Prolactin's Effects on Lipoprotein Lipase (LPL) Activity and on LPL mRNA Levels in Cultured Mouse Mammary Gland Explants

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Abstract. **The *in vitro* effects of prolactin (PRL) on lipoprotein lipase (LPL) activity and on LPL mRNA levels were studied in cultured mammary tissues derived from mid-pregnant mice. Mouse mammary gland tissues were initially incubated for 24 hr in M199 media containing 1 μg/ml insulin and 10^{-7} *M* cortisol. A subsequent treatment of the tissues with 1 μg/ml PRL caused a 76% increase in heparin-releasable LPL (hrLPL) activity after 24 hr. A significant increase in LPL activity was detected 16 hr after PRL addition, but not at earlier times. PRL at 100 ng/ml elicited a maximum stimulation of LPL activity. When Northern hybridization techniques were employed, PRL was also found to increase the tissue content of LPL mRNA; this effect was initially detected after a 6-hr PRL treatment employing PRL concentrations of 50 ng/ml and above. Specificity studies revealed that only lactogenic hormones stimulated LPL activity and LPL mRNA accumulation in cultured mammary tissues. PRL also expressed a small (25% increase), but significant, effect on ATP citrate-lyase activity in mammary tissues cultured for more than 6 hr with the hormone.** [P.S.E.B.M. 1997, Vol 214]

Lipoprotein lipase (LPL) and ATP citrate-lyase (ACL) are two important enzymes involved in lipogenesis. Lipoprotein lipase (EC 3.1.1.34), a glycoprotein with a molecular weight of 34,000–73,000 Daltons (1), bears two N-oligosaccharide side chains. It is present in tissues that utilize triacylglycerol fatty acids; these tissues include heart, adipose tissue, and lactating mammary gland (2). LPL catalyzes the hydrolysis of plasma triacylglycerols and thereby regulates the uptake of fatty acids derived from chylomicrons and vLDL in the circulation (3). In the mammary gland, mammary epithelial cells are known to secrete the lipase into milk; however, recent studies indicate that LPL's primary site of synthesis is in stromal cells, probably primarily in fat cells (4). The cells that synthesize the LPL likely release it for transfer to endothelial sites, where it binds to glycan chains of heparin sulfate proteoglycans (5) and functions within the lumen of capillaries.

LPL has an important role in adipose and muscle tissue in the removal of triacylglycerol fatty acids from the plasma (6). During lactation, the reciprocal effects take place in adipose tissue and mammary gland, where LPL activity is reduced and increased, respectively. LPL activity can be altered in a tissue-specific manner, which is physiologically important because it directs fatty acid utilization according to the metabolic demands of individual tissues. Specific hormones regulate LPL activity. Insulin activates preexisting lipoprotein lipase at a posttranscriptional and posttranslational level, but it does not affect gene expression (7). *In vivo* studies indicate that hormones derived from the pituitary gland suppress LPL activity in adipose tissue and stimulate LPL activity in the mammary gland, thus diverting dietary fatty acids from body fat stores to the mammary gland for the synthesis of milk fat (8). Furthermore, Jensen *et al.* (4) proposed a model for LPL transport in the mammary gland and concluded that LPL is regulated primarily at the translational and posttranslational level. However, no information is available concerning effects of hormones,

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including prolactin (PRL), on LPL activity and LPL mRNA accumulation in cultured mammary tissues.

Animal ACL (EC 4.1.3.8) is a cytosolic enzyme that plays a role in transferring acetyl CoA from mitochondria to cytosol for the synthesis of fatty acids and cholesterol employing citrate as a carrier molecule (9). The reaction catalyzed by this enzyme is as follows:

$$\text{citrate} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{2+}} \text{acetyl CoA} + \text{ADP} + \text{Pi} + \text{oxalocetate}.$$

ACL is widely distributed in animal tissues (10), and its enzymatic activity varies with factors such as diet and age (11). Several papers also report specific hormone effects on phosphorylation and accordingly perhaps activation of ACL (12). We therefore undertook a study to determine if PRL regulates ACL activity in cultured mouse mammary tissues.

