

Expression, activity, and localization of hormone-sensitive lipase in rat mammary gland during pregnancy and lactation

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Abstract We examined the presence of hormone-sensitive lipase (HSL) in mammary glands of virgin, pregnant (12, 20, and 21 days), and lactating (1 and 4 days postpartum) rats. Immunohistochemistry with antibody against rat HSL revealed positive HSL in the cytoplasm of both alveolar epithelial cells and adipocytes. In virgin rats, immunoreactive HSL was observed in mammary adipocytes, whereas diffuse staining was found in the epithelial cells. Positive staining for HSL was seen in the two types of cells in pregnant and lactating rats. However, as pregnancy advanced, the staining intensity of immunoreactive HSL increased in the epithelial cells parallel to their proliferation, attaining the maximum during lactation. An immunoreactive protein of 84 kDa and a HSL mRNA of 3.3 kb were found in the rat mammary gland as in white adipose tissue. Both HSL protein and activity were lower in mammary glands from 20 and 21 day pregnant rats than from those of virgin rats, although they returned to virgin values on days 1 and 4 of lactation. Mammary gland HSL activity correlated negatively to plasma insulin levels. Immunoreactive HSL and HSL activity were found in lactating rats' milk. **The observed changes indicate an active role of HSL in mammary gland lipid metabolism.**—Martín-Hidalgo, A., L. Huerta, N. Álvarez, G. Alegría, M. del Val Toledo, and E. Herrera. **Expression, activity, and localization of hormone-sensitive lipase in rat mammary gland during pregnancy and lactation.** *J. Lipid Res.* 2005. 46: 658–668.

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Mammary glands, besides being among the tissues in the body with the highest lipid content after adipose tissue (1), are one of the most active metabolic tissues in the body during pregnancy and lactation. Milk lipid is an important source of both calories and essential fatty acids for

the newborn. During lactation, women secrete 800 ml of milk per day containing 4% fat, mostly corresponding to triacylglycerols, of which the mammary gland synthesizes ~32 g daily (2). The lactating mouse mammary gland secretes 5 ml of milk per day containing ~30% fat (3). To develop this transitory capacity for handling such a large amount of lipids, the morphology of the mammary tissue changes during pregnancy and lactation. In nonpregnant mammary glands, the predominant cells are adipocytes with epithelial structures interdispersed among them. During pregnancy, in mammary glands there is an extensive proliferation of alveolar structures into the adipocytes, accompanied by differentiation of epithelial cells (4), which show cytoplasmic lipid droplets (5) surrounded by the protein adipophilin (6). During lactation, epithelial cells are the predominant cell type, and only small channels of lipid-filled adipocytes and lipid-depleted adipocytes may be distinguished.

During pregnancy, major changes in maternal lipid metabolism occur. Fat depots accumulate during the early stages of pregnancy and decrease during the late phases (7). Net catabolic changes taking place in adipose tissue during late pregnancy are manifested by an enhanced hormone-sensitive lipase (HSL) activity and decreased LPL activity (8), which result in an increase in maternal plasma lipids both in humans (9, 10) and in rats (11, 12). The increments of triglyceride (TG)-rich lipoproteins (chylomicrons and VLDLs) are among the most pronounced changes in plasma lipids during late pregnancy (10, 13, 14). Reduced adipose tissue LPL activity during late gestation allows blood TG to be diverted from storage in adipose tissue to other tissues, such as mammary glands, where there is an induction in LPL expression and activity, allowing the subsequent hydrolysis and uptake of circulating TG in preparation for lactation (15, 16).

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Besides the enhanced LPL-dependent uptake of TG from plasma TG-rich lipoproteins (17, 18), during lactation mammary epithelial cells synthesize TG from fatty acids obtained from the blood stream and from their de novo synthesis from glucose (19). The cytoplasmic lipid droplets formed move toward the apical surface of the epithelial cells and are budded into the lumen. This process results in secretion of the milk lipid globules that are surrounded by the membrane, composed of both apical plasma membrane and intracellular components (20).

HSL, the key enzyme in lipolysis, is an intracellular neutral lipase that hydrolyzes triacylglycerols, diacylglycerols, monoacylglycerols, and cholesteryl and retinyl esters (21–25). The activity of HSL is regulated posttranslationally by phosphorylation-dephosphorylation reactions. The activation by fast-acting lipolytic hormones (catecholamines, isoproterenol, glucagon, adrenocorticotrophic hormone) involves a hormone receptor-induced increase in the intracellular concentration of cAMP, which activates cAMP-dependent protein kinase A. Protein kinase A then phosphorylates HSL, resulting in an increase in hydrolytic activity (26). Dephosphorylation is affected primarily by protein phosphatases, which are activated by insulin (27).

Although HSL expression is highest in adipose tissue, HSL is also expressed in brown adipose tissue, adrenals, corpus luteum, testis, ovary, and, to a lesser extent, skeletal and cardiac muscle and macrophages (23, 28–31).

The HSL gene is located on chromosome 19q13.3 (32) and was initially described as containing nine exons, which encode the adipocyte protein, spanning 11 and 10 kb in humans (33) and mice (34), respectively. Interestingly, the size of HSL mRNAs is variable. Rat heart, skeletal muscle, placenta, and ovaries express slightly larger HSL mRNAs (3.5 kb) than adipose tissue (3.3 kb) (32). The testis is characterized by expression of an even larger mRNA species of 3.9 kb (32).

The purified adipose tissue enzyme has a molecular mass of 84 and 88 kDa in rats (32) and humans (33), respectively. The testis appears to express two isoforms, one 84 kDa protein that is similar to adipose HSL, and a second larger isoform of ~120–130 kDa, encoded by a single 3.9 kb HSL mRNA in rat testis (30) and by both 3.3 and 3.9 kb HSL mRNAs in human testis (35, 36).

