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Activities of the enzymes of hepatic gluconeogenesis in periparturient dairy cows with induced fatty liver

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The objective was to measure the activities of all the enzymes essential for hepatic gluconeogenesis in dairy cows with induced fatty liver. We aimed to induce severe fatty liver in ten experimental cows by overfeeding them during the dry period while seven control cows were maintained on a restricted diet. To induce a marked negative energy balance, the experimental cows were deprived of feed for 8 h immediately after parturition. In addition, the experimental cows were given a restricted amount of diet during the first 5 d of lactation. Liver samples were collected 1 week before and 1, 2 and 4 weeks after parturition. Before parturition, liver triacylglycerol concentrations did not differ between the two groups. After parturition, the experimental cows developed marked fatty liver as indicated by a higher level of triacylglycerols in the liver compared with the control cows.

Before parturition, all gluconeogenic enzymes in the liver were lower in experimental cows than in control cows. Phosphoenolpyruvate carboxykinase, pyruvate carboxylase and propionyl-CoA carboxylase were significantly lower and fructose 1,6-bisphosphatase and glucose 6-phosphatase tended to be lower in the experimental cows. The activities of two crucial enzymes for gluconeogenesis in ruminants, i.e., phosphoenolpyruvate carboxykinase and propionyl-CoA carboxylase, remained low throughout the sampling period *post partum*. Activities of pyruvate carboxylase and glucose 6-phosphatase in the experimental cows *post partum* were upgraded to values similar to those of the control cows. The results showed that the capacity for hepatic gluconeogenesis before parturition was lower in cows with induced fatty liver than in control cows. After parturition, the low activities of crucial gluconeogenic enzymes indicated insufficient production of glucose. It is suggested that the low gluconeogenic capacity leads successively to low blood glucose concentrations, low insulin levels and high rates of mobilization of fatty acid, causing severe hepatic lipidosis.

Keywords: Gluconeogenesis, negative energy balance, fatty liver, dairy cows.

Metabolism of carbohydrates in ruminant tissues differs in several respects from that in non-ruminants, mainly because ruminants take up very little glucose from the digestive tract (Herdt, 1988). Rumen microbes ferment the bulk of available carbohydrate to the volatile fatty acids, acetate, butyrate and propionate, which are subsequently absorbed. These differences in carbohydrate metabolism between ruminants and non-ruminants are central to the understanding of certain metabolic disorders of the ruminant. A relative shortage of energy occurs in cows after parturition when milk production starts, such that energy requirement is much greater than energy intake, resulting in negative energy balance (NEB). Over-condition at

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parturition predisposes to severe NEB after parturition (Armentano et al. 1991; Grummer, 1993; Van den Top, 1995; Rukkwamsuk, 1999).

For the proper functioning of a number of tissues, it is essential that ruminants have a constant supply of glucose via the circulation. They depend on gluconeogenesis that takes place primarily in the liver (Faulkner & Zammit, 1985; Young, 1977). Of the volatile fatty acids only propionate is a precursor for gluconeogenesis (Leng et al. 1967) but there are lesser contributions from lactate, glucogenic amino acids and glycerol (Grizard et al. 1986). Furthermore, most of the energy requirement of ruminants is met by oxidation of acetate and butyrate. Despite this sparing of glucose, the circulating concentrations of glucose in ruminants are normally lower than in non-ruminants (Kaneko, 1989).

During NEB *post partum* in high-producing dairy cows, fatty acids are mobilized from adipose tissue, leading to increased plasma concentrations of non-esterified fatty acids (NEFA) in plasma (Grummer, 1993; Waltner et al. 1993; Zurek et al. 1995). As a result, there is a large influx of fatty acids into the liver, which are esterified to triacylglycerol (TAG). Secretion of TAG in the form of very-low-density lipoproteins (VLDL) does not keep pace with their formation, leading to accumulation of TAG, i.e., fatty liver (Herdt, 1988). In addition, reduction of fatty acid oxidation following parturition may contribute to accumulation of TAG *post partum* (A Murondoti, unpublished observations). Fatty liver is of clinical and economic importance because it is related to diseases such as mastitis, ketosis, infertility, displaced abomasum and endometritis (Reid, 1980; Gerloff et al. 1986; Grummer, 1995; Oikawa et al. 1997). It might be suggested that abundant availability of glucose would maintain insulin levels, leading to inhibition of adipose tissue lipolysis which in turn would diminish accumulation of TAG in liver. Reported low serum insulin concentrations and low hepatic glycogen levels may indicate low availability of glucose in severe NEB and associated fatty liver (Amaral-Phillips et al. 1993; Holtenius, 1993; Reid et al. 1983; Rukkwamsuk et al. 1999; Studer et al. 1993; Van den Top et al. 1995). Thus, a major question is whether fatty liver in the dairy cow is associated with low rates of gluconeogenesis.

