Regulation of insulin binding to isolated hepatocytes: Correction for bound hormone fragments linearizes Scatchard plots

(membrane receptors/negative cooperativity)

DAVID B. DONNER

Memorial Sloan-Kettering Cancer Center, and Cornell University Graduate School of Medical Sciences, 1275 York Avenue, New York, New York 10021

Communicated by Victor A. McKusick, February 5, 1980

ABSTRACT  Fragments of 125I-labeled insulin (125I-insulin) are rapidly produced after the initial cell binding process. After association of 125I-insulin with hepatocytes, hormone fragments remain bound to cells. At 23°C, ~20% of the label bound at steady state was soluble in trichloroacetic acid. Correction of saturation experiments for the presence of bound trichloroacetic acid-soluble insulin fragments decreased the number and increased the affinity of 125I-insulin-binding sites. Label extracted from cell pellets recovered from saturation experiments was characterized by gel filtration; 59%, 35%, 40%, and 36% of the bound label was from intact hormone after recovery from intact hormone-containing mixtures containing 0.15, 0.60, 4.6, and 7.5 nM applied 125I-insulin, respectively. At high applied 125I-insulin concentrations, the hormone predominantly interacted with lower affinity degradation systems. When binding data were corrected to assay for undegraded 125I-insulin only, curvilinear Scatchard plots were linearized. The insulin receptor is therefore not composed of heterogeneous or negatively cooperative sites. It is necessary to correct for retained fragments of 125I-insulin in order to define mechanisms through which hormone binding and cellular response may be regulated.

Recent characterizations of insulin–receptor interactions have suggested the presence of heterogeneous classes of binding sites on target cells (1, 2). Binding site heterogeneity has been postulated as having significance in regulating cellular response to insulin (1, 2) and to other peptide hormones (2). However, the capacity of bound insulin to produce the cellular response unique to this hormone has been correlated only with putative receptor sites of high affinity (3, 4). The experimental data suggesting the presence of more than a single class of insulin-binding sites are derived from saturation assays yielding curvilinear Scatchard plots (5). These curvilinear plots have been interpreted as demonstrating the presence of two (6, 7), heterogeneous (8), or negatively cooperative sites (9).

Insulin is degraded by every target tissue with which it interacts (10). Degradation of 125I-labeled insulin (125I-insulin) produces iodotyrosoyl fragments of the parent hormone (11). We recently demonstrated that fragments derived from trypic digestion of bovine growth hormone are accumulated, retained, and degraded by hepatocytes (12). This suggested that hormone degradation may produce a heterogeneous distribution of bound label subsequent to the initial binding process. Heterogeneity of bound, labeled substrate may be confused for the presence of more than a single class of receptor sites on target cells. For this reason, the interaction of 125I-insulin with hepatocytes was investigated. The integrity of bound label was asayed to determine whether intact 125I-insulin was the sole labeled species bound to the hepatocyte.

The present studies indicate that fragments of 125I-insulin may remain associated with the cell and affect the interpreta- tion of both equilibrium and kinetic binding studies. When binding data were corrected to assay only for 125I-insulin, curvilinear Scatchard plots were linearized. The insulin receptor in liver is not composed of heterogeneous sites or sites displaying negative cooperativity.

MATERIALS AND METHODS

Materials and the binding assay were as previously described (13). Data analyses, to be described in detail elsewhere, were conducted on curves for total as well as specific binding to ensure quantitative correction for the presence of cell-associated 125I-insulin fragments resulting from hormone degradation. Binding data were fitted to appropriate mathematical expressions by nonlinear least-squares curve-fitting techniques (14). Photographs of computer-generated plots of binding data are presented as indicated in the figure legends; error bars represent SDs of triplicate determinations.

