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INSULIN, INSULIN-LIKE ACTIVITY AND THE HUMAN PLACENTA

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SUMMARY

Specific insulin binding sites can be demonstrated in human placental membranes. Binding is readily reversible. Both the rates of binding and dissociation are temperature sensitive. Insulin analogues bind with affinities which parallel their biological activity. The affinity for insulin is of sufficient magnitude to bind physiologic levels of insulin. Insulin eluted from these binding sites is structurally intact. The binding sites are partially protein in composition and are found in the placental tissue of many species. The binding sites have the properties of biologically significant receptors in well-defined insulin target tissues. This suggests a direct role for insulin in regulating placental function.

Two different soluble insulin degrading enzymes have been described in human placenta. In addition, a third membrane-associated degrading activity can be demonstrated. This enzyme was partially washed out of the membranes under circumstances which did not influence the insulin binding sites. This dissociation of degrading activity and binding suggests that they are not intimately related.

An insulin radioreceptor assay (RRA) was used to follow the purification of an insulin-like peptide (ILAs, 7–12 × 10^3 daltons) from human Cohn fraction IV-4. ILAs had chemical and biological properties like those of somatomedin and NSILAs. It bound in a specific manner to a unique human placental receptor suggesting a role for it in placental function. Using this receptor and [^{125}I]-ILAs, an ILAs RRA was developed and validated. The measurement of ILA in rat serum with the ILAs RRA revealed a large molecular weight crossreacting material whose level was greatly reduced by hypophysectomy. It has not been excluded that this material crossreacts in our assay through an ability to bind [^{125}I]-ILAs. Further study must be aimed at characterizing the nature of insulin-like peptides in the circulation of animals and man.

INTRODUCTION

The present communication summarizes some of our recent data on insulin-placental interactions, and studies on the nature of insulin-like activity and its interaction with the placenta.

Our work demonstrates the presence of insulin receptors in human placental membranes with properties which closely resemble those in established insulin target tissues such as liver [1]. It thus provides a basis for understanding the adverse effect of diabetes on the outcome of pregnancy in terms of direct effects of insulin on placental function.

The characteristics of three different kinds of placental insulin degrading activity are considered. The placenta may contribute to the well appreciated aggravation of diabetes by pregnancy not only through the elaboration of contrainsulin hormones but also by accelerating the catabolism of maternal insulin [2]. The properties of the placental insulin degrading enzymes are thus important in the ultimate understanding of the placenta's role as an insulin-catabolizing site.

The regulation of placental growth remains a mystery. We have employed human placental membranes in an insulin radioreceptor assay (RRA) to isolate an insulin-like peptide from human plasma (ILAs). This peptide closely resembles NSILAs and the somatomedins [3]. Studies pertinent to its potential role as a regulator of placental growth are summarized.

EXPERIMENTAL

Human placental membranes and [^{125}I]-insulin were prepared as previously noted [1]. Membranes were washed by resuspending in 25 mM Tris–10 mM $MgCl_2$, pH 7.4, and centrifuging at 100,000 *g* for 30 min. This procedure led to a considerable loss of protein from the final membrane pellet [4]. Cohn fraction IV-4 was kindly supplied by the Connaught Research Laboratories Willowdale, Ontario. ILAs was extracted from Cohn IV-4 by a modification [4] of the acid–ethanol procedure of Jakob *et al.*[5]. Sephadex (Pharmacia) and carboxymethylcellulose (CM 52, Whatman Biochemicals) were prepared as described elsewhere [4]. Rats were obtained from the Canadian Breeding Laboratories, Laprairie, Quebec. Hypophysectomy was performed under light ether anaesthesia by the parapharyngeal route. Its completeness was validated by establishing that growth had ceased completely and by examining the pituitary fossa postmortem.

