0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 3
Printed in U.S.A.

Insulin-Induced Up-Regulation of Lipoprotein Lipase Messenger Ribonucleic Acid and Activity in Mammary Gland*

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ABSTRACT

The effects of insulin on lipoprotein lipase (LPL) messenger RNA (mRNA) were studied in mammary glands from virgin and late-pregnant rats. Virgin and pregnant rats (at 17 days of gestation) were subjected to a continuous infusion (35 ml/day) with 50% glucose for 72 h to produce a prolonged hyperinsulinemic-euglycemic condition. Gestation causes a 4-fold increase in LPL mRNA accompanied by a 3- to 4-fold increase in total LPL activity. Experimental hyperinsulinemia, generated by the 50% glucose infusion, had a similar effect in both pregnant and virgin rats, by enhancing (2- to 3-fold) both their LPL activity and LPL mRNA. Thus, total LPL activity and LPL mRNA significantly correlated with plasma insulin ($r = 0.789$, $P < 0.001$; and $r = 0.772$, $P < 0.001$, respectively). Furthermore, total LPL activity was correlated with LPL mRNA ($r = 0.765$, $P = 0.001$). In conclusion, the present study shows that insulin participates in the control of LPL expression in mammary glands, revealing its role as a modulator of the enzyme at a mRNA level. (*Endocrinology* **140:** 1089–1093, 1999)

LIPOPROTEIN lipase (LPL) is an enzyme synthesized in the parenchymal cells and transported to its site of action on the capillary endothelium, where it hydrolyzes triglycerides in circulating chylomicrons and very-low-density lipoproteins, thereby facilitating the uptake of hydrolytic products by the subjacent tissues (1). The enzyme expresses high activity in adipose tissue, heart, skeletal muscle, and lactating mammary gland (2, 3). In the lactating mammary gland, the enzyme seems to be synthesized by stromal cells, presumably adipocytes, and transported to the interstitial space, where it is present in very high concentration, as revealed by immunocytochemical analysis (2).

LPL is subjected to complex tissue-specific regulation by dietary and hormonal factors, which modulate LPL activity via transcriptional, posttranscriptional, and posttranslational mechanisms. In adipose tissue, insulin is the principal positive modulator of LPL activity (4, 5), and the decrease in LPL activity that appears during late pregnancy has been associated with the insulin resistance occurring in this situation (6, 7). In contrast to adipose tissue around parturition, LPL activity in mammary glands increases (7) and remains high during lactation (8). This high LPL activity in the mammary tissue during late pregnancy, besides enhancing the use of circulating triglycerides for milk synthesis, is the major factor that causes the decline of maternal hypertriglyceridemia (9). On cessation of lactation (*e.g.* litter removal), LPL activity is diminished in mammary gland when milk synthesis is inhibited (10).

Maternal hyperinsulinemia during late gestation, together with the increase in PRL levels, has been shown to contribute to the induction of LPL activity in mammary glands (7, 11), which is in agreement with the high insulin sensitivity in this tissue (12, 13). Although changes in the messenger RNA (mRNA) expression of LPL in mammary gland have been studied in the transition from pregnancy to lactation (8), the role of insulin on the expression of LPL mRNA is still unknown. Thus, the present work was addressed to study the potential relationship between plasma insulin levels and mammary gland LPL mRNA in pregnant and nonpregnant animals under conditions of prolonged hyperinsulinemia.

Materials and Methods

Animals and tissue collection

Female Wistar rats from our colony were housed at 22–24 C and 12-h light, 12-h dark cycles, from 0800 to 2000 h, with free access to water and to a chow diet (Panlab, Barcelona, Spain). Some animals were mated when weighing between 170 and 180 g, and the beginning of pregnancy was determined by the presence of spermatozoids in vaginal smears. In pregnant rats at day 17 of gestation and in age-matched virgin rats, a SILASTIC catheter (Dow Corning Corp., Midland, MI; 0.02-inch id, 0.037 inch od) was placed into the right jugular vein and another one into the right femoral vein, under ketamine cocktail anesthesia (ketamine, 50 mg/ml; diazepan, 5 mg/ml; and atropine, 1 mg/ml; 5/4/1, vol/vol/vol). After recovery from anesthesia, animals were housed in individual cages and continuously infused for 72 h with either bidistilled water or 50% glucose, through the catheter placed into the jugular vein, at the rate of 35 ml/day. Other methodological details have been previously described (14, 15). After the 72-h infusion period, animals were decapitated, and blood was collected from the neck wound in heparinized tubes for immediate separation of plasma, at 4 C. Mammary glands were rapidly dissected and placed in liquid nitrogen, to be stored at −80 C until processed for LPL activity and mRNA preparation, as described

Received April 6, 1998.