Trichloroacetic Acid Precipitation of Cell-Bound Label. Hepatocytes were incubated in Hanks’ balanced salt solution/1% albumin with 125I-insulin. Binding was assayed as described (13). The precipitability of cell-associated label was determined by addition of 250 μl of a 10% solution (wt/vol) of trichloroacetic acid to cell pellets isolated from the binding assay. The cell pellet was stirred with a fine wire rod until it was well mixed with trichloroacetic acid. The microcentrifuge tube containing the cells was recentrifuged (1 min) and supernatant containing soluble label was siphoned off. The tip of each microfuge tube, containing trichloroacetic acid-precipitable label and the cell pellet, was cut and placed in a glass tube for measurement of 125I.

Trichloroacetic Acid Precipitation of Total Label Applied to Cells. Aliquots of incubation medium (containing free and bound label) were added to 1 ml of Hanks’ solution/1% albumin. Trichloroacetic acid solution (10% wt/vol) was then added, producing a protein precipitate. The solution was centrifuged (1100 X g, 3 min) and the supernatant was recovered from the resulting precipitate pellet. The supernatant and precipitate were assayed for 125I in a gamma counter. The precipitability of the labeled protein was defined as the ratio of cpm in the precipitate divided by the sum of the cpm in the supernatant and precipitate. The concentration of unbound precipitable label was calculated by subtracting the precipitable bound cpm from the total precipitable cpm.

Gel Filtration of Label Extracted from Hepatocyte Pellets. Aliquots (200-μl) of label extracted from hepatocytes (see legend to Fig. 3) were added to Hanks’ solution/1% albumin (400 μl, pH 7.4). This solution was carefully applied to a column (Pharmacia, 0.9 X 28 cm) packed with Sephadex G-50 fine; Hanks’ solution/1% albumin was used as the column eluent. Half-milliliter fractions were collected with a Gilson minirack fraction collector.

Abbreviation: 125I-insulin, 125I-labeled insulin.
RESULTS

At 23°C, the initial rate of binding of a physiologic concentration (1.6 nM) of 125I-insulin to hepatocytes was rapid (Fig. 1). In the total binding system, an apparent steady state was reached after about 40 min and maintained for at least 4 hr. Cell pellets obtained during assay of binding were suspended in trichloroacetic acid solution to precipitate 125I-insulin. This permitted determination of the concentration of precipitable label bound at any time. The binding of 125I-insulin was accompanied by the production of degradation products of the parent hormone, some of which remained associated with the cells. After 10 min of incubation, \( \approx 30\% \) of the bound label was soluble in trichloroacetic acid. At steady state the concentration of trichloroacetic acid-soluble label remaining bound to the cells was a constant proportion (\( \approx 20\% \)) of the total radioactive uptake. At each time assayed during the course of association, a substantial portion of the bound label was not 125I-insulin (which was \( \geq 95\% \) precipitable prior to incubation with hepatocytes).

The specific binding of 125I-insulin increased for the first 50 min of incubation and then decreased. The decrease in specific binding was a consequence of the fact that nonspecific binding was a greater portion of the total radioactive uptake with more extended incubation time. Whether the decrease in specific binding resulted from internalization and degradation of bound label (15) is not known. However, curves for specific binding showed about the same proportion of trichloroacetic acid-soluble label as the curves for total binding (\( \approx 20\% \) trichloroacetic acid-soluble label after 3 hr of incubation). That some of the specifically bound label was not intact 125I-insulin suggested a close relationship between high-affinity binding and subsequent degradative processes. Virtually all of the bound label remained precipitable in the nonspecific system (not shown) due to the capacity of high concentrations of native insulin to inhibit degradation of 125I-insulin.

The observation of trichloroacetic acid-soluble label at steady state suggested that degradation products of 125I-insulin would affect the interpretation of equilibrium binding assays. Increasing concentrations of 125I-insulin were applied to hepatocyte suspensions in a saturation experiment. Cell pellets containing bound label were precipitated with trichloroacetic acid to permit correction of the number and affinity of binding sites for hormone fragments. Scatchard plots of total (Fig. 2 Left) and specific (Fig. 2 Right) 125I-insulin binding contained data for significant amounts of label not precipitable by trichloroacetic acid and were curvilinear. Correction for bound and free trichloroacetic acid-soluble label decreased the number and increased the affinity of binding sites (Table 1). For example, in the total system the number of high-affinity sites was reduced from 27,000 to 18,000. The \( K_d \) of such sites decreased from 0.97 to 0.59 nM. A linear Scatchard plot was not obtained by correction of curves for total or specific binding by use of methodology that fractionated solely on the basis of acid solubility. However, subtraction of the effect of trichloroacetic acid-soluble 125I-insulin fragments from binding curves diminished the curvilinearity of Scatchard plots.

