Lipoprotein lipase in human milk: compartmentalization and effect of fasting, insulin, and glucose

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Abstract The object of this study was to investigate the effect of maternal metabolic state on the activity of lipoprotein lipase (LPL) in human milk. Although the total LPL activity in milk was not significantly affected by up to three cycles of freezing and thawing, the amount of LPL associated with the cream fraction of the milk increased from an average of less than 10% to about 70% after this treatment. The enzyme was relatively stable when the milk was stored on ice, losing activity at a rate of about 1% per hour. At 37°C degradation was more rapid, about 7% per hour. When LPL activity was measured in samples taken at hourly intervals by breast pump, using oxytocin to achieve a complete letdown at each pumping, activity was found to double from the first to the third pumping. Thereafter the activity was stable under fasting conditions. Hyperglycemic and euglycemic, hyperinsulinemic glucose clamp protocols were used to evaluate the effects of glucose and insulin. Both high plasma glucose and high plasma insulin in the presence of normal glucose significantly increased LPL activity within 4 hours. We conclude that, like adipose tissue LPL, mammary LPL is regulated by plasma insulin. —**Neville, M. C., L. J. Waxman, D. Jensen, and R. H. Eckel.** Lipoprotein lipase in human milk: compartmentalization and effect of fasting, insulin, and glucose. *J. Lipid Res.* 1991. **32:** 251–257.

Supplementary key words human lactation • milk lipase • glucose clamp

Human milk contains two lipases (1, 2), a bile salt-stimulated lipase and lipoprotein lipase (LPL). Bile salt-stimulated lipase is present in sufficiently high concentrations to play a role in lipid digestion in the intestine of the infant (3, 4). Lipoprotein lipase is present at high concentration in the milks of many species including the cow (5), mouse (Neville, M. C., unpublished data), and human (2). It is also found in very high concentrations in the lactating mammary gland, reaching levels more than 3 times those of nonmammary adipose tissue in the virgin rat (6–9). The enzyme is bound to heparan sulfate proteoglycans on capillary walls (10, 11) where it hydrolyzes triglycerides in chylomicrons and very low density lipoproteins, making their component fatty acids available for synthesis into milk lipid. There appears to be reciprocal regulation of adipose and mammary LPL and the mammary level declines rapidly after cessation of suckling (7, 9). The function of milk LPL is unknown; the enzyme is probably not active in milk due to the absence of the serum factor, apolipoprotein C-II, necessary for its activity (12).

Although the effects of lactational stage on mammary and adipose LPL have been investigated in a number of laboratories (7, 13–17) and the metabolic regulation of adipose tissue LPL has received wide investigation (11), there has been little systematic study of the metabolic regulation of the enzyme in the mammary gland or in milk. Such information is important because of its possible bearing on the regulation of lipid secretion into milk. For this reason we determined the LPL activity in milk samples collected hourly during a study designed to determine the effects of short-term fasting, as well as maternal levels of insulin and glucose on human milk production (Neville, M. C. and W. W. Hay, Jr., unpublished). We found that the first samples obtained after an overnight fast had much lower LPL activity than later samples. The degradation of human milk LPL at 37°C was investigated to determine whether degradation in the breast could account for this finding. The effects of insulin and glucose suggest that the enzyme in human milk is subject to metabolic regulation: specifically, it is increased in response to increases in plasma insulin.

Abbreviations: LPL, lipoprotein lipase.

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METHODS

Collection and storage of milk samples

For testing of assay and storage conditions as well as stability of human milk LPL, up to 50 ml of milk was obtained by electric breast pump (Medela, Inc., Crystal Lake, IL) from lactating volunteers between 8 and 10 AM after one or two morning breast-feeds. Milk from both breasts was pooled and maintained on ice until used within 2 h for assay or treatment according to the protocol stated. When used, skim milk was obtained by aliquoting the milk into a 1.5-ml microfuge tube and centrifuging at 3,000 *g* at 4°C for 20 min. The skim was extracted with a syringe and needle from the bottom of the tube.

For effects of fasting, insulin, and glucose, milk samples were collected hourly (18), using a Medela breast pump and nasal oxytocin to obtain a complete letdown as described below. Samples from these experiments were kept on ice no longer than 7 h, then stored in 0.5-ml aliquots at −70°C. Samples were thawed on ice immediately prior to assay and were not refrozen. Samples were carefully mixed by gentle and repeated inversion before an aliquot of the whole milk was taken for assay as below.

