Destruction of Insulin Effector System of Adipose Tissue Cells by Proteolytic Enzymes*

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SUMMARY

Insulin did not stimulate glucose utilization or inhibit lipolysis in adipose tissue cells previously incubated with trypsin. The acceleration of glucose oxidation by insulin was also reduced by prior incubation of cells with chymotrypsin, papain, or ficin, but not with purified collagenases or an acidic proteinase of Clostridium histolyticum. The loss of insulin effects was not due to destruction of the hormone. Furthermore, it appeared that trypsin did not digest cells nonspecifically since the treated tissue retained (a) a substantial lipolytic activity that was still responsive to epinephrine and adrenocorticotropin, (b) the capacity to oxidize glucose rapidly when sugar transport was stimulated by a high glucose concentration in the incubation medium, (c) a cell membrane that was not leaky to fat, glucose, or lactate dehydrogenase, and (d) a stereospecific sugar transport mechanism. These results suggest that a site (or sites) on the cell surface containing peptide components is necessary for the action of insulin on glucose transport and lipolysis. The site is probably distinct from the elements involved directly in glucose transport, lipolysis, and turnover of cyclic AMP.

In striated muscle and in adipose tissue, insulin stimulates the utilization of glucose by increasing the rate of the sugar transport across the cell membrane (1-6). In adipose tissue, insulin inhibits lipolysis by reducing the concentration of cyclic AMP (7-10). The initial step in both of these insulin reactions is probably the binding of the hormone to the target cells (11-14), but the details of these control mechanisms are still obscure. These insulin effects have been mimicked by trypsin or certain other proteolytic enzymes at very low concentrations (2 to 10 µg per ml) (15-19). However, these enzymes did not show any such activities at high concentrations (15-19), and trypsin at about 1 mg per ml has been considered to be noninjurious for a variety of cell types, at least during the short incubation period required to dissociate cells from the tissue for culturing (20, 21).

Recently, it has been noted that crude bacterial collagenase was more effective than trypsin in dispersing cells from various types of tissues (22-26), and that fat cells prepared by this method were sensitive to insulin and other hormones (27, 28). Fat cells were also dispersed (29) by a joint action of trypsin and a mixture of purified collagenases. However, as shown in this report, cells dispersed by these purified enzymes were almost unresponsive to insulin. This finding prompted the present studies on the effects of proteolytic enzymes on the insulin-dependent metabolism of rat epididymal adipose tissue cells. A preliminary account of a part of the present work has appeared in a review article (30).

MATERIALS AND METHODS

Two kinds of crude collagenase preparations, both from Clostridium histolyticum, were used. Crude collagenase N (obtained by special order from Nutritional Biochemicals) was a very impure preparation that had been precipitated only once with ammonium sulfate, whereas crude collagenase W (Code, CLS, from Worthington) was a partially purified preparation. Collagenases A-C and B-D (formerly considered to be a single enzyme, clostridiopeptidase A, EC 3.4.4.19) were purified from crude collagenase N as described previously (31). In addition, one of the proteolytic enzymes in the crude collagenase preparation was partially purified (obtained in Fraction I at purification Step 4 (31)), and is referred to as acidic proteinase from C. histolyticum. Trypsin (EC 3.4.4.4), α-chymotrypsin (EC 3.4.4.5), papain (EC 3.4.4.10), soybean trypsin inhibitor (all crystalline), ficin (EC 3.4.4.12, partially purified), and bovine serum albumin (Fraction V of Cohn et al. (32)) were also obtained from Nutritional Biochemicals. Insulin (bovine, from Mann), epinephrine and ACTH (both from Parke Davis), and D-glucose-U-¹⁴C (New England Nuclear) were also obtained commercially.

Male rats of the Sprague-Dawley strain were fed ad libitum, and those weighing 150 to 200 g were anesthetized with Nem

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1 The abbreviation used is: ACTH, adrenocorticotropic hormone.

