Impairment of the liver insulin receptor autoactivation cascade at full-term pregnancy in the rat

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Partially purified liver insulin receptors from full-term pregnant rats show decreased autophosphorylation rates if compared with receptors from virgins. We studied the molecular mechanism of this phenomenon, looking at possible structural and functional changes of several domains. The ATP-binding domain seems to be unaltered in receptors from pregnant rats since K_m for ATP was similar to that observed in virgins. In contrast, the V_{max} is decreased some 45 %, suggesting changes in the kinase domain. Truncation of a fragment of 10 kDa from the C-terminal tail does not normalize the kinase activity in receptors from pregnant rats, suggesting that this domain is not involved in the inhibitory regulation. Treatment with alkaline phosphatase increases the [32 P]P_i incorporation into receptors from pregnant rats; however, the autophosphorylation remains lower than that observed in virgin rats. Tryptic phosphopeptide maps of phosphorylated receptors show that the same phosphopeptides are present in

receptors from virgin and pregnant rats. However, the progression through the autoactivation cascade in the kinase domain is impaired in receptors from pregnant rats. Differences in the cleavage by trypsin at the two alternative sites in the kinase domain were observed, indicating possible structural changes in receptors from pregnant rats that could be related to the impairment of the autoactivation cascade. Integrity of the α - and β -subunits, as well as differential expression of the two receptor isoforms, were shown to be unaltered. We conclude that (1) the decreased autophosphorylation rate of the liver insulin receptor from pregnant rats is associated with the impairment of its autoactivation cascade, probably as a consequence of the basal Ser/Thr phosphorylation; and (2) the inhibition of the autoactivation cascade does not account for the overall inhibition of autophosphorylation observed in receptors from pregnant rats.

INTRODUCTION

The first event in the mechanism of action of insulin involves its binding to the extracellular domain of the insulin receptor, a transmembrane heterotetrameric glycoprotein that links the outside and the inside of the cell. The binding of the hormone stimulates the autophosphorylation of specific tyrosine residues within the cytoplasmic domain of the receptor β -subunit which leads to the activation of the intrinsic tyrosine kinase activity toward endogenous substrates, and thereby triggers a cascade of cellular effects [1,2]. Three of these tyrosine residues, Tyr-1146, Tyr-1150 and Tyr-1151 according to the numbering reported in [3], are included in the so-called tyrosine kinase domain that has been shown to undergo phosphorylation in response to insulin *in vitro* and *in vivo*. Although triphosphorylation of this domain seems to correlate with the receptor kinase activity [4], other reports indicate that biphenosphorylation might be enough for the insulin signalling [5,6]. Additionally, the ATP-binding domain that includes Lys-1018 (whose mutation to alanine impairs insulin action on five different biological effects [7]), the C-terminal tail that contains several serine residues, Thr-1336, Tyr-1316 and -1322, and some other putative serine and tyrosine phosphorylation sites, seem to play an important role in regulating the kinase activity of the insulin receptor [8–13]. Inhibitory regulation of the kinase activity has been proposed as a major mechanism responsible for the decreased receptor

autophosphorylation observed in several cases of insulin resistance [4,14,15]. A second mechanism that could be involved in eliciting a decreased insulin response has been suggested to be the existence of two receptor populations that differ in their abilities to undergo phosphorylation, but not in their insulin-binding activities [16–18]. Moreover, several investigators have also demonstrated the existence of multiple insulin-receptor species differing in the size of the β -subunit [19,20] and O'Hare and Pilch [21] reported the separation of three species with identical binding ability, and showed that only the intact ($\alpha\beta$)₂ form is active as a kinase. Finally, cloning of the insulin-receptor DNA revealed the existence of two receptor isoforms differing in 12 amino acids of the α -subunit encoded by a discrete exon [3,22,23], and it has been shown that the different α -subunit structure of both isoforms determines differences in their respective tyrosine kinase activities, suggesting that altered regulation of alternative splicing could be responsible for the altered kinase activity in situations of insulin resistance [24].

We have previously reported that the initial rate of autophosphorylation, the maximal [32 P]P_i incorporation into the β -subunit, and the tyrosine kinase activity towards exogenous substrates are decreased in rat liver receptors from full-term pregnant rats if compared with those from virgins [25]. In the present paper, we further extend these studies, looking for definite changes in both the α - and the β -subunits of the receptor molecule.

Abbreviations used: WGA, wheat germ agglutinin; DSS, disuccinimidyl suberate; AMV, avian myeloblastosis virus.

