Fat accumulation in the rat during early pregnancy is modulated by enhanced insulin responsiveness

M. P. Ramos, M. D. Crespo-Solans, S. del Campo, J. Cacho, and E. Herrera
Facultad de Ciencias Experimentales y de la Salud, Universidad San Pablo-CEU, E-28668, Madrid, Spain

Ramos, M. P., M. D. Crespo-Solans, S. del Campo, J. Cacho, and E. Herrera. Fat accumulation in the rat during early pregnancy is modulated by enhanced insulin responsiveness. *Am J Physiol Endocrinol Metab* 285: E318–E328, 2003. First published April 15, 2003; 10.1152/ajpendo.00456.2002.— Insulin sensitivity has been implicated in the variation of fat accumulation in early gestation by as-yet-unknown mechanisms. In the present study, we analyzed the insulin sensitivity of lipolysis and lipogenesis in lumbar adipocytes from rats at 0, 7, 14, and 20 days of gestation. In adipocytes of 7-day pregnant rats, we found a twofold decrease in both β-agonist (isoproterenol and BRL-37344)-stimulated lipolysis and β_3-adrenoceptor protein but not in lipolysis initiated by forskolin or isobutylmethylxanthine, suggesting a modification of the lipolytic pathway at the receptor level. Whereas adipocytes from 7-day pregnant rats showed a twofold increase in fatty acid synthesis from glucose, those from 20-day pregnant animals displayed a decreased lipogenic activity. Insulin responsiveness of the lipolytic and lipogenic pathways was analyzed by dose-response experiments, giving evidence for the involvement of improved insulin responsiveness in the enhanced lipogenic and reduced lipolytic activities of adipocytes in early pregnancy. In contrast, insulin resistance is responsible for lower antilipolytic and lipogenic actions of insulin in late pregnant animals. In conclusion, the present study shows that enhanced adipose tissue insulin responsiveness during early pregnancy contributes to maternal fat accumulation, whereas decreased insulin responsiveness during late gestation modulates fat breakdown.

adipose tissue; β_3-adrenoceptor; lipolysis; lipogenesis

PREGNANCY IS A STATE characterized by modifications in maternal adiposity, leading to an increase in adipose tissue mass during the earlier phase and followed by a decrease of fat mass during the late phase (37). These two metabolic stages are associated with adaptations of fat cell functions, resulting in a predominance of lipogenic and lipolytic pathways, respectively. The net anabolic condition present during the first part of gestation seems to be driven by insulin, which is the most efficient anabolic hormone, and its pancreatic concentration and secretion are both enhanced from early pregnancy (43, 54). In addition, clinical studies suggest that, during early pregnancy, the mother has an increased response to insulin (28, 40). It has been proposed that insulin sensitivity may be implicated in

maternal fat accumulation, since women with decreased pregravid insulin sensitivity show a reduced fat accumulation in early gestation (7). However, the mechanism for the relationship between these variables remains unknown. The situation is substantially modified during late gestation, when both hyperinsulinemia and insulin resistance are consistently present (52). This decreased insulin responsiveness might be responsible for several of the metabolic changes taking place during this late stage of gestation (46, 47).

Insulin and catecholamines are of major importance for the endocrine control of lipid mobilization from fat cells. In rats, catecholamines stimulate lipolysis by acting via the β_3-adrenoceptors, activating adenylate cyclase, cAMP-dependent protein kinase (PKA), and hormone-sensitive lipase (HSL), resulting in the hydrolysis of triacylglycerols (for review, see Ref. 34). In contrast, the antilipolytic action of insulin is a multistep process, so acute effects of insulin are related to activation of cGMP-inhibited phosphodiesterase (PDE3) (12), activation of a phosphatase, and/or sequestration of β_3-adrenoceptors from the cell surface (15). The mechanisms by which long-term insulin treatment reduces β-adrenergic sensitivity are less documented, although it is known that long-term exposure to insulin induces a decrease of mRNA β_3-adrenoceptor expression in both isolated adipocytes (14) and 3T3-F442A preadipocytes (18).

Enhanced maternal adipose tissue lipolytic activity during late gestation has been related to autonomic nervous system activation, secondary to hypoglycemia. However, a decreased response of adipocytes to catecholamines has been reported during late gestation in the rat (1, 23) and in the sheep (22, 58). Furthermore, this effect could be a consequence of decreased adrenoceptor activity secondary to maternal hyperinsulinemia, since when nonpregnant animals become hyperinsulinemic by means of the euglycemic hyperinsulinemic clamp the β-adrenoceptor activity is impaired (23).

Because fat accumulation is the result of a balance between lipid synthesis and breakdown, the present study was designed to clarify the modulation of lipogenic and lipolytic responses to insulin in the "anabolic" and "catabolic" phases of pregnancy in the rat. To address this issue, adipocytes from virgin and pregnant

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Address for reprint requests and other correspondence: M. P. Ramos, Facultad de Ciencias Experimentales y de la Salud, Universidad San Pablo-CEU, Ctra. Boadilla del Monte km.5,3, E-28668, Madrid, Spain (E-mail: pramos@ceu.es).

rats (7, 14, and 20 days of gestation) were exposed to different concentrations of the β-agonists forskolin or isobutylmethylxanthine, and lipolysis was quantified as glycerol release into the incubation medium. Furthermore, to investigate the molecular mechanism underlying the observed changes in the responsiveness of the tissue at different stages of pregnancy, the study was extended to determine by means of immunodetection studies whether β_3-adrenoceptor expression is affected during pregnancy, and insulin sensitivity of lipolysis was assessed by isoproterenol-stimulated lipolysis in the presence of various concentrations of insulin. In another set of animals, the dose response of insulin stimulation of lipogenesis was studied by quantifying glucose incorporation into fatty acids. These experiments revealed that adipose tissue lipolysis and lipogenesis become highly responsive to insulin during early pregnancy, whereas a decreased responsiveness to insulin of these pathways emerges at late pregnancy.

MATERIALS AND METHODS

Animals and tissue collection. Female Wistar rats from our colony were housed at 22–24°C on 12:12-h light-dark cycles from 0800 to 2000 and free access to water and a chow diet (Panlab, Barcelona, Spain). Some animals were mated when weighing between 180 and 190 g, and *day 0* of pregnancy was determined by the presence of spermatozoids in vaginal smears. Experimental groups were composed of virgin rats and rats at 7, 14, and 20 days of gestation. After CO_2 anesthesia, animals were decapitated, and blood was collected from the neck wound in heparinized or EDTA tubes for immediate separation of plasma at 4°C. Lumbar adipose pads were rapidly dissected and placed in liquid nitrogen to be stored at −80°C until processed for Western blot analysis as described in *Protein extraction and immunoblotting.* For lipolysis and lipogenesis experiments, fresh lumbar adipose tissue was placed in warm (25°C) 0.9% NaCl and processed fresh. The two uterine horns were immediately dissected and weighed to obtain the whole conceptus weight. This value was subtracted from the mother's body weight to obtain the net maternal body weight. The experimental protocol was approved by the Animal Research Committee of the Faculty of Experimental and Health Sciences, University San Pablo-CEU, Madrid, Spain.

