Functional alterations of type I insulin-like growth factor receptor in placenta of diabetic rats

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The presence of type I insulin-like growth factor (IGF-I) receptors on placental membranes led to the hypothesis that these receptors might play a critical role in the rapid growth of this organ. Diabetes induces fetoplacental overgrowth, but it is not known whether it modifies IGF-I receptor activity in fetal and/or placental tissues. To answer this question, we have partially purified and characterized placental receptors from normal and streptozotocin-induced diabetic rats. In normal rats, binding of ^{125}I -IGF-I to a 140 kDa protein corresponding to the α subunit of the receptor was observed in cross-linking experiments performed under reducing conditions. Stimulation by IGF-I induces the autophosphorylation of a 105 kDa phosphoprotein representing the β subunit of the receptor. In rats made hyperglycaemic and insulinopenic by streptozotocin injection on day 1 of pregnancy, placental IGF-I receptor-binding parameters were not different from controls on day 20 of pregnancy. In contrast, the autophosphorylation and kinase activity of IGF-I receptors of diabetic rats were increased 2–3-fold in the basal state and after IGF-I stimulation. The present study indicates that the rat placental IGF-I receptor possesses structural characteristics similar to that reported for fetal-rat muscle, and suggests that the high-molecular-mass β subunit could represent a type of receptor specifically expressed during prenatal development. In addition, it clearly demonstrates that diabetes induces functional alterations in IGF-I receptor kinase activity that may play a major role in the placental overgrowth in diabetic pregnancy.

INTRODUCTION

Although fetal development is closely dependent on adequate placental functions, metabolism and growth rate, the precise mechanisms regulating placental growth are not fully understood. Specific receptors for insulin-like growth factor (IGF) I and II are present in several fetal tissues and in the placenta (Sara *et al.*, 1983; Deal *et al.*, 1982). In addition, the notion that these tissues can synthesize IGFs has led to the idea that growth factors act through autocrine or paracrine mechanisms within the fetoplacental compartment (D'Ercole *et al.*, 1980). The well-known effects of IGFs to promote mitogenesis in a number of different cell lines (Froesch *et al.*, 1985), as well as their importance in stimulating postnatal growth (Daughaday *et al.*, 1972), suggest that these polypeptides play a role in fetal development. The human placenta contains large amounts of both IGF-I and IGF-II receptors (Daughaday *et al.*, 1981). The placental IGF-I receptors are structurally and functionally similar to receptors characterized in other tissues and cell types, and two types of IGF-I receptors with distinct binding affinities and different immunoreactivity have been observed (Jonas & Harrison, 1984). The high-affinity receptor is composed of two α and two β subunits, with apparent molecular masses of 135 and 96 kDa respectively (Tollefsen *et al.*, 1987). IGF-I stimulates the protein tyrosine kinase activity intrinsic to the receptor, leading to autophosphorylation of the β subunit and subsequent exogenous substrate phosphorylation (Rubin *et al.*, 1988).

Diabetic pregnancy is commonly associated with fetoplacental overgrowth, and alterations in the signalling pathways of insulin and/or IGF-I receptors might be involved in the development of this complication. The tyrosine kinase activity of the insulin receptor is decreased in different tissues from diabetic rats (Kadowaki *et al.*, 1984; Burant *et al.*, 1986), but little is known about the function of the IGF-I receptor in such pathological states. To investigate the role of the placental IGF-I receptor during development, we have purified receptors from rat placental

membranes and studied their tyrosine kinase activity in normal and diabetic pregnant rats.

MATERIALS AND METHODS

Materials

Streptozotocin (STZ), Hepes, dithiothreitol (DTT), proteinase inhibitors and insulin-agarose were from Sigma (St. Louis, MO, U.S.A.). Wheat-germ agglutinin (WGA)-agarose was from ICN (Lisle, IL, U.S.A.). Disuccinimidyl suberate was from Pierce (Rockford, IL, U.S.A.). ^{125}I -[Thr⁶⁹]IGF-I (2000 Ci/mmol), [γ - ^{32}P]ATP (3000 Ci/mmol) and insulin radioimmunoassay kit were from Amersham International (Amersham, Bucks, U.K.). Human recombinant IGF-I was a gift from Eli Lilly Co. (Indianapolis, IN, U.S.A.). Human and rat insulins were from Organon (Paris, France). Reagents for SDS/PAGE were from Bio-Rad (Richmond, CA, U.S.A.). Anti-phosphotyrosine antibody was a gift from Dr. M. White (Joslin Diabetes Center, Boston, MA, U.S.A.). Anti-C-terminal insulin-receptor antibody was generously provided by Dr. K. Siddle (University of Cambridge, Cambridge, U.K.).

