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Changes in the Kinase Activity of the Insulin Receptor Account for an Increased Insulin Sensitivity of Mammary Gland in Late Pregnancy*

J. M. CARRASCOSA, P. RAMOS, J. C. MOLERO, AND E. HERRERA

Department of Biochemistry (J.M.C., J.C.M.), Centro Biología Molecular-Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, E-28049 Madrid, Spain; and Faculty of Experimental and Technical Sciences (P.R., E.H.), Universidad San Pablo-CEU, E-28668 Madrid, Spain

ABSTRACT

Mammary gland is an organ that undergoes cycles of growth, differentiation, and function during pregnancy and lactation. Although it is known that the gland enhances its sensitivity to insulin during lactation, it remains to be investigated whether this increased sensitivity develops during pregnancy and which are the molecular mechanisms underlying such a change. To address this issue, virgin and late-pregnant rats were subjected to a continuous infusion with 50% glucose for 72 h to produce a prolonged hyperinsulinemic-euglycemic condition. Insulin sensitivity in mammary gland was determined as the glucose utilization index by using 2-[^{3}H]-deoxyglucose. Furthermore, binding characteristics and kinase activity were studied by means of both [^{125}I]insulin binding and *in vitro* phosphorylation studies with insulin receptors partially purified from mammary gland.

Whereas the glucose utilization index in mammary gland from nonpregnant rats remained unaffected by hyperinsulinemia, glands from pregnant rats displayed a high insulin-dependent glucose uptake. This effect was not paralleled by changes in the binding characteristics of insulin to the high-affinity receptor, suggesting that the high insulin sensitivity of mammary gland in pregnancy is not accounted for by changes at the level of hormone-receptor interaction. Autophosphorylation studies showed that insulin-stimulated kinase activity of insulin receptors from mammary gland was 6- and 20-fold higher in pregnant than in virgin animals under normo- and hyperinsulinemic conditions, respectively. Moreover, insulin dose-response curves revealed that the efficacy of insulin to stimulate kinase activity of the insulin receptor was markedly higher in pregnant than in virgin rats, whereas its potency ($ED_{50} \sim 15$ nM) was not changed. These data, therefore, show that mammary glands develop increased insulin sensitivity during late pregnancy, caused by an augmented kinase activity of the insulin receptor. (*Endocrinology* **139:** 520–526, 1998)

LATE pregnancy is characterized by the development of insulin resistance in both humans (1–3) and rats (4–6). Among the tissues that most actively contribute to the reduced insulin sensitivity are adipose tissue (4, 7) and skeletal muscle (8, 9). Whereas adipose tissue remains insulin-resistant during lactation (10), lactating mammary gland becomes highly sensitive to insulin (11–13). This enhanced mammary gland insulin sensitivity is rapidly abolished on weaning (12), but nothing is known about insulin responsiveness in the late-pregnancy mammary tissue.

The mammary gland in late pregnancy is characterized by alveolar morphogenesis, disappearance of adipose cells, and structural and functional differentiation of alveolar epithelial cells, enabling them to secrete milk fat and protein during lactation (14, 15). This development and differentiation of the rat mammary gland are further accompanied by changes in the expression of glucose transporters. Whereas in mammary gland of virgin rats, the insulin sensitive isoform of the glucose transporter Glut4 predominates (16), during late pregnancy, expression of Glut4 decreases and is abolished in the lactating mammary tissue (14, 16). Furthermore, lipoprotein lipase activity increases during late pregnancy (17), reaching a maximum during lactation (18). Thus, the morphological and functional changes characteristic for the lactating and mature mammary gland apparently start to develop already during pregnancy. Therefore, the present study was designed to clarify whether the increased insulin sensitivity of mammary gland observed in lactation does develop during pregnancy. To address this issue, virgin and pregnant rats were exposed to a continuous iv infusion with glucose that has been already shown to induce hyperinsulinemia (HI) unaccompanied by hypoglycemia (19, 20). In these animals, insulin sensitivity was assessed using the euglycemic-hyperinsulinemic clamp, and glucose utilization in the mammary gland was quantified by means of the 2-deoxyglucose (2-DOG) technique. These experiments revealed that mammary gland tissue becomes insulin sensitive during pregnancy. To further investigate the molecular mechanism underlying the increased responsiveness of the tissue, the study was extended to determine whether the interaction of insulin with its receptor and/or the insulin receptor kinase activity are affected during pregnancy under basal or hyperinsulinemic conditions, by means of radioligand binding and *in vitro* phosphorylation studies. Finally, insulin-dependent autophosphorylation of the receptors was correlated with the insulin responsiveness of the mammary gland, providing evidence for the involvement of increased receptor kinase activity in the enhanced insulin sensitivity of mammary gland during late pregnancy.