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EXPERIMENTAL

Materials

Porcine insulin and Protein A were purchased from Sigma. [γ - 32 P]ATP and A14- 125 I-monoiodoinsulin were from Amersham. L-1-Tosylamido-2-phenylethylchloromethane ketone (TPCK)-treated trypsin was from Worthington. Wheat germ agglutinin (WGA) coupled to Sepharose was from Pharmacia. Immobilized alkaline phosphatase was prepared by reacting 2 ml of Affi-Gel 10 (Bio-Rad) with 5000 units of alkaline phosphatase (Sigma, P-2276) in 0.1 M Hepes, pH 7.4, as described in the manufacturer's instructions. Triton X-100 was purchased from Merck. Reverse transcriptase was from Seikagazu, *Taq* polymerase was provided by Perkin-Elmer, and synthetic oligonucleotide were purchased from Isogen. Reagents for electrophoresis were from Bio-Rad and Serva. Disuccinimidyl suberate (DSS) was from Pierce. 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. Control of gestational age was as previously reported [25].

Receptor preparation

Isolation of liver plasma membranes and solubilization with Triton X-100 were performed essentially as described previously [25]. Solubilized receptors were added to 1 ml of WGA-Sepharose and rotated end-over-end for 2 h at room temperature. After extensive washing with a buffer containing 50 mM Tris, pH 7.4, 0.05 % Triton X-100, 100 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 100 mM NaF and 10 mM sodium pyrophosphate, receptors were eluted with 300–500 μ l of the same buffer supplemented with 0.3 M *N*-acetylglucosamine.

Binding to WGA-purified receptor

Samples containing 5 μ g of partially purified receptor protein were incubated at 4 °C overnight with 33 pM A14- 125 I-insulin in a total volume of 250 μ l containing 0.004 % Triton X-100, as described [25]. Separation of the free and receptor-bound insulin was achieved by precipitation with 10 % polyethylene glycol/0.5 mg/ml γ -globulin. After 30 min in ice, samples were centrifuged and the pellets were washed twice with 10 % polyethylene glycol, dried, and the amount of receptor-bound insulin was quantified by scintillation counting. Non-specific binding was assessed in the presence of 0.5 μ M unlabelled human insulin (Lilly). Receptor affinity has been shown to be identical in virgin and pregnant animals [25], therefore the insulin-binding activity determined under these conditions was used to compare the relative amounts of receptor polypeptide in phosphorylation experiments.

Affinity cross-linking of 125 I-insulin to partially purified receptor

Insulin-receptor preparations (10 μ g of protein) were incubated overnight at 4 °C in the presence of 0.8 nM 125 I-insulin. Chemical cross-linking was started by addition of 0.1 mM DSS (dissolved in DMSO). After 20 min on ice samples were divided into two aliquots and the reaction was terminated by addition of Laemmli buffer with or without 5 % (v/v) 2-mercaptoethanol. In order to separate the high-molecular-mass insulin-receptor complexes, samples under non-reducing conditions were treated with 20 mM *N*-ethylmaleimide and proteins were resolved in 4 % polyacrylamide gels (acrylamide:bisacrylamide ratio of 100:1) as described by O'Hare and Pilch [21]. Integrity of the α -subunit

was analysed by electrophoresis of the cross-linked samples in SDS/7.5 %-polyacrylamide gels, under reducing conditions.

Autophosphorylation of WGA-purified receptor

Portions containing 10–15 μ g of WGA-purified protein ($\approx$ 250 fmol of insulin-binding capacity) were preincubated overnight at 4 °C in the presence of 0.16 μ M insulin. To assess the K_m for ATP, initial rates of autophosphorylation were determined with different ATP concentrations at constant specific radioactivity (2 μ Ci/nmol) in medium containing 12 mM MgCl₂, 6 mM MnCl₂ and 1 mM sodium orthovanadate. After 15 min on ice, phosphorylation was stopped, receptors were immunoprecipitated with anti-phosphotyrosine antibodies and separated by 7.5 % PAGE [25]. Dried gels were autoradiographed, and labelled bands were quantified by densitometry of autoradiographs whose exposure was in the linear range.

Mild tryptic digestion of purified receptor

WGA-purified receptors (10–15 μ g of protein) were digested with 10 μ g/ml of trypsin for 1 min at room temperature. Digestion was terminated by addition of aprotinin (100 μ g/ml final concentration), and receptors were incubated with or without 160 nM insulin for 1 h at room temperature and phosphorylated as indicated above.

