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Metabolic Characteristic of the Liver of Dairy Cows during Ketosis Based on Comparative Proteomics*

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ABSTRACT : The objective of the present study was to identify differences in the expression levels of liver proteins between healthy and ketotic cows, establish a liver metabolic interrelationship of ketosis and elucidate the metabolic characteristics of the liver during ketosis. Liver samples from 8 healthy multiparous Hostein cows and 8 ketotic cows were pooled by health status and the proteins were separated by two-dimensional-electrophoresis (2D-E). Statistical analysis of gels was performed using PDQuest software 8.0. The differences in the expression levels of liver proteins ($p<0.05$) between ketotic and healthy cows were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF-TOF) tandem mass spectrometry. Five enzymes/proteins were identified as being differentially expressed in the livers of ketotic cows: expression of 3-hydroxyacyl-CoA dehydrogenase type-2 (HCDH), acetyl-coenzyme A acetyltransferase 2 (ACAT) and elongation factor Tu (EF-Tu) were down-regulated, whereas that of alpha-enolase and creatine kinase were up-regulated. On the basis of this evidence, it could be presumed that the decreased expression of HCDH, which is caused by high concentrations of acetyl-CoA in hepatic cells, in the livers of ketotic cows, implies reduced fatty acid β-oxidation. The resultant high concentrations of acetyl-CoA and acetoacetyl CoA would depress the level of ACAT and generate more β-hydroxybutyric acid; high concentrations of acetyl-CoA would also accelerate the Krebs Cycle and produce more ATP, which is stored as phosphocreatine, as a consequence of increased expression of creatine kinase. The low expression level of elongation factor Tu in the livers of ketotic cows indicates decreased levels of protein synthesis due to the limited availability of amino acids, because the most glucogenic amino acids sustain the glyconeogenesis pathway; thus increasing the level of alpha-enolase. Decreased protein synthesis also promotes the conversion of amino acids to oxaloacetate, which drives the Krebs Cycle under conditions of high levels of acetyl-CoA. It is concluded that the livers of ketotic cows possess high concentrations of acetyl-CoA, which through negative feedback inhibited fatty acid oxidation; show decreased fatty acid oxidation, ketogenesis and protein synthesis; and increased gluconeogenesis and energy production. (**Key Words** : Dairy Cow, Ketosis, Liver Metabolic Characteristic, Comparative Proteomics, Metabolic Network)

INTRODUCTION

Ketosis is a major metabolic disorder of dairy cows in early lactation, which develops when dairy cows fall into a condition of excessively negative energy balance caused by insufficient dietary intake and generous lactation, and characterized by relatively high concentrations of the ketone bodies acetoacetate, β-hydroxybutyrate (BHBA) and acetone, and a concurrent low concentration of glucose in the blood (Grummer 1995; Duffield et al., 1998; Melendez et al., 2006; Ingvartsen 2006). A concentration of serum BHBA greater than 1,200 μmol/L is a common standard used for the diagnosis of ketosis (Duffield, 2000; Geishauser et al., 2000). Liver is the main organ of energy and substrate metabolism, which are critical for metabolic disease, especially for ketosis (Grummer, 1993).

During early lactation, the requirement for glucose is increased significantly because the production of lactose rises dramatically (Danfaer et al., 1995). Thus, fat mobilization is increased in adipose tissue and more