Studies *in vitro* suggest a relationship between fatty liver and reduced hepatic function in ruminants because gluconeogenesis from propionate in isolated bovine hepatocytes is depressed in cells infiltrated with lipid (Cadorniga-Valino et al. 1997). There is little information on the effect on gluconeogenesis when cows experience both severe NEB and increased hepatic influx of fatty acids leading to fatty liver. Rukkwamsuk et al. (1999) measured the activities of a few gluconeogenic enzymes in cows with moderate fatty liver and concluded that hepatic gluconeogenesis probably is inadequate during periods of NEB. Hepatic TAG accumulation as induced by the feeding of undegradable protein is associated with a lower activity of glucose 6-phosphatase (G6-Pase), but a higher activity of fructose 1,6-bisphosphatase (FBPase) (Murondoti et al. 2002). The objective of the present study was to collect more information on the activities of all the key enzymes in hepatic gluconeogenesis, i.e., phosphoenolpyruvate carboxykinase (PEPCK), propionyl-CoA carboxylase (PCC), pyruvate carboxylase (PC), FBPase and G6-Pase, before and after parturition in dairy cows with levels of hepatic TAG as high as those seen in clinical practice in animals with fatty liver syndrome, i.e., exceeding 15% (Herdt et al. 1983).

Materials and Methods

Cows and diets

The experimental design was approved by the animal experiments committee of the Utrecht Faculty of Veterinary Medicine. Seventeen pregnant, multiparous Holstein-Friesian cows were allocated to two groups: seven cows were assigned to the control group and ten cows were assigned to the experimental group. Details of the cows (means ± SD) were as follows: age: experimental group, 4·6 ± 0·8 years, control group, 5·5 ± 1·9 years; cumulative 305-d milk production: experimental group, 8312 ± 900 kg, control group, 8535 ± 1403 kg; body weight: experimental group, 734 ± 67 kg, control group, 613 ± 43 kg at the start of the experiment. Body weight was recorded by weighing on a scale. The feeding regimen of the experimental group during the dry period was designed to induce excess body fat at calving and fatty liver *post partum* (Van den Top et al. 1996). The dry period for the experimental group ranged from 62 to 90 d before calving and for the control group from 55 to 94 d before calving. Each group was given the appropriate diet for the entire dry period. Energy requirement for maintenance was taken to be 0·285 MJ net energy for lactation (NE_L)/$kg^{0·75}$. Energy balance (expressed as MJ NE_L) was calculated as [(intake)−(maintenance+milk)] × 0·75. Thus, it was assumed that metabolizable energy (ME) derived from the body was converted to milk energy 25% more efficiently than that derived from the diet.

All cows were offered a total mixed ration (Table 1) in two equal portions at 10·00 and 22·00. For the dry period, control cows were restricted to 6·8 kg DM (47 MJ NE_L) of the total mixed ration whereas the experimental cows had free access to the same diet. From 10 d before calving and during lactation, the diet was also offered to the animals in the control group for consumption *ad libitum*. To induce a large NEB, the experimental cows were fasted for 8 h immediately after parturition and their feed intake restricted to 10·1 kg DM (70 MJ NE_L) of the total mixed ration for the first 5 d of lactation. Thereafter, feed consumption was *ad libitum*. Feed intake was measured as the amount of feed offered minus the recorded orts, on a DM basis. Cows were housed in a stanchion barn with wheat straw as bedding. Cows were fed individually and had free access to tap water. Body condition scores were assessed on a scale of

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Table 1. Composition (g/kg DM unless stated otherwise) of the total mixed ration

Corn silage†	523
Solvent extracted rapeseed meal‡	77
Solvent extracted soyabean meal‡	172
Dried sugar beet pulp‡	218
Minerals and vitamins§	10
Calculated energy content, NE_L/kg DM	6·9 MJ
Calculated crude protein	185

† The analysed composition (319 g of DM/kg) was: (g/kg of DM): crude protein, 74; crude fibre, 186; starch, 361; ash, 46. Calculated energy content on the basis of analysed crude fibre, crude protein and ash was 6·6 MJ NE_L/kg DM (Bedrijfslaboratorium voor Grond- en Gewasonderzoek, Oosterbeek, The Netherlands)

‡ Nutrient composition was taken from tabular data (Centraal Veevoederbureau, 2000)

§ The mineral mix contained the following (/kg): vitamin A, 600 000 IU; vitamin D, 200 000 IU; vitamin E, 2 g; Ca, 150 g; P, 80 g; Na, 80 g; Mg, 100 g; Fe, 0·5 g; Mn, 4 g; Zn, 5 g; Cu, 3 g; Co, 0·1 g; I, 0·15 g; Se, 0·04 g

1 to 5 to the nearest 0·5, higher scores being associated with greater body condition.

