Interrelations Between Glucose-Induced Insulin Response, Metabolic Indicators, and Time of First Ovulation in High-Yielding Dairy Cows

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ABSTRACT

High-yielding dairy cows are more susceptible to metabolic and reproductive disorders than low-yielding cows. Insulin plays a pivotal role in the development of both problems. In the present study, we aimed to assess the glucose-induced insulin responses of dairy cows at different time points relative to calving and to relate this to the metabolic status and the time of first ovulation. Twenty-three healthy, multiparous Holstein-Friesian cows with a high genetic merit for milk yield were studied from 14 d prepartum to 42 d postpartum. Intravenous glucose tolerance tests were performed on -14, 14, and 42 d relative to calving to evaluate the plasma insulin and glucose responses to a glucose load, as estimated by the peak concentration, the area under the curve (AUC), and the clearance rates of insulin and glucose. Blood samples were obtained at 3-d intervals and analyzed for glucose, insulin, and nonesterified fatty acids (NEFA). The time of first ovulation was defined by transrectal ultrasonography and plasma progesterone analysis. Glucose-induced insulin AUC and peak concentration decreased and glucose clearance increased during lactation compared with the dry period. Plasma NEFA concentrations were negatively related to insulin AUC and peak concentrations. Fourteen cows ovulated within 42 d postpartum, and the remaining 9 cows suffered from delayed resumption of ovarian function. Survival analysis demonstrated that cows with lower NEFA concentrations during the dry period tended to have earlier resumption of ovarian activity. In conclusion, our data suggest a decreased plasma insulin response to glucose postpartum in high-yielding dairy cows, possibly contributing to metabolic stress during the early postpartum period. It is hypothesized that NEFA impair glucose-induced insulin secretion in dairy cows. Additionally, our results suggest

the importance of lipolysis during the transition period as a risk factor for delayed ovulation.

Key words: dairy cow, intravenous glucose tolerance test, insulin, first ovulation

INTRODUCTION

Average milk production in dairy cows has increased dramatically over the past decades. Unfortunately, an increase of the incidence of metabolic and reproductive disorders (Opsomer et al., 2000; Lucy, 2001) has been reported in high-yielding dairy cows, causing considerable economic losses (Shrestha et al., 2004b). This worrying trend is challenging scientists worldwide to shed more light on the inverse correlation between yield and fertility.

A large retrospective study has pointed out that 49% of Flemish high-yielding dairy cows suffer from ovarian dysfunctions postpartum (**pp**; Opsomer et al., 2000), resulting in lower conception rates and longer calving intervals (Shrestha et al., 2004b; Walsh et al., 2007). A delay of the first ovulation (**DFO**; no progesterone increase before 50 d pp), caused by inactive ovaries or cystic ovarian disease (**COD**) and a prolonged luteal cycle (ovulation followed by a progesterone increase for >20 d) were by far the most important causes of ovarian dysfunction. Although a prolonged luteal cycle seemed to be mainly related with dystocia and uterine disorders, clinical features indicative for a severe negative energy balance (**NEB**), such as clinical ketosis, severe body condition loss, and diseases affecting dry matter intake, were identified as major risk factors for DFO (Opsomer et al., 2000). Similar results were found by others (Shrestha et al., 2004a; Walsh et al., 2007).

The effect of NEB on fertility has been studied in more detail at our department. Interestingly, low plasma insulin concentrations appear to be associated in vivo with developmental disturbances of the follicle such as COD (Vanholder et al., 2005). This is in agreement with in vivo and in vitro evidence found by other authors. For instance, administering insulinogenic diets results in an earlier resumption of ovarian func-

