



Changes of Plasma Metabolites, Hormones, and mRNA Expression of Liver PEPCK-C in Spontaneously Ketotic Dairy Cows*

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ABSTRACT : The objective of this study was to understand changes of plasma metabolites, hormones, and mRNA level of cytoplasmic phosphoenolpyruvate carboxykinase (PEPCK-C) in liver in spontaneous clinical ketosis; 10 clinically ketotic cows and 10 healthy cows were chosen from the same dairy farm. Eleven blood parameters and liver fat content were measured in all cows, and mRNA levels of PEPCK-C in liver were measured by semi-quantitative reverse transcription (RT) polymerase chain reaction (PCR). In ketotic cows, concentration of plasma glucose decreased ($p < 0.01$), concentration of plasma nonesterified fatty acids (NEFA) and β -hydroxybutyric acid (BHBA) increased ($p < 0.01$), liver fat content (18.8% wet weight) and activity of plasma aspartate aminotransferase (AST) increased ($p < 0.01$), but concentration of plasma total bilirubin (TBIL), γ -glutamyl transpeptidase (γ -GT), and cholinesterase (CHE) increased ($p > 0.05$). In addition, concentration of plasma insulin decreased ($p < 0.05$), concentration of plasma glucagons decreased ($p > 0.05$), and mRNA level of PEPCK-C in liver increased ($p < 0.05$). It is concluded that the adaptative changes of metabolites, hormones, and mRNA level of PEPCK-C in ketotic cows were in favor of the enhancement of gluconeogenesis, the decrease of fat mobilization and the relief of ketosis, but these were still inadequate to relieve ketosis. (**Key Words :** Dairy Cow, Ketosis, Metabolites, Hormones, PEPCK-C, mRNA Expression)

INTRODUCTION

Ketosis is a major metabolic disease of many dairy cows in early lactation, and occurs frequently in high yielding cows. It is extremely detrimental for milk performance and reproductive ability of dairy cows. The decrease of DMI and increase of demand in the transition period may result in negative energy balance (NEB) of dairy cows, which can stimulate gluconeogenesis and fat mobilization. The adaptive accommodation by the metabolic and endocrine system plays an important role in the relief of NEB in the transition period. Once NEB cannot be restored, ketone body production increases and ketosis is unavoidable (Veenhuren et al., 1991; Dale et al., 1997).

Cytoplasmic phosphoenolpyruvate carboxykinase (PEPCK-C) is one of the most important rate-limiting enzymes of gluconeogenesis in the liver (Theera et al.,

1998; Cansu et al., 2002). Hormones and glucose precursors can regulate gluconeogenesis by controlling the activity and mRNA expression of PEPCK (Donkin et al., 1994; She et al., 2000; Colleen et al., 2002).

So far, there have been some reports on changes of plasma metabolites and hormones in periparturient cows, but little is known about the mRNA profile of PEPCK-C in the liver of spontaneously ketotic cows in early lactation. The purpose of this study was to clarify changes in metabolites, hormones, and mRNA level of the PEPCK-C gene in liver in order to reveal the metabolic mechanism of ketosis at a molecular level.

MATERIALS AND METHODS

Animals, samples, and analysis

All experimental procedures were conducted according to Guidelines for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Ten clinically ketotic cows (K) and ten healthy cows (C) were chosen from a 1,000-cow dairy farm located in Daqing city, Heilongjiang province, China. These cows were aged 5-8 yr and of 3-5 parities, and were fed thrice daily at 0500, 1100 and 1800 h a total mixed ration (TMR) which met the

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requirements for energy, protein, minerals, and vitamins during lactation. Ingredient composition of the TMR was 24.00% corn grain (cracked), 12.00% soybean meal, 2.00% cotton seed, 0.90% soybean oil, 0.85% feather powder, 2.00% wheat bran, 0.45% calcium phosphate, 0.40% additive premix, 0.45% stone powder, 0.35% salt, 0.60% baking soda, 24.50% corn silage, 9.50% sugar beet pulp pellets, and 22.00% alfalfa hay. Nutrient composition of the TMR contained 21.75 Mcal/d, 23.98 kg/d DM, 14.65% CP, 19.80% CF, 0.62% Ca, and 0.44% P. All cows were fed alfalfa hay *ad libitum* and were supplied with a concentrate supplement at 1 kg per 3 kg milk daily during lactation.

