Insulin binding to liver plasma membranes from rats rendered diabetic by alloxan

A kinetic demonstration of two classes of binding sites in equilibrium with each other

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The association of ^{125}I -labelled insulin with liver plasma membranes from diabetic rats was consistent with more than one compartment of binding. After a short association period, insulin dissociation comprised rapid and slow phases. After a long association period, dissociation was only at a slow rate. Lower-affinity hormone–receptor complexes were converted to higher-affinity complexes as the time of occupancy lengthened.

The resistance of target tissues to the actions of insulin may be related to pathophysiological states (Crettaz & Jeanrenaud, 1980; Olefsky, 1980). Liver, muscle and adipose tissue from animals rendered diabetic by alloxan or streptozotocin exhibit resistance to insulin (Kobayashi & Olefsky, 1979, and references cited therein). Attempts to demonstrate resistance at the level of the receptor have not been generally successful (Livingston *et al.*, 1978; Olefsky, 1980). However, since the first step in insulin action is binding to surface receptors on target cells (Cuatrecasas, 1974; Czech, 1977), it may still be reasonable to consider this interaction as being related to the lesion leading to resistance to insulin in alloxan-induced diabetes. A decrease in the ability of ^{125}I -labelled insulin to dissociate from liver (Carpentier *et al.*, 1979) and adipose (Kahn & Baird, 1978; Olefsky *et al.*, 1979) cells as the association time was lengthened has been reported. Based upon similar observations, we recently described the conversion of occupied hepatic insulin receptors from a state from which insulin dissociates rapidly (lower-affinity complexes) to a state from which insulin dissociates slowly (higher-affinity complexes) (Donner & Corin, 1980; McCaleb & Donner, 1981; Corrin & Donner, 1982). Regulatory significance was attributed to this phenomenon. It is not known if receptors from alloxan-induced-diabetic animals exhibit this property. We therefore examined the dissociation of ^{125}I -labelled insulin from liver plasma membranes of rats made diabetic with alloxan as a function of the length of association time.

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Materials and methods

The sources of all materials and the preparation of ^{125}I -labelled insulin (specific radioactivity 150–200 Ci/g) were as previously described (Donner & Corin, 1980). Male, Sprague–Dawley rats (125–150 g), rendered diabetic by a single subcutaneous injection of alloxan (175 mg/kg body wt.) after overnight starvation, were used after 7–10 days. Animals were fed Purina laboratory chow *ad libitum*. Diabetes was indicated by high urinary glucose (>2%, w/v), and decreased plasma insulin levels ($8.35 \pm 9.43 \mu\text{units/ml}$; $n = 44$) relative to normal control animals ($82.34 \pm 44 \mu\text{units/ml}$; $n = 13$) as determined by radioimmunoassay. Partially purified plasma membranes were prepared from the livers of diabetic rats as described by Pohl *et al.* (1971).

Experiments were performed at 23°C in Hanks' balanced salt solution, pH 7.4, supplemented with 0, 0.1% or 1.0% (w/v) bovine serum albumin (fraction V) for perfusion, dissociation and association studies, respectively. The specific binding of ^{125}I -labelled insulin to liver membranes was always greater than 90% of the total radioactivity uptake. Binding was assayed by microcentrifugation (Donner, 1980) or filtration (Donner & Corin, 1980) for association and dissociation reactions, respectively.

Non-cyclic liver perfusions were performed as previously described (Donner *et al.*, 1980) with the following modifications: collagenase and hyaluronidase were omitted, the temperature of the perfusion medium was 23°C, and ^{125}I -labelled insulin (1 nM), or ^{125}I -labelled insulin (1 nM) and native in-

sulin ($13\text{ }\mu\text{M}$) were present. After perfusion with hormone for 60 min (flow rate 300 ml/h), Hanks' solution was passed through the liver to wash away rapidly dissociable radioactivity. Data analysis for association (Galper *et al.*, 1977) and dissociation (Donner *et al.*, 1980) data and the extraction and chromatography of membrane-bound radioactivity were as described (Terris & Steiner, 1975).