Animals

Female Wistar rats purchased from IFFA Credo (L'Abresle, France) were housed and fed *ad libitum* in the laboratory. They were mated at a weight of 250 g, and the day of mating was taken as day 1 of gestation. STZ-diabetes was induced on day 1 of gestation by injecting STZ (45 mg/kg) through the saphenous vein under light ether anaesthesia. Diabetes was diagnosed on day 3 after injection by measuring glycaemia, which was then monitored twice a week. Pregnancy was confirmed by abdominal palpation on day 13–14 of gestation.

Preparation of placental membranes

On day 20 of gestation, rats were killed by cervical dislocation, and fetuses and placentas were removed after maternal lap-

Abbreviations used: IGF-I, insulin-like growth factor; STZ, streptozotocin; WGA, wheat-germ agglutinin; DTT, dithiothreitol.

arotomy. Placental membranes were prepared by the procedure described by Harrison & Itin (1980), with some modifications required for rat tissue. Placentas were trimmed of membranes, washed extensively in 50 mM-Hepes pH 7.4, containing 250 mM-glucose, 1 mM-phenylmethanesulphonyl fluoride and 25 mM-benzamidine, and cut into small pieces. Fresh tissue (7–12 g) was homogenized for 60 s in a loose-fitting Dounce homogenizer in 2 vol. of the same buffer supplemented with 1 unit of aprotinin/ml, 10 μ g of leupeptin/ml, 10 μ g of pepstatin/ml and 1 mg of bacitracin/ml. These proteinase inhibitors were added at the same concentrations at each subsequent step of the purification procedure. Homogenates were centrifuged at 600 *g* for 15 min, and the supernatant was centrifuged again at 100 000 *g* for 45 min after addition of 0.2 mM-MgCl₂ and 100 mM-NaCl. The resulting pellet was homogenized, resuspended in 10 vol. of the same buffer supplemented with fresh inhibitors, and centrifuged at 180 000 *g* for 45 min. The pellet, corresponding to the particulate membrane fraction, was resuspended in 1 ml of 50 mM-Hepes, pH 7.4 containing 1 % Triton X-100 and stored at –80 °C until further processed.

Partial purification of IGF-I receptors by using WGA-agarose

Step 1. Placental membranes were quickly thawed, diluted with 4 vol. of 50 mM-Hepes containing 1 mM-phenylmethanesulphonyl fluoride and 1 unit of aprotinin/ml and solubilized by stirring on ice for 60 min after addition of Triton X-100 (final concn. 1 %). A clear supernatant was obtained by centrifugation of the solubilized membranes at 100 000 *g* for 60 min at 4 °C. The supernatant was poured on to a column of WGA-agarose previously equilibrated with 50 mM-Hepes/0.1 % Triton, pH 7.4, and recycled three times through this column. After extensive washing (200 bed vol.) of the column with the same buffer, the glycosylated proteins were eluted with 0.3 M-*N*-acetylglucosamine in Hepes/Triton, and samples were taken for protein and receptor-binding measurements. The fractions containing the highest specific IGF-I and insulin-binding activities were those that also contained the highest protein concentration. Therefore, receptor-binding determinations were later omitted at this step of the purification procedure.

Step 2. The fractions exhibiting the highest IGF-I-binding activity were pooled and applied to a column (2 cm × 3 cm) of insulin-agarose. The first flow-through volume was collected and recycled once more on to the column to ensure total depletion of insulin receptors. In early preparations, insulin-binding activity was assayed in the column effluents, and specific binding was shown to be less than 3 % of total radioactivity. The last insulin-affinity-column effluent was diluted with an equal volume of Hepes/Triton buffer, and the IGF-I receptor activity was concentrated on a second WGA-agarose column by the procedure described in step 1. The partially purified IGF-I receptors eluted from this second WGA-agarose column were stored in 10 % glycerol at –80 °C until used.

