of quantification was 0.072 to 9.2%.

Cows were milked twice daily at 0530 and 1500 h, and milk yield was recorded for 3 consecutive days. Blood samples were collected between 0730 and 0830 h (before feeding) by a coccyleal venipuncture; then the serum was separated after centrifugation at 3,500 × g for 15 min at 4°C. Serum was obtained and stored at −80°C until analysis. Cows with serum FFA concentration >0.6 mM were preclassified as having fatty liver, and those with serum FFA content <0.6 mM were preclassified as healthy.

After blood sample collection, the liver tissue samples were collected from the 11th or 12th right intercostal space by liver puncture needle (Shanghai Surgical Equipment Factory). To obtain liver tissue samples, the intercostal space of cows was shaved, sanitized with iodine scrub and 75% alcohol, and anesthetized with subcutaneous injection of 2% lidocaine HCl (1366013; Sigma-Aldrich Co.; United States Pharmacopeia Reference Standard). A scalpel blade was used to make a 3-mm stab incision in the skin. Collected samples (~200 mg) were immediately frozen in liquid nitrogen. A portion of the liver samples was used to classify the animals, and the rest was used for detection of biochemistry indexes, genes, and proteins.

Measurement of liver TG is the gold standard for diagnosing and staging fatty liver in dairy cows (Bobe et al., 2004). Thus, we used liver tissue after homogenizing in radioimmunoprecipitation assay lysis buffer (C1053; Applygen Technologies Inc.) to determine total protein concentration using the bicinchoninic acid (BCA) assay (P1511; Applygen Technologies Inc.); additionally, a separate portion of supernatant was heated in a water bath (70°C) for 10 min. After cooling at room temperature, samples were vortexed and centrifuged at 2,000 × g for 5 min at 4°C. The supernatant was collected and used for TG assay using an enzymatic kit (E1013; Applygen Technologies Inc.) according to the manufacturer’s instructions. In 10 healthy Holstein cows, hepatic TG content is less than 1% (% g/g of wet weight). In 10 Holstein cows with fatty liver, hepatic TG content is higher than 5%. The intra-assay CV for TG was 3.4%. The BW of each cow was measured via weigh-bridge 1 d before liver biopsy. Body condition score of each cow was assigned based on a 1- to 5-point scale according to previous reports (Edmonson et al., 1989). The basic description of the cows is reported in Supplemental Table S1 (https://doi.org/10.6084/m9.figshare.19681521.v1; Fang et al., 2022).

**Determination of Blood Parameters**

According to the manufacturer’s instructions, serum concentrations of BHB and glucose were determined using a Hitachi 3110 autoanalyzer (Hitachi) with commercially available kits (BHB, cat. no. RB1008; glucose, cat. no. GL3815; Randox Laboratories). For the BHB and glucose, the limits of quantification are 0.1 to 5.75 mM and 0.335 to 34.1 mM, respectively. The intra-assay CV for BHB and glucose were 7.8 and 8.9%, respectively.
**Hepatocyte Isolation, Culture, and Identification**

A total of 5 Holstein calves (1 d old, female, 30–40 kg, and fasting) were used in the present study. Methods for isolation of hepatocytes were described in a previous study (Shen et al., 2021a). Briefly, a scalpel was used to obtain the caudate lobe of the liver from the calf. Subsequently, 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) was used to perfuse the liver at a flow rate of 50 mL/min for 12 min. Then 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 outflow liquid became transparent. Subsequently, liver was digested with collagenase IV solution (cat. no.17104019; Gibco; 68 units/mL, pH 7.2–7.4, 37°C) at a flow rate of 20 mL/min for 15 to 20 min. The liver was moved to a sterile flat plate after digestion, and fetal bovine serum (FBS; Hyclone Laboratories) was added to terminate collagenase digestion.