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nonesterified fatty acids (NEFAs) are absorbed in the mitochondria of liver cells. Elevated concentrations of NEFA increase lipogenesis and ketogenesis in hepatocytes (Cadórniga-Valiño et al., 1997). High concentrations of ketone bodies decrease the rates of β-oxidation of fatty acids (Grum et al., 1996; Cadórniga-Valiño et al., 1997), gluconeogenesis (Cadórniga-Valiño et al., 1997; Rukkwamsuk et al., 1999) and the citric acid cycle in hepatocytes. However, contradictory results on the metabolic changes in the liver during ketosis have been reported, with some studies indicating that gluconeogenesis is unchanged in ketogenic situations (Kronfeld, 1969). What determines whether fatty acids are oxidized completely to CO_2 and water to produce ATP, or only partially oxidized while producing ATP and ketone bodies? The most widespread hypothesis is that acetyl-CoA is directed towards incomplete oxidation (ketogenesis) as a result of low accessibility of oxaloacetate in the mitochondria (Krebs, 1966). However, this theory has been criticized, as it is documented that the activity of phosphoenolpyruvate carboxykinase in the mitochondria and cytosol is unchanged in ketogenic situations, and that the gluconeogenic flux is diminished in fasted animals (Kronfeld, 1969). Zammit (1990) concludes that Krebs' theory that maintaining a low level of oxaloacetate is the cause of ketogenesis is probably correct, but the reasoning for it is wrong. The low concentration of oxaloacetate is achieved by a much higher NADH/NAD^+ ratio in the mitochondria than in the cytoplasm. This should result in a minimal rate of intramitochondrial phosphoenolpyruvate formation under ketogenic conditions, accounting for the lower rate of gluconeogenesis during ketotic periods with inappetance (Zammit, 1990). In recent years, many researchers have investigated the etiology of ketosis, often leading to contradictory opinions (Grummer, 1993; Drackley, 1999; Herdt, 2000; Drackley et al., 2001).

Research of comparative proteomics of the liver in ketotic cows is limited. This technology can provide broad-scale information on the metabolic profile of the livers in ketotic cows, and may help us to understand the etiology of ketosis. The objectives of this paper were to identify differences in the expression levels of liver proteins between healthy and ketotic cows, establish a liver metabolic network of ketosis, and elucidate the metabolic characteristics of the liver during ketosis.

MATERIALS AND METHODS

Sample collection and detection of ketosis

All animals used in this study were treated according to the International Guiding Principles for Biomedical Research Involving Animals. Sixteen multiparous, lactating Holstein cows of the same breed, age, similar milk production and body condition score at calving, were selected from a commercial dairy farm (QingXin) located in the Heilongjiang province, China. Cows were fed the same diet during the dry period and early lactation. All samples, blood and tissues, were collected before feed in the morning after parturition. According to the following serum parameters and clinical signs, eight cows were diagnosed as ketosis (β-hydroxybutyric acid>1.2 mmol/L, glucose<2.5 mmol/L, and triglyceride<0.12 mmol/L), and eight as normal (β-hydroxybutyric acid<0.60 mmol/L, glucose>3.75 mmol/L, and triglyceride>0.16 mmol/L) (Mutlu and Abdullah, 1998). After 28 days of calving, cow liver tissue samples were taken from the 11th or 12th right intercostal space using a liver transfixion pin. All liver tissue biopsies were quickly washed in a cold rinse buffer containing 0.2 mM protease inhibitor cocktail (Protease inhibitor, Roche), oxacillin (25 μg/ml; Sigma-Aldrich Co.), gentamycin (50 μg/ml; Sigma-Aldrich Co.), penicillin (100 U/ml; Sigma-Aldrich Co.), streptomycin (100 μg/ml; Sigma-Aldrich Co.), amphotericin B (0.25 μg/ml; Sigma-Aldrich Co.), and nistatin (50 U/ml; Sigma-Aldrich Co.) to remove cell debris and blood, and were then frozen by immersion in liquid nitrogen. Blood samples were taken from the jugular vein just before the liver biopsies were obtained. Blood samples were centrifuged immediately after collection at 4,000×g for 10 min, and serum was collected and frozen at -20°C. All blood samples were handled similarly and anticoagulant was not used. The concentrations of serum glucose, using the glucose oxidase method, β-hydroxybutyric acid, using an enzymatic-rate method and triglyceride, using an enzymatic method, were analyzed. Serum results for the

Table 1. Concentrations of serum β-hydroxybutyric acid, glucose and triglyceride of sixteen cows

	Ketosis sample [1]								Normal sample [1]							
β-hydroxybutyric acid (mmol/L)[2]	1.80	2.02	1.49	1.54	1.25	2.11	1.75	1.30	0.52	0.47	0.39	0.25	0.26	0.23	0.32	0.26
Glucose (mmol/L)[3]	2.24	2.36	2.48	1.99	2.44	2.35	2.47	2.41	4.14	4.03	3.86	4.20	3.78	4.73	3.76	4.50
Triglyceride (mmol/L)	0.09	0.10	0.06	0.07	0.10	0.11	0.11	0.12	0.29	0.21	0.16	0.20	0.21	0.18	0.30	0.30

[1] The sixteen liver samples (eight from ketotic cows and eight from normal cows), were from cows of the same breed, age, milk productivity and body condition scores at calving, and which had been given the same diets during the dry period and early lactation.