Binding studies

Binding assays were performed on 10 μ l of receptor preparations. Receptors were incubated for 16 h at 4 °C in the presence of ¹²⁵I labelled insulin or IGF-I. Separation of receptor-bound and free ligand was performed by the poly(ethylene glycol) (PEG) method (Desbuquois & Aurbach, 1971). Free iodinated hormone was separated from the bound ligand by centrifugation at 10 000 *g* for 5 min after precipitation in the presence of 25 % PEG and 2 % γ -globulin as carrier, and this step was repeated once with 12.5 % PEG. Non-specific binding was determined in the presence of 0.1 μ M unlabelled insulin or IGF-I. Specific radioactivity was obtained by subtracting non-specifically bound (d.p.m.) from each point. The data from competition binding

studies were analysed by the method of Scatchard using the LIGAND computer program (Munson & Rodbard, 1980).

Affinity labelling of receptors

Partially purified IGF-I receptors (10 μ g of protein) were incubated for 3 h at 15 °C with 60 000 c.p.m. of ¹²⁵I-IGF-I in the absence or presence of 100 nM unlabelled IGF-I in a final volume of 100 μ l. The bound radioligand was cross-linked by incubating the mixture with 0.1 mM-disuccinimidyl suberate for 45 min at 4 °C. The reaction was stopped with 50 μ l of 5 % trichloroacetic acid and 20 μ l of 1 % BSA. After centrifugation, the pellets were washed with acetone, dried and solubilized in 1 × Laemmli sample buffer containing 100 mM-DTT. SDS/PAGE was run on 7.5 % resolving gels (Laemmli, 1970).

Autophosphorylation of the IGF-I receptors

Partially purified IGF-I receptors (6 μ g of protein for the control group matched for binding with the STZ-treated group) were diluted to 40 μ l in a buffer containing 50 mM-Hepes and 0.1 % Triton X-100, pH 7.4, and incubated without or with 0.1 μ M-IGF-I for 45 min at 4 °C in the presence of 5 mM-MnCl₂ and 8 mM-MgCl₂. The phosphorylation reaction was initiated by adding 25 μ M- $[\gamma$ -³²P]ATP, and was continued for 10 min at 22 °C. The reaction was stopped by adding 50 μ l of 5 % trichloroacetic acid and 20 μ l of 1 % BSA. After centrifugation, the pellet of precipitated proteins was rinsed with acetone and dried. Then 60 μ l of 1 × Laemmli buffer containing 100 mM-DTT was added to each tube. The samples were boiled for 3 min and centrifuged before electrophoresis. Proteins were analysed by SDS/PAGE on 7.5 % resolving gels. The gels were stained with Coomassie Blue in 50 % trichloroacetic acid, destained in 6 % acetic acid and subjected to autoradiography.

Immunoprecipitation of the phosphorylated receptors

In some experiments, the phosphorylation reaction was terminated by adding 500 μ l of a stopping solution (final concns. 50 mM-Hepes, pH 7.4, 0.1 % Triton X-100, 100 mM-NaF, 10 mM-sodium pyrophosphate, 5 mM-EDTA, 2 mM-sodium orthovanadate). Anti-phosphotyrosine antibody (2 μ g/ml) was added to each tube, incubated for 16 h at 4 °C, and antibody-receptor complexes were precipitated with 50 μ l of a 10 % Pansorbin solution. The precipitates were washed three times with 50 mM-Hepes (pH 7.4)/1 % Triton X-100/0.1 % SDS/2 mM-sodium orthovanadate. Then 1 × Laemmli sample buffer containing 100 mM-DTT was added, and the samples were heated for 3 min in boiling water before being subjected to SDS/PAGE.