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* This study was supported by Grant 92/407 from the Fondo de Investigaciones Sanitarias de la Seguridad Social of Spain, and by Grants PB92/0918 (to A.M.-H.) and PM88–0050 (to P.R.) from the Dirección General de Investigación Científica y Técnica.

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below. Plasma aliquots were used to measure glucose (16) and insulin using a specific RIA kit for rats (17) (Novo Nordisk A/S, Copenhagen, Denmark). The experimental protocol was approved by the Animal Research Committees of Hospital Ramón y Cajal and Faculty of Experimental and Technical Sciences, University San Pablo-CEU.

RNA preparation

Total cellular RNA was isolated from frozen mammary gland tissue by a single-step extraction with acid guanidinium thiocyanate-phenol-chloroform (18). Briefly, tissues were homogenized in a polytron with 4 M guanidinium thiocyanate, 0.5% sodium *N*-lauroylsarcosine, 25 mM sodium citrate (pH 7.0), and 0.1 M 2-mercaptoethanol. RNA was purified via a series of ethanol precipitations and quantified by OD at 260 nm.

Northern analysis

Equal amounts (5 μg) of total RNA were fractionated on 1% agarose gels containing 2.2 M formaldehyde. Electrophoresis was carried out for 18 h at 50 V in 3-(*N*-morpholino)propanesulfonic acid, pH 7.0, running buffer. RNA was transferred to a nylon membrane (Hybond-N; Amersham, Buckinghamshire, UK) for 1 h in 3 M NaCl, 0.3 M sodium citrate (pH 7.0), by a vacu-aid vacuum blotting system (Bio-Rad Laboratories, Inc., Hercules, CA) and immobilized by cross-linking with UV light (19).

The nylon membranes were prehybridized for 1 h at 60 C in 0.5 M sodium phosphate (pH 7.0), 1 mM EDTA, 7% (wt/vol) SDS, and 1% (wt/vol) BSA. Northern hybridization was performed with denatured ^{32}P-labeled cDNA probes (1×10^6 cpm/ml) for 17–18 h at 60 C in the same buffer as above. cDNA probe (mouse LPL clone ML 2) (20) was radiolabeled, as described by Feinberg and Vogelstein (21), with an oligolabeling kit (LKB, Pharmacia, Uppsala, Sweden). DNA (25–50 ng) was labeled to a specific activity of $1–2 \times 10^9$ dpm/μg^{-1} using [^{32}P]deoxycytidine triphosphate (3,000 Ci/mmol, Amersham). Northern filters were washed twice (20 min per each wash) with 0.3 M NaCl, 30 mM sodium citrate (pH 7.0), 0.1% SDS at room temperature; and twice (20 min per each wash) with 15 mM NaCl, 1.5 mM sodium citrate (pH 7.0), 0.1% SDS at 60 C. Autoradiography was performed with a single intensifying screen at −80 C and quantified by densitometric scanning.

Northern analyzes of whole mammary gland RNA were done for each animal, and bands of 3.6 kb corresponded to LPL mRNA were expressed in glands from all groups. There were no differences in loading, as verified by ethidium bromide staining of the gels. Bands, corresponding to 28S ribosomal RNA (rRNA) were quantified from the photographs of the gels, and these values were used as internal standard. Thus, LPL mRNA is expressed as the ratio: mRNA/28S rRNA.

LPL assay

Total LPL activity (*i.e.* extracellular and intracellular) was measured as previously described (22, 23). Briefly, tissue samples were homogenized in 0.2 M Tris-HCl (pH 8.2) at 4 C and delipidated with acetone-diethyl ether. LPL activity was assayed in triplicate using an egg lecithin-stabilized emulsion of ^{14}C-fatty acid-labeled triolein as substrate (final concentration: 2.5 mM triolein, 2.4% BSA, 0.2 M Tris (pH 8.2), 0.1 M NaCl, and 8% heated rat serum in 0.25 ml), in the absence or presence of 1 M NaCl (high saline conditions). LPL activity was determined by subtracting the non-LPL-dependent activity (high salt) from the total lipolytic activity.