Protocol for effects of fasting, insulin, and glucose

Lactating women were asked to check into the Clinical Research Center in University Hospital at 7:00 AM after an overnight fast. Intravenous lines were inserted into arm or hand veins for blood sampling and infusion of insulin and glucose. Isotonic saline was infused at a rate sufficient to keep the vein open. The breasts were pumped at hourly intervals using a Medela breast pump with two heads to pump both breasts simultaneously. After pumping for 10 min, the subject received one drop of Syntocinon (Sandoz) intranasally and pumping was continued for another 5 min or until milk flow ceased. Milk samples were immediately placed on ice. A 10-ml sample from each breast was retained for later aliquoting and freezing; the remainder was returned to the mother for later use for the infant. This procedure was repeated on an hourly basis for 6 to 9 h. Noncaffeinated diet beverages were permitted during the course of the experiment, but no food or coffee. In order to assess the effects of insulin and glucose, in some subjects infusions of glucose or glucose and insulin were started after 2 h of control measurements had been completed. To assess the effects of insulin, a continuous infusion of 40 mU/m^2 per min of insulin was begun; blood glucose was assayed every 5 min and the rate of glucose infusion was adjusted to maintain the fasting glucose level. In a separate set of experiments, no insulin was infused, but glucose was infused at a rate sufficient to maintain the glucose level at twice the basal concentration.

LPL assay

The technique for routine assay of LPL was modified from Eckel et al. (19). Ten μl of milk was diluted in 2 ml of Krebs-Ringer phosphate buffer containing 4 μg/ml heparin to stabilize the enzyme. One hundred μl of this solution was incubated with 100 μl of an emulsion containing [^{14}C]triolein (0.063 μCi), 7.5% human serum as a source of apo-C-II, 2.0% serum albumin, 3.0 mM triolein, and 0.04 M Tris buffer for 30 min at 37°C. The reaction was stopped with the organic mixture of Belfrage and Vaughn (20) and 50 mM $NaHCO_2$ (pH 10.5) was added to allow extraction of free fatty acid into the aqueous phase. An aliquot of this phase was counted with scintillation fluid on a Beckman β-scintillation counter. A control using [^{14}C]oleic acid was run with each experiment to determine extraction efficiency. The activity of the enzyme was calculated as described by Eckel et al. (19) in nmol of triglyceride hydrolyzed per minute per ml of milk. Krebs-Ringer phosphate buffer was run as a blank.

Other assays

Milk protein was measured by the BCH assay (Pierce Biochemical) as previously detailed (21). Plasma glucose was measured with the Yellow Springs Instrument glucose analyzer and plasma insulin by radioimmunoassay.

Statistical analysis of data

Samples were run in duplicate. The intraassay coefficient of variation was 7–10%. Because of a larger interassay variation, when hourly samples from one woman were analyzed they were always run in the same batch. Values from right and left breasts were averaged prior to further analysis. Small sample *t*-test was used for statistical comparisons. These protocols were approved by the Institutional Review Board for Human Subjects of the University of Colorado Health Sciences Center.

RESULTS

Lipoprotein lipase activity in fresh milk

Fig. 1 shows the LPL activity in fresh whole and skim milk or colostrum samples from five subjects. LPL activity varied considerably from subject to subject, the lowest value being 125 nmol/ml per min and the highest being 711 nmol/ml per min. The reason for the variation between subjects was not readily apparent from these samples. Although the samples were run on separate days, the interassay variation does not account for the nearly sixfold difference. The variation does not seem to be a function of duration of lactation since the colostrum sample (sample 2) fell within the range of the other samples, taken at 3 to 4 months of lactation. Similar values have been reported by others (22, 23).

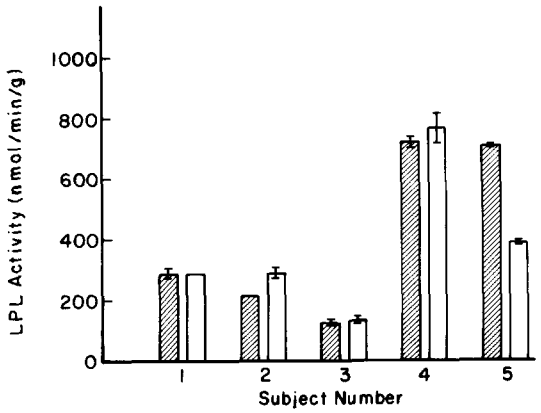

Fig. 1. LPL activity in fresh whole and skim milk samples from five individuals. Milk samples were obtained by breast pump between 7 and 9 AM after the first morning feed and immediately transported on ice to the laboratory. The duration of lactation was 3 months, 3 days, 4 months, 3 months, and 4 months, respectively, for subjects 1 through 5; Hatched bars, whole milk; open bars, skim milk.