125I-Insulin (0.18, 0.60, 4.5, and 7.5 nM) was applied to hepatocytes in a saturation assay. These concentrations corresponded to amounts of insulin lower than, approaching, and greater than the \( K_d \) of the high-affinity binding site (Table 1). After equilibration, bound label was extracted from cell pellets by vigorous agitation in 0.1 M HCl. Extracts of label were passed over columns packed with Sephadex G-50 fine (Fig. 3). After incubation with 0.18 nM 125I-insulin (23°C, 1 hr), two major peaks of radioactivity were recovered from gel chromatography. The first peak (eluting at fraction 22) was 125I-insulin. The ratio of the radioactivity in the first peak to that in the second peak (eluting at fraction 31) was 1.0:0.64. Two smaller peaks of radioactivity were also observed. One peak eluted at the void volume of the column; the other contained material of apparently lower molecular weight than the other species recovered from the column (based exclusively on elution volume). Application of higher 125I-insulin concentrations to hepatocytes resulted in the isolation of an additional species eluting at fraction 27 (between the two major peaks described above). The ratios of the radioactivity in the three major peaks in order of elution were 1.0:0.60:0.59, 1.0:0.50:0.80, and 1.0:0.50:1.10 at 0.6, 4.6, and 7.5 nM 125I-insulin, respectively. The cpm associated with the 125I-insulin peak were divided by the total cpm recovered from each column; 55%, 55%, 40%, and 36% of the bound label was intact hormone after recovery from incubation. Mixtures containing 0.18, 0.60, 4.5, and 7.5 nM 125I-insulin, respectively. The progressive increase in the amount and proportion of bound label that was not 125I-insulin correlated with the deviation from linearity in the Scatchard plots in Fig. 2. Because the concentration of cell-bound hormone fragments increased, the Scatchard plot suggested the presence of more low-affinity binding.

The Scatchard plot in Fig. 2 (total curve, Left) was corrected for bound label that was not 125I-insulin. The total cpm recovered from hepatocytes by extraction at each hormone concentration was calculated. The amount of radioactivity (cpm) corresponding to 125I-insulin was divided by the total cpm recovered to obtain the fraction of label that was intact hormone. Because the fraction of bound label that was not 125I-insulin increased with applied hormone concentration, such correction increased the affinity and decreased the number of insulin receptors. The concentration of free label was also corrected. Approximately 20% of the supernatant label was 125I-insulin at each applied hormone concentration. Thus, degradation in solution was not dependent upon the concentration of 125I-insulin present. This degradation may have resulted in part from enzymes released from the hepatocytes to
the medium (16). Correction for the state of free label decreased the apparent number of sites. The corrected values of bound 
\[ ^{125}\text{I-insulin}/\text{free}^{125}\text{I-insulin} \] are plotted against values of bound 
\[ ^{125}\text{I-insulin} \] in Fig. 4.

Correction for the chemical integrity of bound and free label yielded a linear Scatchard plot of 
\[ ^{125}\text{I-insulin} \] binding, suggesting the presence of a single class of hormone-binding sites in equilibrium with the medium. The number and affinity of binding sites, uncorrected, corrected by trichloroacetic acid precipitation, and corrected to assay for 
\[ ^{125}\text{I-insulin} \] only are summarized in Table 1. Such data should be considered as semiquantitative because the extensive hormone degradation demonstrated here probably precluded attainment of true equilibrium conditions. It is clear, however, that the affinity of binding increased progressively by removal, first, of trichloroacetic acid-soluble fragments and then of all hormone fragments from analyses. Interestingly, assay for 
\[ ^{125}\text{I-insulin} \] binding only (gel filtration) and computer-resolved derivation of high-affinity site number suggested the same number of insulin receptors (36,000 and 26,000 ± 12,000, respectively). It may be that nonlinear regression analysis may be used to define the true number of binding sites in the presence of retained hormone fragments.

