Evaluation of hepatic AMP-activated protein kinase (AMPK), mechanistic target of rapamycin kinase complex 1 (mTORC1) and autophagy-lysosomal pathway in cows with mild or moderate fatty liver

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**ABSTRACT**

The aim of the present study was to investigate the activity of AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin kinase complex 1 (mTORC1) and transcription factor EB (TFEB) transcriptional activity and autophagy-lysosomal function in the liver of dairy cows with mild fatty liver (FL) and cows with moderate FL. Liver and blood samples were collected from healthy dairy cows (n = 10; hepatic triglyceride content < 1% wet weight) and cows with mild FL (n = 10; 1% ≤ hepatic triglyceride content < 5% wet weight) or moderate FL (n = 10; 5% ≤ hepatic triglyceride content < 10% wet weight) that had a similar number of lactations (median = 3, range = 2 to 4) and DIM (median = 6 d, range = 3 to 9). Blood parameters were determined using a Hitachi 3130 autoanalyzer with commercially-available kits. Protein and mRNA abundance was determined using Western blotting and quantitative real-time PCR, respectively. Activity of calcineurin and β-N-Acetylglucosaminidase was measured with commercial assay kits. Data were analyzed using one-way ANOVA with subsequent Bonferroni correction. Blood concentrations of glucose were lower in moderate FL cows (3.03 ± 0.21 mM) than in healthy (3.71 ± 0.14 mM) and mild FL cows (3.76 ± 0.14 mM). Blood concentrations of β-hydroxybutyrate (BHB, 1.37 ± 0.15 mM in mild FL, 1.88 ± 0.17 mM in moderate FL) and free fatty acids (FFA, 0.69 ± 0.05 mM in mild FL, 0.96 ± 0.09 mM in moderate FL) were greater in FL cows than in healthy cows (BHB, 0.76 ± 0.12 mM; FFA, 0.42 ± 0.04 mM). Compared with healthy cows, phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase 1 was greater and lower in cows with mild and moderate FL, respectively. Phosphorylation of mTOR was lower in cows with mild FL compared with healthy cows. In cows with moderate FL, phosphorylation of mTOR and its downstream effectors was greater than in healthy cows and cows with mild FL. The mRNA abundance of TFEB was downregulated in cows with moderate FL compared with healthy cows and mild FL cows. In mild FL cows, the mRNA and protein abundance of TFEB was greater than in healthy cows. Compared with healthy cows, the mRNA abundance of autophagy markers sequestosome-1 and microtubule-associated protein 1 light chain 3-II, the protein and mRNA abundance of lysosome-associated membrane protein 1 and cathepsin D were increased in mild FL cows, but decreased in moderate FL cows. Compared with healthy cows, the mRNA abundance of mucolipin 1 and activities of β-N-acetylglucosaminidase and calcineurin were higher in cows with mild FL, but lower in cows with moderate FL. These data demonstrate that hepatic AMPK signaling pathway, TFEB transcriptional activity and autophagy-lysosomal function are increased in dairy cows with mild FL, hepatic mTORC1 signaling pathway is inhibited in mild FL cows but activated in moderate FL cows, and activities of AMPK and TFEB as well as autophagy-lysosomal function are impaired in moderate FL cows.

**Key words:** fatty liver, energy metabolism, TFEB, autophagy

**INTRODUCTION**

The transition period is characterized by dramatic physiological changes and imposes severe challenges on the cows due to the profound energy requirements at calving that are followed by a delayed increase in DMI (Goff and Horst, 1997). To compensate for the energy deficit, lipolysis of triglyceride (TG) stores in adipose tissue occurs, releasing free fatty acids (FFA) into circulation. In postpartum dairy cows, both blood...
flow to the liver and circulating FFA concentration are elevated, which result in an increase uptake of FFA into hepatic tissue (Lomax and Baird, 1983; Reynolds et al., 2003). When the liver is overwhelmed with FFA, there is increased production of TG, leading to the onset of fatty liver (FL; Bobe et al., 2004). Dairy cows with FL are at an increased risk of developing displaced abomasum and metritis (Heinonen et al., 1987; Rehage et al., 1996), which may increase their risk of removal from the herd during early lactation.

The AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR) signaling pathways serve as signal nexus for regulating cellular metabolism and nutrient homeostasis (Xu et al., 2012; González et al., 2020). The AMPK acts as a cellular energy sensor that is activated under conditions of high intracellular AMP, such as nutrient deprivation (Hardie, 2007). For instance, it has been shown that feed deprivation increased AMPK activity in the liver of dairy cows (Kuha et al., 2009). In contrast, nutrient-rich conditions activate mTOR complex 1 (mTORC1), which is composed of the core subunits mTOR, regulatory-associated protein of mTOR and mammalian lethal with SEC13 protein 8, to promote mitochondrial biogenesis, protein synthesis and hepatic lipogenesis (Laplante and Sabatini, 2012; Morita et al., 2013; Gosis et al., 2022). A more recent study demonstrated that supplementation of media with glucose and amino acids inhibited AMPK and activated mTORC1 and promoted proliferation of cow mammary epithelial cells (Zhang et al., 2018). These results raised the possibility that AMPK and mTORC1 may be implicated in regulating the hepatic energy metabolism in dairy cows experiencing a negative energy balance in early lactation. However, we know little about the activity of hepatic AMPK and mTORC1 in the dairy cows with mild or moderate FL.

Ever since its discovery by Christian de Duve in the 1950s, the lysosome has been classically considered as terminal degradative organelles (De Duve et al., 1955). Recently, growing evidence indicates that the lysosome as a signaling hub, which integrates extracellular and intracellular signals to regulate nutrient sensing and metabolic adaptation (Medina et al., 2015; Baldabio and Bonifacino, 2020; Nanayakkara et al., 2022). Schmitt et al. (2022) revealed that activation of AMPK by upstream kinase liver kinase B1 (LKB1) primarily occurred at the surface of the lysosome, where AMPK and mTORC1 are regulated in a opposite manner by the availability of nutrients (Zhang et al., 2014; González et al., 2020). Moreover, AMPK and mTORC1 also act antagonistically via effects on the transcription factor EB (TFEB) and transcription factor binding to IGHM enhancer 3 (TFE3; Paquette et al., 2021; Gosis et al., 2022), which induce genes involved in autophagy and lysosome biogenesis and function (Settembre et al., 2012; Martina et al., 2014; Balabio and Bonifacino, 2020). Although dairy cows with FL are characterized by energy deficiency, the significant differences in metabolic profile of plasma (Rukkwamsuk et al., 2000; Kalaitzakis et al., 2010; Weber et al., 2013) suggests that the metabolic status may be differ between mild and moderate FL cows.

In the present study, we hypothesized that the metabolic changes of mild and moderate FL cows would affect the activity of AMPK and mTORC1 and further alter the autophagy-lysosomal function in the liver. Thus, our objective was to investigate changes in AMPK and mTORC1 signaling pathway as well as autophagy-lysosomal function in the liver of dairy cows with mild or moderate FL.

