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Identification of novel pathways in pathogenesis of ketosis in dairy cows *via* iTRAQ/MS

Shi Shu[1], Chuchu Xu[1], Cheng Xia[1, 2], Xinhuan Xiao[1], Gang Wang[1], Ziling Fan[1], Yu Cao[1], Yanhui Wang[1], Hongyou Zhang[1]

[1]Department of College of Animal Science and Veterinary Medicine, Heilongjiang BaYi Agriculture University, Daqing 163319, China
[2]Department of Synergetic Innovation Center of Food Safety and Nutrition, Northeast Agricultural University, Harbin 150030, China
xcwlxyf2014@163.com

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Abstract

Introduction: To identify novel pathways involved in the pathogenesis of ketosis, an isobaric tag for relative and absolute quantitation/mass spectrometry was used to define differences in protein expression profiles between healthy dairy cows and those with clinical or subclinical ketosis. **Material and Methods:** To define the novel pathways of ketosis in cattle, the differences in protein expression were analysed by bioinformatics. Go Ontology and Pathway analysis were carried out for enrich the role and pathway of the different expression proteins between healthy dairy cows and those with clinical or subclinical ketosis. **Results:** Differences were identified in 19 proteins, 16 of which were relatively up-regulated while the remaining 3 were relatively down-regulated. Sorbitol dehydrogenase (SORD) and glyceraldehyde-3-phosphate dehydrogenase (G3PD) were up-regulated in cattle with ketosis. SORD and G3PD promoted glycolysis. These mechanisms lead to pyruvic acid production increase and ketone body accumulation. **Conclusion:** The novel pathways of glycolysis provided new evidence for the research of ketosis.

Keywords: dairy cows, ketosis, pathogenesis, proteins, iTRAQ/MS.

Introduction

Ketosis is a serious metabolic disorder in high-producing dairy cows that is characterised by increased concentrations of ketone bodies in blood, urine, and milk (14). Two types of ketosis – clinical and subclinical – can decrease milk production and increase the risk of displaced abomasum and mastitis (29). Economic losses are considerable, due to decreased milk production, cost of treatment, and occasionally death and disposal of affected cows (14). Negative energy balance (NEB) is an important cause of ketosis. In contrast to that of non-ruminant animals, the response of ruminants to NEB, which involves glucose regulation, is very complicated. Because of the lack of carbohydrates in the rumen and/or increasing energy requirements, ruminants mainly rely on non-carbohydrate compounds to meet their energy demands *via* gluconeogenesis (25).

Several studies have reported proteomic profiles in milk and serum of cows infected with different pathogens. The isobaric tag for relative and absolute quantification (iTRAQ) method is a recent proteomic platform. The technology has facilitated many reports of mastitis caused by *Staphylococcus aureus*. Studies showed that a total of 2971 milk proteins were identified and more than 300 were found to be associated with host defence against *Staphylococcus aureus* (12, 19).

Material and Methods

In this study, iTRAQ was used to screen differences in protein expression profiles between healthy cows and those with clinical and subclinical ketosis to identify potentially novel pathways.

Animals. Serum samples were collected from 30 dairy cows from an intensive dairy farm in accordance with the requirements of the Veterinary Medical Ethical Committee of the Local Agricultural Department of Mishan, Heilongjiang province, China. Three groups were separated according to β-hydroxybutyrate (BHBA) concentration and clinical symptoms: group CK – threshold BHBA value >2.0 mM (mean 3.61 ±0.41, n = 10) and clinical symptoms present; group SK – threshold BHBA value >1.0 mM (mean 1.57 ±0.30, n = 10) and no clinical symptoms present; and group C (control) – threshold BHBA value <1.0 mM (mean 0.61 ±0.12, n = 10) and no clinical symptoms present. The samples were pooled in fives to decrease the incidence of individual errors, so that two hybrid samples were produced from each group. There were no significant differences in age or body condition score among the groups.

iTRAQ labelling. Due to very high individual differences in dairy cows, five samples were pooled into one to decrease the differences. There were two experimental replicates used: "set 1" and "set 2." These samples were labelled using the iTRAQ reagent kit (AB Sciex, USA) as follows: in set 1, 114 and 117 were labelled CK1, 115 was labelled SK1, and 116 was labelled C1; in set 2, 114 was labelled CK2, 115 and 117 were labelled SK2, and 116 was labelled C2. Before labelling, the high-abundance proteins were removed using the ProteoMiner kit (Bio-Rad, USA).