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Address all correspondence and requests for reprints to: Emilio Herrera, Facultad de Ciencias Experimentales, Universidad San Pablo-CEU, Ctra. de Boadilla del Monte km 5.3, E-28668 Madrid, Spain. E-mail: e.herrera@offcampus.es.
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Materials and Methods

Animals

Female Wistar rats were housed at 22–24 C, with light cycles from 0800 to 2000 h. They had free access to water and to a chow diet (Letica, Barcelona, Spain). Some animals were mated when they weighed 170–180 g. The beginning of pregnancy was determined by the presence of spermatozoids in vaginal smears. In pregnant rats at day 17 of gestation and in age-matched virgin rats, a SILASTIC brand catheter (Dow Corning, Midland, MI, 0.02 inch ID, 0.037 inch OD) was placed into the right jugular vein and another one into the right femoral vein, under ketamine cocktail anesthesia (ketamine, 50 mg/ml; diazepan, 5 mg/ml; and atropine, 1 mg/ml; 5/4/1, vol/vol/vol). After recovery from anesthesia, animals were housed in individual cages and continuously infused for 72 h with either bidistilled water or 50% glucose, through the catheter placed into the jugular vein, at the rate of 35 ml/day. Other methodological details have been previously described (19, 20). At the end of the 72-h infusion period, some animals from each group were decapitated, blood was collected from the neck wound, and mammary glands rapidly dissected and placed in liquid nitrogen, to be stored at −80 C until processed for insulin receptor studies, as described below. The experimental protocol was approved by the Animal Research Committee of the Faculty of Experimental Sciences, University San Pablo-CEU.

Studies of insulin resistance

Euglycemic clamp studies in the conscious rat. A number of rats from each group were subjected to an euglycemic- hyperinsulinemic clamp to test the insulin sensitivity state of the animals, as described before (20). In brief, after the 72-h infusion, blood samples were obtained from the tail tips for determination of blood glucose (21) and plasma insulin (22). The catheter placed in the jugular vein was connected to a two-way interconnector that received flow from two different infusion pumps (Precidor Infusion Pump Type 5003, Infors HT, Denkendorf, Germany). Human insulin (Actrapid monocomponent, Novo, Copenhagen, Denmark) was infused, by means of one pump at a constant rate of 16 μl/min (0.8 IU $\times$ h^{-1} $\times$ kg^{-1}), for 60 min. Glucose infusion (20%) was given at a variable rate through the other pump via the same catheter, to maintain the blood glucose concentration constant at basal levels. A steady-state glucose infusion was normally achieved within 30 min after starting the clamp experiment. A few additional blood samples (200 μl) were collected to determine the insulin concentration (22) at the steady-state. The glucose disposal rate (M) was estimated as the rate of glucose infusion at the steady-state normalized to the body weight. The insulin sensitivity index (S_{ip}), proposed by Ader and Bergman (23), was measured as described (19, 20).

Estimate of glucose utilization index (GUI) in mammary gland. The method of Sokoloff *et al.* (24), developed for the brain, was adapted for the unrestrained rat (19). Briefly, 2-deoxy-D-[1-^{3}H]glucose (2-DOG; 18.3 Ci/mmol, from Amersham, Buckinghamshire, UK) was administered, as a bolus (30 μCi) through the catheter placed in the jugular vein, to virgin and pregnant rats: 1) at the end of a 72-h infusion with bidistilled water (basal group); 2) after 1 h of an euglycemic-hyperinsulinemic clamp, subsequent to a 72-h infusion with bidistilled water (1-h hyperinsulinemic group); or 3) at the end of a 72-h infusion with 50% glucose (72-h hyperinsulinemic group). In the 1-h hyperinsulinemic group, the clamp, *i.e.* insulin and 20% glucose infusions, was maintained until death. Blood samples (50 μl) were collected from the catheter placed in the femoral vein at 1, 2, 5, 7, 10, 20, 30, 45, and 60 min after the 2-DOG administration. Blood was deproteinized (25), and glucose concentrations (21) and 2-DOG radioactivity were determined in the supernatants. After the last blood collection, animals were decapitated, and blood was collected from the neck wound for plasma insulin determination (22). Mammary gland tissue was immediately dissected and immersed in 0.5 ml of 1 M NaOH to determine its content of 2-DOG-6-phosphate (26) and to estimate the GUI, as previously described (19).

