Relationship between the degree of insulin resistance during late gestation and postpartum performance in dairy cows and factors that affect growth and metabolic status of their calves

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ABSTRACT. This study aimed to investigate the effects of insulin resistance (IR) during the close-up dry period on the metabolic status and performance of dairy cows as well as to determine the effects on body weight (BW) and metabolic status of their calves. An insulin tolerance test (ITT) was conducted by administering 0.05 IU/kg BW of insulin to 34 multiparous Holstein cows at 3 weeks prepartum. Blood samples were collected at 0, 30, 45 and 60 min after insulin injection, and cows were divided into two groups based on the time required for glucose to reach the minimum levels [non-IR (NIR), 45 min (n=28); and IR, 60 min (n=6)]. Blood or milk sampling and body condition score (BCS) estimation were performed twice weekly during the experimental period. Blood samples from calves were collected immediately after birth. Cows with IR showed lower BCS (*P*<0.05) and serum urea nitrogen (*P*<0.05) and glucose concentration (*P*=0.05) before calving, and lower serum non-esterified fatty acid concentration (*P*<0.05) and milk yield (*P*<0.05) and earlier resumption of luteal activity (*P*<0.05) after calving; their calves showed lower BW (*P*<0.05) and plasma insulin-like growth factor-I concentration (*P*<0.001) and higher plasma insulin concentration (*P*<0.05). In conclusion, IR at 3 weeks prepartum in dairy cows is related to postpartum metabolic status and performance along with growth and metabolic status of their calves.
KEY WORDS: dairy cow, dry period, insulin tolerance test, metabolic status, postpartum performance

Insulin resistance (IR) is a physiological condition in which body tissues have a lower response to insulin [18]. It is evaluated by insulin responsiveness, which is the response of insulin to glucose, and insulin sensitivity, which is the responsiveness of tissues to insulin [16]. In general, IR is associated with obesity, fat feeding, hyperinsulinemia, hyperlipidemia, malnutrition and other hormones, such as growth hormone (GH) and thyroxine [16]. Female mammals usually have IR, because of the increase of energy requirements during the peripartum period for fetal growth and lactation [3, 16]. However, severe IR is also correlated with some metabolic disorders, such as glucose intolerance, ketonemia and ketonuria in humans and other animals [18]. Such metabolic disorders might induce fatty liver and ketosis; cows with ketosis show low responsiveness to insulin and thus fall into a vicious circle [26, 36]. The abundance of milk secretion might aggravate the degree of IR and prolong severe negative energy balance (NEB) during the postpartum (pp) period, especially in the modern high-producing dairy cows [26, 36], because the energy required for milk production and maintenance of tissue functions exceeds the energy uptake during this period [2, 23]. Butler and Smith [5] found that the levels of NEB were directly related to the pp interval until first ovulation. In addition, the resumption of ovarian activity plays a crucial role in subsequent fertility, in which earlier resumption of ovarian function is related to higher fertility [10, 20, 34, 35]. Further, in humans, IR of the mother is associated with the thinness of child at birth [1]; in cattle, maternal nutrient intake during gestation has been shown to affect the development of both the placenta and the fetus [24, 30]. Therefore, IR and energy status during gestation are associated with pp energy status, fertility and fetal growth; however, there is little information about these processes in dairy cows.

The insulin tolerance test (ITT) is a tool for evaluating insulin sensitivity in the peripheral tissues of animals. In this method, insulin sensitivity is calculated by injecting insulin into the examinee and measuring the blood glucose level at several time points before and after the insulin injection [6]. However, conducting ITT in cows is difficult, especially in pregnant dairy cows, because of the difficulty in holding cows and the stress caused from prolonged restraint during the ITT. Therefore, the detailed changes in blood glucose in dairy cows during the ITT have not yet been elucidated. Our previous study indicated a relationship between a smaller

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dose of insulin injection at ITT for pregnant dairy cows and their pp performance [22]. As the calving day approached, our data showed that increased decline of blood glucose 120 min after the administration of insulin was associated with liver dysfunction and lower fertility during the pp period owing to the slow glucose recovery [22]. Hence, glucose metabolism was found to be directly affected by IR in pregnant dairy cows rather than insulin sensitivity during the prepartum period; therefore, the evaluation of the recovery of glucose after insulin injection at ITT might be an index of the pp performance in high-producing dairy cows. This study aimed to investigate the effects of IR, which was evaluated as the recovery of glucose after a small dose of insulin, during the dry period on the metabolic status, milk yield and ovarian functions of dairy cows as well as the body weight (BW) and metabolic status of their calves.

