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Insulin Binding to Isolated Liver Nuclei from Obese and Lean Mice†

Jo Alene Goidl

ABSTRACT: Nuclei isolated from the livers of mice are capable of binding [^{125}I]insulin. A class of high-affinity binding sites having a K_d of 1–2 nM and a capacity of ~2000 insulin molecules/nucleus are present on these nuclei. Removal of nuclear membranes by Triton X-100 treatment of the nuclei reduces or eliminates the high-affinity binding sites. Nuclei prepared from livers of the genetically obese mouse (ob/ob) lack, or have markedly reduced numbers of, the high-affinity binding sites whether or not the obese nuclei have been exposed to Triton X-100. The reduced insulin-binding capacity of the obese nuclei correlates with the reported decreased binding of insulin to plasma membranes prepared from target tissue of these animals. The possible physiological significance of nuclear insulin binding is discussed.

Although the theory that insulin does not enter its target cell has been in doubt for some time (Davidson et al., 1973; Katzen & Vlahakes, 1973; Kolb et al., 1975), the concept of an extracellular site as the primary locale for insulin action has remained prevalent. Yet there is reason to doubt a unitary mechanism of hormone action in which insulin mediates its effects solely through binding to receptors at the cell surface. To date a second messenger, comparable to cyclic AMP, which would relay information from the receptors to the intracellular sites where insulin exerts its effects, such as on RNA synthesis (Pilkis & Salaman, 1972) and enzyme induction (Pilkis, 1970; Reel et al., 1970), has not been identified. In addition, correction of defective insulin receptors does not necessarily restore normal metabolic responses to the hormone (Abraham & Beloff-Chain, 1971; Le Marchand et al., 1977).

Recently, the internalization of insulin has been demonstrated (Schlessinger et al., 1978; Carpentier et al., 1978). In addition, there have been several reports characterizing the binding of insulin to various organelles (Bergeron et al., 1978; Horvat et al., 1975; Kahn, 1976; Posner et al., 1978a,b). The findings described in these studies suggest the possibility of intracellular binding sites in the mechanism of action of this important, but still poorly defined, hormone.

In several experimental models of insulin resistance, including the genetically obese mouse (ob/ob), a reduction in the number of insulin receptors associated with the plasma membranes of target tissues has been observed (Goldfine et al., 1973; Harrison et al., 1976; Kahn et al., 1972; Kern et al., 1975; Olefsky & Reaven, 1974; Soll et al., 1975a,b). The present communication describes the binding of insulin to nuclei isolated from livers of these obese mice and their lean littermates. The reduced binding to the "obese nuclei", which correlates with the decreased plasma membrane binding, suggests that the liver cell nucleus may play a physiological role in determining the state of responsiveness to insulin.

Materials and Methods

Female ob/ob mice, their lean littermates, and C57Bl/6J mice were purchased from the Jackson Laboratory, Bar Harbor, ME. [^{125}I]Insulin (~100 μCi/μg) and Aquasol-2 were purchased from New England Nuclear. [2-^{3}H]AMP (18.5 Ci/mmol), [8-^{14}C]adenosine (59 mCi/mmol), and Triton X-100 were obtained from Amersham Corp. Bovine insulin and synthetic somatostatin were products of Calbiochem. Porcine proinsulin, desoctapeptide insulin, and desalanine insulin were gifts from the Eli Lilly Co. Murine EGF[1] was generously provided by C. Richard Savage of Temple University Medical School, Philadelphia, PA. Insulin radioimmunoassay kits and crystallized glucagon were purchased from Schwarz/Mann. Porcine FSH, ovine prolactin, bovine TSH, synthetic oxytocin, and pancreozymin (Grade II) were all products of Sigma. PEI–cellulose F plates for thin-layer chromatography were obtained from EM Laboratories, Inc. Ready-Solv was purchased from Beckman Instruments. Protein assay kits were purchased from Bio-Rad.