Although we and others have previously described the presence of HSL activity in mammary glands of lactating rats (37, 38) and human mammary gland contains HSL mRNA (35), the role of HSL in mammary gland metabolism is largely unknown. To attain a better understanding of the potential function of HSL in the mammary gland, the present work was addressed at determining the cell types that might express this protein and the changes that take place in its expression and activity in the mammary gland during pregnancy and lactation in the rat. In addition, because insulin is well known as the most active antilipolytic hormone (39, 40) and during late pregnancy and lactation the mammary gland remains highly sensitive to it (41, 42), the study was extended to determine the potential relationship between plasma insulin levels and mammary gland HSL activity during those same stages.

MATERIALS AND METHODS

Animals and tissue collection

Female Sprague-Dawley rats from our colony, weighing 180–200 g, were maintained at 22–24°C under standard conditions of illumination (from 8:00 AM to 8:00 PM) and feeding (Purina Chow diet; Panlab, Barcelona, Spain). The experimental protocol was approved by the Animal Research Committee of the Hospital Ramón y Cajal, Madrid, Spain.

The experimental groups were age- and sex-matched virgin, pregnant (days 12, 20, and 21), and lactating (days 1 and 4) rats. At least six rats were analyzed per time point. The rats were mated with normal males, and positive pregnancy was determined by the appearance of spermatozooids in vaginal smears. Litter sizes were adjusted to 9–11 pups at birth. Rats were killed between 10:00 and 11:00 AM by decapitation, and blood from the neck wound was collected in heparinized tubes. Plasma was immediately separated after blood collection by centrifugation at 4°C and kept at –80°C until analysis for immunoreactive insulin, using an ELISA kit specific for rats (Mercodia AB). The left fourth (inguinal) mammary glands and the lumbar adipose tissue were removed, snap frozen in liquid nitrogen, and stored at –80°C until processing. The right inguinal mammary glands were removed and fixed in 4% paraformaldehyde in PBS for 6–24 h at 4°C. The tissues were embedded in paraffin using conventional methods (43) and sectioned at 5 µm, either to be stained with hematoxylin and eosin or to be used for immunohistochemistry.

Milk sample collection

To determine the presence of immunoreactive HSL in milk, milk samples were collected from 15 day postpartum lactating rats ($n = 5$). The mothers were separated from the litters for a period of 4 h (9:00 AM to 1:00 PM) before milking. Milk samples were collected after an intraperitoneal injection of oxytocin (10 IU/rat) by manual expression of the teats while the rats were under pentobarbital anesthesia (35 mg/kg body weight). Milk samples were immediately frozen at –20°C until analyzed.

Milk samples were thawed (37°C) and shaken continuously during pipetting to ensure sample uniformity. Whole milk was sonicated (three bursts, 5 s duration per burst, maximum power) and diluted in an assay buffer before assay. To remove milk lipids that would interfere in the HSL assay, milk samples were centrifuged at 1,500 g for 20 min at 4°C, and infranatants were ultracentrifuged at 110,000 g at 4°C for 45 min and used for the assay.

HSL assay

Total HSL activity was measured as previously described (44). Briefly, tissue samples were homogenized in 3 volumes of 0.25 M sucrose containing 1 mM EDTA, 1 mM dithioerythritol, and 10 µg/ml antipain, pH 7.4. Infranatants were obtained by centrifugation at 110,000 g in a Beckman centrifuge (model TL-100) for 45 min at 4°C. A phospholipid-stabilized emulsion of a dioleoylglycerol ether analog, 1(3)-mono[³H]oleyl-2-*O*-oleoylglycerol, was used to assay the hormone-sensitive diacylglycerol lipase activity (36). Inhibition experiments were performed by preincubation with 100 mM NaF.

Northern blot analysis

Total cellular RNA was extracted from frozen rat mammary glands by use of a single-step acid guanidinium thiocyanate-phenol-chloroform extraction method (45) (Ultraspect RNA; Biotect). Briefly, tissues were homogenized with a Polytron in the presence of homogenization buffer. RNA was purified via a series of ethanol precipitations and quantified by optical density at 260 nm.

Equal amounts (10 µg) of total RNA were denatured and 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) for 1 h in 3 M NaCl, 0.3 M sodium citrate, pH 7.0, by a capillary system and immobilized by cross-linking with ultraviolet light (46).

The nylon membranes were prehybridized for 1 h at 60°C in 0.5 M sodium phosphate, pH 7.0, 1 mM EDTA, 7% (w/v) SDS, and 1% (w/v) BSA. Northern hybridization was performed with denatured ³²P-labeled cDNA probes (1 × 10⁶ cpm/ml) for 17–18 h at 60°C in the same buffer. Full-length rat HSL cDNA probe (generously provided by Dr. Cecilia Holm, University of Lund, Sweden) was radiolabeled as described by Feinberg and Vogelstein (47) using an oligolabeling kit (LKB Biotechnology, Pharmacia). DNA (25–50 ng) was labeled to a specific activity of 1–2 × 10⁶ dpm/μg using [³²P]deoxycytidine triphosphate (3,000 Ci/mmol; Amersham). Northern blot filters were washed twice (20 min each wash) with 0.3 M NaCl, 30 mM sodium citrate, pH 7.0, 0.1% SDS at room temperature and twice (20 min 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 blot analysis of whole mammary gland RNA was done for each animal. There were no significant differences in loading, as verified by ethidium bromide staining of the gels. Bands corresponding to 28S rRNA were quantified from the photographs of the gels, and these values were used as an internal standard.