MATERIALS AND METHODS

The experimental animals, feeding and management: The experimental procedures complied with the Guide for the Care and Use of Agricultural Animals of Obihiro University.

The experiment was carried out at the Field Center of Animal Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine. Fifty multiparous Holstein cows, in which parity was from 1st to 5th at dry period, were used in this study and had calved between September 2011 and August 2012. Parity and body condition score (BCS) of the experimental cows at initiation of the study were 2.4 ± 0.2 and 3.41 ± 0.04, respectively. The study was performed from 3 weeks before the expected parturition to 100 days pp. Cows close to the dry period, about 1 month before the expected calving date, were moved to a paddock and fed a limited total mixed ration [dry matter (DM) basis: 127 g of crude protein (CP)/kg and 6.6 MJ of net energy for lactation (NEL)/kg] consisting of grass silage (3.5 kg, DM basis: 165 g of CP/kg and 5.5 MJ of NEL/kg), maize silage (5.1 kg, DM basis: 86 g of CP/kg and 6.0 MJ of NEL/kg), concentrate for dry cows (2.0 kg, DM basis: 170 g of CP/kg and 6.8 MJ of NEL/kg) and grass hay (DM basis: 125 g of CP/kg and 5.7 MJ of NEL/kg) *ad libitum* until parturition. After parturition, cows were housed in a free-stall barn and fed a lactation diet, which was a mixed ration (DM basis: 155 g of CP/kg and 6.2 MJ of NEL/kg) consisting of grass (6.5 kg, DM basis: 165 g of CP/kg and 5.5 MJ of NEL/kg), maize silage (12.5 kg, DM basis: 84 g of CP/kg and 6.4 MJ of NEL/kg) and concentrate for dairy cows (8.0 kg, DM basis: 180 g of CP/kg and 7.1 MJ of NEL/kg) *ad libitum*. In addition, the diets were supplemented with minerals, and the dairy cow concentrate was prepared according to each cow's specific requirements for milk production. Grass hay (DM basis: 104 g of CP/kg and 5.2 MJ of NEL/kg) and water were available *ad libitum*. Cows were milked twice daily between 05:00 and 06:30 hr and between 17:00 and 18:30 hr.

The experimental ITT and sampling: The ITT was performed 3 weeks before the expected calving date. The cows were weighed the day before the initiation of the ITT, and BW was used to determine the doses of insulin for the ITT.

The ITT was performed 2 hr before feeding. Immediately before the ITT, an extension catheter was inserted into the right or left jugular vein. The ITT was performed by intravenously administering 0.05 IU/kg BW of insulin (Novolin R 100 IU/m*l*; Novo Nordisk Pharma, Tokyo, Japan), followed by administration of 5 m*l* heparinized saline (100 IU/m*l*) [22]. Blood samples were collected via the jugular vein at 0 (before insulin injection), 30, 45 and 60 min relative to the administration of insulin via caudal venipuncture to measure glucose and insulin.

