Metabolic Responses of Lactating Dairy Cows to Single and Multiple Subcutaneous Injections of Glucagon¹

G. Bobe, R. N. Sonon, B. N. Ametaj, J. W. Young, and D. C. Beitz²

Nutritional Physiology Group,
Department of Animal Science,
Iowa State University, Ames 50011-3150

ABSTRACT

Continuous, intravenous infusions of glucagon improve carbohydrate status in lactating dairy cows without increasing concentrations of plasma NEFA. The objective was to test whether single subcutaneous injections and multiple subcutaneous injections of glucagon delivered at 8-h intervals over 14 d improve the carbohydrate status in lactating dairy cows without increasing concentrations of plasma BHBA and NEFA. In a single-injection experiment, four midlactation cows each were injected with 2.5 and 5 mg of glucagon 1 wk apart. In a multiple-injection experiment, nine cows, assigned randomly to three treatments, were injected subcutaneously with 0, 2.5, or 5 mg of glucagon every 8 h for 14 d, beginning at d 8 postpartum. Single subcutaneous injections of glucagon increased concentrations of plasma glucagon and single and multiple subcutaneous injections of glucagon increased concentrations of plasma glucose, with larger increases at the 5-mg dosage. Injections of 5 mg of glucagon increased concentrations of plasma insulin in both experiments, whereas the 2.5-mg dosage increased plasma insulin only in the multiple-injection experiment. The response of glucose and insulin to injections of 5 mg of glucagon persisted throughout the 14-d injection period. Concentrations of plasma NEFA decreased in the single-injection experiment, and concentrations of BHBA decreased after 5 mg of glucagon was injected in the multiple-injection experiment. These results document that both single and multiple injections of 5 mg of glucagon over 14 d consistently improve the carbohydrate status of dairy cows and decrease concentrations of plasma NEFA and BHBA.

(**Key words:** glucagon, injection, metabolism)

INTRODUCTION

Fatty liver (i. e., hepatic lipidosis) is one of the major metabolic disorders of dairy cows in the periparturient period, which affects up to 50% of cows in early lactation (Jorritsma et al., 2001). Fatty liver can negatively affect the productivity, health status, and reproductive performance of high-producing dairy cows (Herdt, 1988; Veenhuizen et al., 1991; Wensing et al., 1997; Bobe, 2002) and can be treated with continuous, intravenous infusions of glucagon for 14 d (Hippen et al., 1999b).

Administration of exogenous glucagon increases concentrations of plasma glucagon, glucose, and insulin in lactating dairy cows (de Boer et al., 1986; Trevisi et al., 1997; Hippen et al., 1999b; She et al., 1999) by stimulating hepatic gluconeogenesis, glycogenolysis, amino acid uptake, and ureagenesis (Flakoll et al., 1994; Donkin and Armentano, 1995). The metabolic response to glucagon depends on the dosage and the age, stage of lactation, and health of cows, with weaker responses at lower glucagon dosages in older, ketotic cows in early lactation (de Boer et al., 1986; Holtenius and Tråvén, 1990; Holtenius and Holtenius, 1996; Steen et al., 1997; Hippen et al., 1999a, 1999b; She et al., 1999; Bobe, 2002).

The effects of glucagon on concentrations of plasma BHBA and NEFA can be both direct and indirect because glucagon directly increases lipolysis and ketogenesis (Brockman, 1976; Zupke et al., 1998) but indirectly decreases lipolysis and ketogenesis by increasing concentrations of plasma glucose and insulin (Brockman, 1978). Therefore, glucagon can either increase or decrease concentrations of plasma BHBA and NEFA depending on responses of glucose and insulin, dosage of glucagon, age, stage of lactation, and health of the cow, with increases being more likely at higher glucagon dosages in younger, ketotic cows in early lactation (de Boer et al., 1986; Hippen et al., 1999a, 1999b; She et al., 1999; Bobe, 2002).

The metabolic response to glucagon also is influenced by the mode of application. Pulsatile glucagon delivery, which is similar to multiple injections, increases hepatic glucose production more effectively than does con-

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Corresponding author: D. C. Beitz; e-mail: dcbeitz@iastate.edu.

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tinuous delivery and more closely resembles the release of glucagon by the pancreas (Lefebvre et al., 1996). We demonstrated previously that the insulin response decreases during continuous infusions of glucagon (Hippen et al., 1999b; She et al., 1999) but not during multiple injections of glucagon (Bobe, 2002). Intravenous infusions of glucagon result in greater responses than do subcutaneous injections (Mühlhauser et al., 1985), which is similar to results obtained in our laboratory (Hippen et al., 1999a, 1999b; She et al., 1999; Bobe, 2002); however, long-term intravenous infusions of glucagon are not practical for on-farm use.