Nanoscale liquid chromatography coupled to tandem mass spectrometry (nano LC-MS/MS). Nano LC-MS/MS analysis was performed using an Orbitrap Velos Pro™ Hybrid Ion Trap Orbitrap Mass Spectrometer (Thermo Fisher Scientific, USA). Differences in protein expression profiles were identified by searching the NCBInr database (http://www.ncbi.nlm.nih.gov/protein) for *Bos taurus* using Mascot Daemon software (version 2.3.02, Matrix Science, USA) (6, 28).

Protein identification and quantitation. To estimate the false discovery rate (FDR) as a measure of identification certainty in each replicate set, an automatic decoy database search was performed. The search was performed using Mascot Daemon software and entailed choosing the decoy checkbox in which a random database sequence was generated and tested for raw spectra along with the real database. The threshold values of relative up-regulation and down-regulation were confirmed by calculating inter-error values *via* Proteomic System Performance Evaluation Pipeline analysis. The ratios of the plots (114/116, 115/116, and 117/116 respectively) for each of the quantified proteins between the two sets generated comparable quantification results, as determined by linear regression analysis with an FDR of 5%. Inter-error values were 0.70 and 0.76 for inter-error sets 1 and 2 respectively. Based on these results, the up-regulation and down-regulation threshold values were set at 1.1 and 0.9 respectively.

Gene ontology (GO) and pathway analysis. Differences in protein expression patterns were analysed using DAVID Bioinformatics Resources software v6.7 (http://david.abcc.ncifcrf.gov/home.jsp) with an enrichment threshold of 0.05. GO analysis was performed with the gene ontology tool (GOTERM_BP_ALL, GOTERM_CC_ALL, GOTERM_MF_ALL). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was subsequently performed using the same software, in which a corrected P value of less than 0.05 was deemed significant.

Results

Differential expression analysis. Samples collected from the three groups (CK, SK, and C) were labelled with 4-plex iTRAQ, and nano LC-MS/MS analysis and database searches against the NCBInr database were performed (Fig. 1).

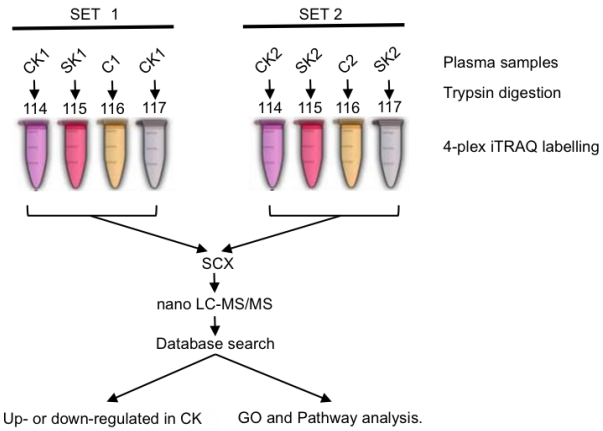

Fig. 1. Flowchart for 4-plex iTRAQ-LC-MS analysis of the three groups

A total of 549 and 405 proteins were identified in sets 1 and 2 respectively. In set 1, 115 and 79 proteins were up- and down-regulated respectively in group CK as compared with group C, and 90 and 96 proteins were up- and down-regulated in group SK as compared with group C. In set 2, there were 100 and 140 proteins in group CK, and 47 and 41 proteins in group SK which were up- and down-regulated as compared with the control group. Due to the large number of proteins overlapping in two sets or two groups, there were 33 differences in protein expression profiles between cows with ketosis and control cows. Of these proteins, 16 were relatively up-regulated and 3 were relatively down-regulated in groups CK and SK, as compared with group C. In addition, differences in expression profiles of some proteins were only observed between groups CK and SK (Table 1).

Annotation of differential proteins and pathway analysis. Of the 33 proteins, 28 were annotated using the DAVID tool. The GO analysis

results are shown in Fig. 2. There were three parts of the analysis: cellular component (CC), biological processes (BP), and molecular functions (MF). For the 28 proteins, enriched CC terms were grouped into nine clusters, BP terms were grouped into 66 clusters, and MF terms were grouped into 13 clusters. According to the number of proteins analysed by BP of GO analysis, metabolic process (GO - 0008152), primary metabolic process (GO - 0044238), macromolecule metabolic process (GO - 0043170), and protein metabolic process (GO - 0019538) were identified. These results indicate the processes of the various proteins.

The pathway analysis results indicated that "Proteasome" was the only term identified. Five different proteins were involved in this pathway: proteasome subunit α type-4, proteasome subunit α type-6, proteasome subunit α type-7, proteasome subunit β type-1, and proteasome subunit α type-2. The P value was $6.96E^{-06}$.