Nuclear Preparation. Mice were sacrificed by cervical dislocation. Nuclei were prepared from mouse livers by using the procedure of Blobel & Potter (1966). Detergent-treated nuclei were prepared by suspending the first 340*g* nuclear pellet in TSM buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, and 3 mM $MgCl_2$) containing 0.5% Triton X-100 and incubating the nuclei at 0–4 °C for 5–10 min. The nuclei were sedimented at 340*g* for 5 min and suspended in TSC buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.5, and 3 mM $CaCl_2$). Subsequent procedures for both detergent-treated and untreated nuclei were identical. The final nuclear pellets obtained after sedimentation through the 2.2 M sucrose cushion were washed in TSM buffer and then suspended in a volume of assay buffer (0.25 M sucrose, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, 2 mM Na_2EDTA, and 5 mg/mL BSA) such that the concentration of DNA was ~1 mg/mL.

High-Speed Supernatant (S100) and Microsomal Preparation. S100 cytosol fractions and microsomal fractions were prepared from the first 340*g* supernatant obtained during nuclear isolation. This low-speed supernatant was centrifuged at 30000*g* for 30 min. The 30000*g* supernatant was then

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[1] Abbreviations used: EGF, epidermal growth factor; FSH, follicle stimulating hormone; TSH, thyroid stimulating hormone; PEI, polyethylenimine; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid; BSA, bovine serum albumin.

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centrifuged at 50 000 rpm for 60 min in a Beckman type 65 rotor. The upper two-thirds of this 100000*g* supernatant was taken as the S100 fraction. At each step care was taken to resuspend as much of the lipid cake as possible. The S100 from lean mouse liver contained 86 mg of protein/mL with an A_{260}/A_{280} of 0.97. The S100 from obese mouse liver contained 49 mg of protein/mL with an A_{260}/A_{280} of 1.0.

The 100000*g* pellet, the microsomal fraction, was suspended in TSM buffer at a concentration of ~100 A_{260} units/mL by gentle stirring at 0–4 °C.

Plasma Membrane Preparation. Plasma membranes were prepared from mouse liver according to the Neville procedure (Neville, 1968) through step 11.

Electron Microscopy. Nuclear samples for ultrastructural examination were fixed in 2.5% glutaraldehyde in Sorensen's phosphate buffer (pH 7.4) and postfixed in phosphate-buffered 1% osmium tetroxide. Samples were dehydrated through ethanol and embedded in Epon 812. Thin sections were stained with uranyl acetate and Reynolds' lead citrate and examined in a Phillips 200 transmission electron microscope.

Enzyme Assays. Glucose-6-phosphatase activity (EC 3.1.3.9) was measured in homogenates and nuclear preparations according to the procedure of Baginski et al. (1974).

For determination of 5′-nucleotidase activity (EC 3.1.3.5), the conversion of [^{3}H]AMP to [^{3}H]adenosine was measured by thin-layer chromatography. Homogenates or nuclear preparations were incubated for various times at 37 °C in the presence of 1.0 mM [^{3}H]AMP with 1.0 mM $MnSO_4$ in a 40 mM veronal buffer. The total volume was 50 μL. The reaction was stopped by the addition of 10 μL of 50% trichloroacetic acid, and the acid-precipitable material was removed by centrifugation. Aliquots of the supernatants were spotted on PEI–cellulose plates, and the chromatograms were developed in 0.2 M LiCl. Chromatograms were cut into 1-mm square strips and counted in 10 mL of Aquasol-2 in a Beckman LS-355 liquid scintillation counter. [^{14}C]Adenosine was run as a marker. Calculations of enzymatic activities were based on initial velocities of the appearance of [^{3}H]adenosine.

Protein was determined by the Lowry procedure (Lowry et al., 1951) by using BSA as a standard or by the Bio-Rad protein assay based on the procedure of Bradford (1976) using bovine γ-globulin as a standard.

DNA was determined according to Burton (1956) by using calf thymus DNA as a standard.

Insulin Binding Assays. The binding of insulin to isolated nuclei was measured by incubating the nuclei in a total volume of 0.5 mL of assay buffer (~0.5 mg of DNA/assay) with 6.7 × 10^{-11} M [^{125}I]insulin (0.4 ng/mL) for 90 min at room temperature (22–26 °C), except where indicated. The reaction was terminated by underlaying the binding assay mixture with 0.5 mL of 0.8 M sucrose and sedimenting the nuclei through the sucrose cushion in a Beckman TJ-6 centrifuge at 3000 rpm for 5 min. The nuclear pellets were suspended in 0.5 mL of TSM buffer and counted in 10 mL of Ready-Solv in a Beckman LS-355 liquid scintillation counter. The efficiency of counting ^{125}I was 40%. Duplicate samples were incubated in the presence or absence of 2.0 μg/mL unlabeled insulin. The difference between [^{125}I]insulin bound in the absence and presence of excess cold insulin represents specific binding.