BCS was assessed twice a week from 3 weeks before the expected parturition to 3 weeks after calving by the same operator by using a 1 to 5 scale with 0.25 intervals, where 1=thin and 5=very fat [13]. Blood samples were obtained by caudal venipuncture twice a week from 3 weeks before the expected parturition to 3 weeks after calving. Blood samples were collected via the jugular vein from the calves immediately after birth. Nonheparinized and silicone-coated 9-m*l* tubes (Venoject, Autosep, Gel + Clot. Act. VP-AS109K; Terumo Corporation, Tokyo, Japan) were used for biochemical analysis, and sterile 10-m*l* tubes containing 200 μl of stabilizer solution (0.3 M EDTA and 1% acetyl salicylic acid, pH 7.4) were used for hormonal analysis. Serum was obtained by centrifuging the blood samples for 15 min at 38°C in an incubator. All the tubes were centrifuged at 2,000 × g for 20 min at 4°C, and plasma samples were maintained at −30°C until analysis. In addition, milk samples were collected twice a week after milking until the onset of luteal activity. The milk samples were centrifuged at 1,500 × g for 15 min at 4°C, and the skim milk samples were stored at −30°C until analysis for progesterone concentration. Daily milk yield was recorded until 100 days pp. Peripartum diseases, such as milk fever, hypocalcemia, ketosis, ruminal acidosis, displaced abomasum, lameness, retained placenta, endometritis and mastitis, were recorded when that has been diagnosed from 3 weeks prepartum to 3 weeks postpartum by veterinarian in the experimental farm.

The experimental measurement of hormones and metabolites: Plasma and skim milk progesterone concentrations were determined using enzyme immunoassay (EIA) after extraction with diethyl ether, as described previously [25]; the extraction efficiency was 90%. The standard curve ranged from 0.05 to 50 *n*g/m*l*, and the 50% effective dose (ED$_{50}$) of the assay was 0.66 *n*g/m*l*. The mean intra-assay and inter-assay coefficients of variation (CVs) were 6.0% and 9.2%, respectively. The total plasma insulin-like growth factor 1 (IGF-1) concentration was determined using EIA by using the biotin–streptavidin amplification technique [19] after protein extraction by using acid ethanol (87.5% ethanol and 12.5% 2 N hydrochloric acid) to obtain IGF-1 free from binding proteins [11]. The IGF-1 standard curve ranged from 0.39 to 50 *n*g/m*l*. Intra- and inter-assay CVs were 5.9% and 6.1%, respectively, and the ED$_{50}$ of this assay system was 7.2 *n*g/m*l*. The plasma GH concentrations were determined using EIA as described previously [19]; the standard curve ranged from 0.78 to 100 *n*g/m*l*, and the ED$_{50}$ was 21 *n*g/m*l*. Intra- and inter-assay CVs were 3.1% and 8.2%, respectively. The plasma insulin concentrations were determined using an

enzyme-linked immunosorbent assay (ELISA) kit (Bovine Insulin ELISA 10-1201-01; Mercodia, Uppsala, Sweden).

The serum concentrations of glucose, non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), total protein (TP), albumin (ALB), blood urea nitrogen (BUN) and total cholesterol (T-CHO) and the activities of aspartate aminotransferase (AST) were measured using a clinical chemistry automated analyzer (TBA120FR; Toshiba Medical Systems Co., Ltd., Tochigi, Japan).

The experimental identification of the onset of luteal activity: When the progesterone concentration in the plasma or skim milk had increased to more than 1 ng/ml, the cows were considered to show luteal activity [37].

The experimental statistical analysis: Sixteen cows were excluded from data analysis, because of the following reasons: a pregnancy period of more than 287 or less than 273 days (n=6), severe mastitis (n=3), twin calving (n=1), blood collection loss at ITT (n=3) and mistakes in insulin injection at ITT (n=3). Cows were divided into two groups based on the time required for glucose to reach the minimum levels after insulin injection. Cows with a minimum glucose at 60 min after insulin injection were considered to have lower insulin sensitivity and/or lower glucose metabolism compared to cows with a minimum glucose level by 45 min after insulin injection. Therefore, cows with a minimum glucose level at 60 min after insulin injection were defined as the insulin resistant group (IR group), whereas those with a minimum glucose level by 45 min after insulin injection were defined as the non-insulin resistant (NIR group) in this study. Before data analysis, BCS, plasma IGF-1, GH and insulin concentrations, and serum metabolite concentrations were averaged weekly. The period of 0–6 days after calving was considered as the parturient week (0 week pp), and the Kolmogorov–Smirnov test (SAS Enterprise Guide version 4.3; SAS Institute Inc., Cary, NC, U.S.A.) was used for statistical testing of normality. In addition, the data were analyzed separately for the prepartum and pp periods. Stat View (Stat View 5.0 software; Abacus Concepts Inc., Berkeley, CA, U.S.A.) was used for data analysis by using the repeated measures of the ANOVA procedure, including time (week), group (NIR or IR) and their interaction in the model as fixed effects. Cows were repeatedly used as the subjects. Diagnosis of peripartum diseases and sex of calves in the NIR and IR groups were analyzed using the chi-square test, and other data, including results for calves between NIR and IR, were analyzed using the Student's t-test or Wilcoxon's signed rank test (SAS Enterprise Guide version 4.3; SAS Institute Inc.). Results are presented as mean ± standard error of the mean (SEM); differences with P<0.05 were considered significant.