For four subjects there was little difference between the LPL activity in whole and skim milk. However, in the fifth subject skim milk had only about half the activity available from whole milk, suggesting substantial association of the LPL with the cream fraction. In some cases we analyzed the cream separately and found the activity to be low (< 10 nmol/ml per min). However, the cream fraction is very difficult to handle and, in any case, competition between the triglycerides of the cream and the substrate cannot be ruled out. For this reason, the association of LPL with the cream is best inferred from the difference between the whole and skim milk fractions. Others have found the majority of LPL to be associated with the cream fraction of human milk (2). However, they used milk that had been frozen and thawed for their determination. For this reason and to reaffirm the results of others that human milk samples could be frozen without substantial loss of LPL activity (22), we next examined the effects of freezing and thawing on LPL activity and distribution.

Effect of freezing and thawing on LPL activity in human milk

The effect of repetitive freezing and thawing on the activity of LPL in four of the fresh milk samples of Fig. 1 was assessed. Although the mean activity fell to 91% of the initial activity with one cycle of freezing and thawing, this fall was not statistically significant and there was no significant further decline in activity. We concluded that milk samples could be stored frozen and thawed without significant loss of activity if the temperature were not allowed to rise during the thawing process. Stored samples were routinely thawed only once prior to assay for LPL. Others have found that LPL activity is stable in human milk samples stored at -70°C (22).

Three of the whole milk samples that were subjected to repeated freezing and thawing were centrifuged after each thawing cycle to obtain the corresponding skim milk fraction. The LPL activity in these samples is compared to the corresponding whole milk activity in **Fig. 2**. After repeated freezing and thawing, only about 30% of the LPL remained in the skim milk fraction; the remainder was presumably associated with the cream fraction. Thus, in samples that have been stored frozen, a substantial amount of LPL can be expected to be associated with the cream fraction. To avoid problems of redistribution of LPL after freezing, in subsequent experiments, we routinely assayed whole milk samples.

Stability of human milk LPL activity at 0°C and 37°C

Fig. 3 shows the effect of incubation at 0°C and 37°C on LPL activity in three of the milks of Fig. 1. The data were normalized to the concentration of LPL present at time zero, then averaged across women and plotted on a semi-log scale. At 0°C activity declined slowly with a first order rate constant of 0.012 (about 1.2% per h). At 37°C the activity declined more rapidly with a rate constant of 0.072, a loss of about 7% per h. Based on these results we concluded that milk samples could be held for up to 8 h on ice with no more than a 10% loss of activity.

LPL activity in fasting women

Eight women who had fasted overnight pumped their breasts at hourly intervals using the protocol described in methods. The mean activity at each time point is graphed in **Fig. 4**. Large milk volumes, up to 200 ml, were usually obtained at the first and second pumpings, representing residual milk stored within the breast (18). This milk had a relatively low LPL activity, 65 ± 19 (SE) (range 1 to 208). From the third pumping on, the volume pumped each hour was constant at about 30 ml and was equivalent

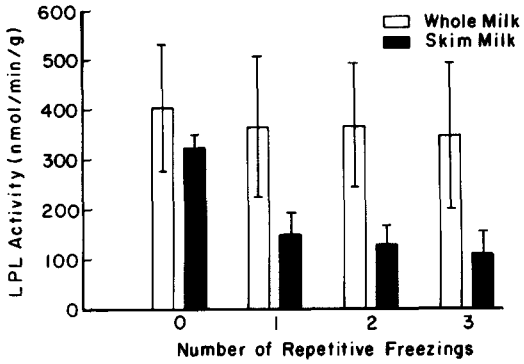

Fig. 2. Effect of freezing and thawing on LPL activity of human milk. Fresh milk samples were frozen at -20°C for 45 min, then thawed on ice just until completely liquid. The whole milks were then assayed directly for LPL, centrifuged to remove the cream prior to assay, or refrozen to repeat the cycle. Three fresh milk samples from different women were subjected to this treatment.