The binding of insulin to plasma membranes was measured according to Freychet et al. (1971).

Results

Purity of Nuclear Preparations. Ultrastructural examination of nuclei prepared from livers of obese and lean mice revealed minimal contamination by cytoplasmic components

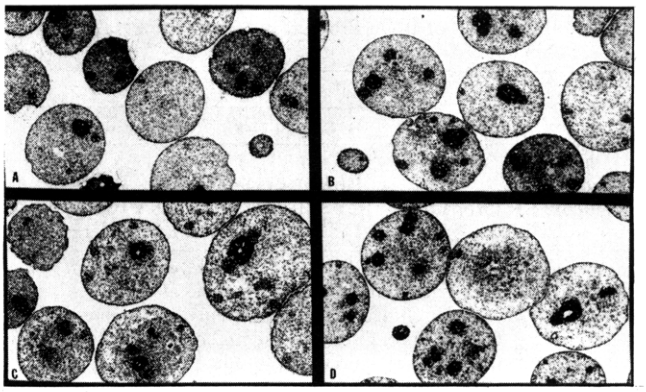

FIGURE 1: Electron micrographs of lean and obese nuclei. Nuclei were prepared from the livers of lean and obese mice with or without Triton X-100 as described under Materials and Methods. (A) Lean nuclei without detergent treatment; (B) lean nuclei with Triton X-100 treatment; (C) obese nuclei without detergent treatment; (D) obese nuclei with Triton X-100 treatment (×1625).

(Figure 1). Nuclei isolated in the absence of detergent (parts A and C of Figure 1) retained outer nuclear membranes. Detergent-treated nuclei were stripped of outer nuclear membranes with only an occasional tab apparent (parts B and D of Figure 1). The electron micrographs also revealed no apparent differences between liver nuclei from lean mice (parts A and B of Figure 1) and nuclei from obese mice (parts C and D of Figure 1). This is in contrast to the morphological differences between hepatocytes from these animals (Kahn et al., 1973). Hepatocytes from obese mice were larger and contained many more vacuoles, presumably containing more lipid and/or glycogen as compared with hepatocytes from lean mice. Although nuclei from both lean and obese mouse liver contained equivalent amounts of DNA (~12 pg/nucleus), the obese mouse liver contained about half as much DNA per gram of liver as the lean mouse liver (data not shown). It therefore seems likely that the two- to threefold greater weight of obese mouse livers, compared with lean mouse livers, represents larger cell size, due primarily to increased lipid and glycogen, rather than differences in cell number.

Nuclear preparations were assayed for two marker enzymes, glucose-6-phosphatase, an enzyme found predominantly associated with endoplasmic reticulum, and 5′-nucleotidase, an enzyme associated almost exclusively with plasma membranes. As shown in Table I, while ~30% of the total DNA found in a liver homogenate was recovered in the purified nuclear fractions, less than 1% of the total glucose-6-phosphatase and 1–2% of the total 5′-nucleotidase were found associated with the nuclei. Marker enzyme recoveries were only slightly altered in detergent-treated nuclei. On the assumption of a 30% recovery of nuclei, the maximal contamination by nonnuclear components would represent less than 6%.

Based on the ultrastructural and enzymatic analyses, it was concluded that the nuclear preparations were of high purity.

Binding of Insulin to Lean Mouse Liver Nuclei. Preliminary studies indicated that binding of insulin to nuclei prepared from livers of lean littermates of the obese mice, i.e., ob/+, and C57Bl/6J (+/+) mice was identical (data not shown). These lean animals have, therefore, been used interchangeably depending on the availability of the animals.

