Adipose tissue proteomic analysis in ketotic or healthy Holstein cows in early lactation¹

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ABSTRACT: Ketosis is a major metabolic disorder of high-yielding dairy cows during the transition period. Although metabolic adaptations of the adipose tissue are critical for a successful transition, beyond lipolysis, alterations within adipose tissue during ketosis are not well known. The objective of this study was to investigate the adipose tissue proteome of healthy or ketotic postpartum cows to gain insights into biological adaptations that may contribute to disease outcomes. Adipose tissue biopsy was collected on 5 healthy and 5 ketotic cows at 17 (± 4) d postpartum and ketosis was defined according to the clinical symptoms and serum β -hydroxybutyrate concentration. Morphology micrographs stained by hematoxylin–eosin showed that adipocytes were smaller in ketotic cows than in healthy cows. The isobaric tag for relative and absolute quantification was applied to quantitatively identify differentially expressed proteins (DEP) in the adipose tissue. We identified a total of 924 proteins, 81 of which were differentially expressed between ketotic and healthy cows ($P < 0.05$ and fold changes > 1.5 or < 0.67). These DEP included enzymes and proteins associated with various carbohydrate, lipid, and amino acid metabolism processes.

The top pathways differing between ketosis and control cows were glycolysis/gluconeogenesis, glucagon signaling pathway, cysteine and methionine metabolism, biosynthesis of amino acids, and the cGMP–PKG signaling pathway. The identified DEP were further validated by western blot and co-immunoprecipitation assay. Key enzymes associated with carbohydrate metabolism such as pyruvate kinase 2, pyruvate dehydrogenase E1 component subunit α), lactate dehydrogenase A, phosphoglucomutase 1, and 6-phosphofructokinase 1 were upregulated in ketotic cows. The expression and phosphorylation state of critical regulators of lipolysis such as perilipin-1 and hormone-sensitive lipase were also upregulated in ketotic cows. Furthermore, key proteins involved in maintaining innate immune response such as lipopolysaccharide binding protein and regakine-1 were downregulated in ketotic cows. Overall, data indicate that ketotic cows during the transition period have altered carbohydrate, lipid metabolism, and impaired immune function in the adipose tissue. This proteomics analysis in adipose tissue of ketotic cows identified several pathways and proteins that are components of the adaptation to ketosis.

Key words: adipose tissue, ketosis, proteomics, transition

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J. Anim. Sci. 2019.97:2803–2815
doi: 10.1093/jas/skz132

¹This work was supported by the National key research and development program (Beijing, China; grant 2016YFD0501007-3), National Natural Science Foundation of China (Beijing, China; grant 31572581 and 31772810), Jilin Province Science Foundation for Youths (Changchun, China;

grant 20160520063JH), and Natural Science Foundation of Jilin Province (Changchun, China; grant 20170101148JC).

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Received February 5, 2019.

Accepted April 12, 2019.

INTRODUCTION

The transition between late gestation and the onset of lactation is a physiologically challenging period for dairy cows and the predominant time for occurrence of metabolic disorders (Goff and Horst, 1997; Drackley, 1999). Dry matter intake (DMI) decreases gradually during the last 3 weeks of gestation, whereas postpartum energy demands for maintenance of body functions and milk production is markedly higher than prepartum, which induces a negative energy balance (Bell, 1995; Drackley et al., 2006). To compensate for insufficient energy intake, a coordinated suite of physiological adaptations including enhanced fat mobilization, hepatic gluconeogenesis, and bone resorption promotes delivery of substrates to support milk synthesis (Reynolds et al., 2003).

Coordination of the metabolism of adipose tissue is an essential part of a successful lactation (Khan et al., 2013) and helps support the overall efficiency of milk production. Several classic studies have outlined the basic metabolic adaptations of adipose tissue to lactation, and specifically aspects of lipogenesis, esterification, and lipolysis (Mcnamara, 1989; McNamara, 1994; Vernon et al., 2003). For instance, it is known that cows with high genetic merit for milk production have increased sensitivity of lipolysis partly due to greater responsiveness to β -adrenergic stimulation and hormone-sensitive lipase (HSL) (Mcnamara, 1989; Martin-Hidalgo et al., 1994; McNamara, 1994). Furthermore, lipolysis is also modulated by posttranscriptional and allosteric changes in the HSL hydrolase pathway in transition dairy cows (Contreras et al., 2017). Recent study has shown that the increase in HSL mRNA only stimulated about 12% to 17% of the variation in lipolysis, which suggested that most of the control of HSL activity is posttranslational (Sumner and McNamara, 2007). Recent studies also demonstrated an important role for transcriptional regulation of anabolic metabolic processes within adipose tissue (Ji, 2012). These changes of metabolic molecules play an important role in the metabolic control of dairy cows during transition.

