Perturbation of the Insulin Receptor of Isolated Fat Cells with Proteolytic Enzymes

DIRECT MEASUREMENT OF INSULIN-RECEPTOR INTERACTIONS*

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SUMMARY

The effects of proteolytic digestion of isolated adipose tissue cells have been studied by simultaneously examining the metabolic and receptor-binding consequences of trypsin digestion. Insulin-receptor interactions in the intact cell are measured directly and independently of the metabolic processes of the cell by determining the specific binding of ¹²⁵I-insulin to cells.

Trypsin digestion of fat cells leads to a selective and profound fall in the affinity of the receptor for insulin. This occurs under conditions where the maximal insulin response (glucose oxidation) and the total amount of receptor are unaffected. These effects are easily detected by either measuring glucose oxidation or insulin binding as a function of increasing concentrations of insulin. More drastic trypsin digestion results in additional and qualitatively different effects. Such digestion either destroys more critical regions of the receptor or prevents the normal transmission of signals from the insulin-receptor complex to the glucose transport mechanisms of the cell membrane. Effects similar to those observed with trypsin are obtained by digesting fat cells with α-chymotrypsin.

Agarose derivatives containing covalently linked trypsin produce the same effects that are observed with trypsin in solution. This is strong evidence that the effects of trypsin digestion result exclusively from perturbations of superficial structures of the cell membrane.

The decreased affinity of the insulin receptor resulting from trypsin digestion does not change during prolonged periods of incubation. There is no evidence that this specific and well characterized effect of trypsin digestion is subject to facile and spontaneous repair. Changes in the basal and insulin-stimulated rates of glucose oxidation are observed during incubation, but these changes are complicated and not easily interpreted.

Digestion of fat cells with phospholipase C or phospholipase A increases quite appreciably the total amount of insulin receptor which is exposed to the medium. The insulin receptor in such cells becomes much more susceptible to trypsin digestion. In these cells trypsin cleaves regions more critical to the binding function of the receptor since severe destruction of the receptor occurs without significant effects on affinity.

No displacement of specific binding of ¹²⁵I-insulin to fat cells is observed with high concentrations of several enzymatically inactive precursors and derivatives of proteolytic enzymes. Trypsin and α-chymotrypsin (at 4°C) do not cause displacement of ¹²⁵I-insulin binding. These enzymes probably cannot specifically interact with the same receptor as insulin in the fat cell.

The insulin receptor of the fat cell is normally located in the membrane in such a way that “peripheral” glycopeptide regions are exposed to the solvent and are susceptible to digestion by proteolytic enzymes and neuraminidases. Cleavage of certain parts of these glycopeptides alters but does not abolish the function of the receptor. The more critical and “central” regions of the receptor structure may normally be hindered from macromolecules in the solvent, perhaps by membrane phospholipids.

The well known ability of insulin to stimulate glucose transport and to inhibit lipolytic processes in isolated adipose tissue cells is mimicked by large agarose beads which contain covalently linked insulin on their surface (1). This is strong evidence for the superficial, cell surface localization of the insulin receptor in these cells. Attempts to study the properties of the insulin receptor by perturbing the intact cell with various chemical (2-4) and enzymic (5-14) probes have been hampered by the inability to experimentally measure the initial insulin-receptor interaction separately from the complicated biological processes which constitute the insulin response.

Procedures have recently been described which permit direct and precise measurements of the interaction of insulin with intact
fat cells by methods which are independent of metabolic events (15). The interaction is a highly specific and reversible bi-
molecular process which can be characterized by classical equilib-
rium and kinetic expressions (15). No significant chemical
alteration of insulin or of receptor results from the interaction.
The receptor exhibits homogeneous kinetic behavior, and it
appears to be localized exclusively on the cell membrane (15).
The assay for specific insulin binding (15) can be used to study
in detail the properties of the insulin receptor in intact and
broken cell preparations. By digesting fat cells with various
neuraminidases it has been possible to dissociate the initial
binding of insulin (unaffected) from metabolic processes (abol-
ished) which are normally specific consequences of the binding
interaction (16). It has been suggested that the insulin receptor
is probably a glycoprotein structure, and that sialic acid residues
at the surface of the cell are essential for transmitting the insulin-
mediated signals for glucose transport and for lipolysis, but are
not essential for insulin recognition (16).

The present report describes the effect of digesting fat cells
with trypsin and other proteolytic enzymes on the insulin recep-
tor and on glucose transport. Trypsin digestion of fat cells has
recently been shown to selectively abolish the insulin-stimulated
glucose transport and antilipolytic processes of these cells (11,
13, 14). It was postulated from indirect evidence that this
enzyme, which presumably does not penetrate the cell interior,
specifically modifies protein moieties of the insulin receptor at
the surface of the cell.