The binding of insulin to nuclei isolated from livers of lean mice was linear for an hour at 22–26 °C (Figure 2). Nonspecific binding represented ~40% of total binding at a level of 5000-fold excess cold insulin. When binding was carried out at 37 °C, total binding of insulin was increased. However, nonspecific binding of the hormone was increased

Table I: Recoveries of DNA, Protein, and Marker Enzymes in Nuclear Preparations

	percent	
	triton − nuclei[a]	triton + nuclei[b]
DNA	31.1 ± 4.8[c] (5)	34.6 ± 5.9[c] (4)
protein	1.0 ± 0.2 (5)	1.0 ± 0.2 (4)
glucose-6-phosphatase	0.8 ± 0.1 (3)	0.8 ± 0.2 (2)
5'-nucleotidase	1.2 ± 0.005 (2)	1.0 ± 0.1 (2)

[a] Nuclei were prepared from livers of lean mice without detergent treatment. Determinations were performed as described under Materials and Methods. [b] Nuclei were prepared from livers of lean mice. The procedure included treatment with Triton X-100 as described under Materials and Methods. [c] Data are expressed as a percentage of DNA, protein, or enzymatic activity in a volume of homogenate equivalent to that from which nuclei were prepared. The values represent the averages of the results of the number of experiments in parentheses ±1 SEM.

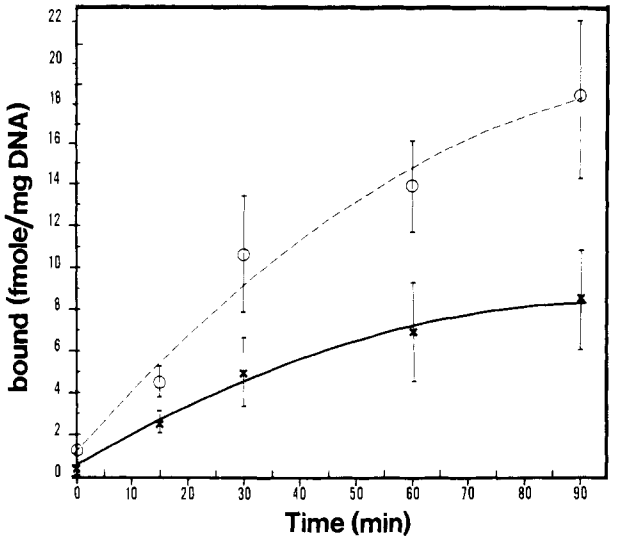

FIGURE 2: Time course of insulin binding. Nuclei were prepared from lean mouse livers without detergent treatment. The nuclei were incubated with 6.7×10^{-11} M [^{125}I]insulin (0.4 ng/mL) in the presence or absence of 2.0 μg/mL unlabeled insulin at 22–26 °C for the indicated times. (O---O) Total binding in the absence of excess cold insulin; (×—×) nonspecific binding in the presence of excess cold insulin. The error bars give the standard error of the mean obtained from three experiments.

to such a level that specific binding was obliterated. Binding at 0 °C represented about one-third of that at room temperature. The rapid loss of specific insulin binding at 37 °C might represent a mechanism by which a transient rather than a sustained response to the hormone could occur under physiological conditions.

The specificity of these nuclear binding sites for insulin is illustrated in Figure 3. Unlabeled insulin and desalanine insulin, an analogue of insulin which has a biological potency identical with native insulin (Rudman et al., 1968), competed for the binding of [^{125}I]insulin to isolated nuclei far more than any other hormone tested. Proinsulin and desoctapeptide insulin, analogues of insulin having reduced biological activity relative to native insulin (Freychet et al., 1971), were less effective in reducing the binding of [^{125}I]insulin. Structurally unrelated polypeptides, EGF, glucagon, and somatostatin, had no inhibitory effect. Several other hormones (FSH, TSH, prolactin, oxytocin, and pancreozymin) were also ineffective in competing for insulin-binding sites on isolated nuclei (data not shown).

