Increased autophagy mediates the adaptive mechanism of the mammary gland in dairy cows with hyperketonemia

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ABSTRACT

Hyperketonemia is a metabolic disease in dairy cows, associated with negative nutrition balance (NNB) induced by low dry matter intake (DMI) and increased nutrient requirements. Hyperketonemia could induce metabolic stress, which might indirectly affect mammary tissue. Autophagy is a highly conserved physiological process that results in the turnover of intracellular material, and is involved in maintaining cellular homeostasis under the challenge of metabolic stress induced by NNB. The aim of this study was to investigate the autophagy status and autophagy-related pathways AMP-activated kinase α (AMPK α) and mechanistic target of rapamycin (mTOR) in the mammary glands of dairy cows with hyperketonemia. Cows with hyperketonemia [CWH, $n = 10$, blood β -hydroxybutyrate (BHB) concentration 1.2 to 3.0 mmol/L] and cows without hyperketonemia (CWOH, $n = 10$, BHB < 1.2 mmol/L) from 3 to 12 DIM were randomly selected from the herd. The mammary tissue and blood samples were collected from these cows between 0630 and 0800 h, before feeding, at 3 to 12 d in milk. Serum concentrations of glucose, BHB, and fatty acids were determined using an autoanalyzer with commercial kits between 0630 and 0800 h, before feeding. Concentrations of fatty acids, BHB (median and interquartile range: CWH, 2.44 and 1.3, 2.82 mM; CWOH, 0.49 and 0.41, 0.57 mM), and milk fat were greater in CWH. The DMI, glucose concentration, milk production, and milk protein levels were lower in CWH. The mRNA abundance of autophagosome formation-related gene, beclin 1 (*BECN1*), phosphatidylinositol 3-kinase catalytic subunit type 3 (*PIK3C3*), autophagy-related gene (*ATG*) 5, *ATG7*, *ATG12*, microtubule-associated protein 1 light chain 3 (*MAP1LC3*, also called LC3) and sequestosome-1 (*SQSTM1*, also called p62) were greater in the mammary glands of CWH.

The protein abundance of LC3-II and phosphorylation level of Unc-51-like kinase 1 (ULK1) were greater in CWH, but the total ubiquitinated proteins and protein abundance of p62 were lower. Transmission electron microscopy showed an increased number of autophagosomes in the mammary glands of CWH. Furthermore, the phosphorylation of AMPK α was greater, but the phosphorylation of mTOR was lower in the mammary glands of CWH. These results indicate that activity of mTOR pathways and autophagy activity, and up-regulation of AMPK α , may be response mechanisms to mitigate metabolic stress induced by hyperketonemia in the mammary glands of dairy cows.

Key words: hyperketonemia, mammary gland, autophagy, AMPK α , mTOR pathways

INTRODUCTION

Hyperketonemia, at either a subclinical or a clinical level, is a common metabolic condition in high-producing dairy cows (Vanholder et al., 2015). High-yielding dairy cows undergo a period of negative nutrition balance (NNB) during early lactation because of energy expenditure resulting from milk production and decreased DMI (Chiho et al., 2016; Du et al., 2018b; Zhang et al., 2019). Negative nutrition balance initiates fat mobilization and a subsequent increase in the blood concentrations of fatty acids (Gross et al., 2013). Excessive fatty acids absorbed by the liver from plasma can be metabolized into ketones (BHB, acetone, and acetoacetate), inducing varying degrees of hyperketonemia (White, 2015; Zhu et al., 2019a).

In hyperketonemia, the mammary tissue is facing intense metabolic challenges such as high concentrations of fatty acids and BHB, low concentration of glucose, and significant changes to insulin, glucagon, and prolactin concentrations (Palmquist, 2006; Silva et al., 2017; Maret et al., 2019). Interestingly, the majority of cows with hyperketonemia (CWH) can cope with the metabolic changes during the transition period (Zhu et al., 2019a, b). The mammary tissue plays a key role in

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response to metabolic stress induced by hyperketonemia. However, the metabolic adaptation mechanisms of mammary tissue in CWH has not been characterized in previous studies.