Studies with insulin receptors

Preparation of partially purified insulin receptors. Pools of mammary gland from each group (7–11 g) were homogenized in 3 vol of 25 mM Tris-HCl (pH 7.4) buffer, containing 0.3 M sucrose, 7 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1.3 mM phenylmethylsulfonyl fluoride (PMSF), 2 mg/ml bacitracin, and 0.3 mg/ml trypsin inhibitor. After centrifugation at 3,000 $\times$ *g* for 15 min, the supernatant was centrifuged at 50,000 $\times$ *g* for 1 h. The microsomal pellet was resuspended in 50 mM Tris-HCl (pH 7.4) buffer, containing 150 mM NaCl and 1 mM PMSF, and stored at −70 C. Microsomes (500 μl) were solubilized for 1 h in 2 ml of 25 mM Tris-HCl (pH 7.4) buffer, containing 1% Triton-X-100, 4 mM EDTA, 7 mM sodium pyrophosphate, 30 mM NaF, 0.6 mg/ml benzamidine, 0.3 mg/ml trypsin inhibitor, 2.5 mg/ml bacitracin, and 1.3 mM PMSF. Insoluble material was removed by centrifugation at 150,000 $\times$ *g* for 1 h, and the clear supernatant was diluted 1:5 with 50 mM Tris-HCl (pH 7.4), containing 0.05% Triton-X-100, 100 mM NaCl, 2.5 mM KCl, and 1 mM $CaCl_2$, added to 0.5 ml of wheat germ agglutinin-Sepharose (WGA-Sepharose, Pharmacia, Uppsala, Sweden) and rotated end-over-end for 2 h at room temperature. The WGA-Sepharose was then washed five times with 20 vol of the same buffer, and receptors were eluted by the addition of 500 μl of 0.3 M *N*-acetyl-glucosamine in the same buffer. The protein concentration was measured (27) in these fractions and always ranged between 0.2 and 0.4 mg/ml.

Binding to solubilized receptors. Aliquots (10 μl) of the WGA-Sepharose eluates were incubated overnight at 4 C, with [^{125}I-Tyr^{A14}]insulin (100 pM, from Amersham) and various concentrations of human monocomponent insulin (0.16–1600 nM) in a final vol of 500 μl. After incubation, receptor-hormone complexes were separated from free insulin by 10% polyethylene glycol precipitation, and measurement of the bound hormone was done as previously described (28). Insulin binding constants [equilibrium dissociation constants (K_d) and maximal binding constants (B_{max})] were estimated by Scatchard analysis.

Autophosphorylation of solubilized receptors. Aliquots (10–15 μg of protein) of WGA-Sepharose purified insulin receptors were incubated at room temperature for 2 h in the *N*-acetyl-glucosamine buffer supplemented with increasing amounts of porcine insulin (Sigma, 0–160 nM). Phosphorylation assays were carried out at 0 C in the presence of [γ-^{32}P]ATP, as previously described (29). Briefly, the phosphorylation reaction was initiated by adding 50 μM [γ-^{32}P]ATP (specific radioactivity ~3300 cpm/pmol), 12 mM $MgCl_2$, 6 mM $MnCl_2$, and 1 mM sodium orthovanadate (final concentrations), to give a final vol of 80 μl. After a 15-min incubation, the reaction was ended by applying 80 μl of a 50 mM Tris-HCl (pH 7.4), containing 20 mM EDTA, 20 mM sodium pyrophosphate, 1 mM ATP, 1 mM sodium orthovanadate, and 1 mM dithiothreitol. Insulin receptors were quantitatively immunoprecipitated with antiphosphotyrosine antibodies prepared as in (30). After immobilization on Protein A (Sigma Chemical Co., St. Louis, MO), the pellets were washed three times with 50 mM TripH 7.4 (containing 0.1% SDS, 0.1% Triton-X-100, and 0.15 M NaCl), and proteins were separated by SDS-PAGE (7% acrylamide). Radioactive proteins were identified by autoradiography, and the ^{32}P-incorporation into the insulin receptor β-subunit was quantified by densitometric scanning.

Statistical analysis

Statistical comparisons were made with ANOVA, followed by the Tuckey test, or by a multiple linear-regression analysis with a 95% confidence interval, using the Systat program (Systat, Inc., Evanston, IL). Results are expressed as means ± SEM.

The K_d and B_{max} were calculated using the nonlinear regression fitting option of the Sigma Plot Program (Jandel Scientific Corp., San Rafael, CA).

Results

Model of the euglycemic-hyperinsulinemic rat

Body weight, circulating components. In the present study, to investigate the response of mammary glands to insulin in late pregnancy, an animal model of prolonged iv glucose infusion in the rat, to attain hyperinsulinemia under euglycemic conditions, was used (19, 20). Virgin and pregnant rats (17 day of gestation) were subjected to a continuous infusion with bidistilled water (control) or 50% glucose for 72 h.

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

	Virgin		Pregnant	
	Control	50% Glucose	Control	50% Glucose
Body weight (g)	223 ± 6^a	209 ± 6^a	306 ± 12^b	292 ± 6^b
Blood glucose (mM)	5.17 ± 0.20^a	5.52 ± 0.37^a	4.09 ± 0.41^b	4.40 ± 0.22^b
Plasma insulin (pM)	183 ± 30^a	447 ± 32^b	465 ± 40^b	1386 ± 83^c

In anesthetized Wistar virgin and pregnant rats (at day 17 of gestation), a catheter was placed into the jugular vein. After recovery from ketamine anesthesia, animals were continuously infused for 3 days (35 ml/day) through the catheter placed in the jugular vein, either with 50% glucose, to generate sustained hyperinsulinemia, or with bidistilled water (control groups). At the end of the 72-h infusion period, blood samples (200 μl) were collected to determine glucose and insulin concentrations (21, 22). Values are mean ± SEM of 6–13 rats/group. Statistical comparison between groups for each parameter is shown by the *superscript letters:* different letters indicate significant difference ($P < 0.05$).