Western blotting or immunoblot analysis

Tissues were homogenized in a buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 20 μg/ml antipain, and 20 μg/ml leupeptin, pH 7.4. Cellular proteins were dissolved in SDS-PAGE sample solution [60 mM Tris, pH 6.5, 10% (w/v) glycerol, 5% (w/v) β-mercaptoethanol, 20% (w/v) SDS, and 0.025% (w/v) bromophenol blue] by boiling (2 × 5 min), sonication (2 × 5 min in a sonication bath), and vortex mixing (2 × 30 s). The total amount of protein loaded in each lane was always 10 μg. After fractionation by SDS-PAGE on slab gels (7 × 14 cm), proteins were electroblotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) using a blotting apparatus (Bio-Rad Laboratories, Hercules, CA). Reference proteins were myosin [molecular weight (M_r) 209,000], β-galactosidase (M_r 137,000), BSA (M_r 84,000), carbonic anhydrase (M_r 44,000), soybean trypsin inhibitor (M_r 32,800), lysozyme (M_r 18,700), and aprotinin (M_r 7,200) (all from Bio-Rad). Blots were blocked for 2 h in 10% defatted dry milk-TBS-0.1% Tween and then incubated for 2 h at room temperature with primary antibody (1:20,000) (chicken polyclonal antiserum directed against HSL from white rat adipose tissue; generously provided by Dr. Cecilia Holm). This was then followed by a 1 h incubation with secondary peroxidase-conjugated antibody (1:20,000) [donkey anti-chicken IgY (IgG) (H+I); RDI Research Diagnostics, Inc.] in 5% defatted dry milk-TBS-0.1% Tween. All steps were performed at room temperature, and blots were rinsed between incubation steps with TBS-0.1% Tween. Immunoreactive bands were visualized using the enhanced chemiluminescence detection method according to the manufacturer's instructions (Amersham, Dübendorf, Switzerland) and subsequent exposure of the membrane to X-ray film. Protein determination was performed using the Bradford dye method (48), with Bio-Rad reagent and BSA as the standards.

Immunohistochemistry

Streptavidin-peroxidase methods. Mammary gland paraffin sections (5 μm thick) from rats were mounted at the indicated times on silanized slides and allowed to dry overnight before immunohistochemical staining. Paraffin was removed with xylene. Sections were hydrated, and endogenous peroxidase activity was inhibited

by incubation with 3% H₂O₂ for 10 min and 0.3% H₂O₂ in methanol for an additional 20 min. Sections were then washed in TBS and incubated in 3% normal goat serum, 0.01% Triton X-100, and 0.1% glycine in TBS, pH 7.6, at room temperature for 30 min to prevent nonspecific binding of the first antibody. Afterward, the sections were incubated for 12 h at 4°C with the primary antibody, anti-HSL (chicken polyclonal antiserum directed against HSL from white rat adipose tissue; 1:100). The sections were washed twice in TBS to remove unbound primary antibody and then incubated with the secondary antibody for 1 h at room temperature. The biotinylated secondary antibody used was goat anti-chicken IgY (1:200) for HSL (Vector Laboratories, Inc., Burlingame, CA). Sections were washed in TBS and incubated with the streptavidin-peroxidase complex (Zymed Laboratories, Inc., San Francisco, CA) for 30 min and then washed in TBS followed by Tris-HCl buffer, pH 7.6. The peroxidase activity was revealed using 3-diaminobenzidine tetrahydrochloride as chromogen (Sigma, St. Louis, MO). The sections were counterstained by Carazzi's hematoxylin. Thereafter, the sections were dehydrated in ethanol, mounted in DePeX (Serva), and observed with a light microscope.

Immunofluorescence methods. Mammary gland sections were processed and incubated with the primary antibodies as described above and then incubated with Alexa Fluor 488 goat anti-chicken IgY (1:300) as secondary antibody (Molecular Probes, Eugene, OR) for 60 min in darkness. After washing in TBS, sections were mounted using Mowiol (Sigma-Aldrich Quimica SA, Madrid, Spain) and observed on Zeiss epifluorescence and Bio-Rad confocal microscopes.

The number and volume of mammary adipocytes stained by hematoxylin and eosin were determined by deconvolution of the images followed by quantitation of the cells using masking algorithms on SlideBook (Intelligent Imaging Innovation, Inc.). Calculated values were based on the analysis of 10–30 randomly selected mammary gland adipocytes from the different days of pregnancy and lactation studied.

Control experiments

The specificity of the immunohistochemical procedures was assessed by means of negative controls as follows: *i*) omitting the primary antibodies; *ii*) using nonimmune serum instead of the primary antibodies; and *iii*) incubating with an inappropriate secondary antibody after the incubation with the primary antibodies at optimal titers.

Expression and statistical analysis of the data

Results are expressed as means ± SEM. Statistical differences between data were analyzed using the Student-Newman-Keuls test. Where indicated, statistical comparisons were made with the ANOVA, followed by the Tukey test, with 95% confidence limits. Simple regressions were analyzed by means of Pearson's correlation coefficient for linear variations.

RESULTS

HSL activity and expression in adult nonpregnant rat mammary gland

Diacylglycerol lipase activity was assayed as an index of total HSL activity that is not influenced by the phosphorylation state. As shown in **Fig. 1**, in virgin rats, HSL activity expressed per gram of fresh tissue was higher in the mammary gland than in white adipose tissue (**Fig. 1A**), whereas it was lower in the former when expressed per milligram of protein (**Fig. 1B**).