Plasma analysis. Plasma aliquots were used to measure glucose by an enzymatic colorimetric test (glucose oxidase, GOD/PAP method, Roche Diagnostics, Barcelona, Spain) (3). Insulin was determined in plasma samples with a specific ELISA kit for rats (Mercodia, Uppsala, Sweden), the values within the detection range of the assay being 0.07–5.5 μg insulin/ml (1.8% intra-assay variation, 3.8% interassay variation). Leptin was assayed by ELISA in diluted plasma samples as indicated by the manufacturer, by use of a commercially available kit specific for rat leptin (Assay Designs, Ann Arbor, MI). Leptin concentrations were within the detection range of the kit, i.e., 0.06–3.6 ng leptin/ml (11.6% intra-assay variation, 11.0% interassay variation). Glycerol was determined in plasma samples by an enzymatic colorimetric test (GPO-Trinder; Sigma-Aldrich, Madrid, Spain), and nonesterified fatty acids (NEFA) were analyzed in EDTA-plasma samples by a colorimetric method (Wako Chemicals, Neuss, Germany).

Oral glucose tolerance test. Glucose tolerance tests were performed in 14-h-fasted rats. After a basal blood sample from the tail was drawn, a bolus of glucose (2 g/kg) was administered orally to the animals. Subsequently, blood samples were collected into heparinized tubes at 2.5, 5, 10, 15, 20, 30, 45, 60, and 90 min after glucose dose and placed on ice. Samples were centrifuged, and plasma was stored at −20°C until processed.

Glucose tolerance (K_g) was estimated as the rate of plasma glucose disappearance (59) corrected by the maximal increase in plasma insulin. Plasma glucose disappearance was calculated as the slope of the plasma glucose concentration vs. time, using the samples obtained from the 2.5- to 15-min period after glucose administration [Δglucose/Δtime $(mg \cdot \mu g^{-1} \cdot min^{-1})$] (30).

Isolation of adipocytes and determination of cell size and number. Adipocytes were prepared from white adipose tissue according to the method of Rodbell (50) with minor modifications. Briefly, freshly isolated adipose pads were cut into small pieces and digested by collagenase A (1 mg/ml; Roche Diagnostics; activity 0.21 U/mg) in Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) containing 4% (wt/vol) BSA (fatty acid free, fraction V; Sigma-Aldrich) and 5.5. mM glucose (KRB-Buffer), for 30 min at 37°C in an O_2-CO_2 atmosphere (19:1, vol/vol) with shaking (60 cycles/min). Subsequently, fat cells were dispersed and filtered through a silk screen, washed three times with KRB-Buffer to eliminate collagenase, and resuspended in the same buffer at a concentration of ~0.4 × 10^6 cells/ml. The size of a fat cell was measured by direct microscopic determination, and the mean adipocyte diameter was calculated from measurements of 100 cells. Because adipocytes have 95% lipid content and are spherical, their volume and weight can be estimated from their diameters (26). Total cell lipid content was determined gravimetrically after organic extraction (13), and the number of fat cells was calculated by dividing the total lipid weight by the mean cell weight.

Lipolysis measurements. Freshly isolated adipocytes were incubated at 37°C in KRB-Buffer containing 1 U/ml of adenosine deaminase (Sigma-Aldrich) in the absence and presence of the indicated concentrations of (±)-isoproterenol hydrochloride, BRL-37344 (Sigma-RBI), forskolin, or 3-isobutyl-1-methylxanthine (all from Sigma-Aldrich). After 90 min of incubation, the tubes were placed on ice, and aliquots of 50 μl of the infranatant were removed for the enzymatic determination of glycerol (GPO-Trinder). Glycerol released into the incubation medium was taken as an index of lipolytic activity and expressed as nanomoles of glycerol released per minute per 100 milligrams of cell lipid (23).

Concentration-effect curves of glycerol release as a function of either agonist were calculated as a percentage of basal lipolysis (i.e., without agonist). Half-maximal effective agonist concentration (EC_{50}) and maximum effect (E_{max}) values were obtained by computer fitting of concentration-effect curves of the agonists to Hill's model (5, 11) by using the Sigma-Plot program (version 4.0; SPSS Jandel Scientific, Erkrath, Germany).

Insulin sensitivity of adipose tissue lipolysis. The antilipolytic effect of insulin was measured in adipocytes isolated from lumbar adipose tissue from nonpregnant and pregnant rats. Cells were incubated in KRB-Buffer for 5 min at 37°C with varying concentrations of insulin (0 to 100 nM) (insulin from bovine pancreas, Sigma-Aldrich) before addition of isoproterenol (100 nM) and incubation for a further 90 min. Subsequently, glycerol release into the incubation medium was quantified.

Insulin sensitivity of adipose tissue lipogenesis. Rat adipocytes freshly isolated from lumbar white adipose tissue of virgin and 7-, 14-, and 20-day pregnant rats were incubated in KRB buffer containing 5 mM glucose in the presence of increasing concentrations of insulin (0 to 1,000 nM). After 30 min, 0.5 μCi/tube of D-[U-^{14}C]glucose (specific activity 12.5 mCi/mmol, New England Nuclear, Madrid, Spain) was added and incubated for a further 90 min at 37°C with shaking. Lipids were extracted from adipocytes in chloroform-methanol (2:1) by the method of Folch et al. (20) with modifications (24). Aliquots of total lipids were saponified in 5 N ethanolic KOH for 1 h at 100°C, and, after acidification with H_2SO_4, fatty acids were extracted with heptane. Radioactivity measurements were expressed as nanomoles of glucose incorporated into fatty acids per 100 milligrams of cellular lipid.

Protein extraction and immunoblotting. To extract protein from the tissues, 200 mg of frozen lumbar adipose tissue were powdered in liquid nitrogen in a mortar precooled to −80°C and disrupted in an ice-cold hypotonic lysis buffer (10 mM Tris·HCl, 2.5 mM EDTA, pH 7.4). Cellular debris was pelleted and discarded after centrifugation at 1,500 *g* for 5 min at 4°C. Supernatants, containing cellular proteins, were centrifuged at 17,000 *g* for 30 min at 4°C. The resulting pellets, containing the crude membranes, were pooled, and after protein determination by the Bradford method (6), they were resuspended in the appropriate volume of Laemmli buffer to give a final protein concentration of 1 mg/ml. After being boiled for 3 min, the insoluble material was pelleted at 15,000 *g* for 20 min. The resulting supernatant was stored at −20°C until use for Western blot analysis.