2 D-Glucose-U-¹⁴C refers to the uniformly labeled compound.
The rate of glucose oxidation by adipose tissue cells was determined by measuring the production of $^{14}$CO$_2$ from uniformly labeled glucose. The cells or tissue from 0.1 g of fresh tissue were incubated for 30 min in 1 ml of buffer containing albumin (2%), and glucose (usually 0.1 pmole), with or without insulin (0.1 unit). The $^{14}$CO$_2$ produced was measured as described by Rodbell (27). The concentration of glucose in the incubation medium was determined by measuring the production of $^{14}$CO$_2$ from uniformly labeled glucose. The cells or tissue from 0.1 g of fresh tissue were incubated for 30 min in 1 ml of buffer containing albumin (2%), and glucose (usually 0.1 pmole), with or without insulin (0.1 unit). The $^{14}$CO$_2$ formed was measured as described by Rodbell (27). The concentration of glucose in the incubation medium was determined by a glucose oxidase method (34), after deproteinization by the Ba(OH)$_2$-ZnSO$_4$ method (35). The lipolytic reaction was followed by measuring the amount of glycerol released into the incubation medium. The incubation was carried out at 37°C and killed. Epididymal adipose tissue was taken out and its metabolism was studied with three types of preparations: (a) intact tissue that had not been treated with any enzymes, (b) partially digested tissue (see below) in which cells were associated in a matrix of certain proteins, and (c) cells dispersed from the tissue with the use of either crude collagenase as described by Rodbell (27) or various purified enzymes under the following conditions. Fresh adipose tissue (0.1 g) was incubated with 1 mg of trypsin (or other enzymes, cf. Table I) in 1 ml of buffer (see below) for 1 hour. The partially digested tissue was washed three times with 1-ml portions of buffer containing albumin (2%) and soybean trypsin inhibitor (0.1 mg per ml), and incubated again for 1 hour with purified collagenases (cf. Table I). Finally, the dispersed cells together with a small amount of incompletely digested tissue fragments were washed by repeated centrifugation and resuspension in a buffer containing albumin (2%). The control tissue was treated in exactly the same way but without the addition of any enzymes. Unless indicated otherwise, all incubations were performed in Krebs-Henseleit bicarbonate buffer (33), pH 7.4, equilibrated with 95% O$_2$-5% CO$_2$ at 37°C in plastic containers that were shaken (two strokes per sec) in a water bath.

The results in Fig. 1 indicate, however, that various cell preparations were obtained with various enzyme systems.

### RESULTS

**Glucose Oxidation by Dispersed Fat Cells**—Under the conditions indicated in Table I, adipose tissue cells were dispersed almost completely by various enzyme systems. In agreement with the previous results (29), free cells were not obtained by the action of either a noncollagenolytic protease or a mixture of the purified collagenases alone. The appearance of fat cells in these preparations was identical by phase microscopy. The content of lactate dehydrogenase in all of these cell preparations was about 80% of that in intact tissue (cf. Table I, Footnote a). This suggested that only a small portion of the fat cells has been broken in making these preparations since lactate dehydrogenase, which is localized entirely in the soluble fraction of adipocytes (38), would have been lost from these washed cell preparations if the cell membrane was injured.

The results in Fig. 1 indicate, however, that various cell preparations were obtained with various enzyme systems. The figure shows the rates of oxidation of glucose (0.3 pmole plus a trace of glucose-U-$^{14}$CO$_2$) to $^{14}$CO$_2$ in the presence or absence of insulin by (A) control adipose tissue and (B to E) free fat cells that had been prepared by dispersion of tissue with (B) trypsin and purified collagenases, (C) crude collagenase N, (D) crude collagenase W, and (E) acidic proteinase from C. histolyticum and purified collagenases. The conditions for the preparation of cells are shown in Table I.