Results

The association of ^{125}I -labelled insulin (0.7 nM) with liver plasma membranes from diabetic animals was a time-dependent reaction and achieved an apparent steady state between 40 and 60 min of incubation at 23°C (Fig. 1, insert). Replotting the association data by a graphical method (Galper *et al.*, 1977) yielded Fig. 1. If such data were monocomponent, the operation of a simple bimolecular interaction (Galper *et al.*, 1977) between insulin and the insulin receptor would be indicated. Our data clearly did not yield such a line (Fig. 1). Scatchard plots of ^{125}I -labelled insulin binding to liver membranes from diabetic animals revealed two classes of binding sites with different affinities and capacities (Davidson & Kaplan, 1977). The multicomponent association reaction described (Fig. 1) is consistent with the presence of two classes of binding sites.

The relationship of the two classes of receptors was assessed by examining the kinetics of ^{125}I -labelled insulin dissociation after 8 and 180 min of association. After 8 min of association, the dilution-induced dissociation of ^{125}I -labelled insulin was

biphasic (Fig. 2) and 40% of the hormone was released at a rapid rate [$k_{-1} = (1.4 \pm 1.0) \times 10^{-3}\text{ s}^{-1}$, $t_{\frac{1}{2}} = 8.25\text{ min}$] whereas 60% dissociated at a slow rate [$k_{-1} = (1.2 \pm 1.0) \times 10^{-4}\text{ s}^{-1}$, $t_{\frac{1}{2}} = 96.2\text{ min}$]. After 180 min of association, dissociation was monophasic (Fig. 2) and hormone was released at a slow rate [$k_{-1} = (2.2 \pm 0.5) \times 10^{-4}\text{ s}^{-1}$, $t_{\frac{1}{2}} = 52.5\text{ min}$]. The fraction of bound ^{125}I -labelled insulin exhibiting a slow dissociation rate increased at the expense of the rapidly dissociable ^{125}I -labelled insulin. This result is consistent with the suggestion that the two classes of binding sites are in equilibrium with each other (Donner & Corin, 1980). Hormone-receptor complexes initially capable of rapidly releasing ^{125}I -labelled insulin (after 8 min of association) are converted (after 3 h) to complexes from which ^{125}I -labelled insulin dissociates slowly.

The above experiments were predicated on the assumption that the changes observed were due to alterations of receptor binding and not a change in the integrity of the ^{125}I -labelled insulin. During experiments *in vitro* no degradation of ^{125}I -labelled insulin by plasma membranes was detected when determined by the precipitation of radioactivity in 5% (w/v) trichloroacetic acid.

To determine if complexes from which ^{125}I -labelled insulin dissociated slowly were formed *in situ*, livers in alloxan-induced-diabetic animals were perfused with ^{125}I -labelled insulin (1 nM) or ^{125}I -labelled insulin (1 nM) plus $13\text{ }\mu\text{M}$ -insulin. Livers perfused with ^{125}I -labelled insulin retained radioactivity ($1.06 \times 10^7\text{ c.p.m./liver}$) even after an extensive wash with hormone-free Hanks' solution. After the

Fig. 1. Association of ^{125}I -labelled insulin with liver plasma membranes

Membranes ($240\text{ }\mu\text{g}$ of protein/ml) were incubated with ^{125}I -labelled insulin (0.7 nM) and specific binding was assayed. At steady state 5.5% of the applied tracer was bound. The insert shows binding as a function of time. Binding at equilibrium and at any time as steady state was approached are represented by B_{eq} and B_t , respectively.