MATERIALS AND METHODS

Animals

The use of animals and experimental procedures were approved by the Ethics Committee on the Use and Care of Animals of Jilin University (Changchun, China). On the first day, dairy cows (n = 75) with similar number of lactations (median = 3, range = 2 to 4) and DIM (median = 6 d, range = 3 to 9) from a commercial dairy farm located in Inner Mongolia (China) milking 3,500 cows with a rolling herd average of 9,300 kg of milk were enrolled in this study. On the same day, dairy cows underwent a thorough clinical examination by the same veterinarian to rule out cows (n = 12) with displaced abomasum, mastitis, endometritis or laminitis. The endometritis can be diagnosed by the presence of purulent vaginal discharge. The lesion characteristics of laminitis are mainly hoofed lesions, such as hemorrhage in the hoof and white line, as well as a sole ulcer. The next day, blood samples from 63 cows were collected between 0700 and 0800 h (before feeding) by a cecocolic venipuncture; then the serum was separated after centrifugation at 3,500 × g for 15 min at 4°C. Given that dairy cows with fatty liver have high circulating concentrations of FFA (Rukkwamsuk et al., 2000), we performed a preliminary screen based on serum FFA. Cows with serum FFA concentration >0.6 mM were preclassified as having FL (n = 48), and those with serum FFA content <0.6 mM were preclassified as healthy (n = 15). During the next 5 d, blood samples and liver tissue samples from 63 cows were collected between 0700 and 0800 h (before feeding). Based on hepatic TG content (% g/g of wet weight), TG content in healthy cows was < 1%, TG content in cows with mild FL was 1 to 5%, TG content in cows with moder-
Determine the hepatic TG content. The whole duration for sample collection takes 1 week. During the period of sample collection, 8 cows with displaced abomasum, mastitis, endometritis or laminitis were excluded from this study. Moreover, 6 cows from mild FL group and 9 cows from moderate FL group developed hyperketonemia (blood BHB concentration ≥1.2 mM). Additionally, 5 cows (1 healthy, 1 mild FL and 3 moderate FL) developed subclinical hypocalcemia (1.5 mM < serum total Ca concentration <2 mM). Dairy cows in FL group that developed hyperketonemia and/or subclinical hypocalcemia were also used for statistical analyses. Cows with FL received 1 L of glucose solution (25%) by intravenous injection and 200 mL of propylene glycol, 15 mg of niacin, and 80 mg of choline orally each day for 5 d. During the experimental period, cows were housed in a tie-stall barn and provided a TMR at 0730 and 1330 h daily with free-access to clean water. The experimental design and timeline of samples collection is shown in Figure 1. The basal diet formulation is described in a previous study (Fang et al., 2022).

**Determination of Blood Parameters**

The serum concentrations of total Ca, FFA, glucose and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a Hitachi 3130 autoanalyzer (Hitachi, Tokyo, Japan) with commercially-available kits. Feed intake and milk yields were recorded during the period that blood samples were collected. The basic description of the cows used is reported in Supplemental Table S1.

The concentration of tumor necrosis factor-α (TNF-α) in serum was measured using a bovine ELISA kit according to the manufacturer’s instruction (SEA133Bo, USCN Life Science Inc.). For TNF-α, the detection range was 7.8 to 500 pg/mL. The intra- and inter-assay coefficients of variation for TNF-α were less than 10% and 12%, respectively. Every sample was analyzed in triplicate, and absorbance values were

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**Figure 1.** Schematic diagram showing the experimental design and samples collection. FFA = free fatty acids; FL = fatty liver.
Liver Tissue Collection and TG Measurement

The liver tissue samples were collected between 0700 and 0800 h (before feeding) from the 11th or 12th right intercostal space by liver puncture needle. The intercostal space was shaved before the liver biopsy, sanitized with iodine scrub and 75% alcohol, and anesthetized with a subcutaneous injection of 2% lidocaine HCl (Sigma-Aldrich Co.). A 3 mm stab incision in the skin was made with a scalpel blade. The puncture needle was then inserted through the intercostal muscle and into the liver. Liver tissue biopsies (~200 mg) were immediately frozen in liquid nitrogen. After biopsy, the incision area was closed with surgical staples and treated with a topical antiseptic (povidone-iodine ointment, 10%). 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.). 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.

Quantitative Real-Time (qRT) PCR Analysis

Total RNA was extracted from liver tissues using RNAiso Plus (9109; TaKaRa Biotechnology Co. Ltd.) according to the manufacturer’s instruction. The purity of RNA was measured by calculating the ratio of UV activity at 260/280 nm using the Nanophotometer N50 Touch (Implen GmbH, Munich, Germany). The RNA integrity was assessed by gel electrophoresis (1% agarose gels) and the results indicated clear 18S and 28S bands without significant degradation. The concentration of purified RNA was determined by UV spectrum at 260 nm. Subsequently, cDNA was generated from total RNA using reverse transcription kit (RR047A; TaKaRa Biotechnology Co. Ltd.). The mRNA expression levels were evaluated by using qRT-PCR technology with the SYBR Green QuantiTect RT-PCR Kit (RR420A; TaKaRa Biotechnology Co. Ltd.) and a 7500 Real-Time PCR System (Applied Biosystems Inc., Waltham, MA, USA). The qRT-PCR was conducted with an initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s), and extension at 72°C for 12 min. The relative quantitation values were normalized against the geometric mean of β-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), both of which were stably expressed in healthy, mild and moderate FL cows. Fold changes in gene expression relative to the mean of control group were calculated using the 2−ΔΔCt method. The primer pairs used in this study were designed using Primer Express software 3.0.1 (Applied Biosystems Inc.) according to bovine reference sequences from National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). The PCR products were evaluated by agarose gel electrophoresis (a signal band of correct size) and melt curve (a signal peak). The primer sequences are listed in Supplemental Table S2.

