Effects of Slow-release Insulin on Production, Liver Triglyceride, and Metabolic Profiles of Holsteins in Early Lactation

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ABSTRACT

The objective of this experiment was to determine whether there is a dose of slow-release insulin (SRI) that decreases concentrations of plasma nonesterified fatty acids (NEFA) and liver triglyceride (TG) without decreasing plasma glucose concentration, dry matter intake (DMI), and milk yield. Forty-three Holsteins weighing 765 ± 70 kg with body condition score of 3.29 ± 0.25 (mean $\pm$ SD) were fed for ad libitum consumption of the same diet from 2 wk before parturition through 6 d postpartum. Cows were blocked according to actual calving date and parity and then assigned randomly to intramuscular injection of a single dose of 0, 0.14, 0.29, or 0.43 IU of SRI per kilogram of body weight (BW) on d 3 postpartum. On the day of injection, cows were fed hourly to minimize fluctuations in blood hormones and metabolites due to feed intake pattern. Blood samples were collected via jugular catheter every hour from 0 to 24 h and every 6 h from 24 to 48 h postinjection. Pre- and postinjection period liver samples were taken on d 2 and 5 postpartum. One cow injected with 0.29 and two cows injected with 0.43 IU of SRI per kilogram of BW could not complete the trial due to severe hypoglycemia (<20 mg/dl). Both DMI and milk yield during d 3 to 5 postpartum tended to increase quadratically by increasing dose of SRI. Concentrations of serum insulin and glucagon increased linearly, concentration of plasma glucose decreased linearly, and concentrations of plasma NEFA and β -hydroxybutyrate decreased quadratically from 0 to 24 h postinjection by increasing dose of SRI. Serum insulin concentrations remained higher in cows injected with SRI (CISRI) than in cows injected with sterile water (CISW; 0 IU of SRI/kg of BW), the quadratic effect of SRI on plasma NEFA concentration continued, and the linear effect of SRI on plasma glucose concentration diminished from 24 to 48 h postinjection. Concentration of hepatic TG for CISRI tended to be lower than for CISW, and increasing dose

of SRI quadratically decreased hepatic accumulation of TG. Increasing dose of SRI tended to increase concentration of hepatic glycogen (GLY) quadratically and decreased the ratio of TG to GLY quadratically. In conclusion, a low dose of SRI (0.14 IU/kg of BW) could be considered for prophylactic use against hepatic lipidosis and ketosis.

(Key words: periparturient cow, insulin, nonesterified fatty acids, liver triglyceride)

Abbreviation key: apoB = apolipoprotein B, CISRI = cows injected with slow-release insulin, CISW = cows injected with sterile water, CPT I = carnitine palmitoyl-transferase I, GLUT = glucose transporter, GLY = glycogen, SRI = slow-release insulin, TG = triglyceride, VLDL = very low-density lipoprotein.

INTRODUCTION

Insulin, a peptide hormone consisting of acidic and basic chains connected by disulphide bridges, is secreted from β -cells of the islets of Langerhans in the pancreas. Despite distinct differences in metabolism of nutrients and in magnitude of responses to exogenous insulin, basic structures (Hsu and Crump, 1989) and major roles (Brockman and Laarveld, 1986) of insulin in nonruminants and ruminants are similar. Insulin is an anabolic hormone and acts to preserve nutrients in their storage forms by stimulating glycogenesis, lipogenesis, and glycerol synthesis and by inhibiting gluconeogenesis, glycogenolysis, and lipolysis (Brockman and Laarveld, 1986). Insulin is also a potent regulator of feed intake and nutrient partitioning in ruminants (Laarveld et al., 1981).

Parturition and transition from gestation to lactation are under homeorhetic control. During late gestation and early lactation, lowered responsiveness and sensitivity of extra hepatic tissues to insulin (Sano et al., 1991) facilitate partitioning of nutrients for the rapidly growing fetus and mammary tissue. Due to an inability to consume adequate amounts of feed, cows mobilize body reserves to meet nutrient requirements for milk synthesis during early lactation. Increased concentrations of serum glucagon and growth hormone, plasma

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NEFA and BHBA, and liver triglyceride (TG) and decreased concentrations of serum insulin, plasma glucose, and liver glycogen (GLY) occur in periparturient cows (Herbein et al., 1985; Vazquez-Anon et al., 1994) and in cases of induced or spontaneous hepatic lipidosis and ketosis (Ballard et al., 1968; DeBoer et al., 1985).

Hepatic lipidosis and ketosis are common metabolic disorders in peripartum cows and increase predisposition to other postpartum health problems. Evidence suggests that factors involved in the etiology of hepatic lipidosis and ketosis are similar, and in both cases liver function is impaired (DeBoer et al., 1985; Drackley et al., 1992). In addition to elimination of negative energy balance, subcutaneous injection of 100 to 200 IU of protamine zinc insulin, a type of slow-release insulin (SRI), with concomitant infusion of dextrose is suggested for treatments of hepatic lipidosis (Pearson and Maas, 1990) and ketosis (Fleming, 1990). Sakai et al. (1993) showed that subcutaneous injection of 200 IU of SRI with concomitant infusion of dextrose compared with infusion of dextrose alone for 4 d enhanced the effectiveness of treatment for ketosis and shortened the recovery period. As reported by Sakai et al. (1993), intravenous injection of 40 to 50 IU of insulin alone (Sato et al., 1986) or intramuscular injection of 120 to 160 IU of insulin as adjunct to infusion of dextrose (Toyoda et al., 1987) for 3 d were effective treatments for hyperlipoproteinemia in dairy cows.

Deetz et al. (1980) showed that intravenous injection of 4 IU of rapid-release insulin per kilogram of BW increased DMI and did not decrease plasma glucose concentration in sheep. Low serum insulin concentration may promote maximal rates of gluconeogenesis and ketogenesis in the liver and lipolysis in adipose tissue during early lactation (Brockman, 1979; Holtenius, 1993). We hypothesized that there may be a dose of SRI that would decrease plasma NEFA and liver TG, without decreasing blood glucose and DMI. Therefore, our objectives were to investigate the effects of a single intramuscular injection of several doses of SRI on production and metabolic parameters of dairy cows during the first week of lactation.