TABLE 2. Effect of sustained hyperinsulinemia-euglycemia on insulin sensitivity in virgin and pregnant rats

	Virgin		Pregnant	
	Control	50% Glucose	Control	50% Glucose
Blood glucose (mM)	5.60 ± 0.20^a	5.78 ± 0.30^a	3.90 ± 0.48^b	4.43 ± 0.18^b
Plasma insulin (pM)	1608 ± 28^a	1920 ± 42^b	1812 ± 48^b	2617 ± 55^c
Glucose disposal rate (M) ($g \cdot min^{-1} \cdot kg^{-1}$)	23.2 ± 0.8^a	14.3 ± 0.8^b	9.4 ± 0.1^c	15.4 ± 0.1^b
Insulin sensitivity index (S_{ip}) ($10^{-4} \cdot dl \cdot min^{-1} \cdot kg^{-1} \cdot U^{-1} \cdot ml$)	9.05 ± 0.59^a	5.16 ± 0.29^b	4.94 ± 0.29^b	9.30 ± 0.84^a

Virgin and pregnant rats were infused with 50% glucose for 72 h (35 ml/day) to generate sustained hyperinsulinemia. Control animals were infused with bidistilled water. Following the 3-day infusion period, animals were subjected to the euglycemic clamp. A catheter placed in the jugular vein was connected to a two-way interconnector that received flow from two different infusion pumps. Insulin was infused by means of one pump at a constant rate of 16 μl/min (0.8 IU $\times$ h^{-1} $\times$ kg^{-1}) for 60 min. Glucose infusion (20%) was given at a variable rate through the other pump to maintain the blood glucose concentration constant at basal levels. Steady-state glucose infusion was achieved within 30 min of the clamp. After 60 min, blood samples (200 μl) were collected to determine glucose and insulin concentrations (21, 22). The glucose disposal rate (M) was calculated as the rate of glucose infusion normalized to body weight, and the insulin sensitivity index (S_{ip}) measured as described (19, 23). Values are mean ± SEM of five rats per group. Statistical comparisons for each parameter were made by ANOVA, followed by a Tuckey test with 95% confidence limits. Significance is shown by *superscript letters: different letters* indicate significant differences ($P < 0.05$).

Animals within each group were of equal weight at the beginning of the infusion and, as shown in Table 1, 50% glucose treatment for 72 h did not affect the body weight of either virgin or pregnant rats. Blood glucose levels were lower in pregnant than in virgin rats and remained the same upon glucose infusion in both groups. Plasma insulin levels were higher in pregnant than in virgin rats, but both groups responded similarly to glucose treatment with a 2- to 3-fold increase in plasma insulin concentration (Table 1). These data, therefore, show that the model of glucose infusion for 3 days is suitable to generate hyperinsulinemic-euglycemic conditions, both in virgin and pregnant rats.

Euglycemic-hyperinsulinemic clamp. To determine how the glucose infusion affected insulin sensitivity, an euglycemic-hyperinsulinemic clamp (0.8 IU insulin $\times$ h^{-1} $\times$ kg $^{-1}$) was performed for 60 min in unrestrained virgin and pregnant rats at the end of the 72-h infusion with either 50% glucose or bidistilled water. Under these conditions, blood glucose remained stable, whereas plasma insulin increased in the four groups (Table 2) when compared with the basal values before the clamp (Table 1). Plasma insulin concentrations during the clamp were higher in the groups receiving the 50% glucose infusion than in their respective controls, and in pregnant than in virgin rats (Table 2). In virgin animals, 72 h of 50% glucose infusion resulted in a significant decrease in both the M and the S_{ip}, by 39% and 43%, respectively, showing the development of insulin resistance under hyperinsulinemic-euglycemic conditions (Table 2). Pregnant control animals clearly exhibited insulin resistance, as evidenced by their low M and S_{ip}-values (41% and 55% of virgin controls, respectively). In contrast to virgin animals, in pregnant rats, 72 h of 50% glucose infusion markedly increased the M by 64% and completely restored the impaired S_{ip} to values that did not differ from those of the virgin control rats, thus indicating the full reversion of the insulin-resistant condition.