Protein Extraction and Western Blotting

Total protein from liver tissues was extracted using a commercial protein extraction kit (C510003; Sangon Biotech Co. Ltd.). The concentration of total protein was determined by the BCA method according to the manufacturer’s instructions (P1511; Applygen Technologies). After denaturing (95°C for 5 min), a total of 20 μg of protein from each sample was separated on 10 or 12% Tris-glycine gels with a known pre-stained protein ladder (26616; Thermo Fisher Scientific). The membranes were blocked in Tris-buffered saline solution with 0.1% Tween-20 (TBST) containing 3% bovine serum albumin (BSA) for 4 h at room temperature. The blocked membranes were incubated overnight at 4°C with primary antibodies against phosphorylated (p)-AMPK (Thr172; 1:1,000; cat. no. 2531, Cell Signaling Technology), AMPK (1:1,000; cat. no. 5831, Cell Signaling Technology), phosphorylated acetyl-CoA carboxylase 1 (p-ACC1; Ser79; 1:1,000; AP0298, ABclonal), cathepsin D (CTSD; 1:2,000; cat. no. 21327–1-AP, Proteintech), lysosome-associated membrane protein 1 (LAMP1; 1:1,000; ab24170, Abcam), cathepsin D (CTSD; 1:2,000; cat. no. 21327–1-AP, Proteintech), phosphorylated mTOR (Ser2448; 1:1,000; ab84400, Abcam), mTOR (1:5,000; ab2833, Abcam), phosphorylated eukaryotic factor 4E-binding protein 1 (p-EIF4EBP1; Thr37/46; 1:1,000; cat. no. 2855, Cell Signaling Technology), EIF4EBP1 (1:1,000; ab227540, Abcam), phosphorylated ribosomal protein S6 kinase B (p-RPS6KB; Ser24; 1:1,000; ab131436, Abcam), phosphorylated ribosomal protein S6 kinase B (p-RPS6KB; Ser24; 1:1,000; ab131436, Abcam), p-TFEB (Ser211; 1:1,000; cat. no. 37681, Cell Signaling Technology), TFEB (1:1,000; ab2636, Abcam) and β-actin (1:5,000; ab8226, Abcam). Then, membranes were washed 3 times with TBST and incubated with the appropriate peroxidase-conjugated secondary antibody for 45 min. Immunoreactive bands were visualized via a Tanon Imaging System (Tanon...
4600, Tianneng Co. Ltd., Shanghai, China) using an enhanced chemiluminescence solution (WBULS0500, Millipore, Bedford, USA). All bands were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD) and β-actin was used as an internal control.

**Calcineurin Activity Assay**

Calcineurin activity was determined via a Calcineurin Cellular Activity Assay Kit (BML-AK816–0001, Enzo Life Sciences) following the manufacturer’s instructions (Zhu et al., 2017). In brief, liver tissues were crashed with frozen pestles in frozen tubes with lysis buffer provided in the kit with addition of protease inhibitors. The extracts were passed through desalting resins to remove free phosphate and then incubated with calcineurin substrate RII phosphopeptide at 30°C for 30 min. After that, BIOMOL Green were added to terminate the reaction and plates were kept at room temperature for 20 min. Sample absorbance values were read at 620 nm using a spectrophotometer (51119100; Thermo Fisher Scientific). The BCA protein assay kit (P1511; Applygen Technologies) was used to determine protein concentration. Calcineurin activity was normalized to protein concentration.

**β-N-Acetylglucosaminidase Assay**

The activity of β-N-acetylglucosaminidase (NAG) was measured with a commercial assay kit (CS0780, Sigma-Aldrich Co.). The assay is based on the hydrolysis of the NAG substrate, 4-nitrophenyl N-acetyl-β-d-glucosaminide, by the enzyme. This enzymatic hydrolysis of the substrate releases phosphorylated nitrophenol, which, upon ionization in basic pH, can be measured colorimetrically at 405 nm. First, liver tissue was lysed in mammalian tissue lysis/extraction reagent (C3228, Sigma-Aldrich Co.); then 10 μg from each sample was normalized to an equal volume and measured for NAG activity following the protocol provided by the supplier. Absorbance at 405 nm was proportional to the activity of NAG.

