Effects of hypothyroidism on mammary and liver lipid metabolism in virgin and late-pregnant rats

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Abstract Untreated maternal hypothyroidism (hypoT) has serious consequences in offspring development that may result from the effect on lactation of maternal metabolism dysfunction. We studied the effects of prolonged propylthiouracyl (PTU)-induced hypoT (0.1% PTU in drinking water starting 8 days before mating until day 21 of pregnancy or for 30 days in virgin rats) on liver and mammary lipid metabolism and serum lipid concentrations. In virgins, hypoT reduced hepatic mRNAs associated with triglyceride (TG) and cholesterol synthesis (including fatty acid synthase and 3-hydroxy-3-methylglutaryl coenzyme A reductase), and induced lobuloalveolar mammary development. Pregnancy increased hepatic mRNAs associated with TG and cholesterol synthesis and uptake (including LDL receptor) and with lipid oxidation, such as acyl CoA oxidase. HypoT decreased mRNAs and the activity of proteins associated with TG synthesis, and mRNAs associated with cholesterol uptake and lipid oxidation. Pregnancy increased mammary mRNAs related to lipid oxidation and decreased cholesterol synthesis, whereas hypoT decreased mRNAs and activities of proteins associated with TG synthesis and decreased epithelial mammary tissue. Virgin and pregnant hypoT rats had increased circulating VLDL + LDL cholesterol. HypoT decreased circulating TGs in pregnant rats. The observed effects of hypoT may result in decreased mammary lipid availability. This, along with the decreased epithelial mammary tissue during lactogenesis, may contribute to the future lactational deficit of hypoT mothers.—Hapon, M. B., S. M. Varas, G. A. Jahn, and M. S. Giménez. **Effects of hypothyroidism on mammary and liver lipid metabolism in virgin and late-pregnant rats.** ***J. Lipid Res.*** **2005.** 46: **1320–1330.**

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Thyroid hormones influence all major metabolic pathways. Their most obvious and well-known action is an increase in basal energy expenditure through actions on protein, carbohydrate, and lipid metabolism. With specific regard to liver lipid metabolism, thyroid hormones stimulate fatty acid and cholesterol synthesis (1, 2), increase mobilization of plasma cholesterol and triglycerides (TGs) (3, 4), and stimulate fatty acid and cholesterol degradation (5, 6). Disturbances in thyroid function are commonly associated with alterations in plasma lipid levels. Experimental hypothyroidism (hypoT) induced by propylthiouracyl (PTU) treatment is characterized by the accumulation of plasma LDL cholesterol, and decreased VLDL and plasma TGs (7), generally reflecting reduced binding activity of the hepatic LDL receptor (LDLR), which can be normalized after substitution therapy with thyroid hormone (3, 8).

Pregnancy is a state of dynamic changes in metabolism and nutrient utilization. The insulin-resistant condition and the increase in plasma estrogen levels occurring during late pregnancy are the main factors responsible for the development of a state of maternal hypertriglyceridemia that has been extensively studied in humans and rats (9–11). This condition benefits the progeny in two ways. First, it supplies essential fatty acids that are critical to normal fetal development and that circulate primarily esterified and associated with lipoproteins. A linear correlation between maternal and fetal plasma TGs has been described that has an important implication in newborn weight (12, 13). Second, it contributes to milk synthesis in preparation for lactation, providing circulating TG in the form of lipoprotein to the mammary gland (MG) for milk lipid synthesis (9).

It has been demonstrated that the induction of hypothyroidism in dairy cows suppresses milk production during the treatment period (14). On the other hand, ad-

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Abbreviations: ACC, acetyl CoA carboxylase; ACO, acyl CoA oxidase; CPT, carnitine palmitoyl transferase; EC, esterified cholesterol; FC, free cholesterol; GPAT, glycerol-3-phosphate acyltransferase; HMG-CoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; hypoT, hypothyroidism; LDLR, LDL receptor; MG, mammary gland; PTU, propylthiouracyl; T_3, triiodothyronine; T_4, tetraiodothyronine; TG, triglyceride.

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ministration of a moderate oral dose of triiodothyronine (T_3) to lactating rat dams induces a higher growth rate in the pups, and this positive effect seems to be mainly due to augmented secretion of milk that, in addition, contains an elevated proportion of TGs (15). The prevalence of subclinical hypothyroidism in women of childbearing ages is 4–5% (16) and, at least in two population-based surveys carried out in areas with different iodine intake, suggests a 2.5% overall prevalence of compensated or uncompensated hypothyroidism during pregnancy (17), making it a significant risk for gestational outcome. The impact of maternal and neonatal hypothyroidism on the offspring is very well documented, and its most marked consequences are low birth weight, stunted growth, and delayed maturation of the newborn, which lead to mental retardation and subnormal height (17). In rats, hypothyroidism has been associated with delayed delivery, subnormal number of fetuses, increased pup mortality, decreased pup growth, and altered circulating hormones (18, 19). Although most of these effects can be related to the hypothyroid state of the infants, we found impairments in milk ejection that may contribute to the growth retardation of the litters (19).

A state of clinical hypothyroidism during pregnancy in rats shows decreased synthesis of TGs in terms of ^{14}C-glucose incorporation and, subsequently, decreases in TGs and enhanced cholesterol in the circulation (20, 21), but we have found no report on the mechanism underlying these changes or on any effect of hypothyroidism on MG metabolism. Pregnancy is characterized by changes in thyroid hormone utilization and metabolism; late pregnancy is considered a state of "physiological" hypothyroidism, with increased metabolism and clearance of thyroid hormones and peripheral tissue resistance to them (22, 23). Thus, a state of clinical or subclinical hypothyroidism may well be aggravated by the pregnant state. In particular, the availability of TGs to the fetus and to the MG that is preparing for lactation could be affected, inasmuch as they are targets of thyroid hormones. Furthermore, maternal hyperlipidemia or hypercholesterolemia, even when limited to pregnancy, may trigger pathogenic events, such as fetal and maternal cardiovascular alterations that could lead to atherosclerosis later in life (24, 25).

We hypothesize that hypothyroidism may produce alterations in lipid metabolism (in particular TGs and cholesterol) at the hepatic, mammary, and systemic levels that may contribute to the lactation deficit observed in hypothyroid mothers. With these objectives, we examined the effects of an approximately 30 day PTU treatment given to late-pregnant rats on lipid content, synthesis, and activity; expression of enzymes related to lipid metabolism in liver and MG; and serum parameters related to lipid metabolism. To differentiate between the effects of pregnancy and those of hypothyroidism, we also included groups of virgin rats given PTU for the same length of time. Thus, we will attempt to gain a better comprehension of the mechanisms underlying the impact of hypothyroidism on the metabolic changes preliminary to the initiation of lactation.

MATERIALS AND METHODS

Chemicals and radioisotopes

[^{3}H]H_2O (3.70 GBq/g), [^{14}C]$NaHCO_3$ (39.2 MBq/mmol) and ^{125}I-(629 GBq/mg) were purchased from New England Nuclear Life Science Products, Inc. (Boston, MA). Lipid standards were acquired from Sigma Chemical Co. (St. Louis, MO). All the other chemicals were of reagent grade and were obtained from Merck Laboratory (Buenos Aires, Argentina) or from Sigma Chemical Co.