Membrane protein (20 μg) was subjected to 7.5% SDS-PAGE and electrophoretically transferred to PVDF membranes (Amersham Pharmacia Biotech, Barcelona, Spain). The membranes were incubated with an anti-β_3 polyclonal antibody raised in goat (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution for 35 min and subsequently with a mouse anti-goat polyclonal antibody conjugated to horseradish peroxidase (Sigma-Aldrich) at a 1:30,000 dilution. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech) and quantified by densitometry (Bio-Rad, Madrid, Spain). In each gel, samples of adipose tissue from the four experimental groups (0, 7, 14, and 20 days of pregnancy) were always run in parallel.

Statistical analysis. Results are expressed as means ± SE of 4–10 animals per group. Data were analyzed for homogeneity of variance with a Levene test. Values were log transformed to equalize the variance between conditions. Statistical comparisons were made by analysis of variance followed by a Bonferroni test with 95% confidence limits (51), using the SPSS program (version 9.0.1). With respect to the log

normal distribution of the EC_{50} values, the statistical analyses were done on the logarithm of these parameters (5).

RESULTS

To evaluate the relationship between insulin and fat accumulation in adipocytes from rats at different stages of gestation, molecular and functional studies were performed on adipose tissue from rats at 0, 7, 14, and 20 days of pregnancy.

Experimental animals and fat depot characteristics. All the pregnant animals included in the present study exhibited hyperphagia, as total daily food intake in nonpregnant rats (19.3 ± 0.6 g/day) was significantly lower than in the pregnant animals (26.0 ± 1.1, 29.5 ± 1.8, and 30.4 ± 1.0 g/day for 7-, 14-, and 20-day pregnant rats, respectively).

As shown in Table 1, maternal body weight progressively increased with gestational time. Although this change partially corresponds to the increase in conceptus mass (mean number of fetuses was 14 ± 0.34 fetuses/mother), the value of this parameter was almost negligible on *day 7*, and most of the increase at this gestational time corresponded to conceptus-free maternal weight (Table 1). The conceptus-free body weight progressively increased until *day 14* of pregnancy, whereas from 14 to 20 days of gestation, corresponding to maximal fetal growth, there was no further increase.

Changes in the weight of lumbar adipose tissue are summarized in Table 1, showing that lumbar fat pad weight increases from the beginning of pregnancy. This increase in fat content was also observed when the values were expressed as percent net body weight (0.38 ± 0.02, 0.40 ± 0.04, 0.54 ± 0.03, and 0.73 ± 0.07% for 0-, 7-, 14-, and 20-day pregnant rats, respectively). The augmented adipose tissue weight parallels an increase in the mean size of the cells but not in adipocyte number ($\approx$3 × 10^6 cells/lumbar fat pads), indicating that fat deposition during gestation is the result of the observed hypertrophy of the tissue.

Plasma glycerol and NEFA during pregnancy. Glycerol and NEFA were determined in plasma samples of rats on different days of gestation. Values for glycerol were 279 ± 18, 342 ± 38, 408 ± 57, and 640 ± 79 μM for 0-, 7-, 14-, and 20-day pregnant rats, respectively. NEFA values were 486 ± 63, 515 ± 59, 662 ± 109, and

Table 1. *Effect of pregnancy on maternal and conceptus weights and white lumbar adipose tissue characteristics*

	Virgin Rats	7-Day Pregnant Rats	14-Day Pregnant Rats	20-Day Pregnant Rats
Total body weight, g	214.3 ± 2.5[a]	255.6 ± 3.5[b]	290.8 ± 4.5[c]	376.7 ± 7.8[d]
Free conceptus body weight, g	214.3 ± 2.5[a]	248.9 ± 3.2[b]	283.2 ± 7.3[c]	285.7 ± 11.1[c]
Conceptus weight, g		0.65 ± 0.02[a]	12.02 ± 0.31[b]	76.00 ± 2.90[c]
Lumbar adipose tissue weight, g	0.96 ± 0.07[a]	1.10 ± 0.08[a]	1.57 ± 0.07[b]	1.97 ± 0.17[b]
Lumbar adipocyte size, μm	64.25 ± 1.25[a]	73.00 ± 5.68[ab]	82.00 ± 2.97[b]	86.80 ± 1.98[c]

Data are means ± SE of ≥5 rats/group. Values of body weight and conceptus body weight were log transformed to equalize the variance between conditions. Comparisons are made by ANOVA, followed by Bonferroni test with 95% confidence limits. Significance is shown by letters, different letters indicating significant difference (*P* < 0.05) between groups.

Table 2. *Effect of pregnancy on plasma glucose, plasma insulin, plasma leptin, and glucose tolerance in rats*

Rats	Plasma Glucose, mg/dl	Plasma Insulin, μg/l	Glucose Tolerance, mg·μg^{-1}·min^{-1}	Plasma Leptin, ng/ml
Virgin	135.4 ± 7.5[ab]	1.1 ± 0.2[ab]	6.5 ± 2.6[a]	3.8 ± 0.8[a]
7-Day pregnant	145.6 ± 6.8[a]	1.0 ± 0.1[a]	26.3 ± 3.4[b]	11.7 ± 4.1[b]
14-Day pregnant	118.7 ± 5.8[bc]	1.9 ± 0.3[b]	5.8 ± 2.1[a]	14.7 ± 3.6[b]
20-Day pregnant	97.6 ± 8.1[c]	2.7 ± 0.9[b]	1.3 ± 0.5[a]	10.3 ± 0.9[b]

Data are means ± SE of ≥5 rats/group. Values of plasma insulin and leptin were log transformed to equalize the variance between conditions. Comparisons are made by ANOVA, followed by Bonferroni test with 95% confidence limits. Significance is shown by letters, different letters indicating significant difference ($P < 0.05$) between groups.

1,120 ± 148 μM for 0-, 7-, 14-, and 20-day pregnant animals, respectively. Plasma levels of both glycerol and NEFA in the 20-day pregnant rats were significantly higher than those of rats at 0, 7, and 14 days of gestation, confirming the overall higher lipolytic activity in late pregnancy.

Plasma glucose, insulin, leptin, and glucose tolerance during pregnancy. Plasma glucose levels were significantly lower in late pregnancy (*day 20*), whereas no change in this metabolite was observed in the 7-day pregnant rats. Late pregnancy was accompanied by hyperinsulinemia with no modification of insulin concentration in early gestation (Table 2). Glucose tolerance (K_g; calculated as the slope of the plasma glucose concentration over time for 2.5–15 min after the oral bolus administration of glucose) was significantly higher in 7-day pregnant animals than in the other groups, suggesting an enhanced insulin responsiveness in early pregnancy. In contrast, the 20-day pregnant rats showed an impairment of glucose tolerance. Gestation was also accompanied by considerable changes in plasma leptin concentration. As shown in Table 2, plasma leptin levels gradually increased until *day 14* of pregnancy and on *day 20* started to decrease, the values at postpartum being similar to those of the virgin animals (data not shown).