MATERIALS AND METHODS

Animals, Treatments, Management, and Diet

The Research Animal and Resource Committee of the University of Wisconsin-Madison approved this experimental protocol. Forty-three Holsteins housed at the University of Wisconsin-Madison Dairy Cattle Research Center were blocked according to actual calving date and lactation number (12 entering the second lactation and 31 entering the third or greater lactation) and then assigned randomly to intramuscular injection

of a single dose of 0, 0.14, 0.29, or 0.43 IU of SRI per kg BW. Slow-release insulin (100 IU/ml; Humilin, Ultralente human insulin rDNA origin in extended zinc suspension; Eli Lilly Co., Indianapolis, IN) was injected into *Musculus semitendinosus* at 0800 h on d 3 postpartum. Cows in control group were injected with 2 ml of sterile water.

Cows were housed in individual box stalls from 2 wk before projected calving date through the first day of lactation and then transferred to a tie-stall facility until the end of experiment on d 6 postpartum. Body weights were measured at 1400 h on d 15 before projected calving date and on d 2 postpartum. Two individuals evaluated BCS on the days BW were measured using a numerical range of 1 to 5 with 0.25 unit intervals. Body weights (mean $\pm$ SD) were 765 ± 70 and 700 ± 63 kg, and BCS were 3.29 ± 0.25 and 3.12 ± 0.27 on d 12 prepartum ($n = 43$; SD = 3.7 d for difference between actual and projected calving dates) and d 2 postpartum ($n = 37$), respectively.

Cows were fed the same diet for ad libitum consumption once daily at 0800 h from 2 wk before projected calving date through 6 d postpartum. The same diet was fed for the entire period to eliminate metabolic changes associated with diet changes. Dry matter intake was recorded daily during the experiment. To increase the likelihood that the experiment was conducted on cows that were healthy and not likely to succumb to metabolic disorders, cows were required to consume at least 6.7 kg of DM on d 2 postpartum. This criterion was based on a previous experiment (Hayirli et al., 2001), in which average DMI was 6.7 kg on d 2 postpartum and DMI increased by 11% on d 3 postpartum. Three cows did not meet this criterion and were removed from the experiment. On d 2 postpartum, DMI expressed as percentage of BW and kilogram per day were 1.82 ± 0.6 and 12.7 ± 4.1 ($n = 37$, mean $\pm$ SD), respectively. On d 3 postpartum, each cow was offered 130% of TMR consumed on d 2 postpartum to accommodate rapidly increasing feed intake and avoid feed restriction. Feed was provided in 24 equal portions to minimize fluctuations in blood hormones and metabolites due to feed intake pattern. We did not record feed intake every hour, but none of the cows emptied the feed bunk by the end of d 3 postpartum.

Cows had free access to water at all times during the experiment and were released outside for approximately 3 h after milking at 0300 and 1500 h, except on d 3 postpartum. Milk yields were recorded daily during the experiment. On d 2 postpartum, milk yield ($n = 37$, mean $\pm$ SD) was 25.1 ± 10.7 kg. Measurements of heart rate and rectal temperature were obtained every 6 h on d 3 and 4 postpartum for monitoring health status of cows.

Dry matter contents of roughages and concentrate were determined weekly to adjust their proportions in the TMR. Ingredient composition of the experimental diet on a DM basis was 51.3% forage (17.6% alfalfa silage and 82.4% corn silage) and 48.7% concentrate (54.6% ground corn, 36.8% soybean meal (44% CP), 3.8% meat and bone meal, 1.2% limestone, 0.6% magnesium oxide, 2.0% trace-mineralized salt, and 0.8% vitamin premix of vitamins A, D, and K). The estimated energy value of the diet was 1.71 Mcal NE_L/kg of DM (NRC, 2001). Forages and concentrates were collected biweekly, dried for 48 h in a 60°C forced-air oven, and then ground to pass through a 1-mm Wiley mill screen (Arthur H. Thomas, Philadelphia, PA). Monthly composites of forages and concentrate were analyzed for concentrations of CP using the micro-Kjeldahl method (AOAC, 1990) and NDF using the filter bag technique (Mertens, 1999). Concentrations of CP and NDF in the experimental diet ($n = 6$ mo, mean $\pm$ SD) were 15.9 ± 1.1 and 22.9 ± 2.8 , respectively.

Tissue Sample Collection and Analytical Procedure

At 1300 h on d 2 postpartum, the neck area was aseptically prepared with 7.5% betadine (providine-iodine, wt/vol), 30% ethyl alcohol (vol/vol), and 7% iodine tincture (wt/vol) and then anesthetized locally with 5 ml of 2% lidocaine hydrochloride (wt/vol). About 10 cm of a previously autoclaved flexible tube (Tygon Microbore Tubing; Norton Performance Plastics, Akron, OH) was inserted into the jugular vein and sutured to skin for sample collection. The catheter was flushed with 10 ml of saline containing 10 IU of heparin every 6 h.

After the treatments were administered at 0800 h on d 3 postpartum, approximately 25 ml of blood was collected every hour before provision of each hourly meal from 0 to 24 h, and blood sampling was extended to every 6 h from 24 to 48 h. Ten milliliters of blood was drawn into Vacutainers containing 100 mg of potassium oxalate and 20 mg of sodium fluoride, and samples were kept on ice. Plasma was obtained after centrifugation at $950 \times g$ for 15 min at 4°C, and aliquots were kept at -20°C until laboratory analyses. Enzymatic assays were conducted in a micro plate reader (EL_x 808 Ultra Plate Reader; Bio-Tek Instruments Inc., Los Angeles, CA) for concentrations of glucose (Raabo and Terkildsen, 1960), BHBA (Williamson et al., 1962), and NEFA (Johnson and Peters, 1993) using commercial kits (Glucose # 510 and B-HBA kits; Sigma Chemical Co., St. Louis, MO; NEFA-C kit; Wako Fine Chemicals Industries USA, Inc., Dallas, TX).