Sampling and assay procedures

Blood and liver samples were collected 1 week before the expected date of parturition and at 1, 2 and 4 weeks after parturition. Blood samples were taken at 10·00 and liver samples at 12·00. Samples were collected and handled as in earlier studies (Van den Top et al. 1995). Concentrations of hepatic TAG (kit number 337-A; Sigma Chemical Co., St Louis, MO, USA), plasma BHBA (kit number RB 1007; Sanbyo BV, Uden, The Netherlands) and plasma NEFA (NEFA C®, kit number 994-75409; Instruchemie BV, Hilversum, The Netherlands) were measured enzymically with commercial kits as directed by the manufacturers. Activities of PEPCK (EC 4.1.1.32), FBPase (EC 3.1.3.11) and G6-Pase (EC 3.1.3.9) were determined as described by Rukkwamsuk et al. (1999). PC (EC 6.4.1.1) and PCC (EC 6.3.4.10) were assayed by incorporation of $[^{14}C]NaHCO_3$ as described by Scrutton et al. (1969) and Tietz & Ochoa (1962), respectively.

Statistical analysis

Results from the control and the experimental groups were compared using Student's *t* test. To take into account the increased probability of a type 1 error because of multiple comparisons, the level of significance was pre-set at $P<0·0125$ instead of 0·05 according to Bonferroni's adaptation.

Results

Performance

In the dry period, control cows consumed 6·8 kg DM/d. The feed consumption of the experimental cows with free access to feed was not recorded in that period. In the 10 d before parturition, i.e., the period in which both groups had free access to feed, experimental cows consumed 10·4±3·5 kg DM/d and control cows 12·7±4·0 kg DM/d. DM intake during the first week after parturition was 9·0±3·0 kg/d for the experimental group and 16·1±5·1 kg/d for the control group. For the 2-week period after parturition, DM intake had increased to 12·8±4·2 kg/d for experimental cows and to 17·2±3·5 kg/d for control cows.

During the dry period, experimental cows consumed more energy than did control cows, and, as a result, experimental cows gained more weight than control cows [103·0±25·0 kg *v.* 75·5±25·0 kg]. At the end of the dry period, body condition scores for the experimental group were higher than for the control group (4·7±0·2 *v.* 3·0±0·4; $P<0·001$). After parturition, mean BW loss (i.e., the difference between BW measured immediately after parturition and that measured 4 weeks later) was greater for experimental cows than for control cows [55·7±41·6 kg *v.* 28·0±45·3 kg]. At 4 weeks *post partum*, body condition scores fell by 33% in experimental cows and by 23% in control cows (3·2±0·5 *v.* 2·3±0·4). From lactation weeks 1 to 4 inclusive, mean milk production was 27·3±5·6 kg/d for experimental cows and 29·6±4·1 kg/d for control cows, and did not differ between the two groups. Energy balance 1 week after parturition in the experimental group (−38·3±15·3 MJ NE_L/d) was significantly more negative ($P<0·009$) than in the control group (−1·4 ±MJ NE_L/d). In the second week *post partum*, energy balance was still significantly more negative ($P<0·022$) in experimental cows (−25·6±11·9 MJ NE_L/d) than in control cows (−9·0±14·9 MJ NE_L/d). Cows in the experimental group were in NEB longer than the control group.

Plasma NEFA, plasma BHBA and liver TAG

Plasma concentrations of NEFA did not differ between the two groups 1 week before parturition but rose sharply after parturition in both groups (Table 1). Compared with the concentration 1 week before parturition, the concentration 1 week after parturition had increased by 165% in the experimental group ($P<0·001$) but increased only by 67% in the control group. Mean plasma BHBA concentrations did not differ between the two groups before parturition. In the control group the concentration did not change after parturition ($P>0·05$). However, the concentration rose significantly in the experimental group 1 week after parturition ($P<0·01$) and remained elevated throughout the experimental period ($P<0·02$) (Table 2). Mean concentrations of liver TAG are presented in Table 2. One week before parturition, liver TAG levels were similar in the two groups of cows. Compared with the value 1 week before parturition, the mean concentrations in the two groups rose sharply 1 week after parturition and remained higher throughout the sampling period *post partum*. The rise was markedly greater in the experimental cows than in the