Therefore, the current objective was to determine whether single subcutaneous injections of glucagon and multiple injections every 8 h over 14 d consistently improve the carbohydrate status in lactating dairy cows without increasing concentrations of plasma BHBA and NEFA. More specifically, the objective was to determine the effects of subcutaneous injections of 2.5 and 5 mg of glucagon on concentrations of plasma glucagon, glucose, insulin, BHBA, and NEFA over a 14-d injection period.

MATERIALS AND METHODS

Experimental Design

Parts of the experimental design and several of the experimental techniques and methods have been described in detail elsewhere (Bobe, 2002). Multiparous Holstein cows housed in a tie-stall barn were used in both experiments. They had free access to a typical NRC-recommended (2001) TMR for high-producing cows that was offered daily at 0800 and 1800 h. The ration consisted primarily of corn, alfalfa hay, cottonseed, and soybean meal and contained 1.72 Mcal/kg of NE_L, 16.8% CP, 16.5% ADF, and 29% NDF (Bobe, 2002).

Using a latin square design for the single-injection experiment, four midlactation cows were injected subcutaneously at 0100 h with either a single dosage of 2.5 or 5 mg of lyophilized bovine glucagon (donated by Eli Lilly & Co., Indianapolis, IN) followed 1 wk later by the other dosage. The glucagon was dissolved before injection in 60 ml of 0.15 M NaCl (final pH 10.25). By using a completely randomized design for the multiple-injection experiment, nine cows (three cows per treatment) were injected subcutaneously with 0 (Saline), 2.5, or 5 mg of glucagon in 60 ml of 0.15 M NaCl every 8 h (6:00, 14:00, and 22:00 h) for 14 d beginning at 14:00 h of d 8 postpartum (Bobe, 2002). Serial samples of blood were collected starting 15 min before and continuing until 8 h after glucagon injections on d 1 at 14:00 h (D1-14:00), on d 1 at 22:00 (D1-22:00), on d 7 at 14:00 h (D7-14:00), and on d 13 at 14:00 h (D13-14:00). All cows were managed and treated in accordance with

guidelines established by the Iowa State University Committee on Animal Care.

Sampling and Analysis

For blood collection, a catheter (Angiocath, 22.1 × 133 mm; Becton Dickinson Infusion Therapy Systems Inc., Sandy, UT) was inserted into the jugular vein 15 min before blood collection started. Insertion of the catheter immediately preceded sampling because catheters tended to move out of the jugular vein or blood coagulated in the catheter. Before catheterization, the area was disinfected, shaved, and anesthetized with an injection of 10 ml of Xylocaine (lidocaine hydrochloride, 2%; Med. Tech. Inc., Elkwood, KS). In both experiments, blood was collected at -15, -10, -5, 15, 30, 45, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360, 420, and 480 min after glucagon injections into 10-ml Vacutainer tubes (Beckton Dickinson and Co., Rutherford, NJ) that contained Na₂-EDTA in the single-injection experiment and K₂-EDTA in the multiple-injection experiment. The first three samples were used to establish a baseline. Between samples, catheters were kept patent by injecting 5 ml of sodium citrate (1% sodium citrate in 0.15 M NaCl) into the catheter. The catheter and catheterization sites were changed after each injection period except for the two d-1 injection and collection periods in the multiple-injection experiment.

Plasma samples were prepared by centrifugation within 20 min after blood collection and stored in polystyrene or glass (for glucagon analysis) tubes at -20°C until analysis. Plasma samples were analyzed for concentrations of glucagon (glucagon kit number KGND1; Diagnostic Products Corp., Los Angeles, CA), glucose (glucose kit number 315; Sigma, St. Louis, MO), insulin (insulin kit number TKIN; Diagnostic Products Corp., Los Angeles, CA), NEFA (NEFA-C kit number 994-75409; WAKO, Richmond, VA), and BHBA (kit number 310-A; Sigma, St. Louis, MO). Before storage of samples, aprotinin (Boehringer-Mannheim, Indianapolis, IN) was added at 5:00 KIU to 1 ml of plasma samples intended for glucagon analysis. Unfortunately, K₂-EDTA interfered with analysis of glucagon samples and, therefore, glucagon concentrations cannot be reported for the multiple-injection experiment.

Statistical Analysis

Data from both experiments were analyzed as a double repeated measures study by using the mixed models procedures of SAS Version 8.2 (2001).

Single-injection experiment. In the single-injection experiment, the dependent variable was the change of the response variable from baseline concen-

trations averaged across -15, -10, and -5 min before glucagon injection to the concentrations at 15, 30, 45, 60, 80, 1:00, 120, 150, 180, 210, 240, 3:00, 360, 420, and 480 min after glucagon injection. The fixed effects were treatment (2.5 or 5 mg), time after injection (15, 30, 45, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360, 420, or 480 min), and treatment $\times$ time after injection interaction. The Kronecker product of a completely unrestricted variance-covariance matrix (for treatment) and a first-order autoregressive variance-covariance matrix (for time after injection) was used to account for double repeated measures taken on individual cows across time.