Regulation of the abundance of intracellular components via recycling is the basis of cellular survival, particularly during abrupt physiological changes (Singh and Cuervo, 2011). Autophagy (Greek for “self eating”) is a highly conserved recycling process that involves degradation of cellular constituents in lysosomes (Dikic and Elazar, 2018). As an adaptative catabolic process under nutrient-deficient conditions, autophagy will generate energy for cells by enhancing the turnover of nonfunctional proteins and organelles (Du et al., 2018a). Therefore, autophagy is crucial for maintaining cellular and organic energy homeostasis and alleviating metabolic stress in NNB conditions. The induction of autophagy is tightly regulated by energy metabolic pathways—for example, AMP-activated kinase α (AMPK α) and mechanistic target of rapamycin (mTOR; Kim et al., 2011; Zhang et al., 2016). The data obtained from mice demonstrated that activation of AMPK α and inhibition of mTOR pathways could significantly induce autophagy and further improve the starvation-induced hepatic metabolism disorder (Zhang et al., 2013; Chauvin et al., 2014; Howell et al., 2017). Impairment of these signaling pathways could inhibit cellular autophagy flux and further aggravate metabolic disorders (Egan et al., 2011; Kim et al., 2011).

Hyperketonemia is an energy metabolism disorder induced by NNB (Zhu et al., 2019a). Consequently, the mammary gland tissue has to face the challenges of metabolic stress. Given the positive role of autophagy in response to metabolic stress, we hypothesized that an induction of autophagy activity triggered by AMPK α and mTOR pathways is required in order to respond to metabolic stress in the mammary gland of CWH. The aim of this study was to investigate the autophagy status and the phosphorylation level of AMPK α and mTOR in the mammary glands of CWH and dairy cows without hyperketonemia (CWOH).

MATERIALS AND METHODS

Animals

The study protocol was approved by the Ethics Committee for the Use and Care of Animals, Jilin University (Changchun, China). The animals received humane care according to the principles and specific guidelines presented in *Guide for the Care and Use of Agricultural Animals in Research and Teaching*, 3rd ed. (FASS, 2010). Dairy cows were selected from a 4,000-cow dairy farm

located in Jilin City, China. A total of 223 lactating Holstein cows (number of lactations: median = 3, range = 2 to 4; DIM: median = 7 d, range = 3 to 12 d) were tested to identify CWH and CWOH between April and May 2018. Furthermore, all cows were subjected to a routine physical examination by experienced veterinarians to ensure absence of other perinatal diseases, such as hypocalcemia and mastitis. Cows were classified as suspected CWH by veterinarians if the nitroprusside powder (GL3097, Beijing Baiaolaibo Co. Ltd., Beijing, China) test for ketone bodies was positive. A negative test was one with no color change. A faint tinge of pink was rated a trace reaction. A somewhat more definite pink color was rated +. A definite purple color was ++. Accordingly, 20 cows suspected hyperketonemic and 20 healthy cows were preselected. Subsequently, blood samples were collected from the jugular vein with a 50-mL syringe (no anticoagulant) between 0630 and 0800 h, before feeding, and centrifuged at $1,800 \times g$ for 15 min to obtain serum. Serum concentrations of glucose, BHB, and fatty acids were determined immediately in a laboratory-based analysis, using a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan) with commercial kits (Randox Laboratories, Crumlin, UK; BHB: Cat. No. RB1008; fatty acids: Cat. No. FA115; glucose: Cat. No. GL3815) between 0630 and 0800 h, before feeding. According to the serum BHB concentration (Zhu et al., 2019a), 10 subclinical CWH with serum BHB concentration higher than 1.2 mM and lower than 3.0 mM, and 10 CWOH with serum BHB concentration less than 1.2 mM, were randomly selected. The productive and physiological parameters of the cows is presented in Table 1. Cows were fed twice a day, at 0830 and 1600 h, and they had ad libitum access to feed. The nutrient composition of forage is shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-16910>). Feed samples were collected twice daily at each feeding on 5 consecutive days, from 3 d before biopsy to 2 d post-biopsy.