**Statistical Analysis**

All data were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene tests, respectively. One-way ANOVA with subsequent Bonferroni correction were performed for multiple comparisons. All data were analyzed using GraphPad Prism 8.0 (Graph Pad Software, San Diego, CA) or SPSS software 23.0 (IBM, Chicago, IL). Data are expressed as means ± SEM. P < 0.05 was considered statistically significant, and P < 0.01 was considered extremely significant.

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**RESULTS**

**Animal Selection**

As shown in Figure 1, 48 cows with serum FFA concentration higher than 0.6 mM were preclassified as having FL and 15 cows with serum FFA content lower than 0.6 mM were preclassified as healthy. According to the hepatic TG content, 10 cows with hepatic TG content < 1% were classified as healthy, those with hepatic TG content of 1 to 5% were classified as having mild fatty liver (n = 10), those with hepatic TG content of 5 to 10% were classified as having moderate fatty liver (n = 10). During the period of sample collection, 8 cows with displaced abomasum, mastitis, endometritis or laminitis were excluded from this study.

**Performance and Blood Parameters**

As shown in Supplemental Table S1, no differences were observed in serum total Ca concentration, BW and BCS across groups. The milk yield and DMI were comparable between cows with mild FL and healthy cows, but lower (P < 0.05) in moderate FL cows than in healthy cows (Supplemental Table S1). Blood concentrations of glucose were lower (P < 0.05) in moderate FL cows than in mild FL and healthy cows (Supplemental Table S1). Conversely, blood concentrations of BHB and FFA as well as activities of ALT and AST were greater (P < 0.05) in FL cows (mild and moderate) than in healthy cows (Supplemental Table S1). In moderate FL cows, the concentration of TNF-α in the serum were greater (P < 0.05) than in mild FL and healthy cows (Supplemental Table S1).

**Activity of AMPK in Liver**

The mRNA abundance of LKB1, an upstream kinase of AMPK, was comparable across groups (Figure 2A). In cows with mild FL, phosphorylation of AMPK and its downstream target ACC1 was greater (P < 0.01) than in healthy cows (Figure 2B, C and D). Furthermore, phosphorylation of AMPK and ACC was lower (P < 0.01) in cows with moderate FL compared with healthy cows and cows with mild FL (Figure 2B, C and D).

**Activity of mTORC1 in Liver**

Phosphorylation of mTOR was lower (P = 0.04) in cows with mild FL compared with healthy cows (Figure 3A and B). In cows with moderate FL, phosphorylation of mTOR and its downstream effectors, including RP-S6KB and EIF4EBP1, was greater (P < 0.01) than in...
healthy cows and cows with mild FL (Figure 3A, B, C and D). Protein levels of p-RPS6KB and p-EIF4EBP1 was mildly lower in mild FL cows than in healthy cows (Figure 3A, C and D).

**Function of TFEB and TFE3 in Liver**

The mRNA abundance of TFEB and TFE3 was downregulated ($P < 0.05$) in cows with moderate FL compared with healthy cows and mild FL cows (Figure 4A and B). In mild FL cows, the mRNA and protein abundance of TFEB was greater ($P < 0.01$), whereas the phosphorylation of TFEB was lower ($P = 0.04$) than in healthy cows (Figure 4A, C, D and E). In moderate FL cows, the protein abundance of TFEB was lower ($P = 0.04$), but the phosphorylation of TFEB was greater ($P < 0.01$) compared with healthy cows (Figure 4C, D and E). Compared with healthy cows, the mRNA abundance of glycoprotein Nmb (GPNMB) and peroxisome proliferator-activated receptor γ coactivator 1 α (PPARGC1A), direct targets of TFEB and TFE3, was upregulated ($P < 0.01$) in cows with mild FL, but downregulated ($P < 0.01$) in cows with moderate FL (Figure 4F and G).