MATERIALS AND METHODS

Crystalline pork zinc-insulin (24 units per mg) was obtained
from Eli Lilly; [U-14C]glucose (10 μCi per μmole) and carrier-free
Na125I in 0.1 M NaOH from New England Nuclear. Phospho-
lipase C from Clostridium perfringens was obtained from Nutri-
tional Biochemical and diisopropyl fluorophosphate-trypsin
(0.75% trypsin), diisopropyl fluorophosphate-α-chymotrypsin
(0.08% α-chymotrypsin, 0% trypsin), pepsinogen, five times
crystallized chymotrypsinogen, trypsinogen, soybean trypsin
inhibitor, and twice crystallized trypsin were purchased from
Worthington. Phospholipase A from Vipera russelli was ob-
tained from Sigma. Microfine silica, QUSO G-32, was obtained
from the Philadelphia Quartz Company, Philadelphia, Pennsyl-
vania, and talc tablets (25 mg) from Gold Leaf Pharmacal Com-
pany, Inc., Englewood, New Jersey.

Isolated fat cells were prepared by the method of Rodbell
(1, 17). The conversion of [U-14C]glucose to 14CO2 was deter-
mined as described earlier (1), using Krebs-Ringer-bicarbonate
buffer containing 2% (w/v) albumin and 0.2 mM [14C]glucose
(specific activity, 5.1 μCi per μmole). With this concentration
of glucose the rate of glucose oxidation reflects accurately glucose
transport since the latter is the rate-limiting step in this process
under these conditions (18-20). The cells were generally incu-
bated for 60 to 90 min at 37°C.

Digestion of fat cells with trypsin or with other proteolytic
enzymes was performed at 37°C in Krebs-Ringer-bicarbonate
buffer containing 1% (w/v) albumin. Trypsin digestions were
stopped by adding soybean trypsin inhibitor and washing the
cells at least three times by centrifugation using the same buffer.
The specific procedural details are presented in legends to the
figures and tables.

125I Insulin was prepared and purified as described previously
(15). The specific activity of the preparations used in these
experiments varied from 1380 to 1620 mCi per μmole, representing
less than 1 atom of iodine per insulin molecule. The binding
assay depends quite critically on having a properly prepared and
purified preparation of 125I-insulin. The criteria used to judge
the quality of the 125I-insulin were the following. (a) Precipita-
tion of radioactivity by 8% trichloroacetic acid, performed in
0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% (w/v)
albumin, must be at least 97%. (b) Adsorption of radioactivity
to talc (25 mg per ml), performed in the same buffer described
above, must be greater than 97%. (c) Adsorption of radioac-
tivity to microfine silica (5 mg per ml), performed in 0.1 M sodium
acetate buffer, pH 4.5, and containing 0.1% (w/v) albumin,
must be greater than 97%. (d) The nonspecific binding of
125I-insulin to the fat cells and to the Millipore membrane (to be
described shortly) must together be less than 0.4% of the total
125I-insulin present in the incubation medium, and less than 20%
of the total uptake of radioactivity when 106 cells are used per 0.5
ml of incubation medium. The most important of these tests
is the last one, since aged or otherwise poor preparations of
125I-insulin result in prohibitively high nonspecific adsorption
of radioactivity to cells and filters.

The assay for specific binding of 125I-insulin to fat cell receptors
is described in detail elsewhere (15). Briefly, isolated fat cells
about 106 per ml) are incubated in disposable, polystyrene
tubes (12 X 75 mm) for 20 min at 24°C in 0.5 ml of Krebs-Ringer-
bicarbonate buffer containing 1% (w/v) albumin and 125I-insulin
(10-16 to 10-24 M). Three milliliters of ice-cold Krebs-Ringer-
bicarbonate buffer containing 0.1% (w/v) albumin are added to
the tubes, the contents are poured on a cellulose acetate EAWP
Millipore filter positioned with vacuum, and the filters are washed
under vacuum with 10 ml of ice-cold Krebs-Ringer-bicarbonate
buffer containing 0.1% albumin. The steps of dilution, filtration,
and washing consume 10 to 15 sec. It has been established
that under these conditions dissociation of insulin from the
receptor is negligible (less than 0.5% of that bound). The filters
are removed, cut in half, and placed in counting vials. One
milliliter of 10% (w/v) sodium dodecyl sulfate is added and the
vials are shaken for 30 to 40 min at room temperature. Radio-
activity is determined by liquid scintillation using 10 ml of TLX
toluene Fluoroloy and 2 ml of Bio-Solv solubilizer BBS-3 (Beck-
man).

Counting efficiency for 125I is 55%.