IGF-I receptor kinase assay

Partially purified receptors were stimulated for 45 min at 4 °C in the absence or presence of 0.1 μ M-IGF-I and 10 mM-magnesium acetate. Preincubation in the presence of 25 μ M-ATP for 10 min at 22 °C was followed by addition of 0.22 mg of poly-(Glu₄/Tyr₁)/ml, in 50 mM-Hepes buffer containing 0.1 % Triton X-100 and 25 μ M- $[\gamma$ -³²P]ATP. After 20 min at 22 °C, the kinase reaction was terminated by addition of 50 μ l of 5 % trichloroacetic acid and 20 μ l of 1 % BSA. The incorporation of phosphate into poly(Glu-Tyr) was measured by using the filter-paper assay (Glass *et al.*, 1978). Mixtures were centrifuged for 5 min at 8000 *g* and samples of the supernatants were spotted on 2 cm² pieces of phosphocellulose paper (Whatman P81). Papers were washed extensively in 75 mM-H₃PO₄, rinsed with acetone and allowed to dry. ³²P incorporation was determined by Čerenkov radiation.

Analytical methods

During the course of pregnancy, maternal blood was sampled

twice weekly from the tail vein of the rats to check glucose concentration in the diabetic group. On the day of membrane preparation, blood was collected from the mother through a puncture in the dorsal aorta and from fetuses via a puncture through the axillary vessels (Girard *et al.*, 1973). Blood glucose levels were determined by the glucose oxidase method (Boehringer Mannheim, Meylan, France). Plasma immunoreactive insulin and IGF-I were determined by radioimmunoassay with rat insulin and human recombinant IGF-I as standards (Leturque *et al.*, 1984). Protein were measured by the method of Bradford (1976) with IgG as a standard.

Statistics

Data are presented as means ± S.E.M. Student's *t* test was used to establish significance between two groups.

RESULTS

Purification of rat placental IGF-I receptors

The major goal of the purification protocol used in our study was to obtain an IGF-I-receptor preparation devoid of insulin receptors. To achieve this purpose, we added an insulin-agarose affinity-column step after the usual step of WGA-agarose purification. By this technique, IGF-I receptors from rat placenta were purified 870-fold as compared with crude membrane fractions. A typical IGF-I receptor preparation is shown in Table

Table 1. Partial purification of IGH-I receptor from rat placentas

For this preparation, 32 placentas from 20-day-pregnant rats (*n* = 3) were processed as described in the Materials and methods section. 100000 *g* supernatant = solubilized receptors loaded on WGA I; insulin-affinity column = run-through from column loaded on WGA II. IGF-I binding activity and protein content were assayed in triplicate in the different membrane fractions.

Step	Volume (ml)	Total protein (mg)	10 ⁻⁶ × IGF-I binding (c.p.m.)	Yield (%)	Purification (fold)
Solubilized proteins	10	491	2.74	100	1
100000 <i>g</i> supernatant	9	87	2.79	100	6
WGA I eluate	3.5	10.2	1.62	59	28
Insulin-affinity column	10	2.5	1.25	45	89
WGA II eluate	1.5	0.2	1.02	37	867

Table 2. Physiological data of STZ-diabetic pregnant rats

The results are means ± S.E.M. of 5-7 determinations in different rats in each group. Blood and plasma parameters were assayed in duplicates on each sample. *****P* < 0.0005, ****P* < 0.005, ***P* < 0.05, **P* < 0.1 compared with controls.

Group	Gestational age (days)	Duration of diabetes (days)	Blood glucose (mg/dl)	Plasma insulin (μ-units/ml)	Plasma IGF-I (ng/ml)	Fetal wt. (g)	Placental wt. (g)	No. of fetuses/litter
Mothers								
Control	15	—	117 ± 11	36 ± 3	168 ± 26			
STZ	15	15	440 ± 20****	9 ± 1****	297 ± 54*			
Control	20	—	73 ± 4	32 ± 3	217 (<i>n</i> = 2)		0.53 ± 0.02	
STZ	20	20	420 ± 15****	12 ± 4**	146 ± 11		0.79 ± 0.05***	
Fetuses								
Control	20	—	16 ± 1	95 ± 4	93 ± 14	3.07 ± 0.02		12 ± 1
STZ	20	—	330 ± 14****	54 ± 14**	54 ± 2	3.05 ± 0.09		12 ± 2

