Tyrosine kinase activity of liver insulin receptor is inhibited in rats at term gestation

Carmen MARTÍNEZ, Pilar RUIZ, Antonio ANDRÉS, Jorgina SATRÚSTEGUI and José M. CARRASCOSA*

Departamento Biología Molecular, Centro Biología Molecular, C.S.I.C., Universidad Autónoma de Madrid, 28049-Madrid, Spain

Late gestation is associated with insulin resistance in rats and humans. It has been reported that rats at term gestation show active hepatic gluconeogenesis and glycogenolysis, and diminished lipogenesis, despite normal or mildly elevated plasma insulin concentrations, indicating a state of resistance to the hormone action. Since autophosphorylation of the insulin receptor has been reported to play a key role in the hormone signal transduction, we have partially purified plasma-membrane liver insulin receptors from virgin and 22-day-pregnant rats and studied their binding and kinase activities. (1) Insulin binding to partially purified receptors does not appear to be influenced by gestation, as indicated by the observed K_D and B_{max} values. (2) The rate of autophosphorylation and the maximal ^{32}P incorporation into the receptor β -subunit from pregnant rats at saturating concentrations of insulin are markedly decreased with respect to the corresponding values for virgin rats. (3) The diminished autophosphorylation rate was due to a decreased responsiveness of the kinase activity to the action of insulin. (4) Phosphorylation of the exogenous substrates casein and poly(Glu⁸⁰Tyr²⁰) by insulin-receptor kinase was also less when receptors from pregnant rats were used. These results show the existence of an impairment at the receptor kinase level of the insulin signalling mechanism that might be related to the insulin-resistant state characteristic of term gestation in rats.

INTRODUCTION

Studies using the glucose-tolerance test, the insulin-tolerance test, and the euglycaemic/hyperinsulinaemic clamp technique have shown that late pregnancy is associated with a state of insulin resistance in rats and humans [1–4]. However, the tissues involved in the overall resistance have been in the past a matter of controversy, since decreased sensitivity to insulin of isolated target tissues was not always observed [5–7]. Studies *in vivo* have demonstrated that rats become resistant to insulin after 16 days of gestation, and that this resistance is due to a decreased sensitivity to the hormone of tissues producing and utilizing glucose [1]. Moreover, measurement of the glucose utilization *in vivo* by different maternal tissues has allowed the identification of some of those involved in this overall resistance [8].

At present, the mechanisms underlying this phenomenon are largely unknown. Although insulin-binding studies have occasionally reported a diminished binding capacity of some maternal tissues during late pregnancy, the main body of evidence suggests that decreased insulin sensitivity in humans and rats is primarily due to the impairment of hormone signalling at a post-binding level [1,3,4]. After insulin binds to its receptor in the plasma membrane, the β -subunit of the receptor molecule undergoes phosphorylation on tyrosine residues, catalysed by its own tyrosine-specific kinase activity (see [9] for a review). This autophosphorylation process has been demonstrated to be essential for the hormone action [10–12]. Furthermore, insulin resistance occurring in several physiological and pathological situations [13–16]

seems to be associated with a diminished tyrosine kinase activity of the insulin receptor, and the same was observed in adipose cells rendered resistant artificially by treatment with isoprenaline or tumour-promoting phorbol esters [17,18].

The aim of the present work was to compare the tyrosine kinase activity of liver insulin receptors from virgin and 22-day-pregnant rats, in order to explore at the molecular level the mechanisms responsible of this insulin resistance. Previous reports demonstrated that rats at term gestation show a marked hepatic insulin resistance, manifested by diminished lipogenesis [19] and increased gluconeogenesis, as well as a lesser glycogen content [20,21], despite moderately higher plasma insulin concentrations than in virgin rats [19].

MATERIALS AND METHODS

Materials

Pig [¹²⁵I-Tyr^{A14}]insulin and [γ -³²P]ATP were from Amersham. WGA–Sepharose 6MB and Percoll were purchased from Pharmacia. PMSF, trypsin inhibitor, bacitracin, benzamidine and Protein A were from Sigma. Pig insulin (Velosulin) was purchased from Nordisk (Denmark). Triton X-100 and casein (Hammarsten type) were from Merck. Reagents for electrophoresis were from Bio-Rad and Serva. All other reagents were of the best grade commercially available.