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Table 2. Concentrations of hepatic triacyglycerol (TAG), plasma non-esterified fatty acids (NEFA) and plasma beta-hydroxybutyrate (BHBA) before and after parturition

Values are means±SEM for $n=7$ for control cows and $n=10$ for experimental cows

	Weeks from parturition			
	−1	1	2	4
		TAG (mg/g tissue)		
Control	22·00±1·96	54·96±12·33	57·53±12·52	48·82±12·58
Experimental	25·59±1·48	170·27±25·69^{b,B}	146·18±27·61^{b}	139·23±33·10^{a}
		NEFA (mmol/l)		
Control	0·36±0·04	0·60±0·12	0·54±0·05	0·33±0·05
Experimental	0·60±0·12	1·59±0·20^{b,B}	0·76±0·08	0·75±0·13
		BHBA (mmol/l)		
Control	0·50±0·04	0·55±0·07	0·59±0·07	0·51±0·05
Experimental	0·55±0·05	1·79±0·35^{a,A}	1·25±0·24	1·62±0·38

[A,B] Indicate significant differences between the two groups at the same sampling time
[a,b] Indicate significant differences within a group between values before and after parturition at different sampling times. $^{a,A}P<0·01$, $^{b,B}P<0·001$

Table 3. Hepatic activities of fructose 1,6-bisphosphatase (FBPase), glucose 6-phosphatase (G6-Pase), pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK) and propionyl-CoA carboxylase (PCC), before and after parturition

Values are means±SEM for $n=7$ for control cows and $n=10$ for experimental cows

	Specific enzyme activity (mmol/min per mg protein)			
	Week from parturition			
	−1	1	2	4
		FBPase		
Control	79·14±5·92	75·57±9·01	65·94±5·59	66·44±6·56
Experimental	63·69±8·62	63·18±5·16	56·53±5·75	62·36±6·90
		G6-Pase		
Control	1·44±0·30	2·48±0·56	2·08±0·55	1·62±0·34
Experimental	1·31±0·13	3·87±0·51^{b}	3·26±0·75	2·69±0·43^{a}
		PC		
Control	35·82±6·41	63·53±5·72^{a}	65·26±3·43^{a}	60·74±2·46^{a}
Experimental	15·79±2·44	64·39±2·56^{b}	56·89±4·91^{b}	64·36±5·32^{b}
		PEPCK		
Control	33·49±2·11	30·31±3·97	27·19±3·01	30·85±3·34
Experimental	25·64±1·30^{A}	17·86±1·70^{a,A}	15·63±1·76^{b,A}	21·60±2·27
		PCC		
Control	10·29±0·51	11·67±0·91	11·04±0·93	11·42±0·73
Experimental	8·49±0·59	8·44±0·52^{A}	7·42±0·67^{A}	8·48±0·66^{A}

[A] Indicates significant differences between the two groups at the same sampling time
[a,b] Indicate significant differences within a group between values before and after parturition at different sampling times. $^{a,A}P<0·01$, $^{b}P<0·001$

control cows, and differed significantly between the two groups at 1, 2 and 4 weeks after parturition.

Gluconeogenic enzyme activities in liver

Gluconeogenic enzyme activities in liver are shown in Table 3. Activities of FBPase did not differ significantly between the two groups. However, the group-mean activities were consistently lower for the experimental cows both before and after parturition. Before parturition, activities of G6-Pase for the two groups were not significantly different. In both groups, the activities of this enzyme increased *post partum* compared with before parturition. The activities increased significantly more in the experimental group. Activities of PC were lower in experimental cows than in control cows at 1 week before parturition. Compared with

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the activities 1 week before parturition, activities 1 week after parturition increased by 77% in the control group, but increased by 308% in the experimental group. Activities remained high throughout the sampling period. There were no significant differences between the two groups *post partum*. Activities of PEPCK in experimental cows were significantly lower 1 week before parturition than in control cows. After parturition, activities dropped even further in the experimental cows whereas the activities in control cows remained unaffected throughout the sampling period. Activities of PCC were significantly lower 1 week before parturition in experimental cows. The differences between the two groups remained throughout the sampling period.