Animals and experimental design

Adult female Wistar rats bred in our laboratory, 3 to 4 months old and weighing 200–230 g at the onset of treatment, were used. The rats were kept in a light- (lights on 6:00 AM–8:00 PM) and temperature- (22–24°C) controlled room. Rat chow (Cargill, Cordoba, Argentina) and tap water or PTU solution were available ad libitum. Hypothyroidism was induced by administration of PTU at a concentration of 0.1 g/l in the drinking water. The treatment was started on the estrus day 8 days before mating for the pregnant group. The presence of spermatozoa in the vaginal smear the morning after caging with a fertile male in the night of pro-oestrus was indicative of pregnancy and this day was counted as day 0 of pregnancy. PTU-treated (hypoT) or control rats were sacrificed after 30 days of PTU treatment or on day 21 of pregnancy at 6:00 PM. Animal maintenance and handling was performed according to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication No 86-23, revised 1985 and 1991) and the UK requirements for ethics of animal experimentation (Animals Scientific Procedures, Act 1986).

After decapitation, trunk blood was collected and serum was separated by centrifugation and stored at −20°C until used. The livers and inguinal MGs from the dams were removed, snap frozen in liquid nitrogen, and stored at −70°C until they were analyzed. The values are means ± SEM for groups of eight rats.

Tissue preparation and enzymatic assays

Liver or MG portions (1 g for 4 ml of buffer) were homogenized in an Ultra Turrax T25 homogenizer in 0.5 M potassium phosphate buffer (pH 7) containing 10 mM EDTA and 10 mM D,L-dithiotreitol (DTT). The homogenates were centrifuged at 100,000 *g* for 1 h to yield the cytosolic fraction in a Beckman model L8-80M ultracentrifuge with a Ti-80 rotor.

Cytosolic FAS activity was determined spectrophotometrically by a modified version of the method of Alberts et al. (26). The reaction mixture contained 0.5 M potassium phosphate buffer (pH 6.6), 1 μmol each of EDTA and DTT, 100 nmol of NADPH, and 0.05 ml of the cytosolic fraction. The reaction was started by adding 100 nmol of malonyl-CoA, and the final assay volume was 1.05 ml. The oxidation of NADPH at 30°C was monitored at 340 nm. FAS activity was expressed as U/mg of cytosolic proteins.

Acetyl CoA carboxylase (ACC) activity in liver or mammary cytosols was measured as described previously by Allred and Rochringer (27). The enzyme activity was measured using a reaction mixture that, in a final volume of 700 μl, contained 60 mM buffer Tris-acetate (pH 7.8), 100 mM potassium acetate, 3 mM DTT, 8.5 mM potassium citrate, 1 mM ATP, 0.6 mg/ml BSA, 0.35 mM acetyl CoA, 8 mM magnesium acetate, 25 mM sodium bicarbonate, and 2 μCi [^{14}C]$NaHCO_3$. For blanks, acetyl-CoA was omitted. The mixture was preincubated for 1 min at 37°C, then 50 μl of the cytosolic fractions was added. After 1 min incubation at 37°C, 50 μl of 70% HCl was added to stop the reaction. A 200 μl aliquot was transferred into the scintillation vial and dried under cold air flow. The dried extract was resuspended with 200 μl 50% ethanol, and the radioactivity was measured in 10 ml scintillation fluid in a Wallac LKB 1409 liquid scintillation analyzer.

ACC activity was expressed as U/mg protein, where 1 unit equals 1 pmol of [^{14}C]bicarbonate incorporated into malonyl-CoA per minute at 37°C. The protein concentration was determined by the method of Lowry et al. (28), using fraction V BSA as standard.

Lipid determinations

The lipids from hepatic or mammary tissue were extracted with chloroform-methanol (2:1) according to the method of Folch, Less, and Sloane-Stanley (29). One aliquot of the lipid extracts was taken to determine total cholesterol and another to separate the different lipid fractions by thin-layer chromatography with an *N*-hexane-diethyl ether-acetic acid (80:20:1; v/v/v) solvent system. The scraped bands were eluted, and aliquots were used for determination of the mass of the different lipids. Free cholesterol (FC) and esterified cholesterol (EC) were determined according to the method of Zack et al. (30) after saponification (31). TGs were quantified by the method of Sardesai and Manning (32), and phospholipids were determined according to Bartlett (33). A recovery from thin-layer chromatography averaging 90% of cholesterol mass was obtained.

Incorporation of ^{3}H from H_2O into lipids

The groups of pregnant rats were fed ad libitum and then injected intraperitoneally with 37 mBq (1 mCi)/rat [^{3}H]H_2O in 1 ml saline. They were killed 2 h later by decapitation to ensure that the newly synthesized lipids in the liver and MG had been labeled. One gram each of liver and MG were extracted with 20 ml of chloroform-methanol (2:1) according to the method of Folch, Less, and Sloane-Stanley (29). The different lipid fractions were separated by thin-layer chromatography (see lipid determinations), the bands were scraped and vacuum dried, and the radioactivity incorporated into each fraction was counted in a β counter (Wallac-Pharmacia 1209). The results are expressed as ng of ^{3}H incorporated/h/g of tissue.

Hormone determinations

Thyroid-stimulating hormone (TSH) was radioiodinated using the chloramine T method and purified by passage through Sephadex G75 (34). The results were expressed in terms of rat TSH RP-3 standard preparation. Assay sensitivity was 0.5 $\mu g/l^{-1}$ serum and the inter- and intra-assay coefficients of variation were <10%.

Insulin was measured in the serum samples from the fasted rats used for lipid and glucose determinations (see below) by heterologous radioimmunoassay using a commercial solid-phase human insulin RIA kit (DPC Coat-a-Count Insulin; Diagnostic Products Corporation, Los Angeles, CA) and rat insulin (Linco rat insulin standard; Linco Research Inc., St. Charles, MO) as standard. Results are expressed as ng/ml rat insulin. Assay sensitivity was <0.6 ng/ml, and intra-assay variation was <5%. All samples were measured in the same assay.

Circulating T_3 and tetraiodothyronine (T_4) were measured by RIA, using commercial kits for total hormones (DSL Total T_3, DSL3100 DSL Total T_4 double-antibody RIAs; Diagnostic Systems Laboratories, Los Angeles, CA).

Serum lipid, protein, and glucose determinations

Serum glucose, total lipids (comprising TGs, phospholipids, FC, EC, and free fatty acids), TGs, phospholipids, total cholesterol, and HDL-cholesterol concentrations were measured by colorimetric or enzymatic methods (kits from Boehringer, Buenos Aires, Argentina) using fresh serum from control or hypothyroid rats that had been fasted for 14 h and bled on day 21 of pregnancy or after 30 days of PTU treatment for the virgin rats. The rats were lightly anesthetized with ethyl ether between 10:00 and 10:30 AM, and a blood sample was obtained from the tail vein. The blood was centrifuged within 1 h of sampling, and serum was separated and kept at 4°C to minimize glucose metabolization by blood cells. Glucose was measured immediately, and total lipids, total cholesterol, and HDL-cholesterol measurements were performed within 4 h of obtaining the samples. HDL-cholesterol was measured in the supernatant after precipitation of other lipoproteins by the dextran sulfate-$MgCl_2$ procedure (35). LDL + VLDL cholesterol was calculated by subtracting the HDL values from total cholesterol values.