Cows had access to a constant supply of fresh water and were milked twice daily at 0800 and 1530 h. Milk yield was measured by an integrated milk meter (Afi-Milk, SAE Afikim, Afikim, Israel), and milk samples were collected at each milking for 5 d and stored at 4°C with a preservative (1 mg/mL potassium dichromate). To take into consideration the daily variation in milk yield as well as the effect of tissue biopsy on milk production, data from the 3 d before biopsy were averaged with data from 2 d post-biopsy, disregarding production data from both the day of biopsy and the first day post-biopsy. This was deemed necessary because the biopsy procedure typically leads to a reduction in milk yield for the first 2 to 3 milkings (Farr et al., 1996). Milk fat,

protein, and lactose were analyzed via infrared analysis with a 4-channel spectrophotometer (Multispec, Foss Food Technology Corp., Eden Prairie, MN).

Percutaneous biopsies from 10 CWH and 10 CWOH were obtained from the right or left rear quarter of the mammary gland according to published procedures (Bionaz and Looor, 2008) between 0630 and 0800 h, before feeding. Briefly, the skin and the mammary capsule were incised to ensure that tissue obtained during the biopsy was mammary parenchyma. Immediately after removal of the biopsy instrument from the capsule, pressure was applied to the wound until visual signs of bleeding were absent. The skin incision was closed with 4 or 5 Michel clips (11 mm; Henry Schein, Melville, NY). The incision site was sprayed with a topical antiseptic (Povidone Iodine Ointment, 10%; Taro Pharmaceuticals, Hawthorne, NY). Health was monitored after surgery by recording rectal temperature, milk yield, and feed intake daily for 7 d. Surgical clips were removed 7 d post-biopsy. No incidence of mastitis was detected for any cow during the 7 d after surgery. Tissue samples were frozen immediately in liquid nitrogen and stored at -80°C for further analysis. Tissue samples for transmission electron microscopy were washed with normal saline and then fixed with 2.5% glutaraldehyde.

Quantitative Reverse-Transcription PCR Assay

Total RNA from mammary tissues was extracted using Trizol (15596026, Invitrogen, Carlsbad, CA) according to the supplier's instructions. The RNA concentration and quality were measured using K5500 MicroSpectrophotometer (Beijing Kaiao Technology Development Ltd., Beijing, China) and electrophoresis (1% agarose gels), respectively. In our study, the optical density (OD) ratio at 260 and 280 nm of the total RNA was 1.85 to 1.97 (Supplemental Table S2; <https://doi.org/10.3168/jds.2019-16910>) and met the specified

purity requirements [an $\text{OD}_{260}:\text{OD}_{280}$ ratio of 1.8 to 2.0 is the threshold for RNA quality according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines]. The agarose gel electrophoresis of total RNA showed 3 bands (28S, 18S, and 5S), as presented in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2019-16910>). Then 2 μg of total RNA was reverse-transcribed to cDNA using a reverse transcription kit, containing gDNA Eraser (RR047A, TaKaRa Biotechnology Co. Ltd., Tokyo, Japan), according to the manufacturer's instructions. To eliminate gDNA contamination, the RNA extractions were treated with gDNA Eraser for 2 min at 42°C . The gDNA Eraser has potent DNA degradation activity. The mRNA abundance was detected using a FastStart Universal SYBR Green Master (ROX; 4913850001, Roche, Norwalk, CT) with the 7500 Real-Time PCR System (Applied Biosystems Inc., Waltham, MA). All melt curves of SYBR-based PCR targets are single discrete peaks, respectively. The conditions were as follows: 95°C for 3 min, 35 cycles of 95°C for 15 s, and 60°C for 1 min. The primers were designed using the Primer-BLAST tool available from the NCBI website (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) according to a previous study (Ye et al., 2012). The primers used for autophagy-related gene (*ATG5*), *ATG7*, *ATG12*, beclin 1 (*BECN1*), phosphatidylinositol 3-kinase catalytic subunit type 3 (*PIK3C3*), microtubule-associated protein 1 light chain 3 (*MAP1LC3*, also called LC3), sequestosome-1 (*SQSTM1*, also called p62), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and *ACTB* are shown in Supplemental Table S3 (<https://doi.org/10.3168/jds.2019-16910>). The cycles-to-threshold values of *GAPDH* and *ACTB* were not different between subclinically ketotic cows and control cows (Farke et al., 2008; Morey et al., 2011; Du et al., 2017; Supplemental Figure S2, <https://doi.org/10.3168/jds.2019-16910>); therefore, the relative

Table 1. Productive and physiological parameters of cows with hyperketonemia (CWH) and cows without hyperketonemia (CWOH)¹