Lipolytic activities stimulated by β-adrenergic agonists. To test catecholamine-stimulated lipolysis during pregnancy, isolated adipocytes from rats at different times of gestation were incubated with various β-adrenergic agonists. Because previous studies had reported that some variables of catecholamine-stimulated lipolysis vary with fat cell size and gestation itself results in increased adipocyte volume, we eliminated the influence of this factor by expressing lipolysis per lipid content in the preparation and corrected as a percentage of the basal value (i.e., without agonist) (5, 23). In our study, we found no significant differences in basal lipolysis (1.9 ± 0.18, 1.97 ± 0.25, 1.57 ± 0.18, and 2.21 ± 0.34 nmol glycerol·min^{-1}·100 mg cell lipid^{-1} for 0-, 7-, 14-, and 20-day pregnant rats, respectively).

Figure 1 summarizes the concentration-response relationships after isolated adipocytes were incubated in the presence of various concentrations (0.01 to 10,000 nM) of isoproterenol (nonspecific β-agonist; Fig. 1*A*) and BRL-37344 (β3-agonist; Fig. 1*B*). The lipolytic response to both agents was substantially decreased in adipocytes from rats at 7 and 20 days of gestation
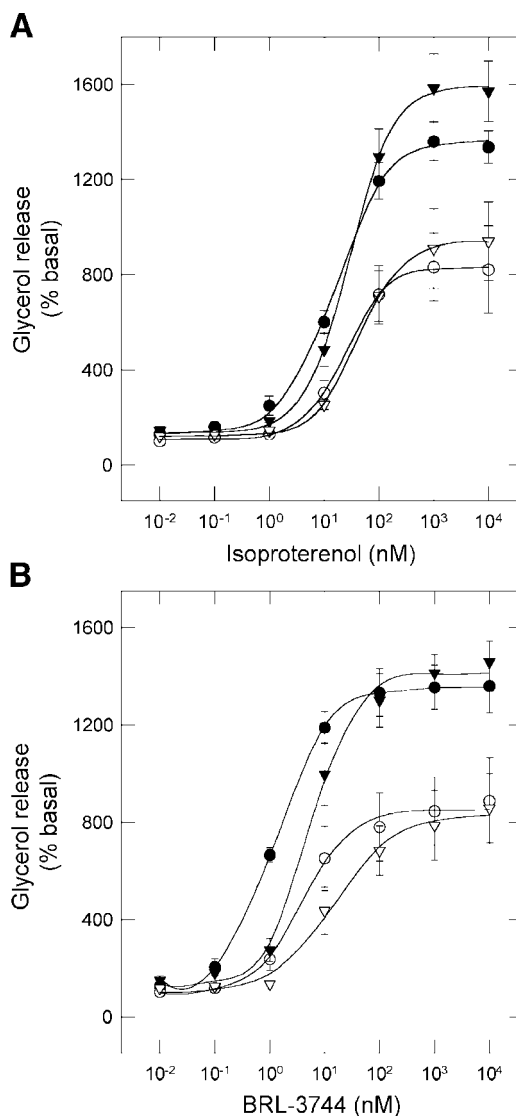

Fig. 1. Concentration-response curves of catecholamine-stimulated lipolysis in adipocytes from pregnant rats. Rat adipocytes freshly isolated from lumbar white adipose tissue from virgin (●) and 7- (○), 14- (▼), and 20-day (▽) pregnant rats were incubated for 90 min at 37°C in Krebs-Ringer bicarbonate buffer in the presence of increasing concentrations of isoproterenol (*A*) and BRL-37344 (*B*). Lipolysis was determined as glycerol release into the incubation medium as described in MATERIALS AND METHODS. Values (means ± SE) represent lipolytic activities normalized in each experiment to basal lipolysis (in absence of agonist) of ≥5 rats/group.

Table 3. *Lipolytic efficacies (E_{max}) and potencies (EC_{50}) of β-agonists on lipolysis of adipocytes from pregnant rats*

| Rats | Isoproterenol | | BRL-37344 | |
	E_{max}, %basal	EC_{50}, nM	E_{max}, %basal	EC_{50}, nM
Virgin	$1{,}373.1 \pm 75.6^{ac}$	17.1 ± 1.8^a	$1{,}398.6 \pm 104.7^a$	1.1 ± 0.2^a
7-Day pregnant	842.8 ± 143.4^b	25.7 ± 2.6^{ab}	753.3 ± 145.9^b	4.5 ± 1.1^b
14-Day pregnant	$1{,}563.6 \pm 108.3^a$	31.1 ± 2.5^b	$1{,}427.4 \pm 72.8^a$	9.7 ± 3.5^{bc}
20-Day pregnant	939.7 ± 169.1^{bc}	43.3 ± 11.0^b	839.2 ± 136.3^b	19.9 ± 6.1^c

Values are means $\pm$ SE of $\geq$5 rats/group. Apparent maximum effect (E_{max}) values (lipolytic efficacies) initiated by isoproterenol and BRL-37344 in lumbar adipocytes from virgin and 7-, 14-, and 20-day pregnant rats were calculated by the Hill equation from each animal with the values presented in Fig. 1. Because of the log-normal distribution of the EC_{50} values, statistical analyses were done on the logarithm of these parameters. Comparisons are made by ANOVA, followed by Bonferroni test with 95% confidence limits. Significance is shown by letters, different letters indicating significant difference ($P < 0.05$) between groups.

compared with those at *days 0* and *14*. To further analyze these activities, the lipolytic efficacies (i.e., E_{max}) and EC_{50} values were obtained from the concentration-response curves. Lipolytic efficacies (Table 3) of both compounds were higher in adipocytes from virgin and 14-day pregnant rats than in those from rats at 7 and 20 days of gestation. The computed EC_{50} values (lipolytic potency) are presented in Table 3. As shown, the lipolytic potency of both isoproterenol and BRL-37344 showed a significant decrease in 14- and 20-day pregnant rats compared with the nonpregnant animals.

We also studied the relationship between the maximal lipolytic effects of isoproterenol and BRL-37344. As shown in Fig. 2, an excellent correlation can be observed between BRL-37344- and isoproterenol-mediated lipolytic effects ($r = 0.98$, $P < 0.0001$). Furthermore, to ensure that such dependence occurs independently of the reproductive state of the animals, linear correlations were also made within each group. As expected, isoproterenol and BRL-37344 E_{max} correlated significantly in all groups ($r = 0.986$, 0.966, 0.89,

and 0.918 for virgin and 7-, 14-, and 20-day pregnant rats, respectively) with a slope close to 1. This result indicates that the reduction of the lipolytic effect of isoproterenol-stimulated lipolysis during pregnancy is associated with a reduction of the β$_3$-mediated component.

Lipolytic activities stimulated by nonadrenergic agents. As the difference in lipolytic responsiveness between the groups could be localized at any step in catecholamine-induced lipolysis from adrenoceptors to the final activation of HSL, lipolysis was stimulated using different agents acting at *1*) the adenylate cyclase level (forskolin, an adenylate cyclase activator) and *2*) at the PDE level (isobutylmethylxanthine, a nonspecific PDE inhibitor).