In addition, the other 28 differentially expressed proteins were individually searched by KEGG database. Two proteins of interest were identified: sorbitol dehydrogenase (SORD) and glyceraldehyde-3-phosphate dehydrogenase (G3PD), which were both up-regulated in groups CK and SK as compared with the control group. SORD was identified with the fructose and mannose metabolism pathway (map no. 00051), and one of the downstream pathways was the glycolysis/gluconeogenesis pathway (map no. 00010). Interestingly, G3PD was identified as an important enzyme in the glycolysis/gluconeogenesis pathway. A possible pathway based on these results is shown in Fig. 3.

Table 1. Identification of differentially expressed proteins among the three groups by iTRAQ

Protein ID	Protein name	Expression [a]	Fold change [b]	
			CK/C	SK/C
gi|115497340	serum amyloid A protein precursor (*Bos taurus*)	CK↑SK↑	7.693	4.5395
gi|84579853	lipopolysaccharide-binding protein precursor (*Bos taurus*)	CK↑SK↑	3.517	2.3365
gi|95147666	periostin precursor (*Bos taurus*)	CK↑SK↑	1.712	1.9295
gi|75832116	inter-α-trypsin inhibitor heavy chain H4 precursor (*Bos taurus*)	CK↑SK↑	1.3325	1.4245
gi|330688463	filamin-A (*Bos taurus*)	CK↑SK↑	1.5915	1.412
gi|77735935	complement C2 precursor (*Bos taurus*)	CK↑SK↑	1.496	1.342
gi|77736269	proteasome subunit α type-2 (*Bos taurus*)	CK↑SK↑	1.208	1.3275
gi|358412958	predicted complement C3 (*Bos taurus*)	CK↑SK↑	1.237	1.3125
gi|84370111	proteasome subunit β type-1 precursor (*Bos taurus*)	CK↑SK↑	1.235	1.3125
gi|77735717	proteasome subunit α type-4 (*Bos taurus*)	CK↑SK↑	1.2405	1.2825
gi|82617550	sorbitol dehydrogenase (*Bos taurus*)	CK↑SK↑	1.8375	1.2805
gi|77735425	proteasome subunit α type-7 (*Bos taurus*)	CK↑SK↑	1.206	1.2795
gi|166159174	angiotensinogen precursor (*Bos taurus*)	CK↑SK↑	1.214	1.2755
gi|60592792	heat shock protein HSP 90-α (*Bos taurus*)	CK↑SK↑	1.3035	1.266
gi|77404273	glyceraldehyde-3-phosphate dehydrogenase (*Bos taurus*)	CK↑SK↑	1.3865	1.2355
gi|77735803	argininosuccinate lyase (*Bos taurus*)	CK↑SK↑	1.4825	1.2045
gi|27807335	cathelicidin-7 precursor (*Bos taurus*)	CK↓SK↓	0.7975	0.815
gi|27806789	transthyretin precursor (*Bos taurus*)	CK↓SK↓	0.695	0.7995
gi|32189338	polymeric immunoglobulin receptor precursor (*Bos taurus*)	CK↓SK↓	0.603	0.6755
gi|27807367	14-3-3 protein ζ/δ (*Bos taurus*)	CK↑	1.4665	-
gi|77736171	hemopexin precursor (*Bos taurus*)	CK↑	1.2955	-
gi|375065868	ceruloplasmin precursor (*Bos taurus*)	SK↑	-	1.253
gi|115497264	carboxypeptidase N catalytic chain precursor (*Bos taurus*)	SK↑	-	1.3295
gi|115496876	hepatocyte growth factor-like protein precursor (*Bos taurus*)	SK↑	-	1.4015
gi|300798390	vinculin (*Bos taurus*)	SK↑	-	1.366
gi|114053135	proteasome subunit α type-6 (*Bos taurus*)	SK↑	-	1.3485
gi|358420568	PREDICTED: complement C4-A-like (*Bos taurus*)	SK↑	-	1.6155
gi|190360741	apolipoprotein F precursor (*Bos taurus*)	CK↓	0.7365	-
gi|95006989	ribonuclease 4 precursor (*Bos taurus*)	CK↓	0.755	-
gi|75832056	apolipoprotein A-I preproprotein (*Bos taurus*)	CK↓	0.812	-
gi|134085882	apolipoprotein A-V precursor (*Bos taurus*)	CK↓	0.7765	-
gi|155372309	mammaglobin-A precursor (*Bos taurus*)	SK↓	-	0.8015
gi|115497814	nucleobindin-1 precursor (*Bos taurus*)	SK↓	-	0.793