Figure 4 shows the binding of insulin to nuclei isolated from livers of lean mice. A Scatchard plot of this data (Figure 5)

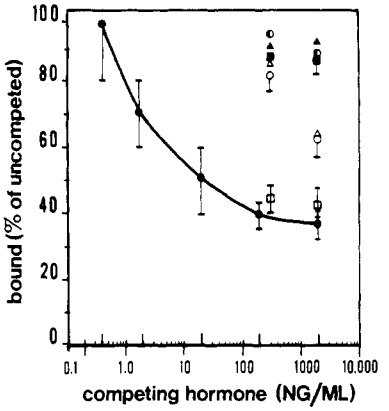

FIGURE 3: Specificity of insulin binding. Nondetergent-treated nuclei from lean mouse livers were incubated with 6.7×10^{-11} M [^{125}I]insulin (0.4 ng/mL) with increasing amounts of unlabeled insulin (●) or with either 400 or 2 μg/mL proinsulin (○), desoctapeptide insulin (△), desalanine insulin (□), EGF (▲), glucagon (■), or somatostatin (◐). The incubation was carried out for 90 min at 22–26 °C. The data are expressed as the amount of [^{125}I]insulin bound in the presence of competing hormone as a percentage of that bound in the absence of competing hormone. The error bars give the standard error of the mean obtained from 3–5 experiments.

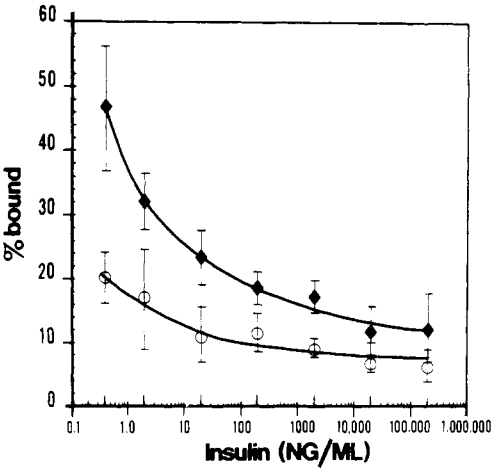

FIGURE 4: Binding of insulin to lean nuclei. Nuclei prepared from livers of lean mice with or without detergent treatment were incubated in the presence of 6.7×10^{-11} M [^{125}I]insulin (0.4 ng/mL) with increasing amounts of unlabeled insulin for 90 min at 22–26 °C. The amount of [^{125}I]insulin bound/mg of DNA is expressed as a percentage of the input [^{125}I]insulin (33.3 fmol). The error bars give the standard error of the mean obtained from 3–5 experiments. (♦) Nuclei prepared without detergent treatment; (○) nuclei prepared with a Triton X-100 treatment.

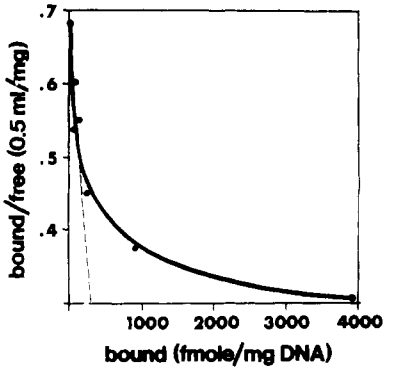

FIGURE 5: Scatchard plot of binding data from lean nuclei without Triton X-100 treatment.

reveals the nonlinear curve typical of insulin binding. The high-affinity portion of the curve indicates the existence of a

Table II: Purification of Nuclei in the Presence or Absence of Additional Plasma Membranes[a]

	membranes added	membranes not added
[^{125}I]insulin specifically bound (fmol)	2.10 ± 0.52	1.85 ± 0.43
protein (mg/assay)	4.92 ± 1.70	4.98 ± 1.74
insulin/protein (fmol/mg)	0.59 ± 0.24[b]	0.51 ± 0.20[b]

[a] Partially purified plasma membranes were added to one-half of a homogenate of lean mouse liver. Nuclei were then prepared from both homogenates, and insulin binding to the nuclei was measured as described under Materials and Methods. The values represent the averages of three experiments ±1 SEM. [b] Not significantly different by paired *t* analysis.

class of binding sites that have a K_d of 1–2 nM and a binding capacity of ~200–300 fmol/mg of DNA. Such a binding capacity represents about 2000 molecules of insulin/nucleus.

When the nuclear preparation procedure included a detergent treatment of the nuclei, the high-affinity binding sites were dramatically reduced in number (Figure 4). Since Triton X-100 is known to remove outer nuclear membranes, and perhaps inner membranes as well (Aaronson & Blobel, 1974), this finding indicates that the high-affinity sites are associated with the nuclear membrane.