RNA isolation and RT-PCR analysis

Total RNA from MGs and livers was prepared using the guanidinium isothiocyanate-acid phenol method (36) as modified by Puissant and Houdebine (37). Ten micrograms of total RNA were reverse transcribed at 42°C using random hexamer primers and Moloney murine leukemia virus RT (Invitrogen/Life Technologies, Buenos Aires, Argentina) in a 20 μl reaction mixture. Aliquots of the reverse transcription reaction mix cDNA corresponding to different quantities of cDNA for each reaction [livers: ACC, 200 ng; FAS, 100 ng; glycerol-3-phosphate acyltransferase (GPAT), 5 ng; acyl CoA oxidase (ACO), 5 ng; carnitine palmitoyl transferase (CPT), 10 ng; 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), 20 ng; and rat ribosomal protein L19, 20 ng; MGs: ACC, 1 μg; FAS, 10 ng; GPAT, 50 ng; ACO, 50 ng; CPT, 100 ng; 7α-hydroxylase LDLR, 100 ng; HMG-CoA, 200 ng; LPL, 10 ng; and L19, 20 ng] were amplified with primers specific for rat FAS, ACC, GPAT, ACO, CPT, LPL (38), 7α-hydroxylase, LDLR (39), and HMG-CoA reductase (HMG-CoAR) (40) modified by us to correspond to the rat sequence (**Table 1**), and L19 (41), used as control (Table 1). Before proceeding with the semiquantitative PCR, the conditions were established for each tissue such that the amplification of the products was in the exponential phase, and the assay was linear with respect to the amount of input RNA. All reactions were carried out for 25 cycles for L19 and 30 cycles for all the rest, with the following cyclic parameters for all the reactions: 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and then terminated with a 5 min extension at 72°C. The conditions and quantities of cDNA added were such that the amplification of the products was in the exponential phase, and the assay was linear with respect to the amount of input RNA. RNA samples were assayed for DNA contamination by PCR without prior reverse transcription. The PCR products were analyzed on 1.5% agarose gels containing 0.5 mg/ml ethidium bromide and photographed with a Polaroid camera. Band intensities of RT-PCR products were quantified using NIH Image software. Relative levels of mRNA were expressed as the ratio of signal intensity for the target genes relative to that for L19 cDNA.

Mammary gland histology

Mammary tissue was fixed in buffered phormol, dehydrated in ethanol, and embedded in paraffin wax. Sections of 3–5 μm were cut with a Reichert-Jung Hn 40 microtome and stained with hematoxilin-eosin. Images were taken with a Zeiss Axioscop 2 light microscope fitted with a Sony CCD-IRIS/RGB video camera. For all the morphological analyses, only the inguinal MGs were used. Six to eight sections from each animal were histologically evaluated for changes in the extent of lobuloalveolar development and supporting adipose tissue, measuring the relative areas in each preparation at 50× magnification. The areas were calculated using NIH Image 1.6.3 software. The size of lipid droplets in the preparations from pregnant rats was measured, taking the greater and smaller diameters of individual lipid droplets within the epithelial cells at 1,000× magnification, using Image Pro Plus 4.5 software. Approximately 100 droplets were taken at random within the epithelial compartment for each individual sec-

TABLE 1. Primer sequences used in the PCR amplification of various cDNAs

Gene	Sense Primer (5′-3′)	GenBank Accession Number	Size of Amplification Product (bp)
L19 S	CGCCAATGCCAACTCTCGTCA	X82202	120
L19 AS	TTCCGTCGGGCCAAAGGTGTTC		
FAS S	GTTTGATGGCTCACACACCT	M84761	515
FAS AS	TACACTCACTCGAGGCTCAG		
ACC S	ACTCCAGGACAGCACAGATC	NM022193	535
ACC AS	TCTGCCAGTCCAATTCTAGC		
GPAT S	TGATCAGCCAGGAGCAGCTG	U36771	508
GPAT AS	AGACAGTATGTGGCACTCTC		
ACO S	GCCCTCAGCTATGGTATTAC	J02752	634
ACO AS	AGGAACTGCTCTCACAATGC		
CPT-1 S	TATGTGAGGATGCTGCTTCC	L07736	629
CPT-1 AS	CTCGGAGAGCTAAGCTTGTC		
LPL S	CCTGAAGACTCGCTCTCAGA	L03294	454
LPL AS	TTGGTTTGTCCAGTGTCAGC		
7α-Hydroxylase S	GCCGTCCAAGAAATCAAGCAGT	X17595	306
7α-Hydroxylase AS	TGTGGGCAGCGAGAACAAAGT		
LDLR S	ATTTTGGAGGATGAGAAGCAG	X13722	931
LDLR AS	CAGGGCGGGGAGGTGTGAGAA		
HMG-CoAR S	GTGATTACCCTGAGCTTAGC	NM013134	462
HMG-CoAR AS	TGGGATGTGCTTAGCATTGA		

ACC, acetyl CoA carboxylase; ACO, acyl CoA oxidase; AS, antisense; CPT, carnitine palmitoyl transferase; GPAT, glycerol-3-phosphate acyltransferase; HMG-CoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDLR, LDL receptor; S, sense.

tion, and the average diameters were calculated for each preparation. For statistical analysis, we compared the means of the averages.

Statistics

Statistical analysis was performed using two-way ANOVA followed by the Bonferroni posthoc test to compare any two individual means or using Student's *t*-test when only two groups were compared (42). When variances were not homogeneous, we performed log transformation of the data. Differences between means were considered significant at the $P < 0.05$ level.

RESULTS

Circulating levels of thyroid hormones and TSH in virgin and pregnant rats

The virgin rats had higher levels of thyroid hormones and of TSH than the rats on day 21 of pregnancy. Administration of PTU in the drinking water produced decreases in circulating T_3 and T_4 and increases in serum TSH in both groups, confirming the hypothyroid state of the rats (**Table 2**), but the decreases in T_3 and T_4 were more profound in the pregnant rats compared with the virgins (Table 2).