Statistical analysis

Results are expressed as means ± SEM. Statistical comparisons were made with the ANOVA, followed by the Tukey test, with 95% confidence limits, using the Systat program (Systat, Inc., Evanston, IL). The relationship between variables was determined by Pearson correlation coefficient using the Systat program (Systat, Inc.). Where indicated, a multiple regression analysis was also performed.

Results

In the present study, a model of prolonged iv glucose infusion in the rat, to attain hyperinsulinemia under euglycemic conditions (12, 14, 15), was used. Pregnant rats (17 days of gestation) and age-matched virgins were subjected to a continuous infusion with water (control) or 50% glucose for 72 h. Animals within each group had equal weight at the beginning of the glucose infusion, and (as shown in Table 1) 50% glucose treatment did not affect the body weight of either virgin or pregnant rats. Blood glucose levels were significantly lower in pregnant than in virgin rats and remained the same upon glucose infusion in both groups. Plasma insulin concentration was higher in pregnant than in virgin rats, but both groups responded similarly to glucose treatment, with a 2- to 3-fold increase in plasma insulin concentration.

The total activity of LPL in mammary glands (*i.e.* intracellular and extracellular) is shown in the *left part* of Fig. 1. LPL activity was significantly higher in mammary glands from pregnant rats than from virgin animals. Although, in both groups, total LPL activity markedly increased upon the hyperinsulinemia generated by the 50% glucose infusion, values were always significantly higher in pregnant than in virgin rats.

Northern hybridization techniques were used to explore the effect of insulin on LPL mRNA extracted from mammary glands, and the results are illustrated in the *right part* of Fig. 1. The *insert* of this figure shows a representative autoradiogram of the hybridization of cDNA probe for LPL mRNA in the four experimental groups. Mammary tissue from pregnant rats showed an accumulation of LPL mRNA, in comparison with nonpregnant animals. Thus, the 3–4 times higher LPL activity of mammary glands of pregnant rats (Fig. 1, *left*) was associated with a 3- to 4-fold increase in their LPL mRNA content (Fig. 1, *right*). Moreover, experimental hy-

TABLE 1. Effect of 50% glucose infusion (35 ml/day), for 3 days in pregnant (days 17–20 of gestation) and virgin rats, on body weight and circulating components

	Virgin		Pregnant	
	Control	Glucose 50%	Control	Glucose 50%
BW (g)	224.6 ± 6.4	209.4 ± 209.4	305.5 ± 11.7[a]	291.7 ± 6.3[a]
Plasma glucose (mM)	7.71 ± 0.19	8.06 ± 0.85	5.33 ± 0.29[b]	5.38 ± 0.69[b]
Plasma insulin (μU/ml)	47.99 ± 2.59	109.61 ± 14.3[c]	68.73 ± 9.81	271.21 ± 30.03[a,d]

Wistar virgin and pregnant rats (at day 17 of gestation) were continuously infused for 3 days (35 ml/day) through the catheter placed in the jugular vein, either with 50% glucose (to generate sustained hyperinsulinemia) or with bidistilled water (control groups). At the end of the 72-h infusion period, blood samples were collected to determine glucose and insulin concentrations. Values are mean ± SEM of 6–11 rats/group.

[a] $P < 0.001$ (statistical comparison between pregnant *vs.* virgin rats).

[b] $P < 0.05$ (statistical comparison between pregnant *vs.* virgin rats).

[c] $P < 0.05$ (statistical comparison between glucose 50%-treated *vs.* control rats).

[d] $P < 0.001$ (statistical comparison between glucose 50%-treated *vs.* control rats).