In animal models and humans, it is now well-established that adipose tissue contributes to systematic inflammation during obesity via the production of adiponectin, leptin, inflammatory, and anti-inflammatory cytokines (Bernabucci et al., 2006; Baranova et al., 2007). Dairy cattle with metabolic disorders such as ketosis or fatty liver display high levels of systemic inflammation (Li et al., 2015), even without signs of microbial infections

or pathological signs (Bertoni et al., 2008). Both of these disease states have increased lipolysis, insulin resistance, and increased inflammation of early lactation.

Recent developments in the field of proteomics have led to a renewed interest in animal disease diagnosis and treatment. isobaric Tag for relative and absolute quantification (iTRAQ) is the most popular differential proteomics technique, and combined with multidimensional liquid chromatography (LC) and tandem MS, it is used to study differentially expressed proteins (DEP) (Sun et al., 2012; Tse et al., 2013; Wang et al., 2016). In view of the changes of glycolipid metabolism and systemic inflammation in ketotic cows, we hypothesize that key proteins and affected pathways that regulate glycolipid metabolism and inflammation in adipose tissue will be differentially expressed and altered in response to ketosis. Therefore, the specific aim of this study was use iTRAQ to investigate the proteomic profiles in adipose tissue during ketosis as a way to identify proteins beyond those related to the classic metabolic pathways in an effort to better understand how this disorder may also encompass other cellular processes.

MATERIALS AND METHODS

Experimental Design and Cows

Holstein dairy cattle from the Shengao Dairy Center (Nenjiang, China) were housed and fed in accordance with the Guiding Principles of Animal Experiments by the Chinese Society of Laboratory Animal Sciences and protocols approved by the Jilin University Animal Care and Use Committee (201803052). The cows were classified as suspected clinical ketosis by veterinarians if feed intake, milk yield, or both were reduced and a nitroprusside test for ketone bodies in milk was positive (Du et al., 2018). Subsequently, the blood concentration of β -hydroxybutyrate (BHB) in these cows was measured. According to the clinical symptoms and serum BHB concentration (Itle et al., 2015; Vanholder et al., 2015; Du et al., 2018), five clinically ketotic cows with serum BHB concentration higher than 3 mM, and five control cows with serum BHB concentration less than 0.6 mM were randomly selected. The basic descriptions of the ketotic and healthy cows are given in Table 1. Blood samples were collected via coccygeal venipuncture with heparin before feeding at 5 a.m. and centrifuged at $1,900 \times g$ for 15 min at 4 °C. The blood concentrations of glucose, nonesterified fatty acid (NEFA), and

Table 1. Characteristics of the dairy cows and samples used in this study

Parameter	Ketosis	Control	<i>P</i>
Parities	Third	Third	
No. of cows	5	5	
Milk production, kg/d	27.43 ± 1.20	38.27 ± 0.80	<0.01
Body weight (kg)	644.80 ± 17.31	610.00 ± 22.37	0.025
DMI (kg/d)	19.88 ± 0.57	21.62 ± 0.51	<0.01
Body condition scores	3.20 ± 0.11	2.70 ± 0.11	<0.01
BHBA (mM)	4.10 ± 0.38	0.48 ± 0.11	<0.01
NEFA (mM)	1.04 ± 0.06	0.29 ± 0.04	<0.01
Glucose (mM)	2.14 ± 0.14	3.65 ± 0.28	<0.01
TNF-α (pg/mL)	188.89 ± 12.51	77.32 ± 19.87	<0.01
IL-1β (pg/mL)	895.34 ± 69.32	158.67 ± 35.44	<0.01

BHB were detected using a Hitachi 7170 autoanalyzer (Hitachi, Tokyo, Japan; lab assay) with commercially available kits (Randox Laboratories, Crumlin, United Kingdom). The blood concentrations of tumor necrosis factor-α (TNF-α) and Interleukin 1 beta (IL-1β) were detected using commercially available kits (SEA133Bo & SEA563Bo; USCN Life Science Inc., Wuhan, China).