Insulin binding assays were conducted in 12 × 75 mm plastic tubes in duplicate or triplicate with constant shaking. The incubation mixture contained 25 mM Tris, pH 7.4, 10 mM $MgCl_2$,

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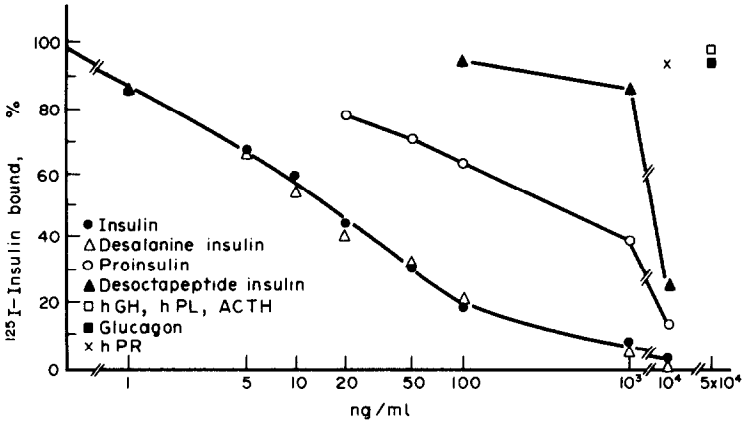

Fig. 1. Specificity of binding of [^{125}I]-insulin to human placental membranes. Maximum binding of 100% was the amount of binding of [^{125}I]-insulin in the absence of unlabelled hormone. The unlabeled hormone concentration on the abscissa refers to that in the standards and was 10 times that in the final incubation. The hormones studied included human growth hormone (hGH), human placental lactogen (hPL) and human prolactin (hPR).

60–120 × 10^3 c.p.m. of [^{125}I]-insulin (S.A. 100–150 μCi/μg), 150 μg membrane protein, 0.1% bovine serum albumin (BSA) and unlabeled insulin in a final vol. of 0.5 ml. All components were preincubated for 1 h at 4°C, and labeled insulin was added for 2 more h of incubation at 4°C. The reaction was terminated by the addition of 3 ml ice-cold 0.1% BSA–Tris–$MgCl_2$ buffer followed by centrifugation at 4°C for 30 min at 750 *g* in an IEC PR-6 centrifuge to separate bound and free labeled hormone. After decantation of the supernatant and drainage in the inverted position the pellet was counted in a Packard Autogamma spectrometer (model 3002) at an efficiency of 40%. All ILA values are reported in ng of porcine insulin (24.4 U/mg) equivalents where for purposes of conversion 1 ng equals 25 μU.

ILAs prepared by carboxymethylcellulose (CMC) chromatography was iodinated with chloramine T. [^{125}I]-ILAs was purified from the iodination mixture by adsorption to and elution from human placental membranes as described in detail elsewhere [4].

[^{125}I]-insulin degradation was determined as noted in the legend to Fig. 2. Protein was measured by the method of Lowry *et al.*[7] or by fluorimetry.

RESULTS

I. *Insulin–placental interactions*

A. *Insulin binding.* In previous studies we have shown that insulin binds specifically to membranes from human placental tissue. The rates of binding and dissociation are temperature sensitive. Binding is more rapid at 25°C than at 4°C but attains a higher plateau level at 4°C than at 25°C. The rate of dissociation is substantially greater at 37°C than at 30°C and is essentially arrested at 0°C [1].

Figure 1 depicts a study of the inhibition of [^{125}I]-insulin binding to human placental membranes by unlabeled insulin, insulin analogues, and unrelated hormones. Insulin, desala insulin, proinsulin, and desoctapeptide insulin inhibit binding with efficacies that parallel their known biological activities [8]. Unrelated hormones at supraphysiologic concentrations have no inhibitory effect. Binding of [^{125}I]-insulin is inhibited by low concentrations of insulin. Thus at 1 ng/ml of standard (0.1 ng insulin/tube) there was 10% inhibition of binding; and at 20 ng/ml of standard (2.0 ng insulin/tube) there was 50% inhibition of binding. Scatchard analyses of these data have

Table 1. Some characteristics of 125-I-insulin eluted from insulin–placental membrane complex

Properties of [^{125}I]-insulin:	Freshly prepared	Eluted from membranes
% precipitated by 10% TCA	95	98
% bound to insulin antibody	87	92
Specific binding to fresh membranes (nmol/mg · protein) at 5×10^{-10} M [^{125}I]-insulin	$18.8 \pm 0.4 \times 10^{-5}$	$15.5 \pm 0.6 \times 10^{-5}$

[^{125}I]-insulin was incubated with placental membranes for 40 min at 25°C.