Insulin responsiveness of mammary gland in late pregnancy: GUI

As shown above, insulin resistance that normally develops during late pregnancy disappears in response to a prolonged hyperinsulinemia caused by the 72 h of 50% glucose infusion. To investigate insulin responsiveness of mammary gland during late pregnancy and how this tissue responds to such prolonged hyperinsulinemic condition, glucose utilization was quantified by measuring the GUI. GUI was assessed by the administration of an iv bolus of 2-DOG and the subsequent analysis of phosphorylated 2-DOG in the mammary tissue. These experiments were performed in pregnant and nonpregnant rats under normoinsulinemia (basal) and under both short- and long-term hyperinsulinemia, generated by means of the euglycemic-hyperinsulinemic clamp (1-h HI) and by the 72-h continuous glucose infusion (72-h HI), respectively. As shown in Fig. 1, in mammary gland of normoglycemic virgin animals (basal), the GUI value was 1.60 ± 0.15 mg $\times$ min^{-1} $\times$ kg^{-1}, and neither short-term (1-h HI) nor prolonged (72-h HI) exposure to hyperinsulinemia affected the GUI in mammary glands of these animals (1.56 ± 0.34 and 1.91 ± 0.34 mg $\times$ min^{-1} $\times$ kg $^{-1}$, respectively). In pregnant rats, under basal conditions, the GUI of mammary gland was

1.31 ± 0.26 mg $\times$ min^{-1} $\times$ kg^{-1} and, thus, did not differ from the value found in the virgin basal group. However, in contrast to virgin rats, mammary glands of pregnant animals responded to hyperinsulinemia with increased glucose utilization (Fig. 1). This effect seemed to be dependent on the duration of hyperinsulinemia, with GUI values increasing to 2.08 ± 0.15 mg $\times$ min^{-1} $\times$ kg^{-1} (1.6-fold) and to 3.08 ± 0.35 mg $\times$ min^{-1} $\times$ kg^{-1} (2.4-fold) upon exposure to hyperinsulinemic conditions for 1 and 72 h, respectively.

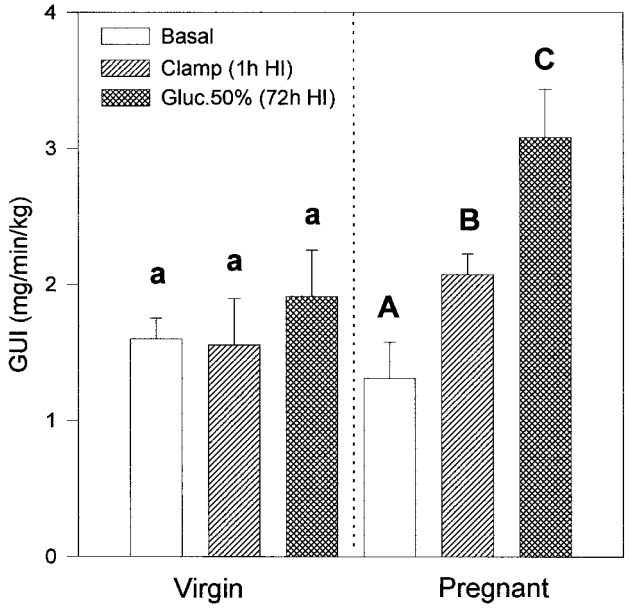

FIG. 1. Effect of hyperinsulinemia (HI) on glucose utilization in the mammary gland of virgin and pregnant rats. GUI in mammary gland of virgin and 20-day-pregnant rats was measured as described in *Materials and Methods* under normoglycemic, short-term, and long-term hyperinsulinemic conditions, *i.e.* in rats at the end of a 72-h infusion with bidistilled water (basal group), after 1 h of an euglycemic-hyperinsulinemic clamp (0.8 IU insulin $\times$ h^{-1} $\times$ kg^{-1}), subsequent to a 72-h infusion with bidistilled water (1-h HI) or at the end of a 72-h infusion with 50% glucose [72 h hyperinsulinemic group (72-h HI)], respectively. Statistical comparisons were made by ANOVA, followed by a Tuckey test with 95% confidence limits. Significance is shown by *letters*: *different letters* indicate significant difference ($P < 0.05$). *Capital letters* are used for the pregnant rats.

Insulin binding characteristics of insulin receptors from mammary gland

To investigate whether the increased insulin sensitivity of mammary gland in late pregnancy can be accounted for by changes in binding of insulin to its receptor, we performed competition binding studies with ^{125}I-labeled insulin and insulin receptors partially purified from mammary glands of virgin and pregnant rats exposed to normo- and hyperinsulinemic conditions (*i.e.* infusion of bidistilled water or 50% glucose for 72 h, respectively). Scatchard analysis of competition binding curves (not shown) revealed that virgin and pregnant rats exhibited both low- and high-affinity sites for insulin. As shown in Table 3, both binding capacity and affinity of insulin receptors were similar in mammary gland of pregnant and virgin rats. The binding data also show that in virgin and pregnant animals, hyperinsulinemia caused by the 50% glucose infusion did not significantly modify either maximal insulin binding to both the high-($B_{max\text{-}1}$, ANOVA $P = 0.68$) and low-affinity ($B_{max\text{-}2}$, ANOVA $P = 0.08$) receptor or the affinity of insulin for the high-($K_{d\text{-}1}$, ANOVA $P = 0.761$) or for the low-affinity binding sites ($K_{d\text{-}2}$, ANOVA $P = 0.203$) (Table 3). Furthermore, the number of receptors per mg of tissue was not significantly affected either by pregnancy or by sustained hyperinsulinemia (ANOVA $P = 0.177$).