Next, the liver was cut open to release hepatocytes, and the liver capsule, blood vessels, and connective tissue were removed. The tissue suspension was filtered sequentially with 100-mesh (150 μm) and 200-mesh (75 μm) cell sieves. The hepatocyte suspension was then washed twice in RPMI-1640 basic medium containing 10% FBS and 1% penicillin/streptomycin. Hepatocytes were seeded into 6-well tissue culture plates at 1 × 10⁶ cells/mL using adherent medium (RPMI-1640 basic medium supplemented with 10% FBS, 10⁻⁶ mol/L of insulin, 10⁻⁶ mol/L of dexamethasone, 10 μg/mL of vitamin C, 100 U/mL penicillin, and 100 μg/mL streptomycin) and incubated at 37°C in 5% CO₂. After isolation, hepatocytes were seeded into 6-well tissue culture plates at 1 × 10⁶ cells/mL using adherent medium (RPMI-1640 basic medium supplemented with 10% FBS, 10⁻⁶ mol/L of insulin, 10⁻⁶ mol/L of dexamethasone, 10 μg/mL of vitamin C, 100 U/mL penicillin, and 100 μg/mL streptomycin) and incubated at 37°C in 5% CO₂. After 4 h, hepatocytes began to adhere and spread, and the medium was replaced with growth medium (RPMI-1640 basic medium containing 10% FBS and 1% penicillin/streptomycin). Cells that failed to adhere were removed during medium exchange. Growth medium was replaced every 24 h.

To characterize isolated hepatocytes, cytokeratin 18 (CK 18) immunofluorescence staining was performed and the morphology of hepatocytes were observed under a phase contrast microscope (Leica DMI6000 B; Leica Microsystems).

**Immunofluorescence Assay**

The cells were fixed with 4% paraformaldehyde (P1110; Solarbio) for 20 min. Antigen retrieval was performed using EDTA-Na₂ at 95°C. After that, the cells were permeabilized using 0.1% Triton X-100 (P1081; Solarbio) for 10 min. Then, cells were incubated with an anti-CK 18 antibody (1:500; ab219271; Abcam) diluted in PBS containing 5% goat serum overnight at 4°C. Next, the cells were incubated with secondary antibody conjugated to fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200; 111-095-003; Jackson ImmunoResearch Laboratories) for 45 min. The nuclear was stained with 4′,6-diamidino-2-phenylindole (C0065; Solarbio). The samples were imaged using laser confocal microscopy (Fluoview FV1200, version 4.2; Olympus).

**FFA Preparation**

The concentrations of FFA used in this study were based on the blood concentrations of FFA in dairy cows with fatty liver (Rukkwamsuk et al., 2000; Contreras et al., 2010). The FFA mixture was prepared according to a previous study (Du et al., 2018a). Briefly, the stock fatty acid solution was prepared by diluting the fatty acid components in 0.1 M KOH at 60°C; then the pH of the solution was adjusted to 7.4 with hydrochloric acid (1 M). The stock fatty acid (52.7 mM) solution included oleic acid (22.9 mM, 43.45%; Sigma-Aldrich Co.), linoleic acid (2.6 mM, 4.93%; Sigma-Aldrich Co.), palmitic acid (16.8 mM, 31.89%; Sigma-Aldrich Co.), stearic acid (7.6 mM, 14.42%; Sigma-Aldrich Co.), and palmitoleic acid (2.8 mM, 5.31%; Sigma-Aldrich Co.). The stock solution of FFA was diluted in RPMI-1640 basic medium containing 2% bovine serum albumin (V900933; Sigma-Aldrich Co.) to prepare a working solution.

**Hepatocytes Treatment**

Specific small interfering RNA for bovine PRKN (NM_001199065.1) was designed based on the bovine PRKN mRNA sequence (NM_001199065.1). The small interfering RNA of PRKN (siPRKN; synthesized by GenePharma Co.) sequences used are as follows: sense 5'-GGUGAAUGUCAGUCUCAATT-3' and antisense 5'UUUGAGACUGACUAUCCACCTTT-3'. The negative control (siControl) was purchased from GenePharma (A06001), and its sequences were as follows: sense 5'-UUCUCGAGCCGAGUCACGAGTTT-3' and antisense 5'-ACGUGACAGUCCGAGAGATT-3'. The PRKN overexpressed and control plasmids were constructed by Wuhan Miaoling Biotechnology Co. Ltd. Full-length bovine PRKN (NM_001199065.1) was subcloned into the pcDNA3.1-EGFP vector (P13688) to construct pcDNA3.1-EGFP-PRKN. The determination of transfection efficiency is shown in Supplemental Figure S1 (https://doi.org/10.6084/m9.figshare.19681521.v1; Fang et al., 2022).