**DISCUSSION**

Insulin is degraded by every target tissue with which it interacts (10, 15). The degradative process is sequential, proceeding through a series of bond cleavages yielding peptides derived from the parent hormone (11). When 
\[ ^{125}\text{I-insulin} \] is bound to target cells, the ultimate degradative products isolated are low molecular weight iodothyrosyl fragments of insulin (11). Characterizations of the relationship between receptor binding and subsequent hormone degradation have generally relied upon assay of peptide fragments released to the medium. Such assay may only indirectly reflect the state of the hormone that remains cell associated.

We recently demonstrated that fragments derived from tryptic digestion of bovine growth hormone may bind to or be taken up by hepatocytes (12). This suggested that 
\[ ^{125}\text{I-labeled} \] fragments of insulin might remain associated with the cells for sufficient time to affect the interpretation of both equilibrium and kinetic binding data. In order to measure this directly, I assayed the integrity of the cell-bound label as well as label released to the medium by precipitation in trichloroacetic acid. Such precipitation does not correct absolutely for hormone degradation because peptide fragments were produced that

**Table 1.** Scatchard analysis of saturation of 
\[ ^{125}\text{I-insulin} \] binding to hepatocytes

<table>
<thead>
<tr>
<th>Incubation procedure</th>
<th>Total binding curves</th>
<th>Specific binding curves</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_d, \text{M} )</td>
<td>Sites/cell</td>
</tr>
<tr>
<td>Uncorrected curves</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H.A.)</td>
<td>9.7 ± 1.5 × 10^{-10}</td>
<td>27,000 ± 4,900</td>
</tr>
<tr>
<td>(L.A.)</td>
<td>5.9 ± 3.2 × 10^{-8}</td>
<td>600,000 ± 250,000</td>
</tr>
<tr>
<td>Corrected for acid-soluble fragments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H.A.)</td>
<td>5.9 ± 1.0 × 10^{-10}</td>
<td>18,000 ± 3,300</td>
</tr>
<tr>
<td>(L.A.)</td>
<td>5.8 ± 2.7 × 10^{-8}</td>
<td>600,000 ± 250,000</td>
</tr>
</tbody>
</table>
| Corrected with gel filtration: 
\[ ^{125}\text{I-insulin binding only} \] | \( 4.0 × 10^{-10} \) | 36,000* (total no. of binding sites) | — | — |

Nonlinear regression analysis was performed on the data within the Scatchard plots in Fig. 2. The presence of two classes of binding sites was assumed: H.A., high affinity; L.A., low affinity. Data corrected to assay 
\[ ^{125}\text{I-insulin} \] binding only were hand fitted to the presence of a single class of binding sites.

* Insufficient data to permit accurate resolution of site number.

† Hand-fitted without computer-assisted curve fitting.
remained precipitable in trichloroacetic acid. However, precipitation in trichloroacetic acid was sufficiently sensitive and convenient to permit partial correction for the effects of degradation. The trends suggested by such correction were confirmed when supplemented by data from gel filtration chromatography assaying for the chemical state of each species of bound label.

Fragments of $^{125}$I-insulin produced subsequent to the initial binding process may remain associated with cells. A substantial fraction of bound label was soluble in trichloroacetic acid ($\approx 50\%$ after 10 min, $\approx 20\%$ at steady state) at each time assayed, suggesting that both equilibrium and kinetic binding data should be corrected for the presence of retained fragments. That a constant proportion of bound label was soluble over a 4-hr association interval suggested that a steady state relating receptor-bound hormone and subsequent interaction with degradative processes was obtained. The presence of soluble label was shown by curves characterizing both the total and specific, but not the nonspecific, hormone binding. There was a close relationship between a fraction of the hormone bound to high-affinity sites and the degradative systems with which it ultimately interacted.