Effect of hypothyroidism on liver lipid metabolism in virgin and pregnant rats

To evaluate the effects of thyroid hormone on lipogenic enzymes, mRNA abundance of ACC, FAS, and GPAT was measured using semiquantitative RT-PCR of total RNA prepared from livers of virgin and pregnant rats. FAS mRNA was increased in the pregnant groups and hypoT decreased it in both groups of rats. Gestation did not alter the levels of liver ACC mRNA, but hypoT decreased them significantly in virgin and pregnant rats (**Fig. 1**). GPAT mRNA was increased more than 2-fold in pregnant liver, compared with the virgin group, and was decreased by hypoT in virgin rats but not in pregnant rats (Fig. 1). These data suggest that gestation stimulates FAS and GPAT expression as a result of the increased requirement for TG synthesis. This gestational requirement was affected by thyroid hormone deficit, in agreement with the results observed by others in male rats (43). Because degradation of lipids is affected by altered thyroid states, we also measured the mRNA concentrations of ACO and CPT, enzymes related to β-oxidation. Liver ACO and CPT mRNA concentrations were increased by gestation, suggesting a stimulation of fatty acid oxidation during pregnancy. Only ACO was diminished in both hypoT groups, in agreement with the observations of Kremser et al. (5). To explore the possible causes of the hypercholesterolemic effect of hypothyroidism, we evaluated the hepatic mRNA contents of HMG-CoAR, the rate-limiting enzyme of cholesterol synthesis, and of LDLR and 7α-hydroxylase, factors involved in cholesterol uptake and metabolization, respectively (Fig. 1). HMG-CoAR was increased in late gestation com-

TABLE 2. Effects of PTU treatment on serum concentrations of T3, T4, and TSH of virgin and pregnant rats

	Virgins		Day 21 of Pregnancy	
	Control	HypoT	Control	HypoT
T3 (ng/dl)	159.8 ± 10.4	131.2 ± 4.8[a]	50.1 ± 11.7[b]	9.3 ± 3.5[a,b]
T4 (μg/dl)	49.5 ± 3.4	23.4 ± 4.9[a]	10.6 ± 1.1[b]	0.4 ± 0.2[a,b]
TSH (ng/ml)	1.1 ± 0.1	14.2 ± 1.0[a]	0.6 ± 0.1[b]	8.2 ± 0.5[a,b]

PTU, propylthiouracyl; T_3, triiodothyronine; T_4, tetraiodothyronine; TSH, thyroid-stimulating hormone. Values are means ± SEM for groups of eight rats. Virgin rats were given PTU for 30 days, pregnant rats.

[a] $P < 0.05$ compared with the respective control groups using two-way ANOVA and the Bonferroni posthoc test.

[b] $P < 0.05$ compared with the respective virgin group using two-way ANOVA and the Bonferroni posthoc test.

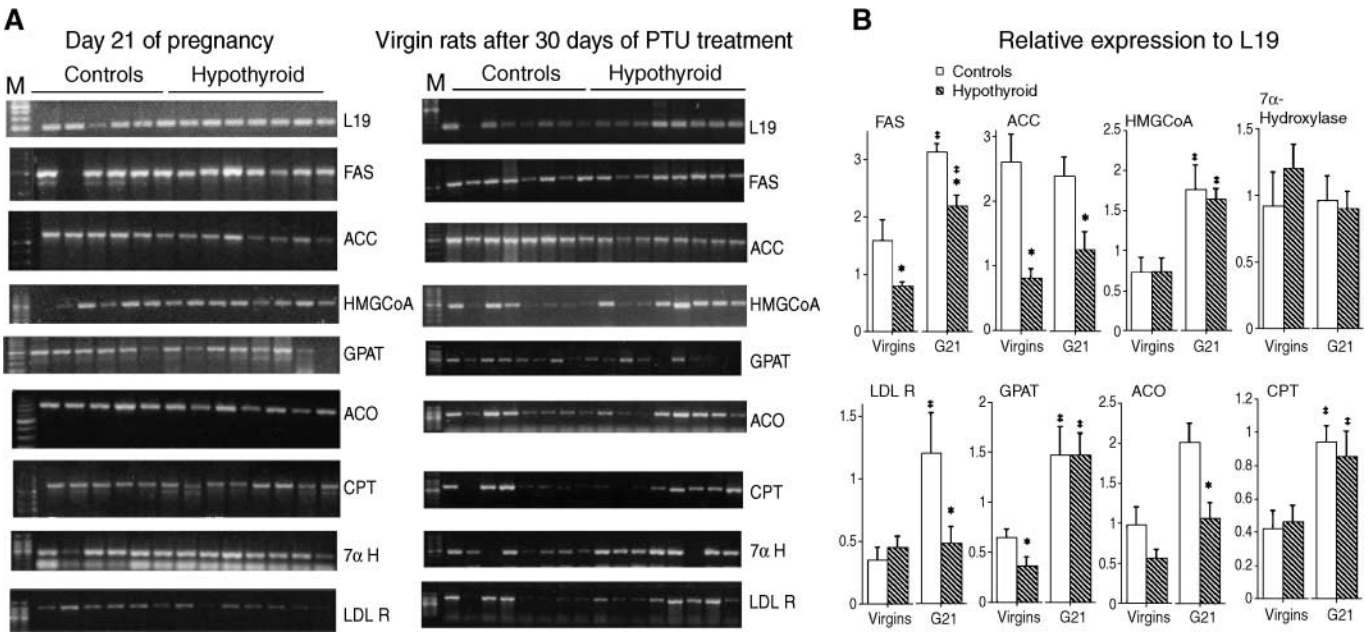

Fig. 1. Expression of liver LDL receptor (LDLR) and enzymes related to lipid metabolism in control and hypoT virgin or day 21-pregnant rats (G21). A: Measurement by RT-PCR of expression of L19, FAS, acetyl CoA carboxylase (ACC), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR), glycerol-3-phosphate acyltransferase (GPAT), acyl CoA oxidase (ACO), carnitine palmitoyl transferase (CPT), 7α-hydroxylase (7αH), and LDLR. Ethidium bromide fluorescence photograph of the gel electrophoresis of the amplification products; Lane M: molecular weight markers. B: Relative expression of FAS, ACC, HMG-CoAR, ACO, CPT, 7α-hydroxylase, and LDLR relative to L19, respectively. The gel photographs were quantified using NIH Image and expressed as arbitrary units. Results are expressed as the average ± SEM. * $P < 0.05$ compared with the respective control groups using two-way ANOVA and the Bonferroni posthoc test. ‡ $P < 0.05$ compared with the respective virgin group using two-way ANOVA and the Bonferroni posthoc test.

pared with virgin rats. This result is in accord with the increased reductase activity observed after parturition (11), suggesting an increased expression of the enzyme that becomes active after delivery in response to increased demand for cholesterol. The changes in HMG-CoAR were not modified by hypoT. The mRNA levels of 7α-hydroxylase were not modified by pregnancy or by hypoT. LDLR mRNA was also increased during late gestation, reflecting a need for increased lipoprotein clearance during and after gestation (11). The decrease observed in the pregnant hypoT rats could complicate this physiological adaptation, leading to the increased circulating LDL cholesterol.

Because FAS and ACC mRNA abundances were significantly diminished in the virgin and pregnant hypoT rats, we investigated whether enzymatic activities in the pregnant groups were similarly affected (**Table 3**). ACC activity was not modified by PTU treatment, suggesting a post-translational upregulation. On the other hand, in accordance with the observed changes in mRNA abundance, FAS activity was significantly decreased.

To have another measure of lipid synthetic activity, we determined the effect of hypoT on the incorporation of $[^3H]H_2O$ into different lipid fractions from livers of pregnant rats (Table 3). We observed a decrease in the incorporation into the TG fraction in the hypoT group, whereas there were no differences in ^3H incorporation into cholesterol or phospholipids.