Six incubation samples (two sets, in triplicate) are performed
for each determination of “specific binding.” Three of these
(Set 1) differ from the other three (Set 2) only by the presence
of native insulin (20 to 40 μg per ml). This is added to the cells
before the 125I-insulin. Total radioactive uptake is obtained
from Set 2, lacking native insulin, and “nonspecific” binding is
obtained from Set 1 tested in the presence of native insulin.
“Specific binding” is determined by difference. It represents
that amount of bound 125I-insulin which can be displaced by
native insulin, and is accurately paralled the biological activation
of the cells by insulin (15). The nonspecific component repres-
ents radioactivity which adsorbs nonspecifically to the filter and
to the cells.

It is imperative to always include the set of samples containing
the high concentration of native insulin since the nonspecific
binding may vary considerably under various conditions, and an
exact correction factor must be available to calculate the specific
binding with precision. For example, the nonspecific component
may vary with various cell preparations, with different filter lot
numbers, with slight changes in the incubation conditions, with

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two-milliliter suspensions of fat cells, containing about 8 x 10^5 cells per ml of Krebs-Ringer-bicarbonate buffer, 1% (w/v) albumin, were incubated at 37° for 15 min with various concentrations of trypsin. Soybean trypsin inhibitor equal to 3 times the amount of trypsin by weight was added. The cells were washed three times with 10 ml of the same albumin buffer, and the cells were suspended in 6 ml of this buffer. Samples (0.25 ml) of these suspensions were incubated at 37° in Krebs-Ringer-bicarbonate buffer (1.0 ml) containing 2% (w/v) albumin and 0.2 mM [3H]-glucose (specific activity, 5.1 μCi per μ mole) in the absence and presence of 10 milliunits of insulin. The production of 3H2O was measured during a 60-min incubation period (O). The specific binding of [3H]-insulin to samples (0.5 ml) of the digested and washed cells was studied as described in the text, using 2.3 X 10−6 M [3H]-insulin.

The apparent paradox of finding total loss of insulin binding with only partial loss of the biological response is not explained by degradation of [3H]-insulin by trypsin. The enzyme is inactivated with soybean trypsin inhibitor, the cells are washed extensively, and the [3H]-insulin in the incubation medium of the digested cells can be separated from the cells and shown to bind to native fat cells identically to native [3H]-insulin.

The explanation for the discrepant loss of binding and biological properties is suggested by the difference in the concentration of insulin which was used in the experiments which measure these two functions (Fig. 1). The concentration of insulin in the study of glucose oxidation was 6 X 10−8 M, whereas that used to measure insulin binding was 2.3 X 10−11 M. This indicates that tryptic digestion of fat cells must result in a profound decrease in the affinity of the receptor for insulin, and that this may at least in part explain the loss of insulin sensitivity which accompanies digestion of cells with this enzyme.

Effect of Insulin Concentration on Glucose Oxidation by Trypsin-treated Cells—The supposition that tryptic cleavage may primarily affect the affinity of fat cells for insulin was examined by studying the [3H]glucose oxidation response at various concentrations of insulin (Fig. 2). It is clear that dramatic changes in the insulin dose-response curves occur upon tryptic digestion of fat cells.

Quantitation of the “loss” of the insulin response is difficult since very different values result depending on the particular concentration of insulin used. The cells treated with 10 μg per ml of trypsin are virtually without insulin response when tested with less than 50 micromoles of insulin per ml, but their maximal response (at 750 micromoles of insulin per ml) is identical with that of undigested cells (Fig. 2). This would indicate that the cells treated with 10 μg per ml of trypsin possess a full complement of insulin receptor but that the affinity of this receptor for insulin is severely compromised. The specificity of this loss of affinity is based on the demonstration that in the absence of factors which independently alter glucose transport or metabolism, the insulin dose-response curve for glucose oxidation reflects accurately the affinity of insulin for the receptor (15). The half-points (Fig. 2) of the insulin saturation curves for native and trypsin-treated (10 μg per ml) cells are 9 and 110 μm units of insulin per ml, respectively; such tryptic digestion may thus result in a 10-fold loss in insulin-receptor affinity.

Digestion of fat cells with high concentrations of trypsin results in even more profound changes in the affinity of the cells for insulin. In addition, however, other effects occur which are qualitatively different from those occurring with the lower concentrations of trypsin (Fig. 2). The maximal insulin response of cells digested with 30 to 200 μg of trypsin per ml appears to be less than that of the normal cells. More drastic tryptic digestion thus may alter structures other than the insulin receptor which are necessary for conveying the signals of the insulin-receptor interaction. However, on the basis of previous studies which demonstrate that tryptic digestion does not substantially alter glucose transport processes (11, 13), it is possible that the loss of the maximal insulin response may reflect direct damage to the receptor structure itself or of a closely related structure.