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tion (Miyoshi et al., 2001; Gong et al., 2002). In vitro research has pointed out that insulin and insulin-like growth factor are major regulators of granulosa cell proliferation and estrogen synthesis (Spicer and Echtenkamp, 1995; Gutierrez et al., 1997). Additionally, low insulin results in lipolysis and elevated peripheral concentrations of NEFA, which, in turn, have a detrimental effect on granulosa cell function (Vanholder et al., 2005) and increase the risk of production diseases such as ketosis, abomasal dislocation, and hepatolipidosis (Roche, 2006). Altogether, these data demonstrate that low insulin concentrations play a pivotal role in the development of reproductive and metabolic disorders. On the other hand, low insulin levels are associated genotypically (Gutierrez et al., 2006) and phenotypically (Ingvarsen and Friggens, 2005) with high milk yield. Although insulin has no direct effects on galactopoiesis, low insulin decreases the uptake of glucose by insulin-sensitive tissues, such as skeletal muscles, and enhances glucose availability for the mammary gland, which is insulin-insensitive (Zhao et al., 1996; Nishimoto et al., 2006; van Knegsel et al., 2007). Therefore, insulin is a very interesting candidate molecule concerning the paradox between yield, metabolic diseases, and reproductive performance.

The factors influencing insulin release in cows remain poorly documented. In humans, prolonged periods of elevated NEFA concentrations, such as obesity, increase the risk of type II diabetes mellitus. This seems to be due to decompensation and exhaustion of the Langerhans islets after prolonged episodes of insulin resistance and hyperinsulinaemia (Golay and Ybarra, 2005). Apart from this, a direct deleterious effect of fatty acids on the pancreatic β -cell insulin secretion and viability has been demonstrated by in vitro studies in man (Zhou and Grill, 1995) and rat (Maedler et al., 2001). Because dairy cows are also exposed to high-NEFA concentrations throughout periods of NEB, we hypothesized that they also suffer from an impaired insulin secretion. The aims of our study were (1) to evaluate glucose-induced plasma insulin responses at several time points in the peripartum period using intravenous glucose tolerance tests (IVGTT), (2) to determine metabolic risk factors associated with low plasma insulin responses, and (3) to relate metabolic factors with the resumption of ovarian cyclicity.

MATERIALS AND METHODS

Selection of the Animals

Twenty-seven multiparous dairy cows with a high genetic merit for milk yield, housed at the research dairy farm of Ghent University (Biocentrum Agrivet, Melle,

Belgium), were studied from 14 d before the expected calving date until 42 d pp. Cows in the herd were taken up in the Flemish Cattle Breeding Association and had an average 305-d fat-corrected milk yield of 11,500 kg in the previous lactation and an average genetic index for production of 306.8 kg above the mean of all cows in the Association. Experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent. Cows were housed in a loose cubicle stable and milked on average 2.68 ± 0.03 times per day by an automated milking system (VMS, DeLaval NV, Ghent, Belgium). Whenever a cow had not been milked for more than 10 h, the dairyman was automatically alerted, and the cow was guided to the milking robot.

In the dry period, the ration consisted of wheat straw, offered *ad libitum* in the bunk and refreshed on a daily basis, approximately 8 kg DM of corn silage, and 1.4 kg DM of soybean meal per day (Aveve, Merksem, Belgium). The dry period ration was supplemented with 25 g of magnesium oxide (Nutreco, Ghent, Belgium) and 200 g of a dry period mineral mixture (Nutreco) per day. During the last 7 to 10 d before the expected calving date, cows were introduced into the herd and offered the lactation roughages and 2 kg of concentrate per day (Aveve). During lactation, cows were fed *ad libitum* a mixed ration consisting of approximately 45% corn silage, 13.5% grass silage, 20% sugarbeet pulp, 12% corncob mix, 8% soybean meal, and 1.5% hay on a DM basis. Overall, the TMR contained approximately 1.606 Mcal of NE_L and 79.6 g of digestible intestinal protein per kilogram of DM. The different components of the ration were weighed before feeding, and total daily DMI of cows was estimated at 15 kg of roughage (corn silage, grass silage, sugarbeet pulp, and hay), 2.25 kg of corncob mix, and 1.5 kg of soybean meal. Based on an expected daily peak milk yield of 45 kg, cows were given maximally 8 kg of concentrate per day. This maximum was gradually reached by 50 DIM; later on, it was adapted individually based on differences in actual milk yield, DMI, expected lactation length, and body condition. Two kilograms of concentrate was offered during robot milking; the rest was taken up voluntarily at automated feed dispensers. More information about the composition of the dry period mineral mixture and the different concentrates and roughages is summarized in Table 1 and Table 2.