Treatment of purified receptor with alkaline phosphatase

WGA-purified receptors (10–15 μ g of protein) were incubated for 1 h at 4 °C with 500 units of immobilized alkaline phosphatase to remove phosphate prior to performing phosphorylation reactions. The supernatant was separated from the phosphatase by centrifugation, and used in phosphorylation assays as indicated. Control samples were prepared by incubation with alkaline phosphatase previously inactivated by boiling for 30 min.

Tryptic digestion of phosphorylated β -subunit and two-dimensional phosphopeptide mapping

WGA-purified receptors (50–80 μ g of protein) were stimulated with 160 nM insulin and phosphorylated as indicated previously. Specific radioactivity of ATP was raised to 25 μ Ci/nmol in order to maximize [32 P] P_i incorporation. Phosphopeptide mapping was carried out essentially as described by Boyle et al. [26] and Tavaré and Denton [27]. The β -subunit was eluted overnight from gel slices in 50 mM NH₄HCO₃ buffer containing 0.1 % SDS and 0.5 % 2-mercaptoethanol. Eluted protein was precipitated with trichloroacetic acid, oxidized with cold performic acid, and lyophilized. Pellets were resuspended in 50 mM NH₄HCO₃, pH 8.0, proteolysed with trypsin (0.2 mg/ml) for 16 h at 37 °C and lyophilized again. Tryptic peptides were resolved by electrophoresis at 1000 V for 1.5 h on TLC-cellulose plates, in medium containing pyridine/acetic acid/water (1:10:185, by vol.) at pH 3.5, followed by chromatography in a solvent containing pyridine/acetic acid/butan-1-ol/water (10:3:15:12, by vol.). TLC plates were autoradiographed and [32 P] P_i incorporation into discrete peptides was quantified by scanning densitometry.

Analysis of insulin-receptor mRNA

Total liver RNA was prepared from virgin and pregnant rats by sequential extractions with guanidine hydrochloride/ethanol and phenol [28]. The first-strand cDNA was synthesized from 3 μ g of total mRNA using AMV (avian myeloblastosis virus) reverse transcriptase as described by Sambrook et al. [29]. The cDNA

was primed with 20 pmol of an antisense oligonucleotide, 5'-AGGCCAGAGATGACAAGTGAC-3', complementary to the rat insulin-receptor mRNA sequence starting at position 2438 [30]. PCR was performed in a total volume of 50 μ l using a dilution 1:20 of reverse transcriptase reaction as template, in medium containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 200 mM of each deoxynucleotide triphosphate, 2.5 units of *Taq* polymerase, 50 pmol of the above-mentioned oligonucleotide, and 50 pmol of a sense oligonucleotide 5'-TTCATTTCAGGAAGACCTTCGA-3' identical to the rat insulin-receptor cDNA sequence from position 2181 [30]. The template was denatured for 5 min at 94 °C followed by 20 cycles of PCR with incubations of 1 min at 55 °C, 2 min at 72 °C, 1 min at 94 °C and a final extension for 10 min at 72 °C. Reaction products were electrophoresed in non-denaturing 5% polyacrylamide gels in 1 $\times$ TBE buffer (90 mM Tris-Borate, pH 8, 2 mM EDTA). Size markers were fragments from phage λ DNA digested with *Bst*II. Gels were stained with ethidium bromide and the UV fluorescence was photographed. Kidney, muscle and fat RNA samples were processed in parallel to confirm that under the reaction conditions used both receptor isotypes could be detected.

RESULTS

Kinetic analysis of insulin-receptor autophosphorylation

Initial rates of insulin-receptor autophosphorylation were determined using different ATP concentrations as indicated in the Materials and methods section. Phosphorylation of receptors from virgin and pregnant rats was carried out simultaneously, and proteins were electrophoresed and autoradiographed in a single gel in order to make densitometric data comparable. Figure 1(a) shows an autoradiograph of the phosphorylated β -subunit from virgin and full-term pregnant rats. Data obtained after densitometric quantification of receptor phosphorylation, corrected for the insulin-binding activity of each preparation, and normalized as percentages of the phosphorylation observed at 60 μ M ATP, showed that [³²P]_i incorporation was higher in receptors from virgin rats. As previously reported by White et al.