[^{125}I]-insulin bound to the membrane pellet, obtained by centrifugation, was eluted to the extent of 95% with 0.2 N HCl containing 10% BSA. The elution mixture was filtered and neutralized with NaOH. The eluted radioactivity was evaluated for integrity by binding to fresh placental membranes, TCA precipitation, and immunoprecipitation as described before [1]. Data from Posner [1], courtesy of the publisher.

generated nonlinear concave up plots [1]. This is consistent with either a heterogeneity of sites or negative cooperativity of binding site interactions [9].

[^{125}I]-insulin bound to placental membranes was eluted from the binding sites to the extent of 95% and shown to be highly intact material by three different criteria (Table 1). This indicates that the radiolabeled insulin was not substantially altered in the course of binding to the membrane.

A number of other properties of the binding sites have been demonstrated. It has been shown that binding is greatly increased in the presence of divalent cations. A marked reduction in binding seen after gentle proteolytic digestion has suggested that the sites are, at least in part, protein in nature. It was also demonstrated that binding occurred to placental membranes from a range of animal species with particularly high levels of binding seen in guinea pig and monkey [1].

B. *Insulin degradation.* The placenta has long been known to have a great capacity to degrade insulin [2]. Two soluble insulin-degrading enzyme activities have been described in human placental tissue [10]. One enzyme activity has properties which very much resemble those of an insulin-glutathione transhydrogenase first described in bovine liver [11]. The other enzyme has properties similar to a sulfhydryl protease first described in rat muscle [12]. Membrane associated degrading activity has been observed in rat liver membranes [13] and in human placental membranes as well [10, 14].

Figure 2 depicts the time course of degradation at 37°C of [^{125}I]-insulin by human placental membranes. The rate and extent of loss of the ability of [^{125}I]-insulin to rebind to fresh membranes is greater than is the loss of immunoreactivity. Trichloroacetic acid (TCA) precipitability is lost least rapidly. This greater sensitivity of rebindability as an index of hormonal integrity has previously been noted. Degradation was slower at 24°C, and was much slower at 4°C, indicating marked temperature sensitivity of the degrading process.

Figure 3 compares degrading activity with binding in washed and unwashed human term placental membranes. Washing resulted in a marked increase in the specific binding activity of the placental membranes (lower panel) but correspondingly in a significant reduction in the degrading activity (top panel). Thus, there was a dissociation between binding and degrading activities. Further washings as described under Experimental did not lead to a further loss of degrading activity. In other studies it has been shown that the degrading activity is relatively, though not completely, specific for insulin, and is inhibited by pCMB (Omori and Posner, manuscript in preparation).

II. *Insulin-like activity (ILA) and the placenta*

A. *Isolation and characterization.* Human placental membranes were used to construct an insulin radioreceptor assay (RRA) essentially as noted in Fig. 1. The assay was sensitive to as little as 0.1 ng (2.5 μU) of

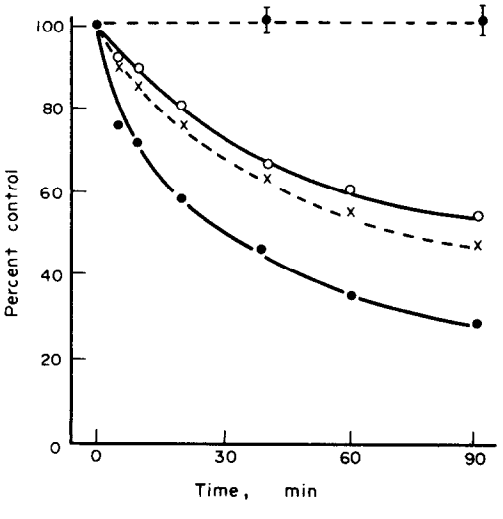

Fig. 2. Time course of degradation of [^{125}I]-insulin by human placental membranes at 37°C. At each time point the reaction mixture was diluted, placed on ice, and centrifuged at 4°C to obtain the supernatant. The integrity of supernatant radioactivity was evaluated by rebinding to fresh membranes (●——●), in the binding assay noted under Experimental, by precipitation with 10% TCA (○——○), and by immunoprecipitation (×——×), as described previously [1]. [^{125}I]-insulin incubated at 37°C in the absence of membranes (●- - -●) remained unaltered. Control refers to values obtained for unincubated [^{125}I]-insulin.