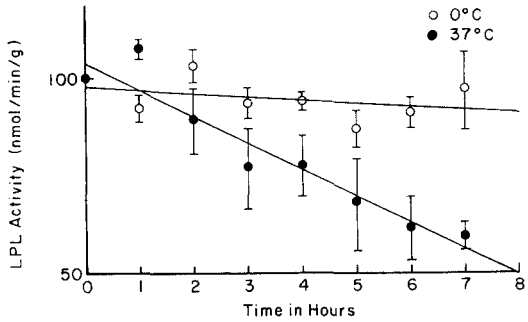

Fig. 3. Effect of storage at 0°C and 37°C on human milk LPL activity. Duplicate milk samples were held on ice or in a 37°C water bath. Aliquots were removed at hourly intervals and the LPL activity was determined. The data were normalized by dividing by the LPL activity present at time zero and multiplying by 100. Each point represents the mean normalized LPL activity in milk samples from three women. The lines are theoretical according to the equation $A_t = A_o \exp(-kt)$ where A_t and A_o are the activities at time t and at the start of the experiment, respectively and k is the first order rate constant amounting to 0.0115 at 0°C and 0.0719 at 37°C. The ordinate scale is logarithmic.

to the milk ingested on the previous day by the infant (determined by test-weighing the infant before and after each feed (18)). There was no effect of fasting on milk volume or proximate composition (M. C. Neville and W. W. Hay, Jr., unpublished data). The LPL activity in the milk began to plateau after 2 h and reached a maximum of 269 ± 54 (SE) (range 151–429) at 6 h (**Fig. 5**). The decline after 5 h was not statistically significant and was more marked in some women than others.

Effect of hyperglycemia and hyperinsulinemia on milk LPL

In order to determine whether milk LPL is affected by maternal metabolic state, we infused either glucose alone (hyperglycemic clamp) or glucose and insulin (euglycemic, hyperinsulinemic clamp) after a 2-h control period in women fasting as described above. The overall pattern of LPL activity during the first 4 h of this experiment was similar to that observed in the fasting women (data not shown). We therefore used the 2-h LPL activity as reference level and determined the ratio of the LPL activity in the milk obtained at 5, 6, and 7 h after the start of the experiment to the activity at 2 h. The data are given in **Table 1** along with the corresponding plasma insulin and glucose levels. The LPL activity is plotted in Fig. 5. Although the insulin infusion had been stopped at 6 h in the hyperinsulinemic clamp, we considered that the milk obtained at 7 h would still reflect the effects of the earlier insulin treatment and have, therefore, included the analysis of the two milk samples that were available.

In the fasting women the insulin levels were around 4 μU/ml and the plasma glucose about 70 mg/dl (Table 1). At hours 5, 6, and 7, LPL activity was not significantly different from hour 2 (see also Fig. 4). With the hyperglycemic clamp the plasma glucose was maintained around 144 mg/dl from hours 4 to 7 in six subjects. This high glucose stimulated the secretion of endogenous insulin bringing the insulin concentration to about 24 μU/ml during hours 5 through 7. By hours 6 and 7 the milk LPL activity was 50% greater than both the activity in the 2-h sample and the corresponding activity of the fasting controls. These observations suggested that either insulin, glucose, or both stimulate the production of mammary LPL as monitored in the milk.

With the euglycemic, hyperinsulinemic clamp, the mean plasma insulin was about 75 μU/ml but the plasma glucose was not different from the control. By hour 6, 2 h after the onset of the insulin infusion, the milk LPL was 40% above the 2-h level in these subjects, a value that was significant at the $P<0.05$ level and significantly above the fasting controls as well ($P<0.1$). By hour 7 the activity had jumped to nearly 3 times the control (Fig. 5).

There was no change in the rate of milk secretion or its composition (M. C. Neville and W. W. Hay, Jr., unpublished data) in response to insulin and/or glucose infusion. The protein content of the milk actually diminished slightly during the experiment in all groups. For example, with the euglycemic, hyperinsulinemic clamp, the milk protein concentration decreased from 9.2 mg/ml at the start of the experiment to 8.2 mg/ml after 4 h of insulin infusion. Changes in the milk protein content of a similar magnitude were observed in fasting women and women undergoing the hyperglycemic clamp. Thus, the effect of insulin on LPL activity is not part of a generalized stimulation of milk protein synthesis.