Fig. 1. Phosphorylation of IGF-I receptors from rat placenta

WGA-purified IGF-I receptors (12 μg of protein) were incubated without or with unlabelled IGF-I (100 nM) for 45 min at 4 °C. Autophosphorylation was carried out with 25 μM-ATP and 4 μCi of [γ -³²P]ATP/tube for 10 min at 22 °C. The reaction was terminated by adding 500 μl of stopping solution. Phosphorylated proteins were immunoprecipitated with antibodies to phosphotyrosine (Anti-*P* Tyr; lanes 1, 2) or to the insulin receptor C-terminus (Anti-IR; lanes 3-6). Insulin receptors (10 μg of protein) prepared from rat mammary gland (Burnol *et al.*, 1990) were autophosphorylated after insulin stimulation (100 nM, 45 min, 4 °C) and were used for testing the efficiency of antibody to insulin receptor (lanes 5, 6).

1. Starting from three pregnant rats with a total of 32 placentas (wet wg. 12.6 g when trimmed from membranes and connective tissue), about 200 μg of partially purified IGF-I receptors was obtained from 0.49 g of solubilized placental membranes, with a final yield of 37%.

The efficiency of purification was further assessed by analysing the immunological properties of the partially purified IGF-I receptor after autophosphorylation of the receptor β subunit. As shown in Fig. 1, after stimulation by IGF-I, a 105 kDa phosphoprotein corresponding to the β subunit of the autophosphorylated IGF-I receptors was readily immunoprecipitated by anti-phosphotyrosine antibody (lanes 1, 2). By contrast, after stimulation by insulin, autophosphorylated receptors were not immunoprecipitated by antibodies to insulin receptor (lanes 3, 4). However, the same antibodies to insulin receptor efficiently immunoprecipitated insulin receptors obtained from rat mammary gland (lanes 5, 6) as described previously (Burnol *et al.*,

1990). These results indicate that the residual insulin-binding activity still detectable in our preparations after the insulin-affinity column (i.e. < 3 % of the total radioactivity) either does not lead to receptor autophosphorylation or cannot be detected by classical immunodetection methods.

Plasma glucose, insulin and IGF-I concentrations in diabetic pregnant rats

Blood glucose levels of STZ-induced diabetic pregnant rats were already elevated on day 3 after STZ injection (303 ± 17 mg/dl, $n = 7$) and remained above 400 mg/dl until the end of pregnancy (Table 2). Similar degrees of maternal hypo-insulinaemia (≤ 12 μ -units/ml) were observed in 15- and 20-day-pregnant rats. Plasma IGF-I concentrations were significantly elevated only in 15-day-pregnant rats (Table 2). After 20 days of maternal diabetes, fetuses from STZ-treated mothers were hyperglycaemic (> 300 mg/dl) and fetal plasma insulin and IGF-I concentrations were decreased 2-fold compared with controls. Fetal weight remained unchanged in diabetic rats, but placental weight was increased by 50 %.

Effect of STZ-diabetes on placental IGF-I-receptor binding parameters

IGF-I receptors prepared from placenta obtained on day 20 of gestation (normal or diabetes) displayed similar binding parameters. As shown on Fig. 2, competition binding curves were similar in both groups. Scatchard plots were linear in each case and revealed an unchanged binding capacity of approx. 0.4 nmol/mg of protein. The K_d values were not significantly different, 1.11×10^{-8} M and 0.80×10^{-8} M for control and diabetic rats respectively. The Hill coefficients were 1.186 and 0.899 respectively, and the regression analysis gave correlation coefficients of -0.928 and -0.937 for the two curves. Thus 20 days duration of maternal STZ-diabetes did not generate binding defects in rat placental IGF-I receptors. These data were further confirmed by affinity labelling experiments (Fig. 3). In both control and diabetic groups, 125 I-IGF-I was cross-linked predominantly to a receptor complex of similar apparent molecular mass > 350 kDa under non-reducing conditions, and under reducing conditions to a 140 kDa species representing the α subunit of the IGF-I receptor. Cross-linking of both species was inhibited to the same extent by an excess of unlabelled IGF-I in the diabetic and control preparations.