**Autophagy-lysosomal Function in Liver**

Compared with healthy cows, the mRNA abundance of autophagy markers sequestosome-1 (SQSTM1) and microtubule-associated protein 1 light chain 3-II (MAP1LC3) were upregulated ($P < 0.01$) in mild FL cows, but decreased ($P < 0.05$) in moderate FL cows (Figure 5A and B). Compared with healthy cows, the protein (Figure 5C, D and E) and mRNA (Figure 5F and G) abundance of LAMP1 and CTSD were increased ($P < 0.05$) in mild FL cows, but decreased ($P < 0.01$) in moderate FL cows. The mRNA abundance of LAMP2 was similar for mild FL and healthy cows, but downregulated ($P < 0.01$) in cows with moderate FL than in healthy cows and mild FL cows (Figure 5H). Compared with healthy cows, the mRNA abundance of mucolipin 1 (MCOLN1) and activities of NAG and calcineurin were higher ($P < 0.05$) in cows with mild FL, but lower ($P < 0.01$) in cows with moderate FL (Figure 5I, J and K).

**DISCUSSION**

During the early lactation period, dairy cows with a poor adaptive response to negative energy balance-induced metabolic stresses may develop FL. Emerging evidence from rodents suggests that AMPK and mTORC1 act as cellular energy sensors and signal transducers that play an essential role in the regulation of metabolic and energy homeostasis (Xu et al., 2012; González et al., 2020; Gosis et al., 2022). In the present study, activation of AMPK signaling pathway and enhanced TFEB transcriptional activity and autophagy-lysosomal function were accompanied by inhibited mTORC1 signaling pathway in the liver of mild FL cows. However, inhibition of AMPK and TFEB and impairment of autophagy-lysosomal function as well as overactivation of mTORC1 were observed in the liver of moderate FL cows. Thus, these data suggest that the liver metabolic status is different between mild and moderate FL cows.

AMPK is phosphorylated and activated by LKB1 in response to an increasing cytosolic ratio of AMP:ATP, which reflects a decrease in energy availability (Woods et al., 2003; Xu et al., 2012). Subsequently, AMPK phosphorylates and inactivates ACC1/2, suppressing fatty acid synthesis and promoting fatty acid oxida-

![Figure 2](image-url)
tion and ATP generation (Fullerton et al., 2013). The present study revealed that hepatic mRNA abundance of LKB1 was similar for healthy, mild and moderate FL cows. Although we did not determine the ratio of AMP:ATP and the activity of LKB1 (due to the small amount of liver tissues available), the finding that phosphorylation of AMPK and ACC1 was increased suggests that hepatic AMPK could have been activated in mild FL cows. In contrast, we found a decrease of protein abundance of p-AMPK and p-ACC1 in the liver of moderate FL cows, suggesting the inhibition of AMPK signaling pathway. Dairy cows with FL generally experience marked negative energy balance, which induces sustained lipolysis and high blood concentrations of FFA (Bobe et al., 2004; Kuhla et al., 2009). Although FFA can serve as sources of energy in dairy cows, it is well established that high concentrations of FFA cause lipotoxicity (Listenberger et al., 2003; Li et al., 2019). Of note, bovine mammary epithelial cells and murine hepatocytes challenged with high levels of FFA had a reduction in phosphorylation of AMPK (Hu et al., 2021; Li et al., 2023). Thus, a higher influx of FFA into liver may be associated with inhibited hepatic AMPK signaling pathway in dairy cows with CK.

At least in nonruminants, AMPK inhibits mTORC1 through phosphorylation of TSC2 complex subunit 2 (TSC2) and RAPTOR (Inoki et al., 2003; Gwinn et al., 2008; González et al., 2020). Although we did not measure the protein abundance of phosphorylated TSC2 or RAPTOR, the finding that phosphorylation of mTOR and its downstream targets RPS6KB and EIF4EBP1 was decreased indicated that hepatic mTORC1 signaling pathway was suppressed in mild FL cows. Thus, activation of AMPK and inhibition of mTORC1 lead to a metabolic switch from anabolism to catabolism in the liver of mild FL cows. These findings of the present study were in agreement with earlier data showing enhanced fatty acids oxidation and autophagy in the liver of dairy cows with high liver fat content (Weber et al., 2013; Du et al., 2018; Chen et al., 2020).