The overall effects of 2.5 and 5 mg of glucagon on concentrations of response variables were evaluated by comparing their estimated changes from baseline concentrations averaged across time after injection (15 to 480 min) with a) each other by using a paired *t*-test and b) the H_0 hypothesis that the changes from baseline concentrations are equal zero (no changes) by using a *t*-test in the ESTIMATE statement. The duration of the effects of 2.5 and 5 mg of glucagon were evaluated by comparing the changes from baseline concentrations at specific times after glucagon injection with a) each other by using a paired *t*-test and b) the H_0 hypothesis that the baseline changes are equal to zero using a *t*-test. The average effects of 2.5 and 5 mg of glucagon for the 8-h postinjection period were evaluated by calculating the area under the curve minus the area below the baseline concentration by using the trapezoidal rule (Granville et al., 1941) divided through 480 min. The estimated average areas of 2.5 and 5 mg of glucagon were compared with a) each other using a paired *t*-test and b) the H_0 hypothesis that the areas are equal to zero using a *t*-test.

Multiple-injection experiment. In the multiple-injection experiment, the dependent variable was the change of the response variable from baseline concentrations (-15, -10, and -5 min before glucagon injection) to the concentrations after glucagon injection (the time points were the same as in the single-injection experiment). For the plasma insulin and NEFA response, the change was calculated from log-transformed concentrations because the statistical model would not converge using the nontransformed values. The fixed effects were treatment (saline, 2.5 mg, or 5 mg), day and time of injection (D1-14:00, D1-22:00, D7-14:00, or D13-14:00), time after injection (same as in the single-injection experiment), and all their two- and three-way interactions. The Kronecker product of a completely unrestricted variance-covariance matrix (for day and time of injection) and a first order autoregressive variance-covariance matrix (for time after injection) was used to

account for double repeated measures taken on individual cows across time.

The overall effects of 2.5 and 5 mg of glucagon were evaluated by comparing their estimated changes from baseline concentrations averaged across time after injection (15 to 480 min) with the corresponding estimated change of the Saline group using a *t*-test in the ESTIMATE statement. The duration of the effects of 2.5 and 5 mg of glucagon were evaluated by comparing the changes from baseline concentrations at specific times after glucagon injection with the corresponding changes of the saline group using a *t*-test in the ESTIMATE statement. The average effects of 2.5 and 5 mg of glucagon for the 8-h postinjection period were evaluated by calculating the area under the curve minus the area below the baseline concentration by using the trapezoidal rule (Granville et al., 1941) divided through 480 min. The estimated average areas of 2.5 and 5 mg of glucagon were compared with the corresponding average area of the Saline group using a *t*-test in the ESTIMATE statement.

In both experiments, to obtain the correct degrees of freedom the KENWARDROGER option was invoked that uses the Satterthwaite adjustment for degrees of freedom with a Kenward-Roger adjustment on standard errors (Tempelman et al., 2002). Means and SEM presented in figures are raw means and SEM. Significance was declared at $P \leq 0.05$.

RESULTS

To characterize the responses of plasma parameters to single and multiple injections of 2.5 and 5 mg of glucagon for the 8-h postinjection period, three parameters were used: overall, average, and duration of response. The overall response is defined as the overall change for the entire 8-h post-injection period, whereas the average response is defined as the average change during the 8-h postinjection period and is not shown in the figures. Duration of response is defined as the time during which concentrations were significantly different at $P \leq 0.05$ from baseline concentrations in the single-injection experiment and from concentrations of the saline-treated group at the same time point in the multiple-injection experiment.

Single-injection experiment. In the single-injection experiment, the midlactation multiparous Holstein cows ($n = 4$) were between 100 and 200 DIM, had a milk production between 32 to 53 kg/d, and seemed to be in positive energy balance. In the single-injection experiment, concentrations after glucagon injections were compared with concentrations before injections. Single subcutaneous injections of 2.5 and 5 mg of glucagon increased plasma glucagon (both $P \leq 0.0001$; Figure

Figure 1. Responses of plasma glucagon, glucose, insulin, and NEFA concentrations to single, subcutaneous injections of 2.5 and 5 mg of exogenous glucagon in four midlactation, multiparous dairy cows during the 8-h postinjection period. A, Response of plasma glucagon to 2.5 mg ($P \leq 0.0001$) and 5 mg of glucagon ($P \leq 0.0001$) and difference in response ($P \leq 0.002$). Concentrations were increased significantly for 4 (2.5 mg) and 3 h (5 mg), with greater increases for the first 2 h after injection of 5 mg of glucagon. B, Response of plasma glucose to 2.5 mg ($P \leq 0.002$) and 5 mg of glucagon ($P \leq 0.0001$) and difference in response ($P \leq 0.0001$). Concentrations were increased significantly for 2 (2.5 mg) and 5 h (5 mg), with greater increases for the first 3 h after injection of 5 mg of glucagon. C, Response of plasma insulin to 2.5 mg ($P \leq 0.72$) and 5 mg of glucagon ($P \leq 0.05$) and difference in response ($P \leq 0.22$). Concentrations were increased significantly for 1 (2.5 mg) and 2.5 h (5 mg), with no significant differences between dosages. D, Response of plasma insulin to 2.5 mg ($P \leq 0.06$) and 5 mg of glucagon ($P \leq 0.03$) and difference in response ($P \leq 0.90$). Concentrations were not affected significantly by 2.5 mg of glucagon, decreased significantly for 4 h after injection of 5 mg of glucagon, and were significantly greater at 8 h after injection of 5 mg of glucagon.

