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Insulin Receptor Interaction in Human Placental Plasma Membranes

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Insulin-receptor interaction in partially purified preparations of human placental plasma membranes from normal mothers at term of pregnancy has been characterized. ^{125}I-insulin became rapidly and reversibly bound to plasma membranes, being time and temperature dependent. The binding readily appeared at 1.0 ng/ml insulin concentration which falls within the physiological range of peripheral blood. Low levels of unlabeled insulin inhibited binding; 20 ng/ml insulin produced fifty per cent inhibition. Scatchard plots of data from competitive insulin binding proved to be curvilinear. The insulin greater hability for binding observed in this preparation can be explained by the purification degree achieved at the plasma membranes. ^{125}I-insulin was less degraded by partially purified placental plasma membranes than by a microsomal-membrane preparation obtained without differential centrifugation in sucrose linear gradient. All these properties strongly suggest that the insulin-binding sites characterized in the plasma membrane fraction of the placenta represent biologically important receptors to hormone.

During pregnancy placental secretions are regulated by hormones and metabolic factors. Previous investigations have demonstrated that starvation increases (12) whereas insulin and glucose decrease the blood level of human placental lactogen (hPL) (1, 22). HAOUR *et al.* (8) suggested that during human pregnancy, glucose and insulin influence the secretion of hPL by placental slices.

On the other hand, placenta was an active site of insulin metabolism: recent work has defined two different soluble insulin-degrading enzyme activities in the human placenta (18). In 1974 it was published the first report that demonstrated the presence of insulin receptors in microsomal-membrane fractions obtained from human placental tissue (9, 19).

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In order to prepare a more purified placental preparation we have modified the methods previously described (9, 19) for the obtention of a biological fraction containing preferently placental plasma membranes. In this work we show our results concerning the interaction of insulin with its specific receptors in a microsomal-membrane fraction and in the placental plasma membranes obtained at term of normal pregnancy.

Materials and Methods

Materials. Purified porcine «monocomponent» insulin (lot S 835158, 27 i.u/mg) were purchased from Novo Research Institute (Copenhagen, Denmark) and was used for iodination as well as unlabeled standard in the binding studies. Carrier-free Na ^{125}I (300 mCi/ml, in NaOH solution, pH 8-11, less than 1 % of ^{126}I) was purchased from the Radiochemical Centre. A guinea-pig anti-insulin serum (20) and talc tablets (Silicosorbe, 50 mg, Dreyfus Herschtel, Paris) were used in experiments measuring degradation (5) of the ^{125}I-insulin. DEAE-cellulose (microgranular DE 52) was from Whatman and bovine serum albumin (BSA) fraction V, Sigma. Other chemicals were of reagent grade.

Placental tissue and tissue fractionation. Human full-term placentas were obtained from healthy mothers after vaginal delivery. From the same placentas tissues were processed simultaneously for the obtention of microsomal-membrane and purified plasma membrane preparations.

Microsomal-membrane preparation. Tissue was processed according to the method described by POSNER (19), with the following modifications: the homogenization was performed in ten volumes of sucrose (0.3 mol/l) in a glass homogenizer with a motor-driven teflon pestle. The homogenate was filtered through two and then four layers of cheesecloth. After centrifugation for 15 min at 600 *g*, the supernatant was centrifuged again at 50 000 *g* for 60 min to obtain the microsomal-membrane pellet.

Purified placental plasma membranes. After the 50 000 *g* centrifugation the pellet was suspended by gentle homogeneization in 0.1 mol/l Tris buffer, pH 7.4, and then delivered into the top of a centrifuge tube containing a 30 % to 60 % (wt/wt) linear sucrose gradient. It was then centrifuged again at 100 000 *g* for 60 min. After this centrifugation we recognized three different layers: the first (F_1), intermediate (F_2) and last (F_3) layers appeared at sucrose densities of 30 %, 38-40 % and 50 %, respectively. These three layers were characterized by electron microscopic examinations, by their 5'-nucleotidase activities and by their ability to bind ^{125}I-insulin. They were resuspended and washed in 0.1 mol/l Tris buffer, pH 7.4, and centrifuged again at 50 000 *g* for 10 min in a Sorvall (RC5-B) centrifuge, and then frozen at —80° C until assay. All the above procedures were carried out at 4° C.