The amount and proportion of bound label that was not $^{125}$I-insulin increased as the concentration of applied hormone was increased from 0.18 to 7.5 nM. Saturation of high-affinity sites favored the interaction of $^{125}$I-insulin with lower affinity degradative systems. At low hormone concentrations, Scatchard plots of saturation data were essentially linear (Fig. 2). At $^{125}$I-insulin concentrations approaching and above receptor saturation ($\approx 5 \times 10^{-10} \text{M}$), interaction with lower affinity degradative sites and curvilinearity in Scatchard plots increased. The portion of $^{125}$I-insulin degradation coupled to receptor binding was not primarily responsible for the curvilinearity in Scatchard plots. The production of insulin fragments subsequent to low-affinity cellular interactions presented the appearance of site heterogeneity.

Saturation studies yielding curvilinear Scatchard plots of $^{125}$I-insulin binding have been the prime experimental methodology suggesting the presence of two (6, 7), heterogeneous (8), or negatively cooperative (9) insulin receptors. Correction of saturation data for label that was not precipitable in trichloroacetic acid decreased the number and increased the affinity of the remaining sites (Table 1). Correction for precipitable as well as nonprecipitable fragments by gel filtration decreased the number of sites and increased receptor affinity further. Linear Scatchard plots were obtained when bound and free hormone fragments were not considered in data analyses (Fig. 4). Heterogeneity of substrate has, therefore, been widely interpreted as demonstrating the presence of multiple classes of insulin binding sites. The linear Scatchard plot presented here suggests the presence of a single, high-affinity insulin receptor class in equilibrium with the medium.

$^{125}$I-Insulin binding conducted at low temperature to minimize degradation (unpublished data) or measured by filtration assay, which promotes the elimination of low-affinity bound fragments (17, 18), suggests a single insulin receptor class in liver and adipose tissue. Terris and Steiner (15) and Gammeltoft et al. (19) minimized degradation by using lower cell concentrations than those used here ($\approx 10 \times 10^6 \text{cells per ml}$). In the latter study, a homogeneous insulin receptor was characterized. The number of sites per cell ($\approx 22,000$) and dissociation constant (0.6 nM) agreed with the values reported here ($\approx 36,000$ sites per cell; $K_d$, 0.4 nM). Were more sensitive techniques, such as re-binding to fresh hepatocytes, used to assay degradation in both studies, the number of sites and dissociation constants obtained might be closer still.

Curvilinear Scatchard plots of insulin saturation experiments were observed in studies with liver membranes (7). In membranes, receptors are uncoupled from cytoplasmic compartments responsible for most degradation. However, insulin may interact with degradative sites within the plasma membrane. The effects of such interaction remain to be determined.

The corrected linear Scatchard plot in Fig. 4 suggests that models of insulin receptor regulation based upon site heterogeneity (1, 2) or negative cooperativity (1, 2, 9) need to be re-
interpreted. Although increased rates of loss of $^{125}$I-insulin from cells in the presence of native insulin is an interesting phenomenon, it does not demonstrate cooperative site-site interactions. We (13) and others (20, 21) have noted that kinetic and equilibrium binding data suggest other interpretations.

Persistent binding of epidermal growth factor (22), thyrotropin (23), and glucagon (24) correlated with the onset and completion of cellular response to these hormones. High-affinity insulin binding correlated with increased glucose uptake in adipose tissue (4). Low-affinity $^{125}$I-insulin uptake, related to the presence of cell-associated hormone fragments, has not been correlated with the production of a biologic response characteristic of insulin. High-affinity, cell-surface receptor binding is probably the primary event associated with the production of cellular response to insulin. Insulin uptake at high concentration seems largely the result of interaction with low-affinity degradative systems unrelated to the important biologic properties of this hormone.

This work was supported by Grants AM 19846, AM 22121, and CA 08748 from the National Institutes of Health. I was the recipient of a Research and Development Award from the American Diabetes Association.