Effect of [3H]-Insulin Concentration on Insulin Binding by Trypsin-treated Cells—The dramatic decrease in insulin affinity resulting from tryptic digestion was confirmed by measuring the specific binding of [3H]-insulin at various concentrations (Fig. 3). Cells digested with 10 μg per ml of trypsin bind no detectable insulin at 1.6 X 10−11 M [3H]-insulin; at 6.4 X 10−11 M [3H]-insulin they bind 15 times less than normal cells, at 3.8 X 10−10 M they
Fig. 2. Effect of increasing the concentration of insulin on the conversion of \( ^{14}C \)glucose to \( ^{14}CO_2 \) by isolated fat cells treated with various concentrations of trypsin. The fat cells from 14 rats were divided equally into four 5-ml samples using Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin. These were incubated in the absence (■) or presence of 10 \( \mu g \) per ml (□), 30 \( \mu g \) per ml (○), and 200 \( \mu g \) per ml (□□) of trypsin for 15 min at 37°. Soybean trypsin inhibitor equal to 3 times the weight of trypsin was added. Each cell fraction was washed three times with 10 ml of the albumin buffer, and the fractions were resuspended in a total volume of 23 ml. Samples (0.5 ml) of these cells were used to measure the specific binding of insulin as a function of the concentration of \( ^{125}I \)-insulin in the medium (data in Fig. 3). Other cell samples (0.25 ml) were used to examine insulin-dependent \( ^{14}C \)glucose oxidation. The cells were incubated for 60 min at 37° in Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin, 0.2 mM \( ^{14}C \)glucose and varying concentrations of native insulin as indicated in the figure.

Fig. 3. Effect of increasing the concentration in the medium of \( ^{125}I \)-insulin on the specific binding to isolated fat cells treated with various concentrations of trypsin. The fat cells used in this experiment were the same as those that were used in the experiment depicted in Fig. 2. The ability of these cells to increase the \( ^{14}C \)glucose oxidation in response to various insulin concentrations is described in Fig. 2. The cells were undigested by enzymes (■), or treated with 10 \( \mu g \) per ml (□) or 30 \( \mu g \) per ml (○) of trypsin for 15 min at 37°. Varying amounts of \( ^{125}I \) insulin were added to 0.5 ml suspensions containing 2.5 \( \times \) \( 10^5 \) cells. These were incubated at 24° for 25 min, followed by filtration and washing on Millipore filters as described in the text.

1 Unfortunately, the loss in affinity with trypsin digestion is so great that it is technically difficult and impractical to obtain the entire binding curve for \( ^{125}I \)-insulin (Fig. 3). This would require prohibitively high concentrations of radioactive insulin, the trypsin-treated (10 \( \mu g \) per ml) cells show a half-maximal insulin saturation for glucose oxidation of 7.5 \( \times \) \( 10^{-10} \) M insulin (Fig. 2) and a half-maximal saturation for \( ^{125}I \)-insulin binding of 6.7 \( \times \) \( 10^{-9} \) M insulin (Fig. 3). The data substantiate the view that under the conditions used here the binding and biological functions reflect closely related processes. It follows by necessity that low trypsin concentrations affect exclusively a selective alteration of the receptor structure which is expressed as a com
promised affinity for insulin. Also, the identity of 125I-insulin and native insulin (15) is again confirmed.

Effect of Digesting Cells with Trypsin-Agarose—All previous studies of tryptic digestion of cells have been performed on the assumption that this enzyme does not penetrate the cell interior, or, that if it does, the biological effects do not result from intracellular proteolysis. The studies presented above indicate that these assumptions are probably correct, at least under conditions of relatively mild tryptic digestion. Under these conditions the only apparent effect is on the affinity of the receptor, a structure known to be located on the cell membrane (1, 15).

It was nevertheless important to demonstrate directly that all the effects of tryptic digestion result exclusively from digestion of superficial cell structures. Studies using trypsin covalently linked to agarose beads show that the same effects are obtained as with native trypsin, although larger quantities of agarose-trypsin are necessary to achieve the same effects (Table 1). In such experiments it is imperative to demonstrate that free trypsin is not somehow released into the medium from the agarose beads during the digestion. This was done by incubating a large number (8 \times 10^6) of fat cells in 3 ml of Krebs-Ringer-bicarbonate buffer containing 1% (w/v) albumin and a large amount (2.5 mg) of trypsin-agarose. After incubation for 30 min at 37°C the suspension was filtered through a Millipore membrane. Fat cells were incubated in the filtrate for 40 min at 37°C. No effect was observed on the specific binding of 125I-insulin (4 \times 10^{-11} M) to these cells. This is a very sensitive test for detecting even slight tryptic damage, provided that low concentrations of 125I-insulin are used. The results of these studies strongly suggest that the biological and binding effects of tryptic digestion result exclusively from perturbation of cell surface structures.