In addition, PTU treatment produced a significant decrease in liver weight relative to body weight in the virgin and pregnant rats without significantly affecting RNA content (**Table 4**). Pregnancy decreased liver protein and TG concentrations, most probably the result of their increased delivery to circulation in the form of lipoproteins. In contrast, late-pregnant rats had increased total lipid, FC, EC, and phospholipid concentrations (Table 4), perhaps reflecting an increased metabolic rate, compared with virgin

TABLE 3. Effects of hypothyroidism on liver and mammary gland FAS and ACC activities and incorporation of ^3H from $[^3H]H_2O$ into the lipids of rats on day 21 of pregnancy

	Control	HypoT
Liver		
FAS (U/mg cytosol protein)	2.50 ± 0.53	0.69 ± 0.18[a]
ACC (U/mg cytosol protein)	33.52 ± 1.51	32.41 ± 2.91
^3H-incorporation		
Into TG (ng ^3H/h/g of liver)	5.26 ± 0.44	3.70 ± 0.36[a]
Into FC(ng ^3H/h/g of liver)	0.99 ± 0.19	1.36 ± 0.12
Into EC (ng ^3H/h/g of liver)	0.51 ± 0.04	0.60 ± 0.08
Into PL (ng ^3H/h/g of liver)	2.37 ± 0.18	2.60 ± 0.38
Mammary gland		
FAS (U/mg cytosol protein)	0.68 ± 0.07	0.36 ± 0.09[a]
ACC (U/mg cytosol protein)	85.5 ± 5.3	69.1 ± 3.4[a]
^3H-incorporation		
Into TG (ng ^3H/h/g of MG)	5.61 ± 0.76	3.44 ± 0.31[a]
Into FC (ng ^3H/h/g of MG)	0.59 ± 0.08	0.62 ± 0.13
Into EC (ng ^3H/h/g of MG)	0.60 ± 0.09	0.70 ± 0.15
Into PL (ng ^3H/h/g of MG)	2.13 ± 0.17	2.15 ± 0.33

EC, esterified cholesterol; FC, free cholesterol; MG, mammary gland. Values are means ± SEM for groups of eight dams.

[a] $P < 0.05$ compared with the respective control groups using Student's *t*-test.

TABLE 4. Effects of hypothyroidism on liver and mammary triglyceride, phospholipids, and cholesterol concentrations of virgin and pregnant rats

	Virgins		Day 21 of Pregnancy	
	Control	HypoT	Control	HypoT
Liver				
Weight (g/100 g body weight)	3.43 ± 0.16	2.94 ± 0.12[a]	3.48 ± 0.14	2.87 ± 0.05[a]
Total RNA (mg/g)	7.17 ± 0.39	5.23 ± 0.18	5.65 ± 1.08	3.98 ± 0.57
Total protein (mg/g)	174 ± 5	177 ± 4	152 ± 2[b]	151 ± 5[b]
Total lipids (mg/g tissue)	44.2 ± 1.3	44.7 ± 2.0	68.1 ± 2.6[b]	68.0 ± 2.6[b]
Triglyceride (mg/g)	32.9 ± 3.2	38.1 ± 4.1	24.2 ± 2.3[b]	23.9 ± 1.4[b]
FC (mg/g)	0.96 ± 0.07	1.14 ± 0.06	2.13 ± 0.05[b]	1.90 ± 0.03[a,b]
EC (μg/g)	196 ± 29	149 ± 25	439 ± 27[b]	464 ± 23[b]
Phospholipids (mg phosphorus/g)	0.49 ± 0.05	0.65 ± 0.02[a]	1.15 ± 0.05[b]	1.24 ± 0.04[b]
Mammary gland				
Weight (g/100 g body weight)	1.02 ± 0.09	1.70 ± 0.15[a]	1.99 ± 0.07[b]	1.65 ± 0.07[a]
Total RNA (mg/g)	0.155 ± 0.037	0.132 ± 0.012	0.414 ± 0.072[b]	0.318 ± 0.031[a,b]
Total protein (mg/g)	61.5 ± 5.0	74.5 ± 6.4	97.2 ± 11.7	80.2 ± 8.6
Total lipids (mg/g tissue)	607 ± 33	630 ± 37	484 ± 25	578 ± 19[a]
Triglycerides (mg/g)	600 ± 34	613 ± 18	425 ± 24	547 ± 30[a]
FC (mg/g)	0.81 ± 0.11	0.82 ± 0.14	1.48 ± 0.11	1.49 ± 0.10
EC (μg/g)	505 ± 69	407 ± 47	460 ± 38	371 ± 30
Phospholipids (mg phosphorus/g)	0.19 ± 0.02	0.30 ± 0.03[a]	0.47 ± 0.05[b]	0.33 ± 0.03[a]

Values are means ± SEM for groups of eight rats.

[a] $P < 0.05$ compared with the respective control groups using two-way ANOVA and the Bonferroni posthoc test.

[b] $P < 0.05$ compared with the respective virgin group using two-way ANOVA and the Bonferroni posthoc test.

rats. HypoT had slight effects on these parameters, only increasing liver phospholipid concentration in virgin rats and slightly decreasing FC in the pregnant rats (Table 4).

Effect of hypothyroidism on mammary lipid metabolism in virgin and pregnant rats

To evaluate the effects of thyroid hormone on mammary lipogenic enzymes, mRNA abundances of ACC, FAS, GPAT, and LPL were measured using semiquantitative RT-PCR of total RNA prepared from MG from virgin and pregnant rats (**Fig. 2**). Gestation did not alter FAS or ACC mRNA content, and hypoT significantly reduced their abundances in the pregnant rats but had no effect on virgin rats. GPAT mRNA abundance, and that of LPL, an enzyme linked to hydrolysis and uptake of TGs from circulation, were not modified by either gestation or PTU treatment. Altogether, these results demonstrate that pregnancy, in contrast with the findings in liver, does not alter mammary lipogenesis, but that PTU treatment has a negative effect in the pregnant MGs. Because there exists