#### Table I

<table>
<thead>
<tr>
<th>Additives, to 0.1 g adipose tissue in 1 ml</th>
<th>Lactate dehydrogenase in the cell preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First incubation (1 hr)</strong></td>
<td><strong>Second incubation (1 hr)</strong></td>
</tr>
<tr>
<td>Crude collagenase N (3 mg)</td>
<td>No second incubation</td>
</tr>
<tr>
<td>Trypsin (1 mg)</td>
<td>Collagenases*</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsin (1 mg)</td>
<td>Collagenases*</td>
</tr>
<tr>
<td>Trypsin (0.5 mg) + $\alpha$-chymotrypsin (0.5 mg)</td>
<td>Collagenases*</td>
</tr>
<tr>
<td>$\Gamma$-papain (1 mg)</td>
<td>Collagenases*</td>
</tr>
<tr>
<td>Ficin (5 mg)</td>
<td>Collagenases*</td>
</tr>
<tr>
<td>Acidic proteinase (2 mg)</td>
<td>Collagenases*</td>
</tr>
<tr>
<td>Trypsin (0.5 mg) + $\alpha$-chymotrypsin (0.5 mg), no Ca$^{2+}$</td>
<td>Collagenases*</td>
</tr>
</tbody>
</table>

* Activity in the cell preparation from 0.1 g of tissue $\times$ (activity in 0.1 g of fresh tissue)$^{-1} \times 100$.

* Mean value $\pm$ standard error of the mean (number of observations).

* Purified collagenases (25 $\mu$g of $\alpha$-collagenase + 50 $\mu$g of $\beta$-collagenase) and 20 mg of albumin.

#### Fig. 1

Glucose oxidation by adipose tissue and fat cells prepared with different enzyme systems. The figure shows the rates of oxidation of glucose (0.3 pmole plus a trace of glucose-U-$^{14}$CO$_2$) to $^{14}$CO$_2$ in the presence or absence of insulin by (A) control adipose tissue and (B to E) free fat cells that had been prepared by dispersion of tissue with (B) trypsin and purified collagenases, (C) crude collagenase N, (D) crude collagenase W, and (E) acidic proteinase from C. histolyticum and purified collagenases. The conditions for the preparation of cells are shown in Table I. The number and vertical line at the top of each bar indicate the number of trials and standard error of the mean. * wet weight of fresh tissue from which the preparation was made.
In some cases, untreated control tissue was incubated under standard conditions (cf. "Materials and Methods") in the presence of Ca++. The partially digested tissue then was washed and incubated with uniformly labeled glucose for 30 min under the standard conditions (cf. "Materials and Methods") in the presence or absence of insulin (0.1 unit per ml). In some cases, untreated control tissue was incubated together with the partially digested tissue (cross-hatched columns).

The above results suggested that trypsin together with a certain component of crude collagenase (see "Discussion") may be harmful for metabolism of fat cells. It therefore was of interest to determine the effects of trypsin alone on fat cells. However, unlike cells in tissue, dispersed fat cells were ruptured easily on exposure to such treatment. Accordingly, effects of individual enzymes on glucose oxidation were studied, as shown in the next section, by incubating adipose tissue with single enzymes (except in case of the collagenases) at concentrations used in the above experiments (Table I), and by determining the metabolic activities of the partially digested tissue preparations.

Glucose Oxidation by Partially Digested Adipose Tissue—The results shown in Fig. 2 indicate that glucose oxidation of adipose tissue in the presence of insulin was little affected by prior treatment of the tissue with purified collagenases or acidic proteinase, but inhibited seriously with trypsin, chymotrypsin, papain, or ficin. Under these conditions, however, inactivation of the cells located deep inside of the tissue might not have been complete since a small insulin effect was usually seen when the cells were prepared from these apparently unresponsive tissue preparations (see Fig. 1B).

It is unlikely that the failure to show insulin effects was due to proteolytic decomposition of insulin since the incubation media stimulated glucose oxidation in untreated tissue that was incubated together with the partially digested tissue (Fig. 2). Furthermore, the media incubated with partially digested tissue for 30 min still retained the capacity to stimulate glucose oxidation in isolated fat cells (Fig. 3). Also, it does not appear that the insulin unresponsiveness was caused by a nonspecific destruction of fat cells since the rate of basal metabolism was not seriously affected. In addition, it has already been shown in Table I that the contents of lactate dehydrogenase were about 80% of the control even after the cells were dispersed from these tissue preparations. In agreement with this view, the data in Table II indicate that decomposition of the cells by the trypsin treatment was negligible.

