Insulin increases 17 β -estradiol production by the dominant follicle of the first postpartum follicle wave in dairy cows

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Abstract

Prolonged anovulation following parturition has a negative impact on fertility in dairy cows. Insulin plays an important role in ovarian function in many species, and is profoundly depressed in dairy cows during early lactation. We hypothesized that hypoinsulinemia during early lactation represents a key indicator of nutritional status, resulting in delayed ovulation. Holstein cows ($n = 10$) were subjected to either a hyperinsulinemic–euglycemic clamp (INS) or saline infusion (CTL) for 96 h, beginning on day 10 after parturition during the first postpartum follicular wave. Insulin was infused continuously ($0.3 \mu\text{g/kg}$ body weight per h) via a jugular catheter, and euglycemia was maintained by infusion of glucose. Circulating insulin concentrations were elevated 2.6-fold in INS cows compared with CTL cows (0.73 ± 0.026 vs 0.28 ± 0.026 ng/ml; $P < 0.001$). Insulin treatment did not affect ($P > 0.05$) luteinizing hormone (LH) pulse frequency, pulse amplitude or mean circulating LH. Circulating estradiol was elevated in INS cows ($P < 0.01$) and circulating testosterone also tended to be higher. The ratio of testosterone to estradiol was not different between treatments for the initial 30 h of infusion, but was significantly reduced thereafter in response to insulin ($P < 0.01$), suggesting that hyperinsulinemia increased follicular aromatase activity. Insulin treatment also resulted in reduced circulating nonesterified fatty acids, and increased circulating total and free insulin-like growth factor-I concentrations. Insulin infusion increased estradiol secretion by the dominant follicle of the first postpartum follicular wave in dairy cows, and this effect appears not to be mediated through changes in pulsatile LH release.

Reproduction (2004) **127** 537–545

Introduction

Intense genetic selection of dairy cattle for increased milk production has been associated with a reciprocal change in reproductive efficiency (Butler & Smith 1989, Darwash *et al.* 1999). It is generally accepted that energy balance (calories consumed minus calories expended), rather than intake of any specific class of nutrient (carbohydrate, lipid or protein), is the main regulator of reproductive status (Butler & Smith 1989, l'Anson *et al.* 1991). During early lactation, the energetic cost of rising milk production is greater than the energy consumed, resulting in a prolonged period of negative energy balance (NEB). Thus, in terms of body tissue mobilization, early lactation is analogous to severe undernutrition; it is typical for lactating cows to lose 30–40% of their initial lipid reserves at parturition (Chilliard *et al.* 2000). There are many changes in the metabolic and endocrine milieu associated with NEB in the periparturient cow that are implicated in the reduced reproductive performance. Pulsatile release of luteinizing hormone (LH) is diminished; circulating levels of insulin, insulin-like growth factor-I (IGF-I) and leptin and glucose are depressed; and nonesterified

fatty acids (NEFA), β -hydroxybutyrate and growth hormone are elevated (for recent reviews, see Butler 2003, Diskin *et al.* 2003).

Insulin, which is secreted from the pancreatic β -cells, plays a central role in the metabolism of the body. Insulin is also well recognized as a signal of energy status to the central nervous system (Ingvarsen & Andersen 2000, Schwartz *et al.* 2000). Insulin receptors have been localized in the arcuate nucleus and medial basal hypothalamus (regions of the brain containing gonadotropin-releasing hormone (GnRH) neurons) of the rat (van Houten *et al.* 1979). *In vitro* culture studies of hypothalamic explants indicate that insulin can stimulate GnRH release (Arias *et al.* 1992), and studies in diabetic rats and sheep indicate an absolute requirement for insulin for normal LH pulsatility and induction of the LH surge (Kirchick *et al.* 1982, Bucholtz *et al.* 2000). Furthermore, dietary treatments known to increase gonadotropin release in sheep are associated with increased circulating and cerebrospinal fluid concentrations of insulin (Miller *et al.* 1998). At the level of the ovary, insulin receptors are widely distributed throughout all ovarian compartments,

including granulosa, thecal and stromal tissues (Poretsky & Kalin 1987). *In vitro* studies have shown that insulin directly stimulates both mitosis and steroid production of cultured bovine granulosa (Gutierrez *et al.* 1997), theca (Stewart *et al.* 1995) and luteal cells (Mamluk *et al.* 1999).