Possible Reversibility of Effects of Tryptic Digestion—Kono (13) has reported that tryptic abolition of insulin-stimulated glucose oxidation by isolated fat cells is reversed spontaneously over an incubation period of 30 to 90 min. In view of this, vigorous attempts were made to detect reversibility of the loss of 125I-insulin binding which results from tryptic digestion. It was felt that the 125I-insulin binding assay would detect even very small changes in receptor affinity, especially if low 125I-insulin concentrations were used.

In these experiments mild tryptic digestions were used. The tryptic digestion was so chosen that the affinity of the cells for insulin was severely depressed, but the maximal response (oxidation) to very large concentrations of insulin was equal to that of undigested cells (as in Fig. 2, 10 pg per ml of trypsin). These conditions were chosen because it is reasonably certain that in

### Table 1

**Trypsin Digestion and Insulin Receptors**

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Conversion of [14C]glucose to [14CO2] (µmoles/mg)</th>
<th>Specific [125I]-insulin bound (µmoles/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No insulin</td>
<td>Insulin*</td>
</tr>
<tr>
<td>Undigested cells</td>
<td>0.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Trypsin-treated cells</td>
<td>0.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Trypsin-agarose-treated cells</td>
<td>0.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Insulin, 0.5 milliunit per ml.
this case the only significant effect of tryptic digestion is on the
affinity of the receptor. Furthermore, it was felt that perhaps
lesser degrees of cell damage may be more readily repaired, thus
facilitating detection of the repair process.

No return in the loss of specific 125I-insulin binding could be
detected under the conditions described here (Fig. 4). Incubation
at 37°c for periods as long as 4 hours does not significantly
alter the amount or affinity of insulin receptor of normal or of
trypsin-treated (and washed) cells.

In contrast to the constancy of the receptor activity during
incubation, changes of complicated character occur in glucose
oxidation in both normal and trypsin-treated cells (Fig. 4). The
base-line (in absence of insulin) rate of glucose oxidation rises progressively during incubation. There is no difference in
this effect between the trypsin-treated and normal cells. The
insulin response of undigested cells falls progressively over the
4-hour period of incubation. Virtually no insulin response is
detected in normal cells after incubating these for 4 hours at 37°.
The insulin response (oxidation) of the trypsin-treated cells
increases to the "normal" value after incubating the cells for 1
hour. Beyond this point the changes in glucose oxidation occurrung in these cells are minimal. Relative to the undigested
cells, however, the changes, although small, appear to represent
an increase in the rate of glucose oxidation.

Interpretation of this data of glucose oxidation is difficult.
Whatever changes occur in the trypsin-treated cells have to be
compared with the control cells, which are themselves changing
during the incubation. However, the increased oxidation of
digested cells observed between 1 and 2 hours (Fig. 4) does not
represent a change in the insulin receptor, since no change in
binding of 125I-insulin occurs. If the spontaneous increase in
base-line oxidation of normal as well as digested cells in a reflection
of trauma and damage to these cells, then it is possible that
the protracted increase in insulin response of the digested cells
might also represent peculiar membrane damage which occurs
with time. It is not clear why this kind of damage with resulting
membrane "leakiness" would not be observed in the insulin
response of the untreated cells. Perhaps trypsin initially changes
membrane structures other than the insulin receptor, the effects
of which are not immediately expressed. Changes might occur,
for example, which decrease the stability of the membrane and
predispose it to physical or thermal stresses.

Attempts were not made to determine whether recovery of
125I-insulin binding would occur after digestion with the higher
concentrations of trypsin used in other studies (11-14). It was
felt that the changes occurring in glucose oxidation would be
even more complicated, and that the probability of significant
repair processes would be even dimmer than in the case described
above.

Effect of Digestion with Phospholipase C on Susceptibility to
Trypsin—Digestion of isolated fat cells with phospholipase C
from Clostridium perfringens causes abolition of the biological
response to insulin but enhances dramatically the capacity for
125I-insulin binding (22). The insulin receptor of phospholipase C-treated cells is very sensitive to destruction by trypptic digestion
(Fig. 5). Concentrations of trypsin which cause no change in
the affinity of fat cells for insulin destroy 90 to 99% of the insulin
receptor of phospholipase C-treated cells. Trypsin concentrations
higher than those shown in Fig. 5 cause further and complete abolition of the receptor in phospholipase-treated cells, but changes also occur in the normal cells at these higher trypsin
concentrations. Digestion with phospholipase A from V. russelli
also results in increased susceptibility to destruction by trypsin
(Table II).