Animals

Albino Wistar rats fed on standard laboratory chow and water *ad libitum* were used for the experiments. The

Abbreviations used: WGA, wheat-germ agglutinin; PMSF, phenylmethanesulphonyl fluoride.

* To whom correspondence should be addressed.

presence of spermatozoa in the vagina was assumed to indicate conception, and gestational age was confirmed by the fetal weight. Age-matched virgin rats were used as controls [20].

Insulin-receptor preparation

Isolation of plasma membranes was performed as described in ref. [22], by using the protease inhibitors PMSF (1 mM), bacitracin (0.1 mg/ml), trypsin inhibitor (0.1 mg/ml) and benzamidine (0.2 mg/ml) throughout all centrifugations. Membranes from two livers were finally suspended in 0.6 ml of 50 mM-Tris (pH 7.4)/2 mM-EDTA/1 mM-PMSF.

Solubilization of membranes was carried out in 1% Triton X-100 in the presence of 1 mM-PMSF, bacitracin (0.1 mg/ml), trypsin inhibitor (0.1 mg/ml), 10 mM-sodium pyrophosphate and 100 mM-NaF, for 1 h at 4 °C. The solubilized membranes were adjusted to a volume of 1 ml with 50 mM-Tris (pH 7.4)/2 mM-EDTA/1 mM-PMSF, and insoluble material was removed by centrifugation at 100 000 *g* for 1 h. The clear supernatant was diluted 1:5 with a solution containing 50 mM-Tris (pH 7.4)/0.05% Triton X-100/100 mM-NaCl/2.5 mM-KCl/1 mM-CaCl₂/1 mM-PMSF/100 mM-NaF/10 mM-sodium pyrophosphate, mixed with 2.5 ml of WGA-Sepharose (Pharmacia) and rotated end-over-end for 1 h at 4 °C. The slurry was then poured into a column, and eluent was recycled four times through the WGA-Sepharose, and, after extensive washing with 100 ml of the former solution, the insulin receptor was eluted with 0.3 M-acetylglucosamine/10% (v/v) glycerol in the above buffer. Insulin receptor was usually collected in the first 1.5 ml fraction. The protein concentration of this fraction was generally 0.25 mg/ml. Recovery of the applied receptors was approx. 90%, as measured by insulin binding.

Binding to solubilized insulin receptor

Portions (30 μ l) of WGA-purified receptor were incubated overnight with [¹²⁵I-Tyr^{A14}]insulin (33 pM) and various concentrations of unlabelled insulin, at 4 °C, in 500 μ l of a medium containing 25 mM-Tris (pH 7.4) and 0.2% bovine serum albumin. The amount of receptor-bound hormone was determined by previously described methods [16].

Autophosphorylation of solubilized receptors

Portions (60 μ l) of WGA-purified insulin receptor were incubated overnight at 4 °C in the absence or presence of increasing insulin concentrations. Phosphorylation assays were carried out with 50 μ M-[γ -³²P]-ATP (sp. radioactivity 3300 c.p.m./pmol) in a medium containing 6 mM-MnCl₂, 1 mM-sodium orthovanadate and 12 mM-MgCl₂, in a total volume of 80 μ l. After incubation at 0 °C, for various periods of time the reaction was stopped as described in ref. [23]. Insulin receptor was immunoprecipitated with anti-phosphotyrosine antibodies prepared as previously reported [24]. Antibodies were immobilized on Protein A, the precipitates washed as in [23], and proteins eluted with 2% SDS/1% glycerol/1% β -mercaptoethanol, boiled for 15 min, and separated by SDS/polyacrylamide-gel electrophoresis (7% acrylamide). Radioactive proteins were identified by autoradiography of the stained and dried gel, and ³²P incorporation was quantified by scintillation counting or densitometry.

Phosphorylation of exogenous substrates

Preincubation of WGA-purified receptor was carried out overnight as described above. Phosphorylation of the insulin receptor was conducted with 50 μ M unlabelled ATP for 15 min at room temperature, and subsequently samples of casein or poly(Glu⁸⁰Tyr²⁰) were added together with 4 μ Ci of [γ -³²P]ATP, and phosphorylation was continued for 10 min more. Final concentrations of substrates were 0.3 mg/ml for casein and 0.1–5 mg/ml for the synthetic peptide. Phosphorylation of casein was analysed by SDS/polyacrylamide-gel electrophoresis (10% acrylamide) and autoradiography. Phosphorylation of synthetic peptide was quantified as in ref. [24]. Treatment of gels with 1 M-KOH was used to remove serine- and threonine-bound radioactive phosphate as previously reported [25].