All animals were fed the same diet during the dry period, starting at approximately 21 d prepartum, formulated to meet nutrient requirements as recommended by National Research Council (2001; [Supplementary Table S1](#)). During lactation, all animals were fed the same diet to requirements (NRC, 2001; [Supplementary Table S1](#)). Intake was not measured in this study.

Biopsies

The adipose tissue biopsies were collected at 17 (±4) d postpartum. Prior to biopsies, the hair on the tail-head and to one side of the tail-head was clipped closely and thoroughly scrubbed with surgical soap. Local anesthesia was applied over the area between the point of the ischium and coccygeal vertebra. A 6- to 8-cm incision was made and the skin pulled back using sterile forceps, exposing the tissue. Samples were taken via blunt dissection using a sterile scalpel blade and forceps. After the sample was taken, pressure was applied with sterile gauze to stop any external bleeding. The incision was closed with 8 to 12 surgical staples (#89063337, Appose ULC Skin Stapler, 35 wide; Henry Schein Inc., Melville, NY). The adipose tissue biopsies were washed with RNase-free PBS. Biopsied tissue (1 to 2 g) was weighed and stored in liquid N₂ and fixed in 4% formalin, respectively. After formalin fixation, tissue specimens were embedded in paraffin blocks using routine procedures, followed by hematoxylin and eosin (HE) staining and

histopathological examination under the microscope to identify morphological changes.

Protein Isolation, Digestion, and Labeling With iTRAQ Reagent

Protein extraction was performed on 5 healthy and 5 ketotic cows. Briefly, the frozen tissue was ground into powder and then dissolved in lysis buffer I (7 M urea, 2 M thiourea, 4% propanesulfonic acid, 40 mM Tris-HCl, pH 8.5) containing complete protease inhibitor. The cells were lysed by sonication at 200 W for 15 min and then centrifuged at 4 °C, 30,000 × *g* for 15 min. The supernatant was mixed with 5× volume of chilled acetone containing 10% (vol/vol) trichloroacetic acid, incubated at −20 °C overnight, and then centrifuged as described. The precipitate was washed 3 times in chilled acetone. The recovered pellet was air-dried and dissolved in lysis buffer II (7 M urea, 2 M thiourea, 4% Nonidet P40, 20 mM Tris-HCl, pH 8.0–8.5). The suspension was sonicated, centrifuged as described, and transferred to another tube. The disulfide bonds were reduced by treatment with 10 mM dithiothreitol, whereas cysteines were blocked by treatment with 55 mM iodoacetamide. The supernatant was then washed in acetone and centrifuged as described previously. Recovered pellets were air-dried, dissolved in 500 μL of 0.5 M tetraethylammonium bromide (TEAB; Applied Biosystems, Milan, Italy), and sonicated once more. Lastly, samples were centrifuged, and the supernatant was transferred to a new tube and quantified using the Bradford method. The proteins in the supernatant were kept at −80 °C for further analysis.

Total protein (100 μg) was measured from each sample solution and the protein was digested at 37 °C for 16 h with Trypsin Gold (Promega, Madison, WI) at a protein-to-trypsin ratio of 30:1. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 8-plex iTRAQ reagent (Applied Biosystems). Briefly, 1 unit of iTRAQ reagent was thawed and reconstituted in 24 μL of isopropanol. Samples were labeled, respectively, with different isobaric tags and incubated at room temperature for 2 h. The labeled peptide mixtures were then pooled and dried by vacuum centrifugation.

Liquid Chromatography–Electrospray Ionization Tandem Mass Spectrometry Analysis

The iTRAQ-labeled peptide mixtures were fractionated by strong cation exchange chromatography

in a LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan) using a 4.6×250 mm Ultremex SCX column containing 5- μ m particles (Phenomenex, Torrance, CA). The peptides were eluted at a flow rate of 1 mL/min with a gradient of buffers as follows: buffer A [25 mM NaH_2PO_4 in 25% acetonitrile (ACN), pH 2.7] for 10 min, 5 to 60% buffer B (25 mM NaH_2PO_4 , 1 M KCl in 25% ACN, pH 2.7) for 27 min, and 60 to 100% buffer B for 1 min. The system was then maintained at 100% buffer B for 1 min before equilibrating with buffer A for 10 min before the next injection. Under a monitoring absorbance of 214 nm, 20 fractions were collected, desalted with a Strata X C18 column (Phenomenex), and vacuum-dried.