RESULTS

In 28 of the 34 experimental cows, the time required for glucose to reach the minimum level was 45 min after insulin injection with one exception (30 min; n=1, 45 min; n=27, NIR group). The remaining experimental cows (n=6) required 60 min after insulin injection to attain the minimum glucose levels (IR group). Serum glucose concentrations at
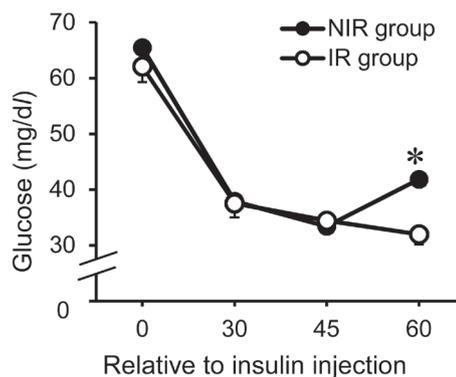

Fig. 1. The change in serum glucose concentration after insulin injection at insulin tolerance test (ITT) in the NIR (n=28) and IR (n=6) groups. Values are mean ± SEM: solid, NIR; open, IR. *indicates differences of P<0.05 between the NIR and IR groups.

60 min after insulin injection were higher in the NIR group than in the IR group, although glucose levels at the other time points did not differ between the NIR and IR groups (Fig. 1; P<0.05).

Table 1 shows the parity, calving difficulty, sex of calves, peripartum disease diagnosis, luteal activity onset and milk yield until 100 days pp in the NIR and IR groups. Days until the onset of luteal activity in the IR group were fewer than those in the NIR group (P<0.05). In addition, the average (P<0.05) and total (P<0.05) milk yields until 100 days pp were lower in the IR group than in the NIR group. Peripartum diseases were diagnosed as mastitis (n=6), hypocalcemia (n=1) and milk fever (n=2) in NIR group, and as mastitis (n=1) in IR group, and there was no significant difference in the number of cows with the peripartum diseases between NIR and IR groups. No significant difference was noted in other factors between the NIR and IR groups.

Figure 2 shows the circulating serum metabolite concentrations, enzyme levels, plasma metabolic concentrations and BCS during the experimental period. During the prepartum period, BCS (P<0.05), and serum BUN concentrations (P<0.05) were lower, whereas serum glucose (P=0.05) and ALB concentrations (P=0.10) tended to be lower in the IR group than in the NIR group. During the pp period, cows of the NIR group had higher serum NEFA (P<0.05) and BHBA (P=0.09) concentrations than those in the IR group. In addition, treatment and time effects were observed (P<0.05) for BCS during the pp period: BCS at 0 (P=0.08) and 1 (P<0.05) week pp were lower in the IR group than in the NIR group. No significant differences were noted in the other factors between the NIR and IR groups in each period.