RESULTS

Insulin binding to partially purified insulin receptor

Scatchard analysis was used to determine the kinetic parameters of the hormone-receptor interaction. Curves in Fig. 1 were obtained by fitting the binding data by the program described in [26], and they show no significant differences in insulin binding to receptors from virgin and term-pregnant rats. Computer analysis gave *K_D* values of 0.55 ± 0.23 and 0.5 ± 0.17 nM (mean of seven experiments) for the high-affinity insulin receptors from virgin and term-pregnant rats respectively. The numbers of high-affinity binding sites in virgin (8.8 ± 1.6 fmol of insulin/ μ g of protein) and pregnant animals (6.3 ± 1.3 fmol of insulin/ μ g of protein) were also very similar.

The low-affinity component of the binding showed *K_D* values of 10.3 ± 3.2 and 7.5 ± 0.9 nM and *B_{max}* of 15.4 ± 3.8 and 18.7 ± 6.8 fmol of insulin/ μ g of protein for virgin and pregnant rats respectively.

Fig. 1. Scatchard analysis of insulin-binding data

WGA-purified insulin receptors from virgin (●) and 22-day-pregnant (○) rats (approx. 7.5 μ g of protein) were incubated with ¹²⁵I-insulin (33 pM; 25000 c.p.m.) together with increasing amounts of unlabelled insulin in concentrations between 0.05 and 50 nM. Curves are the result of fitting the data as described in the Materials and methods section, and show a representative experiment.

Fig. 2. Insulin-receptor autophosphorylation

Samples (10–15 μ g of protein) of WGA-purified insulin receptor were incubated in the presence of different insulin concentrations as described in the Materials and methods section. Phosphorylation was started by addition of 50 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and, after 15 min, reaction was stopped and receptor immunoprecipitated with anti-phosphotyrosine antibodies as indicated in the Materials and methods section. Lanes A–F of the autoradiogram correspond to receptor from virgin rats, and lanes G–L show the autophosphorylation of insulin receptors from pregnant rats. Insulin concentrations used were: A, G, 0; B, H, 0.16 nM; C, I, 1.6 nM; D, J, 16 nM; E, K, 0.16 μ M; F, L, 1.6 μ M.

Receptor autophosphorylation

Autoradiography of immunoprecipitated phosphotyrosine-containing proteins obtained after preincubation of equal amounts of partially purified liver insulin receptors from virgin and term-pregnant rats with different insulin concentrations is shown in Fig. 2. A 95 kDa protein, corresponding to the receptor β -subunit, was phosphorylated in an insulin-dependent manner in

both cases. Receptor phosphorylation in samples from pregnant rats (Fig. 2, lanes G–L) appears to be clearly diminished as compared with that observed in virgin rats (Fig. 2, lanes A–F). Incorporation of ^{32}P into the 95 kDa band was quantified by densitometric scanning of the resulting autoradiographs. As shown in Fig. 3, the maximal initial rate of autophosphorylation for receptors from pregnant rats was only 28 % of that calculated for the receptor from virgin animals. However, dose-response curves for both receptors appear to be superimposable, showing only a minor difference in insulin concentrations giving half-maximal stimulation (5.1 nM for virgin rats, 6.6 nM for pregnant rats). Time courses of

Fig. 3. Dose-response curves of insulin action on receptor autophosphorylation in virgin (●) and pregnant (○) rats

Phosphorylation assays were conducted as indicated in Fig. 2, and the ^{32}P incorporated into the 95 kDa band was quantified by densitometry. Curves ● (virgins) and △ (pregnant rats) show the ^{32}P incorporation expressed as percentage of the maximal phosphorylation observed in receptors from virgin rats (100 % = 100 fmol of P_i /15 min per 10 μ g of protein). Basal values of phosphorylation were considered as 0 %. Curve ○ represents the phosphorylation data for the pregnant-rat receptor expressed considering its maximal phosphorylation as 100 %.