Ten milliliters of blood was drawn into additive-free vacutainers and samples kept at room temperature. Serum was obtained after centrifugation at $2000 \times g$

for 15 min at 20°C and analyzed for concentration of insulin by radioimmunoassay (Coat-a-Count; Diagnostic Products Corp., Los Angeles, CA). An additional 3 ml of blood was drawn into Vacutainers containing 0.068 ml of K₃-EDTA (7.5% wt/vol) and 1500 kallikrein inactivating units of aprotinin (ICN Biomedicals Inc., Aurora, OH). Samples were protected from light and kept on ice. Serum was obtained after centrifugation at $2000 \times g$ for 15 min at 4°C and analyzed for concentration of glucagon by double-radioimmunoassay (Double Antibody Glucagon kit; Diagnostic Products Corp., Los Angeles, CA). Intra- and interassay coefficients of variations were 5.4 and 7.5% for insulin and 4.5 and 6.9% for glucagon assays, respectively.

At 1200 h on d 2 and 5 postpartum, samples of liver were taken pre- and postinsulin injection by puncture biopsy between the 11th and 12th rib after aseptic preparation and administration of 10 ml of 2.0% lidocaine. Liver samples were rinsed in saline, frozen in liquid nitrogen, and then stored at -20°C until laboratory analyses. After homogenizing approximately 0.1 g of liver sample and isolating the total lipid content (Folch et al., 1957), the amount of TG was determined by colorimetric assay using glycerol as a standard (# 339-11; Sigma Chemical Co., St. Louis, MO) as described by Foster and Dunn (1973). After digestion of approximately 1 g of liver tissue homogenate with 2 ml of 30% KOH (wt/vol) and precipitation with 2.4 ml of 95% ethyl alcohol (vol/vol), the amount of GLY was determined as described by Lo et al. (1970). A fluorometric assay (Labarca and Paigen, 1980) was used to determine hepatic DNA.

Statistics

Correlations among response variables were determined using the Corr procedure (SAS, 1998). Dry matter intake, milk yield, and liver TG and GLY data obtained on d 2 postpartum, along with blood hormone and metabolite data obtained prior to SRI injection at 0800 h on d 3 postpartum, was used as covariates for statistical analyses of corresponding response variables. Covariates were excluded from models when their probability of significance was greater than 0.25. This only occurred for parameters that were expressed as a ratio (e.g., serum insulin:glucagon). Analysis of variance was initially conducted using the Mixed procedure (SAS, 1998) as repeated measures with first-order autoregressive covariance structure in time being subplot (Littell et al., 1996). However, the large number of repeated measures resulted in a dataset that exceeded the capacity of the computer to conduct the analysis using first-order autoregressive covariance structure. Therefore, the repeated statement with first-order aut-

regressive covariance structure was dropped, and the conservative degree of freedom method was used (Greenhouse and Geisser, 1959). The linear model to test the effects of SRI on DMI, milk yield, and blood hormones and metabolites was as follows:

$$Y_{aijk} = \mu + b_0 + b_1(\text{Cov}_a) + B_i + \text{TRT}_j + \text{Error A} \\ + t_k + (\text{TRT} \times t)_{jk} + \text{Error B}$$

where Y_{aijk} = response variable, μ = population mean, b_0 = intercept, b_1 = slope, Cov_a = covariate (a = corresponding response variable), B_i = block ($i = 1$ to 10), TRT_j = treatment ($j = 0, 0.14, 0.29$, and 0.43 IU of SRI/kg BW), Error A = whole plot error, t_k = time ($k = d$ relative to parturition or h relative to administering treatment), $(\text{TRT} \times t)_{jk}$ = treatment j and time k interaction, and Error B = subplot error. The linear model to test the effect of SRI on liver metabolites was as follows:

$$Y_{aij} = \mu + b_0 + b_1(\text{Cov}_a) + B_i + \text{TRT}_j + e_{aij},$$

where Y_{aij} = response variable, μ = population mean, b_0 = intercept, b_1 = slope, Cov_a = covariate (a = corresponding response variable), B_i = block ($i = 1$ to 10), TRT_j = treatment ($j = 0, 0.14, 0.29$, and 0.43 IU of SRI/kg BW), and e_{aij} = residual error.

The number of cows completing the experiment was 10, 10, 9, and 8 for treatments 0, 0.14, 0.29, and 0.43 IU of SRI per kg BW, respectively. A contrast to compare the mean response variables for cows injected with SRI (**CISRI**) versus cows injected with sterile water (**CISW**) and polynomial contrasts to test linear and quadratic effects of increasing dose of SRI were computed. Coefficients for polynomial contrasts were calculated as demonstrated by Carmer and Seif (1963), and probabilities of significance were generated using Satterthwaite approximation (Littell et al., 1996) because of unequal replication of treatments. Effects of SRI on response variables were considered to be significant or to have a tendency towards significance at $P \leq 0.10$ and $0.10 < P \leq 0.15$, respectively.

RESULTS AND DISCUSSION

One cow injected with 0.29 and two cows injected with 0.43 IU of SRI per kilogram of BW suffered from hypoglycemic shock within 12 to 16 h following injection. Hypoglycemic shock was characterized by low plasma glucose (<20 mg/dl), anorexia, unconsciousness, tonic extensor spasms, coldness in the ears, excessive foamy salivation, and disturbed behavior such as pica and seizure. Within 1 to 3 h following intravenous infusion of 1 L of 50% dextrose, they recovered and started

eating and ruminating, but they did not remain on experiment.