BW, plasma metabolic hormone levels and serum glucose concentrations at birth in the calves of cows of the NIR and IR groups are shown in Table 2. BW at birth in the calves of the IR group was lower than that in the calves of the NIR group (P<0.05). Furthermore, the calves of the IR group showed lower plasma IGF-1 concentration (P<0.001) and higher plasma insulin concentration (P=0.06). No significant differences were noted in the plasma GH and serum glucose

Table 1. Parity, calving difficulty, sex of calves, peripartum disease, luteal activity onset and milk yield in the NIR and IR groups[a]

	NIR group (n=28)	IR group (n=6)	*P*-value
Parity at the onset of experiment	2.4 ± 0.3	2.2 ± 0.7	0.460
Calving difficulty[b]	1.1 ± 0.1	1.0 ± 0.0	0.700
Sex of calves (male/female)	14/14	3/3	1.000
Diagnosis of peripartum disease[c]	9/28 (32%)	1/6 (17%)	0.645
Days to the onset of luteal activity (days)	38.3 ± 3.8	20.3 ± 3.6	0.039
Average of daily milk yield between days 7 and 100 pp (kg)	41.4 ± 0.9	35.9 ± 2.0	0.013
Total milk yield from days 7 to 100 pp (kg)	3,888.1 ± 81.2	3,375.5 ± 185.9	0.013

Values are the mean ± SEM. a) NIR group; cows with a minimum glucose level by 45 min after insulin injection. IR group; cows with a minimum glucose level at 60 min after insulin injection. b) 1, unassisted birth (natural, without human assistance); 2, easy calving with human assistance; 3, difficult calving with a few humans; 4, dystocia (requiring considerably more force than normal); and 5, surgical treatment or death of cow. c) Milk fever, hypocalcemia, ketosis, ruminal acidosis, displaced abomasum, lameness, retained placenta, endometritis and mastitis from 3 weeks prepartum to 3 weeks postpartum.

levels at birth between the calves of the NIR and IR groups.

DISCUSSION

In this study, the six cows that reached the minimum glucose levels at 60 min after insulin injection were considered to be IR; the reason for IR was thought to be the slow recovery of glucose after insulin injection, which is consistent with the findings of a previous study by Lee *et al.* [22]. In general, BCS and blood glucose and BUN concentrations are known to be associated with energy status and feed intake [7, 8, 38]. During the prepartum period, IR cows showed lower energy status and feed intake owing to the lower BCS and glucose and BUN concentrations. Although IR by ITT was confirmed at 3 weeks before calving, a difference in energy status between IR and NIR cows was noted. In particular, BCS cannot be evaluated on the basis of the change in energy status of the real-time feed intake [28]; therefore, in this study, IR cows might have become insulin resistant during an earlier time. Malnutrition causes imbalance in glucose homeostasis, and the decrease of insulin in circulation induces the reduction of feed intake in dairy cows [16]. Therefore, the feed intake reduction in IR cows might have inhibited the volatile fatty acid production in the rumen and thus suppressed gluconeogenesis in the liver [9]. Lower energy status, such as lower BCS, before calving of IR group in this study might be caused by long-term malnutrition from previous lactation. However, in this study, the reasons for the lower energy status in the IR cows were not clear; thus, further studies are warranted to confirm the onset of insulin resistance in pregnant dairy cows.

BCS at 0 and 1 week pp were lower in the IR group than in the NIR group. NIR cows showed higher serum NEFA and BHBA concentrations than those in IR cows, although the levels of metabolic hormones did not differ between the IR and NIR cows. Furthermore, the average and total milk yield until 100 days pp were lower in the IR group than in the NIR group. Higher NEFA and BHBA indicate greater mobilization of adipose tissue and failure of lipid metabolism in the