[2] Ketotic cows were defined by a β-hydroxybutyric acid >1.2 mmol/L. Normal cows were defined by β-hydroxybutyric acid <0.6 mmol/L (Ingvartsen 2006; Melendez et al., 2006).

[3] Ketotic cows had low glucose (glucose of every sample <2.5 mmol/L). Normal cows had standard glucose (glucose of every sample >3.75 mmol/L).

eight ketotic and eight normal cows are shown in Table 1.

Sample preparation for 2D-E analysis

Sample preparation and solubilization was performed using a slight modification of the SWISS-2D PAGE sample preparation procedure (Reymond, 1997). Liver samples from 8 healthy multiparous Holstein cows and 8 ketotic cows were pooled by health status, with the same weight of liver tissue from each cow. Pooled samples are suitable for comparative proteomics research and are advantageous for studies of disease pathogenesis, as they can avoid the influence of individual differences (Welch et al., 2005; Zhang et al., 2006). Frozen ketotic and normal group samples (approximately 20 mg per group) were crushed in a mortar containing liquid nitrogen, and mixed with 2.0 ml of a solution containing 8 M urea, 4% CHAPS (w/v), 65 mM DTT, 50 mM Tris-HCl and a trace of bromophenol blue. The protein concentrations of ketotic and normal sample groups were determined according to Bradford (1976) using BSA to generate a standard curve. Each sample was run on three separate 2D-E gels. Pictures were analyzed using PDQuest software (Applied Biosystems) (Moura et al., 2006; Qin et al., 2006) according to the procedure described in the manual for PDQuest software.

Electrophoresis and silver staining

Samples containing 50 μg of total protein were separated in a horizontal 2D-E setup (IPGphor, Amersham Biosciences) (Fabio et al., 2003). Isoelectric fractionation (IEF) was performed using nonlinear immobility pH gradient (IPG) strips (0.5×180 mm, pH 4.0-10.0), run at 50 V for 12 h; 200 V for 1 h; 500 V for 1 h; 1,000 V for 1 h; 5,000 V for 1 h; and 8,000 V for 8 h. After equilibration, reduction and alkylation, the IPG strips were transferred onto 12% second-dimension gradient slab gels, and then run on a discontinuous SDS-PAGE system at 60 mA for 5 h (Görg et al., 1988). Protein detection was achieved using a sensitive ammoniacal silver stain. The normalization process of silver staining was as follows: The gel was fixed (50% methanol and 10% glacial acetic acid) for 40 min, sensitized (70 ml of methanol, 10 ml of 10% Sodium thiosulfate, 17 g of NaAC, diluted to 250 ml with dH_2O) for 30 min for each gel and rinsed in dH_2O 3 times for 5 min each. The gel was stained in stain solution (25 ml of 5% $AgNO_3$, 0.1 ml of formaldehyde, diluted to 250 ml with dH_2O) for 30 min for each gel rinsed in dH_2O 3 times 1 min each and placed in developer (250 ml of dH_2O, 6.25 g Na_2CO_3, 50 ml of formaldehyde) for 200 sec. Development was stopped (3.75 g EDTA in 250 ml dH_2O) and scanned using ImageScanner. All procedures were in parallel at 22°C. There were no saturated spots in gels (Shevchenko et al., 1996). Three replicates for each sample were conducted for statistical analysis.

Image analysis

Six gels were scanned using an Image Scanner and LabScan software 3.01 (Bio-Rad) linked to a Macintosh computer. The 2D-E image analysis was carried out using the PDQuest 8.0 software package (Moura et al., 2006; Qin et al., 2006). Spots were detected and quantified using PDQuest 8.0 software. Spots which were significantly different between groups and not significantly different between the three replicate samples were identified by MALDI-TOF-MS/MS. All statistical analyses were performed with the PDQuest 8.0 software package (Beresini et al., 1990). To identify valid spots, PDQuest spot detection software was used with appropriate selection of the faintest and the smallest spots and a large representative section of the image containing spots, streaks, and background gradations was used to make corrections for noise filtration. After the background subtraction, spot detection and matching, one standard gel for each sample was obtained. These standard gels were then matched to yield information about the spots of differentially expressed proteins. Difference expression of protein was defined as $p<0.05$ and if the change of photodensity of the protein spot between ketotic and normal samples was more than 1.5 fold, this process could be completed automatically by PDQuest software.