Each fraction was dissolved in buffer (2% ACN, 0.1% formic acid) and centrifuged at $20,000 \times g$ for 10 min; the final concentration of peptide was on average 0.5 $\mu\text{g}/\mu\text{L}$. Ten microliters of supernatant was loaded on a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) by the autosampler onto a 2-cm C18 trap column (i.d. 75 μm). The peptides were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (MS/MS) in a Q Exactive (Thermo Fisher Scientific, San Jose, CA) coupled online to the HPLC. Intact peptides were detected in the Q Exactive orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using high-energy collision dissociation operating mode with a normalized collision energy setting of 27.0; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between 1 MS scan followed by 15 MS/MS scans was applied for the 15 most-abundant precursor ions above a threshold ion count of 20,000 in the MS survey scan with a dynamic exclusion duration of 15 s. The electrospray voltage applied was 1.6 kV. Automatic gain control was used to optimize the spectra generated by the orbitrap. The automatic gain control target for full MS and MS2 was 3E6 ion counts and 1E5 ion counts, respectively. The m/z scan range was 350 to 2,000 Da. For MS2 scans, the m/z scan range was 100 to 1,800 Da.

Data Analysis

Raw MS data were converted into Mascot generic file (MGF) using Proteome Discoverer 1.2 (PD 1.2, Thermo Fisher Scientific), and the MGF data file was searched by using Mascot search engine (version 2.3.02, Matrix Science, London, United Kingdom) to identify proteins. Each protein identification involved at least 1 unique peptide. For

protein quantification, a protein had to contain at least 2 unique spectra. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot (http://www.matrixscience.com/help/quant_statistics_help.html). We only used ratios with $P < 0.05$, and we set fold-changes >1.5 or <0.67 for adipose tissue as the criteria to determine up- or down-regulated proteins, respectively.

Bioinformatics Analyses

Pathway analysis was performed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The gene Ontology (GO) database was used to facilitate the biological interpretation of the identified proteins. The DEP of GO were divided into 3 categories as follows: biological process, molecular function, and cellular component.

Functional annotation of proteins was performed using Blast2GO program against the nonredundant protein database (NR, NCBI; [http://www.ncbi.nlm.nih.gov/protein/?term=txid9913\[Organism:noexp\]](http://www.ncbi.nlm.nih.gov/protein/?term=txid9913[Organism:noexp])). Gene Ontology is an international standardization of gene function classification system. It has 3 ontologies that can describe molecular function, cellular component, and biological processes, respectively. The main types of annotations were obtained from the GO consortium website (<http://david.abcc.ncifcrf.gov/>). The DEP were compared by a hierarchical cluster analysis using Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>). The data were displayed using Tree View software (<https://sourceforge.net/projects/jtreeview/>). Differential protein abundance from western blots was analyzed by ANOVA with the PROC TTEST procedure in SAS v. 9.4 (SAS Institute Inc., Cary, NC). The model included the fixed effect of health status (healthy or ketosis) and the random effect of cow.

Western Blot and co-immunoprecipitation Assay

Western blotting was performed on a subset of DEP to verify the accuracy of iTRAQ results. The same adipose tissue samples used for iTRAQ were used for Westerns. Total protein was extracted from the biopsy adipose samples and cultured cells using a commercial protein extraction kit (250 mM Tris [pH 7.5], 40 mM NaCl, 10 mM EDTA, 5 mM NP-40, and protease and phosphatase inhibitors; C510003; Sangon Biotech Co., Ltd, Shanghai, China) according to the manufacturer's instructions. The protein concentration was determined using the BCA Protein Assay Kit (P1511;