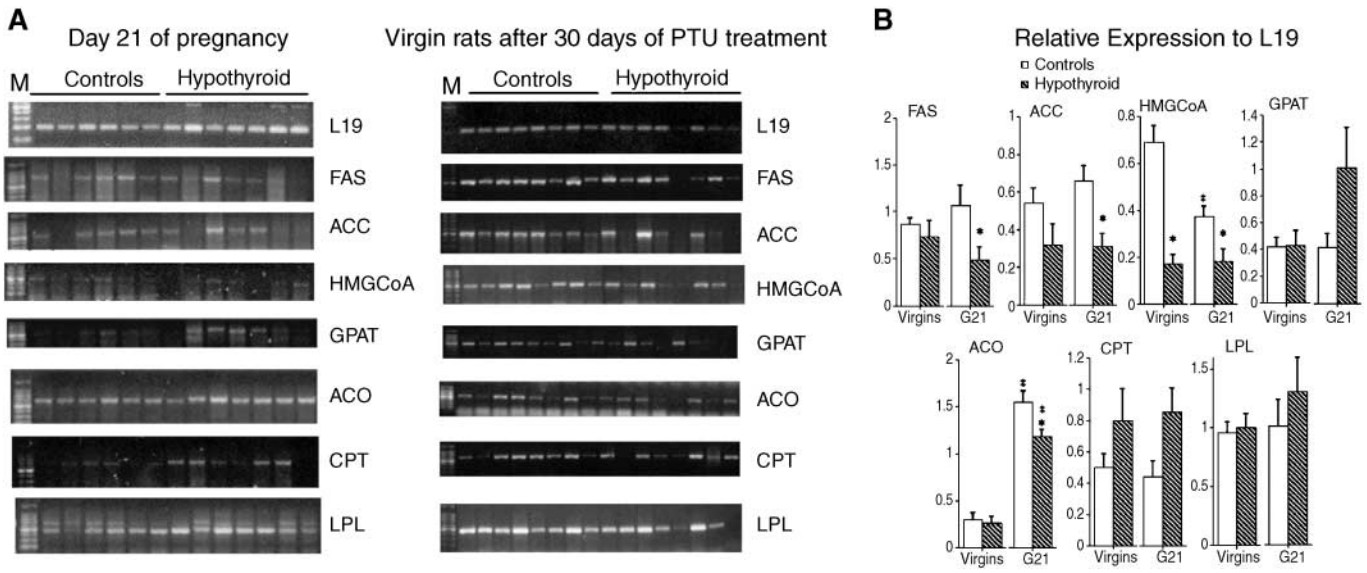

Fig. 2. Expression of mammary enzymes related to lipid metabolism in control and hypoT virgin or day 21-pregnant rats (G21). A: Measurement by RT-PCR of expression of L19, FAS, ACC, HMG-CoAR, GPAT, ACO, CPT, and LPL. Ethidium bromide fluorescence photograph of the gel electrophoresis of the amplification products; Lane M: molecular weight markers. B: Relative expression of FAS, ACC, HMG-CoAR, ACO, CPT and LPL relative to L19, respectively. The gel photographs were quantified using NIH Image and expressed as arbitrary units. Results are expressed as the average ± SEM. * $P < 0.05$ compared with the respective control groups using two-way ANOVA and the Bonferroni posthoc test. ‡ $P < 0.05$ compared with the respective virgin group using two-way ANOVA and the Bonferroni posthoc test.

no information about the regulation of fatty acid oxidation in mammary tissue, we decided to measure expression of ACO and CPT, enzymes related to fatty acid β-oxidation. Mammary ACO mRNA was increased by gestation in a way similar to that in liver, suggesting a stimulation of fatty acid oxidation during pregnancy. In contrast, CPT mRNA was not modified by gestation, suggesting a different physiological regulation. HypoT produced a diminution of ACO mRNA abundance but had no significant effect on CPT. The hypercholesterolemic effect of hypothyroidism was evaluated also in the MG through measurement of HMG-CoAR mRNA abundance. In contrast to the result observed in liver, HMG-CoAR was decreased by gestation and hypoT produced a further decrease, most probably the result of the increased availability of cholesterol from circulation in the hypoT rats (Fig. 2).

The changes produced by hypoT in mammary lipogenic enzyme mRNA expression in the pregnant rats were confirmed by measuring FAS and ACC activities, where we also observed decreases (Table 3). We also measured the effect of hypoT on the incorporation of $[^3H]H_2O$ into different lipid fractions from MGs of pregnant rats (Table 3). As in livers, we observed a decrease in ^3H incorporation into the mammary TG fraction in the hypoT group, whereas there were no differences in ^3H incorporation into cholesterol or phospholipids.

PTU treatment had opposite effects in inguinal mammary weight (relative to body weight) in virgin and pregnant rats; virgin rats had significantly heavier MGs (Table 4), most probably due to the mammary development produced by the PTU treatment, which was accompanied in some cases by synthesis of milk components and the presence of lipid droplets within the epithelial cells (**Fig. 3**) (19), while pregnant mammary weights were decreased by PTU (Table 4). In virgin rats, hypoT produced no differences in mammary concentrations of proteins or any lipid fraction (Table 4), with the exception of phospholipids, which were increased, most probably reflecting the increased epithelial compartment (Fig. 3).

Table 4 shows that pregnant rats had increased total RNA, proteins, FC, and phospholipids and decreased total lipids and TGs, probably as a consequence of the lobuloalveolar development induced by pregnancy (cf. Fig. 3, **Fig. 4**). In pregnant rats, in contrast with the lack of effect observed in virgin rats, hypoT produced increases in TGs and, consequently, in total lipid content (Table 4) and decreases in total RNA and in phospholipids, without modifying FC or EC. These findings suggest a lower proportion of mammary lobuloalveolar epithelial tissue in the hypoT pregnant rats.

To determine whether the observed differences in lipid and TG content in mammary tissue during late pregnancy are due to differences in the epithelial or adipose compartment of the MGs, we measured the ratio of epithelial to adipose tissue and the average size of the lipid droplets in the epithelial cell in histological preparations stained with hematoxylin-eosin. The average volume of the lipid droplets in the epithelial cells was estimated by measuring the greater and smaller diameters of the droplets. We found that in the hypoT rats, both measures were significantly smaller compared with the size of the droplets in the control pregnant rats (Fig. 4). On the other hand, the ratio of adipose to epithelial tissue was greater in the hypoT mothers (Fig. 4). These results are in agreement with the significantly higher lipid content of the PTU mothers, which reflects the increase in adipose tissue, accompanied by a decrease in lipid synthesis, enzyme activities, and expression, and which takes place primarily in the epithelial mammary cells.

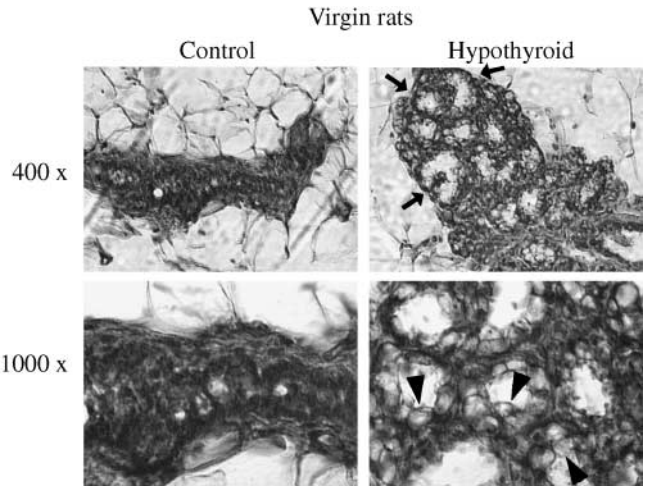

Fig. 3. Histology of mammary glands (MGs) from control and hypothyroid virgin rats. MGs from controls or rats treated for 30 days with propylthiouracyl (PTU) (0.1 g/l) in the drinking water were fixed and stained with hematoxylin-eosin. The images were obtained at 400× and 1,000× magnification. See Materials and Methods for further details. Although control rats show mainly ductal development, with no alveoli, hypothyroid rats show moderate lobuloalveolar development, with signs of active secretion evidenced by distended alveoli (black arrows) and the presence of numerous lipid droplets within the cells and in the alveolar lumen (black arrowheads).