Bovine insulin was not commercially available for use in this experiment. Only enzymatically modified porcine insulin and recombinant human insulin were available. However, insulin produced by different species has similar structure. Threonine located in position 30 of the B chain in human insulin ($\text{C}_{257}\text{H}_{383}\text{N}_{65}\text{O}_{77}\text{S}_6$, MW of 5807.7) is replaced with Ala in porcine ($\text{C}_{256}\text{H}_{381}\text{N}_{65}\text{O}_{76}\text{S}_6$, MW of 5777.6) and bovine insulin ($\text{C}_{254}\text{H}_{377}\text{N}_{65}\text{O}_{75}\text{S}_6$, MW of 5733.6). Also, The and Ile in positions 8 and 10 of the A chain in human insulin are replaced with Ala and Val in bovine insulin (Hsu and Crump, 1989).

Effect of Slow-release Insulin on Production and Health Parameters

Feed intake. Average DMI (kg/d) for CISW and CISRI during d 3 to 5 postpartum was not different ($P < 0.74$ [Table 1]). However, increasing dose of SRI tended to increase DMI (kg/d) quadratically ($P < 0.13$). We cannot explain the quadratic response; however, cows did not increase feed intake to overcome hypoglycemia. There were no effects of treatment on DMI expressed as a percentage of BW (Table 1). Our data and those of others (Baile and Mayer, 1968; Deetz and Wangsness, 1980; Deetz and Wangsness, 1981) indicate that the effects of insulin on feed intake of ruminants has been variable. Subcutaneous injections of goats with 100 and 160 IU of SRI for a day (short-term) and 80 IU for 14 d (long-term) caused hyperinsulinemia and hypoglycemia without altering DMI (Baile and Mayer, 1968). Deetz and Wangsness (1981) reported that five intravenous injections of 6 IU of rapid-release insulin per kilogram of BW during 24 h decreased DMI by 8.5% compared with intravenous injections of saline in sheep. In another study using sheep, Deetz and Wangsness (1980) reported that intravenous injection of a very high dose of rapid-release insulin (2000 IU/kg of BW) caused severe hypoglycemia without affecting DMI, whereas intravenous injection of a moderate dose of rapid-release insulin (6 IU/kg of BW) decreased DMI without causing hypoglycemia. Increasing doses of rapid-release insulin (0, 2, 4, and 6 IU/kg of BW) via intraportal delivery linearly decreased DMI in sheep (Deetz et al., 1980). In contrast, increasing doses of rapid-release insulin via intrajugular delivery quadratically increased DMI; DMI was the maximum in sheep injected with 4 IU per kg BW (Deetz et al., 1980). These studies suggest that the effect of insulin on feed intake of ruminant depends on type or dose of insulin or means of delivery or a combination of both. Moreover, it appears that

Table 1. Effects of a single dose of slow-release insulin on production and health parameters of Holsteins during the first week of lactation.

Response variable ³	Insulin dose, IU/kg BW ¹					Significance of insulin effect ($P > F$) ²		
	0	0.14	0.29	0.43	SEM	Insulin	Linear	Quadratic
DMI, kg/d ⁴	15.1	16.6	15.6	14.3	0.9	0.74	0.49	0.13
DMI, % of BW ⁵	2.15	2.39	2.20	2.11	0.12	0.59	0.65	0.18
Milk, kg/d	30.1	34.7	31.9	29.3	2.6	0.59	0.72	0.15
Heart rate, beats/min ⁵	76.5	75.0	78.8	74.6	1.6	0.84	0.87	0.48
Rectal temperature, °C ^{6,7}	38.9	38.9	38.8	38.8	<0.1	0.29	0.12	0.85

¹Data were covariately adjusted with the least squares means. Treatments were applied at 0800 h on d 3 postpartum.

²Probability values were Satterthwaite approximates. Contrasts: Insulin = cows intramuscularly injected with sterile water versus cows intramuscularly injected with slow-release insulin; Linear = linear effect of increasing doses of slow-release insulin; Quadratic = quadratic effect of increasing doses of slow-release insulin.

³Dry matter intake and milk yield were recorded on d 3, 4, and 5 postpartum, and heart rate and rectal temperature were measured every 6 h on d 3 and 4 postpartum.

⁴Time effect ($P < 0.02$).

⁵Time effect ($P < 0.01$).

⁶Time effect ($P < 0.001$).

⁷Treatment $\times$ time effect ($P < 0.11$).

ruminants do not overcome hypoglycemia by increasing feed intake.

Milk yield. Although average milk yield during d 3 to 5 postpartum did not differ between CISW and CISRI ($P < 0.59$), increasing dose of SRI tended to increase milk yield quadratically ($P < 0.15$ [Table 1]). Unlike DMI, milk yield responses to exogenous insulin are consistent in the literature. Intramuscular injections of cows with 0.2 to 0.6 IU (Kronfeld et al., 1963) and 0.35 IU (Schmidt, 1966) of rapid-release insulin per kilogram of BW depressed both milk and lactose yields. Milk yield was restored following infusion of dextrose, suggesting that depression of milk yield is due to hypoglycemia, not a direct effect of insulin per se (Kronfeld et al., 1963; Schmidt, 1966). Insulin partitions nutrients toward more insulin responsive tissues (i.e., adipose and muscle tissues) for storage. As a result, exogenous insulin may lead to decreased availability of glucose for mammary tissue because insulin does not regulate uptake of glucose through facilitative glucose transporter (GLUT) 1 that predominates in the mammary gland (Laarveld et al., 1981; Zhao et al., 1993). In this study, the quadratic effect of increasing dose of SRI on milk yield (Table 1) could be related to the quadratic increase in DMI ($r = 0.40$, $P < 0.02$), suggesting that the effect of insulin on milk synthesis was indirect.

Heart rate and rectal temperature. Heart rates tended to change during d 3 to 4 postpartum (time effect, $P < 0.11$ [Table 1]). There were no effects of insulin on heart rates. Potassium is required for transport of glucose and insulin and plays a role in regulation of heart rhythm (Genuth, 1993). Hypokalemia is an adverse effect of administering excessive insulin during

diabetic ketoacidosis and can cause an inversion of the T wave and a prolongation of the Q to T interval during an electrocardiogram (Goth, 1968). In this study, mean heart rate (76 beats/min) was close to the upper level of the normal range (80 beats/min).