Materials and Methods

Incubation System. Midpregnant (10–14 days of pregnancy) Swiss-Webster mice were purchased from Harlan Laboratories (Indianapolis, IN). Ovine PRL (NIH-P-S-14) was a gift from NIH. Other substances were from the following sources: cortisol from Charles Pfizer (New York, NY); medium 199 with Earle's salts (M199) and Hanks' balanced salt solution (HBSS) from GIBCO Laboratories (Grand Island, NY); porcine insulin, penicillin, and streptomycin from Eli Lilly Co. (Indianapolis, IN); glycerol trioleate, egg lecithin, 10% fatty-acid-poor albumin, ATP, CoA, DPNH, malic dehydrogenase, and dithiothreitol (DTT) from Sigma Chemical Co. (St. Louis, MO); TRI reagent and BCP from Molecular Research Center, Inc. (Cincinnati, OH); and [α-^{32}P] CTP from Dupont Corp. (Wilmington, DE). The plasmids containing the mouse cDNA probes were gifts from the following: mouse β-actin from Dr. David Smith of Wayne State University; and LPL from Dr. Michael C. Schotz of Wadsworth Medical Center. Methods used to culture tissues in our laboratory were described earlier (13). Briefly, mice are sacrificed by cervical dislocation, and the caudal and inguinal pair of mammary glands are removed aseptically and placed in HBSS. The glands are cut into pieces weighing 3–5 mg and placed on siliconized lens paper floating on 6 ml of M199 containing insulin (1 μg/ml) and cortisol (10^{-7} M) in sterile tissue culture dishes. All incubations are carried out at 37°C in an atmosphere of 95% air/5% CO_2 (v/v). All explants initially undergo a 24-hr incubation in M199 containing insulin and cortisol. Experiments are then begun by adding fresh culture medium alone or plus 1 μg/ml PRL.

LPL Assay. LPL activity was assessed by the following methods. Mammary gland tissue from three to five plates was collected and placed in ice-cold Krebs-Ringer phosphate buffer (KRP: 0.13 *M* NaCl, 5.14 m*M* KCl, 1.7 m*M* $CaCl_2$, 1.3 m*M* $MgSO_4$, 1.3 m*M* KH_2PO_4, and 10 m*M* Na_2HPO_4, pH 7.4) (3). Tissues were minced with scissors into pieces less than 1 mm^3 in this solution and maintained at 0°–4°C for 30 min. The tissues were then incubated at 37°C for 45 min in 400 μl of KRP containing 3.9 μg/ml sodium heparin to release the bound enzyme. In preliminary studies (3), this concentration of heparin was shown to produce maximal LPL release from mouse mammary gland homogenates. The heparin-releasable LPL fraction was designated extracellular lipase. The enzyme reaction mixture (1 ml total) contained 0.9 ml enzyme preparation plus 0.1 ml activated substrate solution. Each 6-ml substrate mixture contained 270 mg glyceryl trioleate (20 μCi 2-^{3}H glyceryl trioleate), 6.2 mg egg lecithin, 3.0 ml 10% fatty-acid poor albumin (pH 8.0), 1.6 ml 2 *M* Tris-HCl (pH 8.2). The substrate was sonicated for 3 min and then activated *via* incubation at 37°C for 30 min with overnight-fasted rat serum in the ratio of 1:2. LPL activity was assessed by the extent of hydrolysis of ^{3}H triolein in the presence of serum, which contains the essential LPL activator, apolipoprotein II (14). LPL activity in tissue extracts was expressed as the amount of fatty acid formed from serum-activated triacylglyceride per gram protein per hour. Protein content was determined by the method of Bradford (15).

ACL Assay. For ACL determination, tissues were homogenized using a glass homogenizing tube and loose fitting Teflon pestle in 10 volumes ice cold 0.1 *M* potassium phosphate buffer (pH 7.0) containing 10% glycerol, 1 m*M* $MgCl_2$, 0.1 m*M* EDTA, 2% rabbit serum, and 1 m*M* dithiothreitol (10). The homogenates were centrifuged at 7000*g* for 15 min, after which the supernatant was centrifuged at 20,000*g* for 30 min. The supernatant fluid was referred to as the enzyme extract. All the steps above were carried out at 0°–4°C. ACL activity was assayed by the malate dehydrogenase-coupled procedure (16). The assay mixture contained 100 μmol Tris-HCl (pH 8.7), 20 μmol potassium citrate, 10 μmol dithiothreitol, 10 μmol $MgCl_2$, 500 units malate dehydrogenase, 0.33 μmol CoA, 0.15 μmol DPNH, 5 μmol ATP, and the enzyme in a total volume of 1.0 ml. Reactions were initiated with addition of ATP, and the rate of DPNH oxidation was assessed *via* a densitometric determination at 340 nm in a Beckman DU-50 spectrophotometer. All assays were carried out at 25°C. One unit of enzyme activity is defined as the amount necessary to catalyze the oxidation of 1 μmol of DPNH/min under the assay conditions employed. The specific activity is expressed as units per milligram of protein. Protein concentration was measured according to the methods of Bradford (15).