Applygen Technologies). Total protein (20 µg) was loaded on 12% SDS-PAGE gels and then transferred onto 0.45-µm Polyvinylidene Difluoride (PVDF) Membranes (Millipore Corp., Billerica, MA). After blotting, the membrane was blocked with 5% skim milk and incubated with primary antibody and second antibody at 37 °C for 60 min, respectively. The incubated membrane was then washed in 0.05% PBS Tween 3 times. Detection was performed using an enhanced chemiluminescent (ECL) reagent (Pierce Biotechnology Inc., Chicago, IL) and recorded by film exposure. β -Actin was used as a loading control. The maximum intensity of each band was quantified using ImageJ software. Ratios of pyruvate kinase 2 (PKM2), pyruvate dehydrogenase E1 component subunit α (PDE1 α), lactate dehydrogenase A (LDHA), phosphoglucosmutase 1 (PGM1), and 6-phosphofructokinase 1 (PFK) were normalized to β -actin. Antibodies against perilipin-1 (PLIN1), protein kinase A (PKA), and p-HSL were used to detect the expression and phosphorylation state of critical regulators of lipolysis. Antibodies against PKM2 (ab137791), PDE1 α (ab110334), PGM1 (ab223469), and PLIN1 (ab3526) were products of Abcam (Cambridge, United Kingdom). Antibodies against LDHA (NBP1-48336) were from Novus Biologicals (Littleton, CO). Antibodies against PFK (TA308655) were from OriGene Technologies, Inc. (Rockville, MD). Antibodies against PKA (06-903) were from EMD Millipore, Inc. (Hong Kong, China). Antibodies against p-HSL (#4139) and anti-rabbit IgG antisera were from Cell Signaling Technology, Inc. (Danvers, MA).

Co-immunoprecipitation (Co-IP) assays were performed as described previously (Wang et al., 2015). A portion of the same adipose tissue samples used for iTRAQ analysis were washed with ice-cold PBS and lysed in Co-IP lysis buffer (150 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and proteinase inhibitor cocktails, pH 7.4). The supernatants were collected after centrifugation at $12,000 \times g$ for 30 min at 4 °C. A small proportion of the total lysate was kept for determining the protein expression levels, and all of the remaining lysate was used for Co-IP by adding immunoprecipitating antibodies and protein A/G beads (Santa Cruz). After mixing at 4 °C overnight, the beads were pelleted, and the supernatant was removed. Beads were further washed 3 times with Co-IP lysis buffer, and the proteins on the beads were extracted by adding protein-loading buffer and boiling.

RESULTS

Morphology and iTRAQ Analysis of Adipose Tissue

A representative section showing typical morphologic changes observed in the adipose tissue specimens from cows with ketosis is shown in Figure 1. The HE staining results showed that adipocytes were smaller in ketotic cows than in healthy cows in early lactation (Figure 1).

Protein concentrations for ketosis and control samples were 2.5 and 3.2 µg/µL per Bradford assay, respectively. A total of 217,420 spectra and 14,670 unique spectra were used to assign 5,027 unique peptides, representing 1,381 proteins. The iTRAQ quantification and LC-MS/MS analysis identified relative changes in a total of 1,381 proteins, of which 924 were quantifiable (data not shown). Using fold changes of >1.5 or <0.67 in the level of protein abundance as a benchmark for potential physiologically significant changes ($P < 0.05$) resulted in 81 DEP representing 8.7% of the total proteins detected in the adipose tissue. Of these DEP, the expression of 60 of them was upregulated in ketosis vs. control adipose tissue, whereas 21 DEP had lower expression in ketosis compared with controls (Figure 2 and Supplementary Table S2).

Functional Classifications of DEP

Functional annotation of DEP was initially performed using Blast2GO. Three main types of annotations were obtained from the GO consortium website: cellular component, metabolic function, and biological processes (Supplementary Table S3). Enrichment analysis of biological processes indicated that cellular, single-organism, and metabolic process differed in tissue from ketotic cows (Supplementary Figure S1A). Molecular function enrichment analysis also identified ion and protein binding as significantly enriched during ketosis (Supplementary Figure S1B). Furthermore, cellular component-based enrichment analysis identified DEP as being located in the intracellular compartment (Supplementary Figure S1C).

The analysis identified protein ortholog classifications via the COG database, allowing us to predict the possible functions of these proteins and potentially uncover further functional classifications (Supplementary Table S4). The functional class of R (general function prediction only) had the highest number (182/1004, 15.42%) of proteins, O (posttranslational modification, protein

Figure 1. Morphologic changes in adipose tissue of ketotic (A) and healthy (B) dairy cows investigated following hematoxylin and eosin (HE) staining (16.8 $\times$). Micrographs of the bovine adipose tissue showing morphologic changes observed during ketosis (A) and normal tissue (B). Scale bar = 50 μ m.

turnover, and chaperones) had 150 (14.94%) proteins, J (Translation, ribosomal structure, and biogenesis) had 80 (7.97%), and N (cell motility) had the lowest number of proteins (**Figure 3**).