Item	CWH		CWOH		P-value
	Median	IQR	Median	IQR	
BW (kg)	638	608, 662	621	593, 640	0.55
Milk production (kg of milk/cow per day)	28.2	26.7, 31.8	31.1	28.4, 34.3	0.06
DMI (kg/d)	19.7	18.1, 21.3	22.1	20.5, 24.1	0.07
Milk fat (%)	4.2	4.01, 4.55	3.5	3.24, 3.73	0.04
Milk protein (%)	2.87	2.53, 3.03	3.25	3.16, 3.38	0.12
Milk lactose (%)	4.21	3.82, 4.55	4.05	3.77, 4.23	0.59
Serum glucose (mM)	2.85	2.63, 3.27	3.84	3.68, 4.04	0.01
Serum fatty acids (mM)	0.52	0.45, 0.57	0.41	0.37, 0.49	0.05
Serum BHB (mM)	2.44	1.3, 2.82	0.49	0.41, 0.57	0.01

¹According to serum BHB concentrations, 10 Holstein CWH with serum BHB concentration higher than 1.2 mM and lower than 3.0 mM and 10 Holstein CWOH with serum BHB concentration less than 1.2 mM were randomly selected. IQR = interquartile range.

expression of target genes was normalized to *GAPDH* and *ACTB*. The relative expression of target genes was determined through the $2^{-\Delta\Delta CT}$ method.

Protein Extraction and Western Blotting

Western blotting assays were performed as described by Li et al. (2019). Mammary tissues were minced into small pieces. Pre-cooled extraction reagents contained protease and phosphatase inhibitors were added, and the tissues were homogenized 3 times for 20 s at 5,500 rpm with 30-s intervals using a tissue homogenizer (Tian-Gen Biotech, Beijing, China). Then the total protein of homogenized tissues was extracted, using a commercial protein extraction kit (BC3640-50T; Solarbio Science and Technology Co. Ltd., Beijing, China) in accordance with the manufacturer's instructions. The protein concentration was measured via the BCA method (P1511; Applygen Technologies, Beijing, China). A total of 25 μ g of protein from each sample was separated by 12% SDS-PAGE. The molecular location of bovine target protein on gel was consistent with the manufacturer's statement of antibody (molecular weight of the target protein) by referring the molecular weight marker (26616; Thermo Fisher Scientific, Waltham, MA). The target protein on gel was electrophoretically transferred to a polyvinylidene difluoride membrane (PVDF). The membranes were blocked in 3% BSA/Tris-buffered saline/Tween (TBS-T) buffer for 4 h, and were then incubated overnight at 4°C with primary antibodies against AMPK α (1:1,000; 2532, Cell Signaling Technology, Danvers, MA), phosphorylated (Thr172)-AMPK α (1:1,000; 2532, Cell Signaling Technology), phosphorylated (Ser555)-Unc-51-like kinase 1 (**ULK1**; 1:2,000; 5869; Cell Signaling Technology), ULK1 (1:1,000; 8054; Cell Signaling Technology), ATG5 (1:500, NB110, Novus Biologicals, Littleton, CO), p62 (1:2,000; ab101266; Abcam, Cambridge, MA), LC3 (1:1,000; ab48394; Abcam), β -actin (1:2,000; ab8226; Abcam), phosphorylated (Ser2448)-mTOR (1:1,000; ab84400, Abcam), or mTOR (1:1,000; ab2732, Abcam), respectively. In addition, the abundance of ubiquitinated proteins from total tissue proteins by using antibody against Ubiquitin (1:200; sc-271289; Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, the PVDF membranes were washed with TBS-T 3 times and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin at room temperature for 45 min. Immunoreactive bands were visualized by enhanced chemiluminescence solution (Millipore, Bedford, MA). β -Actin was used as a reference protein in this study. Lastly, all Western blot bands were analyzed using Image-pro Plus (Media Cybernetics, Rockville, MD). The antibodies of ATG5, actin, p62, LC3, AMPK α ,

p-AMPK α , mTOR, and p-mTOR can react with bovine target proteins according to the manufacturer's statement. The species reactivities of ULK1 antibody shown on the manufacturer's statement are human, mouse, rat, and monkey but not bovine. However, the protein homology of ULK1 between human and bovine is 92%. In this study, the Western blotting result showed that the cow ULK1 protein could be determined using ULK1 antibody.