The activity of mTORC1 is dynamically regulated by several environmental cues, including energy levels, growth hormone (GH) and stresses (Laplante and Sabatini, 2012; González et al., 2020). Although dairy cows with FL are characterized by energy deficiency, our present study revealed that hepatic mTORC1 was overactivated in dairy cows with moderate FL cows. Moreover, Fang et al. (2022) shown that FFA, BHB, proinflammatory cytokines and GH could activate mTORC1 in bovine hepatocytes. Thus, these factors might be partly responsible for overactivation of hepatic mTORC1 in moderate FL cows.

TFEB along with TFE3 were recently identified as master transcriptional regulators of autophagy and lysosome function (Sardiello et al., 2009; Martina et al., 2014). Upon nutrient depletion, TFEB and TFE3 are translocated from the cytosol into the nucleus, where they regulate target genes expression through binding to coordinated lysosomal expression and regulation motifs (Sardiello et al., 2009; Napolitano and Ballabio, 2016). It has been reported that AMPK phosphorylated TFEB on the Ser466, Ser467 and Ser469, which further increased mTORC1-mediated TFEB de-phosphorylation (Ser211 and Ser142) and nuclear translocation (Medina et al., 2015; Paquette et al., 2021). In the present study, decreased abundance of phosphorylated TFEB (Ser211) and increased abundance of TFEB, as well as upregulated TFEB target genes (TFEB is its own target gene), suggesting that the transcriptional activity of TFEB was increased in the liver of mild FL cows.

![Figure 3. Activity of hepatic mechanistic target of rapamycin kinase complex 1 (mTORC1).](image-url)

(A) Representative blots of hepatic protein abundance of the phosphorylated (p)-mechanistic target of rapamycin (mTOR), mTOR, ribosomal protein S6 kinase B (RPS6KB), p-RPS6KB, eukaryotic factor 4E-binding protein 1 (EIF4EBP1), p(EIF4EBP1) in the liver of healthy cows (n = 6), cows with mild fatty liver (FL; n = 6), and cows with moderate FL (n = 6). (B-D) Quantification of protein levels of p-mTOR/mTOR, p-RPS6KB/RPS6KB and p(EIF4EBP1)/EIF4EBP1. Data were analyzed using one-way ANOVA with subsequent Bonferroni correction and expressed as means ± standard error of the mean.)
FL cows. In contrast, the transcriptional activity of TFEB and TFE3 was decreased in moderate FL cows, as evidenced by downregulated GPNMB, PPARGC1A, TFEB and TFE3 and elevated phosphorylation of TFEB. Considering the role of upstream kinases in regulating transcriptional activity of TFEB and TFE3 (Settembre et al., 2012; Paquette et al., 2021), changes of AMPK and mTORC1 signaling pathway could partly explain the function of these 2 transcription factors in dairy cows with different degrees of FL.

In the present study, the protein and mRNA abundance of TFEB was elevated in mild FL cows, whereas the mRNA abundance of TFE3 did not alter between healthy and mild FL cows. Previous studies revealed that TFEB and TFE3 had overlapping or compensatory functions in regulating lysosomal function and biogenesis (Pastore et al., 2016; Pastore et al., 2019). For instance, both TFEB overexpression in TFE3 knockout mice and TFE3 overexpression in TFEB liver-specific knockout mice rescued high fat diet-induced obesity (Pastore et al., 2017). Thus, increased transcriptional activity of TFEB might compensate for TFE3 in dairy cows with mild FL.

Autophagy is a lysosome-dependent catabolic process that contribute to the clearance of defective or excess organelles and unwanted proteins to maintain energy homeostasis (Nanayakkara et al., 2022; Xie et al., 2023). LAMP1 and LAMP2 are estimated to contributed to about 50% of all proteins of the lysosome membrane (Eskelinen, 2006). The lysosomal proteinase CTSD is involved in the regulation of proteolytic activity of the lysosomes (Haidar et al., 2006). In the present study, the higher abundance of genes in autophagy markers (SQSTM1 and MAP1LC3), LAMP1, LAMP2 and CTSD and greater activity of lysosome enzyme NAG suggest that autophagy-lysosomal function was enhanced in the liver of mild FL cows, which could partly alleviate negative energy balance. Additionally, impaired autophagy-lysosomal function was observed in moderate FL cows. It is noteworthy that knockout of hepatic TFEB downregulates autophagy and lysosomal biogenesis genes and leads to an increase in hepatic steatosis and injury in mice (Chao et al., 2018). Thus,