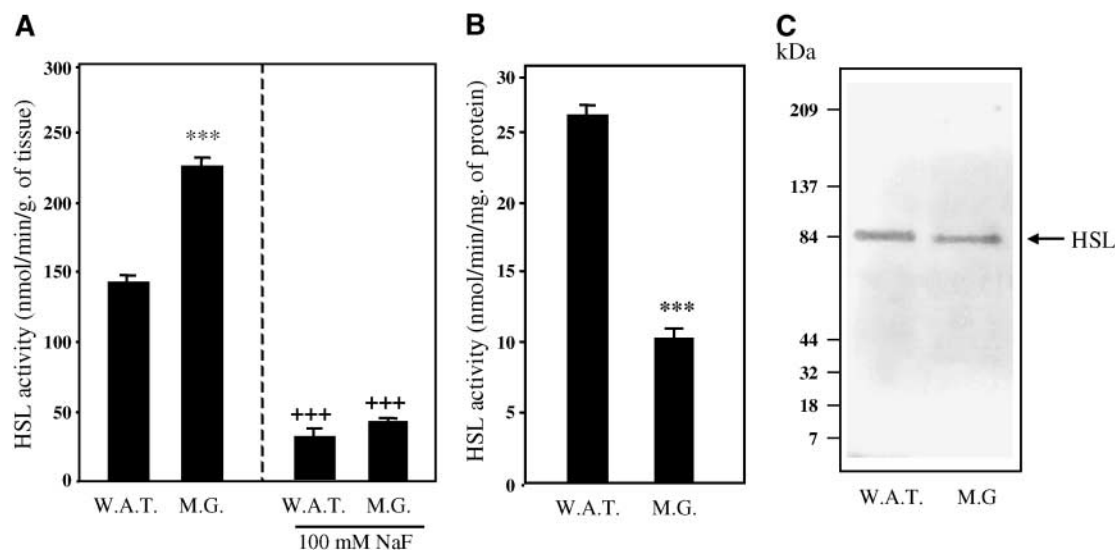


Fig. 1. Hormone-sensitive lipase (HSL) activity and protein expression in adult nonpregnant rat mammary gland. HSL activity (A, B) in the absence (A, left) or presence of the inhibitor NaF (A, right) and protein expression (C) in adult nonpregnant rat white adipose tissue (W.A.T.) and mammary gland (M.G.). HSL activity was determined as described in Materials and Methods and is expressed in nmol/min/g fresh tissue (A) or nmol/min/mg protein (B). HSL protein expression was determined by Western blot analysis (for details, see Materials and Methods). Ten micrograms of protein was always loaded in each lane. Reference proteins were myosin [molecular weight (M_r) 209,000], β -galactosidase (M_r 137,000), BSA (M_r 84,000), carbonic anhydrase (M_r 44,000), soybean trypsin inhibitor (M_r 32,800), lysozyme (M_r 18,700), and aprotinin (M_r 7,200). The position of the 84 kDa HSL band is indicated. Values are means \pm SEM ($n = 6-7$ animals). Statistical comparisons were made by ANOVA, followed by a Tukey test, with 95% confidence limits. *** Significant differences between lumbar adipose tissue and mammary gland HSL activity ($P \leq 0.001$); +++ significant differences between activities measured in the presence and absence of NaF ($P \leq 0.001$).

To verify the identification of the HSL, the effect of 100 mM NaF, which is known to inhibit white adipose tissue HSL activity (22), was studied. It was found that NaF markedly inhibited the HSL activity in both rat mammary gland and adipose tissue extracts (Fig. 1A, right panel), indicating that most of the assayed diacylglycerol lipase activity corresponded to HSL.

HSL protein expression was determined by Western blotting in fat-depleted infranats of both mammary gland and white adipose tissue. One major immunoreactive protein of 84 kDa was identified by the polyclonal chicken anti-HSL antibody in both tissues (Fig. 1C). The level of expression was lower in the mammary gland than in white adipose tissue.

HSL mRNA expression in adult nonpregnant rat mammary gland

Figure 2 shows the results of Northern blot analysis of samples from adult virgin rat white adipose tissue and mammary gland. As in previous studies, rat white adipose tissue yielded one HSL mRNA transcript of ~ 3.3 kb. Total RNA rat mammary gland analysis using a full-length rat white adipose tissue HSL cDNA probe also revealed the expression of one messenger of ~ 3.3 kb (Fig. 2), the same size as rat white adipose tissue HSL mRNA.

Immunolocalization of HSL in adult nonpregnant rat mammary glands

Immunohistochemistry was performed to determine the localization of HSL in virgin rat mammary glands. Both streptavidin-peroxidase (Fig. 3B) and immunoflu-

orescence (Fig. 3C) by immunohistochemical methods gave similar results, whereas no staining was found in any of the negative controls performed (Fig. 3A, D). Immunohistochemistry on sectioned mammary gland with the antibody directed against rat HSL revealed positive HSL-like immunoreactivity in the cytoplasm of alveolar epithelial cells (e in Fig. 3B, C) and adipocytes (a in Fig. 3B, C). Higher magnification views were used to examine individual alveoli (Fig. 3E, left panel) and adipocytes (Fig. 3E, right panel). With anti-HSL serum, unequivocal positive staining of the cytoplasm of mammary epithelial cells and adipocytes was observed.

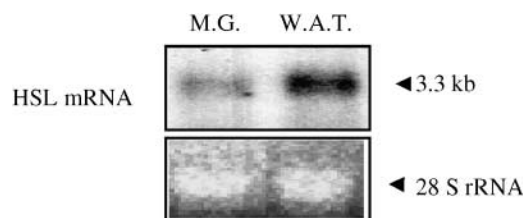


Fig. 2. Expression of HSL mRNA in adult nonpregnant rat mammary gland. Northern blot analysis of HSL mRNA expression in white adipose tissue (W.A.T.) and mammary gland (M.G.) of six to seven rats. Equal amounts (10 μ g) of total RNA were run on an agarose gel, blotted onto nylon membranes, and hybridized with rat HSL cDNA probe. The figure shows the results of a representative experiment, in which the expression of one messenger of ~ 3.3 kb was observed in mammary gland and white adipose tissue. The band of 28S rRNA, corresponding to the loading internal standard, is also shown.