Transmission Electron Microscopy

The ultrastructural characteristics of autophagosomes were visualized through transmission electron microscopy. Mammary parenchyma was fixed with 2.5% glutaraldehyde (in 0.1 M PBS) for 12 h and washed with 0.1 M PBS, and then postfixed in 1% osmium tetroxide for 1 h. Subsequently, mammary parenchyma was dehydrated in a graded series of ethanol (30, 60, 70, 90, and 100%) and infiltrated with Spurr's resin. Ultrathin sections (50 nm) were cut and stained with 4% uranyl acetate and 0.2% lead citrate. The autophagosomes were observed by an H-7650 electron microscope (Hitachi, Japan). Autophagosomes are double-membraned vesicles that contain cellular material slated to be degraded by autophagy. Autophagosomes were counted and quantified from at least 10 random cells in each sample and expressed as the number of autophagosomes per cell.

Statistical Analysis

Baseline characteristics are expressed as the median and interquartile range, and other data are expressed as the mean $\pm$ standard error of the mean. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). All data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene tests, respectively. For data with Gaussian distribution, parametric statistical analysis was performed using the independent-samples *t*-test; for data with skewed distribution, nonparametric statistical analysis was performed using the Wilcoxon test. We considered $P < 0.05$ to be statistically significant and $P < 0.01$ to be highly significant.

RESULTS

Baseline Characteristics and Blood Parameters

As shown in Table 1, we found no significant difference in body weight between CWH and CWOH ($P = 0.55$). Percentages of milk protein ($P = 0.12$) and lactose ($P = 0.59$) also did not differ between groups. The DMI ($P = 0.07$) and milk production ($P = 0.06$)

of CWH were lower than those of CWOH. The milk fat percentage of CWH was greater than that of CWOH ($P = 0.04$). The blood concentrations of fatty acids and BHB were higher ($P = 0.05$ and $P = 0.01$) in CWH compared with CWOH. In contrast, the concentration of glucose was markedly lower ($P = 0.01$) in CWH.

Expression of Autophagosome Formation-Related Genes in the Mammary Glands of Cows

The mRNA abundance of autophagosome formation-related genes *BECN1*, *PIK3C3*, *ATG5*, *ATG7*, and *ATG12* was higher in the mammary glands of CWH (Figure 1A, $P < 0.05$) than in those of CWOH. Although the protein abundance of ATG5 was slightly high in the mammary gland of CWH, we observed no statistically significant differences (Figure 1B and C, $P = 0.2523$). The phosphorylation level of ULK1 was greater in the mammary gland of CWH than of CWOH (Figure 1B and D, $P = 0.0150$).

Changes of Total Ubiquitinated Proteins, Expression of MAP1LC3 and SQSTM1, and Autophagosomes in the Mammary Glands of Cows

The total ubiquitinated proteins were lower ($P = 0.0013$) in the mammary glands of CWH than of CWOH (Figure 2). The mRNA abundance of *MAP1LC3* (Figure 3A, $P = 0.0068$) and its protein (Figure 3B and D, $P = 0.0195$) were greater in the mammary glands of CWH than in those of CWOH. It is noteworthy that mRNA expression of *SQSTM1* was greater in the mammary glands of CWH (Figure 3A, $P = 0.0031$). However, the protein expression of p62, which is required for autophagic degradation of ubiquitinated proteins, was significantly lower in the mammary glands of CWH than of CWOH (Figure 3B and C, $P = 0.0270$). Transmission electron microscopy showed a significant increase in the number of autophagosomes in the mammary glands of CWH (Figure 4, $P = 0.0079$).

Changes of Autophagic Pathways in the Mammary Glands of Cows

The phosphorylation levels of AMPK α were greater in the mammary glands of CWH than in CWOH (Figure 5 A and B, $P = 0.0062$). By contrast, the phosphorylation level of mTOR was lower in the mammary glands of CWH (Figure 5 A and C, $P = 0.0147$).