RNA Isolation. Total RNA from cultured mammary tissues was extracted by a procedure employing the guanidium thiocyanate-phenol-chloroform method described by Chomcyzynski and Sacchi (17). The tissues were minced in a denaturing TRI reagent (a monobasic mixture of guanidium thiocyanate and phenol). The samples were then homogenized at 4°C with 25 sec pulses in a polytron homogenizer. To each sample was added 0.1 volumes of BCP for each 1 ml of TRI reagent. The samples were then centrifuged at 10,000*g* for 15 min at 4°C. To the guanidine isothiocyanate layer, 0.5 volume of isopropyl alcohol was

then added for each 1 ml of TRI reagent used. After 15 min at 4°C, the samples were centrifuged at 10,000*g* for 15 min. One milliliter of 75% ethanol was added to the pellet and the samples were centrifuged at 7500*g* for 5 min at 4°C. The pellet was air dried for 5–10 min, after which 100 μl DEPC (diethyl pyrocarbonate) treated sterile water was added. This mixture was then placed in a water bath at 68°C for 10–30 min. Optical densities of the samples were then read at 260 and 280 nm employing a 1:100 dilution of each sample. Samples having OD260:280 ratios of 1.4–2.0 were used for Northern blot analysis.

Northern Blotting and Hybridization. Aliquots of total RNA (30 μg) were subject to electrophoresis in 1.2% agarose/10.65 *M* formaldehyde gel, and then transferred to a nylon membrane. The membranes were hybridized overnight at 68°C with [^{32}P]-labeled cDNA probes for LPL and β-actin. Labeling of the probes was accomplished with a kit purchased from Gibco BRL Life Technologies, Inc. After hybridization, the membranes were exposed to film for appropriate times at 4°C. The films were then developed and the bands quantitated *via* laser densitometry. Results are expressed as a ratio of the density of the LPL band to that of the β-actin band.

Statistical Analysis. Statistical comparisons were made with Student's *t* test or an analysis of variance (ANOVA) followed by Scheffe's test where appropriate.

All values represent the mean ± SEM of four observations.

Results

Effect of PRL on Heparin-Releasable LPL (hrLPL) Activity. Figure 1 reports a time-course for the PRL regulation of LPL activity. A 76% increase in heparin-releasable (hrLPL) activity was observed at 24 hr while an initial PRL effect was seen at 16 hr. Results of dose-response studies are shown in Figure 2. Addition of 0.1 μg/ml PRL significantly increased LPL activity, whereas PRL concentrations of 10 ng/ml or less were without effect. PRL concentrations above 100 μg/ml did not further increase the magnitude of the PRL response.

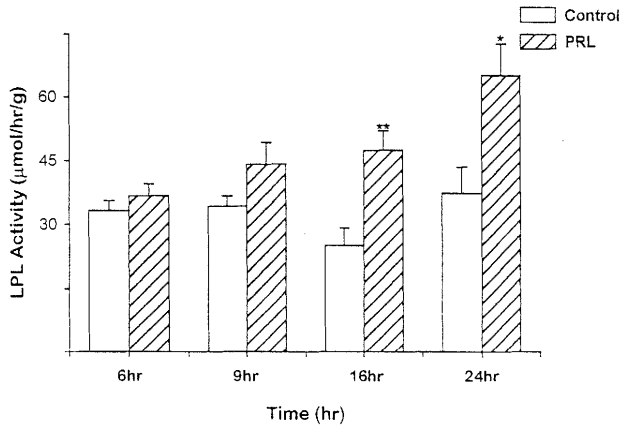

Figure 1. Time-course of PRL effect on LPL activity. Explants were incubated for 24 hr in culture medium containing insulin (1 μg/ml) and cortisol (10^{-7} *M*). Time course was begun by adding fresh culture medium alone or with prolactin (1 μg/ml), and incubation continued for indicated times. At the end of this incubation, explants were collected and the specific activity of LPL was determined. LPL enzyme activity was expressed as μmol fatty acid hydrolyzed/g protein/hr. *Significantly greater than control with $P < 0.05$; **Significantly greater than control with $P < 0.01$.