Protein identification

For protein identification, selected spots were excised from the 2D-E gel, triturated, and washed with water. Proteins were reduced with 10 mM DTT in 100 mM NH_4HCO_3 (45 min, at 55°C), and S-alkylated with 55 mM iodoacetamide in 100 mM NH_4HCO_3 (30 min, at 25°C, in the dark). Gel particles were washed with 50 mM NH_4HCO_3 and acetonitrile, dried, and rehydrated with digestion solution (12.5 ng/μl of trypsin in 50 mM NH_4HCO_3). After incubation for 1 h at 4°C, the supernatant of the digestion solution was replaced with 50 mM NH_4HCO_3, and gel particles were incubated overnight at 37°C. Gel particles were further extracted with 25 mM NH_4HCO_3/acetonitrile (1:1 v/v), and the resulting peptide mixtures were freeze-dried.

Peptides were dissolved in 5 μl of 2, 5-dihydroxyhenzoic acid. A digested aliquot (1 μl) was analyzed by MALDI-TOF-TOF tandem mass spectrometry using a 4700 Proteomics Analyzer (Mass spectra, Applied Biosystems) (Moura et al., 2006; Qin et al., 2006), and data were run in the IPI.BOVIN.FASTA database using GPS software. The specific parameters were: error = 100 ppm; index mode = combined (MS+MS/MS); searching database parameter = trypsin; max missed clearage = one; variable modifications = acetyl (N-term), carbamidomethyl (C), and Oxidation (M); MS/MS Fragment Toleration = 0.2 Da;

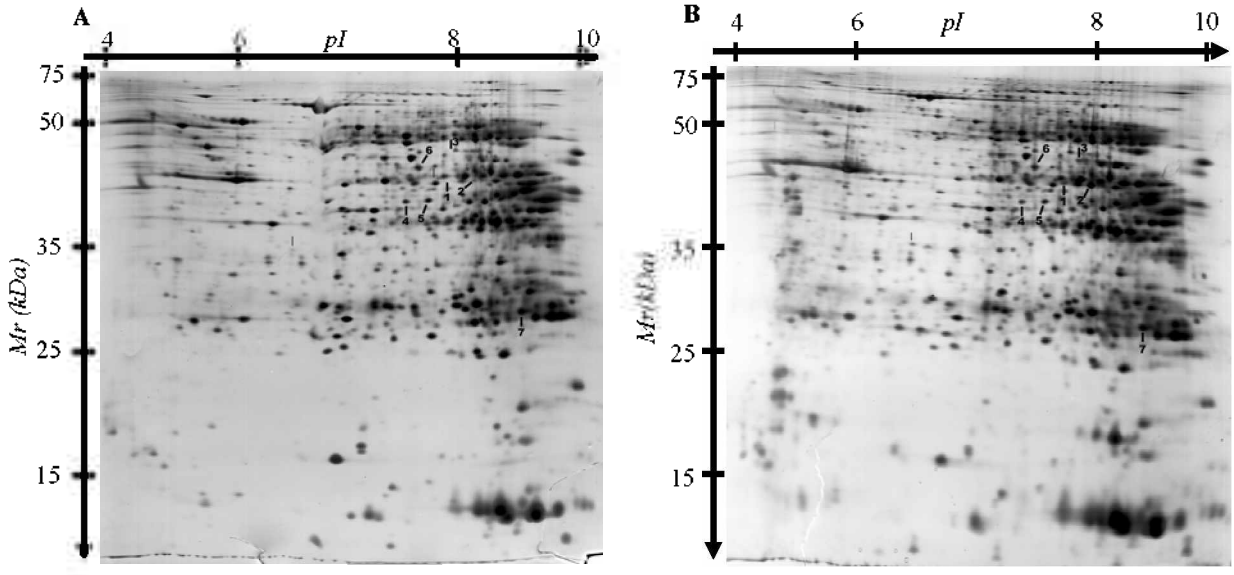

Figure 1. Silver-stained 2D-E gel of (A) normal cow liver and (B) ketotic cow liver. A total of 50 μg of protein was loaded, and 2D-E was performed using a pH range of 4-10 in the first dimension, and SDS-PAGE (12% T) in the second dimension. Marked spot numbers refer to numbers in Table 2.