The health status of the cows was estimated subjectively by observation of individual milk yield, milk composition and concentrate intake, conversation with the herd manager, and regular clinical inspections. Four cows with disorders that severely affected feed intake and milk yield, such as abomasal dislocation followed by surgery and dystocia followed by caesarean section, were excluded from the study, reducing the number of

Table 1. Composition of the dry period mineral mixture¹

Compound	Concentration
Vitamins	
Vitamin A	500,000 IU/kg
Vitamin D ₃	100,000 IU/kg
Vitamin E	8,000 IU/kg
Choline chloride	5,000 IU/kg
Minerals	
Sodium selenite	12.8 mg/kg
Organic selenite	7 mg/kg
Calcium	4%
Phosphorus	5%
Sodium	6.2%
Magnesium	7.5%

¹Nutreco, Ghent, Belgium.

cows in the study to 23. All data of those 4 cows were omitted from statistical analyses.

IVGTT

On 14 d before the expected calving date and on 14 and 42 d pp, an IVGTT was performed. At these time points, cows were expected to be in positive energy balance, at the nadir of NEB, and recovering from NEB (Jorritsma et al., 2003). Because the gestation length of most cows was slightly longer than expected, the interval between the first IVGTT and actual calving date ranged from 9 to 28 d (mean: 18 ± 1.32 d). From 0630 h, the selected cows were automatically separated and restrained after milking. Cows that had not been milked by 0800 h were guided to the milking robot and separated. All IVGTT were performed around 1000 h, within 2 to 3.5 h after concentrate intake during milking. A 12-gauge catheter (Intraflon, Vygon, Brussels, Belgium) was inserted in the jugular vein and fixed to the skin. Cows were allowed to rest for 30 min after insertion of the catheter until blood sampling was started. Stress was avoided as much as possible; cows

generally continued to ruminate and appeared relaxed during the test. The body weight was estimated based on the average dry period weight of the herd and the size and condition score of the cow and varied between 520 and 750 kg. Blood samples were obtained from the catheter in evacuated gel-coated and fluorinated blood tubes (Vacutainer, Terumo Europe NV, Leuven, Belgium) on -10, 0, 4, 8, 12, 18, 25, 36, 45, and 60 min relative to the infusion of 150 mg of glucose per kilogram of body weight (aqueous solution of 300 mg/L; glucose dextrose, Eurovet, Heusden-Zolder, Belgium), according to the protocol described by Holtenius et al. (2003). The catheter was rigorously flushed after every infusion or sampling. Blood samples were centrifuged ($2,000 \times g$, 30 min) within 1.5 h after collection, and serum and plasma were stored at -25°C until analysis for glucose and insulin. As measures for the strength of the insulin response to a glucose load, the area under the curve (AUC; $\mu\text{U/mL}\cdot\text{min}$) of insulin concentration was computed as the total insulin increment above basal levels during the 60 min after infusion, as described by Holtenius et al. (2003). Similarly, AUC of glucose ($\text{mmol/L}\cdot\text{min}$) was calculated. Clearance rates of insulin ($\mu\text{U/mL}\cdot\text{min}^{-1}$) and glucose ($\text{mmol/L}\cdot\text{min}^{-1}$) were considered linear and were defined by calculating the steepness of the downward slope of concentration in time. Insulin and glucose peak concentrations were determined.

Blood Sampling for Glucose, Insulin, and NEFA

Blood samples were obtained on 14 d before the expected calving date (mean interval to true calving date: 18 ± 1.32 d) and on 4, 8, 12, 15, 18, 21, 25, 28, 32, 35, and 39 d pp. After milking, cows were separated, restrained, and sampled as described above. Agitation of the cows was avoided, because stress may interfere with endocrine and metabolic variables. Also, blood

Table 2. Contents of the different feed components in the ration¹

Nutrient	Contents			
	DM, %	Fiber/kg of DM, g	NE _I /kg of DM, Mcal	DIP ² /kg of DM, g
Concentrate	87	105	1.584	115
Soybean meal	87	100	1.535	200
Corn silage	30.1	190	1.520	48
Grass silage	38	250	1.485	68
Beet pulp	20.9	197	1.733	104
Corn cob mix	61	28	1.988	69
Hay	83	260	1.363	82
Wheat straw	84	420	0.713	3

¹Concentrate and soybean meal were purchased (AVEVE, Merksem, Belgium). All other nutrients had been produced on the farm.