Early resumption of ovulatory estrous cycles following parturition is associated with improved fertility in dairy cows (Darwash *et al.* 1997, Westwood *et al.* 2002, Butler 2003). Previous reports have indicated that follicular activity during the early postpartum period is characterized by a large incidence of dominant follicles that appear to grow at a normal rate, but have compromised estradiol synthetic capacity (Beam & Butler 1997, 1998). This results in a disproportionate percentage of follicles undergoing atresia (>40%) rather than ovulation (Beam & Butler 1999). It has been posited that the capacity of the dominant follicle to produce estradiol, stimulate an LH surge, and ovulate is dependent on (i) the frequency of LH pulses during follicular growth and (ii) the circulating concentrations of insulin and IGF-I (Beam & Butler 1999), both of which act synergistically with gonadotropins to stimulate steroidogenesis. Gong *et al.* (2002) recently demonstrated that dietary-induced increases in circulating insulin resulted in improved reproductive performance in dairy cows. Collectively, these observations led us to hypothesize that the depressed circulating levels of insulin observed in the early lactation period represent an important metabolic signal linking nutritional status to the reproductive axis in dairy cows. To test this hypothesis, dairy cows were subjected to either a hyperinsulinemic–euglycemic clamp or a saline infusion for 4 days during the first postpartum follicular wave. The results of the study indicate that elevated circulating insulin stimulates ovarian steroidogenesis independently of any apparent effect on pulsatile LH release.

Materials and Methods

Animals and treatment

All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee. The experimental procedures were similar to those previously described (Butler *et al.* 2003). Ten mature lactating Holstein cows (of 620 ± 20 kg body weight ($\pm$ S.E.M.) at 10 days postpartum) had *ad libitum* access to a total mixed ration (1.63 Mcal NE_L/kg) formulated for nutritional support of early lactation cows. The average parity of the cows was 3 (range 2–6). Feed was offered every 2 h to minimize postprandial variations in nutrient supply, and water was freely available at all times. Daily samples of the feed offered were composited on a monthly basis for nutrient analysis (Dairy One Cooperative, Ithaca, NY, USA). Feed refusals were weighed and discarded at noon each day. Cows were milked at 0600 h and 1800 h

daily, milk yield was determined, and milk samples were collected for compositional analysis twice weekly (Dairy One Cooperative).

On day 8 postpartum, cows were randomly assigned to either a hyperinsulinemic–euglycemic clamp (INS (insulin)) or saline infusion (CTL (control)) treatment ($n = 5/\text{treatment}$). For both treatments, three indwelling jugular catheters were inserted (Tygon Microbore Tubing, Norton Performance Plastic, Akron, OH, USA); two catheters on one side were used for infusion of solutions (insulin and glucose or saline), and the catheter on the contralateral side was used to collect blood samples. Baseline measurements (four blood samples/day) were taken on days 8 and 9, and the treatments were imposed for a 96-h period, starting at 1500 h on day 10 and finishing at 1500 h on day 14. As a prophylactic measure, animals were treated with penicillin G procaine (9×10^6 units/day; Butler Company, Columbus, OH, USA). For the INS group, the target glycemia for each cow ($\pm 10\%$) was based on the average blood-glucose concentration for that individual cow determined during the baseline measurements. The insulin infusate was prepared for each cow by dissolving purified bovine pancreatic insulin (I-5500, lot 109H0967, 28.3 USP units/mg; Sigma) in 0.01 M HCl, followed by dilution with sterile saline-containing plasma (1.25%) from that cow. The insulin solution prepared for each cow was calculated to provide an infusion rate of $0.3 \mu\text{g/kg}$ body weight per h, and was infused via a syringe pump (model SE 400; Vial Medical, Grenoble, France). During the insulin infusion, euglycemia was maintained by infusion of glucose (50% w/v dextrose solution; Butler Company) from sterile bottles at variable rates with a peristaltic pump (Micro/Macro Plum XL; Abbott Laboratories, Morgan Hills, CA, USA). Blood samples were collected hourly during the 96-h infusion period. Blood-glucose concentrations were determined immediately (SureStep Blood Glucose Monitoring System; Lifescan, Milpitas, CA, USA), and the infusion rate of glucose was adjusted if necessary. For CTL animals, sterile saline was infused at a rate of 100 ml/h for 96 h, and blood glucose was measured every 4 h.