Pathway mapping of data using KEGG revealed that 81 DEP belong were linked to 150 pathways (data not shown). Most relevant molecular and cellular pathways are shown in **Figure 4**, and **Supplementary Table S5**. The most relevant pathways affected by ketosis include carbohydrate and lipid metabolism (e.g., glycolysis/gluconeogenesis, glucagon signaling pathway carbon metabolism, pyruvate metabolism, the citrate cycle, 2-oxocarboxylic acid metabolism, cGMP–PKG signaling pathway, tight junction, and regulation of lipolysis in adipocytes), pathways involved in amino acid metabolism (e.g., cysteine and methionine metabolism and biosynthesis of amino acids), inflammatory immune response (e.g., chemokine signaling pathway and NF-kappa B signaling pathway), and oxidative stress (e.g., HIF-1 signaling pathway).

Validation of Changes in Protein Level by Western Blot and Co-immunoprecipitation Assay

Western blot analysis of PKM2 (FC = 5.512), PDE1 α (FC = 1.625), LDHA (FC = 2.434), PGM1 (FC = 2.998), and PFK (FC = 2.359) is reported in **Figure 5A** and **Supplementary Table S2**. The protein abundance of PKM2, PDE1 α , LDHA, PGM1, and PFK was greater in the adipose tissue of ketotic compared with control cows (**Figure 5B** and **C**). These data confirmed that the ratios (ketosis over healthy) of the chosen were consistent with those obtained from the iTRAQ analysis (**Figure 5A**).

The lipid-droplet coat protein PLIN1 (FC = 2.472; **Supplementary Table S2**) is a serine-phosphorylated protein and a substrate for PKA, which prompted us to examine its alteration during ketosis via PKA-mediated phosphorylation. After immunoprecipitating PLIN1 from adipose tissue, the phosphorylation of PLIN1 was detected with antibodies against PKA substrates. The results showed that the PKA-mediated phosphorylation of PLIN1 was greater in adipose tissue of ketotic cows. Furthermore, the phosphorylation state of HSL also was greater in ketotic cows (**Figure 5D**).

DISCUSSION

Ketosis is a metabolic disorder that is characterized by elevated concentrations of the ketone bodies BHB, acetoacetate and acetone in blood (hyperketonemia), urine, and milk. This disease mainly occurs in early-lactation dairy cows, when body reserves are used to support lactation. Ketosis is prevalent in dairy herds worldwide, causing significantly economic losses resulting from increased susceptibility to mastitis and endometritis and decreased reproductive performance and fertility (**Sheldon et al., 2009**). Understanding the molecular mechanisms of ketosis at the protein level is likely to contribute to the design of new methods in disease control and fertility enhancement. According to the results of iTRAQ analysis, we identified over 924 proteins in the adipose tissue of dairy cows, 81 of which were differentially expressed between ketotic cows and healthy cows in early lactation. Furthermore, through KEGG, we identified the main canonical pathways that differed between ketotic and control cows.

Figure 2. Heat map of all differentially expressed proteins between control (N) and ketosis (K) adipose tissue in dairy cows. The distinctive pattern of protein expression is shown in control adipose tissue (lanes 1–5) and ketosis adipose tissue (lanes 6–10).

The high abundance of ATPase components [ATP synthase protein 8 (F-ATPase8, FC = 3.860)] and 2 mitochondrial enzymes [PDE1- α and isocitrate dehydrogenase (NADP, FC = 2.112)] during ketosis likely contributed to supporting glucose utilization and lipogenesis (Bénit et al., 2001; Martín et al., 2005; Hourel et al., 2012). This idea is further supported by the upregulation of DEP in several carbohydrate metabolism-related pathways (glycolysis/gluconeogenesis, pyruvate

metabolism, citrate cycle, and 2-oxocarboxylic acid metabolism).

Although the catabolic state triggered by ketosis is closely associated with lipolysis, the fact that lipid anabolic pathways would be upregulated might have been associated with a mechanism to preserve some tissue functionality. Among glycolysis/gluconeogenesis-related components, 4 upregulated DEP (PGM1, PFK, PKM2, and LDHA) appear critical for glucose oxidation during ketosis. PGM1

Figure 3. Clusters of orthologous groups (COG) database function classification of cell sequence. The x-axis shows the COG classification entry, and the y-axis shows the number of the corresponding proteins by functional classification.