1A), with an average response for the 8-h postinjection period of 92 ± 17 pg/ml at the 2.5-mg dosage ($P \leq 0.03$) and 206 ± 43 pg/ml at the 5-mg dosage ($P \leq 0.04$). The overall response of plasma glucagon was greater at the 5-mg dosage ($P \leq 0.002$; Figure 1A). Concentrations of plasma glucagon were increased significantly for the first 4 and 3 h after the injection of 2.5 and 5 mg of glucagon, respectively, with a stronger increase for the first 2 h at the 5-mg dosage (Figure 1A). The fact that the duration of the response was shorter at the 5-mg dosage, although raw means of plasma glucagon were higher at the 5-mg dosage (Figure 1A), can be explained by greater variation of responses of plasma glucagon.

The response of plasma glucose and glucagon to single injections of glucagon was similar (Figure 1). Plasma glucose concentrations were increased overall by subcutaneous injections of 2.5 ($P \leq 0.007$) and 5 mg of glucagon ($P \leq 0.0001$), with a stronger increase at the 5-mg dosage ($P \leq 0.0001$; Figure 1B). The average

response for the 8-h postinjection period was 4.3 ± 0.9 ($P \leq 0.02$) for 2.5 and 14.1 ± 2.7 mg/dl ($P \leq 0.01$) for 5 mg of glucagon injected. Concentrations of plasma glucose were increased significantly for the first 2 and 4 h after injections of 2.5 and 5 mg of glucagon, respectively (Figure 1B).

The shape of the response curve for plasma insulin, glucagon, and glucose to single injections of glucagon were similar (Figure 1), but the response of insulin was smaller than responses of plasma glucagon and glucose because concentrations of insulin decreased below baseline at 4 h after the injection (Figure 1C). Subcutaneous injections of 5 mg of glucagon ($P \leq 0.05$) but not injections of 2.5 mg of glucagon ($P \leq 0.72$) increased overall concentrations of insulin. Concentrations of plasma insulin were increased significantly for 1 and 2.5 h after injection of 2.5 and 5 mg of glucagon, respectively (Figure 1C).

Subcutaneous injections of both 2.5 and 5 mg of glucagon decreased overall plasma NEFA concentrations at $P \leq 0.06$ and $P \leq 0.03$, respectively, with significant decreased concentrations for the 4 h at the 5-mg dosage but not at the 2.5-mg dosage (Figure 1D). The pattern of responses of plasma NEFA to single injections of glucagon were mirror images of responses of glucagon, glucose, and insulin, except that the rates of decrease were less steep and more persistent and concentrations of NEFA increased at 7 to 8 h postinjection (Figure 1).

Multiple-injection experiment. In the multiple-injection experiment, the multiparous Holstein cows ($n = 9$) were visually healthy before parturition and had BCS between 3.5 and 4.25. After calving, all cows developed mild to moderate fatty liver (1 to 7% liver triacylglycerol/wet tissue) and lost BW throughout the first 3 wk postpartum. The average DMI $\pm$ SEM for the saline group for wk 1, 2, and 3 postpartum was 25.1 ± 4.1 , 33.4 ± 4.0 , and 37.1 ± 4.4 kg/d, respectively. The average DMI $\pm$ SEM for the 2.5-mg glucagon group for wk 1, 2, and 3 postpartum was 32.6 ± 1.5 , 40.4 ± 3.2 , and 39.3 ± 4.0 kg/d, respectively. The average DMI $\pm$ SEM for the 5-mg glucagon group for wk 1, 2, and 3 postpartum was 31.0 ± 3.6 , 40.3 ± 0.8 , and 43.2 ± 2.2 kg/d, respectively. The average milk production $\pm$ SEM for the Saline group for wk 1, 2, and 3 postpartum was 21.2 ± 3.6 , 33.5 ± 5.5 , and 36.4 ± 5.8 kg/d, respectively. The average milk production $\pm$ SEM for the 2.5-mg group for wk 1, 2, and 3 postpartum was 31.2 ± 1.9 , 40.0 ± 3.9 , and 41.1 ± 4.0 kg/d, respectively. The average milk production $\pm$ SEM for the 5-mg group for wk 1, 2, and 3 postpartum was 30.5 ± 2.8 , 43.0 ± 1.7 , and 43.8 ± 0.5 kg/d, respectively.