precursor tolerance = 0.2 Da; peptide charge = +1; maximum peptide rank = 10; minimum ion score C.I.% = 0.

RESULTS

The 2D-E gels of normal and ketotic liver samples are shown in Figure 1. Every 2D-E gel contained approximately 1,000 distinct proteins. Protein spots were imaged, analyzed and quantified using PDQuest software. There were 1,086 spots in the images of 2D-E gels that were different between the liver samples of ketotic and normal cows. As a consequence of gel-to-gel variation, the match rate of three gels was 88%, which means that 88% spots in any one gel could be found in the three gels. This result indicates that the results of 2D-E and silver stain were reproducible and reliable (Jia et al., 2006). Five spots that were expressed at significantly different levels between the liver samples of normal and ketotic cows were subsequently subjected to identification by MALDI-TOF-MS/MS, and five enzymes/proteins were identified, as shown in Table 2. The cut-off value for difference in protein spots was 1.5, and all difference protein spots were consistent with *t*-test ($p<0.05$). The expression levels of 3-hydroxyacyl-CoA dehydrogenase type-2 (HCDH), acetyl-Coenzyme A acetyltransferase 2 (ACAT) and elongation factor Tu were down-regulated, and alpha-enolase and creatine kinase were up-regulated in the livers of ketotic cows. The 2D-E results for HCDH and ACAT were confirmed by Western blotting (data not shown in this paper). These data demonstrated that liver proteins can be separated by 2D-E highly qualitatively, and that there was reproducibility among the three replicates for each sample.

Based on these differences in the expression levels of liver proteins, we established a liver metabolic network.

Table 2. List of differences in the spots/proteins identified by MALDI-TOF-TOF tandem mass spectrometry analysis of bovine liver samples separated by 2D-E gels

Spot[1]	Fold change[2]	Protein name[3]	IPI number[4]	Accession number[5]	Exp.Mr[6]	Exp.pI[7]
1	9,020	Creatine kinase M-type	IPI00685709.2	60097925	42,961.8	6.63
2	4.55	Creatine kinase M-type	IPI00685709.2	60097925	42,961.8	6.63
3	8,198	Alpha-enolase	IPI00707095.2	87196501	47,165.4	6.37
4	0.69	Acetyl-Coenzyme A acetyltransferase 2	IPI00685191.2	76626357	41,172.2	6.46
5	0.61	Acetyl-Coenzyme A acetyltransferase 2	IPI00685191.2	76626357	41,172.2	6.46
6	0.64	Elongation factor Tu	IPI00696052.1	27806367	49,367	6.72
7	0.41	3-hydroxyacyl-CoA dehydrogenase type-2	IPI00699128.1	27805907	26,992.2	8.48

[1] The spot number labeled in Figure 1.

[2] Fold change: the expression ratios (ketosis group/normal group) between the means of spot value (% vol) resulting from digital image analysis.

[3] Protein name: identified by results of MALDI-TOF-MS/MS and the protein scores >59, Total Ion C.I.% >95%.

[4] IPI number comes from EBI Databases. [5] Accession number comes from NCBI entry or SWISS-PROT.

[6] Protein molecular weight value. [7] Protein isoelectric point.