The entire in vitro experiment was divided into 2 sections: (1) calf hepatocytes were transfected with
Determination of Malondialdehyde Content

Contents of malondialdehyde (MDA) in liver tissue and hepatocytes were measured using thiobarbituric acid reactive substances assay by a spectrophotometric diagnostic kit (A003-I; Nanjing Jian Cheng Institute of Bioengineering), according to the manufacturer’s protocols. Briefly, liver tissue or hepatocytes were homogenized with radioimmunoprecipitation assay lysis buffer and centrifuged at 12,000 × g for 15 min at 4°C, and the supernatant was collected for analysis. A standard solution (0.15 mL) was added to each sample followed by thiobarbituric acid (2.5 mL), shaking, and incubation at 95°C for 40 min. Then, each specimen was cooled to room temperature and centrifuged at 1,125 × g for 15 min. Finally, the supernatant in each tube underwent a colorimetric assay at 532 nm. Total protein concentration was measured using the BCA method. To avoid the effects of the liver tissue weight or the number of cells on MDA content, MDA concentration was normalized to mass of protein and expressed as nanomoles per milligram of protein. The intra-assay CV for MDA was 9.7%.

Determination of Hydrogen Peroxide (H_2O_2) Content

Content of H_2O_2 in liver tissue and hepatocytes was measured using a spectrophotometric diagnostic kit (A064; Nanjing Jian Cheng Institute of Bioengineering) according to the manufacturer’s protocols. Briefly, liver tissue or hepatocytes were homogenized in ice-cold mitochon-
dria isolation buffer with 1 mM phenylmethylsulfonyl

Isolation of Mitochondria

Mitochondria were isolated from fresh liver tissue or hepatocytes using a mitochondrion isolation kit (C3606; Beyotime Institute of Biotechnology) according to the manufacturer’s instructions. Briefly, tissue or hepatocytes were homogenized in ice-cold mitochondria isolation buffer with 1 mM phenylmethylsulfonyl
fluoride and centrifuged at 1,000 × g for 5 min at 4°C. Subsequently, the supernatants were transferred to another centrifuge tube and centrifuged at 3,500 × g for 10 min at 4°C, and the sediment was mitochondria. Extracted mitochondria were treated in ice-cold mitochondrial lysis buffer with phenylmethylsulfonyl fluoride and then centrifuged at 12,000 × g for 10 min at 4°C to obtain mitochondrial protein. Concentrations of mitochondrial protein were determined by the BCA (P1511; Applygen Technologies Inc.) method according to the manufacturer’s instructions.

### Western Blotting

In the present study, all the antibodies were validated by using positive and negative controls as manufacturer’s instruction suggested: HeLa cells (TCHu187; Cell Bank of the Chinese Academy of Sciences) were used as positive controls for p62, LC3, and β-tubulin; the SQSTM1 knockout 293T cell lysate (RM01791; Abclonal), MAP1LC3B knockout 293T cell lysate (RM01782; Abclonal), and mitochondrial lysate were used as negative controls for antibodies p62, LC3, and β-tubulin, respectively; HepG2 cells (SCSP-510; Cell Bank of the Chinese Academy of Sciences) were used as positive controls for PINK1 and complex IV (COX IV); PINK1 knockout 293T cell lysate (ab266393; Abcam) and COX4II knockout 293T cell lysate (RM02354; Abclonal) were used as negative controls for PINK1 and COX IV, respectively; 293T cells (SCP-502; Cell Bank of the Chinese Academy of Sciences) and HeLa cells (TCHu187; Cell Bank of the Chinese Academy of Sciences) were used as positive and negative controls for Parkin, respectively.

A total of 30 μg of protein from each sample was separated on 10 to 15% Tris-glycine gels with a known prestained protein ladder (26616; Thermo Fisher Scientific). Membranes were blocked in 3% BSA/Tris-buffered saline/Tween buffer for 4 h at room temperature. Blocked membranes were incubated overnight at 4°C with primary antibodies against PINK1 (1:1,000; sc-517353; Santa Cruz Biotechnology), p62 (1:1,000; ab101266; Abcam), Parkin (1:5,000; ab15954; Abcam), LC3 (1:1,000; ab48394; Abcam), COX IV (1:1,000; ab14744; Abcam), and β-tubulin (1:5,000; ab78078; Abcam). Membranes were washed with Tris-buffered saline/Tween buffer and incubated with horseradish peroxidase-conjugated anti-rabbit (1:5,000; ab205718; Abcam) or anti-mouse (1:5,000; ab205719; Abcam) Ig at room temperature for 45 min. Finally, immunoreactive bands were visualized by enhanced chemiluminescence solution (WBULS0500; Millipore). All bands were analyzed using Image-Pro Plus 6.0 (Media Cybernetics) and target proteins normalized to COX IV, which is stably expressed in the inner membrane of mitochondria and used previously as an internal control (Dalmonte et al., 2009; Cheng et al., 2021; Martin-Sancho et al., 2021).