Discussion

Experimental cows gained more body weight than did the control cows that were fed restricted amounts of feed, as reported by others (Grummer et al. 1995; Van den Top et al. 1995; Rukkwamsuk et al. 1999). Experimental cows lost more body weight during the first few weeks *post partum*, indicating that these cows were in greater NEB than control cows. This observation is supported by the fact that the experimental cows had significantly higher TAG levels in the liver. Mean hepatic TAG concentrations in cows given free access instead of restricted access to feed during the dry period was 170 mg/g of liver in the first week *post partum*, which was more than 3-times higher than in the control cows. The level of liver TAG in the experimental cows was of the same order of magnitude as that seen in clinical practice in animals with fatty liver syndrome (Herdt et al. 1983), and was markedly higher than in the study of Rukkwamsuk et al. (1999), in which hepatic activities of some enzymes of gluconeogenesis were measured. In the study by Rukkwamsuk et al. (1999), the mean TAG concentration in experimental cows 1 week *post partum* was about 120 mg/g of liver. This comparison indicates that in the current study, experimental cows developed a greater NEB leading to a higher rate of lipolysis and a subsequent higher hepatic accumulation of TAG. Higher hepatic TAG levels in the present study were probably caused by the restricted feeding of the experimental cows in the first 5 d after parturition and by the composition of the diet.

Before parturition, activities of all gluconeogenic enzymes were lower in the experimental cows than in the control cows. These lower activities are not related to fatty infiltration of the liver because massive influx of NEFA in the liver had not yet occurred. The lower activities suggest that free access of a high-energy diet before parturition suppressed gluconeogenesis. Control animals had a high enzyme capacity to cope with the demand for glucose. The high feed intake of the experimental animals may have provided glucose in the form of ileal-digestible, but rumen-undegradable starch. Meeting the sudden glucose demand at the onset of lactation would be difficult in animals with low enzyme capacity for gluconeogenesis, i.e., the experimental cows. Hence, the glucose concentration in the blood of the experimental cows would have dropped more than in the control cows (Van den Top et al. 1995), resulting in more lipolysis in adipose tissue and contributing to greater accumulation of TAG in the liver of the experimental cows.

Activities of FBPase in experimental cows tended to be lower than those in control cows at all sampling times. Despite the greater availability of glycerol (Van den Top et al. 1995), which was liberated during the intense lipolysis, activities of FBPase were not upgraded. It is likely that activities of FBPase, which were the highest of all gluconeogenic enzymes (Table 2), were sufficient to cope with the increase in the amount of substrate.

Before parturition, cows consuming feed *ad libitum* during the dry period accumulate more hepatic glycogen than cows fed restricted amounts of feed (Van den Top et al. 1995; Rukkwamsuk et al. 1999). After parturition, when the demand for glucose increases sharply, this glycogen store is rapidly depleted (Rukkwamsuk et al. 1998). Higher activities of G6-Pase in the experimental cows *post partum* coincided with the rapid drop in glycogen. Therefore, higher activities of G6-Pase in the experimental cows *post partum* may be linked to a faster mobilization of glycogen. The increased expression of enzyme activity may compensate also for decreased availability of the substrate, glucose 6-phosphate. In that way the production of free glucose (in the face of a decreased glucose concentration in the blood) can better compete with glucose 6-phosphate consumption in the pentose phosphate pathway.

PC is required for the entry of lactate and some of the glucogenic amino acids into the gluconeogenic pathway. Before parturition, the activity of this enzyme was lower in experimental cows than in control cows, contributing to the afore-mentioned low hepatic capacity for gluconeogenesis in experimental cows at the onset of lactation. However, 1 week *post partum* the activity in both groups was upgraded to the same level. Therefore, it is unlikely that the activity of PC explains the difference between the two groups.

PCC is a crucial enzyme for gluconeogenesis in ruminants. Microbial fermentation produces volatile fatty acids of which propionate is the only gluconeogenic precursor (Leng et al. 1967). After uptake from the rumen, propionate is acylated to propionyl-CoA in the liver and is subsequently acted upon by PCC. The next key gluconeogenic enzyme to come into play is PEPCK. Both enzymes are essential for utilization of propionate, the major gluconeogenic substrate in ruminants. Before parturition, both enzyme activities were significantly lower in experimental cows than in control cows and were not upgraded in experimental cows at the onset of lactation. Hence, gluconeogenesis probably was inadequate in the experimental cows at the onset of lactation when the demand for glucose increases dramatically.

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In conclusion, our findings show that the capacity for hepatic gluconeogenesis before parturition was lower in cows consuming feed *ad libitum* than in cows with restricted access to feed during the dry period. After parturition, low activities of crucial gluconeogenic enzymes indicated insufficient production of glucose in experimental cows with severe hepatic lipidosis. It appears that cows with fatty liver have diminished hepatic gluconeogenic capacity for metabolism of propionate.

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