The loss of 125I-insulin binding which results from trypctic diges-
tion of phospholipase-treated cells appears to be qualitatively dif-
ferent and more severe than the loss observed after trypptic diges-
tion alone. The latter results primarily in a change in affinity
of the receptor. In contrast, the receptor capacity which is lost by
trypsin after phospholipase digestion is complete and does not
respond to increasing concentrations of 125I-insulin (Table II).
It appears that in the latter situation the receptor molecules
affected by trypsin are completely destroyed whereas those not
affected (or spared) retain nearly normal affinity for insulin.
The proportional increase in the residual binding upon incuba-
tion with increasing 125I-insulin concentrations is similar to that
of the normal cells. It thus appears that phospholipase digestion
alters the receptor environment so that more critical or
"central" regions of the receptor become exposed or susceptible
to proteolytic cleavage.

Effect of Digesting Cells with Chymotrypsin and Papain—
Digestion of isolated fat cells with α-chymotrypsin results in changes
in glucose oxidation and binding of 125I-insulin that are very
similar to those observed after trypptic digestion (Fig. 6). With
a low concentration of 125I-insulin there is an apparent, nearly
complete loss of insulin binding in conditions where insulin
responsiveness is unaffected. The reasons for these findings are
the same as the explanations given in the case of trypptic diges-
tion. The predominant effect of chymotryptic digestion is to
decrease the affinity of the receptor for insulin. This effect is
even easier to demonstrate with chymotryptic digestion, since
**Table II**

<table>
<thead>
<tr>
<th>Enzymatic digestion</th>
<th>Specific $^{125}$I-insulin bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inulin, $4.1 \times 10^{-10}$ M</td>
</tr>
<tr>
<td>No enzymatic digestion</td>
<td>5.7</td>
</tr>
<tr>
<td>Phospholipase A only</td>
<td>13.4</td>
</tr>
<tr>
<td>Phospholipase C only</td>
<td>22.1</td>
</tr>
<tr>
<td>Trypsin, 2 $\mu$g per ml</td>
<td>5.9</td>
</tr>
<tr>
<td>Trypsin, 4 $\mu$g per ml</td>
<td>5.5</td>
</tr>
<tr>
<td>Trypsin, 8 $\mu$g per ml</td>
<td>2.0</td>
</tr>
<tr>
<td>Phospholipase C + Trypsin, 2 $\mu$g per ml</td>
<td>7.8</td>
</tr>
<tr>
<td>Phospholipase C + Trypsin, 4 $\mu$g per ml</td>
<td>4.9</td>
</tr>
<tr>
<td>Phospholipase A + Trypsin, 2 $\mu$g per ml</td>
<td>1.7</td>
</tr>
<tr>
<td>Phospholipase A + Trypsin, 4 $\mu$g per ml</td>
<td>4.6</td>
</tr>
</tbody>
</table>

**Effect of Inactive Proteolytic Enzyme Derivatives on Insulin Binding**

One of the more interesting effects of proteolytic enzymes on adipose tissue is the enhancement of glucose oxidation which is observed when low concentrations of these enzymes are added to the tissue or cells (5–13). This insulin-like activity is generally observed under milder conditions of digestion than those that have been presented in this report. There is speculation that the insulin-like activity of the proteolytic enzymes may not be related to their catalytic function since alkaline-denatured pepsin and pepsinogen both stimulate glucose uptake and glycogen formation in muscle (23). A possibility exists that, under certain conditions, some of these enzymes may share the same receptor as insulin.

Very large concentrations of various catalytically inactive proteolytic enzyme precursors, and of selectively inactivated proteolytic enzymes, do not effectively compete with insulin for receptor binding even when low concentrations of $^{125}$I-insulin are used (Table III). Any insulin-like activity which these proteolytic derivatives may have, therefore, cannot easily be explained by postulating interaction with the insulin receptor.

**Table III**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Specific binding of $^{125}$I-insulin $\mu$moles $\times 10^3$/10$^6$ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.1 $\pm$ 0.2 $^a$</td>
</tr>
<tr>
<td>Diisopropyl fluorophosphate-trypsin</td>
<td>4.5 $\pm$ 0.3</td>
</tr>
<tr>
<td>Diisopropyl fluorophosphate-chymotrypsin</td>
<td>4.1 $\pm$ 0.1</td>
</tr>
<tr>
<td>Pepsinogen</td>
<td>4.2 $\pm$ 0.1</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>4.1 $\pm$ 0.2</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>4.4 $\pm$ 0.1</td>
</tr>
</tbody>
</table>

$^a$ Mean value $\pm$ standard error of the mean (for three observations).

The effects of on oxidation seen after digesting cells with even higher enzyme concentrations. This probably means that more drastic chymotryptic digestion is less likely to affect more critical receptor bonds, or to affect other membrane structures concerned with transmission of signals from the insulin-receptor complex, than is comparatively drastic tryptic digestion.