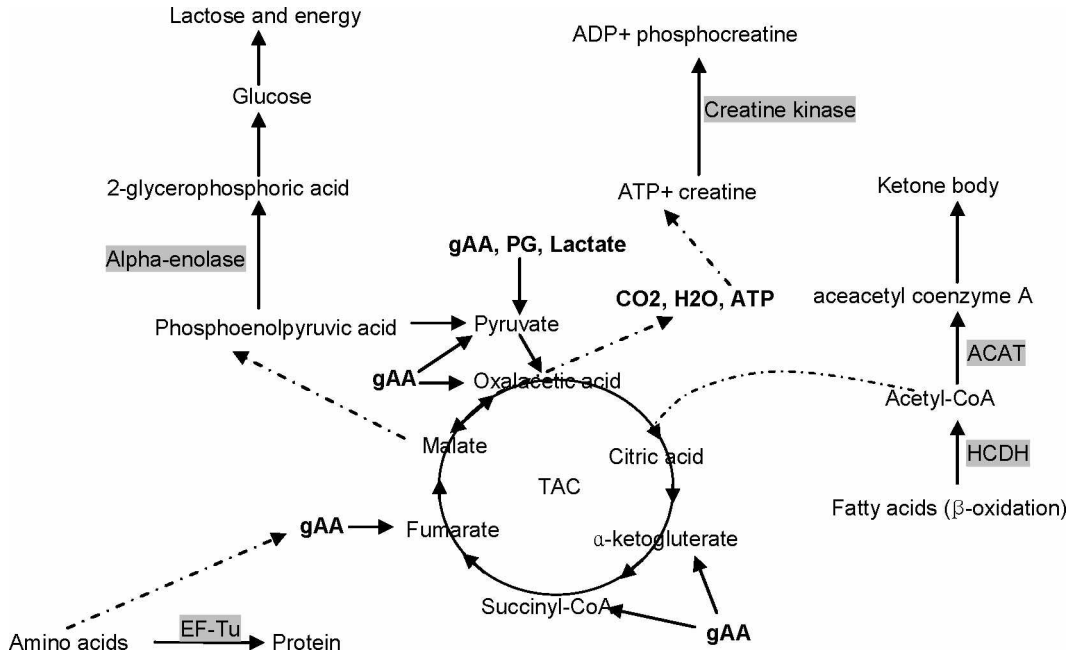

Figure 2. Overview of metabolic interrelationships in liver between ketotic and healthy cows. When clinical ketosis was observed in cows, fatty acid β-oxidation, ketogenesis and protein synthesis in hepatic cells were weakened; gluconeogenesis was increased; ATP was profusely stored as phosphocreatine in hepatic cells and acetyl-CoA was accumulated. Abbreviations: HCDH, hydroxyacyl-CoA dehydrogenase type-2; ACAT, acetyl-Coenzyme A acetyltransferase 2; PG, propylene glycol. The glucogenic amino acids are included via pyruvate (Ala, Cys, Gly, Hyp, Ser, Thr), α-ketoglutarate (Arg, His, Gln, Pro), succinyl-CoA (Ile, Met, Val), Fumarate (Tyr, Phe) and oxaloacetate (Asp). ⇢ links between different metabolic pathways.

When ketosis occurred (Figure 2), the ability of ketogenesis may be weakened owing to the high concentrations of ketone bodies, which may result in the accumulation of acetyl-CoA. Accumulated acetyl-CoA depressed the level of ACAT and decreased the production of ketone bodies. High concentrations of acetyl-CoA in the liver may have inhibited fatty acid β-oxidation, so that expression of HCDH was decreased. Lower liver metabolic function due to acetyl-CoA may also cause feedback inhibition of the dehydrogenase reactions of fatty acid oxidation. As a result, expression of HCDH was decreased. The low expression levels of elongation factor Tu in the livers of ketotic cows minimize protein synthesis, indicating that most glucogenic amino acids take part in the Krebs cycle and gluconeogenesis, which has limited the capacity for protein synthesis so that the protein synthetic machinery is down-regulated. Decreased protein synthesis may be also regulated by low insulin (Figure 2); high concentrations of acetyl-CoA and glucogenic amino acids accelerated the Krebs Cycle and produced high levels of ATP, which resulted in increased expression of creatine kinase and ATP was stored as the form of phosphocreatine, but this result needs more support (Figure 2); glucogenic amino acids also sustained the gluconeogenesis pathway, explaining why alpha-enolase was increased (Figure 2).