Blood samples were collected at 10-min intervals for 8 h immediately prior to commencement (BASE) and termination of infusions (END). In addition, 10-min blood samples were collected from INS cows for a further 8 h immediately following the commencement of the insulin infusion (START).

Ovarian follicular activity was examined daily in all cows from day 8 postpartum until the end of the first follicular wave by linear array ultrasonography with a 7.5 MHz transrectal transducer (Aloka 210; Corometrics Medical Systems, Wallingford, CT, USA). A follicular wave had commenced in all cows by day 8, and measurements of follicular growth continued until the fate of the first postpartum follicular wave had been determined.

Plasma measurements

Plasma was collected and stored at -20°C four times daily during days 8 and 9 (baseline period) and every hour during the 4-day infusion period. The samples were subsequently assayed for insulin, estradiol, testosterone, IGF-I, free IGF-I, IGF-binding proteins (IGFBP) and NEFA. Insulin concentrations were quantified daily on days 8 and 9 and every 2 h during the 96-h infusion period by a double-antibody radioimmunoassay (RIA; Linco Research, St Louis, MO, USA), with a sensitivity of 0.05 ng/ml, as previously described (McGuire *et al.* 1995). Plasma estradiol concentrations were quantified daily by RIA (Serono Maia, Cortlandt Manor, NY, USA) on days 8 and 9, and every 6 h during the infusion period, as described previously by Beam and Butler (1997), with the following exceptions: the amount of primary antibody added to each tube was 25 μl , the standard curve extended from 0.063 to 10 pg estradiol/tube, and total binding of tracer was greater than 30%. Plasma testosterone levels were determined using a commercially available kit (DSL Inc., Webster, TX, USA). Because the expected values in plasma were lower than the declared sensitivity of the assay (0.05 ng/ml), a larger aliquot of plasma (500 μl) was extracted in 5 ml of 2:1 benzene–hexane, the tubes were dried, the extract was reconstituted in 50 μl of gel-PBS (0.1% gelatin, 0.14 M NaCl, 3.5 mM $\text{NaH}_2\text{PO}_4 \cdot 1\text{H}_2\text{O}$, 7 mM Na_2HPO_4 anhydrous, 3 mM NaN_3 , pH 7.1), and the assay was conducted according to the kit instructions. LH concentrations were determined by RIA (sensitivity = 0.2 ng/ml) on all samples taken during the frequent sampling periods using USDA-bLH-B-6 for iodination and standard, and rabbit antiovine LH (R#16; a gift from R.B. Staigmiller), as previously described (Price *et al.* 1987). Pulses were identified as values that were greater than two standard deviations above the mean LH concentration, and had at least one adjacent elevated point. Pulse amplitude is reported as the peak LH pulse value minus the baseline LH concentration. Circulating levels of total IGF-I were determined during the baseline period, and at 0, 48 and 96 h in a single assay following ethanol:acetone:acetic acid (60:30:10) extraction, as described by Enright *et al.* (1989), and complete removal of all binding proteins was verified by Western ligand blot. Sensitivity of the assay was 12 ng/ml, and primary antibody and IGF-I for iodination and standards were sourced as previously described (Butler *et al.* 2003). Circulating levels of free IGF-I were determined during the baseline period and at 0, 48 and 96 h relative to the start of infusion in a single assay, using a commercially available sandwich-type IRMA (sensitivity = 0.03 ng/ml; DSL Inc.). The plasma IGFBP profile was determined by Western ligand blot with ^{125}I -labeled IGF-I at 0, 48 and 96 h, using two gels in a single run on a double-gel unit, and IGFBP-3 and IGFBP-2 were identified by molecular mass, as previously described (Butler *et al.* 2003). Plasma NEFA concentrations were quantified daily on days 8 and 9, and every

12 h during the infusion period by enzymatic assay (Wako Pure Chemical Industries, Osaka, Japan). Inter- and intra-assay coefficients of variation were 11.3 and 4.4%, 12.5 and 7.1%, 15.3 and 4.0%, and 5.4 and 4.8% for the estradiol, insulin, LH, and testosterone RIA respectively.