Kinase activity of insulin receptor from mammary gland

Because the binding data did not provide an explanation for the development of increased insulin sensitivity in the mammary gland during late pregnancy, we speculated that pregnancy might modify the kinase activity of the mammary gland insulin receptor. To address this issue, *in vitro* phosphorylation studies, with insulin receptors partially purified from mammary glands of control and glucose-infused virgin and pregnant rats, were performed; and the kinase activity of the receptor was determined as insulin-dependent autophosphorylation of its 95 kDa α-subunit. Representative au-

TABLE 3. Effect of 50% glucose infusion (35 ml/day) for 3 days on insulin binding to the mammary gland insulin receptor of virgin and pregnant rats

	Virgin		Pregnant	
	Control	50% Glucose	Control	50% Glucose
Maximal insulin binding (pg of insulin bound/μg protein)				
High-affinity ($B_{max\text{-}1}$)	18 ± 10	55 ± 33	53 ± 21	37 ± 12
Low-affinity ($B_{max\text{-}2}$)	8428 ± 2699	7296 ± 1874	4254 ± 1600	1358 ± 296
Binding affinity -K_d (nM)				
High-affinity ($K_{d\text{-}1}$)	1.36 ± 0.92	0.51 ± 0.22	1.40 ± 0.70	1.18 ± 0.38
Low-affinity ($K_{d\text{-}2}$)	210 ± 40	680 ± 170	800 ± 260	810 ± 170
Receptor number ($\times 10^{-6}$ sites/μg tissue)	5.4 ± 1.7	3.6 ± 0.7	3.4 ± 1.5	1.5 ± 0.3

Virgin and pregnant rats were infused with 50% glucose for 72 h (35 ml/day) to generate sustained hyperinsulinemia. Control animals were infused with bidistilled water. Following the 3-day infusion period, animals were killed and mammary glands rapidly dissected. Insulin receptors were partially purified as described in the *Materials and Methods* section. Receptor preparations (10 μl), were incubated overnight with ^{125}I-insulin (100 pM) and increasing concentrations of unlabeled insulin (0.16–1600 nM). Hormone-receptor complexes were separated from free insulin by 10% polyethylene glycol precipitation. B_{max} (maximal insulin binding) and K_d (dissociation constant) for both high- and low-affinity sites were derived from Scatchard plots with the nonlinear regression fitting option of the Sigma Plot program. Number of receptors per amount of fresh tissue was determined from the total binding in each experiment. Values are mean ± SEM of four experiments/group. Statistical comparisons for each parameter were made by ANOVA with a 95% confidence interval. No significance was found for any of them.

toradiographs of the phosphorylated and immunoprecipitated mammary gland insulin receptors, in the presence (160 nM) or the absence of insulin, are depicted in the *upper part* of Fig. 2. To quantify the kinase activity of the insulin receptor under the different experimental conditions in each preparation, ^{32}P-incorporation into the β-subunit was determined by scanning densitometry and normalized to its insulin binding activity (IBA). As shown in Fig. 2, insulin receptors from mammary glands displayed basal autophosphorylation that was approximately 20-fold higher in pregnant than in virgin rats. Although, in both groups, autophosphorylation was markedly increased upon incubation with 160 nM insulin, phosphorylation levels were always higher in the pregnant than in the virgin group. The effect of glucose infusion also was different in virgin and pregnant animals. The quantitative analysis shown in Fig. 2 revealed that in receptors from mammary gland of glucose-infused virgin rats (*i.e.* rats kept hyperinsulinemic for 72 h), insulin (160 nM)-stimulated kinase activity was only 38% of that found in control animals. Hence, whereas in virgin rats the glucose infusion reduced the insulin-dependent phosphorylation even more, in pregnant rats it led to a slight (although not significant) increase in insulin-stimulated autophosphorylation. Thus, in the insulin receptors of mammary glands from pregnant rats, the insulin-stimulated kinase activity was 6- and 20-fold higher under normo- and hyperinsulinemic conditions, respectively, as compared with virgin animals.