Autophosphorylation and kinase activity of IGF-I receptors from diabetic rats

WGA-purified placental IGF-I receptors from normal and diabetic rats were compared for their ability to autophosphorylate. In each group, basal and IGF-I stimulated incorporation of 32 P into a protein of 105 kDa was analysed. This species is believed to be the β subunit of the IGF-I receptor, since it was precipitated by anti-phosphotyrosine antibody after stimulation by IGF-I and because our preparation was essentially devoid of insulin receptors. In normal placentas, the time course of IGF-I receptor autophosphorylation was linear until 10 min and increased more slowly up to 60 min (Fig. 4). Half-maximal stimulation of autophosphorylation was achieved at 15 min. The kinetics of autophosphorylation was not significantly modified by diabetes. To analyse further the difference between the autophosphorylation rate of the placental IGF-I receptor in diabetic and control rats, we performed a dose-response experiment. The autoradiogram presented in Fig. 5 shows that, in the basal state, the autophosphorylation of the IGF-I receptor β subunit was increased 2–3-fold in the diabetic group relative to control and that this difference persisted under stimulation by 0.1 μ M-IGF-I. Similar results were obtained with two different

Fig. 2. Competition curves of 125 I-IGF-I binding

IGF-I binding capacity of placental IGF-I receptors partially purified from normal ($\square$) and STZ-diabetic ($\blacksquare$) rats was analysed in competition-binding experiments. Binding assays were performed with 12.5 μ g protein/assay and increasing amounts of unlabelled IGF-I in the presence of 125 I-IGF-I (60000 c.p.m.) in a final volume of 100 μ l. Data are expressed as the fraction of maximal specific binding. Non-specific binding measured in the presence of 100 nM unlabelled IGF-I has been subtracted from each point. Values are means of triplicate determinations. Scatchard plots (insert) and regression analysis were performed by the LIGAND computer program (Munson & Rodbard, 1980).

Fig. 3. Affinity cross-linking of 125 I-IGF-I to its partially purified receptor from rat placentas

WGA-purified IGF-I receptors from control or diabetic preparations (with same binding activity) were incubated for 3 h at 15 $^{\circ}$ C with 125 I-IGF-I without (lanes A, C, E, G) or with 100 nM unlabelled ligand (lanes B, D, F, H). Cross-linking was carried out with 0.1 mM-disuccinimidyl suberate for 45 min at 4 $^{\circ}$ C. Samples were analysed by gel electrophoresis either under reducing conditions in the presence of 100 mM-DTT (7.5 % resolving gels) or in the absence of disulphide reduction (5 % resolving gels). SDS/PAGE and autoradiography were as described in the Materials and methods section. Arrows point to labelling of > 350 kDa and 140 kDa receptor bands on non-reduced and reduced gels respectively. The presence of a 68 kDa band on the reducing gel was a constant finding, and corresponds to BSA (present in our incubation buffer) linked to 125 I.

amounts of IGF-I receptor-binding activity i.e. 25 and 50 % of total binding in each experimental group (results not shown). These results were confirmed by quantification of 32 P incorporation into the β subunit after scanning densitometric analysis

Fig. 4. Time course of autophosphorylation of rat placental IGF-I receptors

Samples of partially purified IGF-I receptors normalized for binding activity were obtained from control (□) and STZ-treated (■) rats. After incubation with or without 100 nM-IGF-I for 45 min at 4 °C, the autophosphorylation was initiated by adding 4 μCi of [γ-³²P]ATP and processed at 20 °C for the indicated time intervals. Reaction was stopped with 5% trichloroacetic acid and 20 μl of 1% BSA. SDS/PAGE was run on 7.5% resolving gels after elution of the proteins with 1 × Laemmli sample buffer containing 100 mM-DTT. Results represent arbitrary units of scanning densitometry of 2–3 autoradiograms.