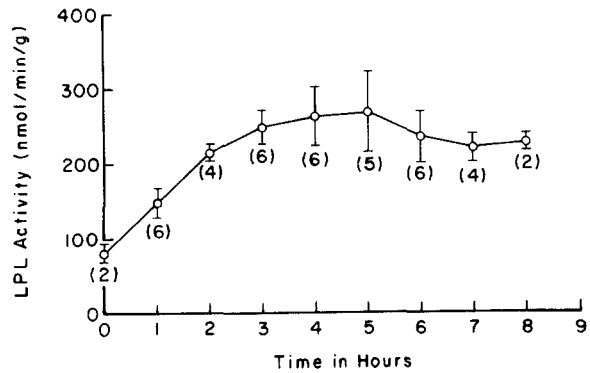

Fig. 4. Time course of LPL activity in milk from fasting women. Seven women were asked to fast overnight. They were then admitted to the Clinical Research Center of University Hospital where their breasts were pumped hourly as described in Methods. Milk samples were held on ice until they were aliquoted, frozen, and stored at −70°C until assay. In these experiments samples were available from both right and left breasts and samples were assayed separately. Since there was no consistent difference, the activity in samples from the right and left breasts was averaged for each time point. The numbers under the curve represent the numbers of women whose milks were averaged for that time point.

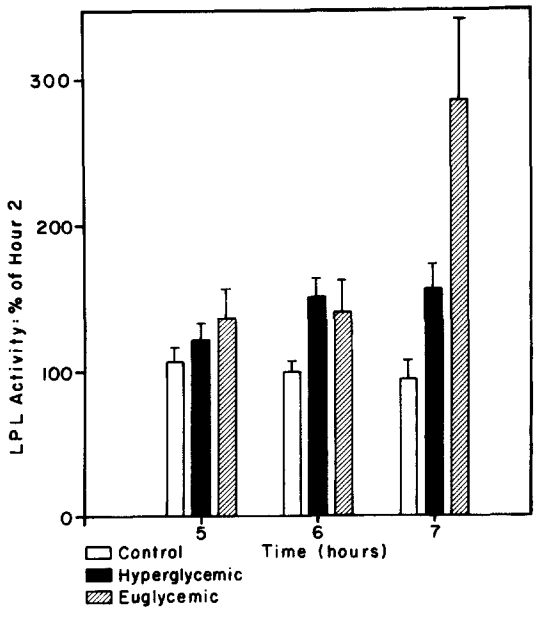

Fig. 5. Effect of insulin and glucose on milk LPL in fasting women. Women were infused with saline (□), glucose (■), or glucose and insulin (▨) and milk samples were taken hourly as described in the text. The data were normalized to the milk LPL activity in the 2-h sample taken prior to beginning the infusions. Plasma glucose and insulin levels are shown in Table 1.

DISCUSSION

The relation between the concentrations of LPL in milk and mammary tissue is unknown. Clearly there are species differences since, in rats, milk LPL is nearly undetectable, although there are high concentrations of LPL in the tissue itself (7). In most other species milk contains substantial LPL activity. In humans, earlier studies have shown that LPL is not present in the antepartum secretion of the mammary gland (4). During pregnancy the rate of milk synthesis is very slow; it is possible that the small amounts of substrate needed to provide the long chain fatty acids present in the prepartum secretion could be obtained from circulating free fatty acids bound to serum albumin. It is also possible that any LPL present is degraded during the relatively long sojourn of the milk within the ducts and mammary alveoli. Milk LPL increases immediately postpartum as milk fat synthesis is initiated (24). The decrease in milk volume attendant on gradual weaning is associated with a substantial decrease in LPL activity in the milk (25) suggesting a relation between the rate of milk fat synthesis and LPL concentration in the milk. Milk from mothers of preterm infants had lower LPL activity than milk from full-term mothers (24). Whether this is related to a lower volume of milk production and therefore a lower rate of lipid synthesis in the preterm mothers is not clear. In any case animal studies to determine the relation between milk and mammary tissue LPL would be useful.