In the multiple-injection experiment, responses to glucagon injections in the 8-h postinjection period were compared with those after saline injections. For time and day-of-injection effects, data from responses to multiple injections of 5 mg of glucagon on D1–14:00, D1–22:00, D7–14:00, and D13–14:00 were used. Single and multiple subcutaneous injections of glucagon affected concentrations of plasma glucose in a similar way (Figure 1B vs. Figure 2A and B). The only differences in the multiple-injection experiment were that baseline concentrations of glucose were lower (55 vs. 73 mg/dl; Figures 1B and 2A) and that concentrations of glucose were increased less after injections of glucagon (6.6 ± 2.1 mg/dl at the 2.5-mg dosage and 3.6 ± 2.1 mg/dl at the 5-mg dosage). Multiple subcutaneous injections of glucagon increased overall concentrations of glucose after injections during the 14-d injection period at both dosages ($P \leq 0.002$ for 2.5 mg and $P \leq 0.0001$ for 5 mg), but the increase was larger at the 5-mg dosage ($P \leq 0.02$; Figure 2A). Multiple injections of glucagon significantly increased concentrations of plasma glucose for the first

2 (2.5 mg) and 4 h (5 mg) after injections during the 14-d injection period, and the increase was larger for the first hour after injection of the 5-mg dosage (Figure 2A).

Day and time of glucagon injection both affected the response of plasma glucose to multiple injections of 5 mg of glucagon (Figure 2B). The overall response of glucose was larger after injection of 5 mg of glucagon at 14:00 h on d 1, 7, and 13 ($P \leq 0.0001$, $P \leq 0.004$, and $P \leq 0.0001$) than at D1–22:00 ($P \leq 0.54$; Figure 2B), which caused a time of injection interaction on d 1 ($P \leq 0.02$). The average response of glucose to injections of 5 mg of glucagon for the 8-h post-injection period increased numerically from 6.6 ± 4.9 mg/dl ($P \leq 0.23$) on D1–14:00 to 10.7 ± 3.1 mg/dl ($P \leq 0.01$) on D13–14:00 and concentrations of glucose remained elevated significantly longer (2 h at D1–14:00 in comparison to 4 h at D13–14:00; Figure 2B). The greater variation of responses of plasma glucose at D1–14:00 in comparison to D13–14:00 can explain why the raw means of the response of plasma glucose were greater but less statistically significant for average response at D1–14:00 (Figure 2B).

The responses of plasma insulin and glucose to multiple subcutaneous injections of glucagon were very similar (Figure 2). In comparison to the single-injection experiment (Figure 1C), concentrations of insulin were increased more significantly and for a longer duration in the multiple-injection experiment ($P \leq 0.03$ and 2 h for 2.5 mg and $P \leq 0.0002$ and 3 h for 5 mg; Figure 2C). The responses of plasma insulin and glucose were similar for multiple injections of 5 mg of glucagon (Figures 2B and D) because the duration of the insulin response increased over the 14-d injection period (2 h at D1–14:00 and 3 h at D13:14:00) and the insulin response was greater at 14:00 h ($P \leq 0.01$) than at 22:00 h on d 1 of injection ($P \leq 0.15$; Figure 2D). Baseline concentrations of insulin and, to a smaller extent, of glucose were higher at D1–22:00 than at D1–14:00 (Figures 2B and D).

In contrast to the single-injection experiment (Figure 1D), injections of 2.5 and 5 mg of glucagon every 8 h for 14 d had no overall significant effects on concentrations of plasma NEFA for the 8-h post injection period in the multiple-injection experiment ($P \leq 0.80$ and $P \leq 0.63$; Figure 3A). Concentrations of plasma NEFA for single and multiple subcutaneous injections of glucagon decreased similarly (Figures 1D and 3A); however, concentrations of plasma NEFA also decreased for the saline-treated group in the multiple-injection experiment ($P \leq 0.01$). Concentrations of plasma NEFA were decreased after the injection of 5 mg of glucagon on D1–14:00 ($P \leq 0.007$), when baseline concentrations of NEFA were highest (Figure 3B). Concentrations of plasma NEFA were decreased significantly for the first