Figure 3 shows the results of a study in which the specificity of the PRL effect on hrLPL activity was determined. T_3 and bGH did not significantly increase LPL activity whereas all the lactogenic hormones (PRL, hPL, and hGH) elicited significant responses.

Effect of PRL on LPL mRNA Accumulation. In subsequent experiments, Northern hybridization techniques were employed to explore the effect of PRL on LPL mRNA levels in cultured mammary tissues. Figure 4 shows the results of a time-course experiment (selected from three experiments where similar results were observed). Panel A of Figure 4 shows the results of typical blots of LPL and β-actin mRNA autoradiograms. After quantitation of the blots via laser densitometry, the time-course shows an abrupt increase in the ratio of LPL:β-actin mRNA after a 6-hr PRL treatment. Dose-response studies (Fig. 5) revealed that PRL at concentrations of 10 ng/ml and above increased LPL mRNA accumulation in mammary tissues after a 24-hr PRL treatment.

Figure 6 shows the specificity of the PRL effect on LPL mRNA accumulation. PRL and hGH clearly expressed the greatest responses.

Effect of PRL on ACL Activity. In further studies (Table I), PRL was also shown to cause a small but significant increase (about 30%) in ACL activity in cultured mammary tissues. The onset of this effect occurs between 6 and 13 hr after PRL addition to the mammary tissues.

Discussion

Circulating lipids are an important source of long chain fatty acids for the synthesis of milk triacylglycerol (18).

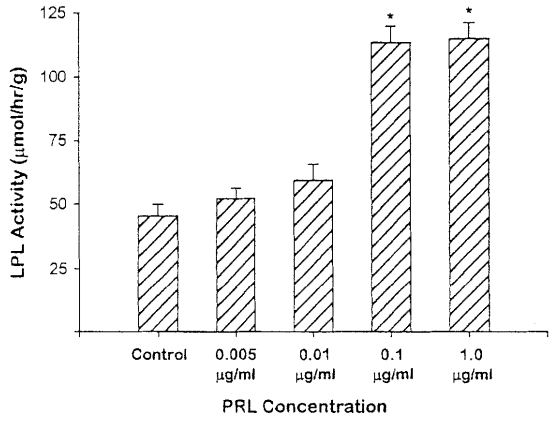

Figure 2. Dose-response of PRL stimulation of LPL activity. Explants were incubated in medium containing insulin (1 μg/ml) and cortisol (10^{-7} *M*) for 24 hr and then incubated in fresh culture medium alone or with the addition of the indicated amount of PRL for another 24 hr. At the end of incubation, explants were collected and the enzymatic activity of LPL was determined. *Significantly greater than control with $P < 0.05$.

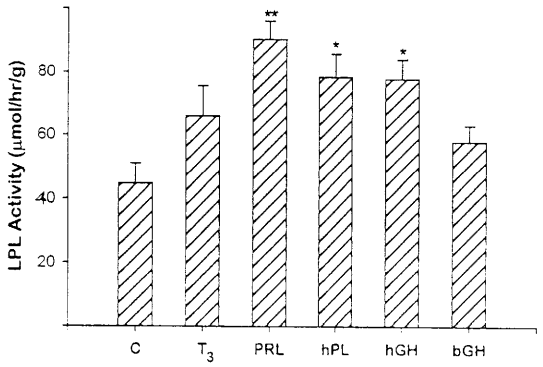

Figure 3. Specificity of PRL effect on LPL activity. Explants were incubated in medium containing insulin (1 μg/ml) and cortisol (10^{-7} *M*), then incubated in fresh culture medium alone (C) or with prolactin (1 μg/ml), T_3 (1 μg/ml), hPL (1 μg/ml), hGH (1 μg/ml), or bGH (1 μg/ml) for 24 hr. At the end of this incubation explants were collected and the specific activity of LPL was determined. *Significantly greater than control with $P < 0.05$. **Significantly greater than control with $P < 0.01$.

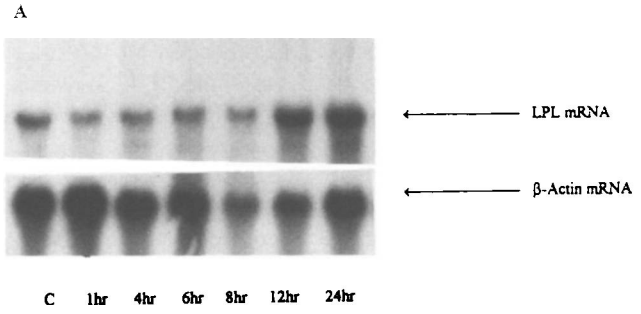

Figure 4. Time-course of PRL effect on LPL:β-actin mRNA levels. Tissues were treated the same way as in Figure 1. (A) Autoradiograms of hybridization of cDNA probes for LPL and β-actin mRNA. (B) Time-course of PRL response on ratio of LPL mRNA to mouse β-actin mRNA.