DISCUSSION

Changes in the protein composition of mammalian tissues and fluids have been associated with different physiological and pathological conditions (Marton et al., 1998; Lockhart et al., 2000). In classical veterinary studies in the cow, single enzymatic activities or genetic anomalies have been specifically associated with physiological and pathological conditions. However, systematic studies have not been performed to evaluate the entire repertoire of proteins in tissues and fluids associated with physiological and pathophysiological phenomena, and there have only been a very limited number of proteomic studies in bovine species (Hogarth et al., 2004; Chiara et al., 2005; Connolly et al., 2006). The present study is the first report utilizing proteomic technology in the ketotic cow.

The metabolic characteristics in the livers of ketotic cows were tested based on the comparative proteomics technique. Five enzymes/proteins were identified as being differentially expressed, as shown in Table 2. The metabolic network in liver cells is showed in Figure 2. 3-hydroxyacyl-CoA dehydrogenase type-2 (HCDH), which is involved in fatty acid β-oxidation metabolism, was decreased in the livers of ketotic cows. During early lactation, cows are in an excessively negative energy balance, so that fat mobilization and fatty acid β-oxidation are increased

acutely. However, activation of mitochondrial ACAT is associated with elevated ketone body levels (Treberg, 2006). When ketosis occurred, high levels of ketone bodies inhibited the activity of ACAT, so decreased the transport of acetyl-CoA to ketone bodies and acetyl-CoA may have accumulated quickly. A high concentration of acetyl-CoA may have inhibited the fatty acid β-oxidation pathway through negative feedback when ketosis occurred, and this may be a cause of the down-regulated expression of HCDH in the livers of ketotic cows. The reduction in fatty acid oxidation following parturition may contribute to postpartum ketosis, because of the decreased activity of 3-hydroxy-acyl-CoA dehydrogenase, which impairs the fatty acid β-oxidation pathway in the liver after parturition (Murondoti, 2004). The decreased expression of 3-hydroxyacyl-CoA dehydrogenase type-2 in the liver tissues of ketotic cows suggests that the ability to utilize fatty acids is decreased. As a consequence, fatty acids may accumulate in hepatic cells (Jonas et al., 1978), resulting in hepatic lipidosis. Grum and Cadórniga-Valiño concluded that a high concentration of ketone bodies may decrease the rates of β-oxidation of fatty acids, and that a high concentration NEFA increased lipogenesis in liver cells (Grum et al., 1996; Cadórniga-Valiño et al., 1997). Lower liver metabolic function for acetyl-CoA may also cause feedback inhibition of dehydrogenase reactions of fatty acid oxidation. As a result, expression of HCDH was decreased and acetyl-CoA accumulated. Decreased 3-hydroxyacyl-CoA dehydrogenase activity in the liver has also been confirmed in postpartum cows under circumstances of increased serum NEFA concentration and serum BHBA levels (Murondoti, 2004). In ketotic cows, high concentrations of acetyl-CoA and down-regulated expression of 3-hydroxyacyl-CoA dehydrogenase in hepatocytes may be related to hyperketoemia caused by ketosis (Figure 2).

Acetyl-Coenzyme A acetyltransferase 2 (ACAT) transforms acetyl-CoA into acetoacetyl-CoA and can increases the rate of ketogenesis. The activity of ACAT is linearly correlated with the level of acetoacetyl-CoA. Under conditions of saturating acetyl-CoA and acetoacetyl-CoA, the activity of ACAT was down-regulated (Huth, 1975; Jonas, 1978). In the present study, a decreased level of ACAT was observed in livers of ketotic cows, and this contributed to negative feedback induced by the increased concentration of acetyl-CoA and acetoacetyl-CoA (Huth, 1975; Jonas, 1978). However, activation of mitochondrial ACAT is associated with elevated ketone body levels (Treberg, 2006). During early lactation, the levels of ketone bodies may be increased by enhanced levels of ACAT. In ketotic cows, the decrease in ACAT levels was inhibited by high levels of ketone bodies. High concentrations of acetyl-CoA and acetoacetyl-CoA are also in accord with the liver metabolic characteristic of ketotic cows. On the one hand, high concentration of acetoacetyl-CoA promotes the production of ketone bodies and results in ketosis; on the other hand, high concentrations of acetoacetyl-CoA inhibit the transformation of acetyl-CoA to acetoacetyl-CoA and decrease ACAT expression, resulting in the accumulation of acetyl-CoA (Huth, 1975; Jonas, 1978). These results imply that acetoacetyl-CoA and acetyl-CoA accumulated when ketosis occurred, and that ketogenesis was decreased because of the down-regulation of ACAT. Acetyl-CoA may be utilized partly through the Krebs Cycle (Figure 2).