Figure 2. Responses of plasma glucose and insulin concentrations to multiple, subcutaneous injections of 2.5 and 5 mg of exogenous glucagon every 8 h for 14 d in early lactation, multiparous dairy cows ($n = 3$ in each treatment group) during the 8-h postinjection periods. A, Overall response of plasma glucose to 2.5 mg ($P \leq 0.002$) and 5 mg of glucagon ($P \leq 0.0001$) and difference in response ($P \leq 0.02$) over the 14-d injection period. Concentrations were increased significantly for 2 (2.5 mg) and 4 h (5 mg), with greater increases for the first hour after injection of 5 mg of glucagon. B, Response of plasma glucose to 5 mg of glucagon on D1–14:00 ($P \leq 0.0001$; SEM = 0.9 to 6.8), D1–22:00 ($P \leq 0.54$; SEM = 0.4 to 6.0), D7–14:00 ($P \leq 0.004$; SEM = 1.2 to 8.3), and D13–14:00 ($P \leq 0.0001$; SEM = 1.6 to 7.6). Concentrations were increased significantly for 2 h on D1–14:00 and D7–14:00 and 4 h on D13–14:00 and were not affected significantly on D1–22:00. C, Overall response of plasma insulin to 2.5 mg ($P \leq 0.03$) and 5 mg of glucagon ($P \leq 0.0002$) and difference in response ($P \leq 0.06$) over the 14-d injection period. Concentrations were increased significantly for 2 (2.5 mg) and 3 h (5 mg), with greater increases for the first h after injection after injection of 5 mg of glucagon. D, Response of plasma insulin to 5 mg of glucagon on D1–14:00 ($P \leq 0.01$; SEM = 0.5 to 8.5), D1–22:00 ($P \leq 0.15$; SEM = 0.3 to 1.4), D7–14:00 ($P \leq 0.05$; SEM = 0.3 to 2.6), and D13–14:00 ($P \leq 0.03$; SEM = 0.5 to 2.8). Concentrations were increased significantly for 2 h on D1–14:00 and D7–14:00 and 3 h on D13–14:00 and were not affected significantly on D1–22:00.

4 h (Figure 3B). Over the 14-d injection period, multiple subcutaneous injections of 5 mg of glucagon decreased the baseline concentrations of plasma NEFA (Figure 3B).

Multiple subcutaneous injections of 5 mg of glucagon every 8 h for 14 d decreased concentrations of BHBA for the 8-h postinjection period ($P \leq 0.008$) in contrast to injections of 2.5 mg of glucagon ($P \leq 0.45$), which resulted in significant dosage differences for responses of plasma BHBA ($P \leq 0.05$; Figure 3C). The overall decrease of plasma BHBA concentrations was greatest after the injection on D7–14:00 ($P \leq 0.02$), when baseline concentrations of BHBA were highest (Figure 3D). The shape of the responses of BHBA and NEFA to multiple subcutaneous injections of 5 mg of glucagon were similar (slow and persistent decrease), except that concentrations of plasma BHBA did not increase 8 h after the

injection of 5 mg of glucagon on D1–22:00 (Figures 3B and D).

DISCUSSION

The objective of the current study was to determine whether single subcutaneous injections of glucagon and multiple injections every 8 h for 14 d improve the carbohydrate status in lactating dairy cows without increasing concentrations of plasma BHBA and NEFA. The results document that subcutaneous injections of 5 mg of glucagon increase concentrations of plasma glucagon to similar peak concentrations in dairy cows as shown for intravenous infusions of 10 mg/d of glucagon (Figure 1A; Hippen et al., 1999b; She et al., 1999). The response to glucagon is also similar to results in humans when subcutaneous and intravenous administrations of glu-

Figure 3. Responses of plasma NEFA and BHBA concentrations to multiple, subcutaneous injections of 2.5 and 5 mg of exogenous glucagon every 8 h for 14 d in early lactation, multiparous dairy cows ($n = 3$ in each treatment group) during the 8-h postinjection periods. A, Overall response of plasma NEFA to 2.5 mg ($P \leq 0.80$) and 5 mg of glucagon ($P \leq 0.63$) and difference in response ($P \leq 0.82$) over the 14-d injection period. Concentrations were not increased significantly at either dosage. B, Response of plasma NEFA to 5 mg of glucagon on D1–14:00 ($P \leq 0.007$; SEM = 9 to 162), D1–22:00 ($P \leq 0.44$; SEM = 42 to 203), D7–14:00 ($P \leq 0.79$; SEM = 10 to 1:00), and D13–14:00 ($P \leq 0.30$; SEM = 8 to 133). Concentrations were decreased significantly for 4 h on D1–14:00 and were not affected significantly on D1–22:00, D7–14:00, and D13–14:00. C, Overall response of plasma BHBA to 2.5 mg ($P \leq 0.45$) and 5 mg of glucagon ($P \leq 0.008$) and difference in response ($P \leq 0.05$) over the 14-d injection period. Concentrations were decreased significantly for 15 min (2.5 mg) and 2 h (5 mg), with greater decreases for the first 2 h after injection of 5 mg of glucagon. D, Response of plasma BHBA to 5 mg of glucagon on D1–14:00 ($P \leq 0.37$; SEM = 0.11 to 2.96), D1–22:00 ($P \leq 0.91$; SEM = 0.27 to 2.50), D7–14:00 ($P \leq 0.02$; SEM = 0.60 to 4.40), and D13–14:00 ($P \leq 0.18$; SEM = 0.44 to 2.40). Concentrations were decreased significantly for 1 h on D13–14:00 and were not affected significantly on D1–14:00, D1–22:00, and D7–14:00.

cagon are compared (Mühlhauser et al., 1985). Concentrations of plasma glucagon were halved when a single dosage of 2.5 instead of 5 mg of glucagon was injected, indicating that the transfer of glucagon into plasma was not limiting at the concentrations injected.