Figure 1 ATP-dependent phosphorylation of insulin-receptor β -subunit

(a) Insulin-stimulated receptors from virgin and pregnant rats were incubated with increasing ATP concentrations at constant specific radioactivity, as described in the Materials and methods section. An autoradiograph of phosphorylated β -subunit after immunoprecipitation with anti-phosphotyrosine antibodies is shown. The insulin-binding activity in preparations from virgin rats was 1.4-fold higher than from pregnant rats in this experiment; (b) Densitometric values from autoradiographs were processed as indicated in the text, fitted to a sigmoidal function [31], and V_{max} and K_m values were obtained. Figure 1(b) shows the V_{max} observed in four independent experiments, and the corresponding mean values $\pm$ S.E.M. These were calculated to be significantly different using Student's *t* test ($P < 0.025$).

Figure 2 Insulin-dependent autophosphorylation of the insulin receptor purified from virgin and pregnant rats, before and after mild trypsinization

Autophosphorylation of WAG-purified receptors was performed before and after trypsinization as indicated in the Materials and methods section. Trypsinized and non-trypsinized receptors were preincubated with 160 nM insulin for 1 h as indicated. The Figure shows an autoradiograph of immunoprecipitated receptors. This experiment was repeated four times using receptors from four different pairs of animals.

[31], computer-assisted statistical analysis (Sigma Plot software, Jandel Corporation) showed a better fit of the data to a sigmoidal velocity curve than to the hyperbolic Michaelian equation. Consequently, densitometric data from each experiment were fitted to the sigmoidal equation indicated in [31] and V_{max} and K_m values were calculated. Figure 1(b) shows the V_{max} observed in four separate experiments. Despite some variability, in all four cases the V_{max} observed in receptors from pregnant rats was lower than in virgins, the mean V_{max} for pregnant rats being some 56% of that calculated for virgins. On the contrary, only minor differences were observed for the mean K_m^{ATP} values between both groups (59 ± 3.9 and 49 ± 11 μ M for the virgin and pregnant-rat receptors respectively). Thus, these results confirm the previously reported differences in autophosphorylation rate between both types of receptors and indicate that they are due to different intrinsic kinase activities rather than to alterations in the ATP-binding domain of the receptor molecule.

Autophosphorylation after mild trypsinization

Trypsinization of the insulin receptor under the conditions indicated in the Materials and methods section is known to remove a 10 kDa fragment from the C-terminal tail of the β -subunit. This fragment contains Thr-1336, a target site for protein kinase C [8,9], as well as other putative serine phosphorylation sites. Several authors have suggested that Ser/Thr phosphorylation of this domain is the cause of the diminished insulin-dependent receptor phosphorylation in insulin-resistant states [14,15], and in one case at least, i.e. the starved rat, removal of this fragment eliminates the differences in receptor kinase activity between fed and starved rats [32]. Figure 2 shows the autophosphorylation of equal amounts of WGA-purified insulin receptors from virgin and pregnant rats, treated or not with trypsin. Trypsin-treated β -subunits migrated as expected, as an 85 kDa band whose phosphorylation was insulin-dependent. Trypsin treatment caused a decrease in insulin-stimulated phosphorylation of 17% and 25% for receptors from virgin and pregnant rats respectively (means of four experiments), probably as a consequence of the loss of Tyr-1316 and Tyr-1322. As can be seen in Figure 2, trypsin treatment of the receptors does not eliminate the inhibition of autophosphorylation in pregnant-rat receptors (45% and 53% before and after trypsinization respectively; means of four experiments),

Figure 3 Effect of treatment with alkaline phosphatase on receptor autophosphorylation

Equal amounts of receptors from virgin and pregnant rats were incubated with alkaline phosphatase as indicated in the Materials and methods section. Control incubations (—) were performed with inactivated enzyme. After treatment, samples were stimulated with 160 nM insulin and phosphorylated as indicated previously. (a) This shows an autoradiograph of immunoprecipitated receptors. (b) This shows the densitometric quantification of the autoradiograph (hatched bars, receptors treated with inactivated alkaline phosphatase; open bars, receptors treated with the active enzyme).

suggesting that the difference in kinase activity is not due to the contribution of the C-terminal domain of the insulin receptor.