### Statistical Analysis

In vivo results, normally distributed, were analyzed using parametric statistical analysis with an independent sample t-test; moreover, for data with non-normal distribution, non-parametric statistical analysis was performed using the Wilcoxon test. Results from silencing of PRKN were analyzed with an independent sample t-test; additionally, other in vitro results were analyzed using 2-way ANOVA followed by Tukey’s tests. All analyses were performed using GraphPad Prism 8.0 (Graph Pad Software) or SPSS software 23.0 (IBM). Data are expressed as means ± standard error of the mean, P < 0.05 was considered statistically significant, and P < 0.01 was considered extremely significant.

## RESULTS

### Performance, Blood Parameters, and Status of Hepatic ROS Content

We screened healthy cows and cows with fatty liver according to their serum concentrations of FFA (Supplemental Table S1) and hepatic TG contents (Figure 2A). The results show that BW of cows with fatty liver was higher than that of healthy cows (Supplemental Table S1; P < 0.01), whereas DMI, BCS, and milk yield did not differ (Supplemental Table S1). In addition, cows with fatty liver displayed higher concentrations of BHB (Supplemental Table S1; P < 0.01). Furthermore, hepatic MDA and H2O2 content in the liver of cows with fatty liver was greater than that in healthy cows (Figure 2B and C; P < 0.01).

### CK 18 Immunofluorescence Staining and Morphology of Hepatocytes

The results of immunofluorescence staining and morphology of hepatocytes showed that all cells were positive for CK 18 staining (Figure 3A) and displayed an epithelioid shape (Figure 3B).

### Abundance of Mitophagy-Related Proteins in Hepatic Mitochondrial Fractions

Protein abundance of PINK1 and Parkin were lower in hepatic mitochondrial fractions from cows with fatty liver (Figure 4A–C; P < 0.01). In addition, cows with fatty liver displayed lower protein abundance of p62.
and LC3-II in hepatic mitochondrial fractions (Figure 4A, D, and E; \( P < 0.01 \)).

**Effects of Silencing of PRKN on Mitophagy, Lipid Accumulation, and Oxidative Stress**

Transfection with siPRKN downregulated mRNA abundance of \( PRKN \) (Figure 5A; \( P < 0.01 \)). Silencing of \( PRKN \) downregulated protein abundance of p62 and LC3-II in the mitochondrial fraction from calf hepatocytes (Figure 5B–D; \( P < 0.01 \)). In contrast, TG content was greater in the siPRKN group than the siControl group (Figure 5E; \( P < 0.01 \)). Knockdown of \( PRKN \) also increased MDA and \( H_2O_2 \) content in calf hepatocytes (Figure 5F and G; \( P < 0.01 \)).

**Effects of High FFA on Mitophagy, Lipid Accumulation, and Oxidative Stress**

Protein abundances of PINK1 and Parkin were lower in mitochondrial fractions from calf hepatocytes treated with 1.2 mM FFA than in hepatocytes treated with 0.3 mM FFA (Figure 6A–C; \( P < 0.01 \)). Compared with the 0.3 mM FFA group, protein abundance of p62 and LC3-II were lower in mitochondrial fractions from calf hepatocytes treated with 1.2 mM FFA (Figure 6A, D, and E; \( P < 0.01 \)).

In calf hepatocytes, treatment with 1.2 mM FFA increased the content of TG, MDA, and \( H_2O_2 \) (Figure 6F–H; \( P < 0.01 \)).

**Effects of PRKN Overexpression on Mitophagy, Lipid Accumulation, and Oxidative Stress**

Transfection with pcDNA3.1-EGFP-PRKN upregulated protein abundance of Parkin in the mitochondrial fraction from calf hepatocytes treated with 0.3 mM FFA (Figure 6A and C; \( P < 0.01 \)). Furthermore, in the presence of 0.3 mM FFA, protein abundance of PINK1, p62, and LC3-II in mitochondrial fractions of calf hepatocytes transfected with pcDNA3.1-EGFP-PRKN were greater than the pcDNA3.1-EGFP transfection group (Figure 6A, B, D, and E; \( P < 0.01 \)), whereas the TG (Figure 6F), MDA, and \( H_2O_2 \) content was lower (Figure 6G and H; \( P < 0.05 \)).