Energy balance determination

Energy balance (EB) was calculated daily from measurements of milk yield and dry matter intake, body weight (weekly measurement), milk-fat percentage (twice-weekly measurement) and the calculated NE_i value of the diet (Beam & Butler 1997). Daily net energy consumed ($\text{NE}_{\text{consumed}}$) was increased for the animals on the INS treatment by a variable amount depending on the infusion rate of glucose required to maintain euglycemia, and this increment in $\text{NE}_{\text{consumed}}$ was calculated as described previously (Butler *et al.* 2003).

Statistical analysis

All data were analyzed using statistical software from SAS (SAS Institute, Cary, NC, USA). Baseline values for each variable measured were used for covariate adjustment. When appropriate, hormone, metabolite and production data were analyzed as repeated measures within cow using an autoregressive plus random effect covariance structure, as described by Littell *et al.* (1998). When the interaction between treatment and time was significant ($P < 0.05$), pair-wise comparisons of individual means were carried out with the Tukey–Kramer test. LH data were analyzed as follows: (i) within each cow using a paired *t*-test comparing values measured during BASE with values measured during START and END; and (ii) between treatments using one-way ANOVA.

Results

Insulin and glucose

The temporal profiles of plasma insulin and blood-glucose levels are presented in Fig. 1. During the infusion period, plasma insulin concentrations (means $\pm$ S.E.M.) were elevated 2.6-fold in INS cows over values in CTL cows (0.73 ± 0.026 vs 0.28 ± 0.026 ng/ml; treatment, $P < 0.001$). Euglycemia was maintained in INS cows during the infusion period via infusion of exogenous glucose, resulting in blood glucose values that were not different from CTL cows. To maintain euglycemia, the glucose infusion started at a rate of 40 g/h, increased gradually over the first 72 h, and remained stable at 97 ± 0.5 g/h for the final 24 h.

Ovarian steroids

Circulating estradiol concentrations were elevated in INS compared with CTL cows during the infusion period (treatment by time, $P < 0.01$; Fig. 2). There was no difference between treatments for the initial 30 h, after which

Figure 1 Mean plasma insulin and blood glucose concentrations in dairy cows during the infusion period. (a) INS cows were infused with insulin ($0.3 \mu\text{g/kg}$ body weight per h) at a constant rate commencing at 0 h and concluding at 96 h, whereas CTL cows were infused with saline (100 ml/h). Plasma insulin levels were measured every 2 h during the infusion period. The insulin infusion resulted in increased ($P < 0.001$) circulating insulin (pooled S.E.M. = 0.026 ng/ml). (b) Blood glucose was measured every hour during the infusion period in INS cows and every 4 h in CTL cows (4-h mean values were calculated for INS cows). Blood-glucose levels were not different between the treatments (pooled S.E.M. = 0.8 mg/dl).

estradiol declined in the CTL cows, but increased and remained elevated in the INS cows. The plasma estradiol concentrations were significantly different by 48 h, and this difference was essentially maintained for the remainder of the infusion period. A treatment by time interaction ($P = 0.052$) was also observed for plasma testosterone (Fig. 2). As with estradiol, there was no difference between treatments during the initial 30 h, but circulating testosterone tended to be higher in the INS cows thereafter. The mean ratio of testosterone to estradiol was not different between treatments during the initial 30-h period (15.7 ± 2.8 vs 15.3 ± 3.2), but during the final 66 h of infusion, the mean ratio was significantly reduced in INS cows relative to CTL cows (10.3 ± 1.9 vs 17.9 ± 2.0 ; $P < 0.01$).

LH pulsatility

The plasma LH pulse data is summarized in Table 1. For the INS cows, no differences in LH pulse frequency, mean plasma LH, baseline LH, or pulse amplitude were detected either immediately after the start of infusion (START) or during the final stages of infusion (END) relative to values measured prior to the start of infusion

Figure 2 Mean plasma estradiol and testosterone in the INS and CTL cows during the 96-h infusion period. INS cows were infused with insulin ($0.3 \mu\text{g/kg}$ body weight per h) at a constant rate from 0 h until 96 h. CTL cows were infused with saline (100 ml/h). (a) Plasma estradiol was measured every 6 h during the infusion period (pooled S.E.M. = 0.48 pg/ml). A significant treatment by time interaction ($P < 0.01$) was observed. (b) Plasma testosterone was measured every 6 h during the infusion period. A treatment by time interaction was observed ($P = 0.052$; pooled S.E.M. = 1.1 pg/ml).