Cows had increased plasma BHBA concentrations (>1.5 mmol/L), which were the obviously clinical symptoms of ketosis (Veenhuren et al., 1991; Smith et al., 1997).

Blood samples were collected from K and C cows by coccygeal venipuncture. Plasma was prepared immediately by centrifugation, and frozen at -20°C until analyzed. Liver tissue samples (around 50 mg) were taken between right-side ribs 11-12 by liver transfixion pin, frozen immediately in liquid nitrogen, transported to the laboratory, and stored at -70°C until analyzed for mRNA level.

Plasma glucose (Glu) was measured by the oxidase method, nonesterified fatty acids (NEFA) by the acyl-CoA synthetase/oxidase method, total bilirubin (TBIL) by the dimethyl sulfoxide method, aspartate aminotransferase (AST) by an enzyme velocity method, and γ -glutamyltranspeptidase (γ -GT) and cholinesterase (CHE) by colorimetric methods, with a commercially available kit (Yulan Biotechnology Research Institute, Shanghai, China). Plasma β -hydroxybutyric acid (BHBA) was measured with HPLC-10AVP. Liver fat content was measured as modified by Rukkwamsuk et al. (1999). Plasma insulin (Ins) and glucagons (Gln) were assayed by radioimmunoassay (RIA) using a commercially available kit (Tianjin Xiehe Medicine Technology Limited Company, China); intra- and inter-assay coefficients of variation (CV) for glucagons and insulin were less than 10% and 15%, and 8.1% and 10%, respectively.

Semi-quantitative RT-PCR for PEPCK-C mRNA Level

The relative level of PEPCK-C mRNA in liver tissue was analyzed by semi-quantitative RT-PCR. Total RNA

(8.32 ± 2.10 μg) was prepared by the guanidium isothiocyanate/phenol method as described by Puissant and Houdebine (1990), and quantified by absorbance at 260 nm in a GeneQuant (Pharmacia Biotech, Ltd Company, Buckinghamshire, UK). The ratio of RNA of all samples at 260 nm to 280 nm must be above 1.90 for further analysis. The primers of PEPCK-C and β -actin were designed based on bovine sequences deposited in GenBank (Table 1). First-strand complementary DNA (cDNA) was synthesized in Tpersonal 2000 PCR (Biometra, German) from 1.0 μg of total RNA (5.0 μl) in a 20 μl reaction volume containing 10 pM/ μl Oligo (dT)15 primer (5.0 μl), 10 mM dNTP mixture (2.0 μl), 5 \times RT buffer (4.0 μl), 20 U/ μl RNase inhibitor (0.5 μl), 5.0 U/ μl AMV reverse transcriptase (1.0 μl), and RNase-free distilled water (2.5 μl). The reaction was carried out at 42°C for 90 min, 95°C for 5 min, and then cooled for 5 min in ice. Aliquots of 1.0 μl of the first strand cDNA reaction were amplified in a 25 μl reaction volume containing 10 \times PCR buffer (2.5 μl), 10 mM dNTP mixture (2.0 μl), 5.0 U *Taq* DNA polymerase (0.125 μl), 10 pM/ μl of each primer (1.0 μl), and distilled water (17.375 μl). Following an initial pre-denaturation at 95°C for 3 min, PCR was performed for 26 cycles of denaturation at 94°C for 45 s, specific annealing temperature (Table 1) for 50 s, extension at 72°C for 1 min, and a final extension of 72°C for 10 min in the last cycle. Amplified PCR products were purified by separation on a 2% agarose gel and checked for an expected band size of 480 bp for PEPCK-C and 909 bp for β -actin as described by Xia et al. (2006). A ratio of PEPCK-C to β -actin, calculated by the intensity of two bands on a lane, represented a relative mRNA level of liver PEPCK-C as a percent (%), and ten samples from each group were measured by semi-quantitative RT-PCR (Xia et al., 2006).

Statistical analysis

Data were analyzed by ANOVA using the SPSS 10.00 software. When the main effect of treatment was found to be significant ($p < 0.05$) or very significant ($p < 0.01$), differences among means were determined by Fisher's protected least significant difference post-hoc test. Data are presented as means \pm SEM.