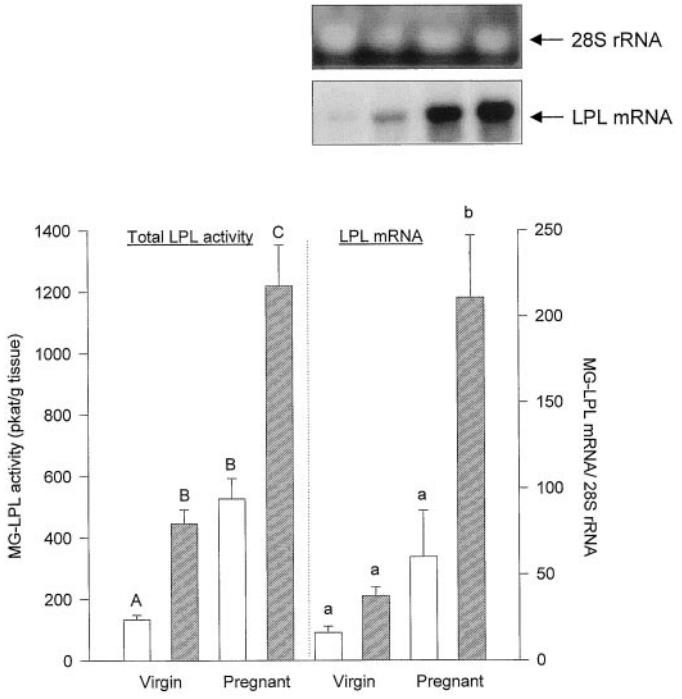

FIG. 1. Effect of hyperinsulinemia on total LPL activity and mRNA in mammary gland of virgin and pregnant rats. Virgin and 20 day-pregnant rats were infused with 50% glucose *(slanted rule bars)* for 3 days to generate hyperinsulinemia, while preserving euglycemia. Control rats were infused with bidistilled water (*open bars*). Total LPL activity (*left side* of the figure) and LPL mRNA (*right side* of the figure) in mammary gland homogenates were measured as described in *Materials and Methods*. The *insert* of the figure shows a representative autoradiogram of hybridization of cDNA probes for LPL mRNA, and a photograph of the gel showing the band corresponding to the 28S rRNA. Statistical comparisons were made by ANOVA, followed by a Tukey test, with 95% confidence limits. Significance is shown by letters: different letters indicate significant differences ($P < 0.05$). *Capital letters* are used for total LPL activity. MG, Mammary gland; pkat, pmol of substrate transformed per second.

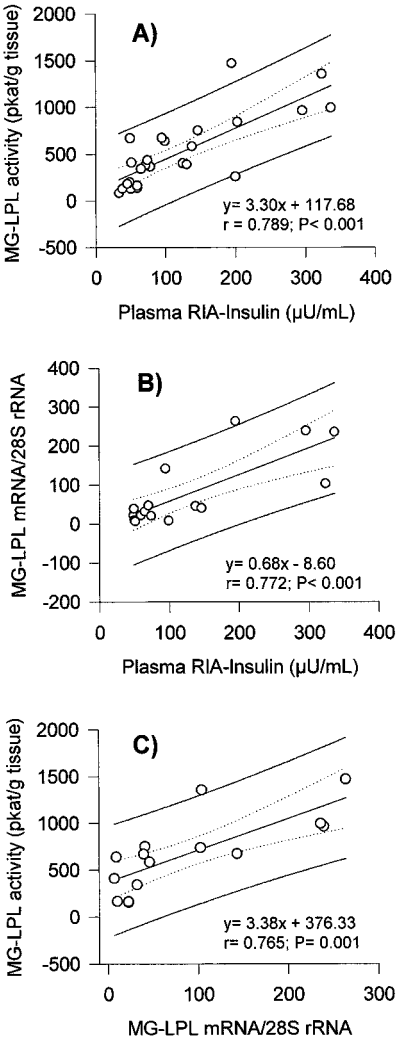

FIG. 2. Linear correlations for virgin and 20-day pregnant rats, infused for 72 h with either 50% glucose or bidistilled water, between: A) plasma insulin and total LPL activity in mammary glands; B) Plasma insulin and LPL mRNA levels in mammary glands; and C) LPL mRNA and total LPL activity in mammary glands. For each regression analysis, the 95% confidence interval is represented by *broken lines*, and the 95% prediction interval by *continuous lines*.

perinsulinemia, generated by 50% glucose infusion, had a similar effect in both pregnant and virgin rats, by augmenting (2- to 3-fold) their LPL mRNA content.

To further investigate the parallelism observed between circulating insulin and mammary gland LPL activity, as well as mRNA content, a correlation analysis was performed with all individual values. As shown in Fig. 2A, total LPL activity correlated very closely with plasma insulin levels. To find out whether this was an independent relationship, a multiple regression analysis, including body weight and plasma glucose, was also performed. From this model, it was found that insulin was the only variable that contributed to LPL activity ($P < 0.001$), whereas plasma glucose or body weight did not ($P = 0.455$ and $P = 0.154$, respectively). Furthermore, to ensure that such a dependence, between total LPL activity and plasma insulin levels, occurs independently of the reproductive state of the animals, linear correlations were also done including only virgin or pregnant rats. As expected, mammary gland LPL activity and plasma insulin levels also correlated significantly in both groups of animals ($r = 0.657$, $P = 0.008$; and $r = 0.776$, $P = 0.005$ in virgin and pregnant rats, respectively).