Fig. 4. Time course of autophosphorylation of liver insulin receptor from virgin (●) and pregnant (○) rats

Insulin receptors were preincubated with maximal stimulating insulin concentrations and phosphorylated for various periods of time under the conditions described in the Materials and methods section. Values are expressed as percentages of the maximal ^{32}P incorporation and are means of two separate experiments.

the autophosphorylation reaction with liver insulin receptors from virgin and term-pregnant rats, preincubated overnight with $0.16 \mu\text{M}$ -insulin, are presented in Fig. 4. Autophosphorylation was linear for approx. 20 min, reaching a maximum by 40–60 min in both cases. The maximal ^{32}P incorporation into the β -subunit of the insulin receptor was 3-fold higher in virgin than in term-pregnant animals.

Phosphorylation of exogenous substrates

To illustrate further the differences reported above in tyrosine kinase activity of the insulin receptors from virgin and term-pregnant rats, phosphorylation of casein and the synthetic polypeptide poly(Glu⁸⁰Tyr²⁰) was studied. Phosphorylation of casein by insulin receptors from virgin (Fig. 5, lanes A, B, E and F) and 22-day-pregnant rats (Fig. 5, lanes C, D, G and H) showed an insulin-dependence. Casein phosphorylation was also higher with insulin receptors from virgin rats (Fig. 5, lanes A and B) than if equal amounts of receptors from term-pregnant rats were used (Fig. 5, lanes C and D). Autophosphorylation of the 95 kDa band corresponding to the β -subunit of the insulin receptor could also be observed in Fig. 5 to be higher in virgin than in pregnant animals. Specific tyrosine phosphorylation of casein was assessed after gel treatment with 1 M-KOH [25]. Again, ^{32}P incorporation into casein tyrosine residues was higher with receptors from virgin than with those from term-pregnant rats. In fact, densitometric scanning of lanes F and H showed that casein tyrosine phosphorylation was approx. 7-fold higher with insulin receptors from virgin rats (results not shown).

Phosphorylation of the synthetic peptide is shown in Table 1. Basal phosphorylation was similar for both receptor preparations. Insulin stimulated the activity of receptor from virgin rats 14.3-fold, but only a 4.4-fold stimulation was observed if receptor from pregnant animals was used.

Fig. 5. Phosphorylation of casein by rat liver insulin receptor

Phosphorylation assays were performed as indicated in the Materials and methods section, in the presence of $0.16 \mu\text{M}$ -insulin (lanes B, D, F, H) or in its absence (lanes A, C, E, G). Equal amounts of insulin-binding capacity from virgin (A, B, E, F) and pregnant rats (C, D, G, H) were used to phosphorylate $30 \mu\text{g}$ of casein in a volume of $100 \mu\text{l}$. Lanes A–D show the total ^{32}P incorporation into proteins, whereas lanes E–H show the remaining ^{32}P after KOH treatment of the gels, assumed to be bound to tyrosine residues [24]. KOH-treated gels were over-exposed with respect to the non-treated gels.

Table 1. Phosphorylation of poly(Glu⁸⁰Tyr²⁰) by WGA-purified liver insulin receptor

Phosphorylation assays were carried out as described in the Materials and methods section, at a peptide concentration of 1 mg/ml . After stopping the reaction, samples were applied on filter papers and protein material was precipitated with 20% (w/v) trichloroacetic acid. Control incubations were performed in the absence of peptide. Incorporation of ^{32}P into synthetic polypeptide is expressed in c.p.m. Specific radioactivity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was 1650 c.p.m./pmol . Insulin was either absent (–) or present (+) at maximal stimulating concentrations ($0.16 \mu\text{M}$) during preincubation of samples.

Animals	Insulin		Insulin effect (fold)
	–	+	
Virgin	229	3280	14.3
Pregnant	286	1264	4.4

To pursue the mechanisms responsible for the decreased kinase activity of insulin receptors from pregnant rats, we examined the kinetic properties of the peptide phosphorylation reaction. To measure the K_m of the insulin receptor for poly(Glu⁸⁰Tyr²⁰), different concentrations of peptide ($0.1\text{--}5 \text{ mg/ml}$) were phosphorylated by using insulin-stimulated and phosphorylated receptors as indicated in the Materials and methods section, and initial rates of ^{32}P incorporation were determined. The results presented in Fig. 6 indicate that the affinity for the synthetic peptide of insulin receptors from virgin and pregnant rats is very similar ($K_m \approx 0.74 \text{ mg/ml}$).