Table 1. Forward and reverse primers & reaction conditions of PCR for PEPCK-C, β -actin

Target gene ¹	Primers	AT ($^{\circ}\text{C}$)	Cycle number	Length (bp)
PEPCK-C (Accession No. AY 145503)				
Forward	5'-CCCTACTCTCCCGGGATGGAAAGT-3'	57	26	480
Reverse	5'-GCCCTCCGAAGATGATGCCCTC AA-3'			
β -actin (Accession No. AY 141970)				
Forward	5'-TGTGCTGTCCCTGTATGCCTCTG- 3'	57	26	909
Reverse	5'-TTGGGAATGCTCGATCCAACCG- 3'			

¹ PEPCK-C = Cytoplasmic phosphoenolpyruvate carboxykinase.

Table 2. Concentration of plasma metabolites and hormones in experimental cows

Groups ¹	Glu (mM)	NEFA (μ M)	BHBA (mM)	Ins (μ U/ml)	Gln (pg/ml)
K	1.67 \pm 0.42**	658 \pm 211**	3.46 \pm 1.40**	10.4 \pm 4.97*	181 \pm 69
C	2.90 \pm 0.30	343 \pm 137	0.60 \pm 0.12	11.8 \pm 3.69	198 \pm 75

¹ K = Ketosis, C = Control, Glu = Glucose, NEFA = Nonesterified fatty acids, BHBA = β -hydroxybutyric acid, Ins = Insulin, Gln = Glucagon.

* Means within a row with different superscripts differ ($p < 0.05$).

** Means within a row with different superscripts differ ($p < 0.01$).

RESULTS AND DISCUSSIONS

Metabolites, liver function, and hormones

Compared with healthy cows (Table 2), concentration of plasma glucose decreased ($p < 0.01$) in ketotic cows (1.67 \pm 0.42 mM vs. 2.90 \pm 0.30 mM), but concentration of plasma NEFA and BHBA increased ($p < 0.01$) (658 \pm 211 μ M vs. 343 \pm 137 μ M; 3.46 \pm 1.40 mM vs. 0.60 \pm 0.12 mM, respectively). Furthermore, concentrations of plasma insulin were lower ($p < 0.05$) in ketotic cows than in healthy cows (10.4 \pm 4.97 μ U/ml vs. 11.80 \pm 3.69 μ U/ml, respectively). These results are similar to previously reported characteristics of clinical ketosis including hypophagia, hyperketonemia, hypoglycemia, hypoinsulinemia, increased hepatic triglycerides, and increased NEFA in plasma (Chelikani and Mohamed, 2004).

It is generally accepted that ketosis is related to the high energy requirement for more milk production, which often cannot be met in high-producing cows by the normal intake of energy during early lactation (Dale et al., 1997). Smith et al. (1997) reported that concentration of plasma NEFA gives an indication of mobilization of body reserves after a decrease of caloric intake, combined with a low concentration of plasma glucose.

Fat content of liver in ketotic cows was 18.8% (Table 4). At 1 wk after calving, a liver fat content above 20% is considered mild fatty liver, 20-40% is moderate fatty liver, and more than 40% is severe fatty liver; a liver fat content of less than 10% is considered normal (Grummer, 1993). Plasma NEFA increases rapidly after calving, suggesting that both a rapid decrease in glycogen and an increase in triglyceride content of liver are prerequisites for susceptibility to ketosis (Grum et al., 1996; Rukkamsuk et al., 1998). Veenhuren et al. (1991) have indicated the probable importance of periparturient NEFA concentration to the development of ketosis and fatty liver, and the inverse relationship of carbohydrate status with hepatic ketogenesis

and TG accumulation. Unlike other metabolites, liver triglycerides disappear very slowly, even when cows are returned to normal feeding and management (Holtenius et al., 2003). These relationships suggest that changes in metabolic capacities of liver tissue for increased oxidation and decreased esterification of NEFA might be key factors in the decreased hepatic lipid accumulation around parturition.

In addition, the activity of plasma AST increased significantly, and there was a rising tendency for plasma γ -GT, TBIL, and CHE in clinically ketotic cows (Table 3). Usually the parameters of liver function, in particular AST, are unaffected by ketosis, but increased activity of plasma AST is closely related with the degree of triglyceride (TG) in liver (Bobe et al., 2004). A severe degree of fat infiltration in liver often accompanies enhanced parameters of liver function, such as plasma AST, γ -GT, TBIL, and CHE in early lactating cows. Therefore, it is confirmed that there is a mild fatty liver in clinically ketotic cows, as well as abnormal activity of plasma AST which might be concerned with fatty liver.