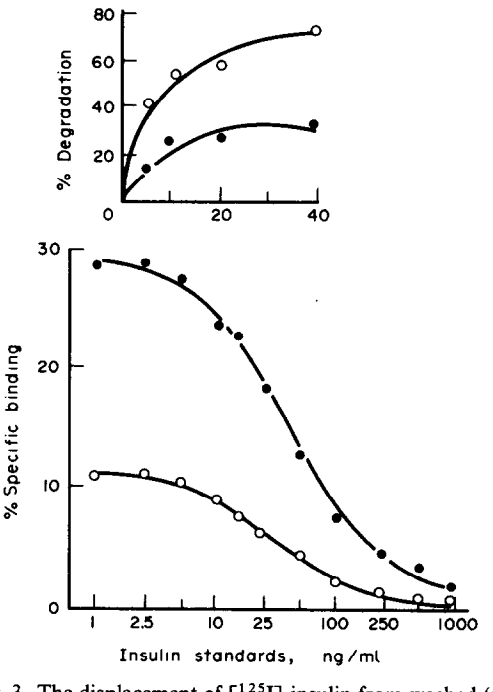

Fig. 3. The displacement of [^{125}I]-insulin from washed (●) and unwashed (○) human placental membranes (bottom), and the degradation of [^{125}I]-insulin with time by these same membranes (top). Degradation was evaluated only by the loss in rebinding ability.

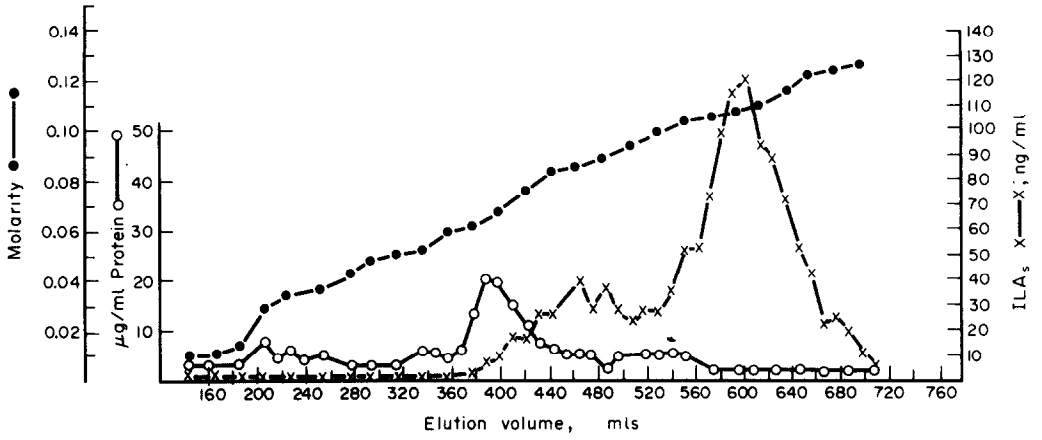

Fig. 4. Elution profile of ILAs and protein on CMC chromatography. The ILAs peak, obtained by chromatography of the acid-ethanol extract of Cohn IV-4 on G-75 sephadex in 1 M acetic acid, was concentrated by Amicon ultrafiltration (UM 02 filters), and dialysed against 0.01 M ammonium acetate pH 5.6. The material was applied to a 100 ml CMC column previously equilibrated in the same buffer. The column was washed with 4 vol. of buffer and eluted with a linear salt gradient (0.05 to 0.20 M ammonium acetate). Protein (O) was measured by fluorimetry, ILAs (×) by RRA and ionic strength (●) by conductometry.

insulin, comparable to what has been reported for bioassays [15] but less sensitive than an insulin radioimmunoassay (RIA) [16]. It has been previously noted that NSILA and somatomedin are very similar in many properties [3], and that somatomedin inhibits insulin binding to its receptors in fat, liver, and placental membranes [17, 18]. We have employed the human placental RRA for insulin to follow the purification of ILAs from human serum and Cohn fraction IV-4 plasma concentrates [4]. In confirmation of the observation of other investigators [3], Cohn fraction IV-4, was found to be especially enriched in ILAs. ILAs, extracted by an acid–ethanol procedure similar to that of Jakob *et al.*[5], was separated from the bulk of extracted proteins by chromatography on G-75 sephadex in 1 M acetic acid. The peak, identified by RRA, migrated at a Kav of 0.56 ± 0.01 (mean ± S.E.M., $N = 12$), distinctly faster than porcine insulin which had a Kav of 0.70. The peak was concentrated dialysed and applied to a column of carboxymethylcellulose. ILAs was eluted with a salt gradient as noted in Fig. 4. The peak of ILAs represents our most purified preparation to date and several such preparations ranged in specific activity from 215 to 408 mU/mg protein as measured in the insulin RRA.