Although the low recovery of 5′-nucleotidase activity in nuclei (Table I) suggests that plasma membrane contamination is minimal, it is possible that the enzyme is masked or inactivated during the nuclear isolation procedure. Therefore, another experiment was designed to determine if the binding observed in the purified nuclear fraction is due to plasma membranes which have copurified with the nuclei. Partially purified mouse liver plasma membranes [floated particles; see Neville (1968)] were added to a homogenate from an equivalent amount of mouse liver, and the nuclei were purified. Table II shows that the binding of insulin to nuclei prepared in the presence or absence of additional plasma membranes is identical. It therefore seems unlikely that nuclear binding represents plasma membranes adventitiously associated with nuclei. In a similar experiment in which a microsomal preparation was added to crude nuclei and the nuclei were subsequently purified, the binding of insulin to nuclei was not altered (data not shown).

Binding of Insulin to Obese Mouse Liver Nuclei. Figure 6 shows the binding of insulin to nuclei prepared from livers of obese mice. Even nuclei with intact nuclear membranes had reduced binding, representing ~30% of the maximal binding observed with lean nuclei. As noted with detergent-treated nuclei from lean mouse livers (Figure 4), it appears that there was a dramatic loss of high-affinity binding sites on obese nuclei, primarily those on the nuclear membranes, rather than a change in the affinity of the binding of the hormone. The fact that there was some competition with unlabeled insulin noted in obese nuclei and detergent-treated lean nuclei suggests that this binding is not merely nonspecific but rather represents low-affinity insulin-binding sites. Presumably because of the limited number of high-affinity binding sites, Scatchard analyses of these data were not possible. The lack of an effect of detergent on the nuclei from obese mouse liver supports the conclusion that the high-affinity sites are associated with the nuclear membrane in lean mouse liver nuclei but are reduced in number or absent in obese mouse liver nuclear membranes.

In order to rule out the possibility that the reduced nuclear binding was a result of binding sites already occupied by endogenous insulin, we assayed obese and lean nuclei for the

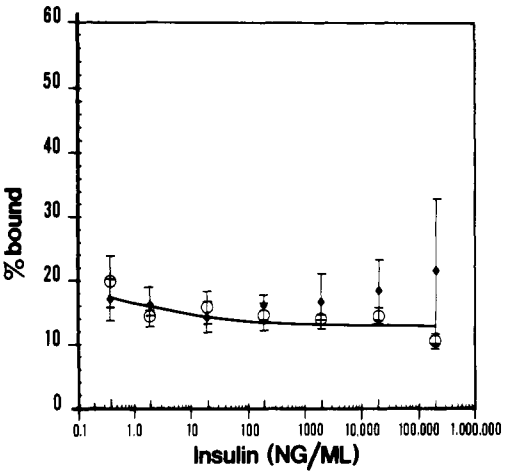

FIGURE 6: Binding of insulin to obese nuclei. Nuclei prepared from livers of obese mice with or without detergent treatment were incubated in the presence of 6.7×10^{-11} M [^{125}I]insulin (0.4 ng/mL) with increasing amounts of unlabeled insulin for 90 min at 22–26 °C. Data are expressed as described in Figure 4. Standard errors were obtained from 2–5 experiments. (♦) Nuclei prepared without detergent treatment; (○) nuclei prepared with a Triton X-100 treatment.

presence of insulin by radioimmunoassay. Although sera from the mice contained levels of insulin characteristic of the normal (lean) and hyperinsulinemic (obese) mice, 29 ± 5.4 μunits/mL and 293 ± 64 μunits/mL, respectively, insulin was not detectable in nuclear preparations from either obese or lean mouse livers (data not shown). Kahn et al. (1973) injected lean mice with insulin prior to preparing liver plasma membranes. Insulin binding to these plasma membranes was indistinguishable from that of untreated lean mice. Thus, it seems unlikely that the reduction in insulin binding to plasma membranes or to nuclear membranes is due to the elevated circulating insulin levels of the obese mouse.