(BASE). There was a decline in the LH pulse frequency in the CTL cows from BASE to END during the infusion period (4.0 vs 2.4 pulses per 8 h in BASE vs END; S.E.M. = 0.6 , $P = 0.056$), but there was no change in the mean plasma LH, baseline LH or pulse amplitude. No differences were observed between the treatments for any of the LH parameters measured ($P > 0.2$).

Follicle growth

The pattern of ovarian dominant follicle development in individual cows is illustrated in Fig. 3. Mean follicle diameter was slightly larger in INS cows throughout the infusion period, but this difference was significant ($P < 0.05$) only on day 10 at the time of the start of infusion. There were no significant effects of treatment ($P > 0.1$) or treatment by time ($P > 0.1$). No difference was observed between INS and CTL cows in cumulative dominant follicle growth between days 10 and 14 (2.0 ± 0.9 vs $3.1 \pm 0.8 \text{ mm}$; $P > 0.3$). None of the dominant follicles of the first postpartum follicle wave ovulated or developed into cysts, and all ultimately became atretic.

IGF-I, IGFBP and NEFA

Circulating total IGF-I levels were not different between treatments at the beginning of the infusion period, and

Table 1 LH pulse data from the frequent blood samples collected prior to and during the infusion period.

	Insulin treatment							CTL treatment			
	BASE	START			END			BASE	END		
		Δ	S.E.M.	<i>P</i>	Δ	S.E.M.	<i>P</i>		Δ	S.E.M.	<i>P</i>
LH pulses (no./8 h)	3.60	0.20	0.37	0.62	0	0	1	4	-1.6	0.6	0.056
Mean LH (ng/ml)	0.56	-0.02	0.05	0.75	0.03	0.10	0.77	0.58	-0.07	0.05	0.24
Base LH (ng/ml)	0.38	0.01	0.03	0.62	0.03	0.03	0.40	0.38	0.005	0.02	0.85
Pulse amplitude (ng/ml)	0.57	-0.06	0.08	0.48	0.25	0.18	0.27	0.63	-0.06	0.12	0.64

BASE, START and END represent measurements taken during the 8 h period immediately prior to start of infusion, immediately after the start of infusion, and the final 8 h of infusion respectively. Treatment effects were examined as paired differences within individuals: START-BASE and END-BASE.

Δ = change relative to BASE.

remained constant in the CTL cows during the 96-h infusion period. Plasma total IGF-I concentration increased in response to insulin, and was 70% and 116% greater than values in CTL cows at 48 and 96 h respectively (treatment, $P < 0.05$; treatment by time, $P < 0.05$; Fig. 4). Circulating levels of free IGF-I were similar in both treatments at the start of the infusion period and remained constant in CTL cows during the infusion period, but increased in response to insulin (treatment, $P < 0.05$; treatment by time, $P < 0.05$; Fig. 4). At 48 h, free IGF-I in INS cows was approximately double that in CTL cows ($P < 0.01$).

The temporal profile of circulating levels of IGFBP is shown in Table 2. No differences between treatments

were observed for any of the binding proteins at 0 h. Plasma IGFBP-3 (40–44 kDa doublet) was significantly increased in response to insulin (treatment, $P < 0.05$; treatment by time, $P < 0.07$). Circulating levels of IGFBP-2 (34 kDa) were reduced in response to insulin (treatment, $P < 0.06$; treatment by time, $P < 0.001$). Insulin treatment also resulted in a reduction in the 29 kDa (treatment by time, $P < 0.001$) and 24 kDa binding proteins (treatment by time, $P < 0.05$).