Monoiodination of insulin. Insulin was iodinated directly with 0.8-1.0 I atom per molecule (specific activity 300-380 μCi/μg or 1.800-2.280 Ci/mmol) using the chloramine T method (7). Iodinated products were chromatographed on DEAE-cellulose (4), except that urea was omitted because of the small quantity of polypeptide applied to the ion exchanger. The main peak of ^{125}I-insulin eluted at the same ionic strength as the biologically active monoiodoinsulin did and its affinity for binding to specific sites in liver membranes was indistinguishable from that of native insulin (4, 5).

Binding studies. Studies of binding of ^{125}I-insulin to microsomal-membrane frac-

tion and to plasma membranes (F_1) were conducted at the temperatures and for the times indicated in the legends to figures, in Krebs Ringer Phosphate (KRP) buffer, pH 7.5, in a final volume of 0.4 ml per incubation tube, that contained ^{125}I-insulin at 0.03-0.2 nmol/l, unlabeled insulin at various concentrations from 0 to 17 μmol/l, placental proteins at 0.30-0.40 mg/ml and BSA at 10 mg/ml. At the times indicated, duplicate 150 μl-aliquot samples from each incubation tube were transferred to microfuge tubes (Beckman) and membrane-bound ^{125}I-insulin was isolated by a ten minutes centrifugation at 50 000 *g* in a Sorvall centrifuge. Except when stated otherwise, data are reported as specific binding: this is obtained by subtracting from the total binding the amount of labelled insulin which is not displaced by a great excess (17 μmol/l) of unlabeled insulin. Each value is the mean obtained from two-four microfuge tubes, and is considered as a single value for all further statistical calculations.

Other analytical studies. The degree of insulin degradation was measured by the precipitation with 5% trichloroacetic acid (TCA), binding to anti-insulin antibody, and ability to bind to specific receptors in rat liver plasma membranes prepared according to the NEVILLE method (15). Protein was measured by the LOWRY procedure (13) and 5'nucleotidase assays were carried out at 37° C (24) and with a TCA deproteinization step before addition of the molybdate reagent (10).

Results

Characterization of placental preparations. The 5'-nucleotidase activity was much greater in the 100 000 *g* F_1 fraction than in the others. In F_1 the purification degree obtained for this enzyme activity was twice that in 50 000 *g* pellet. Specific binding of ^{125}I-insulin paralleled 5'nucleotidase activity and was significantly higher in F_1 fraction. The microsomal-membrane pellet (P.P) and partially purified plasma membrane (F_1) were used in all subsequent studies. Electron microscopic examination showed that the plasma membrane fraction consisted of vesicles of various shapes and sizes which showed a triple-layered structure at high magnification (data not shown).

Studies of insulin binding. Time course. Both the rate and the amount of insulin binding were time- and temperature-dependent. With ^{125}I-insulin at 1 ng/ml (0.16 nmol/l) the binding was rapid at 37° C and reached a peak at 5 to 10 min of incubation. With identical concentration of membrane and ^{125}I-insulin the reaction was slower to reach steady-state at 4° C, requiring at least, more than 120 min. At 24° C steady-state was obtained by 30