The response of plasma glucose to single and multiple injections of glucagon depended on the dosage of glucagon injected, with stronger responses at the 5-mg dosage (Figures 1B and 2A), which can be attributed to higher concentrations of plasma glucagon (Figure 1A). In midlactation dairy cows, the average glucose response for the 8-h postinjection period was an increase of 14.1 mg/dl at the 5-mg dosage (see Results section), which was similar to 14.3 mg/dl after continuous, intravenous infusions of 10 mg/d glucagon (Hippen et al., 1999a), indicating a similar efficiency in increasing concentrations of plasma glucose.

The response of plasma glucose to exogenous glucagon also depended on the stage of lactation, with greater increases in midlactation cows than in early lactation cows (Figure 1B vs. 2A), which also has been demonstrated previously with intravenous infusions of glucagon (Hippen et al., 1999a, 1999b; She et al., 1999). The decreased response of plasma glucose to exogenous glucagon in early lactation can be explained by limited hepatic glycogen reserves in early-lactation cows (Holtenius and Holtenius, 1996; Bobe, 2002). The increase in response of glucose to 5 mg of glucagon over the 14-d injection period (Figure 2B), which coincides with increased concentrations of hepatic glycogen (Bobe, 2002), supports this hypothesis as well. Greater variability in responses of plasma glucose and insulin to injections of 5 mg of glucagon were observed at D1–14:00 in comparison to D7–14:00 and D13–14:00, which

can explain why the responses at D1–14:00 were higher but statistically less significant than those on D7–14:00 and D13–14:00 (Figures 2B and D). Greater variability in responses of glucose and insulin to injections of glucagon has been reported previously for cows in early lactation with metabolic diseases (Holtenius and Holtenius, 1996; Steen et al., 1997).

On d 1 of the multiple injection period, the responses of plasma glucose to 5 mg of glucagon were smaller after injections at 22:00 than at 14:00 h (Figure 2B), which can be explained partly by feeding times, because feeding times influence diurnal variations of insulin concentrations (Bines et al., 1983; de Boer et al., 1985; Sutton et al., 1988). Fröhli and Blum (1988) saw a similar decreased response of plasma glucose when they intravenously infused epinephrine. Both effects can be explained by increased concentrations of plasma insulin after the evening feeding, which decreases hepatic gluconeogenesis (Donkin and Armentano, 1995). Feeding, particularly in the evening, increases concentrations of plasma insulin for 4 to 5 h (Bines et al., 1983; de Boer et al., 1985; Sutton et al., 1988). Such increases also are shown in the current study, where cows were fed at 8:00 and 18:00 h and plasma insulin concentrations were higher at 22:00 than at 14:00 h (Figure 1D).

The response of plasma insulin to single and multiple injections of glucagon followed a trend similar to that of plasma glucose (Figures 1 and 2), which can be explained by the stimulatory effect of glucose on insulin secretion by the pancreas (Pipeleers et al., 1985). The response of plasma insulin to glucagon was statistically smaller than that of plasma glucose in the single-injection experiment because baseline concentrations of insulin decreased in the post-injection period of the single-injection experiment (10:00 to 18:00 h with feeding at 8:00 and 18:00 h), as can be seen also in Figure 1C. In contrast to continuous, intravenous infusions of glucagon (Hippen et al., 1999b; She et al., 1999), the response of insulin to multiple injections of 5 mg of glucagon did not decrease statistically over the 14-d injection period (Figure 2D), because injection of glucagon every 8 h caused fluctuations in the concentration of plasma glucagon, which were similar to the pulsatile release of glucagon in nature (Lefebvre et al., 1996).

There was a concern that the responses of plasma glucose and insulin to injections of glucagon every 8 h would decrease over the 14-d injection period, because the cows might become resistant to multiple injections of glucagon, similar to development of insulin resistance in type II diabetes. This concern was further supported by the decrease in concentrations of plasma insulin during continuous intravenous infusions of glucose and glucagon (Holtenius and Holtenius, 1996; Hippen et al., 1999b). Responses of plasma glucose and insulin

to 5 mg of glucagon did not decrease but rather increased over the 14-d injection period (Figures 2B and D), which indicates that resistance to multiple injections of glucagon did not develop because concentrations of glucagon fluctuate when glucagon is not administered continuously (Lefebvre et al., 1996).