Although not studied as extensively, papain digestion of fat cells also results in loss of binding which is disproportionate to the loss of insulin-stimulated glucose oxidation. It appears very likely, therefore, that a variety of proteolytic enzymes have very similar effects on the isolated fat cell. They may selectively modify regions of the membrane receptor for insulin in such a way that the affinity for insulin is decreased.

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Very large concentrations of various catalytically inactive proteolytic enzyme precursors, and of selectively inactivated proteolytic enzymes, do not effectively compete with insulin for receptor binding even when low concentrations of $^{125}$I-insulin are used (Table III). Any insulin-like activity which these proteolytic derivatives may have, therefore, cannot easily be explained by postulating interaction with the insulin receptor.

**Effect of Proteolytic Enzymes on $^{125}$I-Insulin Binding at $4^\circ$**

The considerations presented above do not exclude the possibility that catalytically active proteolytic enzymes share a common receptor with insulin, by virtue either of catalytic or of conformational similarities (5, 23, 24) with the hormone. This thesis was tested by studying the possible competition by trypsin and chymotrypsin of binding of low concentration of $^{125}$I-insulin to fat...
Effect of trypsin and α-chymotrypsin on binding of 125I-insulin to fat cells at 4°C

Isolated fat cells, 0.6 × 10^6 cells in 0.5 ml of Krebs-Ringerbicarbonate buffer containing 1% (w/v) albumin, were placed in an ice bath for 10 min. Samples of trypsin and chymotrypsin (kept at 4°C) were added to tubes, followed immediately by 125I-insulin (3.8 × 10^{-10} M). After another 5 min in ice the samples were filtered and the specific binding was determined as described in the text.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific 125I-insulin bound to cells, nM × 10^3/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additions</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>Trypsin, 50 μg/ml</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>Trypsin, 100 μg/ml</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>Chymotrypsin, 400 μg/ml</td>
<td>5.0 ± 0.2</td>
</tr>
</tbody>
</table>

* Mean value ± standard error of the mean (for three observations).

The specific binding of 125I-insulin to fat cells is a saturable process which parallels very closely the dependence of glucose transport on the concentration of native or of 125I-insulin (15). A number of other polypeptide hormones do not compete with 125I-insulin for binding even at concentrations 2 × 10^4 times greater than that of 125I-insulin. Biologically inactive derivatives of insulin, such as the reduced or alkylated chains of insulin, or desoctapeptide insulin, do not compete for specific binding of insulin. Proinsulin, which can produce similar effects on glucose transport but only at concentrations 20 times greater than native insulin, binds to fat cells with an affinity constant which is 20 times lower than native insulin (25). Insulin interacts with intact fat cells in a simple reversible manner which is kinetically and qualitatively similar to the formation of a precatalytic enzyme-substrate complex (15). Complex formation does not result in detectable chemical alteration of the insulin molecule or of the receptor structure.

The kinetics of the insulin-cell interaction are consistent with the presence of a homogeneous and unique receptor structure. The rate and affinity constants of the interaction indicate values consistent with the known physiological concentrations of insulin, and with the affinity constants predicted from the in vivo effects of insulin on the rate of glucose transport of fat cells. For these reasons it is postulated that the specific binding of 125I-insulin to cells reflects interactions with biologically significant receptors on the surface of the cells (15).

The rate of 125I-insulin association with the fat cell receptor is 4.9 ± 0.2 nM/10^6 cells. At 4°C the catalytic activity of these proteases is severely depressed. Although the possible rate of binding of the proteases to the receptor would be expected to decrease at 4°C, the rate of insulin binding at this temperature is known to be decreased considerably. It should be considered that the enzyme concentrations used in this experiment are about 10^4 times greater than the concentration of 125I-insulin. The lack of competition for binding might indicate that these enzymes can specifically bind to and activate the insulin receptor, the dissociation constant must be at least 10^4 times greater than that of insulin, which is about 5 × 10^{-11} M (15). It is much more likely that small amounts of the proteolytic enzymes can simulate the action of insulin by cleaving membrane glycopeptides, as will be discussed shortly. This may result in membrane alterations which somehow resemble the events which ordinarily follow the initial recognition of insulin by the receptor.

**DISCUSSION**

There is strong evidence that the biological effects of insulin are initiated by the specific interaction of this hormone with specific receptors located exclusively on the surface of these cells (1). The availability of a specific and sensitive means of measuring the interaction of insulin with intact adipose tissue cells by criteria which do not depend on a biological response of the hormone (15) has permitted direct examination of some important properties of what is likely to be the biologically significant insulin receptor.