Table 2 summarizes these chemical properties of ILAs and various bioactivities of this material that have been tested to date. It is evident that the material closely resembles NSILA-s and somatomedin in a number of properties [3].

B. *ILAs binding sites and their application.* ILAs was iodinated with chloramine T and the iodinated material was adsorbed from the reaction mixture with placental membranes. Subsequently the adsorbed radioactivity was eluted with 0.01 N HCl [4]. This procedure resulted in the preparation of [^{125}I]-ILAs free from excess ^{125}I. The iodinated material showed the same Kav on G-75 sephadex as uniodinated ILAs.

Figure 5 depicts the binding of [^{125}I]-ILAs to human placental membranes and its displacement by unlabeled ILAs. Small amounts of ILAs can be readily detected (*viz.* 1 ng/ml standard yields about 18% displacement). In contrast insulin has been shown to be a far poorer inhibitor of [^{125}I]-ILAs binding. A variety of other materials including nerve growth factor, epidermal growth factor, human growth hormone and glucagon had no significant inhibitory effect [4]. In addition to showing that bind-

Table 2.

Characteristics of serum ILAs

1. *Chemical properties*
   (a) Extracted by acid–ethanol
   (b) Small molecular weight (7–12 × 10^3 daltons by sephadex)
   (c) Cross-reactive in Insulin RRA but unreactive in Insulin RIA
   (d) S.A. of CMC Peak, 215 to 410 mU/mg.
2. *Bioactivities*
   ILA increased incorporation of
   (a) Glucose into rat adipose tissue triglyceride
   (b) [^{35}S]-Sulphate into porcine cartilage
   (c) [^{35}S]-Sulphate into purified proteoglycans of rabbit chondrocytes
   (d) [^{3}H]-thymidine into human fibroblast DNA

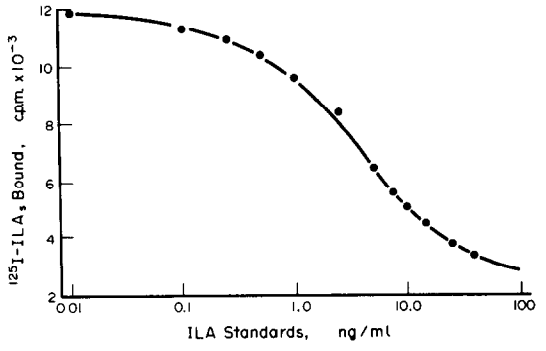

Fig. 5. Inhibition of [^{125}I]-ILAs binding to human placental membranes by unlabeled ILAs. Each incubation tube contained 30 × 10^3 c.p.m. of [^{125}I]-ILAs in a final 0.5 ml vol. The ILAs standards are denoted in terms of ng porcine insulin equivalents as determined by assay in the insulin RRA.

ing was highly specific it was shown that binding increased with an increase in either membrane or ILAs concentration [4].

Figure 6 demonstrates that ILAs chromatographed on G-75 sephadex can be identified and comparably quantitated by either the insulin or ILAs RRA.

In the study depicted in Fig. 7 serum from normal and hypophysectomized rats was chromatographed on G-75 sephadex at pH 2.0. The individual fractions were assayed in the ILAs RRA. Under these acid conditions of chromatography [^{125}I]-ILAs migrated at a Kav = 0.60 whereas under neutral conditions of chromatography (Fig. 7, inset) the [^{125}I]-ILAs migrated in association with the bulk of serum proteins at the void vol. of the column. It is evident that