The results in Fig. 2B showed that LPL mRNA also correlated significantly with plasma insulin levels. Moreover, from a multiple regression analysis, it was found that insulin was the only variable that contributed to LPL mRNA expression ($P = 0.014$), whereas plasma glucose or body weight did not ($P = 0.735$ and $P = 0.480$, respectively). Similar (but somehow weaker) correlations to those found with all animals (Fig. 2B) were seen when only virgin or pregnant rats were studied separately ($r = 0.673$, $P = 0.068$; and $r = 0.695$, $P = 0.054$, respectively). As expected from the results presented above and as shown in Fig. 2C, the total LPL activity of mammary glands had a very close correlation with the LPL mRNA content in the tissue ($r = 0.765$, $P = 0.001$). The same strong correlation was observed when only values of virgin or pregnant rats were plotted separately ($r = 0.867$, $P = 0.025$; and $r = 0.695$, $P = 0.038$, respectively).

Discussion

Rats, under euglycemic-hyperinsulinemic condition obtained by 50% glucose infusion for 3 days, were used to study the effect of insulin on LPL in mammary gland. Our results show that hyperinsulinemia, caused by either pregnancy or the prolonged glucose infusion, markedly enhanced total LPL activity in the mammary gland (7). Because insulin effects are initiated by the stimulation of the insulin receptor tyrosine kinase after insulin binding, the induction of LPL activity and expression by insulin at late pregnancy agrees with our recent finding that the insulin-stimulated kinase activity of the insulin receptor is augmented in mammary glands of late-pregnant rats (12). This increased LPL activity in mammary glands at the end of gestation, together with the decrease in LPL activity in adipose tissue (24), drives the triglyceride-rich lipoproteins to the mammary gland (9), contributing actively to the synthesis of milk in preparation for lactation.

LPL is subjected to complex tissue-specific regulation by hormonal factors, which modulate LPL activity via transcriptional, posttranscriptional, and posttranslational mechanisms. Studies in other tissues, *e.g.* adipose tissue, have shown that insulin regulates LPL gene expression mainly at the mRNA level (25–27); and accordingly, it has been shown that LPL mRNA content is inversely correlated with the degree of insulin resistance (28). Furthermore, during late pregnancy, a condition characterized by an impaired insulin responsiveness of adipose tissue (29), the decrease in LPL activity in adipose cells has also been shown to parallel changes in mRNA (24). Because it is known that LPL is synthesized in mammary interstitial cells (more likely, adipocytes) (8), it already has been suggested that mammary gland LPL should be regulated similarly to adipose tissue LPL (30). Therefore, to clarify whether, as in adipose tissue, insulin regulates LPL in mammary gland at the mRNA level, LPL mRNA was determined in the same probes used for quantification of the enzymatic activity. Present results clearly show that long-term hyperinsulinemia induces LPL mRNA accumulation in mammary glands of both virgin and late-pregnant rats. Moreover, it was found that total LPL activity changes in parallel to the LPL mRNA content, this effect being independent of the reproductive condition of the animal. Similar long-term response has been observed in mammary gland development during lactation (8); whereas, in transition from fed- to fasted-state LPL in mammary gland from lactating mice, it is regulated at a posttranslational level, with no changes in LPL mRNA (8). Consequently, it seems that acute changes in mammary gland LPL are mediated by posttranscriptional events, whereas long-term responses may involve changes at the mRNA level.

Besides hyperinsulinemia and the high insulin sensitivity in mammary glands during pregnancy (12), another positive mediator of LPL may be involved in this condition; PRL is a prime candidate, because its concentration rises around parturition (31), and it is essential for milk production and terminal differentiation of alveolar epithelial cells (31). In a recent study, it has been shown that, in cultured mammary gland explants derived from midpregnant mice, PRL increases both LPL activity and mRNA (32). However, it remains to be established how PRL regulates LPL in these cells, because adipocytes lack PRL receptors (33).

In conclusion, maternal hyperinsulinemia, during late gestation, contributes to the induction of LPL prior parturition by enhancing LPL mRNA. Whether the augmented mRNA levels are caused by a stimulation of LPL gene transcription and/or a decreased rate of degradation of the LPL mRNA, is yet to be established.

Acknowledgments

We acknowledge the excellent technical assistance of Ms. Milagros Morante. We thank Drs. C. Holm and M. C. Schotz for the gift of mouse LPL cDNA probes. The authors thank Beatriz Ramos for her editorial help.

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