The effects of trypsin on certain cell types are affected by divalent cation (29). However, it was shown (data are not shown) that removal of divalent cations (Ca++ or Mg++) or both from the incubation medium had little effects on glucose metabolism in either the control or trypsin-treated adipose tissue.

Characterization of Effects of Trypsin on Glucose Uptake and Metabolism—As production of CO2 accounts for only about 30%
of the total glucose utilization in isolated adipose tissue (30, 40),
effects of trypsin on glucose uptake from the incubation medium
were studied. The results in Table III indicate that insulin did
not stimulate the uptake of exogenous glucose by the treated
tissue. The presence of insulin in the incubation medium was
confirmed by adding untreated tissue to the medium.

When the concentration of glucose in the incubation medium
was increased from 0.3 to 3.0 mM (Table IV), the rate of glucose
oxidation was increased substantially both in the control and in
the trypsin-treated tissues. However, the metabolism of the
controlled tissue was not stimulated by insulin at either glucose
concentration.

The data in Table IV further indicated that the metabolism of
trypsin-treated tissue in the high glucose medium was strongly

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**Table II**

**Composition of control and trypsin-treated tissue**

The tissue (0.1 g) was incubated with or without trypsin (1 mg) in 1 ml of calcium-free Krebs-Henseleit bicarbonate buffer
for 1 hour, rinsed, and subjected for analyses.

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantities found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control tissue</td>
</tr>
<tr>
<td></td>
<td>units/100 mg fresh tissue</td>
</tr>
<tr>
<td>Lipid, dry weight (mg)</td>
<td>81.2 ± 3.8 (4)</td>
</tr>
<tr>
<td>Nonlipid, dry weight (mg)</td>
<td>3.6 ± 0.4 (4)</td>
</tr>
<tr>
<td>Lactate dehydrogenase (units)</td>
<td>406 ± 26 (4)</td>
</tr>
</tbody>
</table>

* See Table I, Footnote b.
  ** Millimicromoles of NADH formed in 1 min.

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**Table III**

**Glucose uptake by adipose tissue preparations**

The tissue (0.1 g) was treated with or without trypsin under standard conditions and then incubated with 0.5 μmole of glucose
and 20 mg of albumin in 1 ml of buffer. The rate of glucose
uptake was estimated by measuring the glucose concentration
in the incubation medium at 0 and 60 min by the glucose oxidase
method (cf. "Materials and Methods"). In the third experiment,
untreated control tissue was incubated together with trypsin-
treated tissue.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Glucose taken up from the medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minus insulin</td>
</tr>
<tr>
<td></td>
<td>mpmoles x (1 hr)⁻¹ x (0.1 g⁻¹)</td>
</tr>
<tr>
<td>Control tissue</td>
<td>36 ± 7 (6)</td>
</tr>
<tr>
<td>Trypsin-treated tissue</td>
<td>33 ± 14 (4)</td>
</tr>
<tr>
<td>Control + trypsin-treated tissue</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

* Weight of fresh adipose tissue from which the preparation
  was made.
  ** See Table I, Footnote b.

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**Table IV**

**Effects of glucose concentration and phloretin on glucose oxidation by trypsin-treated tissue**

The tissue treated with or without trypsin under standard conditions in the calcium-free buffer was incubated with either 0.3 or
3.0 mM glucose (plus a trace of glucose-U-¹⁴C), and the rate of glucose oxidation was estimated by standard procedures (cf. "Materials
and Methods").