Fig. 5. IGF-I-stimulated autophosphorylation of placental IGF-I receptors from normal and diabetic rats

Samples of receptors from control and diabetic rats with identical IGF-I-binding activity were used in each lane. After incubation with the indicated concentrations of IGF-I for 45 min at 4 °C, autophosphorylation was initiated by addition of [γ-³²P]ATP, continued for 10 min at 20 °C and stopped by adding 500 μl of stopping solution. Phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies (0.4 μg/ml), electrophoresed on 7.5%-polyacrylamide gels under reducing conditions (100 mM-DTT), and subjected to autoradiography. The autoradiogram represents a typical experiment. This experiment was repeated four times with different receptor preparations, and analysis of the data was performed after scanning densitometry of the autoradiograms.

of three autoradiograms (Fig. 5, lower panels). Half-maximal stimulation of autophosphorylation was observed with 10 nM-IGF-I, indicating that receptor sensitivity to IGF-I was similar in both groups. Under stimulation with 10 μM-IGF-I, maximal receptor autophosphorylation was 2-fold higher in diabetics relative to controls, in agreement with a change in responsiveness to IGF-I. The effects of diabetes on IGF-I receptor kinase

Table 3. Effect of diabetes on placental IGF-I receptor kinase activity

Partially purified IGF-I receptors from control and diabetic rats were matched for binding activity and incubated in the absence or presence of 0.1 μM-IGF-I. Preincubation was performed with 25 μM-ATP, then poly(Glu₄-Tyr₁) was added with 25 μM-[γ-³²P]ATP to the receptor solutions. Incorporation of phosphate was measured by the filter-paper assay. Results are means ± S.E.M. of 3 independent experiments: * *P* < 0.001 relative to controls.

Insulin concn.	³² P incorporation into poly(Glu ₄ -Tyr ₁) (c.p.m.)	
	Control	Diabetic
0	3010 ± 1185	8790 ± 980*
0.1 μM	4820 ± 1350	12160 ± 1040*

activity are reported in Table 3. A modest increase in kinase activity (40%) between the basal (no IGF-I) and the IGF-I stimulated conditions was observed in each group. However, similarly to the modifications in receptor autophosphorylation, we found a 2.5-fold increase in IGF-I receptor kinase activity in diabetics relative to the control group, both in the basal state (no IGF-I) and after stimulation by 0.1 μM-IGF-I.

DISCUSSION

Insulin-deficient diabetes is associated with defects in insulin active at both receptor and post-receptor levels (Kobayashi & Olefsky, 1979; Kahn & Cushman, 1987; Nishimura *et al.*, 1989). In animal models such as STZ-induced diabetes, insulin-receptor autophosphorylation and kinase activity are decreased in several tissues despite an increase in insulin-receptor number (Kadowaki *et al.*, 1984; Burant *et al.*, 1986; Okamoto *et al.*, 1986). Although the IGF-I receptor displays a high degree of structural and functional homology with the insulin receptor (Ullrich & Schlessinger, 1990), it has not been studied under similar pathological conditions. In the present study, we have partially purified and characterized the placental IGF-I receptor from normal and diabetic rats. We have identified a unique 105 kDa phosphoprotein representing the β subunit of the IGF-I receptor. This protein is mainly phosphorylated on tyrosine residues in response to IGF-I, as shown by its precipitation with antibodies to phosphotyrosine and its ability to phosphorylate a synthetic polymer of tyrosine (Fig. 1 and Table 3). In placenta from normal rats, the IGF-I receptor displays an intrinsic tyrosine kinase activity stimutable by IGF-I similarly to that characterized in the human placenta (Jacobs *et al.*, 1983; Rubin *et al.*, 1988; Bhaumick *et al.*, 1988). In placentas from rats made severely diabetic during the entire course of pregnancy, IGF-I-receptor autophosphorylation and tyrosine kinase activity were significantly increased compared with control rats, both in the basal state and after IGF-I stimulation. To the best of our knowledge, the present study is the first report characterizing the IGF-I receptor from rat placenta and its functional alterations in diabetic animals.