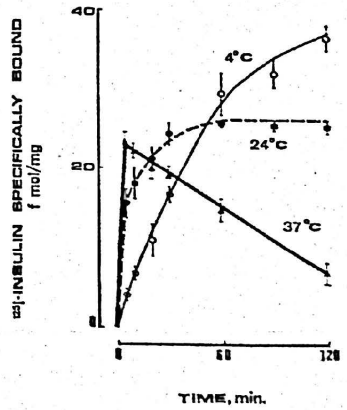

Fig. 1. *Time course of binding of ^{125}I-insulin to the microsomal-membrane fraction of the human placenta.*
Insulin at 0.16 nmol/l was incubated with P.P. at 0.4 mg protein/ml for the times indicated at 4° C, 24° C and 37° C, in the absence and in the presence of unlabeled insulin at 17 μmol/l. The latter was used to determine the proportion of non-specific binding, which has been subtracted from each experimental point. Each point is the mean ± SEM of 5 experiments.

to 120 min (fig. 1). These results were similar to those reported for insulin binding to rat liver and heart plasma membranes (3, 11) and to human lymphocytes (6) and fat cells (16). Because the binding was more stable at 24° C than 37° C all subsequent binding studies were conducted at the steady-state of binding at 24° C after a 60 min incubation period.

Under identical experimental conditions and throughout the entire time course, microsomal-membrane fraction bound only 45-50 % as much insulin as did plasma membranes (fig. 2). This difference could not be attributed to a different non-specific binding that represents 5-10 % of total binding for both F_1 and P.P fractions. In the fraction F_2 the specific binding of ^{125}I-insulin was less than in both F_1 and P.P. However, the non-specific binding in F_2 was greater than in previous fractions and reach about 60 % of total binding.

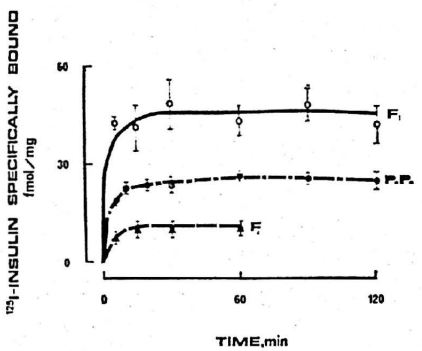

Fig. 2. *Time course of binding of ^{125}I-insulin to purified placental plasma membranes (F_1), microsomal-membranes fraction (P.P.) and F_2 fraction.*
Insulin at 0.16 nmol/l was incubated at 24° C with 0.4 mg protein/ml for the times indicated. Non-specic binding has been subtracted from each experimental point. Each point is the mean ±SEM of 5 experiments. The differences between F_1 and P.P. were significant at 1 per cent level (Student't test).

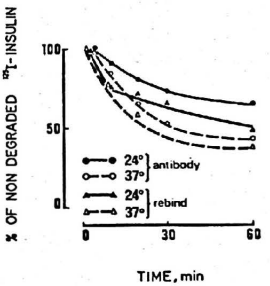

Fig. 3. *Degradation of ^{125}I-insulin as a function of time and temperature of incubation.* ^{125}I-insulin at a concentration of 0.16 nmol/l was incubated with 0.4 mg protein of P.P. in KRP buffer pH 7.5, 1 % BSA, for the times indicated at 24° C or 37° C. The suspension was centrifuged (50,000 × *g*, 10 min) and the supernatant radioactivity was tested for binding to an antiinsulin antibody and to rat liver membranes. Controls of degradation consisted of identical reaction mixtures without membranes. Each point is the mean ± SEM of 5 experiments.

Insulin degradation by placental tissue. The degradation of ^{125}I-insulin by the P.P. fraction was studied at 24° C as well as 37° C. 0.4 mg/ml protein degraded more than 50 % of ^{125}I-insulin at 60 min of incubation as measured by rebinding to rat liver membranes as well as with an anti-insulin serum. At 24° C the degradation was less, but reached 40-50 % after sixty minutes of incubation (fig. 3). As described previously the more sensible method for measuring was the ability of radioactivity free in the medium (after exposure to P.P. fraction) to bind to specific receptors in rat liver membranes (5). Using this method, the degradation of ^{125}I-insulin at 24° C was significantly less with F_1 than with P.P. fraction (fig. 4).