Figure 4. Most relevant molecular and cellular pathways in the postpartum bovine adipose tissue affected by ketosis in early lactation. Top affected pathways were identified through KEGG pathway analysis. Numbers in parentheses represent the number of proteins in the pathway that are differentially expressed ($P < 0.05$ and fold change >1.5 or <0.67) and control cows.

catalyzes the bi-directional interconversion of glucose 1-phosphate (G-1-P) and glucose 6-phosphate (G-6-P) (Boros et al., 2002), which is one of

key step during glycolysis. PFK is one of the most important regulatory enzymes of glycolysis and is able to regulate the pathway through allosteric

Figure 5. Validation of changes in protein level by western blot and co-immunoprecipitation (Co-IP) assay. (A) Abundance of key proteins in the most relevant pathways that are differentially expressed between ketosis and control (healthy) cows. Cows with ketosis (grey bars) or healthy (black bars) in early lactation. Data presented as mean abundances. Error bars represent standard errors of the mean. * $P < 0.05$. All proteins were higher in abundance in the adipose tissue of ketosis cows than in control cows. (B) Western blot of PKM2, PDE1 α , LDHA, PGM1, and PFK protein expression. (C) The densitometric intensity of immunoblots. β -actin served as the internal control. Band intensity was normalized to β -actin. Cows with ketosis (gray bars) or healthy (black bars) in early lactation. Error bars represent SEM. * $P < 0.05$; ** $P < 0.01$. (D) Western blot and Co-IP assay of key proteins involved in lipolysis in adipose tissue (p-PLIN1, PLIN1, and p-HSL), the phosphorylation of PLIN1 was detected by antibodies against PKA.

inhibition, and in this way, the cell can increase or decrease the rate of glycolysis in response to the cell's energy requirements (Wegener and Krause, 2002). In non-ruminants, PFK is activated by fructose 2, 6-bisphosphate, whose role is to supersede ATP inhibition, thus allowing greater sensitivity to regulation by hormones like glucagon and insulin (Usenik and Legiša, 2010). Another upregulated enzyme PKM2 contributes to catalyzing the dephosphorylation of phosphoenolpyruvate to pyruvate. Because of its positioning at the last step of glycolysis, overexpression of PKM2 would stimulate the ATP production within the glycolytic sequence (Mazurek et al., 2005; Gupta and Bamezai, 2010). LDHA catalyzes the interconversion of pyruvate and L-lactate with concomitant interconversion of NADH and NAD⁺ (Echigoya et al., 2009). Collectively, the upregulation of these proteins in ketotic cows during early lactation suggests that the metabolism of adipose tissue is altered toward more active carbohydrate metabolism and ATP synthesis likely as a way to prevent excessive losses of adipose tissue.

Among the most relevant molecular and cellular pathways within lipid metabolism were cGMP–PKG signaling pathway, tight junction, and regulation of lipolysis in adipocytes, all of which were enhanced in ketotic cows. These adaptations would suggest more active remodeling of the adipose tissue during the massive catabolic state that occurs postpartum. Within regulation of lipolysis in adipocytes, PLIN1 and HSL (FC = 1.510) are critical for lipid droplet formation and hydrolysis. Upon activation by PKA, HSL associates with fatty acid binding protein 4 (FABP4) to form a complex that localizes on the lipid droplet (Miyoshi et al., 2008; Lass et al., 2011). A complete activation of the lipolytic process requires not only the activation of HSL, but also the phosphorylation of PLIN1. PLIN1 has a complex role in regulating both basal and stimulated adipocyte lipolysis (Duncan et al., 2007). Under unstimulated conditions, the presence of PLIN1 coating the lipid droplet functions as a protective barrier that restricts access of triacylglycerol lipases to neutral lipid substrates to prevent unrestrained basal lipolysis (Brasaemle

et al., 2000). Because PKA-dependent phosphorylation of PLIN1 may facilitate interaction with HSL on the lipid droplet, the role of PLIN1 is clearly different from HSL during stimulated lipolysis (Miyoshi et al., 2006).