Effect of alkaline phosphatase treatment on receptor autophosphorylation

To determine if the decrease in receptor tyrosine autophosphorylation might be related to an increased basal level of Ser/Thr phosphorylation in other domains apart from the C-tail of the β -subunit, WGA-purified proteins were incubated with immobilized alkaline phosphatase before stimulation by insulin and phosphorylation. Control preparations were treated with inactivated alkaline phosphatase. As can be seen in Figure 3, alkaline phosphatase treatment induced a 140% increase in phosphorylation in receptors from pregnant rats, suggesting that removing Ser/Thr-bound phosphate promotes the incorporation of P_i into the β -subunit. However, phosphorylation of receptors from virgin rats treated with inactivated alkaline phosphatase remains higher, indicating that basal Ser/Thr phosphorylation is not the only cause of inhibition of the insulin-dependent autophosphorylation of pregnant-rat receptors. Surprisingly, treatment with alkaline phosphatase of receptors from virgin animals results in a slight decrease ($\approx 10\%$) in autophosphorylation, in contrast with observations elsewhere [32]. These results suggest that Ser/Thr phosphorylation in residues different from those in the 10 kDa C-terminal fragment could be involved in inhibiting the autophosphorylation of liver receptors from full-term pregnant rats.

Tryptic peptide maps of the phosphorylated β -subunits

In order to determine whether the different autophosphorylation rates could be explained by differences in the so-called autophosphorylation cascade [33] we performed tryptic digestion, and obtained tryptic phosphopeptide maps of the β -subunits. Figure 4 shows a key diagram of the main phosphopeptides observed, together with representative peptidic maps obtained from receptors of virgin and pregnant rats. The names of the peptides are the same as those originally proposed by Tavaré and

Figure 4 Two-dimensional TLC analysis of tryptic phosphopeptides

Insulin receptors were phosphorylated after preincubation with 160 nM insulin as indicated in the Materials and methods section. Following phosphorylation, receptors were immunoprecipitated with anti-phosphotyrosine antibodies, and β -subunits were separated by SDS/PAGE and identified by autoradiography. Elution of proteins, digestion with trypsin, and two-dimensional separation were carried out as indicated in the Materials and methods section. The figure shows autoradiographs of phosphopeptides corresponding to receptors from virgin and pregnant rats, and the upper part is a key to the identification of the ^{32}P -labelled peptides. Arrow heads indicate the origin of sample application. In each case ≈ 2000 c.p.m. of labelled peptides were loaded. These maps are representative of five experiments.

Denton [27], with the exception of B0 which was not described in that paper. Peptides A2, B3 and C1 were reported to be the tris-, bis-, and monophosphorylated forms of the tryptic product DIYETDYYRK resulting from cleavage at Arg-1143 and Lys-1153, whereas A1 and B2 are the tris- and bisphosphorylated forms of the same domain when trypsin cleaves at the next lysine residue, Lys-1156 (sequence DIYETDYYRKGGK). In more recent papers it has been proposed that the two alternative cleavage sites would be Arg-1152 and Lys-1153; however, the electrophoretic migration of the intact phosphopeptides, as well as that of their V8 digestion products (J. C. Molero and J. M. Carracosa, unpublished work), better fits with the originally proposed cleavage sites.

Table 1 Ratios of [32 P]P_i incorporation into different tryptic phosphopeptides from receptors of virgin and pregnant rats

Autoradiographs of Figure 3 were quantified by scanning densitometry, and densitometric data were used directly to calculate the A2/A1 and B3/B2 ratios. For the ratio kinase/C-terminal, the sum of densitometric values for A2, A1, B3, B2 and C1 peptides (kinase domain peptides) was divided by that of B1 + B0 (C-terminal peptides). The progression through the autoactivation cascade was determined considering that peptides A2 and A1 contain three phosphates, B3 and B2 contain two phosphates and C1 contains only one phosphate. These results are consequently expressed as percentages of the total phosphorylated kinase domains (Tris, Bis and Mono indicate the amounts of tris-, bis and monophosphorylated forms of the kinase domain). Results are means $\pm$ S.E.M. of five different experiments. Statistical significance: ** $P < 0.025$; * $P < 0.05$

	P _i incorporation ratios			Degree of phosphorylation (% of total)		
	A2/A1	B3/B2	Kinase/C-terminal	Tris	Bis	Mono
Virgin rats	1.37 $\pm$ 0.21	2.47 $\pm$ 0.88	2.09 $\pm$ 0.44	67 $\pm$ 2	16 $\pm$ 2	17 $\pm$ 3
Pregnant rats	2.87 $\pm$ 0.62**	1.48 $\pm$ 0.29	2.11 $\pm$ 0.27	53 $\pm$ 6*	16 $\pm$ 1	31 $\pm$ 6*

Peptide B1 was previously shown to be part of the C-terminal domain, containing two phosphorylated tyrosines (1328 and 1334). Peptide B0 seems likely to derive from the same domain on the basis of the following findings: (a) phosphoamino acid analysis revealed that it contains phosphotyrosine exclusively (J. C. Molero and J. M. Carracosa, unpublished work); (b) this peptide is not observed in peptidic maps from receptors of isolated rat adipocytes truncated at the C-terminal domain by mild trypsinization (P. Ruiz and A. Andrés, unpublished work). The other peptides indicated in Figure 4, C3, C2' and C2, are not yet characterized but probably correspond with those reported in [27] as being derived from the juxtamembrane domain. These peptides were not consistently observed and were not studied further.