Although the etiology of ketosis is complicated, hormones play an important role in its development (James 1999). A low ratio between insulin and glucagons due to hypoinsulinemia which occurred in ketotic dairy cows (Table 2), and which often appears during negative energy balance in early lactation, would stimulate fat mobilization (Herdt, 2000) leading to a great amount of NEFA being mobilized from fat tissue and, exceeding the oxidative capacity of the liver, increased ketone body formation, and occurrence of ketosis (Hayirli, 2002).

mRNA level of PEPCK-C in livers

The abundance of liver PEPCK-C mRNA was higher ($p < 0.05$) in ketotic cows than in healthy cows (68 \pm 25 vs. 55 \pm 3) (Table 4). Blood glucose in ruminants mainly comes from gluconeogenesis, in which PEPCK-C is one of the

Table 3. Levels of plasma parameters for liver function in the experimental cows

Groups ¹	AST (U/L)	γ -GT (U/L)	TBIL (μ mol/L)	CHE (U/L)
K	88 \pm 15*	19 \pm 5	5.36 \pm 2.25	326 \pm 32
C	70 \pm 10	15 \pm 4	3.95 \pm 1.63	290 \pm 38

¹ K = Ketosis, C = Control, AST = Aspartate aminotransferase, CHE = Cholinesterase, γ -GT = γ -glutamyl transpeptidase, TBIL = Total bilirubin.

* Means within a row with different superscripts differ ($p < 0.05$).

Table 4. mRNA level in liver for PEPCK-C in the experimental cows

Groups ¹	PEPCK-C ² (%)	Liver fat ³ (%)
K	68±25*	18.86±5.32**
C	55±3	8.31±2.25

¹ K = Ketosis, C = Control, PEPCK-C = Cytoplasmic phosphoenolpyruvate carboxykinase.

* Means within a row with different superscripts differ (p<0.05).

** Means within a row with different superscripts differ (p<0.01).

² A relative mRNA expression level of PEPCK-C in liver was represented by a ratio of PEPCK-C to β -actin according to the intensity of the two bands on a lane expressed as a percent (%).

³ Liver fat content was represented by a ratio of liver fat content (mg) to liver wet weight (mg) expressed as a percent (%).

most important rate-limiting enzymes. Overexpression of PEPCK-C can enormously increase the export of glucose from the liver and stabilise the concentration of blood glucose, so activity of PEPCK-C in liver would determine the whole level of gluconeogenesis (She et al., 2000; Cansu et al., 2002). However, activity and gene expression of PEPCK-C are modulated with substrates and hormones, particularly insulin and glucagon. Insulin limits the mRNA expression of PEPCK-C by inhibiting the PEPCK-C transcription promoter, and glucagon stimulates the gene transcription of PEPCK-C by cAMP action, which could be subdued notably by insulin (Grizard et al., 1986; Shen et al., 2003). Therefore, in this study, mRNA level of PEPCK-C in the liver of ketotic dairy cows did not decrease but increased (Table 4), which might be related to the hypoinsulinemia and low ratio of insulin to glucagons.

Adaptative changes of insulin, glucagons, and mRNA level of PEPCK-C occurred in ketotic dairy cows, which were in favor of increased gluconeogenesis and restoration of NEB. However, cows in clinical ketosis are still actually in a hypoglycemic state, which might be related to an insufficiency of gluconeogenic substrate, which is often due to decreased dry matter intake (Greenfield et al., 2000). In the present study, fat infiltration in the liver did not result in a decreased level of PEPCK-C mRNA in ketotic dairy cows, even though activity of plasma AST increased, which means a rising possibility of liver dysfunction. However, the activity of liver PEPCK is influenced and the velocity of liver gluconeogenesis decreases in ketosis (Theera et al., 1999). The response to insulin becomes weakened in the dairy cow with liver fatty infiltration, which might result in insulin resistance and hamper the normalization of blood glucose level (Veenhuren et al., 1991; Grummer et al., 1993).

In brief, the adaptive change of mRNA level in the PEPCK-C genes was helpful for the enhancement of gluconeogenesis and for decreased fat mobilization and ketone body production.

CONCLUSIONS

In this study, the increase of PEPCK-C mRNA level in the liver of ketotic cows was in favor of the augmentation of hepatic gluconeogenesis and relief of negative energy balance, but the decreased concentration of glucagons and the increased concentration of NEFA and BHBA in plasma of the diseased cows meant that the above adaptive changes were still insufficient to correct the metabolic confusion of ketosis. The mechanism of regulation of mRNA level of PEPCK-C in the liver of ketotic cows is still unclear and worthy of further research.

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