Compared with other periods of the lactation cycle in dairy cows, PLIN1 and HSL phosphorylation is increased during the first 3 wk after parturition (Koltes and Spurlock, 2011), which is in agreement with the increased lipolysis and higher phosphorylation state of PLIN and HSL in adipose tissue of ketotic cows in the present study. Lipolysis produces a large amount of fatty acids, which enter the liver with the blood and are oxidized for energy supply. It is noteworthy that the expression of fatty acid synthase (FAS, FC = 3.577) was upregulated in ketotic cows. This is a lipogenic enzyme that supports storage of fatty acids through de novo lipogenesis within the adipocyte (Zachut, 2015). In the current study, the upregulation of FAS may have generated fatty acids for re-esterification during lipolysis, i.e., as part of the remodeling mechanism. At least in nonruminants, during lipolysis it is estimated that up to two-thirds of hydrolyzed fatty acids from TG are re-esterified (Leibel et al., 1985; Edens et al., 1990; Nye et al., 2008). Thus, this may be an adaptive mechanism for acute regulation of adipose tissue homeostasis. A previous study (Chitraju et al., 2017) revealed that re-esterification plays a crucial role in protecting adipocytes from lipid toxicity under such FA-liberating conditions. Similar changes of several key protein in lipid metabolism, including PLIN, HSL, and FAS were also identified in a previous study with periparturient cows (Zachut, 2015), which further supports our idea in ketotic cows that PLIN1, HSL, and FAS play an important role in overall lipolysis and remodeling of adipose tissue in ketotic cows.

Adipose tissue is a significant regulator of systemic amino acid metabolism (Mcmanaman and Neville, 2003). The present study revealed that altered amino acid metabolism within ketotic adipose tissue, particularly cysteine and methionine metabolism and biosynthesis of amino acids, was altered in adipose tissue. Furthermore, X1 subunits of the enzyme glutamate–cysteine ligase catalytic (GCLCX1) were downregulated (FC = 0.583) in the adipose tissue of ketotic cows. Glutamate–cysteine ligase (GCL) is the first enzyme of the cellular glutathione (GSH) biosynthetic pathway (Franklin et al., 2009). Thus, the downregulation of GCL showed its limitation role in metabolizing GSH into its constituent amino acids (glutamate, cysteine, and glycine; Lu, 2009). Furthermore, these

changes may have negative impact on ketotic cows in response to oxidative stress.

Pathways involved in inflammatory responses such as chemokine and nuclear factor-kappa B (NF- κ B) signaling were upregulated in cows with ketosis. Our previous results (Zhang et al., 2018) also confirmed a significant increase in the phosphorylation level of NF- κ B and the expression of inflammatory factors in adipose tissue of ketotic cows. Within the NF- κ B signaling pathway, there was downregulation of a soluble acute phase protein, lipopolysaccharide-binding protein (LBP, FC = 0.536), in adipose tissue of ketotic cows. The acute phase proteins are primarily synthesized by hepatocytes as part of the acute-phase response, which is in turn part of the early innate immune response to different stimuli including infection (Gray et al., 1993). Lipopolysaccharide-binding protein binds to bacterial lipopolysaccharide (LPS) to elicit immune responses by presenting the LPS to important cell surface pattern recognition receptors called CD14 and TLR4 (Yu et al., 1997; Muta and Takeshige, 2001). Studies in mice suggest that the protein encoded by LBP is necessary for the rapid acute-phase response to LPS. LBP-deficient mice are more susceptible to bacterial infection (Fierer et al., 2002). Chemokines are small chemoattractant peptides that provide directional cues for cell trafficking and are vital for a protective host response (Struyf et al., 2001b). Regakine-1 belongs to the chemokine signaling pathway and was downregulated (FC = 0.335) in the adipose tissue of ketotic cows. This protein is believed to recruit myeloid cells into the circulation, whereas its synergy with other neutrophil chemoattractants suggests that it also enhances the inflammatory response to infection (Struyf et al., 2001a; Gouw et al., 2002). Collectively, in dairy cows with ketosis, the lower abundance of LBP and regakine-1 might have impaired the innate immune response of the adipose tissue, thus, diminishing the capacity of the organ to fight stressors or exogenous pathogens.

CONCLUSIONS

This proteomic analysis revealed alterations in protein abundance in the adipose tissue of transitioning cows in ketotic or healthy conditions during early lactation. Ketotic cows have impaired immune function and altered carbohydrate, lipid, and amino acid metabolism in the adipose tissue, as indicated by the top canonical pathways and biological functions identified by KEGG and GO. Based on differential abundance of proteins in these pathways,

ketotic cows have increased ATP synthesis, shifts in precursor supply for gluconeogenesis, massive lipolysis, and impaired inflammatory immune response in their adipose tissue that may contribute to infectious diseases. Thus, these changes in adipose tissue function may be associated with transition-related diseases and poor lactation performance. These findings can facilitate further studies to better understand the molecular mechanisms through which altered proteins may promote inflammation and hence ketosis.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

Conflict of interest statement. None declared.

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