In the presence of 1.2 mM FFA, \( PRKN \) overexpression increased protein abundance of PINK1, p62, and

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**Figure 2.** Triacylglycerol (TG) content and status of oxidative stress in the liver. (A) Hepatic content of TG, (B) malondialdehyde (MDA), and (C) hydrogen peroxide (\( H_2O_2 \)) were measured in healthy cows (n = 10) and cows with fatty liver (n = 10). Data were analyzed with independent samples \( t \)-tests and expressed as mean ± SEM.

**Figure 3.** Cytokeratin 18 (CK 18) and DAPI (4′,6-diamidino-2-phenylindole) immunofluorescence staining and morphology of hepatocytes. (A) Representative image of CK 18 immunofluorescence staining; scale bar represents 20 \( \mu m \). (B) Morphology of hepatocytes; scale bar represents 20 \( \mu m \).
DISCUSSION

A growing body of evidence indicates that impairment of PINK1/Parkin-mediated mitophagy is associated with hepatic oxidative stress and steatosis in humans and mice (Williams et al., 2015; Liu et al., 2018). The low abundance of mitophagy markers in dairy cows with fatty liver agreed with data from nonruminants. Furthermore, the impairment of mitophagy along with increased ROS production in hepatocytes challenged with FFA emphasized a direct link. This was supported by data from PRKN overexpression studies in which PINK1/Parkin-mediated autophagy was enhanced and ROS overproduction and lipid accumulation alleviated. Thus, together, results demonstrated that impaired PINK1/Parkin-mediated mitophagy as a result of FFA overload may potentially promote ROS overproduction and contribute to the development of fatty liver in dairy cows.

Mitophagy, the selective autophagy of mitochondria, is an important quality control mechanism that eliminates damaged mitochondria and is mainly mediated by PINK1/Parkin (Youle and Narendra, 2011). It was reported recently that hepatic total protein abundance of PINK1 and Parkin was lower in ketotic dairy cows (Shen et al., 2021b) experiencing hepatic steatosis (Shen et al., 2019). Our previous study also demonstrated that hepatic autophagic activity was reduced in dairy cows with fatty liver (Du et al., 2018b), which underscored a potential link between impaired hepatic mitophagy and fatty liver. Thus, decreased localization of PINK1 and Parkin on mitochondria of cows with fatty liver in the current study provided direct evidence for this linkage.

Results of the present study are consistent with previous findings that a loss of PINK1 or PRKN led to ac-
accumulation of dysfunctional mitochondria in the livers of mice and pulmonary microvascular endothelial cells of rats (Bueno et al., 2015; Ning et al., 2018; Zhang et al., 2019). The protein p62 accumulates on mitochondria and binds to Parkin-ubiquitinated OMM proteins and LC3-II to facilitate degradation of damaged mitochondria (Lemasters and Zhong, 2018). Wang et al. (2021) reported that knockdown of PRKN decreased colocalization of autophagosome and mitochondria in renal proximal tubule epithelial cells. Furthermore, p62 overexpression increased recruitment of Parkin on mitochondria and promoted the removal of dysfunctional mitochondria (Narendra et al., 2010). It is noteworthy that mRNA abundance of LC3 and p62 was lower in the liver of cows with fatty liver (Du et al., 2018b). Thus, the lower protein abundance of p62 and LC3-II observed in the hepatic mitochondrial fraction of cows with fatty liver agrees with those findings.

Because mitochondria are both a generator of and target for ROS, oxidative stress is closely linked to mitochondrial dysfunction (Nolfi-Donegan et al., 2020; Silwal et al., 2020) and likely impairs mitophagy, resulting in a negative feedback loop that renders the liver more susceptible to oxidative stress (Grummer et al., 2004). This idea is supported by the increased levels of MDA and H2O2 in calf hepatocytes in which PRKN was silenced. At least in rodents, evidence also suggests that defective mitophagy reduces hepatic glutathione content, a classic antioxidant, thereby aggravating hepatic oxidative stress (Wang et al., 2019). Thus, a high content of MDA and H2O2 in dairy cows with fatty liver may be associated with impaired mitophagy.

Accumulation of damaged mitochondria due to impaired mitochondria reduces β-oxidation of FFA (Su et al., 2019), resulting in FFA re-esterification into TG and accumulation in the liver (Andersen et al., 2002). Williams et al. (2015) reported greater hepatic steatosis in PRKN knockout mice after acute binge alcohol treatment. In line with their results, TG content was greater in calf hepatocytes treated with siPRKN. Taken together, our data combined with previous studies demonstrated that low abundance of mitophagy markers is associated with excessive ROS production and steatosis in dairy cows.