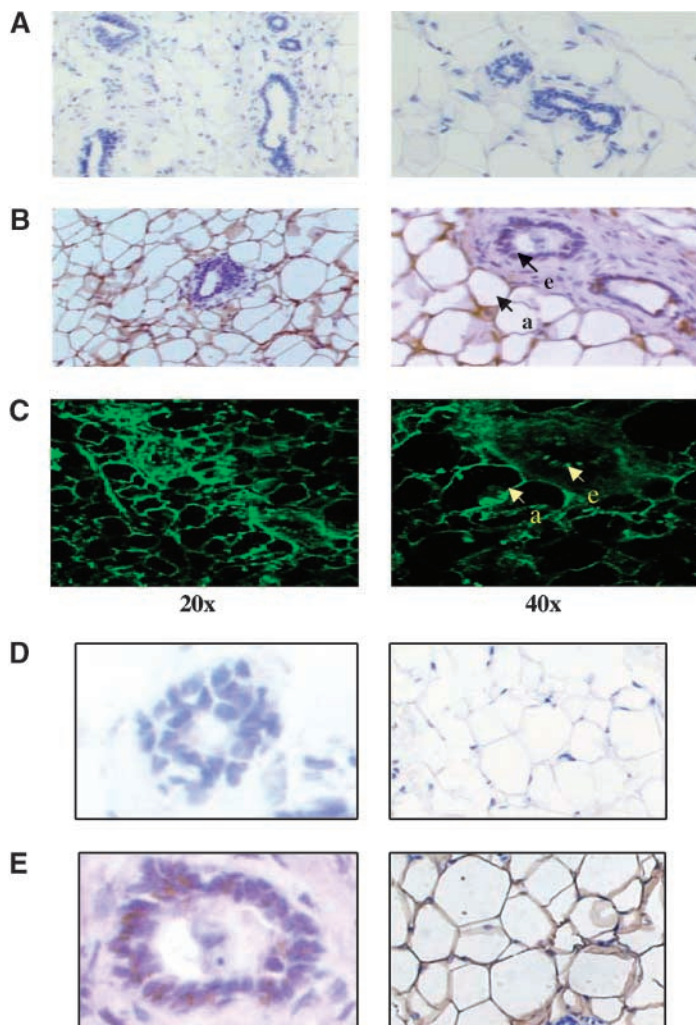


Fig. 3. Immunolocalization of HSL protein in adult non-pregnant rat mammary gland. Mammary glands were removed from nonpregnant rats, embedded in paraffin, sectioned, incubated with HSL primary antibody, and counterstained with Carazzi's hematoxylin. In adult nonpregnant rat mammary gland, adipocytes (a) are noticeable with few epithelial elements (e) present (B, 20 \times , 40 \times). Immunohistochemistry with polyclonal anti-HSL antibody followed by a secondary antibody conjugated with biotin (B, 20 \times , 40 \times) or Alexa Fluor 488 (C, 20 \times , 40 \times) shows positive staining (arrows) in the cytoplasm of adipocytes (a) and alveolar epithelial cells (e) of the adult non-pregnant rat mammary gland. At higher magnification (D, E), immunoreactive HSL occurs in the cytoplasm of the mammary epithelial cell (E, 60 \times , left panel) and mammary adipocytes (E, 60 \times , right panel). As a negative control, nonimmune serum was used as the primary antibody and no staining was found (A, D). Immune and nonimmune slides were photographed at the same exposure settings.

Morphological changes in rat mammary glands during pregnancy and lactation

Mammary gland sections were counterstained with hematoxylin and eosin to study changes in the morphology of the tissue. As shown in Fig. 4A, in mammary glands from virgin rats, the predominant cell types seen were adipocytes with few epithelial structures interdispersed among them. At mid pregnancy (day 12), morphological changes were apparent as epithelial structures began to proliferate, although a substantial proportion of adipocytes were still present. At late pregnancy (days 20 and 21), however, the epithelial cells became the predominant cell type (Fig. 4A). At early lactation (day 1), the epithelial cells were the predominant cell type and only small channels of lipid-filled adipocytes could be distinguished, and on the 4th day of lactation, the proportion of epithelial cells further increased at the expense of a manifest reduction of adipocytes, some of which could be seen to be practically depleted of their lipid stores (Fig. 4A).

HSL immunohistochemistry in rat mammary gland during pregnancy and lactation

An immunohistochemical method based on streptavidin-peroxidase and the use of anti-HSL polyclonal anti-

body were applied in the rat mammary gland to determine the location of HSL. Preliminary experiments showed no differences when samples were formalin-fixed, paraffin-embedded, or studied frozen, and no staining was found in any of the negative controls performed (data not shown).

As shown in Fig. 4B, positive HSL-like immunoreactivity was found in the cytoplasm of alveolar epithelial cells and adipocytes in mammary glands from virgin, pregnant (12, 20, and 21 days), and lactating (1 and 4 days postpartum) rats. Interestingly, the distribution of HSL staining changed parallel to the morphological changes of the mammary glands during pregnancy and lactation. In virgin rats, intensely stained adipocytes and light, diffusely stained epithelial cells were seen. During pregnancy and lactation, although the positive staining for HSL remained in the two types of cells, its intensity and the amount of positive immunoreactivity for HSL progressively increased with the time of pregnancy, reaching its highest intensity during lactation (Fig. 4B). Thus, although the mammary adipocytes were the most intensely stained cells in the virgin rat, epithelial cells were the predominantly stained cells during pregnancy and lactation.