²DIP = digestible intestinal protein.

samples were taken before any other manipulations took place (e.g., rectal examination) to avoid stress. The blood sampling was performed by puncture of the coccygeal vein or artery using evacuated gel-coated and fluorinized blood tubes. Blood samples were centrifuged and stored as described before and analyzed for glucose, NEFA, insulin, and progesterone.

Gynecological Examination

Every third day, cows were subjected to visual inspection, rectal palpation, and transrectal ultrasonographic examination of the genital tract to assess the time of ovulation and uterine involution and to diagnose disorders. Ultrasound examination was performed using a 7.5-MHz linear array probe (Pie Medical, Maastricht, the Netherlands). Ovulation was defined by the disappearance of the dominant follicle followed by the formation of a corpus luteum accompanied by an increase in blood progesterone above 2 ng/mL, as mentioned by Gutierrez et al. (2006). Endometritis was diagnosed by the presence of vaginal discharge with foul odor or color, or both, in combination with an enlarged uterus. Cystic ovarian disease was diagnosed by the presence of a follicular structure of ≥ 2.5 cm in the absence of a corpus luteum for at least 10 consecutive days, in agreement with the definition of Woolums and Peter (1994).

Analyses

All analyses were performed semiautomatically at the Clinical Chemistry Laboratory of the University Hospital, Ghent, Belgium. Interassay CV of the analyses were calculated on a large number of control samples integrated daily in the runs over a long period of time; the intraassay CV was computed on a regular basis on a smaller number of control samples within 1 run. Control samples within the physiological and the pathological range were used.

Glucose concentrations (mmol/L) were defined by the hexokinase method followed by colorimetric analysis using the Cobas GLUC2 kit (Roche Diagnostics, Indianapolis, IN). The detection limit of this kit was 0.11 mmol/L. The inter- and intraassay CV of the analysis were <1.15 and <0.8%, respectively. Concentrations of NEFA (mmol/L) were measured via an enzymatic method followed by colorimetric analysis using the NEFA C Kit (Wako Chemicals, Richmond, VA). The interassay CV of this kit was <1.75%. Insulin ($\mu\text{U/mL}$) and progesterone (ng/mL) analyses were performed by electrochemiluminescence immunoassay using the Cobas Insulin and Progesterone kits (Roche Diagnostics).

Detection limits of insulin and progesterone were 0.200 $\mu\text{U/mL}$ and 0.095 ng/mL, respectively. Inter- and intraassay CV were <3.8% and <2% for insulin and <5.2% and <3% for progesterone, respectively.

Statistics

All statistical analyses were performed using SPSS (version 14.0; SPSS Inc., Chicago, IL) or S-PLUS (Version 7.0, Insightful, Seattle, WA). Descriptive statistics are expressed as the mean $\pm$ standard error. Data were tested for normality using the Kolmogorov-Smirnov method and transformed whenever necessary.