Results are summarized in Table 4, where both forskolin- and isobutylmethylxanthine-stimulated lipolysis are shown. A similar lipolytic activity stimulated by forskolin or isobutylmethylxanthine was found in the adipocytes of virgin and 7-, 14-, or 20-day pregnant rats. The lack of differences in forskolin and isobutylmethylxanthine suggests the presence of modifications in the lipolytic cascade located at the receptor level.

Characterization of β$_3$-adrenoceptor protein expression in white adipocytes from pregnant rats by immunoblot. Because in 7- and 20-day pregnant rats a decrease, not only in sensitivity but also in the maximal
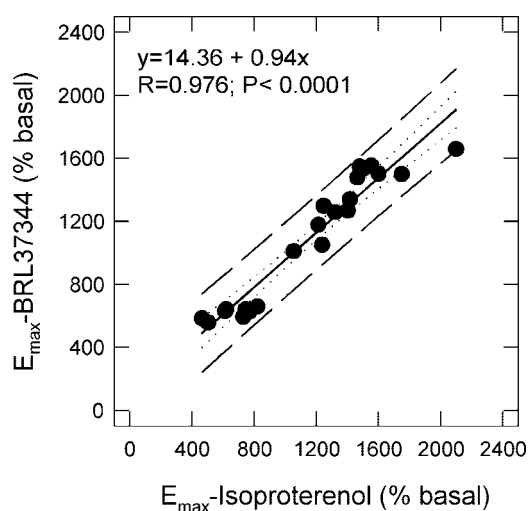

Fig. 2. Correlation between lipolytic efficacies of isoproterenol and BRL-37344 in adipocytes from pregnant rats. Linear correlation between maximum lipolytic responses (E_{max}) initiated by isoproterenol and BRL-37344 in lumbar adipocytes from virgin and 7-, 14-, and 20-day pregnant rats. 95% Confidence interval is represented by dotted lines and the 95% prediction interval by discontinuous lines.

Table 4. *Lipolysis initiated by non-β-adrenergic agonists in adipocytes from pregnant rats*

Rats	Forskolin, 10 μM	IBMX, 10 μM
Virgin	$1{,}096.5 \pm 103.2$	$1{,}161.6 \pm 274.3$
7-Day pregnant	999.9 ± 68.8	$1{,}255.6 \pm 89.0$
14-Day pregnant	$1{,}200.7 \pm 126.4$	$1{,}105.7 \pm 97.5$
20-Day pregnant	881.5 ± 70.1	954.1 ± 101.9

Values are means $\pm$ SE of 5 rats/group. Rat adipocytes freshly isolated from lumbar white adipose tissue from virgin and 7-, 14-, and 20-day pregnant rats were incubated for 90 min at 37°C in Krebs-Ringer bicarbonate buffer in the absence or presence of forskolin or 3-isobutyl-1-methylxanthine (IBMX). Lipolysis was determined as glycerol release into the incubation medium as described in MATERIALS AND METHODS. Values represent lipolytic activities normalized in each experiment to the basal lipolysis (in absence of agonist) and expressed as percentage of basal values. Comparisons were made by ANOVA. No significant differences were found between groups.
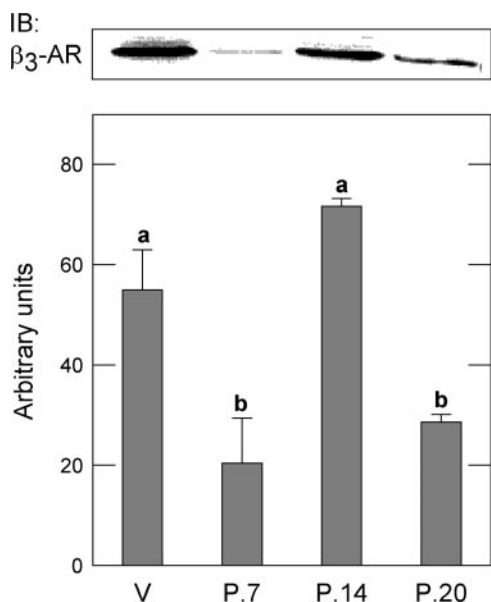
Fig. 3. β₃-Adrenoceptor (β₃-AR) protein in adipocytes from pregnant rats. β₃-Adrenoceptor protein in lumbar adipose tissue of virgin (V) and 7- (P.7), 14- (P.14), and 20-day (P.20) pregnant rats was determined by Western immunoblotting (IB), as described in MATERIALS AND METHODS. *Top*: representative Western blot. *Bottom*: quantitation by densitometric analysis of β₃-adrenoceptor proteins. Each bar represents the mean ± SE of 4 rats/group. Comparisons were made by ANOVA, followed by Bonferroni test with 95% confidence limits. Significance is shown by letters, different letters indicating significant difference ($P < 0.05$) between the groups.

Concentration-dependent effect of insulin on isoproterenol-stimulated lipolysis. We next examined the concentration-dependent capacity of insulin to decrease the lipolytic action of catecholamines. After stimulation with varying concentrations of insulin, cells were treated with a submaximal concentration of the β-agonist isoproterenol (100 nM). We found a concentration-dependent inhibition of lipolysis by insulin in adipocytes from all groups (Fig. 4A). However, the degree of inhibition differed among them. The maximum antilipolytic effect of insulin was found in adipocytes from
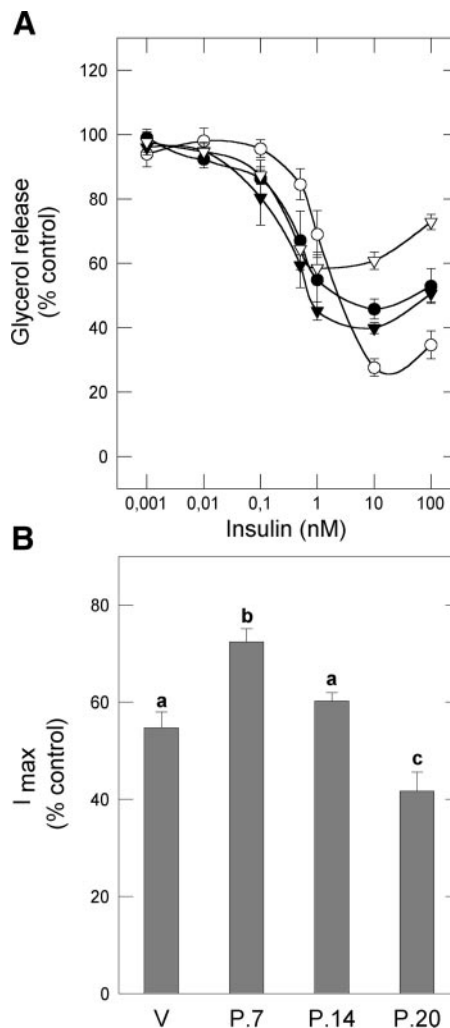
response to the β-agonist, was observed (Fig. 1), a decline in the membrane receptors was expected. To determine whether the impairment in catecholamine action in adipocytes from 7- and 20-day pregnant rats was associated with changes in the β₃-adrenoceptor component, immunoblotting was performed with specific antibodies against the rat β₃-adrenoceptor. β₃-Adrenoceptor protein determined in lumbar white adipose tissue from rats at different times of gestation (0, 7, 14, and 20 days) is shown in Fig. 3. The *inset* of this figure shows a representative autoradiogram of β₃-adrenoceptor protein in a single nonpregnant and in 7-, 14-, and 20-day pregnant rats. The immunoreactive band of β₃-adrenoceptor had an apparent molecular mass of 68 kDa. The quantity of immunoreactive band was clearly decreased in the adipose tissue membranes of 7- and 20-day pregnant animals.