liver [14, 15]. However, cows with lower BCS had sustained reduced plasma NEFA and BHBA concentrations after calving compared to cows with higher BCS [28, 33]. Cows with lower BCS produce milk by protein mobilization, because of the limited body fat; thus, fat-corrected milk yield in those cows was lower than moderate and fat cows [28]. Conversely, it was indicated that higher BCS cows have ability to mobilize fat to maintain energetic homeostasis after feed restriction [33]. Additionally, Roche *et al.* [32] have concluded that BCS at calving had positive effect on milk yield, and optimal BCS at calving was 3.5 in the 5-point scale. In the present study, greater BCS and better gluconeogenesis in NIR group might produce greater milk yield compared with IR group, although the differences of them between NIR and IR groups were not so greater. Days to the onset of luteal activity in the IR group were fewer than in the NIR group. In dairy cows, lowered energy status during the peripartum period is known to delay the first ovulation after parturition [2, 19]. Butler and Smith [5] showed that a negative energy balance was directly related to the pp interval to the first ovulation and that the differences in the energy balance were reflected in the milk yield. In addition, cows with a delayed first ovulation showed higher NEFA and BHBA concentrations after parturition [21, 31, 39]. Therefore, in this study, higher NEFA and BHBA concentrations of NIR cows during the pp period might have delayed the onset of luteal activity, and the lowered milk yield of IR cows might induce earlier resumption of ovarian activity.

The maternal endocrine and metabolic milieu transferred through the placenta during late pregnancy affects the environment of the fetus [17, 24, 30]. In humans, IR of the mother is associated with low birth weight of the infant [1]; in cattle, maternal malnutrition during gestation is related to the lowered development of both the placenta and the fetus [24, 30]. Further, in ewes, restricted maternal feeding during gestation was related to lower BW and plasma IGF-1, insulin and glucose concentrations in the fetus, although maternal IGF-1 concentrations were not affected [27]. In the present study, calves of the IR cows showed lowered BW at birth
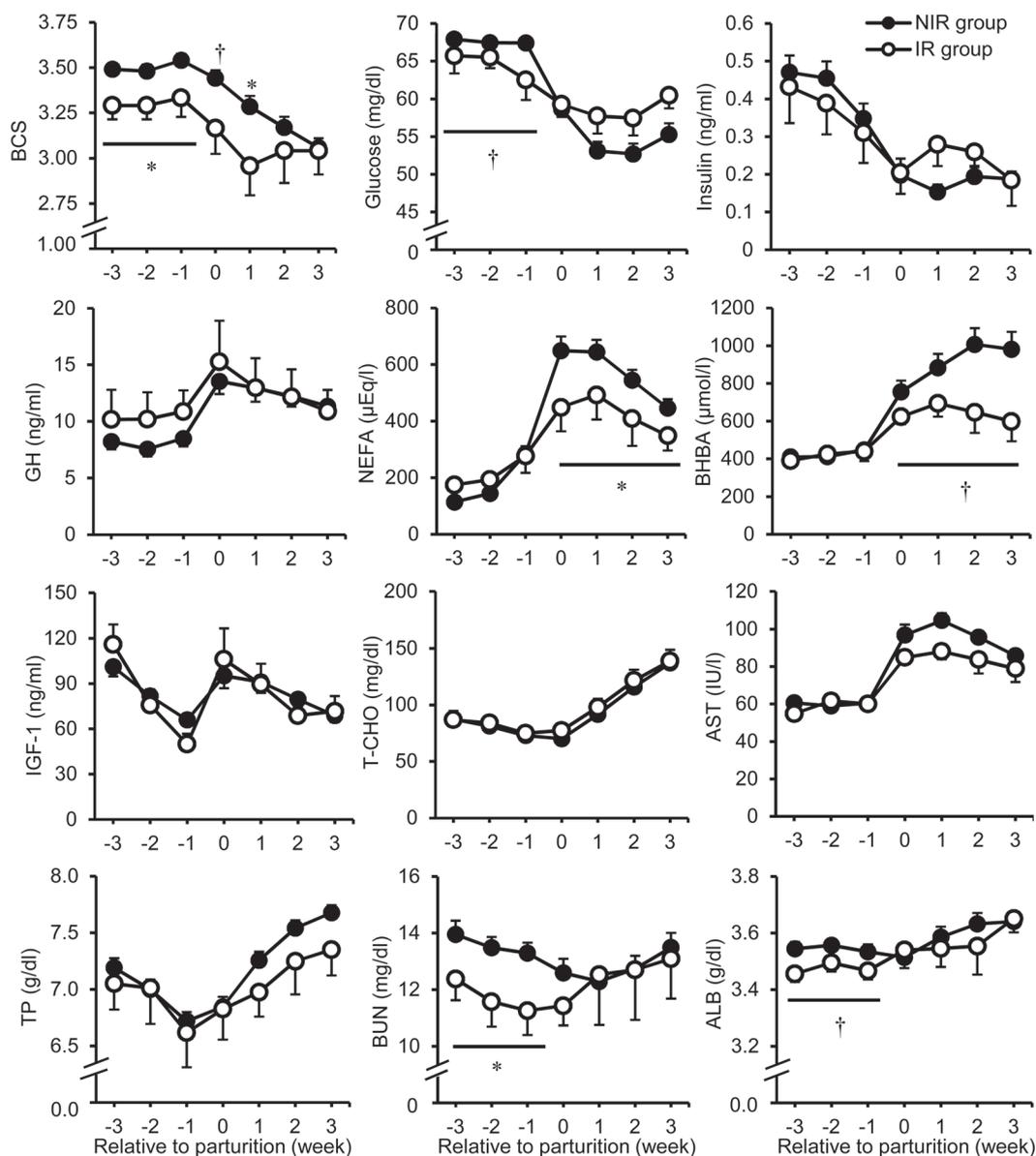
Fig. 2. Serum metabolite concentrations, activities of enzymes and plasma metabolic hormones, and BCS during the experimental period [mean ± SEM: solid, NIR (n=28); open, IR (n=6) groups]. *Indicates differences of $P<0.05$, and † indicates differences of $P<0.1$ between the NIR and IR groups.