IVGTT. The associations between lipolysis and 6 IVGTT outcome variables (AUC, peak concentrations and clearance rates for insulin and glucose, referred to as AUC_{ins} , AUC_{gluc} , Peak_{ins} , $\text{Peak}_{\text{gluc}}$, Cl_{ins} , and Cl_{gluc} , respectively) at different time points relative to calving were studied. However, some adjustments needed to be made first. To correctly evaluate the insulin response to a certain amount of glucose, we decided to calculate the $\text{AUC}_{\text{ins}}:\text{AUC}_{\text{gluc}}$, and the $\text{Peak}_{\text{ins}}:\text{AUC}_{\text{gluc}}$ ratios and to use these corrected terms, $\text{AUC}_{\text{ins}}^*$ and $\text{Peak}_{\text{ins}}^*$, as estimators of insulin response. Furthermore, averages (NEFA_{av}) were computed for the NEFA concentrations from the first analysis until the time of IVGTT to represent more closely the chronic exposure to elevated NEFA concentrations. In the final mixed effect model, cow was considered a random factor, whereas time (categorical: dry period, 14 d pp and 42 d pp; repeated measurements) and NEFA_{av} (continuous) were fixed factors. Pairwise comparisons between different time categories were made. Linear correlations between $\text{AUC}_{\text{ins}}^*$ on -14, 14, and 42 d pp were computed. Finally, linear correlations between milk yield and Cl_{gluc} were computed on 14 and 42 d pp.

Glucose, Insulin, and NEFA. Glucose, insulin, and NEFA concentrations during the dry period and on 4 d pp were compared using a dependent-samples *t*-test. Glucose, insulin, and NEFA concentrations during lactation were investigated over time (repeated measurements) using a mixed effect model. Cow was included as a random factor and time (continuous variable) as a fixed factor. Possible nonlinear associations were studied by including a quadratic term, time^2 , in the different models.

Gynecological Examination. The effects of prepartum and pp lipolysis and AUC_{ins} on the time of first ovulation were investigated using nonparametric survival analysis. Ovulation was defined as the event, and day pp was considered as the time factor. Cows that had not ovulated before 42 d pp were censored. Cows were categorized as in high or low prepartum

lipolysis (NEFA_{dry,high} and NEFA_{dry,low}), in high or low pp lipolysis (NEFA_{high} and NEFA_{low}), and with high or low AUC*_{ins} (AUC*_{high} and AUC*_{low}), relative to the median of the 23 cows studied. Kaplan-Meier graphs were generated.

RESULTS

IVGTT

Insulin Response. All IVGTT outcome variables were transformed logarithmically (Ln) to obtain better normality of the data. Results of the different IVGTT measurements in time are displayed in Table 3.

Both Ln (AUC*_{ins}) and Ln (Peak*_{ins}) were lower on 14 and 42 d pp compared with the dry period ($P < 0.005$), and Ln (AUC*_{ins}) was lower on 14 d compared with 42 d pp ($P < 0.05$). There was a positive correlation between Ln (AUC*_{ins}) during the dry period and on 14 d pp ($R = 0.461$; $P < 0.05$) and between 14 d pp and 42 d pp ($R = 0.478$; $P < 0.05$). The factor NEFA_{av} was negatively related to Ln (AUC*_{ins}) and Ln (Peak*_{ins}) ($P < 0.001$). It was found that Ln (Cl_{ins}) had a tendency to decrease on 14 and 42 d pp compared with the dry period ($P = 0.051$ and 0.054 , respectively) but was not related to NEFA_{av} ($P = 0.31$).

Glucose Response. It was found that Ln (AUC_{gluc}) was lower and Ln (Cl_{gluc}) was greater on 14 and 42 d pp compared with the dry period ($P < 0.01$). It was also found that Ln (Peak_{gluc}) had a tendency to decrease pp ($P = 0.054$). Glucose AUC, peak concentration, and clearance rate were not related to NEFA_{av} ($P = 0.48$, $P = 0.15$, and $P = 0.13$, respectively). Milk yield tended to be positively related to Ln (AUC_{gluc}) ($R = 0.252$; $P = 0.072$).

Milk Yield, Glucose, Insulin, and NEFA

For all of the pp glucose, insulin, and NEFA analyses, the quadratic term for time was not significant; hence, it was omitted from the different models. The evolution of the different concentrations over time was therefore considered to be linear.

Milk Yield Average daily milk yields (L) during the first to the seventh week of lactation were 29.17 ± 1.26 , 40.11 ± 1.16 , 43.06 ± 1.18 , 44.96 ± 1.44 , 46.11 ± 1.40 , 46.09 ± 1.30 , and 46.07 ± 1.48 , respectively.