Positive staining for HSL was also observed around the

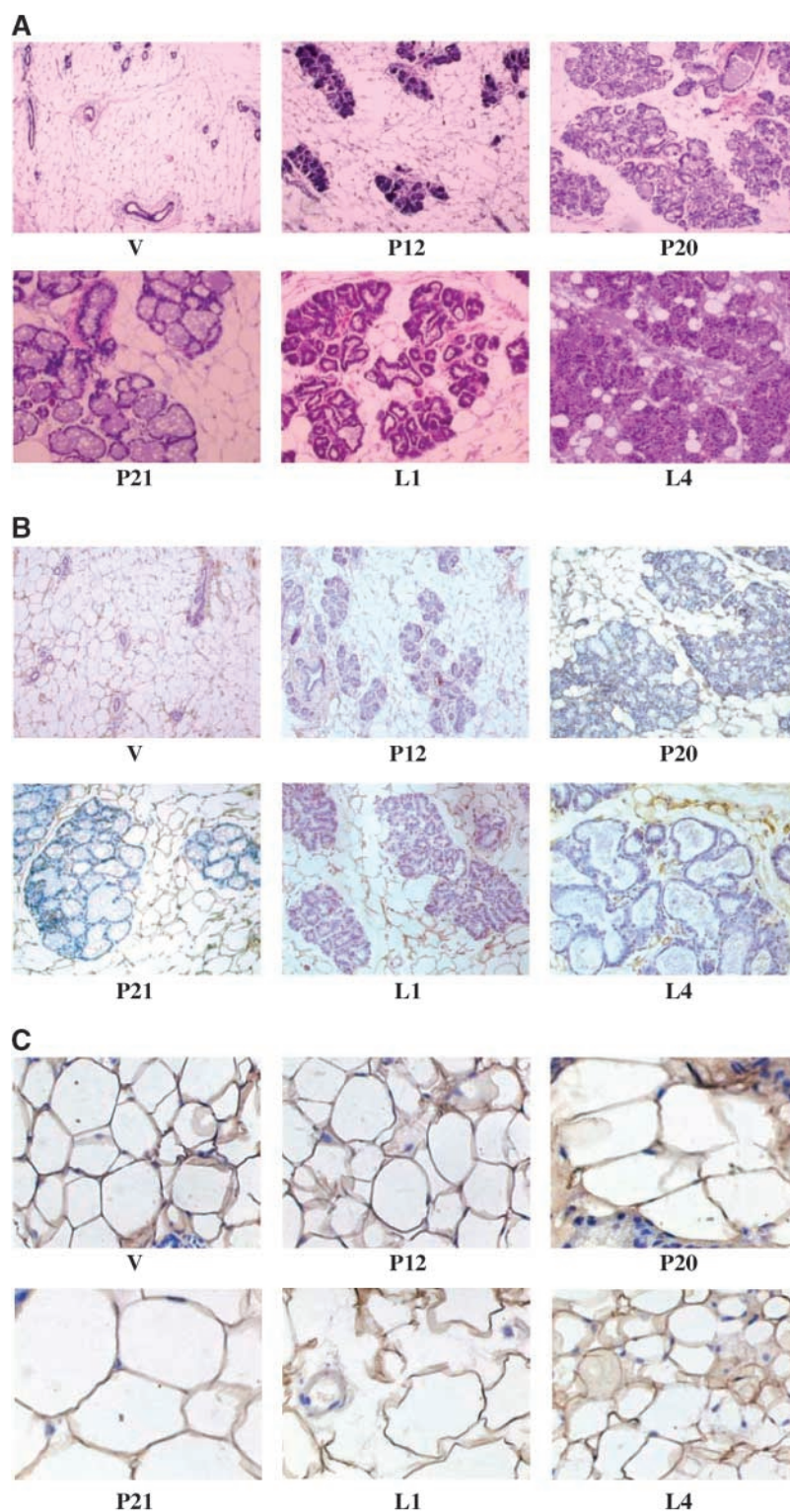


Fig. 4. Changes in rat mammary gland morphology, HSL protein localization, and adipocyte size during pregnancy and lactation. Mammary glands were removed from virgin (V) rats and rats on days 12, 20, and 21 of pregnancy (P12, P20, and P21) and days 1 and 4 of lactation (L1 and L4). They were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (A, 40 \times) or incubated with HSL primary antibody and counterstained with Carazzi's hematoxylin (B, 40 \times). Changes of mammary adipocyte size during pregnancy and lactation are shown in C (60 \times).

milk globules inside the lumen of the mammary gland alveoli in lactating rats (Fig. 4B, L1 and L4).

Changes in the size of the mammary gland adipocytes during pregnancy and lactation

Sections of mammary tissue were observed with an optic microscope to study the changes of mammary adipocytes during pregnancy and lactation. As shown in Fig. 4C, during late pregnancy there was a substantial increase in the size of mammary gland adipocytes compared with those of virgin rats. The morphology of the mammary adipocytes greatly changed on days 1 and 4 of lactation, progressively decreasing in size and becoming depleted of lipids, crumpling their membrane (Fig. 4C).

HSL activity and protein expression in rat mammary gland during pregnancy and lactation

Mammary gland HSL activity and protein were studied. As shown in Fig. 5, HSL activity expressed per unit of fresh tissue weight progressively decreased with pregnancy, becoming significant on days 20 and 21, compared with that of virgin rats. Just after parturition (days 1 or 4 of lactation), mammary gland HSL activity returned to values that did not differ from those in virgin animals (Fig. 5).

Western blot analyses were used to explore the changes

of HSL protein expression in mammary glands during pregnancy and lactation, and a representative autoradiogram of the 84 kDa band is illustrated at the top of Fig. 5. Mammary gland tissue from 20 and 21 day pregnant rats clearly showed less accumulation of HSL protein than did that from virgin rats, whereas on days 1 and 4 of lactation there was a return to values similar to those seen in virgin rats. Despite the parallel change seen in HSL activity and HSL protein, tissue total protein per gram greatly increased in pregnant and lactating rats compared with virgin rats. The HSL activity expressed per milligram of protein in 20 and 21 day pregnant rats and in 1 and 4 day lactating rats decreased compared with that in virgin animals (data not shown).

Plasma insulin levels and their relation to mammary gland HSL activity

To relate the changes observed in mammary gland HSL activity to plasma insulin levels, this variable was also measured. As shown in Fig. 6, plasma insulin levels increased significantly on day 12 of pregnancy, remained high on days 20 and 21 of pregnancy, and declined just from parturition. Values attained on day 4 of lactation were already significantly lower than those in virgin rats. The inverse trend found in plasma insulin levels expressed as mi-