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ments were made with nonsaturating concentrations of insulin, and since the conditions (cell concentration, time of incubation, etc.) used to measure lipolysis and glucose oxidation are different, it is likely that the precise insulin and receptor concentrations in these experiments were not comparable. Also, in the absence of controlled hormone concentrations, the observation that tryptic digestion modifies the biological responses of fat cells to insulin and glucagon differently from the responses to epinephrine and adrenocorticotropin (13) is difficult to interpret.

More drastic digestion of fat cells with trypsin results in more complicated effects which are qualitatively different from those discussed above. In addition to a more dramatic effect on insulin affinity, more drastic digestion causes either a definite loss of insulin binding or damages the capacity of the glucose transport system to respond to formation of the insulin-receptor complex. These alternatives are very difficult to distinguish experimentally. In view of the reportedly intact nature of the glucose transport mechanisms in fat cells treated with higher concentrations of trypsin (13), it seems likely that under more drastic conditions trypsin may be cleaving other, more important regions of the insulin receptor. However, these effects may be similar to those seen after digesting cells with moderately high concentrations of neuraminidase (16). The latter appears to prevent proper transmission of the signals of the insulin-receptor complex to the transport processes.

Digestion of fat cells or fat cell membranes with phospholipase C or phospholipase A leads to a striking (4- to 8-fold) increase in the total quantity of insulin receptor (22). The insulin-receptor equilibrium constant, and the rate constants for association and for dissociation, are not altered (22). Removal of membrane phospholipids therefore appears to unmask a substantial amount of insulin-receptor which is qualitatively and kinetically indistinguishable from that normally expressed and accessible to insulin in the medium. It is interesting, moreover, that removal of membrane phospholipids also increases the susceptibility of the insulin receptor to destruction by trypsin. Those receptor structures which are normally exposed to the solvent and those that are unmasked by phospholipase treatment are both highly susceptible to proteolytic attack after removal of phospholipids. The destruction of the receptor under these conditions occurs with concentrations of trypsin which ordinarily do not appreciably affect the receptor of normal cells.

Furthermore, tryptic digestion of phospholipase-treated cells causes a qualitatively different type of destruction of the insulin receptor than is seen after digestion of normal cells. Trypsin appears to destroy the receptor, and not to affect the affinity for insulin. The usual effect of trypsin on affinity of the receptor for insulin is not observed in the phospholipase-treated cells. Those receptor structures which escape digestion after controlled, very mild trypsin treatment, appear to behave relatively normally in reaction to increasing concentrations of insulin. It seems, therefore, that new peptide bonds become exposed which are much more sensitive and susceptible to trypsin than are those which are cleaved in the normal cells. The former are then more susceptible, and they are cleaved under digestive conditions which are too mild to affect those bonds which would ordinarily be cleaved in the normal cells.

The peptide bonds of the insulin receptor structure which are exposed by phospholipase digestion are not only highly susceptible to trypsin cleavage, but they are also more "central" and critical to the function of the receptor since their cleavage results in total loss of binding capacity. All of these observations together suggest that, under normal circumstances, the insulin receptor is somewhat hindered in its location on the membrane so that proteolytic enzymes modify "peripheral" portions of the receptor structure which are important but not essential for the recognition function (for insulin) of the receptor. It is not clear what the function might be of the large amount of insulin receptor which is normally completely inaccessible to insulin but which can be exposed by removal of membrane phospholipids. It cannot yet be said whether this "reservoir" of receptor is ever overtly expressed biologically under certain physiological states or during some stages of morphogenesis.

The results of studies on the neuraminidase digestion of fat cells (16) are also consistent with the view that trypsin cleaves peptide located "peripherally" on the insulin receptor structures. Trypsin releases from the cell membrane sialopeptides which probably contain the same sialic acid residues which are released when the cells are digested with neuraminidase alone. Whatever factors are involved in making these glycopeptides highly accessible and susceptible to neuraminidase may also be responsible for their susceptibility to trypsin and perhaps other proteolytic enzymes.

The available data do not explain the enhanced rate of glucose oxidation frequently observed with very low concentrations of various proteolytic enzymes (5-10). It is very unlikely, however, that the insulin-like activity of these enzymes is explained by a potential ability of these enzymes to activate the insulin receptor by virtue of some conformational or catalytic similarity with the hormone. The activation which occurs after exposing the cells to low concentrations of neuraminidase appears to be related to removal of the sialic acid residue from the same peptide which is released by very mild trypptic cleavage (16). The stimulatory changes resulting from very mild proteolytic digestion have not yet been studied in detail.

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Perturbation of the Insulin Receptor of Isolated Fat Cells with Proteolytic Enzymes: DIRECT MEASUREMENT OF INSULIN-RECEPTOR INTERACTIONS
Pedro Cuatrecasas


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