Glucose. Glucose concentrations were normally distributed. Glucose concentrations were lower on 4 d pp compared with dry-off ($P < 0.001$). During lactation, glucose concentrations increased over time ($P < 0.001$, Figure 1).

Insulin. Insulin concentrations were not normally distributed and underwent square root transformation. Transformed insulin concentrations, $\sqrt{\text{Ins}}$, were lower on 4 d pp compared with the moment of drying-off ($P < 0.001$). Insulin concentrations increased over time during lactation ($P < 0.001$, Figure 2).

NEFA. Nonesterified fatty acid concentrations were not normally distributed and underwent logarithmic transformation. Transformed NEFA concentrations, Ln (NEFA), were greater ($P < 0.001$) at the onset of lactation compared with dry-off. During lactation, NEFA concentrations decreased over time ($P < 0.001$, Figure 3).

Gynecological Examination

Fourteen out of 23 cows (60.9%) ovulated within 42 d pp. Within the ovulating cows, the mean interval from parturition to first ovulation was 22.21 ± 1.80 d.

Table 3. Different measurements of the intravenous glucose tolerance tests on different time points relative to calving¹

Item ²	Days relative to calving			Overall <i>P</i> -value ³
	-14	+14	+42	
Insulin response				
Ln (AUC* _{ins}), μU/mL·min	2.09 ± 0.11 ^a	1.14 ± 0.12 ^b	1.37 ± 0.12 ^c	<i>P</i> < 0.0001
Ln (Peak* _{ins}), μU/mL	-1.20 ± 0.12 ^a	-1.71 ± 0.10 ^b	-1.71 ± 0.10 ^b	<i>P</i> < 0.0001
Ln (Cl _{ins}), μU/mL·min ⁻¹	-1.35 ± 0.13	-1.60 ± 0.10	-1.61 ± 0.12	<i>P</i> = 0.081
Glucose response				
Ln (AUC _{gluc}), mmol/L·min	3.93 ± 0.03 ^a	3.71 ± 0.03 ^b	3.79 ± 0.03 ^b	<i>P</i> < 0.0001
Ln (Peak _{gluc}), mmol/L	0.31 ± 0.02	0.23 ± 0.02	0.27 ± 0.03	<i>P</i> = 0.054
Ln (Cl _{gluc}), mmol/L·min ⁻¹	-4.16 ± 0.07 ^a	-3.91 ± 0.03 ^b	-3.90 ± 0.04 ^b	<i>P</i> < 0.0001

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹Data are presented as mean $\pm$ standard error.

²AUC*_{ins} = area under the curve of insulin concentration in time, corrected for glucose area under the curve; Peak*_{ins} = peak concentration of insulin, corrected for AUC_{gluc}; Cl_{ins} = clearance rate of insulin; AUC_{gluc} = area under the curve of glucose concentration in time; Peak_{gluc} = peak concentration of glucose; Cl_{gluc} = clearance rate of glucose.

³The P -value of the overall effect of time on all items is included.

Figure 1. Glucose concentrations at different time points relative to calving. Results are displayed as mean $\pm$ standard error.

Figure 2. Insulin concentrations at different time points relative to calving. Results are displayed as mean $\pm$ standard error.

The remaining 9 cows suffered from DFO, and COD was diagnosed in 3 of them. Three cows suffered from endometritis; however, 2 of these cows managed to ovulate before 42 d pp. According to the survival graph (Figure 4), cows in mild lipolysis during the dry period (NEFA_{dry,low}) had a tendency to ovulate earlier in lactation and reach a greater proportion of ovulation by 42 d compared with cows in greater prepartum lipolysis (NEFA_{dry,high}; $P = 0.080$). The NEFA concentrations during the lactation and AUC_{ins} were not related with the onset of ovulation in this study (data not shown).

DISCUSSION

The central aim of our study was to evaluate the insulin response to a glucose load at different time points relative to calving. The nadir concentrations of glucose and insulin occurred on 8 and 12 d pp, respectively, whereas NEFA peaked at 4 d pp. These data suggest that the nadir of NEB in these cows was situated on average between 4 and 12 d pp, which is in agreement with the 2.5 to 12 d range reported by Jorritsma et al. (2003). We estimate that the time point of 14 d pp was well chosen to perform the IVGTT.