[a] Difference in protein expression in group CK or SK as compared with controls (C). ↑ – up-regulated expression; ↓ – down-regulated expression;
[b] - no significant difference between the corresponding two groups

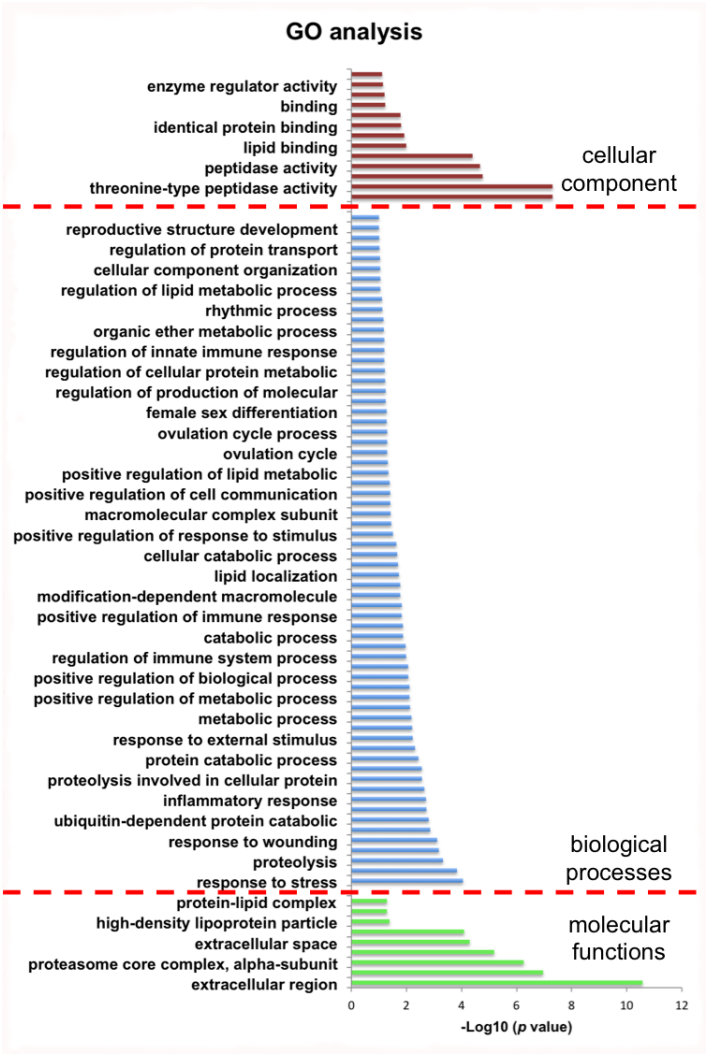

Fig. 2. Cellular component, biological processes, and molecular functions assignments of the identified proteins using DAVID (http://david.abcc.ncifcrf.gov/). CC – cellular component; BP – biological processes; MF – molecular functions

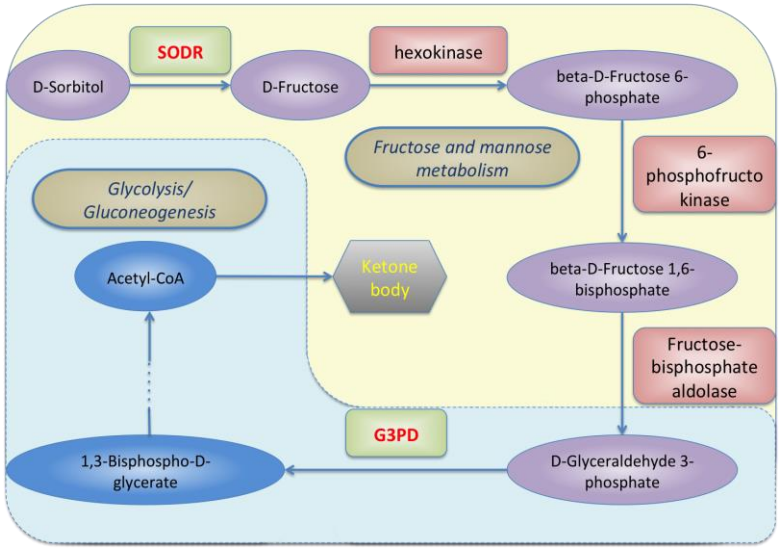

Fig. 3. The result of pathway analysis using the KEGG pathway database. Enzymes are depicted in rectangles, chemical compounds are shown in ovals, and pathways are shown in italics in stadia. The arrows indicate activation and the arrows with broken lines indicate reaction processes. Relatively up-regulated proteins are indicated in red