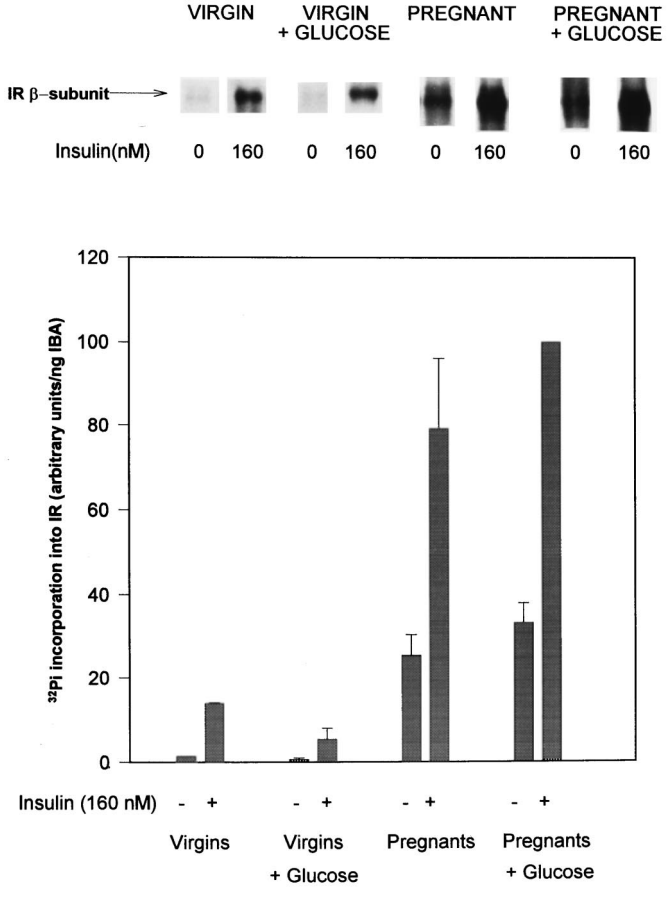

FIG. 2. Insulin receptor (IR) β-subunit autophosphorylation. Insulin receptors, partially purified from mammary gland, were phosphorylated after incubation in the absence or presence (160 nM) of insulin. Phosphorylated receptors were immunoprecipitated with antiphosphotyrosine specific antibodies and separated by SDS-PAGE. The autoradiographs shown in the *upper part* of the figure identify a 95-kDa band corresponding to the receptor β-subunit. Equal amounts of WGA-purified protein were used in the four groups. The *lower part* of the figure shows the absolute ^{32}P-incorporation into the insulin receptor. Autoradiographs were quantified by scanning densitometry, and data were corrected according to the IBA of each preparation. ^{32}Pi-incorporation was expressed as percentage of maximal phosphorylation, which was always observed in the presence of 160 nM insulin, with receptors obtained from glucose-infused pregnant rats. Data are mean ± SEM of three experiments.

To determine whether pregnancy and/or hyperinsulinemia are accompanied by changes in the insulin-responsiveness of the kinase activity of the mammary gland insulin receptor, phosphorylation experiments were carried out in the presence of increasing concentrations of insulin (0–160 nM), and the degree of autophosphorylation of the receptor was quantified (by scanning densitometry of the obtained autoradiographs) and normalized to IBA. The dose-response curves (Fig. 3) show that the insulin concentrations required for half-maximal stimulation of receptor autophosphorylation were virtually identical ($ED_{50} \sim 15$ nM) under the various experimental conditions, *i.e.* virgin and pregnant rats under control or glucose-infused conditions. These curves clearly show that the efficacy of insulin to stimulate the kinase activity of the mammary gland insulin receptor is markedly higher in pregnant than in virgin rats and that the highest insulin-dependent phosphorylation was detected in the glucose-infused pregnant rat group (Fig. 3).

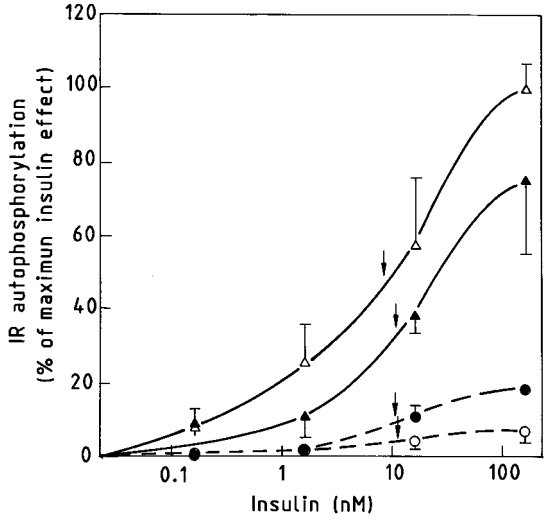

FIG. 3. Insulin dose-response curves for stimulation of receptor β-subunit autophosphorylation. Autoradiographs like those in Fig. 2, but using different insulin concentrations, were quantified by scanning densitometry, and data were corrected according to the IBA of each preparation. Basal values of phosphorylation are considered 0%. Curves show the insulin-dependent phosphorylation (above basal), and data are represented as a percentage of the maximum insulin effect observed with glucose-infused pregnant-rat receptors. Insulin concentrations required for half-maximal stimulation (ED_{50}) are shown by *arrows*. Values are mean ± SEM of three experiments. △, Glucose infused pregnant rats; ▲, pregnant control rats; ○, glucose-infused virgin rats; ●, virgin control rats.