DISCUSSION

During the transition from pregnancy to lactation, high-production dairy cows are at risk of developing

hyperketonemia. The incidence of hyperketonemia in dairy cows ranges from 26.4 to 55.7% in the United States (McArt et al., 2011). Perinatal dairy cows, especially obese cows characterized by higher body weight, experience greater decreases in feed intake and more pronounced NNB, resulting in low milk production

Figure 1. Abundance of autophagosome formation-related molecules in mammary glands of cows with hyperketonemia (CWH) and cows without hyperketonemia (CWOH). According to the serum BHB concentration, 10 Holstein CWH with serum BHB concentration higher than 1.2 mM and lower than 3.0 mM and 10 Holstein CWOH with serum BHB concentration less than 1.2 mM were randomly selected. (A) Abundance of autophagosome formation-related genes in mammary glands of CWH and CWOH. (B) The protein abundance of ATG5, p-ULK1, and ULK1 in mammary glands of CWH and CWOH. (C and D) Quantification of protein expression of ATG5 and p-ULK1. A total of 25 μ g of protein from each sample was separated using 12% SDS-PAGE. The phosphorylation (p) site of ULK1 is Ser555. Data were analyzed with independent-samples *t*-tests and expressed as mean $\pm$ SEM. ATG5 = autophagy-related gene 5; ULK1 = Unc-51-like kinase 1.

(Stockdale, 2001). Thus, fat mobilization of obese cows is increased more than that of cows with normal body weight (Rukkwamsuk et al., 1998). Consequently, excessive fatty acids are released from adipose tissue and are incompletely oxidized into ketone bodies in the liver (McNamara, 2000). Therefore, obese cows are highly susceptible to hyperketonemia. Our data also showed that the body weight of CWH was slightly greater than that of CWOH. However, the DMI and milk production of CWH were lower than those of CWOH. Therefore, control of prepartal body weight is recommended for cows, to prevent hyperketonemia.

Hyperketonemia induces severe metabolic stress, such as low blood concentration of glucose and high blood concentrations of fatty acids and BHB. The majority of CWH can cope with the metabolic changes induced by hyperketonemia during the transition period. In this process, the mammary gland plays a crucial role in responding to the challenges of metabolic stress (Gross and Bruckmaier, 2019; Marett et al., 2019). Autophagy is an evolutionarily conserved physiological process that alleviates metabolic stress and maintains cellular energetic balance during starvation (Singh et al., 2009; Dikic and Elazar, 2018). Accordingly, autophagy may play a role in regulating metabolic stress in mammary epithelial cells of CWH. In this study, the autophagy and its regulation pathways AMPK α and mTOR were upregulated in mammary tissue of CWH, suggesting that increased autophagy is one of the adaptive

mechanisms to respond to the hyperketonemia-induced metabolic stress in the mammary glands of dairy cows.

Autophagy involves the formation of a double-membrane structure that engulfs cytoplasmic material and closes to form an autophagosome, which fuses with the lysosome, leading to degradation of the sequestered material. In this process, LC3-II, ubiquitination, and p62 are markers for the evaluation of autophagic flux (Mizunoe et al., 2018). Formation and lengthening of the autophagosome are initiated by LC3-II (Hwang et al., 2017); ubiquitination is a post-translational modification in which ubiquitin, a small polypeptide, is attached to intracellular misfolded proteins or organelles (Shaïd et al., 2013); and p62 acts as a selective autophagy receptor that recognizes and shuttles ubiquitinated proteins to the autophagosome for degradation (Bjørkøy et al., 2005). Mann et al. (2016) reported that the level of LC3-II was upregulated in the skeletal muscle of dairy cows with negative energy balance during the transition period; theirs is the first study showing a role of autophagy in the homeorhetic adaptation to negative energy balance. Furthermore, in vivo studies of bovine mammary gland physiology suggested that the enhanced process of autophagy may be observed at the end of lactation and during dry periods (Zarzynska and Motyl, 2008; Wohlgemuth et al., 2016), indicating that autophagy might be associated with mammary epithelial cells' physiological adaptation to lactation period. However, autophagy was not char-

Figure 2. Expression of ubiquitinated proteins in mammary glands of cows with hyperketonemia (CWH) and cows without hyperketonemia (CWOH). Selection criteria for CWH (Holstein, $n = 10$) and CWOH (Holstein, $n = 10$) are shown in Figure 1. (A) Expression of ubiquitinated (Ub) proteins in mammary glands of CWH and CWOH. Representative blots are shown. (B) Quantification of expression of ubiquitinated (UB) proteins. A total of 25 μ g of protein from each sample was separated using 12% SDS-PAGE. Data were analyzed with independent-samples t -tests and expressed as mean $\pm$ SEM.