Concentrations of plasma NEFA were decreased in the single-injection experiment (Figure 1D) and after the first injection of 5 mg of glucagon in the multiple-injection experiment (Figure 3B), and concentrations of plasma BHBA decreased after injection of the 5-mg dosage in the multiple-injection experiment (Figure 3C). The decreased concentrations of plasma NEFA and BHBA can be explained by glucagon increasing the concentrations of plasma glucose and insulin. Glucagon directly increases lipolysis and ketogenesis (Brockman, 1976; Zupke et al., 1998), but it also indirectly decreases lipolysis and ketogenesis by increasing concentrations of plasma glucose and insulin, which decreases lipolysis and ketogenesis (Brockman, 1978). The hypothesis of an indirect effect of glucagon on plasma NEFA and BHBA is further supported by the shape of their response to glucagon injections, because the responses of plasma NEFA and BHBA are slower and more persistent than those of plasma glucose and insulin.

Another possible explanation for decreased concentrations of plasma NEFA is that baseline NEFA concentrations were elevated because of handling stress during catheterization (Reynaert et al., 1976), which was done within minutes before the first blood sample was taken. This explanation is unlikely, however, because concentrations of plasma NEFA also decreased after the first injection in the multiple-injection experiment (Figure 3D) and in the companion study (Bobe, 2002), where in both cases responses were compared with a control group.

Subcutaneous injections of 5 mg of glucagon did not decrease concentrations of plasma NEFA in the multiple-injection experiment (Figure 3A) as 5 mg of glucagon did in the single-injection experiment (Figure 1D). The decrease of baseline concentrations of plasma NEFA (Figure 2B) and the results of a companion study (Bobe, 2002) demonstrate overall, however, that multiple injections of 5 mg of glucagon decrease concentrations of plasma NEFA. Another effect of glucagon, which we also demonstrated in the companion study (Bobe, 2002), is that responses of NEFA and BHBA were greater at elevated concentrations of plasma NEFA and BHBA, respectively (Figure 3B and D). The stronger decrease in concentrations of plasma NEFA and BHBA can be explained by glucagon indirectly preventing excess lipolysis and ketogenesis by increasing concentrations of plasma glucose and insulin (Brockman, 1978).

The elevated concentrations of plasma NEFA 4 to 8 h after the second injection on d 1 of the multiple-injection experiment (Figure 3B) and 8 h after the single injection of 5 mg of glucagon can be explained by the short-term negative energy balance associated with the long interval of 6 to 12 h since the last meal (Bines et al., 1983; de Boer et al., 1985; Sutton et al., 1988). Another possible explanation is that the elevated concentrations of plasma NEFA are associated with the end of the promotion of gluconeogenesis by glucagon, similar to what could be observed at the end of long-term glucagon administration (Bobe; Hippen et al., 1999a). This explanation is unlikely, however, because concentrations of glucose and insulin were not below baseline concentrations in either experiment (Figures 1B and 2B and D) and because concentrations of BHBA did not increase at the end of the 14-d glucagon administration period (Figure 3C and D) in contrast to what is observed at the end of long-term glucagon administration (Bobe, 2002; Hippen et al., 1999a).

Concentrations of most plasma parameters examined were elevated significantly for only 4 h after subcutaneous injections of 5 mg of glucagon (Figures 1 and 2). Therefore, subcutaneous injections of 5 mg of glucagon every 4 h instead of every 8 h might improve further the carbohydrate status of lactating dairy cows. The 4-h injection interval, however, was not tested because a) it is less practical for on-farm usage, b) oscillating glucagon and glucose concentrations might be more beneficial, and c) responses of most plasma parameters were increased significantly for the 8-h post-injection period.

In summary, subcutaneous injections of 5 mg and, to a smaller extent, 2.5 mg of glucagon in lactating dairy cows increased plasma concentrations of glucagon, glucose, and insulin and decreased concentrations of NEFA and BHBA. The results are indicators of an improved carbohydrate status. Further, there was little or no change in effectiveness of injections given three times daily during the 14-d injection period. Therefore, it can be concluded that subcutaneous injections of 5 mg of glucagon not only improve the carbohydrate status of dairy cows but also may improve their metabolic and health status (Bobe, 2002).

CONCLUSIONS

The current study demonstrates that both single subcutaneous injections of 5 mg of glucagon and multiple injections every 8 h for 14 d consistently improve the carbohydrate status of dairy cows and decrease concentrations of plasma NEFA and BHBA similar to long-term intravenous infusions of 10 mg/d of glucagon for 14 d, which are not practical for on-farm use. Therefore,

subcutaneous injections of 5 mg of glucagon every 8 h for 14 d seem to be as beneficial and are more practical than continuous, intravenous infusions of 10 mg of glucagon daily to improve the carbohydrate status of dairy cows in early lactation, and thereby possibly prevent or treat hepatic lipidosis in such cows.

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