Fig. 2. Dissociation of ^{125}I -labelled insulin from liver plasma membranes

Membranes ($240\text{ }\mu\text{g/ml}$) were incubated with ^{125}I -labelled insulin (0.7 nM) for 8 (●) or 180 (○) min. Dissociation was initiated by dilution into 200 vol. of medium and binding was assayed at the indicated times after dilution. Specific binding is shown as the percentage bound relative to the zero-time point (□).

liver was homogenized for membrane preparation, radioactivity was extracted from an aliquot of the homogenate and analysed by gel filtration (Fig. 3). The radioactivity was composed of ^{125}I -labelled insulin and a substantial fraction (27.3%) of low-molecular-weight degradation products of ^{125}I -labelled insulin in fractions 28–48 (Fig. 3). This observation was expected since hepatocytes degrade ^{125}I -labelled insulin cytoplasmically (Terris & Steiner, 1975) and transiently retain the hormone fragments (Donner, 1980). A small peak (5%) of high-molecular-weight non-insulin material (fractions 12–16) was also present. The composition of this radioactivity is not known (Terris & Steiner, 1975).

Membranes prepared from livers perfused with ^{125}I -labelled insulin (1 nM) also retained radioactivity (5×10^4 c.p.m./liver). The presence of native insulin (13 μM) during perfusion with ^{125}I -labelled insulin reduced the amount of radioactivity retained by membranes isolated from livers of matched diabetic animals by greater than 90%. Radioactivity was extracted from membranes obtained from liver perfused with ^{125}I -labelled insulin and characterized by gel filtration (Fig. 3). The membrane-bound (extracted) radioactivity eluted as ^{125}I -labelled insulin (Fig. 3). Thus only intact hormone was bound to membranes, a finding consistent with our previous report that intact insulin binds *in vitro* to liver plasma membranes from normal rats (Donner & Corin, 1980). Dissociation of radioactivity from such membranes occurred at a slow rate *in vitro* (results not shown).

Fig. 3. Gel filtration of radioactivity extracted from membranes prepared after liver perfusion

The liver of a diabetic animal was perfused with 1 nM- ^{125}I -labelled insulin for 60 min, then for 20 min with Hanks' solution. The liver was removed and plasma membranes were prepared. Approx. 95% of the radioactivity was extracted from an aliquot of liver homogenate (●) or partially purified membranes (○) and passed through a column of Sephadex G-50 (fine grade).

Discussion

Kinetic experiments indicate (Figs. 1 and 2) the presence of two classes in insulin binding sites on liver plasma membranes from alloxan-induced-diabetic rats. Liver plasma membranes from normal rats also possess two classes of insulin binding sites (Kahn *et al.*, 1974). In this regard diabetic tissue resembles normal tissue. We (Donner & Corin, 1980) previously established a relationship between the two classes of receptors on normal hepatic membranes. It was demonstrated that prolonged exposure of receptors to ^{125}I -labelled insulin resulted in the accumulation of a receptor state from which insulin dissociates slowly, with the loss of receptor complexes from which insulin dissociates rapidly (Donner & Corin, 1980). In this report we demonstrated a similar relationship between the lower and higher affinity states of occupied insulin receptors on liver plasma membranes from diabetic rats (Fig. 2). It was also shown that receptor complexes from which insulin dissociates slowly are probably formed *in situ*. ^{125}I -labelled insulin is the predominant radioactive species bound to membranes under such conditions (Fig. 3). This suggests that the formation of high-affinity receptor complexes may be of physiological significance. The decreased rate of insulin dissociation may result from the receptor-mediated internalization and degradation of bound hormone (Olefsky *et al.*, 1979; Carpentier *et al.*, 1979), the down-regulation of insulin receptors (Kahn & Baird, 1978; Carpentier *et al.*, 1979), or the uncoupling of receptors from membrane effector functions (Donner & Corin, 1980). None of these suggestions are mutually exclusive. Several interesting points emerge from our description of the conversion between binding states of insulin receptors. The ability of unoccupied receptors to undergo this conversion *in vitro* is not apparently affected by ambient plasma insulin or nutrient levels (both are dramatically altered in the diabetic condition) in the animal prior to perfusion and/or membrane preparation. This suggests that the conversion represents an intrinsic property of membranes and/or receptors. Our results also allow speculation that insulin receptors from normal and diabetic liver plasma membranes are similar with respect to hormone-binding characteristics. Our data indicate no obvious explanation for resistance to insulin at the receptor level in alloxan-induced diabetes. However, changes in receptor activity induced by the diabetic state may not be detectable by the experiments employed herein.

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