Figure 3 Individual temporal profiles of growth of the dominant follicle during the study period. INS cows (top) were infused with insulin (0.3 μ g/kg body weight per h) and CTL (control) cows (bottom) were infused with saline (100 ml/h) for 96 h starting on day 10 post-partum. Follicles were measured by ultrasound once daily. Effects of treatment ($P > 0.1$) and treatment by time ($P > 0.1$) on mean follicle diameter were nonsignificant (pooled S.E.M. = 0.8 mm). The solid black bar represents the infusion period.

Figure 4 Plasma total and free IGF-I concentrations in INS and CTL cows during the 96-h infusion period. INS cows were infused with insulin (0.3 μ g/kg body weight per h), and CTL cows were infused with saline (100 ml/h). (a) Total IGF-I concentration in plasma was increased in response to insulin treatment (treatment, $P < 0.05$; treatment by time, $P < 0.05$). (b) The plasma concentration of free IGF-I was increased in response to insulin. Significant effects of treatment ($P < 0.05$) and treatment by time ($P < 0.05$) were observed. *Treatments differed significantly ($P < 0.05$) at the specified time. §Within-treatment differences ($P < 0.05$) relative to 0 h.

Table 2 Changes in plasma insulin-like growth factor-binding proteins (IGFBP) in hyperinsulinemic–euglycemic clamp and saline-infused cows over the 96 h infusion period.

	Insulin treatment			CTL treatment			Pooled S.E.M.	TRT	TRT × time
	0 h	48 h	96 h	0 h	48 h	96 h			
IGFBP-3	5224	6655 ^{‡§}	6356 ^{‡§}	4481	4956	4723	368	0.015	0.061
IGFBP-2	3722	1609 ^{‡§}	742 ^{‡§}	2881	3176	2499	315	0.054	0.001
29 kDa	396	297	195 [§]	298	335	289	38	0.8	0.001
24 kDa	286	208	155 [§]	210	210	219	11	0.9	0.012

Abundance of IGFBP is expressed in arbitrary densitometric units.

[‡] Treatments differ at corresponding time points ($P < 0.05$).

[§] Within-treatment differences compared to 0 h ($P < 0.05$).

Circulating NEFA levels were reduced in response to insulin ($P < 0.001$; data not shown). Over the duration of the infusion period, plasma NEFA levels in INS cows were reduced by 50% relative to values at 0 h, whereas in CTL cows NEFA levels averaged 96% of the value at 0 h.

Production data

Over the duration of the infusion period, average milk production (39.3 ± 0.6 vs 41.1 ± 0.6 kg/day; treatment, $P > 0.05$; treatment by time, $P > 0.5$) and dry matter intake (13.8 ± 0.6 vs 15.6 ± 0.6 kg/day; $P > 0.05$; treatment by time, $P > 0.4$) were not different between INS and CTL cows respectively. Energy balance was not different between treatments at the beginning of the infusion period (-17.9 ± 2.6 vs -19.4 ± 2.6 Mcal/day; $P = 0.7$). During the 96-h infusion period, energy balance was significantly improved in INS cows compared with CTL cows (-11.1 ± 2.6 vs -19.1 ± 2.6 Mcal/day; treatment, $P < 0.05$; treatment by time, $P < 0.05$). The greatest difference was detected at the end of the infusion period (-5.1 ± 2.6 vs -18.9 ± 2.6 Mcal/day; $P < 0.001$).

Discussion

The most striking observation in this study was the sustained increase in circulating estradiol in response to insulin treatment in the absence of a change in LH pulsatility. Possible mechanisms to explain this observation are as follows: (i) insulin had a direct stimulatory effect on overall ovarian steroid production; (ii) insulin had a direct stimulatory effect specifically on aromatization of androgen to estrogen; (iii) insulin increased ovarian responsiveness to another circulating factor (such as LH or IGF-I); (iv) insulin had an indirect effect via altering circulating levels of another factor (such as NEFA or IGF-I).