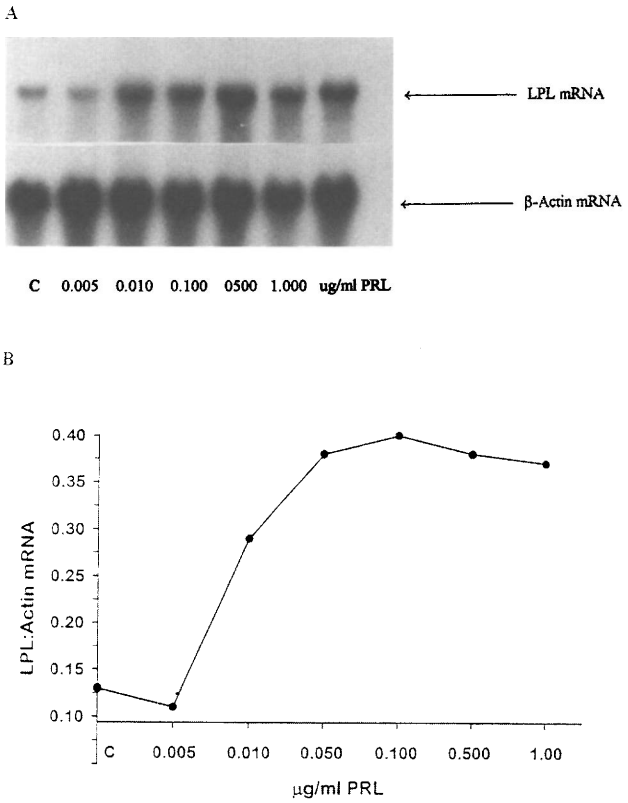

Figure 5. Dose response of PRL effect on LPL:β-actin mRNA levels. Tissues were treated the same way as in Figure 2. (A) Autoradiograms of hybridization of cDNA probes for LPL and β-actin mRNA. (B) Dose-response of PRL effect on ratio of LPL mRNA to mouse β-actin mRNA.

Some of the milk-destined free fatty acids are transported in the blood as triacylglycerol present in chylomicrons and very low density lipoproteins (vLDL), and FFA uptake by mammary tissue involves hydrolysis of triacyglycerol by lipoprotein lipase (LPL) functioning in the lumen at the mammary capillaries (18). LPL, upon activation by apoprotein CII, catalyzes the rate-limiting step in the degradation of vLDL and chylomicron triglycerides (19). During this hydrolysis by LPL, some of the lipoproteins give rise to HDL, while the chylomicron remnants are cleared by the liver and the vLDL remnants are converted to further LDL. Thus, LDL plays a pivotal role in the overall metabolic cascade of lipoproteins (20). In mammary tissue, the resulting fatty acids are subsequently processed by mammary alveolar cells into triglycerides which accumulate in lipid droplets for secretion into milk (21).

In this *in vitro* study, a 24-hr PRL treatment caused a 76% increase in heparin-releasable LPL activity, whereas a 6-hr PRL treatment caused a 2.5-fold increase in LPL mRNA accumulation. The increased LPL mRNA levels could be due to a stimulation of transcription from the LPL gene, an enhanced rate of processing of the LPL gene transcripts, a decreased rate of degradation of the LPL mRNA, or a combination of these. The accompanying increase in extracellular LPL activity (heparin-releasable fraction) could be explained as the result of an elevated rate of translation of LPL mRNA, regulation of post-translational processes, or a combination of these. According to Jensen *et al.* (3), changes in the level of LPL mRNA are seen only during the transition from pregnancy to lactation, and these changes tend to follow, rather than precede, changes in enzyme activity. Our studies clearly show that a PRL effect on LPL mRNA accumulation precedes by several hours the