Insulin is a peptide hormone that is synthesized and secreted by pancreatic β -cells in response to propionate, butyrate, and glucose (Mashek et al., 2001) and metabolized by the liver and the kidneys (Valera Mora et al., 2003). It needs to be stressed that the insulin AUC, peak concentrations, and clearance rates are the result of a dynamic relation between secretion and excretion and the simultaneous secretion of other gastrointestinal hormones, as stated by Golay and Ybarra (2005). Therefore, secretion and excretion cannot be regarded as 2 different and isolated processes. However,

by interpreting all IVGTT measurements collectively, some assumptions can be made about which process is stimulated or inhibited.

Insulin AUC and peak concentrations were corrected for the concurrent glucose AUC to avoid the possibility that fluctuations in the estimated body weight, and hence, the administered glucose dose, would interfere with the insulin response. Both corrected terms, AUC_{ins}^{*} and Peak_{ins}^{*}, were lower pp compared with the dry period. These findings correspond to the conclusions drawn by Holtenius et al. (2003). Several (steroid) hormones, such as progesterone, have greater clearance rates during lactation due to the increased metabolic activity of the liver (Sangsritavong et al., 2002). However, in our study, insulin clearance rates had a tendency to decrease during the pp period. Hence, the lower insulin AUC seems to be solely due to a lower insulin secretion and not to an increased insulin clearance rate. There was a large diversity among cows regarding the insulin outcome variables. A positive correlation was found between insulin AUC during the dry period and on 14 d pp and between 14 and 42 d pp. In other words, cows that responded poorly to a glucose load in the dry period seemed to do so during lactation as well. As stated by Jorritsma et al. (2003), persistent low insulin levels during NEB may be indicative for poor adaptation of the cow to increased energy needs, with an increased risk for reproductive and metabolic disturbances.

Low peripheral insulin concentrations induce a glucose-sparing status and enhance glucose availability for the mammary gland (van Knegsel et al., 2007). Insulin concentrations after parturition have been reported to have a moderate heritability (Swali and Wathes, 2006), and it has been suggested that

Figure 3. Nonesterified fatty acid concentrations at different time points relative to calving. Results are displayed as mean $\pm$ standard error.

high-yielding dairy cows may have a genetic tendency to lower insulin levels (Veerkamp et al., 2003; Gutierrez et al., 2006). Indeed, very recently, Hammon et al. (2007) observed a lower glucose-dependent insulin response in heifers selected for milk production compared with beef heifers. Interestingly, this difference was only apparent during lactation and not during the dry period. Taken together, there are suggestions in the literature that the insulin response to glucose may be defined by both genetic and metabolic factors. Our current findings support this hypothesis: NEFA concentrations were negatively correlated to insulin AUC and peak concentrations, suggesting that elevated NEFA concentrations during lactation may interfere with glucose-induced insulin secretion. Other studies have pointed out that NEFA have a detrimental effect on several bovine cell types, including granulosa cells (Vanholder et al., 2004), oocytes (Leroy et al., 2005), and liver cells (Roche, 2006), but also on human and rat pancreatic β -cells. In man, NEFA are suggested to play a crucial role in the development of pancreatic insufficiency in type II diabetes (Mason et al., 1999). Additionally, Bobe et al. (2004) mentioned pancreatic involution as a complication of fatty liver syndrome in dairy cows. In our study, however, only a correlation but no causal relationship between NEFA and insulin response has been demonstrated, and an equally plausible explanation would be that low-insulin concentrations stimulate lipolysis and elevate NEFA levels. To elucidate the exact mechanism of the lower insulin response in cows, further *in vitro* studies are required.