acterized in the mammary gland of CWH during the transition period in previous studies. We found that the mRNA abundance of autophagosome formation-related genes *BECN1*, *PIK3C3*, *ATG5*, *ATG7*, and *ATG12*, the protein expression of LC3-II, and the number of autophagosomes were greater in the mammary gland of CWH, demonstrating that autophagy is activated and autophagosome formation is increased in the mammary gland of CWH. Furthermore, the low abundance of p62 and total ubiquitinated protein further indicated that autophagic flux is higher in the mammary gland of CWH. Taken together, these results indicate increased autophagic activity in mammary glands of CWH. The mammary tissue of CWH faces intense metabolic challenges, such as high concentrations of fatty acids and BHB and low concentration of glucose. High concentrations of fatty acids and BHB cause lipotoxicity that can damage mitochondria and induce endoplasmic reticulum stress and, further, induce oxidative stress and cell death (Song et al., 2014; Gao et al., 2018; Zhu et al., 2019b). Autophagy can partly relieve those stresses. The detailed reasons are as follows: (1) Autophagy can remove damaged mitochondria to prevent an increase in reactive oxygen species (Bingol and Sheng; 2016). Excess reactive oxygen species can lead to cellular dam-

age, induction of proapoptotic proteins, and cell death (Niizuma et al., 2009; Song et al., 2014). Furthermore, autophagy can maintain normal mitochondrial function and increase the β oxidation of fatty acids to increase ATP production and attenuate low glucose-induced energy deficiency (Kaushik et al., 2010). (2) Autophagy is also important to engulf damaged endoplasmic reticulum in the unfolded protein response (Bernales et al., 2006) and degrade protein aggregates that may improve fatty acid-induced endoplasmic reticulum stress. (3) Autophagy is essential to limit DNA damage and genomic instability, possibly through modulation of protein aggregates and the adaptor protein p62 (Mathew et al., 2009). Accordingly, enhanced autophagy increased the ability of mammary epithelial cells in dairy cows to respond to hyperketonemia-induced metabolic stress. Therefore, increased autophagy at the molecular level may partly explain the inherently homeorhetic adaptation mechanism of mammary tissue in CWH.

Autophagy is highly regulated by AMPK α and mTOR pathways (Kim et al., 2011; Zhang et al., 2016). In the autophagy process, AMPK α directly phosphorylates ULK1 at Ser317, Ser555, and Ser777 to activate the pro-autophagy Vps34 complex, which is critical for its function in autophagy (Egan et al., 2011). Energy

Figure 3. Hepatic autophagy status in mammary glands of cows with hyperketonemia (CWH) and cows without hyperketonemia (CWOH). Selection criteria for CWH (Holstein, $n = 10$) and CWOH (Holstein, $n = 10$) are shown in Figure 1. (A) mRNA abundance of *SQSTM1* and *MAP1LC3* in mammary glands of CWH and CWOH. (B) Protein levels of LC3 and p62. Representative blots are shown. (C and D) Quantification of protein levels of p62 and LC3-II. A total of 25 μ g of protein from each sample was separated using 12% SDS-PAGE. Data were analyzed with independent-samples t -tests and expressed as mean $\pm$ SEM *MAP1LC3* or LC3, microtubule-associated protein 1 light chain 3; *SQSTM1* or p62, sequestosome-1.

Figure 4. Representative transmission electron microscopy images of sections of mammary epithelial cells of cows with hyperketonemia (CWH) and cows without hyperketonemia (CWOH). Selection criteria for CWH (Holstein, $n = 10$) and CWOH (Holstein, $n = 10$) are shown in Figure 1. (A) Representative images of autophagosomes (white arrows) in mammary epithelial cells of CWOH (a) and CWH (b and c). Image c is the magnification of image b. Scale bars: 2 μm (a, b), 1 μm (c). (B) Numbers of autophagosomes per cell. Autophagosomes were counted from at least 10 random cells in each sample. Data were analyzed with independent-samples t -tests and expressed as mean $\pm$ SEM.