There was a diurnal variation in rectal temperature (time effect, $P < 0.001$ [Table 1]). Although there was no difference in rectal temperatures between CISW and CISRI ($P < 0.29$), increasing dose of SRI tended to decrease rectal temperature linearly ($P < 0.12$). However, mean differences were extremely small and probably not of biological significance.

Effect of Slow-release Insulin on Blood Parameters

Serum insulin concentration. Serum insulin concentrations for CISW remained relatively constant during 0 to 24 h following injection (Figure 1). Serum insulin concentrations for cows injected with a single dose of 0.14, 0.29, and 0.43 IU of SRI per kilogram of BW increased by 3.3, 7.0, and 10.2 times during 0 to 12 h postinjection, respectively, and then gradually decreased. Concentrations were still 1.4, 1.6, and 2.3 times greater than preinjection concentrations at 24 h following injection, respectively (time effect, $P < 0.003$ and treatment $\times$ time interaction, $P < 0.07$ [Figure 1]). Average serum insulin concentration for CISRI was greater than for CISW ($P < 0.0001$), and increasing dose of SRI linearly increased serum insulin concentration ($P < 0.0001$) during 0 to 24 h following injection (Table 2). Moreover, serum insulin concentrations for CISRI remained higher than for CISW ($P < 0.08$), and increasing dose of SRI quadratically increased serum insulin

Figure 1. Serum insulin concentrations for cows intramuscularly injected with sterile water (—■—) or 0.14 (—▲—), 0.29 (—●—), or 0.43 (—◆—) IU of slow-release insulin per kilogram of BW at 0800 h on d 3 postpartum. Pooled standard errors were 1.36 and 0.46 for data obtained during 0 to 24 h and 24 to 48 h following injection, respectively. Probabilities of significance for effects of time and treatment $\times$ time interaction during 0 to 24 h were $P < 0.003$ and $P < 0.07$ and during 24 to 48 h postinjection were $P < 0.24$ and $P < 0.86$, respectively.

concentration ($P < 0.04$) during 24 to 48 h following injection (Table 2).

Duration and promptness of insulin actions vary depending on the method of delivery and type of insulin (Reynolds, 1989). According to pharmacokinetics, insulin is categorized as rapid-release (crystalline or regular), intermediate-release (Isophane and Lente), and slow-release (protamine zinc insulin or UltraLente) insulin (Drug Facts and Comparisons, 2000). In humans, serum concentrations of insulin from subcutaneous delivery of short-, intermediate-, and slow-release insulin peak from 0.5 to 1.5, 4 to 12, and 10 to 24 h following injection, respectively, and the effects of insulin last for 7, 24, and 36 h, respectively (Reynolds, 1989). Time to achieve peak serum insulin concentration and to reach basal serum insulin concentration (the duration of action) were shorter for CISRI compared with those measured in humans administered SRI subcutaneously (Reynolds, 1989). Due to the enriched vascular network around muscle tissue compared to adipose tissue, intramuscular injection decreases time between injection and effectiveness of insulin (Reynolds, 1989).

Plasma glucose concentration. Similar to serum insulin, plasma glucose concentrations for CISW remained relatively constant during 0 to 24 h following injection (Figure 2). However, plasma glucose concen-

Table 2. Effects of a single dose of slow-release insulin on blood metabolites and hormones of Holsteins during the first week of lactation.

Response variable ³	Insulin dose, IU/kg BW ¹				SEM	Significance of insulin effect (<i>P</i> > <i>F</i>) ²		
	0	0.14	0.29	0.43		Insulin	Linear	Quadratic
0–24 h								
Insulin, μU/ml	2.0	3.5	5.7	7.6	0.7	0.0001	0.0001	0.81
Glucose, mg/dl	47.5	42.3	35.8	36.7	1.7	0.0001	0.0001	0.12
Glucose:insulin	0.27	0.13	0.08	0.07	0.02	0.0001	0.0001	0.002
Glucagon, pg/ml	93.2	108.4	103.1	111.2	7.1	0.08	0.12	0.55
Insulin:glucagon	0.13	0.26	0.54	0.55	0.08	0.0006	0.0001	0.50
NEFA, μEq/L	576	512	539	643	41	0.89	0.28	0.05
BHBA, mg/dl	11.0	7.9	8.7	8.6	0.7	0.003	0.04	0.03
24–48 h								
Insulin, μU/ml	2.0	2.8	3.1	2.4	0.3	0.08	0.33	0.04
Glucose, mg/dl	48.4	49.9	48.1	44.7	2.0	0.68	0.18	0.24
NEFA, μEq/L	576	545	565	808	56	0.26	0.01	0.02
Glucose:insulin	0.23	0.18	0.15	0.17	0.02	0.02	0.05	0.13

¹Data were covariately adjusted with the least squares means. Treatments were applied at 0800 on d 3 postpartum.

²Probability values were Satterthwaite approximates. Contrasts: Insulin = cows intramuscularly injected with sterile water versus cows intramuscularly injected with slow-release insulin; Linear = linear effect of increasing doses of slow-release insulin; Quadratic = quadratic effect of increasing doses of slow-release insulin.

³Statistical analyses for measurements obtained during 0 to 24 and 24 to 48 h were computed separately. Serum insulin, plasma glucose, and plasma NEFA concentrations were measured every hour during 0 to 24 h and every 6 h during 24 to 48 h following injection. Plasma BHBA and serum glucagon concentrations were measured at 2, 4, 6, 8, 10, 12, 18, and 24 h following injection. Glucose:insulin = mmol:pmol. Insulin:glucagon = pmol:pmol.