As can be seen in Figure 4 the same phosphopeptides are observed in both types of receptors, excluding the possibility that one or more phosphorylation site(s) was impeded during incorporation of phosphate in receptors from pregnant rats. Phosphate incorporation into different peptides was quantified by densitometric scanning and the data were used to study possible differences in the phosphorylation mechanism of both types of receptor. Table 1 shows that the ratio between the phosphate incorporation into the kinase (peptides A1, A2, B3, B2 and C1) and the C-terminal (peptides B1 and B0) domains is identical for receptors from virgin and pregnant rats. Possible structural differences were studied by analysis of the preferential tryptic cleavage site in the kinase domain. As indicated by the ratio B3/B2 the bisphosphorylated form of the kinase domain is cleaved by trypsin in both cases preferentially at the first lysine Lys-1153, a result which agrees with previous observations [27]. For the ratio A2/A1 a value of 1.37 was observed in virgin-rat receptors, indicating an approximately similar probability for trypsin to hydrolyse at both cleavage sites. On the contrary, when the ratio A2/A1 was calculated for receptors from pregnant rats a significant increase was observed, suggesting the existence of some structural changes in these receptors that favour Lys-1153 as a target site for trypsin.

The state of progression of the phosphorylation cascade was calculated from the densitometric data. When allowance is made for the number of tyrosines being phosphorylated, it can be observed that 67 % of the kinase domains are trisphosphorylated in receptors from virgin rats, whereas only 53 % reach the maximal phosphorylation in pregnant rats. These results indicate that the phosphorylation cascade in pregnant rats is impeded. The degree of trisphosphorylation observed in our receptor preparations is well over that previously reported by Issad et al. [10]. The reasons for this discrepancy are unknown, but could be

Figure 5 Cross-linking of insulin receptor to 125 I-insulin

Equal amounts of WGA-purified proteins from virgin and pregnant rats were cross-linked to 125 I-insulin with DSS in the absence or presence of 1 μ M unlabelled insulin. (a) Aliquots of cross-linked receptors were boiled in Laemmli buffer without 2-mercaptoethanol, treated with 20 mM *N*-ethylmaleimide, and proteins were resolved in 4 % polyacrylamide gels as indicated in the Materials and Methods section. Autoradiographs show three labelled bands in receptors from virgins (lane a) and pregnant rats (lane c), whose molecular masses correspond with the three forms previously described in [21]. In the presence of unlabelled insulin none of the three bands was observed (lanes b and d) indicating that cross-linking was specific. The different total radioactivity incorporated in lanes a and c correlates with the different insulin-receptor concentration in both preparations (2.5-fold higher in virgin extracts). (b) Aliquots of cross-linked receptors were boiled in Laemmli buffer containing 5 % 2-mercaptoethanol and proteins were separated in SDS/7.5 % PAGE. After autoradiography, receptor α -subunit appears as a single band of 135 kDa in virgin and pregnant rats (lanes a and c respectively). The presence of unlabelled insulin during the cross-linking reaction eliminates the labelling of the α -subunit in both cases (lanes b and d for virgin and pregnant rats respectively).

due to the different experimental protocols used for the autophosphorylation reaction.

Integrity of the receptor β -subunit

As demonstrated by O'Hare and Pilch [21], WGA-purified insulin receptors are a mixture of intact $\alpha_2\beta_2$, hybrid $\alpha\beta\alpha\beta'$, and degraded $\alpha_2\beta_2'$ receptors, where β' represents a C-terminal-degraded β -subunit. Since only the intact $\alpha_2\beta_2$ receptors have kinase activity,

Figure 6 PCR analysis of insulin-receptor mRNA transcripts

Primers flanking the rat insulin-receptor cDNA sequence positions 2181 to 2438 were used for PCR amplification with *Taq* polymerase (see the Materials and methods section). Reaction products were separated by electrophoresis in non-denaturing 5% polyacrylamide gels, stained with ethidium bromide and photographed using UV shadowing. The size of both PCR products (258 and 222) and of DNA marker (224) is indicated in base pairs in the middle and at the right margin respectively. The figure shows the products obtained from livers of two virgin and three full-term pregnant rats. RNA samples from kidney (K), muscle (M) and fat (F) were used as controls.