Discussion

In high-yielding dairy cows, the metabolic status around the time of calving and the onset of lactation exhibit undeniable parallels to human ketosis. Ketosis occurs when levels of ketones – acetone, acetoacetic acid, and β-hydroxy butyric acid – increase within the body due to incomplete metabolism of sugars and lipids, resulting in clinical symptoms that include poor appetite and loss of activity. Subclinical ketosis is characterised by an increased concentration of circulating ketone bodies without the presence of clinical signs of ketosis (1).

In this study, serum samples from 30 dairy cows were used for iTRAQ-based quantitative proteomic analysis, which identified 33 differentially expressed proteins, 28 of which were annotated using the DAVID tool for GO analysis. Five proteins were involved in the proteasome pathway according to the pathway analysis. Ketosis is a nutritional and metabolic disease. After calving, milk production dramatically increases and constraints on intake of dry matter force dairy cows to mobilise body fat to meet energy requirements. This mobilised overload of fat is metabolised by the liver and converted into ketone bodies (27). The major portion of carbohydrates available to the ruminant is supplied by gluconeogenesis, thus there must be a continuous and rapid flux through this pathway even in the fed state (2). Particular interest in the effect of glucocorticoids on gluconeogenesis in the cow arises from the fact that glucocorticoid administration is an effective therapy for treatment of bovine ketosis (11). However, according to the findings of this study, activation of the gluconeogenesis pathway is not the only mechanism activated in the process of ketosis development in dairy cows. Glycolysis is an alternative glucose metabolic pathway. In this pathway, glucose can be rapidly converted to pyruvic acid and acetyl coenzyme A *via* pyruvate dehydrogenase, and acetyl coenzyme A is the precursor molecule of ketone bodies.

Two interesting proteins, SORD and G3PD, were relatively up-regulated in the ketosis groups as compared with the control group. According to the pathway analysis results, these two special proteins appear to play various important roles in the glycol metabolism pathway.

G3PD, initially well characterised for its role in glycolysis, is now recognised as a novel multifunctional protein exhibiting new functions including the regulation of mRNA stability (4–6, 13, 20, 31), intracellular membrane trafficking (26), iron uptake and transport (17), haeme metabolism (7), maintenance of genomic integrity (3, 8, 15), regulation of gene expression (10, 30), and nuclear tRNA export (23). SORD is a key enzyme in the polyol metabolic pathway. SORD and G3PD are involved in the fructose/mannose metabolism and glycolysis/ gluconeogenesis pathways respectively (Fig. 3). In the integrated pathway, SORD catalyses D-sorbitol to activate D-fructose (18). Up-regulated SORD expression and activity would enhance the production of fructose. Fructose is also several times more effective than glucose in promoting intracellular non-enzymatic glycation (9, 16, 24), and advanced glycation end products may contribute to the vascular complications of diabetes and other pathologic conditions (6, 16, 21, 22). Then, D-fructose is converted to D-glyceraldehyde 3-phosphate through a series of enzymatic reactions. The product of these enzymatic reactions, D-glyceraldehyde 3-phosphate, then enters the glycolysis/gluconeogenesis pathway. In the first step of this pathway, D-glyceraldehyde 3-phosphate is converted to 1,3-bisphospho-D-glycerate *via* G3PD enzymatic activity. In this study, SORD and G3PD were relatively up-regulated. Generally, the speed of enzymatic reactions is in direct proportion to the concentration of the enzymes. The concentration of these two enzymes increased and, hence, the velocity of two enzymatic reactions rose. As a final result, the concentration of the end-product – acetyl-CoA – is raised and a portion is converted to ketone bodies that accumulate in the liver.

This evidence suggests that the enzymatic activities of SORD and G3PD were increased, resulting in ketone body accumulation, and that increased SORD and G3PD activities could aggravate clinical or subclinical ketosis in dairy cows by promoting ketone body accumulation.

In summary, this study is the first to explore the plasma proteomics of cows with CK or SK and indicates the possibility of an alternative pathway leading to ketosis. Two proteins which can possibly explain this novel pathogenesis were identified for the first time using iTRAQ. The findings of this study may contribute to further understanding of the novel pathogenesis of ketosis in dairy cows.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The study was performed in accordance with the guidelines established by the Ethical Committee for Animal Experiments of Northeast Agricultural University, Harbin, China.

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