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Glucose concentration</th>
<th>Additives</th>
<th>Conversion of glucose to CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td></td>
<td>Minus insulin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>mpmoles glucose x (0.5 hr)⁻¹ x (0.1 g⁻¹)</td>
</tr>
<tr>
<td>Control tissue</td>
<td>0.3</td>
<td>None</td>
<td>2.3 ± 0.3 (7)</td>
</tr>
<tr>
<td>Control tissue</td>
<td>3.0</td>
<td>None</td>
<td>26.7 ± 3.5 (4)</td>
</tr>
<tr>
<td>Trypsin-treated</td>
<td>0.3</td>
<td>None</td>
<td>1.9 ± 0.2 (3)</td>
</tr>
<tr>
<td>Trypsin-treated</td>
<td>3.0</td>
<td>None</td>
<td>18.1 ± 2.0 (5)</td>
</tr>
<tr>
<td>Trypsin-treated</td>
<td>3.0</td>
<td>Minus albumin</td>
<td>14.9 ± 1.2 (5)</td>
</tr>
<tr>
<td>Trypsin-treated</td>
<td>3.0</td>
<td>Minus albumin + phloretin, 1 was</td>
<td>2.7 ± 0.4 (5)</td>
</tr>
</tbody>
</table>

* See Table III.
  ** See Table III.
inhibited by phloretin. This compound strongly inhibits glucose transport across the cell membrane in the absence of albumin (6). The metabolism was also inhibited by 3-O-methyl-d-glucose and d-xylene but not by l-xylene (Fig. 4). The first two compounds, but not the last, compete effectively with glucose for transport across the cell membrane in muscle and adipose tissue (6, 41).

Effects of Trypsin Treatment on Lipolysis—As shown in Fig. 5, epinephrine and ACTH stimulated lipolysis in tissue that had been incubated with trypsin. The amounts of glycerol released from the trypsin-treated tissue were approximately 70% of the controls (Fig. 5). The tissue treated with purified collagenase, acidic proteinase, chymotrypsin, or papain (under the conditions shown in Fig. 2) was also sensitive to epinephrine, and the amounts of glycerol released were 70 to 100% of the control.

When the concentration of epinephrine was suboptimal (0.1 µg per ml), insulin inhibited lipolysis in the control tissue as originally described by Jungas and Ball (7), but not in the trypsin-treated tissue (Fig. 6). In agreement with previous results (8–10), epinephrine increased the intracellular level of cyclic AMP, and insulin reduced the concentration of this compound (Fig. 7). In the trypsin-treated tissue, however, insulin failed to reduce the level of cyclic AMP which was accumulated by the effect of epinephrine (Fig. 7).

DISCUSSION

It has been noted previously that fat cells treated with phospholipase C (42) or liver cells disassociated from the tissue by removing calcium ions (43–45) did not utilize glucose rapidly. In these cases, however, the low activities could be attributed to damage to the cell membrane since the fat or liver cells lost substantial amounts of fat (42) or soluble enzymes including lactate dehydrogenase (43–45). In contrast, adipose tissue cells treated with trypsin retained substantially all of their fat (Table II) and lactate dehydrogenase (Tables I and II).

It would appear from the following considerations that trypsin impairs insulin responsiveness but does not seriously damage glucose transport and subsequent metabolic steps. In the absence of insulin, the uptake of glucose and its conversion to CO₂ are limited by transport of glucose across the cell membrane (5, 6), and acceleration of this step accounts for the increase in glucose uptake and CO₂ production caused by the hormone (5, 6). Thus, the failure of insulin to stimulate glucose uptake and its conversion to CO₂ after trypsin treatment of the tissue could be due (a) to damage to the insulin “effector system,” as discussed below, (b) to the transport systems itself, or (c) to subsequent intracellular metabolic steps. However, damage to the latter two seems to be unlikely on the following grounds. First, the basal rate of glucose transport, as indicated by rates of glucose
uptake (Table III) or production of labeled CO₂ (Table IV), was
not reduced. Second, the transport system could be accelerated
many fold by raising the glucose concentration in the incubation
medium (Table IV), and this response was not greatly different
from the response of untreated cells. This shows that the trans-
port system and subsequent metabolic steps in the treated cells
had ample capacity to respond to insulin if the hormonal signal
had been transmitted. Third, the integrity of the cell mem-
brane and the transport system after trypsin treatment was
indicated by the fact that glucose metabolism was inhibited by
certain sugars which characteristically compete with glucose at
the transport level (Fig. 4) and by the strong inhibition produced
by phloretin (Table IV). The latter compound, which is a well
known transport inhibitor, was so effective that it eliminated the
existence of any substantial entrance of sugar by simple diffusion
caused by “leakiness” of the treated cell membrane.