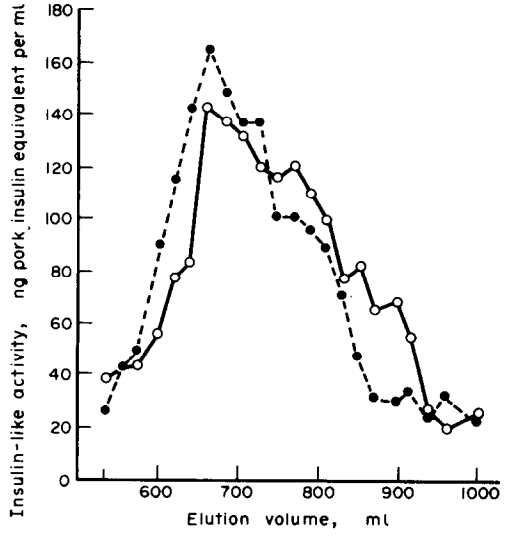

Fig. 6. Measurement of the ILAs peak of G-75 sephadex chromatography by both ILAs (○——○) and insulin (●---●) radioreceptor assays. The acid-ethanol extract of Cohn IV-4 was lyophilized and the powder resuspended in 1 M acetic acid and applied to and eluted from G-75 sephadex in 1 M acetic acid. The fractions were neutralized and assayed by insulin and ILAs RRAs. Only that portion of the chromatogram where the ILAs elutes is depicted.

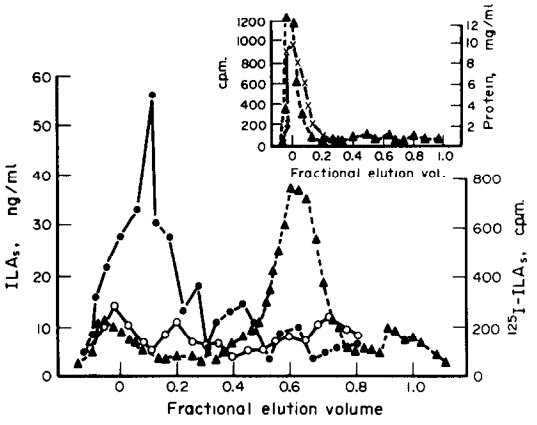

Fig. 7. Elution profile of [^{125}I]-ILAs (▲) and of ILA of normal (●) and hypophysectomized (○) rat sera. Five ml of pooled normal male rat serum (5 rats) were applied to and eluted from 160 ml G-75 sephadex in 1 M acetic acid. Individual fractions were neutralized and assayed in ILAs RRA. Five ml of pooled hypophysectomized male rat serum (5 rats) were chromatographed and assayed in the same manner. [^{125}I]-ILAs was mixed with normal serum prior to chromatography at acid pH and radioactivity in the elution fractions was determined. Inset: Elution profile of [^{125}I]-ILAs in normal rat serum chromatographed on G-75 sephadex at neutral pH. [^{125}I]-ILAs was mixed with normal male rat serum prior to chromatography on G-75 sephadex in 0.1 M ammonium acetate pH 7.4. ILAs radioactivity (▲---▲); Protein (×——×).

a large peak of cross reactive material, distinct from ILAs was readily observable in normal serum but comparable material was virtually absent in hypox serum. The peak material which crossreacts in the ILAs RRA was completely unreactive in the insulin RRA, and diluted in a nonparallel fashion to the ILAs standard curve depicted in Fig. 5.

DISCUSSION

The insulin binding sites of human placental membranes have properties expected of biologically important receptors as delineated in established insulin target tissues [8, 19]. The receptor is clearly capable of binding insulin at physiological levels. Though the significance of these receptors remains to be clarified it is possible that they mediate direct effects of insulin on placental function. Such direct effects have been difficult to identify with certainty [20, 21].

Insulin degrading activity in the placenta is due to several different enzymes. The two soluble degrading activities may serve a barrier function, and prevent maternal insulin from reaching the fetus. A large portion of membrane-associated degrading activity is not tightly coupled to the binding sites, since it can be removed from the membranes by washing. Without further study of more highly purified membrane preparations we cannot exclude the possibility that the residual membrane-associated degrading activity is not intrinsic to the membrane fractions bearing insulin receptors. The role of membrane-associated

degrading enzyme(s) may be to participate in the termination of hormone action and/or to regulate hormone concentration in the vicinity of the receptor.

The ILAs which we have isolated from human Cohn fraction IV closely resembles NSILAs and somatomedin [3]. There is not good reason to believe that it is different from NSILAs except that it has been isolated in a different laboratory using an insulin RRA rather than an insulin bioassay as was employed in the purification of NSILAs [5]. Since this group of substances has been implicated in the regulation of growth it is tempting to consider that ILAs may play a role in regulating placental growth. Two conditions must be met in order to consider ILAs as having a possible influence on placental function. First, the capacity for ILAs to interact with the placenta must be demonstrable. Second, ILAs must be present in the circulation in a form which is capable of interaction with the tissue.