despite the fact that all three forms show normal insulin binding, it has been proposed that an increased amount of kinase-deficient receptors with normal insulin-binding capacity might explain the diminished kinase activity observed in insulin-resistant states. Figure 5(a) shows that after ^{125}I -insulin cross-linking all three receptor forms are detectable in WGA-purified proteins from virgin and pregnant rats, under non-reducing conditions. Densitometric quantification does not reveal significant differences between both types of receptors, the proportion of intact receptors being 35 and 37% for virgin and pregnant rats respectively. Figure 5(b) demonstrates that the different forms of receptors are not a consequence of the presence of a degraded α -subunit, since under reducing conditions only one labelled band is observed, whose molecular mass corresponds to that of the intact receptor α -subunit.

Expression of receptor isoforms

Two insulin-receptor isoforms differing in the presence (IR-B isotype) or absence (IR-A isotype) of exon 11 have been reported to be expressed in humans and rats in a tissue-specific manner [30,34]. *In vitro* autophosphorylation of both receptors has been shown to be markedly different [24], and alterations in the expression of both isoforms have been associated with the pathogenesis of type-II diabetes [35,36]. In order to investigate whether this mechanism could play a role in the different autophosphorylation rates of insulin receptor from virgin and pregnant rats, we determined the expression of both isotypes using total liver RNA. As shown in Figure 6, PCR products obtained indicate that the IR-B isoform is predominant in rat liver in both virgin and pregnant rats. These results are in agreement with previously published data for rat liver [30] and suggest that no difference in the relative abundance of the two mRNA transcripts occurs during pregnancy.

DISCUSSION

Impaired insulin-receptor phosphorylation has been observed in several cases of insulin resistance, but there are few reports concerning the molecular mechanisms responsible for the abnormal receptor function. In this paper we have confirmed previous data on the inhibition of liver insulin-receptor kinase during rat pregnancy [25] and further investigated possible structural and functional changes in the receptor molecule that could lead to the inhibition of its kinase activity.

Kinetic studies provided evidence of the slightly sigmoidal character of the ATP-dependent autophosphorylation curves in

both types of receptors, in good agreement with previous observations in rat hepatoma cell insulin receptors [31]. Changes in the ATP-binding domain that could result in decreased affinity for the substrate were not observed, as indicated by the similar K_m^{ATP} values. On the contrary, significant differences were found in the V_{max} of autophosphorylation between both groups (Figure 1), suggesting that the decrease in the kinase activity of receptors from pregnant rats is more likely to be due to changes in the structure, function and/or regulation of the kinase domain.

One of the mechanisms proposed to cause the inhibition of the kinase domain is the Ser/Thr phosphorylation of the receptor β -subunit. Thus, uncharacterized kinases [37], protein kinase C [8,9,13–15], as well as cyclic AMP-mediated processes [38,39] have been shown to be involved in the impairment of insulin-receptor kinase and other insulin-stimulatable enzymes. For the full-term pregnant rat we have reported [40] that the liver concentration of cyclic AMP is significantly increased, and we speculate that this fact could result in the Ser/Thr phosphorylation of the insulin receptor in pregnant rats.

The results presented in Figure 2 indicate that the mechanism of regulation of receptor function in pregnancy does not involve the Ser/Thr phosphorylation of the C-terminal domain. In at least one case with insulin-receptor-diminished kinase activity, i.e. the starved rat, the elimination of the C-terminal domain by mild trypsinization results in the normalization of the kinase activity [32]. In the starved rat, however, an increased protein kinase C activity was detected in membrane and cytosol, whereas the protein kinase C activity in pregnant and virgin rats is identical (C. Martinez, unpublished work) which could explain the different regulatory mechanisms in both cases. Our results agree with those previously observed in adipocytes of aged rats [41] where the truncation of the receptor C-terminal domain does not normalize its kinase activity. Treatment with alkaline phosphatase, however, suggests that Ser/Thr phosphorylation in other sites different from those in the C-terminal domain plays a role in the inhibition of receptor autophosphorylation in pregnant rats. Thus, removal of phosphate prior to phosphorylation increases by 1.4-fold the $[\text{P}^{32}\text{P}]_i$ incorporation into receptors from pregnant rats, whereas phosphorylation of virgin rat receptors is even slightly decreased. Despite the fact that phosphorylation increases in receptors from pregnant rats it remains lower than in virgin rats, suggesting that this is not the only mechanism involved in the impairment of insulin-receptor kinase activity at full-term pregnancy.