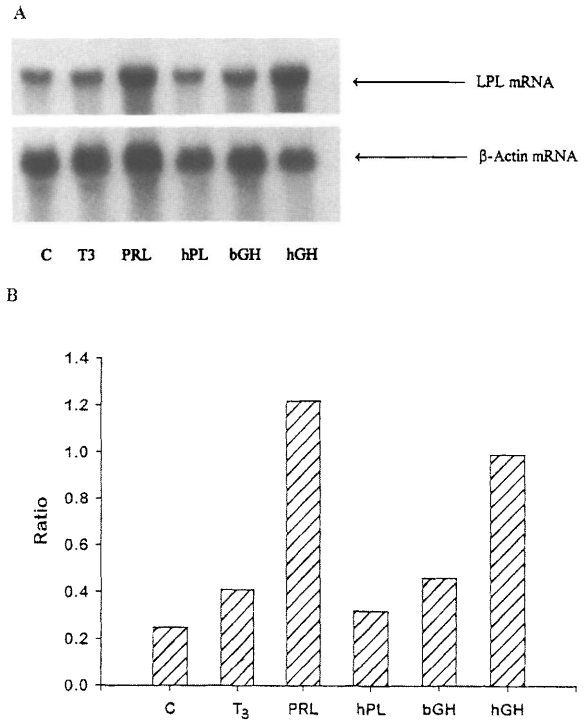

Figure 6. Specificity of lactogenic hormone effects on LPL:β-actin mRNA. Tissues were treated the same way as in Figure 3. (A) Autoradiograms of hybridization of cDNA probes for LPL and β-actin mRNA. (B) Effects of various hormones on ratio of LPL mRNA to mouse β-actin mRNA.

Table I. Effect of PRL on ACL Activity

Time	ACL activity (10^{-2} units/mg protein)[a]	*P*
0 hr	1.22 ± 0.03	—
4 hr	1.30 ± 0.02	NS
6 hr	1.10 ± 0.06	NS
13 hr	1.45 ± 0.05	<0.05
17 hr	1.54 ± 0.07	<0.05
21 hr	1.55 ± 0.03	<0.05

Note. Mammary gland explants were treated for 24 hr with cortisol (10^{-7} *M*) and insulin (1 μg/ml), then cultured for the times indicated above with the addition of 1 μg/ml PRL. ACL activity was then determined.

[a] Values represent the mean ± SEM of four observations.

PRL stimulation of hrLPL activity. It would therefore seem likely that PRL stimulation of hrLPL activity is caused, at least in part, *via* the increased level of LPL mRNA available for translation to the LPL enzyme.

In earlier studies it was reported that other hormones exert profound effects on LPL activity *via* different mechanisms. In adipose tissue the glucocorticoids stimulate LPL activity by a transcriptional mechanism involving increased LPL mRNA synthesis. In contrast, insulin increases LPL activity in adipocytes by activating preexisting lipoprotein lipase at a post-transcriptional and post-translational level, but it does not affect gene expression nor mRNA processing (7). Whether PRL regulates LPL activity in mammary tissues *via* any post-transcriptional mechanisms remains to be determined. The fact that hPL was shown to stimulate LPL activity, but had no effect on its mRNA accumulation (Figs. 3 and 6), may indicate that this hormone functions post-translationally to increase LPL activity.

Since LPL functions on the luminal side of endothelial cells lining the capillaries of tissues, the manner by which LPL in mammary tissues gets to the capillaries is of great interest. Studies by Jensen *et al.* (3) indicate that the LPL is synthesized in mammary stromal cells, most likely fat cells. Assuming this is true, it remains to be explained how PRL regulates LPL production in these cells, since PRL receptors are present on alveolar epithelial cells but are not known to exist on fat cells in mice. It may be that the PRL regulation of LPL production in the mammary gland may involve paracrine factors derived from the alveolar epithelial cells. Subsequent to the assembly of LPL in the stromal cells, it is not yet known how the LPL is transported to the capillary endothelial cells and whether PRL regulates this process in mammary tissues.

The observation that PRL also stimulates ATP-citrate lyase activity in cultured mammary tissues may also be of further importance regarding the PRL regulation of milk lipid synthesis. Although only a 30% increase in ACL activity was observed in these studies, it is likely that the magnitude of increased activity is considerably larger in the PRL-responsive alveolar epithelial cells. A significant portion of the cells in the mammary explants are fat cells which also contain ACL. Subtracting this basal ACL activity from the total in the tissue should increase the magnitude of the PRL response in the mammary parenchymal cells. ACL is clearly an important enzyme involved in the hormonal regulation of lipogenesis in adipose cells, and it may be equally important in regulating lipogenesis in mammary cells.

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