Serum parameters related to lipid metabolism

Because circulating insulin, glucose, and lipids play a role in the regulation of lipid tissue content, metabolism, and secretion, we measured the concentrations of insulin, several lipids, and glucose in the serum of virgin or day 21-pregnant control and hypoT rats fasted on the previous night (**Table 5**).

Circulating glucose concentration was diminished in both groups of pregnant rats, as has been previously shown (44). The decreased glucose is most probably responsible for the decreased circulating insulin observed in the pregnant rats (Table 5). HypoT significantly diminished circulating glucose and insulin in the virgin rats, but had no effect on the pregnant groups (Table 5), indicating that the changes observed in tissue lipid metabolism in these rats are not the result of changes in circulating insulin or glucose, but are a direct effect of the hypothyroid state.

In the pregnant rats, total lipids, phospholipids, TGs, and cholesterol were markedly elevated in comparison to the virgin rats, as has been already demonstrated (11, 12).

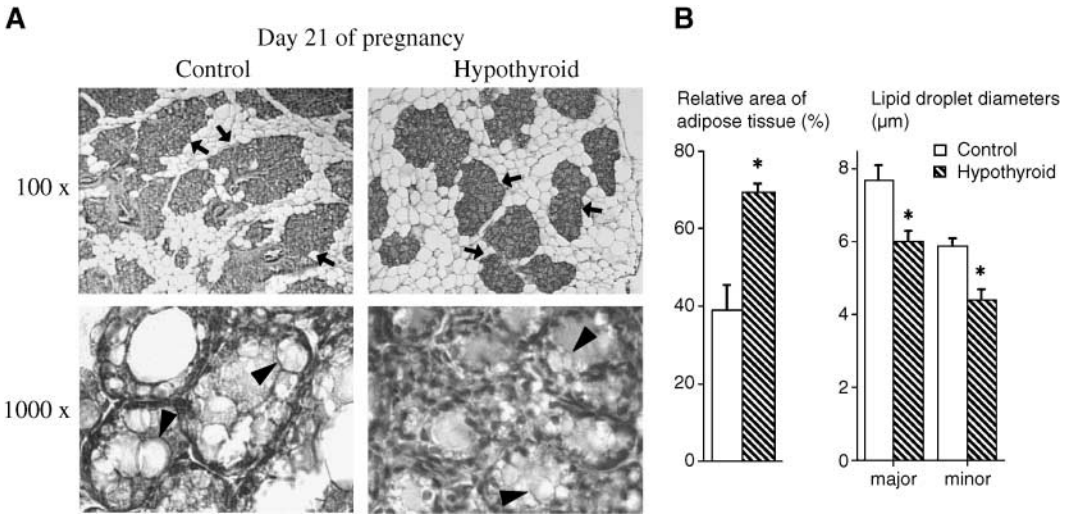

Fig. 4. A: Histological evaluation of lipid content of MGs from control and hypothyroid rats on day 21 of pregnancy. MGs from controls or rats given PTU (0.1 g/l) in the drinking water 8 days before mating were fixed and stained with hematoxylin-eosin. The images were obtained at a low (100×) magnification for evaluation of relative proportions of adipose versus epithelial tissue and a high (1,000×) magnification for measurement of mean lipid droplet size. Note the lower proportion of the lobuloalveolar trees (black arrows) and smaller size of the lipid droplets (black arrowheads) between control and hypoT rats. B: Left, relative proportion of adipose and epithelial tissue in MGs was quantified measuring the areas using NIH Image software and expressing them as percent of the total (epithelial + adipose) area; right, mean size of lipid droplets within the epithelial cells at 1,000× magnification. To estimate the lipid droplet size, we measured minor and major diameters using Image Pro Plus 4.5 software. (Each lipid droplet was selected as an area of interest with the appropriate elliptical shape, then the measurement command was selected to measure the maximum and minimum diameter—the length of the longest and shortest lines, respectively, that can be drawn to pass through the centroid position and join two points on each object's perimeter.) Approximately 100 droplets were taken at random within the epithelial compartment for each individual section, and the average diameters were calculated for each preparation. See Materials and Methods for further details. Results represent the means ± SEM of groups of six rats. * $P < 0.05$ of the hypoT compared with the respective control groups using one-way ANOVA and the Student's *t*-test.

HypoT induced increases in total lipids and total cholesterol in virgin rats, whereas in the pregnant rats, the increases in cholesterol were maintained but there was a decrease in circulating TGs (Table 5). The increased LDL + VLDL cholesterol fraction in the hypoT groups was responsible for the increase in total cholesterol, and in the pregnant rats, the HDL-cholesterol fraction was slightly decreased (Table 5). hypoT had no effects on serum phospholipids.

DISCUSSION

Lipid metabolism is very sensitive to alterations in the thyroid state, and because mammary lipid synthesis and utilization are essential for the normal function of the gland and for milk synthesis, the effects of the hypothyroid state on lipids at the end of pregnancy may have important consequences for the well-being of the mother and newborn. A clinical state of hypothyroidism during late pregnancy may limit the capacity of the maternal or-

TABLE 5. Effects of PTU treatment on serum concentrations of glucose, insulin, proteins, and different lipids of virgin and pregnant rats

	Virgins		Day 21 of Pregnancy	
	Control	HypoT	Control	HypoT
Glucose (mg/ml)	1.05 ± 0.03	0.84 ± 0.04[a]	0.55 ± 0.06[b]	0.54 ± 0.05[b]
Insulin (ng/ml)	7.19 ± 0.81	3.66 ± 0.68[a]	3.82 ± 0.51[b]	3.06 ± 0.29
Total lipids (mg/ml)	1.58 ± 0.16	2.39 ± 0.15[a]	6.65 ± 0.63[b]	6.10 ± 0.51[b]
Triglycerides (mg/ml)	0.47 ± 0.04	0.49 ± 0.05	4.12 ± 0.41[b]	2.53 ± 0.25[a,b]
Phospholipids (mg/ml)	0.51 ± 0.08	0.61 ± 0.09	1.98 ± 0.14[b]	2.16 ± 0.17[b]
Total cholesterol (mg/ml)	0.33 ± 0.02	0.63 ± 0.02[a]	0.64 ± 0.03[b]	0.87 ± 0.05[a,b]
HDL-cholesterol (mg/ml)	0.21 ± 0.01	0.22 ± 0.01	0.23 ± 0.01	0.17 ± 0.02[a,b]
LDL + VLDL cholesterol (mg/ml)	0.15 ± 0.04	0.40 ± 0.02[a]	0.38 ± 0.02[b]	0.66 ± 0.06[a,b]

Values are means ± SEM for groups of eight rats.

[a] $P < 0.05$ compared with the respective control groups using two-way ANOVA and the Bonferroni posthoc test.

[b] $P < 0.05$ compared with the respective virgin group using two-way ANOVA and the Bonferroni posthoc test.

ganism to sustain itself and the fetus adequately and to prepare the mammary tissue for the subsequent lactation, thus compromising delivery and nutrition of the newborn (19).