Figure 2. Plasma glucose concentrations for cows intramuscularly injected with sterile water (—■—) or 0.14 (—▲—), 0.29 (—●—), or 0.43 (—◆—) IU of slow-release insulin per kilogram of BW at 0800 h on d 3 postpartum. Pooled standard errors were 2.16 and 2.17 for data obtained during 0 to 24 h and 24 to 48 h following injection, respectively. Probabilities of significance for effects of time and treatment $\times$ time interaction during 0 to 24 h were $P < 0.0001$ and $P < 0.07$ and during 24 to 48 h postinjection were $P < 0.02$ and $P < 0.08$, respectively.

trations for cows injected with a single dose of 0.14, 0.29, and 0.43 IU of SRI per kilogram of BW decreased by 21, 38, and 35%, respectively, during 0 to 12 h postinjection and then gradually increased and reached 103, 97, and 96% of preinjection concentration at 24 h following injection, respectively ($P < 0.0001$ for time effect, $P < 0.07$ for treatment $\times$ time interaction [Figure 2]). Average plasma glucose concentration for CISW was higher than for CISRI ($P < 0.0001$); plasma glucose concentration decreased linearly ($P < 0.0001$) and tended to decrease quadratically ($P < 0.12$) by increasing dose of SRI during 0 to 24 h following injection (Table 2). There were effects of time ($P < 0.02$) and treatment $\times$ time interaction ($P < 0.08$) on plasma glucose concentration during 24 to 48 h following injection (Figure 2). However, mean plasma glucose concentration did not differ between CISRI and CISW, and both the linear and quadratic effects of increasing SRI dose during 0 to 24 h were diminished during 24 to 48 h following injection (Table 2). Average molar ratio of plasma-glucose-to-serum-insulin for CISW was greater than for CISRI during both 0 to 24 h ($P < 0.0001$) and 24 to 48 h ($P < 0.02$) following injection. Increasing dose of SRI also decreased molar ratio of plasma glucose to serum insulin during both 0 to 24 h (linear effect, $P < 0.0001$ and quadratic effect, $P < 0.002$) and 24 to 48 h (linear effect, $P < 0.05$ and quadratic effect, $P < 0.13$) postinjection (Table 2).

Similar to serum insulin, feeding every hour maintained plasma glucose concentrations for CISW (Figure 2, Table 2). Although serum insulin concentrations reached basal level by 24 h postinjection and there were no differences in plasma glucose concentrations between CISRI and CISW during 24 to 48 h postinjection, the lower ratio of plasma glucose to serum insulin concentrations for CISRI than for CISW during 24 to 48 h following injection (Table 2) suggests that there was a carryover effect of SRI. Baile and Mayer (1968) showed that subcutaneous injection of SRI similar to the low and medium doses tested in the present study caused hypoglycemia (<15 mg/dl in serum) in goats. Intravenous injection of rapid-release insulin (6 IU/kg of BW) decreased plasma glucose concentration in sheep (Deetz and Wangsness, 1980). Insulin facilitates entry of glucose into cells by speeding up the basal rate of exocytosis and slowing down the basal rate of endocytosis of GLUT 2 in the liver and pancreas and GLUT 4 in adipose and muscle tissues (Katzung, 1995). The hypoglycemic effect of insulin may be the consequence of increased oxidation of glucose by extrahepatic tissues, decreased production of glucose by hepatic tissue via inhibition of gluconeogenesis and glycogenolysis, and increased storage of glucose by hepatic and extrahepatic tissues via stimulation of glycogenesis, or a combination of any of the above (Brockman and Laarveld, 1986).

Serum glucagon concentration. Serum glucagon concentrations for CISW decreased by 15% during 0 to 24 h postinjection and then increased to 95% of preinjection concentrations at 24 h following injection (Figure 3). When cows were injected with a single dose of 0.14, 0.29, and 0.43 IU of SRI per kilogram of BW, serum glucagon concentrations increased by 11, 12, and 19%, respectively, during 0 to 12 h postinjection and then gradually decreased to 98, 95, and 88% of preinjection concentrations by 24 h following injection, respectively (time effect, $P < 0.08$ and treatment $\times$ time interaction, $P < 0.15$ [Figure 3]). Mean serum glucagon concentration for CISW was lower than for CISRI ($P < 0.08$), and increasing dose of SRI tended to increase serum glucagon concentrations linearly ($P < 0.12$) during 0 to 24 h following injection (Table 2). Moreover, average molar ratio of insulin-to-glucagon for CISRI was greater than for CISW ($P < 0.0006$), and increasing dose of SRI linearly increased this ratio ($P < 0.0001$ [Table 2]) during 0 to 24 h following injection.

Insulin and glucagon usually act reciprocally to regulate metabolism, and their concentrations in blood change reciprocally in nonruminants (Genuth, 1993). In contrast, because of reliance on gluconeogenesis and glycogenolysis for glucose production, glucagon concentration does not change dramatically under normal

Figure 3. Serum glucagon concentrations for cows intramuscularly injected with sterile water (—◆—) or 0.14 (—■—), 0.29 (—▲—), or 0.43 (—●—) IU of slow-release insulin per kilogram of BW at 0800 h on d 3 postpartum. Pooled standard error was 8.49 and probabilities of significance for effects of time and treatment $\times$ time interaction during 0 to 24 h postinjection were $P < 0.08$ and $P < 0.15$, respectively.

physiological conditions in ruminants (Herbein et al., 1985). However, increased serum glucagon concentrations were shown during spontaneous ketosis (Brockman, 1979), experimentally induced ketosis by feed deprivation (Drackley, 1992), and early lactation (Hayirli, 2001), when serum insulin concentrations were low, suggesting that cows may meet the energy demand for milk synthesis in part by pancreatic secretion of glucagon. In the present study, increased serum glucagon concentrations by SRI (Figure 3; Table 2) could be related to stimulation of glucagon secretion, probably through insulin's hypoglycemic effect (Genuth, 1993).

Plasma nonesterified fatty acid concentration. Sampling time affected plasma NEFA concentrations in a sine-wave manner during both 0 to 24 h and 24 to 48 h following injection (time effect for both phases, $P < 0.007$ and $P < .001$ [Figure 4]). Increasing dose of SRI did not alter the magnitude of diurnal effects on plasma NEFA concentrations during both 0 to 24 h (treatment $\times$ time interaction, $P < 0.26$) and 24 to 48 h (treatment $\times$ time interaction, $P < 0.28$) postinjection (Figure 4). There were no differences in mean plasma NEFA concentrations between CISRI and CISW during 0 to 24 h ($P < 0.89$) and 24 to 48 h ($P < 0.26$) following injection (Table 2). Increasing dose of SRI quadratically decreased mean plasma NEFA concentrations during 0 to 24 h ($P < 0.05$) and 24 to 48 h ($P < 0.02$) following injection (Table 2).