or nutrient starvation could increase the intracellular AMP:ATP ratio and further increase AMPK α activity by triggering its phosphorylation (Li et al., 2013). In addition, mTOR is a crucial regulator for the induction of autophagy via phosphorylation and interaction of ATG13 and ULK1 (Kim et al., 2011). Nutrient starvation, such as low levels of amino acid and glucose, could inhibit the activity of mTOR and further induce autophagy in eukaryotes (Yuan et al., 2013). Importantly, the activated AMPK indirectly activates autophagy by suppressing the activity of mTOR under glucose starvation (Kim et al., 2011). In this study, the phosphorylation of AMPK α (Thr172) and ULK1 (Ser555)

was higher but mTOR phosphorylation (Ser2448) was lower in the mammary gland of CWH, which indicates that the AMPK α and mTOR autophagic pathways are upregulated and involved in the activation of autophagy. The mammary gland of CWH is in an NNB state. Consequently, enhanced autophagy increases catabolism and improves NNB of the mammary gland in CWH, thereby attenuating metabolic stress. Findings from in vitro studies further validated our results that glucose and amino acid deficiency augmented AMPK α phosphorylation and inhibited mTOR phosphorylation in mammary epithelial cells of dairy cows (Burgos et al., 2013; Zhang et al., 2018). These responses were

also observed in the livers of nonruminants (mice) under food restriction (Zhang et al., 2013; Chauvin et al., 2014). Collectively, under conditions of hyperketonemia-induced metabolic stress, activation of AMPK α and inhibition of mTOR intensified the induction of autophagy, to improve metabolic stress. Therefore, AMPK α -mTOR-mediated autophagy may be a potential therapeutic target for prevention and treatment of hyperketonemia-induced metabolic stress and NNB in early-lactating dairy cows.

Cows with hyperketonemia often display low blood concentration of glucose and high concentrations of fatty acids and BHB, as well as significant changes to insulin, glucagon, growth hormone, prolactin, and so on (Peel and Bauman, 1987; Steen et al., 1997; Du et al., 2018b). Previous studies have demonstrated that these signal molecules and hormones all play important roles

in the regulation of AMPK α and mTOR pathways and autophagy (Liu et al., 2016; Sinha et al., 2017). Therefore, the high autophagic activity in the mammary glands of CWH might be due to the combined roles of the above signal molecules and hormones. However, the underlying mechanism and crosstalk roles need to be further investigated.

Furthermore, cows with hyperketonemia displayed decreased milk production in the early postpartal period, which is closely associated with decreased DMI, lipotoxicity of high levels of fatty acids and BHB, and changes to insulin, IGF, and somatotropin. For instance, high levels of fatty acids and BHB could induce apoptosis (Song et al., 2014, 2016) and might impair the proliferation and differentiation abilities of mammary epithelial cells. Additionally, low levels of IGF-1 and insulin have negative effects on the proliferation and differentiation abilities of mammary epithelial cells (Ha et al., 2016), resulting in decreased milk yield. A limitation of this study is that the physiological and metabolic status may differ among cows with different parities and DIM; therefore, the role of autophagy in hyperketonemia-induced decreased milk production in dairy cows merits further study.

CONCLUSIONS

The autophagy-related pathways AMPK α and mTOR and autophagy activity were higher in the mammary glands of CWH. This increased autophagy activity may be a mechanism for the mammary gland to respond to the challenge of metabolic stress induced by hyperketonemia in dairy cows.

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Figure 5. Changes to the AMPK α and mTOR autophagic pathways in mammary glands of cows with hyperketonemia (CWH) and cows without hyperketonemia (CWOH). Selection criteria for CWH (Holstein, $n = 10$) and CWOH (Holstein, $n = 10$) are shown in Figure 1. (A) AMPK α protein, phosphorylated-AMPK α , mTOR, and phosphorylated-mTOR in mammary glands of CWH and CWOH. (B and C) Phosphorylation levels of AMPK α and mTOR. A total of 25 μ g of protein from each sample was separated using 12% SDS-PAGE. The phosphorylation site of AMPK α is Thr172, and that of mTOR is Ser2448. Data were analyzed with independent-samples t -tests and expressed as mean $\pm$ SEM. AMPK α = AMP-activated kinase α ; mTOR = mechanistic target of rapamycin.

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