The compiled values of each group show (Fig. 3) that the β₃-adrenoceptor was reduced by >50% in adipocytes from 7- and 20-day pregnant rats, an effect that is on the same order of magnitude as the observed decrease in the lipolytic efficacies of β-agonists (Table 3). These results, together with the absence of changes in forskolin- and isobutylmethylxanthine-stimulated lipolysis, indicate that, during pregnancy, catecholamine-stimulated lipolytic activity is modulated at the β₃-adrenoceptor level and that the response is highly impaired in 7- and 20-day pregnant rats but not in the 14-day pregnant animals.

Fig. 4. Concentration-dependent inhibition of isoproterenol-induced lipolysis by insulin. *A*: rat adipocytes freshly isolated from lumbar white adipose tissue from virgin (●) and 7- (○), 14- (▼), and 20-day (▽) pregnant rats were incubated in Krebs-Ringer bicarbonate buffer in the presence of increasing concentrations of insulin. After 5 min, 100 nM isoproterenol was added and incubated for a further 90 min at 37°C. Lipolysis was determined as glycerol release into the incubation medium. Data represent lipolytic activities normalized in each experiment to the lipolytic value in the absence of insulin (control). *B*: maximal inhibitory effect (I_max) of insulin on lipolysis initiated by 100 nM isoproterenol in lumbar adipocytes from virgin and 7-, 14-, and 20-day pregnant rats. Values are means ± SE of ≥4 animals performed in duplicate. Comparisons were made by ANOVA, followed by Bonferroni test with 95% confidence limits. Significance is shown by letters, different letters indicating significant difference ($P < 0.05$) between groups.

the 7-day pregnant animals and the lowest in those from the 20-day pregnant rats. The response of the adipocytes of the 14-day pregnant rats was similar to that from virgin animals (Fig. 4*B*).
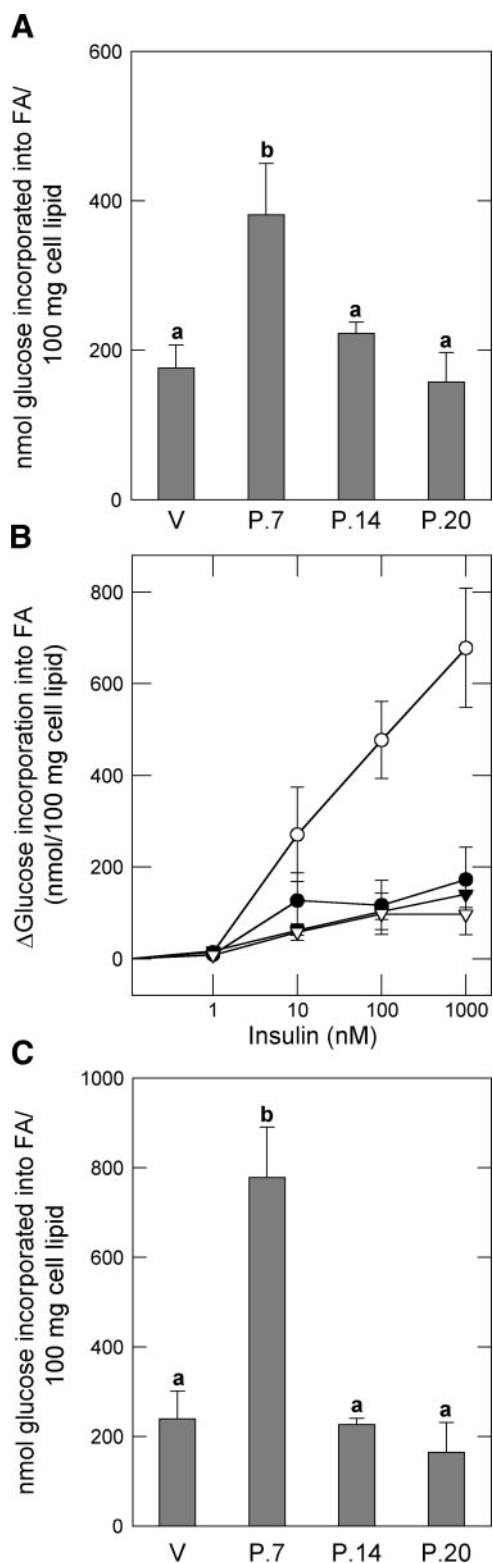

Fig. 5. Concentration-dependent activation of lipid synthesis from glucose by insulin. Rat adipocytes freshly isolated from lumbar white adipose tissue from virgin (●) and 7- (○), 14- (▼), and 20-day (▽) pregnant rats were incubated in Krebs-Ringer bicarbonate buffer containing 5 mM glucose in the presence of increasing concentrations of insulin. After 30 min, 0.5 μCi/tube of [U-^{14}C]glucose was added and incubated for a further 90 min at 37°C. Lipid synthesis was determined as glucose incorporation into cellular fatty acids (FA) after lipid extraction and saponification. *A*: synthesis of FA from glucose in lumbar adipocytes from virgin and 7-, 14-, and 20-day pregnant rats in the absence of insulin. *B*: dose-response relationship between insulin and lipogenesis. Data represent lipogenic activities normalized in each experiment to the FA synthesis in the absence of insulin. *C*: effect of 100 nM insulin on lipogenesis from glucose in lumbar adipocytes from virgin and 7-, 14-, and 20-day pregnant rats. Values are means ± SE of ≥4 animals performed in duplicate. Comparisons were made by ANOVA, followed by Bonferroni test with 95% confidence limits. Significance is shown by letters, different letters indicating significant difference ($P < 0.05$) between groups.

Concentration-dependent effect of insulin on lipogenesis. To analyze the lipogenic activity of adipose tissue during pregnancy, the formation of labeled fatty acids from [U-^{14}C]glucose was examined. Figure 5*A* summarizes the results obtained during incubation of adipocytes from pregnant rats for 90 min in KRB containing [^{14}C]glucose in the absence of insulin (basal state). In this condition, glucose incorporation into fatty acids was significantly higher at 7 days of pregnancy compared with the other experimental groups, which gave values similar to one another. To investigate the insulin sensitivity of fatty acid synthesis, dose-response experiments were performed. As shown in Fig. 5, *B* and *C*, the stimulation of fatty acid synthesis by insulin was maximal at 7 days of gestation, whereas the lowest activation of lipogenesis by insulin takes place in the late-pregnant rats.