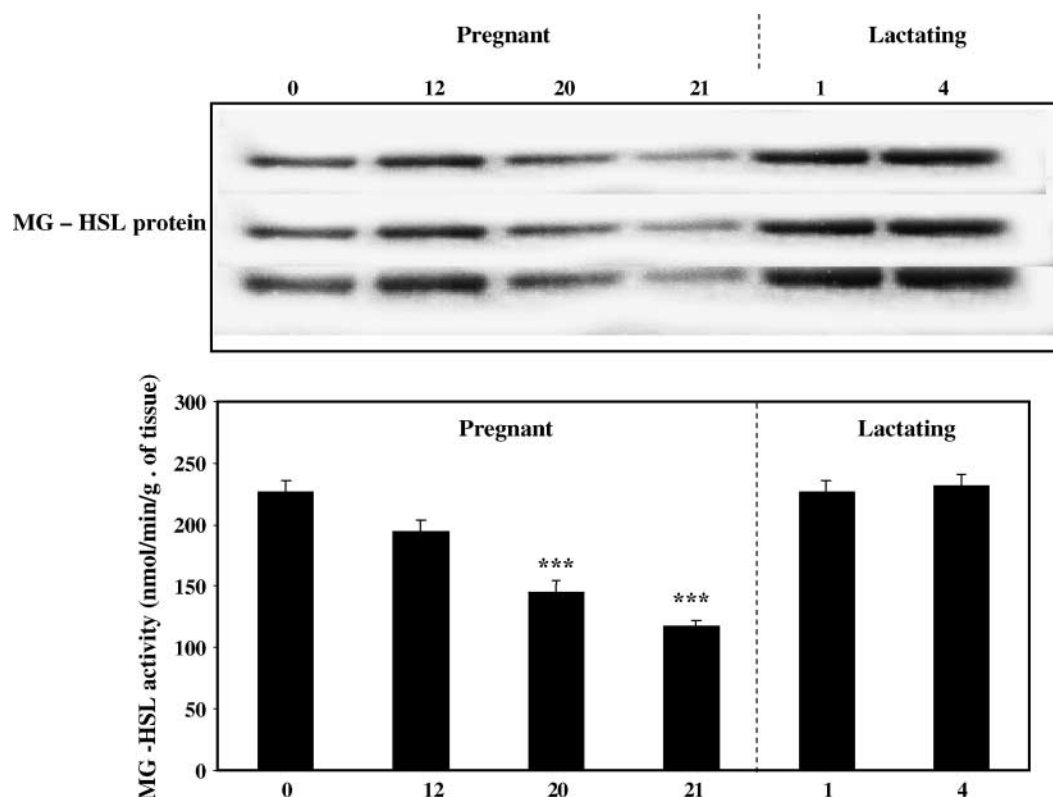


Fig. 5. Changes in HSL activity and protein expression in rat mammary gland (MG) during pregnancy and lactation. HSL activity (bottom panel) and HSL protein expression (top panel) in rat mammary glands from virgin (0) rats and rats at days 12, 20, and 21 of pregnancy (12, 20, and 21) and days 1 and 4 of lactation (1 and 4). HSL activity was determined as described in Materials and Methods. Western blot analysis was performed with anti-rat HSL in the mammary gland. Ten micrograms of protein was always loaded in each lane. Values are means \pm SEM ($n = 6-7$ rats/group). Statistical comparisons were made by ANOVA, followed by a Tukey test, with 95% confidence limits. *** $P \leq 0.001$.

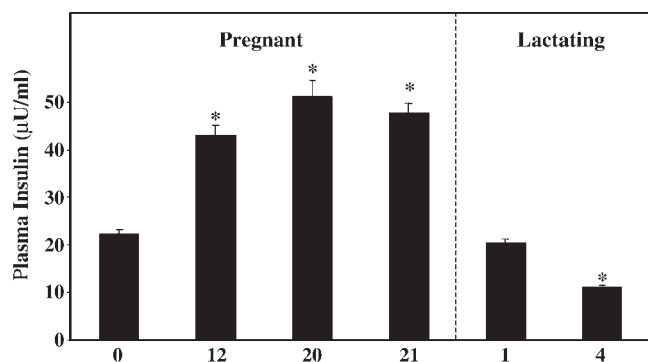


Fig. 6. Plasma insulin levels. Plasma insulin levels in rat mammary glands from virgin (0) rats and rats at days 12, 20, and 21 of pregnancy (12, 20, and 21) and days 1 and 4 of lactation (1 and 4). Values are means \pm SEM ($n = 6-7$ rats/group). Statistical comparisons were made by ANOVA, followed by a Tukey test, with 95% confidence limits. * $P \leq 0.05$ versus virgin rats.

crounits per milliliter (Fig. 6) and mammary gland HSL activity expressed as nanomoles per minute per gram of tissue (Fig. 5) prompted us to determine the linear correlation between these variables. When all of the individual values were considered, it was found that these two variables correlated negatively [insulin = -1.3502 (HSL activity) + 238.63; $r = -0.6114$, $n = 33$]. The correlation was statistically significant ($P \leq 0.001$).

Presence of HSL in milk

To determine whether mammary gland HSL was secreted with the milk, its activity and protein expression were measured in milk from rats on day 15 of lactation. As shown in Fig. 7, HSL activity was 1.35 ± 0.00 nmol/min/mg protein (Fig. 7A) and 26.8 ± 1.7 nmol/min/ml (Fig. 7B). Western blot analyses were used to explore the presence

of HSL protein in rat milk. The results are illustrated in Fig. 7C. Total proteins contained in the infranatant of milk were analyzed on SDS-PAGE gels stained with Coomassie Brilliant Blue, and a band of 84 kDa was clearly observed (Fig. 7C, bar 1). Western blot analyses performed with anti-rat HSL revealed that the immunoreactive band of 84 kDa in the infranatants of milk from lactating rats corresponded to HSL (Fig. 7C, bar 2).

DISCUSSION

In this study, we have demonstrated the presence of HSL in mammary gland adipocytes and epithelial cells from nonpregnant, pregnant, and lactating rats, observing variations in HSL activity and expression during pregnancy and lactation. The presence of HSL in alveolar epithelial cells has previously been reported in lactating rat mammary tissue (37, 38), although this is the first time that its presence has been reported in mammary gland adipocytes. HSL in the mammary gland is known to be regulated, as in other tissues, by cAMP-dependent protein kinase phosphorylation-dephosphorylation and has been described as a neutral cholesteryl ester hydrolase in the microsomal and cytosolic subcellular fractions from rat lactating mammary glands (49, 50). The cellular origin of HSL in mammary tissue is thought to be the mammary epithelial cell, the predominant cell type in the lactating gland. These cells are equipped with a competent cAMP signaling system (51) and express the cAMP-activated protein kinase (52). However, adipocytes are also present in the mammary glands, and HSL is well known to be produced by adipocytes in other sites of the body (53). By immunohistochemistry, we observed a positive reaction to HSL in the cytoplasm of both mammary gland epithelial