The response of the lipolytic system to insulin was also de-
stroyed by trypsin treatment, and the arguments localizing the
site of the enzyme action are analogous to those just reviewed.
It has been known that the lipase system in the cell is activated
by cyclic AMP (8–10), and that the production of this substance
is stimulated by epinephrine or other hormones (8–10). Insulin
reduces the concentration of cyclic AMP in intact cells, particu-
larly when the level of the compound is already high (8–10).
However, it is not certain (9) whether the hormone suppresses
the formation of cyclic AMP by adenylyl cyclase or enhances its
hydrolysis catalyzed by cyclic adenylyl diesterase. The present
data indicate that the trypsin-treated cells retained their lipolytic
response to epinephrine or ACTH (Fig. 5) and showed about the
same rise in cyclic AMP as untreated cells (Fig. 7). It would
seem likely then that trypsin had not seriously damaged the
lipolyte pathway involving the adenylyl cyclase, diesterase,
and lipase systems. The responsiveness of these systems to insulin,
however, was gone entirely (Figs. 6 and 7), indicating that tryp-
sin had damaged a part of the effector system that was distinct
from the above enzyme systems.

One can propose, therefore, as a working hypothesis that
trypsin damages a component necessary for transmission of the
insulin signal to both the transport and lipolytic systems. For
want of a better term, one can suggest the term “insulin effector
system” (Fig. 8) to include the site at which insulin is bound to
the cell and the unknown further elements which may be neces-
sary to generate and transmit appropriate signals to the glucose
transport and adenylyl cyclase-diesterase systems.

Since the effect of trypsin on the insulin system is shared by a
number of other proteolytic enzymes and persists after washing
(Fig. 2), it is very likely that the component modified is a peptide
and that proteolysis is involved. As trypsin treatment does not
cause any apparent damage to intracellular metabolic systems,
it appears that the above-mentioned peptide is localized on the
cell surface. The possibility that trypsin prevents the initial
binding of the hormone to the cell (11–14), by proteolytic attack
on the binding site, is under study.

The present results appear to be in conflict with earlier reports
(15–19) that trypsin and certain other proteolytic enzymes have
insulin-like effects on fat cells. The discrepancy, however, is
accounted for, as will be presented in a separate report, by the
difference in the concentrations of enzymes used; low concen-
trations induce insulin effects whereas high concentrations prevent
them. One can speculate in this regard that progressive damage
first induces and subsequently prevents allosteric changes that

the insulin molecule normally brings about on membrane com-
ponents.

It has been noted in the past that the response to insulin of fat
cells prepared with different batches of crude collagenase is
unpredictable (e.g. see Fig. 1). This can now be understood since
crude collagenase preparations contain an enzyme (clostridio-
peptidase B, EC 3.4.4.20) which has a substrate specificity
similar to but not identical with trypsin (46–48). Because of
this specificity, the enzyme, if present in sufficient quantities,
may impair the insulin responsiveness of fat cells. On the other
hand, the acidic proteinase, which is also in crude bacterial col-
lagenase (31), was unique among the enzymes tested (Fig. 2) in
that it digests the proteins which bind cells together without im-
pairing insulin responsiveness of the cell. This enzyme rapidly
hydrolyzes casein and gelatin at pH 6.5 to 7.0 in the presence of
magnesium or calcium ions, but is practically inactive against
native collagen and either native or heat-denatured albumin.4

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