DISCUSSION

It has been proposed that maternal insulin sensitivity is associated with fat deposition in early gestation. However, the mechanism for the relationship between increased insulin sensitivity and adipose tissue weight gain remains unknown. The present study demonstrates for the first time that, in early pregnancy in the rat, there is an augmented insulin responsiveness of adipose lipolysis and lipogenesis. This adaptation leads to the fat accumulation in the mother that is known to take place during the first half of pregnancy (37). This accretion of fat makes available sufficient nutrients to warrant normal fetal development. In fact, conditions that impair maternal capacity for fat accumulation during early gestation are known to cause smaller fetuses, as is the case with hypothyroidism (4) and severe diabetes (16) in the rat.

During early gestation, both glucose and insulin concentrations in the mother remain unchanged. This happens even when already by *day 6* of gestation the pancreatic content of insulin is significantly enhanced (43), indicating that the augmented release of insulin takes place only under an intense insulinotropic stimulus such as food intake. The results presented here show that 7-day pregnant rats have a higher overall

response to insulin than nonpregnant animals, as deduced from the glucose tolerance test. Different clinical studies have also suggested an enhanced insulin sensitivity in early gestation in women. In a recent report, it was observed that, in normal healthy women in the first trimester of gestation, maternal glucose falls (40). Furthermore, type 1 diabetic women in the late first trimester of pregnancy have a transient decrease in their insulin requirement (28), and it has been proposed that an increased insulin sensitivity is responsible for such a decline (53). Our results show that the augmented insulin responsiveness present in early gestation completely switches to a normal condition by *day 14* of pregnancy, a situation previously observed in rats on *day 15* of gestation (43). This is comparable to what occurs in late second trimester of pregnancy when insulin requirements in type 1 diabetic women are similar to those before pregnancy (10). Therefore, at this stage of gestation, normal sensitivity to insulin in the presence of hyperphagia and hyperinsulinemia sets the appropriate scene for the active anabolic condition present in the mother. As pregnancy proceeds, insulin sensitivity is impaired, and the situation of insulin resistance becomes maximal in late gestation. This insulin resistance state is one of the most characteristic features of late pregnancy both in human and in rat, as with the hyperinsulinemic euglycemic clamp technique (8, 46) it has been demonstrated that insulin-mediated glucose disposal decreases as much as 40–60% from nonpregnancy or early pregnancy to late gestation.

Although the occurrence of insulin resistance in late pregnancy is well documented, the underlying mechanisms causing the changes in adipose tissue insulin responsiveness during pregnancy are still unclear. Ongoing work in our laboratory and data from other groups (19, 55) indicate that there is virtually no decrease in insulin receptor number and that an impaired insulin receptor tyrosine kinase activity could account for the insulin resistance state at late pregnancy (Ramos MP, Crespo-Solans MD, Martinez JI, Herrera E, unpublished data). In addition, preliminary data from our laboratory, investigating the largely unknown molecular mechanisms that account for enhanced insulin responsiveness at early gestation, point to changes in postreceptor protein function (Ramos MP, Crespo-Solans MD, Martinez JI, Herrera E, unpublished data). However, the molecular events responsible for both enhanced insulin responsiveness in early pregnancy and insulin resistance in late gestation remain to be established.

In agreement with previous reports (19), our results show that gestation is a stage that results in increased adipocyte volume, and it is known that adipose fat depots are the net balance of synthesis and hydrolysis of triacylglycerols via lipogenesis and lipolysis. Furthermore, the present study demonstrates that pregnancy causes a striking change in catecholamine-stimulated lipolysis in isolated rat adipocytes, with 50% decreased activity at both 7 and 20 days of gestation with a temporary normalization at midpregnancy (14

days). It has been reported previously that an enlargement in fat cell size causes an increase in lipolysis (27, 57). However, although adipose cells from 20-day pregnant rats are significantly larger than those from nonpregnant animals, their isoproterenol responsiveness is significantly lower. Thus differences in cell size cannot completely explain the perturbations in fat cell metabolism associated with pregnancy.

In rat adipocytes, catecholamines stimulate lipolysis by acting via the β_3-adrenoceptors activating adenylate cyclase; then cAMP promotes lipolytic activity by activating PKA, which phosphorylates HSL (for review, see Ref. 34). To study the molecular factors regulating adipose tissue lipolytic activity during pregnancy, we performed immunodetection of the β_3-adrenergic receptor by using a specific antibody. We observed that β_3-adrenoceptor protein was reduced by >50% in adipocytes from both 7- and 20-day pregnant rats. Similarly, catecholamine-stimulated lipolysis was reduced in these two groups by 50%. In different cell systems, due to the existence of spare receptors, only a fraction of the receptors has to be occupied to obtain a full biological effect after hormone stimulation. Previous studies have observed that such a receptor reserve also exists for β-adrenergic-induced lipolysis in human fat cells (2). According to this hypothesis, a small reduction in receptor number is accompanied by a shift to the right of the lipolytic dose-response curve without a change in its amplitude (49). However, when a larger receptor fraction is inactivated, an additional reduction of the responsiveness should be found. Even though present results show a 50% reduction on the isoproterenol responsiveness, we cannot exclude the possibility that the observed decrease in the lipolytic activity to catecholamines in the adipocytes of 7- and 20-day pregnant animals is a postreceptor defect. In our study, forskolin and isobutylmethylxanthine responses revealed similar lipolytic efficacies in virgin and pregnant rats, suggesting that differences in lipolytic signals are due to adaptations at the level of the plasma membrane with a decreased number of β_3-adrenoceptors.

In adipose cells, insulin and catecholamines are of major importance for the endocrine control of lipid mobilization. Present findings, showing an augmented adipose tissue responsiveness to the antilipolytic action of insulin at early pregnancy, provide an explanation for the decrease in both β_3-adrenoceptor protein and activity, since these variables are known to be interconnected (14, 18). This is in agreement with results showing that the lipolytic response to catecholamines is impaired in biopsies of subcutaneous femoral adipose tissue in late first trimester pregnant women (48). This higher antilipolytic action of insulin in early gestation should play a very important role in the maintenance of fat deposition. Previous studies with rat fat cells have hypothesized that the degree of antilipolysis induced by insulin depends on the rate of lipolysis before the addition of the hormone (56). Thus the antilipolytic effect is more pronounced when the lipolysis is raised. Our study does not support this

hypothesis, since the lipolytic activity in absence of insulin is even higher in virgin than in the 7-day pregnant rats but the antilipolytic effect of insulin is ~30% higher in the latter group. Others have found a positive correlation between the antilipolytic effect of insulin and the size of human fat cells (44). However, we do not find such a relationship in our study, so differences in fat cell size cannot explain this higher response in the 7-day pregnant rat.