Insulin binding to steady-state. ^{125}I-insulin specifically bound to P.P. and F_1 fractions was linearly related to the proteins concentrations, at least between 100

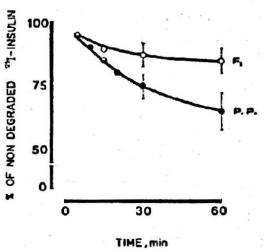

Fig. 4. *Degradation of ^{125}I-Insulin as a function of placental preparation.*
^{125}I-insulin (0.16 nmol/l) was incubated with 0.4 mg protein of F_1 and P.P. at 24° C. The suspension was centrifuged and the supernatant tested for binding to fresh rat liver membranes. Controls of degradation consisted of identical reaction mixtures without placental membranes. Each point is the mean ± SEM of 5 experiments.

and 1 000 μg/ml. This range includes the concentrations employed in the binding studies and permits normalization of the data on the basis of protein concentration.

Unlabeled insulin at physiological concentrations readily inhibited the binding of ^{125}I-insulin in a similar extent for both F_1 and P.P. fractions. With 0.8 nmol/l insulin (5 ng/ml or 125 μU/ml) 20 % inhibition was observed. 50 % inhibition of ^{125}I-insulin binding was obtained with insulin at 3.2 nmol/l (20 ng/ml or 500 μU/ml). Scatchard analysis (21) indicated that the specific binding fits a curvilinear model in which at least two orders of binding sites can be distinguished with respect to the equilibrium constant: at 24° C the high-affinity site has a K_a of about 1.0×10^9 mol/l^{-1}.

Over a wide range of hormone concentrations, from insulin as low as 0.16 nmol/l (1 ng/ml or 25 μU/ml) to saturating concentrations of the hormone above 17.5 nmol/l (100 ng/ml or 2500 μU/ml) the P.P. fraction bounds only 40-50 % as much insulin as did F_1 fraction (fig. 5). Since at any insulin concentration including saturation of the binding sites, the amount of insulin bound was two fold lower for P.P. than for purified placental plasma membranes, we conclude that a decrease in the number of binding sites is the main factor leading to the decreased insulin binding. A similar conclusion is reached when the binding data are analysed by the method of Scatchard: the binding capacities are two fold lower in P.P. than in F_1 fraction whereas the apparent affinity constants are similar in both types of preparation.

In order to exclude the possibility that the insulin binding studies might be influenced by the presence of blood elements in the placenta we performed the following control experiment: one normal

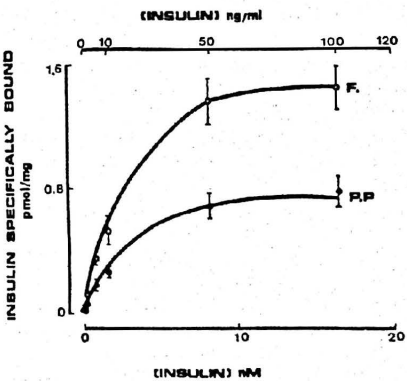

Fig. 5. *Binding of Insulin to F_1 and P.P.*
In 5 separate experiments carried out with five different membrane preparations, the binding of insulin (^{125}I-insulin at 0.16 nmol/l in the absence and in the presence of increasing concentrations of unlabeled insulin) was simultaneously studied in P.P. and in F_1 fractions, after 60 min of incubation at 24° C. The nonspecific binding has been subtracted from the total binding and amounts of hormone specifically bound are plotted as a function of total insulin concentration. Each point is the mean ± SEM of 5 separate experiments. The difference sbetween F_1 and P.P. were significant at 1 per cent level (Student't test).