Glucose AUC was highest in the prepartum period and decreased on 14 and 42 d pp. Glucose clearance rates were greater on 14 and 42 d pp compared with

Figure 4. Cumulative survival chart. Proportion of cows that have ovulated over time in the groups of cows with a low (NEFA_{dry,low}) and a high rate of lipolysis (NEFA_{dry,high}) during the dry period, expressed by their respective NEFA levels prepartum. The NEFA_{dry,low} cows tended to reach a greater proportion of ovulation after 42 d compared with NEFA_{dry,high} cows ($P = 0.080$).

the dry period. Several authors consider peripheral insulin resistance, caused by excessive NEFA and growth hormone concentrations, as a crucial risk factor for metabolic and reproductive diseases during the transition period (Herzog, 2001; Oikawa and Oetzel, 2006; Pires et al., 2007). Intracellular glucose uptake occurs by facilitated transport via glucose transporter molecules (GLUT) incorporated into the cellular membrane. There are 7 different types of GLUT, which are all expressed under different biological stimuli. Skeletal muscles and adipose tissue contain only GLUT 4, the expression of which is strictly insulin-dependent, whereas udder and liver tissue contain predominantly GLUT 1 and 3, expressed independently of insulin (Zhao et al., 1996; Nishimoto et al., 2006). The glucose clearance rate reflects both the insulin-dependent and the insulin-independent glucose consumption. Because insulin concentrations and insulin sensitivity of the peripheral tissues are reported to decrease during NEB (Bell and Bauman, 1997; Holtenius et al., 2003), the greater rate of glucose clearance found in our study is likely to be explained by greater insulin-independent glucose consumption, mainly representing glucose uptake by the mammary gland during lactation. The fact that glucose clearance rates tended to be positively re-

lated to milk yield supports this statement. Therefore, based on the IVGTT results only, no conclusions can be drawn concerning peripheral insulin sensitivity.

In the present study, 39.1% of the cows did not ovulate before 42 d pp. This is markedly greater than the 21.5% DFO reported by Opsomer et al. (2000) and the 13.2% observed by Shrestha et al. (2004a). Resumption of cyclicity tended to occur earlier pp in cows with lower NEFA levels during the dry period (NEFA_{dry,low} group). This can be seen in the context of the findings by Kokkonen et al. (2005), who have demonstrated that cows mobilizing more adipose tissue before calving continued to suffer from a more severe NEB during the first weeks of lactation. As stated before, length and severity of the NEB affect the time of first ovulation. The relevance of a single NEFA measurement during the dry period has been demonstrated before by LeBlanc et al. (2005), who stated that the risk for abomasal dislocation is significantly greater in cows mobilizing more fatty acids prepartum. In our study, statistical significance was not reached, and the number of cows was small; a study on a larger number of cows might shed more light on the use of prepartum NEFA measurements as a tool for prediction of fertility pp. In contrast, NEFA concentrations during lactation did not affect the time of ovulation. This may have been due to the small number of cows and to considerable fluctuation of NEFA both between and within cows. It was found that AUC_{ins}^{*} did not seem to have any effect on the time of first ovulation. In vitro cultured granulosa cells respond to insulin by estrogen synthesis and proliferation in a dose-dependent manner (Gutierrez et al., 1997). However, besides absolute insulin concentrations, ovarian sensitivity to insulin also needs to be taken into account, because insulin and IGF-1 receptors on granulosa cells undergo considerable changes as the follicle develops (Spicer and Echternkamp, 1995). Further research on granulosa cell insulin sensitivity under different metabolic circumstances is required to fully understand the interactions between insulin and the ovarian function.

CONCLUSIONS

Our study demonstrates a decrease of the glucose-induced insulin AUC and peak concentrations during lactation compared with the dry period. These findings, taken together with the fact that the insulin clearance rate did not increase during lactation, suggest that the low AUC is likely to be caused by low insulin secretion. This may present an additional metabolic challenge that the cow has to face during the delicate period of adaptation to lactation. Insulin AUC and peak concentrations were negatively related to concurrent NEFA

concentrations. These findings should support further research about the potential negative effects of elevated plasma NEFA concentrations on bovine pancreas cells, as reported in other species.

We have found a high incidence of ovarian dysfunctions in the early pp period. Survival analysis suggested that cows mobilizing less NEFA during the dry period had a tendency to have earlier resumption of ovarian activity. This tendency deserves further investigation in a larger number of cows.

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