Discussion

The model of the euglycemic-hyperinsulinemic rat, obtained by 50% glucose infusion for 3 days, was used to study mammary gland insulin sensitivity during pregnancy and to shed light on the molecular mechanism underlying the responsiveness of this tissue to insulin. It was found here, in agreement with previous reports (19), that sustained euglycemia-hyperinsulinemia decreases overall insulin sensitivity in virgin rats and reverts the insulin resistance condition (19) normally present in pregnant animals (5, 6, 31, 32). Furthermore, the present study shows, for the first time, that in pregnant rats, mammary gland displays high insulin-dependent glucose uptake under hyperinsulinemia, caused by either the euglycemic-hyperinsulinemic clamp or by the continuous glucose infusion. In contrast, glucose utilization by the glands from nonpregnant rats is not affected under the same conditions. The increased insulin responsiveness in mammary glands of pregnant rats may be accounted for by changes in the binding characteristics of the insulin receptor, by an altered kinase activity and/or phosphorylation status of the receptor, or by postreceptor events. The data obtained from radioligand binding studies argue against changes in the hormone-receptor interaction in late pregnancy or in response to hyperinsulinemia, because both maximal insulin binding and affinity to both high- and low-affinity sites remain unaffected. These data agree with previous reports (33, 34), showing that the number of insulin receptors expressed per amount of protein in wheat-germ agglutinin eluates is similar in mammary gland tissue of untreated virgin and pregnant rats, without relevant differences in affinity values between the partially purified insulin receptors from both groups. Thus, similar to the situation found in liver (29), the moderate hyperinsulinemia characteristic of pregnancy apparently does not result in a down-regulation of insulin binding in mammary gland.

In various physiological and pathological situations, insulin resistance has been associated with changes in insulin receptor function (for review, see Ref. 35) and, more specifically, to an impaired tyrosine kinase activity of the receptor (for review, see Refs. 36, 28, and 29) or with the existence of postreceptor defects (12). Supporting the hypothesis that changes in the insulin receptor kinase activity also may account for the increased insulin sensitivity of mammary gland during late pregnancy, it was found here that insulin-induced autophosphorylation of the receptor β-subunit of pregnant rats was approximately 6-fold higher than in virgin animals. In addition, basal autophosphorylation of the insulin receptor, determined in the absence of insulin, was also markedly higher in mammary glands from pregnant rats than in those from virgin animals. These differences in basal autophosphorylation may be caused by changes in the receptor structure or in phosphatase levels. However, this increased basal autophosphorylation does not modify the glucose uptake in the pregnant mammary gland, as compared with the nonpregnant tissue. Thus, it seems that the differences in the basal phosphorylation are physiologically irrelevant. Dose-response curves confirmed that the concentration of insulin giving the half-maximal stimulation of autophosphorylation (ED_{50} ~15 nM) of the insulin receptor in mammary gland was similar in the virgin and pregnant groups. These results indicate that the increased kinase activity in pregnant rats is mainly caused by an enhanced responsiveness to insulin and not to an altered sensitivity to the hormone.

The hyperinsulinemic condition caused by the prolonged glucose infusion further impairs insulin stimulation of receptor β-subunit phosphorylation in virgin rats. Incubation of adipocytes with high concentrations of glucose has been reported to inhibit insulin receptor kinase activity (37). Because adipocytes are the predominant cells in the mammary gland of virgin rats (14, 15), it may be proposed that, although glucose homeostasis is maintained during the glucose infusion, the increased availability of glucose might induce an inhibitory mechanism, impairing insulin receptor autophosphorylation in the mammary gland of virgin rats.

It could be argued that the observed differences in insulin sensitivity of mammary gland between pregnant and virgin rats are mainly caused by the insulin-sensitive epithelial cells, which are the major cellular type in the mature gland of the pregnant rat. However, it is known that lipoprotein lipase activity is associated with adipose cells in the mature mammary gland (15, 18) and becomes extremely sensitive to hyperinsulinemia in both pregnant (17) and lactating rats (10, 38). This, therefore, suggests that the enhanced insulin sensitivity of mammary gland tissue seen in late pregnancy results from the increased activity of insulin receptors from both adipose and epithelial cells. Furthermore, because adipose tissue is one of the most insulin-sensitive tissues, the absence of insulin response in virgin rat mammary gland (which is formed mainly by adipose cells) can be explained only by postulating the existence of mammary gland-specific mechanisms that inhibit the kinase activity of insulin receptors in virgin, but not in pregnant or lactating, animals.

In conclusion, present findings indicate that, opposite to the insulin resistance present in most tissues of late-pregnant rats, mammary glands exhibit increased insulin sensitivity. Furthermore, we provide evidence that the increased response to insulin of the mature mammary gland in late pregnancy is not accounted for by changes at the level of the hormone-receptor interaction but by up-regulation of insulin receptor kinase activity.

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