Fatty acid synthesis is an important metabolic pathway that is under complex hormonal and nutritional control. Thyroid hormones regulate lipogenesis by altering levels of ACC and FAS mRNAs (43). We have previously found that in virgin and lactating rats, the administration of thyroid hormones produces an increase in hepatic FAS and ACC activities (45, 46). In the present study, we found a decrease in hepatic lipid synthesis evidenced by the diminished incorporation of $^{3}H[H_2O]$ into TGs and by expression and activity of FAS and ACC in virgin and pregnant hypothyroid rats consistent with the described inhibitory effects of hypothyroidism in male rats (47, 48).

Late pregnancy is a state of increased VLDL production, caused by the maternal insulin-resistant condition and the augmented estrogen concentration, which lead to enhanced adipose tissue lipolytic activity and decreased adipose tissue LPL activity, which, in turn, contribute to the augmented plasma levels of VLDL-TGs during gestation (10, 44). The increased hepatic cholesterol synthesis in pregnant rats may also contribute to the elevated circulating VLDL levels, because cholesterol can drive VLDL production (49, 50).

In the hypothyroid mothers, the reduced hepatic TG synthesis associated with their decrease in serum and the diminished serum concentration of estradiol (19) could indicate less secretion of VLDL into circulation. In agreement with our results, congenitally hypothyroid mice show alterations in apolipoprotein B (apoB) RNA editing that switch hepatic production from apoB-100 to apoB-48 isoform (3), whose conformational competence directs the assembly of hepatic VLDL more effectively. The higher circulating TGs observed in late-pregnant rats compared with virgins may be caused by the fact that at the end of pregnancy, peripheral TG catabolism is reduced in order to direct TGs to the fetal and mammary compartments, resulting in their elevation in circulation. Thus, the decreased liver TG synthesis in the late-pregnant hypoT rats may be responsible for the decrease in circulating TGs, compromising the fulfillment of the gestational TG requirements.

Liver metabolism

The liver is central to cholesterol metabolism, balancing hepatic cholesterol synthesis and the uptake of plasma lipoproteins from the circulation against the excretion of hepatic cholesterol and bile acids (11). Thyroid hormones can influence the metabolism of cholesterol at several critical steps in the liver, namely increasing LDLR, which mediates cholesterol uptake from the circulation, HMG-CoAR, which controls cholesterol biosynthesis, and cholesterol 7α-hydroxylase (CYP7AI), the rate-limiting enzyme in the synthesis of bile acids (51–53). The increase in LDL + VLDL cholesterol observed in virgin and pregnant hypoT rats may be due to the LDL fraction, because it is well known that hypothyroidism increases cholesterol through an enhancement of this fraction (7, 54). The elevated serum cholesterol observed in pregnant rats may be the result of higher levels of LDL, in agreement with the decreased expression of hepatic LDLR, making it a risk factor for developing atherosclerosis later in life, even when hypercholesterolemia is temporary and limited to the third trimester in humans. The increased circulating cholesterol and LDL + VLDL cholesterol observed in the hypothyroid rats could also be indirect, in response to the diminished growth hormone (GH) and corticosterone serum concentrations observed in the hypothyroid dams (19), because it has been found that GH positively regulates LDLR expression in the liver (55), whereas corticoids diminish LDL-VLDL fraction and increase HDL (56).

Thyroid hormones have profound effects on liver fatty acid oxidation. Oxidation of long-chain fatty acids and ACO activity are increased in the hyperthyroid state (57, 58), whereas total oxidation of free fatty acids is decreased in the hypothyroid state (57). In accordance with this, we found that hypoT decreased liver ACO mRNA levels but did not modify mitochondrial (CPT-I) fatty acid β-oxidation. Because CPT is regulated by the availability of malonyl CoA, the diminished ACC activity may result in a decrease in malonyl CoA that may compensate for the effects of the hypothyroid state, resulting in no overall change in expression, as observed in vitro by Muller, Koster, and Seitz (59).

During pregnancy, glucose is metabolized to supply energy that is used to promote fetal growth and development and that is converted to fetal liver glycogen to ensure neonatal survival (60). The role of thyroid hormone in carbohydrate metabolism is exerted at various levels, ranging from modulation of insulin secretion and receptor levels to direct actions on glucose uptake and utilization (61, 62). Hypothyroidism lowers blood glucose in male (61) and in nonpregnant females (this study), but in late-pregnant rats, the hypothyroid state had no effect on either blood glucose or insulin concentrations, suggesting that the adaptive changes of pregnancy may have attenuated the consequences of hypothyroidism on these parameters. Therefore, the effects produced by hypoT on lipid metabolism are not a consequence of altered glucose or insulin concentrations.

Mammary metabolism

In MGs, hypothyroidism seemed to have different effects in virgin and pregnant rats, most probably because the mammary development induced by PTU in the virgin rats may have compensated for the effects of the hypothyroid state. The decrease in lipid synthesis and lipogenic enzyme activity and expression, and the increase in the proportion of adipose tissue observed in the hypoT pregnant rats, may reflect increased lipid accumulation resulting from the diminished lipid metabolism characteristic of the hypothyroid state. Although our results suggest a lower proportion of lobuloalveolar epithelial tissue in the pregnant hypoT rats, the degree of differentiation of the epithelial cells on day 21 of pregnancy did not seem to be

overly compromised, because the mammary content of lactose was similar and casein was elevated in the hypoT mothers (19), both markers for the initiation of milk synthesis. Thus, in the pregnant rats the effects observed on mammary TG synthesis and content may be due solely to the hypothyroid state.

The observed effects on the pregnant MG may be a consequence of the hypothyroid state per se and not indirect consequences of the previously described increase in circulating prolactin (19), because, at least in mammary tissue, the increased prolactin should have stimulated lipid synthesis. On the other hand, the increased levels of progesterone, which inhibits lactogenesis, may have contributed to the decrease in activity and expression of the lipogenic enzymes in mammary tissue, because it is well known that ACC and milk synthesis are induced during lactogenesis (63).

The decreased lipogenic enzyme activity and expression observed in the pregnant hypoT rats may be responsible for the differences in milk quality observed during the subsequent lactation. Unpublished results from our laboratory show that TG content of rat milk roughly doubles from day 2 to day 21 of lactation in control rats, while in hypoT mothers, this increase does not take place, resulting in severely diminished milk TG content during mid and late lactation (M. B. Hapon et al., unpublished observations), which may be an important factor contributing to the severe growth deficit of the pups.

In conclusion, hypothyroidism affected liver and mammary TG metabolism, and produced changes in some serum lipid profiles in virgin and pregnant rats. These alterations may be produced directly by thyroid dysfunction. In particular, the decrease in liver TG synthesis and in circulating TGs at the end of pregnancy may result in a decreased availability of these lipids to the MG and fetal compartments. This, along with the decreases observed in the proportion of epithelial mammary tissue and in lipid synthesis at the time when the initiation of milk synthesis is about to proceed, may contribute to the future lactation deficit of hypothyroid mothers (19).

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