Numerous reports from multiple species indicate that insulin has direct stimulatory effects on *in vitro* granulosa cell estradiol production, and indirect stimulatory effects via amplification of gonadotropin action (Poretsky & Kalin 1987, Spicer & Echternkamp 1995, Gutierrez *et al.* 1997, Poretsky *et al.* 1999, Silva & Price 2002). However, reports demonstrating similar stimulatory effects *in vivo* are less

abundant. This is probably due, at least in part, to insulin-induced hypoglycemia when insulin is administered without cotreatment with glucose. The associated counterregulatory neuroendocrine responses to hypoglycemia may override any beneficial effects of insulin. Nevertheless, in beef cows, twice daily injections of insulin concomitant with a superovulation regimen resulted in a fivefold increase in estradiol concentration in large follicles and a twofold increase in the percentage of estrogen-active follicles compared with cows injected with saline (Simpson *et al.* 1994). Similarly, insulin administration to goats prior to or during a superovulation protocol resulted in a marked increase in follicular estradiol output (Selvaraju *et al.* 2003). Pigs treated with insulin for 2 days had increased ^{125}I -hCG binding to granulosa cells, and increased follicular fluid content of estradiol and testosterone (Matamoros *et al.* 1990). Daily insulin administration to rats for 22 days significantly reduced the ratio of androstenedione to estrone (an indicator of aromatase activity; Poretsky *et al.* 1988). Similarly, in the current study, the ratio of testosterone to estradiol was reduced, indicating that aromatase activity was increased. Interestingly, in the study of Poretsky *et al.* (1988), specific ^{125}I -insulin binding to ovarian tissue homogenates was lower in insulin-treated than saline-treated rats, whereas specific ^{125}I -labeled IGF-I binding was significantly increased. IGF-I and IGF-II are more potent than insulin at inducing LH receptors on granulosa cells and stimulating steroidogenesis (Davoren *et al.* 1986). Thus, insulin induced upregulation of type I IGF receptors would facilitate increased responsiveness to combined stimulation of ovarian steroidogenesis by IGF-I, IGF-II and insulin.

Circulating total and free IGF-I was increased in the current study in response to insulin infusion, and the attendant improvement in energy balance, in agreement with our previous findings using a higher insulin dose (Butler *et al.* 2003). In contrast to our earlier report, where an eightfold increase in insulin elevated circulating IGF-I by 400%, we found that a more moderate 2.6-fold increase in insulin required longer to stimulate higher circulating IGF-I, and the effect observed was more moderate (116% increase). The latter, more modest increase in IGF-I would more closely resemble the slow rate of recovery in circulating IGF-I typically observed in postpartum dairy cattle.

Of note, alterations in circulating IGFBP were only slightly reduced in the current study compared with the previous higher dose. It is likely that the shift in relative proportions of IGFBP-2 (decreased) and IGFBP-3 (increased), with half-lives of 30–90 min and 12–15 h respectively (Jones & Clemmons 1995), contributed to the increased circulating IGF-I in this study. The temporal profile of the alterations in free IGF-I are similar to our previous report (Butler *et al.* 2003). It is clear that increased IGF-I is beneficial to ovarian steroidogenesis, and thus may represent an important indirect means by which insulin increased estradiol synthesis.

Plasma NEFA is markedly elevated in dairy cows during early lactation, but glucose is the principal metabolic fuel of the ovary, and NEFA does not appear to be used in normal circumstances (Rabiee *et al.* 1997). Concentrations of NEFA in plasma and follicular fluid are closely related, and a negative relationship between follicular concentrations of NEFA and estradiol has been demonstrated (Comin *et al.* 2002, Jorritsma *et al.* 2003). Insulin action in target tissues is impaired by high circulating NEFA (Bajaj *et al.* 2002, Boden *et al.* 2002). Importantly, Glut4 – the insulin-responsive glucose transporter – is found in both granulosa and theca cells (Williams *et al.* 2001), indicating an important role for insulin in stimulating ovarian glucose uptake. During NEB, when both insulin and glucose are depressed and elevated NEFA is antagonistic to insulin action, ovarian activity may be suppressed due to inadequate uptake of oxidizable fuel. In addition, it has been demonstrated that the saturated fatty acids palmitate and stearate induce apoptosis in granulosa cells (Mu *et al.* 2001). In the current study, insulin-induced suppression of plasma NEFA may have removed inhibitory effects of NEFA.