Although animals were fed every hour to minimize fluctuation in blood hormones and metabolites, diurnal

Figure 4. Plasma NEFA concentrations for cows intramuscularly injected with sterile water (—◆—) or 0.14 (—■—), 0.29 (—▲—), or 0.43 (—●—) IU of slow-release insulin per kilogram of BW at 0800 h on d 3 postpartum. Pooled standard errors were 61.03 and 73.69 for data obtained during 0 to 24 h and 24 to 48 h following injection, respectively. Probabilities of significance for effects of time and treatment $\times$ time interaction during 0 to 24 h were $P < 0.007$ and $P < 0.26$ and during 24 to 48 h postinjection were $P < 0.001$ and $P < 0.24$, respectively.

effects on plasma NEFA were as profound as or more profound than the antilipolytic effects of insulin. Increases in plasma NEFA concentrations coincided with times of milking, which may reflect adrenalin secretion during cow movement. Diurnal effects on plasma NEFA concentrations were observed during late gestation, early lactation, induced ketonemia, and following recovery from induced ketonemia (DeBoer et al., 1985). Insulin enhances fatty-acid synthesis and storage of fatty acids in the form of TG in adipose tissue through stimulation of lipogenic enzymes (lipoprotein lipase, acetyl-CoA carboxylase, and fatty acid synthase). Additionally, insulin suppresses lipolysis by lowering the level of cAMP and inhibiting activities of protein kinase A and hormone sensitive-lipase (Brockman, 1979; Genuth, 1993). Failure to obtain a linear decrease of plasma NEFA could have been related to the quadratic increase in DMI as dose of SRI was increased ($r = -0.67$ between NEFA and DMI, $P < 0.0001$). Increasing blood insulin may cause down regulation of insulin receptors (Garvey et al., 1986). Additionally, increased plasma NEFA concentration may adversely affect physiological action of insulin by disabling insulin receptors and compromising translocation of GLUT 4 (Van Putten et al., 1985; Van Epps-Fung et al., 1997). During early lactation, nutritional and metabolic conditions to support lactation favor increased plasma NEFA and ketones as a result of lipolysis and ketogenesis, which may also lead

Figure 5. Plasma β -hydroxybutyrate concentrations for cows intramuscularly injected with sterile water (—◆—) or 0.14 (—■—), 0.29 (—▲—), or 0.43 (—●—) IU of slow-release insulin per kilogram of BW at 0800 h on d 3 postpartum. Pooled standard error was 0.96 and probabilities of significance for effects of time during 0 to 24 h postinjection was $P < 0.07$.

to lowered adipose tissue responsiveness to insulin (Holtenius, 1993).

Plasma β -hydroxybutyrate concentration. Plasma BHBA concentrations for CISW were relatively constant during 0 to 24 h following injection (Figure 5). When cows were injected with 0.14, 0.29, and 0.43 IU of SRI per kilogram of BW, plasma BHBA concentrations decreased continuously to 30, 20, and 35% of preinjection concentrations by 24 h postinjection, respectively (time effect, $P < 0.07$ [Figure 5]). During 0 to 24 h following injection, average plasma BHBA concentration for CISRI was lower than for CISW ($P < 0.003$), and increasing dose of SRI linearly ($P < 0.04$) and quadratically ($P < 0.03$) decreased plasma BHBA concentrations (Table 2).

Excessive uptake of NEFA by the liver results partially in fatty-acid oxidation to ketones (Cadorniga-Valino et al., 1997). Plasma NEFA concentrations decreased quadratically, whereas plasma BHBA decreased both linearly and quadratically with increasing SRI dose (Table 2). Moreover, there was a positive correlation between concentrations of plasma NEFA and BHBA ($r = 0.52$, $P < 0.002$). Brockman (1979) summarized direct and indirect antiketogenic effects of insulin, which include decreasing liver NEFA uptake through stimulating lipogenesis and inhibiting lipolysis in adipose tissue, enhancing peripheral tissue ketone utilization, and altering enzyme activities and availability of substrates that are involved in ketogenesis in the liver. Insulin decreases activity of carnitine palmitoyltrans-

ferase I (CPT I) and increases affinity of CPT I for malonyl-CoA (Grantham and Zammit, 1988). In the liver, this enzyme is a potent regulator of translocation of long-chain fatty acids from the cytoplasm into the mitochondria, where they partition for esterification and oxidation (Genuth, 1993). Moreover, insulin's inhibitory effect on ketogenesis is also related to its stimulatory effect on activity of acetyl-CoA carboxylase or formation of malonyl-CoA that inhibits activity of CPT I (Zammit, 1996).

Effect of Slow-release Insulin on Liver Metabolites

Liver triglyceride. During the pre- and postinjection period, CIWS deposited more TG in the liver than CISRI ($P < 0.10$, [Table 3]). Increasing dose of SRI quadratically decreased the percentage increase in liver TG between d 2 and 5 postpartum ($P < 0.02$). On d 5 postpartum, average liver TG concentration for CISRI tended to be lower than for CISW ($P < 0.11$). Liver TG values were lower than those previously reported (15–25 μg TG/ μg DNA, [Hartwell et al., 2000]); perhaps effects of SRI would have been more dramatic in cows with more extensive TG accumulation after calving. Nonesterified fatty acids mobilized from adipose tissue are the primary precursors for hepatic TG. Major factors causing hepatic lipidosis are suggested to be increased supply of long-chain fatty acids and impairment of TG export as very low-density lipoprotein (VLDL) transport (Kleppe et al., 1988). Depression in DMI and consequently decreased serum insulin and increased plasma NEFA concentrations around parturition coincide with increased TG in the liver. Hepatic lipidosis may become significant when the export of TG as VLDL from the liver cannot keep pace with increased NEFA uptake and TG synthesis by the liver (Grummer, 1993). Therefore, limiting fat mobilization from adipose tissue and improving TG export ability of the liver are essential strategies for alleviation of hepatic lipidosis.