Compartmentalization and storage of human milk

A puzzling finding has been that the LPL in human milk appears to be associated with the lipid fraction of the milk (2, 26) rather than the aqueous fraction as is the case with bovine milk (5). The results of the current study suggest that in the majority of fresh human milk samples the lipoprotein lipase resides in the aqueous fraction, becoming associated with the lipid fraction only after freezing and thawing. This treatment breaks down the plasma membrane surrounding the milk fat globules (27), expos-

TABLE 1. Effect of glucose and insulin on LPL activity in human milk

Treatment	Fasting	Hyperglycemic Clamp	Euglycemic Clamp
Plasma insulin (μU/ml)			
Hour 5	4.1 ± 0.5 (8)	25.6 ± 6.3[a] (6)	76.9 ± 8.4[a] (10)
Hour 6	4.4 ± 0.6 (8)	26.1 ± 2.7 [a] (6)	78.1 ± 6.4 [a] (10)
Hour 7	3.4 ± 0.6 (5)	22.2 ± 3.7 [a] (6)	–[b]
Plasma glucose (mg/dl)			
Hour 5	72.3 ± 1.5 (8)	145.3 ± 3.7[a] (12)	73.0 ± 6.4 (10)
Hour 6	69.1 ± 1.7 (8)	143.6 ± 2.7[a] (12)	74.5 ± 8.3 (10)
Hour 7	70.0 ± 2.1 (5)	144.7 ± 3.2[a] (5)	–[b]
LPL activity (percent of hour 2)			
Hour 5	106.6 ± 9.6 (5)	121.7 ± 11.4 (10)	136.3 ± 20.0 (9)
Hour 6	99.2 ± 7.3 (6)	151.0 ± 12.8[a] (9)	140.7 ± 21.7[c] (8)
Hour 7	94.3 ± 13.2 (4)	156.4 ± 16.9[a] (7)	284.3 ± 65.1[a] (2)

All numbers are mean ± SE (number of women).
[a]Different from fasting control $P < 0.005$.
[b]No blood samples were available at 7 h from the euglycemic clamps as the insulin infusion had been stopped at 6 h.
[c]Different from fasting control $P < 0.1$.

ing the triglyceride core to the enzyme. Presumably association of the enzyme with the naked milk fat droplet is facilitated by the lipid binding site of the enzyme (28).

Although freezing and thawing changed the distribution of the enzyme within the milk, it did not significantly alter the activity of the enzyme in whole milk, confirming the earlier findings of Berkow et al. (22). This also assured us that the samples we had stored at −70°C were adequate for LPL analysis.

Stability of LPL in human milk

The loss of LPL activity at 37°C can probably be explained by the presence of endogenous proteases that gradually break down the enzyme as shown by Soccorro and Jackson (29). This breakdown may provide a major explanation for the marked variability of LPL activity between samples of human milk as demonstrated in the samples shown in Fig. 1. If LPL in milk within the breast breaks down at the same rate as in vitro, the activity would be reduced by one-half within 10 h. This time is comparable to the time that milk may remain in the breast, particularly in the absence of a night feed. Thus, part of the variability of LPL in human milk can be explained by the time since the breast was last emptied. However, not all variability can be explained in this way since the LPL activity in the milks of the fasting subjects varied nearly threefold even after several episodes of hourly pumping.

Effect of fasting

Once the LPL activity had stabilized in the milks of the fasting women (about the third episode of pumping), there appeared to be little further decline in activity over the succeeding 6 h of the experiment. Whether this indicates that mammary LPL is less affected by fasting than adipose tissue LPL or whether the activity had, in fact, stabilized after the overnight fast is not clear. However, it is worth noting that the mean level of LPL in the women who had not fasted (Fig. 1) was about double the activity in the milks of the fasting subjects. LPL activity in adipose tissue from human volunteers was low in the morning after an overnight fast and increased by 50% after several small meals (30).

Effects of hyperglycemia and hyperinsulinemia on milk LPL

A previous study suggested that milk LPL was subject to diurnal variation with an increase in activity toward mid-day (23). The basis of this observation may be changes in plasma insulin attendant on eating since, in the present study, increases in both glucose and insulin were associated with significant increases in milk lipoprotein lipase, compared to fasting subjects. These observations suggest that mammary lipoprotein lipase is regulated by plasma insulin concentrations. Insulin is thought to be an important regulator of LPL in adipose tissue. In normal weight humans, 6 h of infusion of insulin and glucose increased adipose tissue LPL two- to fivefold (31). Insulin has also been observed to stimulate the rapid release of LPL from 3T3-L1 cells, an action that appears to be independent of energy metabolism and protein synthesis (32, 33). It also increased LPL synthesis and mRNA in adipocytes (11, 34). Thus the finding in this study that milk LPL increases in response to insulin suggests that mammary LPL is regulated in a manner similar to adipose tissue LPL.

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