Because the cytosol from obese mouse liver clearly contains much more lipid and glycogen than lean mouse liver cytosol, it seemed possible that the reduced nuclear binding observed with nuclei from obese mice might be due to a detergent-like effect of the obese cytosol during the nuclear purification procedure. In order to examine this possibility, we performed the following experiment. S100 fractions were prepared from obese and lean livers. Obese and lean crude nuclear pellets from the first low-speed spin (see Materials and Methods) were then suspended in homologous or heterologous S100 and incubated for 10 min at 0–4 °C. Purified nuclei were then prepared as usual and insulin-binding assays performed. In two separate experiments neither homologous nor heterologous S100 had any effect on the binding of insulin to obese or lean mouse liver nuclei (data not shown).

Discussion

Recently, Goldfine and his associates have described the binding of insulin to nuclei isolated from rat liver and cultured human lymphocytes (Goldfine & Smith, 1976; Goldfine et al., 1977a; 1978). The association of insulin with nuclei was observed whether the binding was carried out in vitro by using isolated nuclei or by using nuclei isolated from lymphocytes cultured in the presence of [^{125}I]insulin. The nuclear binding sites were immunologically distinct from plasma membrane binding sites (Goldfine et al., 1977b). The nuclear binding sites have been localized primarily to the nuclear membrane, and, in fact, binding of insulin can be demonstrated by using purified nuclear membranes (Horvat, 1978; Vigneri et al., 1978a). That the nuclear binding sites are regulated by plasma

insulin concentrations in a manner similar to that observed with plasma membranes has also been demonstrated (Vigneri et al., 1978b). In rats made hypoinsulinemic by streptozotocin treatment, nuclear binding of insulin was elevated 25% over that of controls. Conversely, rats made hyperinsulinemic with insulin injections exhibited a 36% decrease in nuclear binding as compared with controls. The present study confirms and extends the findings of Goldfine and his co-workers and of Horvat to the hyperinsulinemic obese mouse. The reduced binding to "obese nuclei" relative to the binding to "lean nuclei" suggests that nuclear insulin binding represents "down regulation" and is a physiologically significant phenomenon rather than an artifact of the in vitro system.

The binding of insulin to Golgi fractions prepared from two strains of obese mice (ob/ob and db/db) and from their lean littermates has been examined by Posner et al. (1978b). While insulin binding was reduced in Golgi vesicles from obese mouse liver comparable to the decrease in plasma membrane binding, Golgi cisternae prepared from either obese (ob/ob) or lean mouse liver bound insulin to the same extent. In fact, Golgi cisternae isolated from the db/db mouse displayed increased hormone binding relative to "lean Golgi cisternae". These investigators concluded that cisternae insulin receptors are regulated in a manner different from those associated with the plasma membrane. They propose that the Golgi binding sites are precursors for those on the plasma membrane and that the Golgi apparatus, in binding the hormone, may participate in the regulation of cell surface hormone receptors.

At present, the functional significance of nuclear binding of insulin is unknown. While it is possible that the relative nuclear binding capacity is merely reflective of the metabolic state of the cell, it has been suggested (Goldfine & Smith, 1976) that the nuclear binding sites may play a role in the long-term effects of insulin, such as protein synthesis. It has been demonstrated that the restoration of normal binding of insulin to liver and adipose plasma membranes does not necessarily restore normal metabolic function in these tissues (Le Marchand et al., 1977). Possibly the binding of hormone to nuclear sites may correlate better with the metabolic state of the tissue than is apparently the case with plasma membrane binding. In another animal model of insulin resistance, the hyperinsulinemic Zucker rat (fa/fa), insulin binding to hepatocytes was reduced relative to lean animals (fa/+) only when the rats had been starved for 24 h (Broer et al., 1977). Here again, the nuclear binding capacity for insulin may provide some clue as to the hormonal regulation of liver metabolism.

Recently Krupp & Livingston (1978) reported the solubilization of two insulin-binding proteins from fat cell membranes. In the presence of insulin there was evidence that the high-affinity species was converted to the lower affinity species. Such a process might explain the reduction or absence of high-affinity binding sites associated with plasma membranes and nuclear membranes of the hyperinsulinemic mouse. It will be interesting to determine whether proteins similar to those described by Krupp & Livingston are present in the nuclear membranes and whether, in fact, the high-affinity species are lacking in the nuclear membranes of the obese mouse liver.