Table 2. BW and plasma metabolic hormones and serum glucose concentrations at birth in the calves of the NIR and IR groups[a]

	Calves of NIR	Calves of IR	P-value
	(n=28)	(n=6)	
BW at the birth (kg)	47.2 ± 0.9	42.1 ± 1.7	0.020
Plasma GH concentration (ng/ml)	13.6 ± 1.3	15.2 ± 4.8	0.653
Plasma IGF-1 concentration (ng/ml)	121.5 ± 6.3	69.8 ± 5.6	0.001
Plasma insulin concentration (ng/ml)	0.3 ± 0.0	0.7 ± 0.2	0.061
Serum glucose concentration (mg/dl)	77.4 ± 5.2	72.1 ± 14.8	0.684

Values are the mean ± SEM. a) NIR group; cows with a minimum glucose level by 45 min after insulin injection. IR group; cows with a minimum glucose level at 60 min after insulin injection.

and a lower plasma IGF-1 concentration, supporting the findings of previous studies. In addition, they showed higher insulin levels than those of NIR cows, despite the similar glucose levels. In the late gestation, fetal growth is mainly regulated by IGF-1, and the dominant regulator of IGF-1 production in the fetus is fetal glucose and insulin [4]. Thus, the differences in blood metabolic hormones and glucose concentrations between the calves of the NIR and IR groups might be attributed to the fetal nutritional condition that was affected by maternal endocrine and metabolic milieu. In humans, lower BW at birth is known to be associated with a wide range of adverse outcomes later in life, including diabetes [12]; further, obese children with low birth weight have higher blood insulin to glucose concentration and show higher insulin resistance as revealed by the homeostasis model assessment compared with obese children with normal birth weight [29]. Therefore, calves of IR cows might develop insulin resistance in the future.

In conclusion, the findings of the present study suggest that IR at 3 weeks before parturition in dairy cows is related to the pp metabolic status, milk production and resumption of ovarian activity along with growth, as well as the metabolic status of their calves. Therefore, IR evaluated on the basis of the recovery of glucose after an injection of a small dose of insulin during the dry period might be an indication of the pp performance of pregnant dairy cows, as well as the growth, fertility and milk production of their calves. In addition, the reason for IR in the present study was thought to be the slow recovery of glucose after insulin injection as well as the previous study [22]. Therefore, the enhancement of the gluconeogenesis in the liver by energy supplementation, such as glycerol, or hepatic stimulant, such as amino acids, should be confirmed in order to improve the IR.

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