Effects of insulin on synthesis and degradation of apolipoprotein B (apoB) and on export of TG as VLDL from the liver are conflicting. Inhibitory effects of insulin on hepatic VLDL output and apoB synthesis were demonstrated in diabetic humans (Gibbons, 1990). A study conducted in rats (Chirieac et al., 2000) supports that insulin suppresses hepatic VLDL and apoB secretions, whereas another study conducted in rats (Satoh et al., 1987) supports that insulin enhances TG secretion rate. Bremner et al. (2000) provided evidence that activity of bovine microsomal TG transfer protein that is required for assembly and secretion of apoB containing VLDL is not affected by insulin.

Increasing the dose of SRI quadratically decreased concentrations plasma NEFA (Table 2) and liver TG

Table 3. Effects of a single dose of slow-release insulin on liver metabolites of Holsteins during the first week of lactation.

Response variable	Insulin dose, IU/kg BW ¹					Significance of insulin effect ($P > F$) ²		
	0	0.14	0.29	0.43	SEM	Insulin	Linear	Quadratic
Triglyceride (TG)								
d5 minus d2, $\mu\text{g}/\mu\text{g}$ DNA	4.5	-0.6	1.2	1.2	2.0	0.10	0.34	0.21
Relative change, %	38.0	-6.0	21.0	40.0	16.0	0.17	0.93	0.02
d5, $\mu\text{g}/\mu\text{g}$ DNA	16.9	11.8	13.5	13.7	2.1	0.11	0.38	0.23
Glycogen (GLY)								
d5 minus d2 $\mu\text{g}/\mu\text{g}$ DNA	2.5	6.2	6.0	2.1	2.5	0.44	0.96	0.14
Relative change, %	160.0	122.0	181.0	108.0	72.0	0.75	0.79	0.80
d5, $\mu\text{g}/\mu\text{g}$ DNA	9.6	14.2	13.5	8.9	2.5	0.41	0.93	0.08
TG:GLY	4.7	1.3	2.0	5.6	2.0	0.47	0.84	0.08

¹Data were covariately adjusted with the least squares means for response variables measured at 1200 h on d 5 postpartum. Treatments were applied at 0800 h on d 3 postpartum.

²Probability values were Satterthwaite approximates. Contrasts: Insulin = cows intramuscularly injected with sterile water versus cows intramuscularly injected with slow-release insulin; Linear = linear effect of increasing doses of slow-release insulin; Quadratic = quadratic effect of increasing doses of slow-release insulin.

(Table 3). A high correlation between concentrations of plasma NEFA and liver TG ($r = 0.57$, $P < 0.0008$) suggests that until factors impeding hepatic VLDL-TG export are identified, limiting uptake of NEFA by the liver through the antilipolytic effect of insulin can be an important strategy to prevent development of hepatic lipidosis.

Liver glycogen. Glycogen was deposited in the liver during d 2 to 5 postpartum (Table 3). Although the amount of GLY deposited in the liver did not differ between CISRI and CISW ($P < 0.44$), increasing dose of SRI tended to quadratically increase the amount of GLY deposited ($P < 0.14$). However, on a percentage basis, changes in liver GLY concentration for CISRI and CISW during d 2 to 5 were not different ($P < 0.75$). On d 5 postpartum, there were no differences in GLY concentrations between CISRI and CISW ($P < 0.41$), but increasing dose of SRI tended to increase concentrations of GLY quadratically ($P < 0.08$).

In contrast to plasma glucose concentrations, increasing dose of SRI quadratically increased GLY deposition and GLY concentration (Table 3). Insulin suppresses activities of gluconeogenic enzymes (pyruvate carboxylase and phosphoenolpyruvate carboxykinase) (O'Brien and Granner, 1990), enhances activity of glycogen synthase that stimulates glycogenesis (Genuth, 1993), and inhibits activity of glycogen phosphorylase that stimulates glycogenolysis (Genuth, 1993) in the liver. In insulin-resistant monkeys, these enzymes responded to insulin in liver but not in muscle (Ortmeyer and Hansen, 1998), indicating that degree of resistance may vary among tissues, with the liver being a less resistant tissue.

In the present study, changes in liver GLY content were inversely related with changes in TG content ($r =$

-0.55 , $P < 0.0007$). Although there were no differences in ratio of TG to GLY in the liver between CISRI and CISW ($P < 0.47$), increasing dose of SRI quadratically decreased the ratio of TG to GLY ($P < 0.08$; Table 3). Drackley et al. (1992) showed that liver function was impaired during induction of ketosis and suggested that the ratio of TG to GLY is a predictor of a cow's likelihood to develop ketosis.

CONCLUSIONS

This study was conducted to determine whether there is a dose of SRI that decreases plasma NEFA and liver TG, without decreasing plasma glucose concentration. There was no dose of SRI that did not cause hypoglycemia (<45 mg glucose/dl). In fact, SRI had more dramatic effects on plasma glucose concentration than plasma NEFA concentrations. This suggests that immediately after parturition, lipolysis in adipose tissue is more resistant to effects of insulin than glucose metabolism in tissues such as the liver. However, the lowest dose of SRI increased DMI, milk yield, and concentrations of serum insulin and liver GLY and decreased the ratio of hepatic TG to GLY, hepatic TG, and plasma NEFA and BHBA. Many responses to insulin were quadratic, some of which may have been related (e.g., plasma NEFA and liver TG). Quadratic responses are difficult to explain but may be related to down regulation of receptors at highest doses of SRI. Administration of the low dose of SRI (0.14 IU/kg BW) could be considered as a prophylactic against hepatic lipidosis and ketosis during the periparturient period. In this study, the effects of SRI were monitored in healthy cows; effects of SRI in cows prone to lipid-related metabolic disorders need to be evaluated.

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