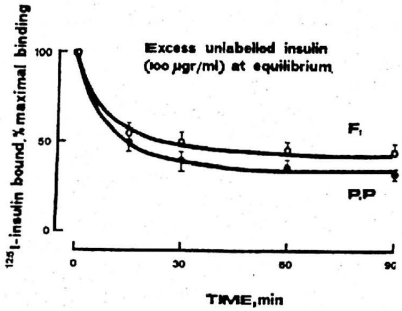

Fig. 6. *Dissociation of bound ^{125}I-insulin.* This was studied in F_1 and P.P. at 24° C by the addition of 100 μg/ml of unlabeled insulin at steady-state of the reaction (60 min). Each point is the mean ± SEM of three experiments.

placenta was extensively perfused using cold saline via the two umbilical arteries and the placental cotyledons used for the homogeneization were also thoroughly washed. The insulin binding capacity of the placental plasma membranes prepared after this perfusion procedure was not significantly different from that found in plasma membranes prepared without placental perfusion.

At steady-state, addition of excess unlabeled insulin resulted in the rapid dissociation of ^{125}I-insulin bound from both F_1 and P.P. fractions in a similar extent (fig. 6). Dissociation was noted within 15 min and was 50 % complete after 30 min.

Several hormones unrelated to insulin were examined for their ability to compete with ^{125}I-insulin for binding to P.P. and F_1 fractions. Porcine glucagon and human growth hormone at concentration as great as 1 μg/ml showed no competition.

Discussion

We have demonstrated specific insulin receptors in a partially purified preparation of placental plasma membranes obtained at term of pregnancy from normal mothers. ^{125}I-insulin was time and temperature dependent, and was readily demonstrated at 1 ng/ml (25 μU/ml) a concentration within the physiological range for insulin in the peripheral blood. Scatchard plots of data obtained from competitive insulin binding studies to both microsomal-membrane and plasma membrane fractions were curvilinear, in agreement with previous reports (14, 17). The binding was inhibited by low levels of unlabeled insulin. Specific binding of insulin paralleled 5'nucleotidase activity and was significantly higher in F_1 fraction. Structurally unrelated hormones such as glucagon and growth hormone did not compete for insulin binding. Although excess of this last hormone have been associated with clinical insulin resistance during pregnancy (25) this does not result from competition with insulin for binding to its receptors in placenta.

The purification degree obtained for the F_1 fraction contributes to explain the higher amount of binding observed in this preparation as compared to microsomal-membrane fraction. Indeed, though the placental tissue were shown to possess insulin degrating activity (18), with purified plasma membranes the ^{125}I-insulin degradation was less than in microsomal-membrane preparation. All these properties strongly suggest that the insulin binding sites characterized in the enriched plasma membrane fraction of the placenta represent biologically important receptors for this hormone.

Despite the presence of high affinity insulin-receptors in placenta, the role of insulin in placental physiology has yet to be defined. High concentrations of insulin increased glucose utilization and glycogen synthesis in human placental slices (23) and activated glycogen synthetase in placental organ cultures (2). The specific binding of insulin reported in the present study suggest from a teleological point of view that insulin could act directly at the placental level.

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Resumen

Se caracteriza la interacción insulina-receptor en una preparación parcialmente purificada de membranas de células placentarias humanas, obtenidas a partir de placentas a término de embarazadas normales. La insulina-I^{125} se une de modo rápido y reversible a dichas membranas. La capacidad de unión es dependiente del tiempo y de la temperatura de incubación y es detectable a 1,0 ng/ml de insulina, concentración situada entre los niveles detectables en sangre periférica. La unión de la insulina-I^{125} a las membranas placentarias se desplaza en presencia de pequeñas concentraciones de insulina, observándose el 50 % de desplazamiento con 20 ng/ml de insulina. La representación de los resultados de los estudios de inhibición competitiva según el método de Scatchard muestra una morfología curvilínea. El grado de purificación alcanzado en la preparación de las membranas placentarias justifica la mayor capacidad de unión de la insulina detectada en esta preparación. Las membranas placentarias parcialmente purificadas degradan menos insulina-I^{125} que las preparaciones microsomales obtenidas sin centrifugación diferencial en gradiente de sacarosa. Todas estas propiedades sugieren que los lugares de unión para la insulina caracterizados en las membranas celulares placentarias representan receptores hormonales biológicamente importantes.

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