Heterogeneity in the molecular mass of the α and β subunits of the IGF-I receptor is well documented (reviewed by Rechler & Nissley, 1985). The molecular mass of the α subunit of the IGF-I receptor varies between 130 and 140 kDa, whereas that of the β subunit ranges between 90 and 98 kDa in different tissues and cell types. We found that the β subunit of the rat placenta IGF-I receptor has a molecular mass of 105 kDa. Interestingly, a 105 kDa β subunit has also been found for the IGF-I receptor of fetal-rat muscle (Alexandrides & Smith, 1989), and seems to be

specific for developing tissue, since it disappears during the first weeks of postnatal life. An altered form of IGF-I receptor β subunit has also been described in a leukaemic cell line (Kellerer *et al.*, 1990), and in human glioma cells (Gammeltoft *et al.*, 1988), suggesting that malignant transformation confers to the cell a signalling transduction system resembling that found in early development. This structural difference would be consistent with increasing amounts of data indicating that there may be more than one type of IGF-I receptor in different tissues and cell types, including placenta (Jonas & Harrison, 1984), liver (Morgan *et al.*, 1986), brain (Garofalo & Rosen, 1989), adipocytes (Kasuga *et al.*, 1981) and lymphocytes (Jacobs *et al.*, 1983).

When placental IGF-I receptors were prepared from diabetic rats (with a duration of diabetes equal to the duration of gestation), we found an increase in IGF-I receptor autophosphorylation and kinase activity over the full range of receptor activation. This was characterized by a higher basal autophosphorylation rate as well as an enhanced responsiveness to IGF-I (Fig. 5 and Table 3). On the basis of the specific physiological and biochemical features required for adequate placental development, it is tempting to speculate on several potential mechanisms involved in these diabetic alterations. The pathways responsible for up-regulating receptor tyrosine kinase activity are not fully understood. The changes in circulating IGF-I and insulin levels are unlikely to explain the increase in IGF-I-receptor tyrosine kinase activity. Hypoinsulinaemia, as found in this study in the diabetic mothers and fetuses, typically results in an up-regulation of insulin receptor number (Kadowaki *et al.*, 1984; Nishimura *et al.*, 1989). However, this type of up-regulation was not observed for the placental IGF-I receptor, since the alterations in the kinase activity were not associated with changes in the apparent affinity or binding capacity of the receptors (Fig. 2), despite lower plasma IGF-I levels. Alternatively, modifications of the phosphorylation status of the receptor involving phosphorylation/dephosphorylation reactions would provide a possible regulatory mechanism. Concerning the dephosphorylation process, it has been shown that hepatic phosphotyrosine phosphatase activity towards insulin receptor is decreased in diabetic-rat liver (Meyerovitch *et al.*, 1989). However, we have obtained recent evidence that phosphotyrosine phosphatase activity towards insulin and IGF-I receptors is increased in placental membranes from diabetic rats (Hauguel-de Mouzon, unpublished work). It is also possible that a decreased phosphorylation on serine residues plays a role in the elevated basal activity and responsiveness of placental IGF-I receptors of diabetic rats. Previous studies have shown that the number of phosphorylated serine residues is greater in the β subunit of the IGF-I receptor than in the insulin receptor (Jacobs & Cuatrecasas, 1986), suggesting that additional kinases are involved in regulating IGF-I receptor function. Although several pieces of evidence support an impairment of protein kinase C activity in diabetes and other insulin-resistant states (Van de Werve *et al.*, 1987; Okumura *et al.*, 1988; Heydrick *et al.*, 1991), diabetes-induced alterations in serine phosphorylation in diabetic placenta remain to be demonstrated. Elevated circulating levels of non-esterified fatty acids and triacylglycerols are a common feature of diabetes and pregnancy, and may serve as precursors for synthesis *de novo* of diacylglycerol, eventually leading to an increase in protein kinase C activity. This would be consistent with the fact that the activity of protein kinases such as insulin receptor (Freidenberg *et al.*, 1985) or protein kinase C is increased by hyperglycaemia (Lee *et al.*, 1989a,b). Although they do not allow one to establish causal relationships, these data suggest a role of hyperglycaemia in insulin resistance.

In conclusion, diabetes induces functional alterations in rat placental IGF-I receptor. The pathological implication of these

modifications is supported by the increased placental weight of diabetic rats and is in agreement with the observations of fetoplacental overgrowth in diabetic pregnant women.

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