Autophosphorylation of the insulin receptor is known to proceed through a sequential mechanism in which Tyr-1150 or Tyr-1151, and Tyr-1146 are first phosphorylated, generating a bisphosphorylated form. This form becomes fully activated as a kinase after phosphorylation of the third tyrosine. In type-II diabetic patients it has been demonstrated that a defective intramolecular autoactivation cascade is involved in the reduced receptor kinase activity of skeletal muscle [4]. Quantitative analysis of tryptic phosphopeptide maps in this work revealed that around 67% of the *in vitro*-phosphorylated kinase domains of rat liver insulin receptor are in the trisphosphorylated form. On the contrary, receptors from pregnant rats show only 53% of the kinase domains in the fully activated form, and a correspondingly increased proportion as monophosphorylated, indicating that the intramolecular process of autoactivation is impeded. At present there are no data about possible structural or regulatory changes that could prevent the efficient transition from the bisphosphorylated to the trisphosphorylated form of the kinase domain. However, the evidence presented here regarding the preferential cleavage of the kinase domain by trypsin in pregnant-rat-derived receptors at the site yielding the shortest

peptide form, compared with virgin-rat-derived receptors, where trypsin cleaves equally at both sites after full phosphorylation is reached (Table 1 in this paper and [27]), allows us to speculate that this domain has some structural alterations in pregnant rats whose nature is at present unknown. One possibility suggested by data in Figure 3 would be that Ser/Thr phosphorylation of the β -subunit modifies the kinase domain structure, inhibiting its full phosphorylation.

From data in Table 1 it is possible to calculate the theoretical inhibition that should be expected in receptor phosphorylation in pregnant rats. Interestingly, this should not be higher than 12 %, in contrast with the mean 45 % inhibition observed (Figure 1). This discrepancy might be explained by the existence of a subpopulation of receptors with normal binding activity but which are completely refractory to phosphorylation, which would be more abundant in extracts from pregnant rats. In fact, other authors have reported [17,18] that around 60 % of the insulin-binding activity *in vitro* does not become phosphorylated. O'Hare and Pilch [21] have proposed that this silent receptor subpopulation originates from differences in β -subunit integrity. In this study we were able to detect three forms of ^{125}I -insulin-labelled receptors (Figure 5a). The portion of intact receptors amounts to 35 % of the total labelled proteins, in good agreement with the results from other groups mentioned above, however, no differences could be observed between virgin and pregnant rats. Furthermore, the results shown in Figure 5(b) indicate that the α -subunit is intact in both cases and consequently the differences in autophosphorylation are unlikely to be derived from a deficient coupling between both receptor subunits. This is also in concordance with previous data [25] which demonstrated no differences in insulin-binding affinity between both types of receptors.

Finally, we have tested possible changes in receptor isotype expression during pregnancy. Receptor isotype expression is a tissue-dependent regulated process that has been reported to be altered in type-II diabetics [35]. Both isotypes differ by only 12 amino acids in the α -subunit, a difference that could not be observed in the electrophoresis shown in Figure 5(b). Moreover, the binding characteristics of both isoforms are not markedly different, and would not be detected in preparations containing a mixture of the two isotypes. However, *in vitro* phosphorylation has been shown to be higher for the IR-B isoform, a fact that opens the possibility that a reduced proportion of this receptor isotype in pregnant rats results in the diminished autophosphorylation observed. The results in Figure 6, however, argue against this hypothesis, since only the IR-B isoform was detected after PCR amplification of total RNA.

In conclusion, our results confirm the inhibition of the insulin-receptor autophosphorylation in liver from full-term pregnant rats, and demonstrate that this inhibition is associated with the impairment of the intramolecular autoactivation cascade. This impairment appears to be due to basal Ser/Thr phosphorylation in residues not included in the 10 kDa C-terminal tail of the β -subunit. These changes in the receptor molecule of pregnant animals induce some alterations in the kinase domain, manifested in the different sensitivity to tryptic cleavage, but do not result in variations at the level of the ATP-binding domain. Additionally, our results prompt us to postulate that in pregnant rats there is a more abundant subpopulation of non-phosphorylated receptors, which is not originated by a higher receptor degradation or an altered expression pattern of receptor isoforms.

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