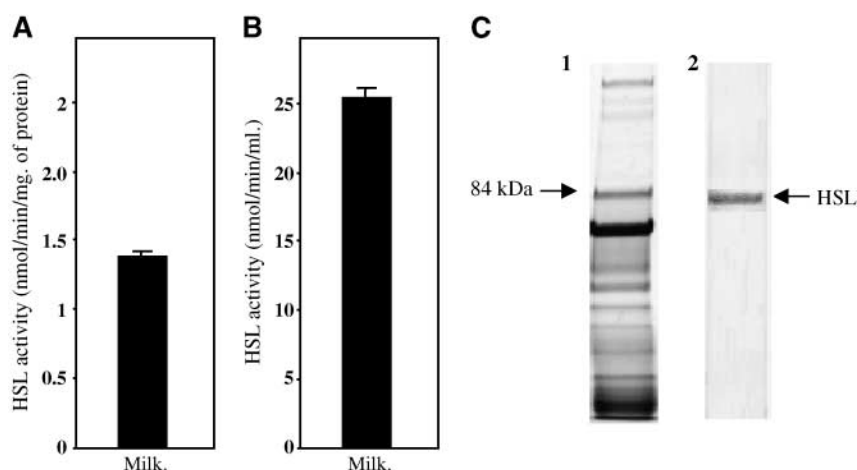


Fig. 7. HSL enzyme activity and protein expression in rat milk. HSL activity expressed as nmol/min/mg protein (A) or as nmol/min/ml (B) and protein expression (C) on an SDS-PAGE gel stained with 0.2% Coomassie Brilliant Blue in methanol-acetic acid-water (C, bar 1) and Western blot analysis performed with anti-HSL (C, bar 2) in the infranatants of milk from lactating rats are shown. Milk infranatants were obtained after ultracentrifugation (110,000 g) as described in Materials and Methods. The samples were preincubated for 60 min at 37°C in 20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM dithioerythritol, and 0.2 mg/ml albumin and then assayed for lipase activity. Values are means \pm SEM ($n = 5$ animals). The position of the HSL 84 kDa band is indicated.

cells and adipocytes. By Western blot analysis, we detected one immunoreactive band of 84 kDa to HSL antibody in the extract of protein from rat mammary glands. The size of this protein was identical to the HSL 84 kDa subunit found in rat white adipose tissue (28, 29), and the transcribed mRNA of 3.3 kb was found here to be expressed in rat mammary gland as well as in white adipose tissue. These results indicate that the HSL protein synthesized in rat mammary gland is identical to the HSL protein previously described in white adipose tissue. The fact that in the nonpregnant rat HSL protein and mRNA expression and activity were lower in mammary gland than in white adipose tissue is consistent with functional differences between these tissues.

The present study also demonstrates that gestation and lactation in the rat causes different responses of HSL activity and protein levels in mammary glands. By immunohistochemistry, we determined that HSL is localized in the cytoplasm of the mammary gland adipocytes and alveolar epithelial cells of nonpregnant, pregnant, and lactating rats. The proportion of stained epithelial cells increases during pregnancy and reaches its highest value during lactation. The mammary gland adipocytes are also positively stained for HSL, and these cells become enlarged during pregnancy to become depleted of lipids during lactation. The decreased HSL activity and protein expression observed in rat mammary gland during late pregnancy indicates a decreased lipolytic activity in this tissue, which would contribute to its increased lipid storage. On the contrary, the increased HSL activity and protein expression in lactating rat mammary gland, together with the decreased adipocyte size, indicates an increased lipolytic activity that would favor the mobilization of the lipid stores. These results indicate that HSL is involved in the regulation of the transitory fat accumulation followed by fat mobilization in rat mammary gland adipocytes during pregnancy and lactation. Therefore, it is proposed that HSL is responsible for the release of FFAs from triacylglycerols, diacylglycerols, and/or cholesterol from the cholesteryl esters stored in the lipid droplet of mammary gland adipocytes, to provide lipids for milk synthesis and secretion by the epithelial cells.

The inverse linear correlation found here between plasma insulin levels and mammary gland HSL activity suggests a direct relationship between these two variables. Different from the insulin-resistant condition that is normally present during late pregnancy in adipose tissue (54, 55) and skeletal muscle (55–57), the mammary gland shows increased insulin sensitivity caused by an augmented kinase activity of the insulin receptor (42). It has also been shown that insulin participates in the enhanced expression of LPL in the mammary gland during late pregnancy (58), whereas it is responsible for the decrease of LPL activity normally seen in maternal white adipose tissue (59, 60). Thus, it is proposed that maternal hyperinsulinemia and enhanced mammary gland insulin sensitivity during late pregnancy are responsible for the decline in HSL, both in activity and expression, found here in the mammary gland before parturition.

The presence of HSL in milk at mid lactation found here indicates that a certain proportion of the enzyme synthesized in the mammary gland is released into milk and becomes available to the digestive tract of the suckling newborn. This action may contribute to the digestion process of milk fat [i.e., the hydrolysis of cholesteryl ester, tri-(di-)acylglycerides, and fat-soluble vitamin esters] in the suckling neonate.

In conclusion, we report here the presence of HSL in both mammary gland adipocytes and epithelial cells. Its activity decreases during late pregnancy and is recovered just at the initiation of lactation. In fact, it is suggested that the reduction of HSL expression and activity found in mammary gland during late pregnancy corresponds to its decreased expression in adipocytes, which does not compensate by its increased expression in epithelial cells. Only with the onset of lactation do HSL expression and activity increase, corresponding to its presence in epithelial cells plus that released into milk globules, inside the lumen of the alveoli. Although the physiological role of these changes remains to be investigated, it is proposed that they, together with the well-known induction of LPL in the mammary gland at parturition, may actively contribute to the net accumulation of lipids for milk synthesis in the mammary gland during late pregnancy. The rapid recovery that takes place in the mammary gland HSL at the initiation of lactation may facilitate its presence in milk and may contribute to the digestion of milk fat in the suckling neonate. ■

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