Figure 4. Function of hepatic transcription factor EB (TFEB) and transcription factor binding to IGHM enhancer 3 (TFE3). (A, B) Relative mRNA abundance of TFEB and TFE3 in the liver of healthy cows (n = 10), cows with mild fatty liver (FL; n = 10), and cows with moderate FL (n = 10). (C) Representative blots of hepatic protein abundance of the phosphorylated (p)-TFEB and TFEB in the liver of healthy cows (n = 6), cows with mild FL (n = 6), and cows with moderate FL (n = 6). (D, E) Quantification of protein levels of TFEB/β-actin and p-TFEB/TFEB. (F, G) Relative mRNA abundance of glycoprotein Nmb (GPNMB) and peroxisome proliferator-activated receptor γ coactivator 1 α (PPARGC1A) in the liver of healthy cows (n = 10), cows with mild FL (n = 10), and cows with moderate FL (n = 10). Data were analyzed using one-way ANOVA with subsequent Bonferroni correction and expressed as means ± standard error of the mean.
hepatic autophagy-lysosomal dysfunction may potentially promote liver injury and steatosis in dairy cows with moderate FL.

Hypocalcemia is a metabolic disorder of dairy cows occurring most frequently in the peripartal period. Although 3 cows from moderate FL group developed subclinical hypocalcemia, no differences were observed in serum total Ca concentration between healthy and FL groups. Intracellular Ca\(^{2+}\) concentration widely varies depending on its location (Bagur and Hajnóczk, 2017). It was reported that Ca\(^{2+}\) released from lysosomes via MCOLN1 activates calcineurin, which in turn binds and dephosphorylates TFEB, thus promoting its nuclear import (Medina et al., 2015). Moreover, MCOLN1 is a direct transcriptional target of TFEB (Medina et al., 2011; Medina et al., 2015). In the pres-

![Figure 5](image-url)

**Figure 5.** Hepatic autophagy-lysosomal function. (A, B) Relative mRNA abundance of sequestosome-1 (SQSTM1) and microtubule-associated protein 1 light chain 3-II (MAP1LC3) in the liver of healthy cows (n = 10), cows with mild fatty liver (FL; n = 10), and cows with moderate FL (n = 10). (C) Representative blots of hepatic protein abundance of the lysosome-associated membrane protein 1 (LAMP1) and cathepsin D (CTSD) in the liver of healthy cows (n = 6), cows with mild FL (FL; n = 6), and cows with moderate FL (n = 6). (D, E) Quantification of protein levels of LAMP1/β-actin and CTSD/β-actin. (F-I) Relative mRNA abundance of LAMP1, CTSD, LAMP2 and mucolipin 1 (MCOLN1) in the liver of healthy cows (n = 10), cows with mild FL (n = 10), and cows with moderate FL (n = 10). (J and K) Activities of β-N-acetylglucosaminidase (NAG) and calcineurin in the liver of healthy cows (n = 10), cows with mild FL (n = 10), and cows with moderate FL (n = 10). Data were analyzed using one-way ANOVA with subsequent Bonferroni correction and expressed as means ± standard error of the mean.

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ent study, the downregulation of MCOLN1 along with decreased calcineurin activity in moderate FL cows agrees with reduced transcriptional activity of TFEB. Because activation of TFEB upregulated MCOLN1 expression and increased calcineurin activity in mouse cardiomyocyte (Pan et al., 2020), it could be possible that a similar mechanism exists in dairy cows with mild FL.

CONCLUSIONS

Our results revealed that activated AMPK signaling pathway in the liver of cows with mild FL is associated with inhibited mTORC1 and increased TFEB transcriptional activity and autophagy-lysosomal function. Conversely, suppressed AMPK along with activated mTORC1 impaired TFEB transcriptional activity and autophagy-lysosomal function in the liver of moderate FL cows.

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