The present study demonstrates that ILAs, like somatomedins A and C [18, 22] binds to a class of sites in human placenta which is different from the insulin receptor. Thus the potential for an influence of ILAs on placental function has been elicited.

The nature of ILA in both human and rat sera is being studied in our laboratory. The general approach undertaken has been summarized in Fig. 7. It is noteworthy that [^{125}I]-ILAs binds to serum protein(s) at neutral but not acid pH. At acid pH the large bulk of material which cross reacted in our RRA for ILA was large molecular weight. It was distinguishable from ILAs not only on the basis of size but also on the basis of nonparallelism in the RRA. We have not excluded the possibility that this cross-reactive material is a binding protein for ILAs which is measured in our RRA on the basis of its competition with the placental receptor for the binding of [^{125}I]-ILAs. This latter possibility must be especially considered since, as seen in the present study and in the work of others [23], ILAs and identical or related peptides are bound at neutral pH to serum protein(s).

The level of crossreactive material was very much reduced by hypophysectomy. Recent studies in our laboratory (Posner and Guyda, unpublished) have shown that treatment of hypophysectomized rats with bovine growth hormone alone restored the level of crossreacting material to normal. The growth hormone dependency of the large molecular weight material is compatible with the view that it is involved in the modulation of the growth response. Further work is required to define the nature of this material more precisely, especially in relation to the characteristics of ILAs, and to evaluate it as either causative or reflective of the growing state.

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DISCUSSIONS

Naftolin. I wonder if you could tell us a little more about the placenta. What it was in fact that you were looking at, what part of the placenta. We have talked about this before it is not just the placenta. Can you describe a little bit better what you were really looking at, what tissue.

Posner. I would like to be able to help, I think what you are getting at if I may take the liberty of interpreting your question, is where these receptors are to be located and we would really like to be able to establish that. Up to now we have not done so. We have focused on rather limited recourses on certain aspects of the problem and not that aspect. That is clearly a very important question, we suspect in view of the richness and the extent of the material that it must be associated with the trophoblastic membrane but it obviously remains to be established with certainty. The way in which we prepare the material, is, we take fresh placenta, we strip away the membranes and we cut it into wedge shaped sections and we freeze it at -20° and we find we can continue to use that material, we simply thaw it on ice and homogenize it by chopping it up and homogenizing the whole wedge and preparing our material from that as I have showed you. It seems to be, if we keep it frozen, stable in terms of membrane protein and in terms of the kind of binding activity we observe. Once the membranes are prepared they can be frozen and thawed several times. I think beyond two or three times they begin to deteriorate in binding activity but if we store them in aliquots they seem to be stable for a very long time.

Naftolin. Do you know where the decidua binds?

Posner. No, we have not specifically examined separate components and individual fractions.

Naftolin. Do you know if there is a sex difference? As you know there are boy and girl placentas.

Posner. We have not correlated that in any systematic way.

Naftolin. This is a very confused area. I wonder if you can expand on proinsulin versus nerve growth factor.

Posner. Proinsulin is clearly a very different thing from ILA. Proinsulin cross reacts reasonably well in an insulin radioimmunoassay; this material does not cross react at all. Proinsulin, in terms of its capacity to displace ILA from membranes, is weaker than insulin. Also the spectrum of bioactivities is very different. Proinsulin is not particularly potent in terms of stimulating thymidine incorporation into DNA. In fact, like insulin, it is required in very large concentrations in order to do that. Epiderm growth factor is an entirely different entity. It is of larger molecular weight and has a different spectrum of bioactivities; nor does it cross react at all in the insulin radioreceptor assay.

Postenvinhenvis. Dr Posner are you sure there is an increasing binding capacity of the placenta membranes, binding of insulin, with age of gestation. Can you comment on that, with another example of the modulation of sites by the genes hormone.

Posner. I have no way of evaluating that in this situation. I would emphasise that one should be cautious about looking at binding sites in tissues at different stages of development. We may be looking at different relative distributions of cell populations rather than changes in receptor concentrations in a particular cell type. Striking changes are seen in guinea pig fetal tissues during development (*Endocrinology* **96** (1974) 532–539). In regard to insulin binding guinea pig tissues the binding pattern as a function of fetal age changes in a different manner for the different tissues studied. This suggests that there must be tissue specific factors that are very important in determining local receptor levels.