Elongation factor Tu plays an important role in global protein biosynthesis, transforming amino acids into proteins. In the present study, the decreased expression of elongation factor Tu indicated that protein synthesis was decreased and that amino acids may be utilized by other pathways in the livers of ketotic cows. The substrates for gluconeogenesis include propionate, glucogenic amino acids (included *via* pyruvate or *via* the Krebs cycle), lactate and glycerol. The quantitatively most important substrates are glucogenic amino acids in early lactation (Danfaer et al., 1995; Adachi, 2006). As shown in Figure 2, when ketosis had occurred, a main cause of decreased protein synthesis appears to be because amino acids may be utilized by gluconeogenesis and the Krebs cycle (including *via* pyruvate or *via* oxalacetic acid or *via* fumarate or *via*α-ketoglutarate or *via* succinyl-CoA) (Ingvartsen, 2006). Most glucogenic amino acids and accumulated acetyl-CoA promote the Krebs cycle in the livers of ketotic cows, simultaneously, and produce a considerable amount of H_2O, CO_2 and ATP (Ingvartsen, 2006). Schei et al. (2005) demonstrated that increased amino acid supply is an important factor in stimulating milk yield in early lactation and avoiding ketosis when cows are in a negative energy balance. In the condition of ketosis, the protein synthesis pathway becomes weak and glucogenic amino acids take part in the Krebs cycle, producing considerable amounts of ATP. The amino acids also act as substrates of gluconeogenesis (Lee, 2008).

Mitochondria are the main site of phosphocreatine synthesis in tissues with the creatine kinase system (Saks et al., 1975), which catalyzes the reaction: ATP+creatine = ADP+phosphocreatine. In the present study, the expression of creatine kinase M-type was up-regulated in the livers of ketotic cows, which suggested that excess ATP, produced by Krebs cycle as mentioned above, may also come from the protein synthesis pathway because inhibited protein synthesis would decrease the demand for ATP which was stored in the form of phosphocreatine. This is the most likely reason why creatine kinase is up-regulated in the livers of ketotic cows (Figure 2). The excess generation of phosphocreatine from ATP seems to imply that ketotic cows display a highly active energy metabolism in order to relieve suffering from a potentially serious negative energy balance. Research on ketotic cows in a condition of excess

energy is limited and may be contrary to traditional concepts on ketosis; further study is required to fully understanding these data.

Alpha-enolase is a significant enzyme of the glycolytic pathway, and enolase-encoding DNA sequences have been found in all organisms tested so far (Verma M, 1994). Alpha-enolase, which is involved in gluconeogenesis, catalyzes the transformation of phosphoenolpyruvate to 2-glycerophosphoric acid. In the present study, alpha-enolase was up-regulated in the livers of ketotic cows, indicating that the gluconeogenesis pathway was enhanced. As shown in Figure 2, the substrate for gluconeogenesis was mostly supplied by glucogenic amino acids through the Krebs cycle (including *via* oxalacetic acid or *via* fumarate or *via*α-ketoglutarate or *via* succinyl-CoA) or pyruvate (Danfaer et al., 1995), because of an insufficient supply of the glucogenic precursor propionate, owing to decreased appetite. Increased gluconeogenesis through glucogenic amino acids can, to a certain extent, correct the low blood glucose levels in ketotic cows.

CONCLUSIONS

The metabolic characteristics of the liver during bovine ketosis were revealed by differences in the expression levels of liver proteins. Fatty acid β-oxidation, ketogenesis and protein synthesis may be weakened, and gluconeogenesis may be increased, in the livers of ketotic cows, but the diseased animals still remain in a hypoglycemic and hyperketonemic condition, while ATP may be plentifully stored as phosphocreatine and acetyl-CoA may accumulate in hepatic cells. These presumptive changes need to be further examined in order to identify the metabolic characteristics of the livers of ketotic cows and elucidate their significance.

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