From a physiological standpoint, a reduction of autophagic activity in the liver of dairy cows with fatty liver arises in part from high blood concentrations of FFA (Du et al., 2018b). Although FFA clearly can serve as sources of energy in dairy cows, it is well established that excessive influx of FFA into liver causes lipotoxicity (Listenberger et al., 2003). For instance, murine and human hepatocytes challenged with high levels of palmitic acid, a main component of circulating FFA, had a reduction in colocalization of PINK1, Parkin, and mitochondria (Liu et al., 2018; Li et al., 2020). Furthermore, Shen et al. (2021a) demonstrated that high concentrations of FFA reduced autophagic activity in calf hepatocytes. Thus, available data, including decreased protein abundance of PINK1, Parkin, p62, and LC3-II due to challenge with FFA,

Figure 5. Effects of parkin RBR E3 ubiquitin protein ligase (PRKN) knockdown on mitophagy, lipid accumulation, and oxidative stress in calf hepatocytes. Calf hepatocytes were transfected with small interfering RNA of PRKN (siPRKN) or negative control (siControl) for 48 h. (A) Relative mRNA abundance of PRKN in calf hepatocytes. (B) Representative blots of sequestosome-1 (p62) and microtubule-associated protein 1 light chain 3 (LC3) in the mitochondrial fraction of calf hepatocytes. LC3-I is formed by the removal of the C-terminal 22 amino acids from newly synthesized LC3. LC3-II is a phosphatidylethanolamine (PE)-conjugated form of modified and processed LC3-I. (C) Quantification of protein levels of p62/complex IV (COX IV) and (D) LC3-II/COX IV. (E) Triacylglycerol (TG) content of calf hepatocytes. (F) Malondialdehyde (MDA) and (G) hydrogen peroxide (H2O2) content of calf hepatocytes. Data were analyzed with independent samples t-tests and expressed as mean ± SEM.
indicate that FFA impaired mitophagy in hepatocytes and leads to ROS overproduction and lipid accumulation.

Zhou et al. (2019) reported that activation of mitophagy by promoting expression of Parkin alleviated high-fat diet-induced hepatic steatosis in mice. Furthermore, overexpression of PINK1 reduced ROS production in Kuppfer cells (Xu et al., 2020). Consistent with those studies, PRKN overexpression attenuated ROS overproduction and lipid accumulation in FFA-treated calf hepatocytes. Thus, previous studies and our present data demonstrate that PINK1/Parkin-mediated mitophagy may be a novel target for preventing or alleviating the potential for FFA to cause an impairment of mitochondrial function that can aggravate hepatic lipid metabolism and oxidant status.

In the present study, protein abundance of mitophagy markers were detected to assess mitophagy in vivo and in vitro. Of note, transmission electron microscopy is a better method to display the ultrastructure of autophagosome and autolysosome and subsequently evaluate the status of mitophagy. In addition, the thiobarbituric acid reactive substances assay is an assay that is not specific, as it can detect aldehydes other than MDA (Palmieri and Sblendorio, 2007; Celi, 2011; Ho et al., 2013). Furthermore, the total H2O2 content, not specific mitochondrial-derived ROS, was assessed, and activities of antioxidant enzymes were not determined in the present study. Thus, based on the present data, we cannot fully measure the status of oxidative stress in liver and hepatocytes. Those are limitations in present study.

CONCLUSIONS

Mature dairy cows with fatty liver displayed low abundance of mitophagy markers, excessive TG deposition, and ROS overproduction. Knockdown of PRKN or treatment with FFA impaired PINK1/Parkin-mediated mitophagy, induced ROS overproduction, and lipid accumulation in female calf hepatocytes, whereas overexpression of PRKN enhanced mitophagy and alleviated FFA-induced ROS overproduction and lipid accumulation. These findings will further improve our understanding of the pathologic mechanisms leading to fatty liver.

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Journal of Dairy Science Vol. 105 No. 9, 2022

P.


Journal of Dairy Science Vol. 105 No. 9, 2022

P.


Journal of Dairy Science Vol. 105 No. 9, 2022

P.


Journal of Dairy Science Vol. 105 No. 9, 2022

P.


Journal of Dairy Science Vol. 105 No. 9, 2022

P.


Journal of Dairy Science Vol. 105 No. 9, 2022

P.


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