An elegant study by Downing *et al.* (1999) on ewes with an autotransplanted ovary failed to indicate any positive effect of insulin on steroidogenesis or LH release. However, that report was different from the current study in a number of key aspects. Firstly, the insulin infusion lasted only 13.5 h. In our study, we did not notice an effect of insulin on steroid production until beyond 30 h of infusion, implying that the positive effects of insulin are of a chronic rather than an acute nature. This would be in accordance with observations from others (Poretsky *et al.* 1988). Second, the infusions carried out with the autotransplanted ovary specifically affect the local arterial and venous system associated with ovarian tissue, with minimal effects on the peripheral concentrations of insulin. Thus, if an insulin-induced alteration of another factor (such as IGF-I or NEFA) is involved in increasing ovarian steroid output, the closed arterial infusion would not allow such an effect to be observed. Third, the cows in our study were experiencing the most severe period of NEB encountered during a gestation–lactation cycle; consequently, insulin levels were at their lowest when the infusions were carried out. Positive effects of insulin *in vivo* may not be observed above some threshold circulating level.

It is also possible that insulin itself acted as a survival signal for follicles that were otherwise destined to undergo atresia at an earlier time, as occurred in the saline-treated cows. Both insulin and IGF-I directly suppress apoptosis in rat preovulatory follicles (Chun *et al.* 1994). Treating pigs twice daily with insulin injections reduced the number and percentage of atretic follicles (Matamoros *et al.* 1990). In diabetic gilts, withdrawal of insulin therapy results in a significant increase in atretic follicles when compared with insulin-treated or nondiabetic gilts (Edwards *et al.* 1996).

It has recently been demonstrated that central actions of insulin are necessary for GnRH synthesis and/or secretion in mice (Brüning *et al.* 2000). Studies in sheep have indicated that dietary supplementation with lupin grain (a highly digestible energy and protein source) stimulates gonadotropin release. The increase in LH pulse frequency is associated with increased circulating and cerebrospinal fluid concentrations of insulin (Miller *et al.* 1998), and intracerebroventricular infusion of insulin to rams on a maintenance diet mimicked the effect of the lupin grain supplement (Miller *et al.* 1995). These results clearly imply that insulin is involved in the increase in LH pulse frequency in the wake of improved nutritional status in sheep. We found no evidence to indicate that a chronic elevation in circulating insulin during early lactation has a beneficial effect on LH pulsatility in dairy cows. In agreement with our findings, comprehensive studies in macaques (Schreihöfer *et al.* 1996, Williams *et al.* 1996) indicate that the depressed plasma insulin and glucose encountered during undernutrition are not responsible for the reduced LH pulsatility. Those authors found that nutrient intake per se, rather than circulating concentrations of glucose or insulin, plays the principal role in restoring LH pulsatility following undernutrition. However, the status of the early-lactation dairy cow is different from fasting or undernutrition insofar as intake is actually increasing, but energy balance remains negative for an extended period due to the energetic costs of rising milk production. There is evidence to indicate that a lingering effect of pregnancy acts to restrain GnRH/LH release regardless of energy balance status in early lactation (Canfield & Butler 1991). Further work is necessary to characterize the causal agent(s) responsible for the suboptimal GnRH/LH release during early postpartum NEB in the cow.

In conclusion, this study demonstrated that a chronic increase in circulating insulin during lactation-induced NEB results in reduced circulating NEFA, and increased circulating concentrations of both total and free IGF-I in conjunction with a shift from low MW IGFBP to the ternary complex. This altered metabolic environment during the first postpartum follicular wave resulted in increased circulating estradiol associated with a reduced ratio of testosterone to estradiol, indicating that aromatase activity was increased. This increase in circulating estradiol occurred independently of any change in LH pulsatility,

implying that hypoinsulinemia is not responsible for the reduced LH pulse frequency observed in early lactation.

Acknowledgements

Partial support of this study was provided by funds allocated to US Department of Agriculture regional project NE-161. Technical advice from D.E. Bauman and D.A. Dwyer and assistance from W. English and B. Berggren-Thomas are greatly appreciated. Antisera to human IGF-I were obtained through NHPP, NIDDK and Dr A.F. Parlow. Antisera to bovine LH was a generous gift from R.B. Staigmiller. S.T. Butler was the recipient of a Walsh fellowship from Teagasc (Irish Agriculture and Food Development Authority) in support of his PhD program.

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Received 6 November 2003

First decision 20 January 2004

Accepted 12 February 2004