The differences in kinase activity among the two groups of rats seem to be accounted for by differences in the

Fig. 6. Lineweaver-Burk plot of poly(Glu⁸⁰Tyr²⁰) phosphorylation by insulin-receptor kinase from virgin (●) and pregnant (○) rats

The values are the means of two experiments conducted in duplicate for each peptide concentration.

$V_{\max}$ of the reaction. $V_{\max}$ values of approx. 606 fmol of phosphate/min and 125 fmol of phosphate/min were measured for the phosphorylation reaction carried out with receptors from virgin and pregnant rats respectively.

DISCUSSION

According to our present knowledge of the insulin signalling mechanism, an impairment of hormone action could be located at the levels of receptor binding, receptor phosphorylation, or at a post-receptor site. The findings reported here indicate that the binding characteristics (K_D and maximal binding capacity per μg of membrane protein) are similar for liver insulin receptors from virgin and term-pregnant rats. On the contrary, insulin receptors from 22-day-pregnant rats showed a lower rate of autophosphorylation at maximal stimulating insulin concentrations, suggesting the existence of an inhibitory regulation mechanism of this activity. Analysis of the insulin dose-response curves and of the half-maximal stimulation values of insulin concentration suggest that the diminished kinase activity in receptor preparations from term-pregnant rats is not due to its decreased insulin sensitivity, but rather to a lesser responsiveness to the hormone action. Since this decreased responsiveness is also observed with exogenous and synthetic substrates for receptor kinase, it is reasonable to suggest that a minor autophosphorylation of the insulin receptor could result in an impaired phosphorylation of unknown physiological substrates in late pregnancy.

Concerning the mechanism of receptor kinase inhibition, several possibilities should be considered. The 95 kDa band was occasionally resolved in our autoradiograms as a doublet. It has been suggested that this finding, also reported by others [27], might reflect a partial degradation of the β -subunit, leading to the formation of a low-molecular-mass fragment with lower kinase activity. Since both receptor preparations were obtained under the same conditions, a different degradation might reflect the existence of a higher endogenous proteolytic activity in liver from term-pregnant rats. This activity, if it exists, however, does not appear to fulfil the characteristics of that described in [27], since inclusion of 5 mM-iodoacetamide in homogenization buffers did not block the generation of the low-molecular-mass fragment (results not shown).

Kinetic studies provided some insight into the mechanism responsible for the inhibition of kinase activity in receptors from pregnant rats. As shown in Fig. 4, the maximal autophosphorylation of the receptor β -subunit in pregnant rats, at maximal stimulating insulin concentrations and sub-saturating ATP levels, amounts to only 35% of that found in virgins. If, as discussed in [28], insulin binding to its receptor results in phosphate-acceptor sites becoming accessible to the catalytic domain, it could be speculated that in receptors from pregnant animals (a) phosphorylation site(s) is/are somehow modified and impeded from incorporating phosphate. The study of the phosphorylated tyrosines in receptors from pregnant rats will allow us to determine whether its decreased phosphorylation is due to specific inhibition of one autophosphorylation site, or whether it reflects a proportionate inhibition of all phosphorylated tyrosine residues.

Further kinetic studies on peptide phosphorylation presented in Fig. 6 demonstrated that the affinity of the

receptor for poly(Glu⁸⁰Tyr²⁰) is not altered in pregnancy, as indicated by the identical K_m values. The $V_{\max}$ of phosphorylation, however, is markedly decreased if receptors from pregnant rats are used. Since the magnitude of the inhibition ($\approx 79\%$) is similar to that found for the autophosphorylation reaction, and the peptide phosphorylation was carried out with insulin-stimulated and phosphorylated receptors, it seems likely that the minor ^{32}P incorporation into poly(Glu⁸⁰Tyr²⁰) is a consequence of the decreased phosphorylation of the receptor molecule and not due to a lesser accessibility of the peptide-recognition site to the catalytic site, as proposed for other insulin-receptor modulation mechanisms [28].

In conclusion, our results show that in term-pregnant rats an impairment of the insulin signal-transduction mechanisms exists at the level of receptor autophosphorylation, whereas the binding capacity remains unaffected. The kinase activity shows a decreased responsiveness to insulin, and this could be responsible for the observed hepatic insulin resistance of late pregnancy.

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