Enhanced maternal adipose tissue lipolytic activity during late gestation has been related to autonomic nervous system activation, secondary to hypoglycemia. However, a decreased response of lumbar adipocytes to norepinephrine or isoproterenol has been reported during late gestation in the rat (1) and in the sheep (22, 58). In addition, it is known that lipolysis varies to a different degree in the various fat depots (27). In this context, we have shown in a previous study (37) that there is a 30% decrease in subcutaneous adipose tissue weight from *days 15* to *20* of gestation. Consequently, the overall decrease in maternal fat in late pregnancy should be the result of the contribution of the different adipose stores. Thus the results of the present study, which focuses specifically on lumbar adipose tissue, do not exclude the possibility that, in late pregnant rats, other fat depots, such as subcutaneous adipose tissue, exhibit an enhanced fat breakdown. A study performed in rabbits reveals that the β_3- and α_2-adrenoceptor components are enhanced in late pregnancy (5). These results are not in disagreement with ours, since they show an increase in the α_2/β ratio, and this corresponds to a higher antilipolytic component in adipocytes from late pregnant rabbits (5). Furthermore, because the β-adrenoceptor activity is impaired when nonpregnant animals become hyperinsulinemic by means of the euglycemic hyperinsulinemic clamp (23), we can speculate that the lower catecholamine-stimulated lipolysis in late gestation could be a consequence of decreased adrenoceptor activity secondary to maternal hyperinsulinemia. The antilipolytic effect of insulin is considered the most sensitive of its metabolic actions (56), so it requires a much lower insulin concentration (1 nM in our study) than other response sites like the stimulation of glucose transport (>10 nM for glucose uptake and lipogenesis). Hence, even in insulin resistance states, in which glucose transport is impaired, sensitivity to insulin's antilipolytic effect is relatively preserved. This is in agreement with the hypothesis that insulin resistance is not a general phenomenon but is confined to specific effector systems (33). This could explain that, although insulin resistance of glucose transport is a characteristic of late pregnancy (insulin stimulation of glucose uptake is reduced by 50%) (36), hyperinsulinemia accounts for the decreased catecholamine-stimulated lipolysis found in late pregnant rats (insulin inhibition of lipolysis is impaired by only 25% in 20-day pregnant rats), resulting in the maintenance or expansion of adipose stores. In support of this hypothesis, it has been found that adipose tissue from late pregnant women retains its sensitivity to the antilipolytic effect of insulin (9). Thus, similar to the results obtained in pregnant rats fed a different fat diet (23), hyperinsulinemia occurring in late pregnancy might be responsible for decreasing (2-fold) both lipolytic activity and β_3-adrenoceptor protein in adipose cells.

Together with the changes observed in lipolysis, it was found here that basal lipid synthesis from glucose is significantly increased in the 7-day pregnant rat. In midpregnancy (*day 14*), the values returned to those found in the nonpregnant condition, whereas in late pregnancy there was a tendency for the values to decrease. The tendency of adipose tissue lipogenesis to decrease in late pregnancy is in agreement with the sharp decline observed in Wistar rats on *day 21* of gestation (45). In this context, it should be mentioned that, in late pregnancy, the mother has hypoglycemia, so the "in vivo" availability of glucose for lipid synthesis is even lower. These results, together with a decreased hydrolysis and uptake of circulating triglycerides resulting from an impaired lipoprotein lipase activity (38), reflect the net breakdown reduction of the various adipose tissue depots seen around delivery (37).

It could be suggested that plasma insulin per se is responsible for the modulation of lipid synthesis from glucose during pregnancy. However, in our study, the highest lipogenic activity of adipose tissue was detected in early pregnancy, when rats are normoinsulinemic and the lowest in the 20-day pregnant rats despite these animals being hyperinsulinemic. Furthermore, the dose-response relationship between insulin and activation of lipogenesis reveals that insulin responsiveness is drastically enhanced in the 7-day pregnant rats. Thus modulation of lipogenesis during pregnancy can be better explained on the basis of insulin responsiveness. Fat accumulation in early gestation is caused by enhanced adipose tissue lipogenesis (17, 32), which is related to maternal hyperphagia because it does not happen in food-restricted rats (41). Our results support this hypothesis because after a meal, when insulin secretion and glucose availability are enhanced, the higher insulin responsiveness of the mother favors accumulation of glucose into lipids, contributing to the hypertrophy of adipose tissue and increased maternal fat depots.

Alternatively, given its participation in the regulation of energy homeostasis, leptin might be involved in fat accretion during pregnancy. Biological effects of leptin include inhibition of food intake and impairment of metabolic action of insulin, including stimulation of glucose uptake, lipogenesis, and inhibition of isoproterenol-stimulated lipolysis (42). However, the present study does not support this interaction in pregnancy, as no relationship between leptin concentration and lipogenesis, lipolysis, or insulin responsiveness was observed. Data from our present study are in agreement with observations indicating that both adipose tissue and plasma leptin levels peak ~12–19 days of gestation and subsequently display a pronounced decline to values similar to those of nonpregnant animals by *day 21* of gestation (29). Furthermore, in a previous

study from our group (25), we have already reported that plasma leptin levels parallel changes in fat mass during pregnancy and lactation. These data suggest that the maternal fat depots contribute, together with placenta (35, 39), to the change in maternal leptin levels during pregnancy.

Modulation of insulin sensitivity in pregnancy might be due to changes in gestational hormones. Specifically, a decline in progesterone, an anti-insulin hormone, during early pregnancy has been proposed to be responsible for lower insulin requirements (28, 31, 32). In addition, it has been suggested that low concentrations of 17β-estradiol, similar to early pregnancy, could be responsible for the enhanced insulin sensitivity in muscle and adipose tissue by increasing the number of insulin receptors (21). On the other hand, high 17β-estradiol concentrations, as found in late pregnancy, could favor insulin resistance by decreasing the insulin receptor number in peripheral tissues (21). Nevertheless, the hormonal and molecular environmental factors responsible for such changes remain to be established.

In conclusion, to our knowledge, this is the first study providing evidence that, at early gestation, an enhanced insulin sensitivity accounts for increased activation of adipose tissue lipid synthesis and inhibition of lipolysis. Normoinsulinemia in the presence of augmented insulin responsiveness, therefore, may drive the anabolic tendencies of the mother during the first two-thirds of gestation, and this accounts for increased fat deposition. This mechanism should have special relevance after a meal, when insulin secretion is enhanced and there is enough glucose availability. With this metabolic adaptation, the mother ensures that circulating glucose is actively taken up by adipose tissue and converted into triglycerides, which are not actively hydrolyzed. In this way, the mother develops a net fat depot that is known to have a major impact on the appropriate availability of substrates that warrant the normal development of the fetus at late pregnancy.

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DISCLOSURE

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