Acknowledgments

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Analysis of the Molecular Species of the Chick Oviduct Progesterone Receptor Using Isoelectric Focusing†

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ABSTRACT: Conditions are described for the preparative isoelectric focusing in flat beds of Sephadex of the progesterone receptor from the chick oviduct. The method allows the fractionation of the receptor into two molecular species, one focusing at p*I* 6 and the other at p*I* 7 with good purification and recovery. The p*I* 6 and p*I* 7 receptor species were purified 2- and 26-fold, respectively. The assaying of the focused fractions with the charcoal binding method provides an accurate identification and quantitation of the [^{3}H]progesterone receptor. The method is reproducible in recovery, quantitation, and resolution of the two receptor species. The receptor with an apparent p*I* of 6 sediments at ~4 S on linear sucrose gradients, while the receptor with an apparent p*I* of 7 sediments at ~3.5 S. On the basis of the sedimentation values and elution patterns from diethylaminoethyl (DEAE) chromatography, the p*I* 6 component is equivalent to the "B" receptor species and the p*I* 7 component is equivalent to the "A" receptor species described previously [Schrader, W. T., & O'Malley, B. W. (1972) *J. Biol. Chem. 241*, 51–59].

Isoelectric focusing has proven to be a useful technique for the separation of proteins and other amphoteric substances which possess different isoelectric points. Many earlier studies on the isoelectric focusing of steroid receptor proteins have utilized predominantly vertical columns containing gradients of sucrose as the media to support the pH gradient and the zones of focused protein (Schrader & O'Malley, 1972; Sherman et al., 1974). However, IEF[1] in sucrose gradients has several inherent disadvantages which include (1) limitations in the amount of sample which can be successfully focused, (2) failure of the gradient to support proteins which precipitate at their p*I*, (3) extended times required for focusing proteins to equilibrium (24–72 h), and (4) diffusion and mixing of focused zones during the elution procedure (Radola, 1973a, 1975; Sherman, 1975). These technical difficulties have been essentially circumvented by the use of either polyacrylamide gel or granulated gel as the support media (Radola, 1973a,b, 1975). The granulated gel has advantages over polyacrylamide with respect to improved recovery of proteins.

This paper describes an IEF method for fractionating the [^{3}H]progesterone–receptor complex into two molecular species which correspond to the "A" and "B" receptor species described previously (Schrader & O'Malley, 1972; Schrader et al., 1972). Partially purified [^{3}H]P–R isolated from the oviducts of estrogen-pretreated immature chicks was focused on flat beds of Sephadex G-75 superfine resin by using select conditions of temperature, buffers, and periods of focusing. This method separates the [^{3}H]P–R into two molecular species in a relatively short period (8 h) with good recovery, reproducibility, resolution, and partial purification.

Materials and Methods

Steroids. [1,2-$^3H_2$]Progesterone (40–60 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Unlabeled progesterone was obtained from Sigma Chemical Co. (St. Louis, MO). The degree of chemical purity of these steroids was checked by high-pressure liquid chromatography using a linear gradient of 30–65% acetonitrile in water over a 1-h period in a reverse phase C_{18} μBondapak column (Waters Associates, Milford, MA). The radioactivity eluting from the column was collected by a fraction collector and the fractions were counted. Greater than 90% purity was found for all preparations used. The stock [^{3}H]progesterone solution in benzene–ethanol (9:1) was frozen, freeze-dried, and redissolved to the original volume with ethanol. When needed, this stock was diluted 1:5 with water to give a 4 μM [^{3}H]progesterone solution in 80% water–20% ethanol (v/v). This diluted stock solution of [^{3}H]P was added directly to the cytosol preparations to obtain labeled progesterone–receptor complexes as described below.

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[1] Abbreviations used: [^{3}H]P, [^{3}H]progesterone; [^{3}H]P–R, [^{3}H]-progesterone–receptor; IEF, isoelectric focusing; Tris, tris(hydroxymethyl)aminomethane; EDTA, disodium ethylenediaminetetraacetate; $(NH_4)_2SO_4$, ammonium sulfate.

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