Insulin-like Effects of Trypsin on Fat Cells

LOCALIZATION OF THE METABOLIC STEPS AND THE CELLULAR SITE AFFECTED BY THE ENZYME

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SUMMARY

The mechanism of the insulin-like action of trypsin was studied. When fat cells were exposed to trypsin (1 mg per ml) for 15 sec, glucose metabolism in the cells was stimulated. This stimulation (like that by insulin) was inhibited by phloretin or 3-O-methyl-d-glucose. By the above treatment with trypsin, cellular lipolysis induced by epinephrine was blocked to a large extent, but that induced by caffeine was only partially inhibited. These effects of trypsin on lipolysis were similar to those of insulin at a low concentration (20 to 30 micromolars per ml). Trypsin (like insulin) did not inhibit lipolysis induced by N6,O2'-dibutyryl adenosine 3',5'-monophosphate.

When fat cells treated with trypsin were incubated with buffer for a few hours after inactivation of the enzyme, the cells had recovered from the insulin-like effects of the enzyme. The recovery was not significantly inhibited by cycloheximide. The "recovered" cells were less sensitive to both insulin and trypsin as compared with untreated fat cells. This decrease in sensitivity was partially prevented when a large dose of insulin was added to the incubation medium prior to the trypsin treatment.

These data were consistent with an assumption that trypsin (a) interacts initially with the insulin receptor site of fat cells, (b) activates the receptor to produce the metabolic responses characteristic of insulin, and (c) eventually modifies the receptor to render the cells less sensitive than normal to both insulin and trypsin.

Insulin stimulates glucose transport across the cell membrane of muscle and fat cells and inhibits lipolysis in fat cells (cf. References 1 and 2). These effects of insulin are mimicked by certain proteolytic enzymes at low concentrations (2 to 10 µg per ml) (3-9). Although the latter observations are potentially important in the understanding of the mechanism of insulin action, it has been obscure whether or not the hormone and these proteolytic enzymes regulate the cellular metabolism by the same mechanism. Accordingly, the present work was initiated to examine whether insulin and a proteolytic enzyme, trypsin, regulate the same metabolic steps and, if they do, whether the two effectors regulate the metabolic reactions upon interaction with a common cellular element (or receptor).

We were particularly interested in the insulin-like effects of trypsin on fat cells since it was recently observed in this laboratory that when fat cells are incubated with trypsin at a high concentration, the enzyme mimics the effects of insulin at the beginning of incubation but subsequently renders the cells unresponsive to insulin, presumably by proteolytic modification of the insulin effector system (10, 11). Accordingly, in the present work, fat cells were incubated with trypsin at a high concentration (1 mg per ml), and the initial insulin-like effects of the enzyme were compared not only with the effects of insulin but also with the subsequent effects of the enzyme itself. A preliminary account of part of the present work has been published (12).

MATERIALS AND METHODS

Trypsin, soybean trypsin inhibitor (chromatographically purified), and crude bacterial collagenase were purchased from Worthington. According to the manufacturer, 1 mg of the soybean trypsin inhibitor preparation had a capacity to inhibit 1.7 mg of trypsin. Dibutyryl cyclic AMP was obtained from Boehringer Mannheim. Crude bovine serum albumin (Fraction V) was purchased from Nutritional Biochemicals and from Schwarz-Mann. Bovine insulin crystallized 10 times was a gift from Dr. Schlichtkrull of Novo Laboratories. The specifications of other reagents have previously been described (11). The medium used for incubation of fat cells was Krebs-Ringer bicarbonate buffer (13), pH 7.4, supplemented with penicillin, streptomycin (both 20 µg per ml), and bovine serum albumin (Fraction V, 20 mg per ml).

The isolated fat cells were prepared by the collagenase method of Rodbell (14) from rat epididymal adipose tissue. Rats used were of the Sprague-Dawley strain; the body weights were 150 to 200 g. The insulin-like effects were induced in fat cells by exposing the cells to trypsin (1 mg per ml) for 15 sec (t1) at 37°. The enzyme reaction was terminated by adding soybean trypsin inhibitor (1 mg per ml). Usually, the treated cells were incubated for an additional 10 min (t2; see "Results") before metabolic activities were tested.

1 The abbreviations used are: dibutyryl cyclic AMP, N6,O2'-dibutyryl adenosine 3',5'-monophosphate; cyclic AMP, adenosine 3',5'-monophosphate; DFP, diisopropyl phosphofluoridate; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone.
Fig. 1. Stimulation of glucose utilization in fat cells by trypsin. Aliquots of fat cells were incubated with trypsin ($T$: 1 mg per ml) for $t_1$ minutes, and the enzyme reaction was terminated with soy bean trypsin inhibitor (STI) (see the schedule at the top right corner of the figure). Then, $t_2$ minutes later, the reaction mixture was supplemented with labeled glucose ($G^*$), and the amount of labeled CO$_2$ formed during the next $t_3$ minutes was determined (in the absence of insulin). Panel A shows the effects of $t_1$ on the rate of glucose oxidation when $t_2$ was 10 min. Panel B shows the effects of $t_2$ when $t_1$ was 15 sec. In both cases, $t_3$ was 10 min. As controls, untreated fat cells were incubated with labeled glucose for 10 min either in the presence (●) or absence (○) of insulin (1 milliunit per ml). Note: Unless stated otherwise, each point and small bar in this and the following figures represent the mean value of three observations and the standard error, respectively.

Fig. 2. Effects of glucose transport inhibitors on glucose utilization by trypsin-treated fat cells. Aliquots of fat cells were treated with trypsin under standard conditions. The rate of glucose utilization was determined without or with the addition of 1 mm phloretin, 20 mm 3-O-methyl-D-glucose, or 20 mm L-xylose as shown, each either in the presence (●) or absence (○) of insulin. As controls, aliquots of untreated (intact) cells were incubated with glucose.

Fig. 3. Antilipolytic effect of trypsin. Since the sensitivity of fat cells to epinephrine increases with time the cells in this experiment were incubated for a total of 130 min. At appropriate time intervals, aliquots of cells were treated with trypsin for 15 sec (●; see Fig. 1), and the incubation was continued after the enzyme was inactivated with soybean trypsin inhibitor. Then, $t_2$ minutes later, the cells were supplemented with epinephrine (0.655 pm) and further incubated for an additional 10 min ($t_3$). The accumulation of glycerol was determined as usual, the data were corrected for the appropriate blank values, and the amounts of glycerol accumulation during the last 10 min were plotted against the $t_2$ values as shown. As a control, the epinephrine-induced accumulation of glycerol by untreated (intact) cells was determined under the same conditions. Each point in the plot represents the mean value of two observations.

The rate of glucose utilization by fat cells was estimated from the rate of conversion of $\nu$-[U-14C]glucose into $^{14}$CO$_2$ and $[^{14}$C]-lipid (10). Since the rates of formation of CO$_2$ and lipid were always parallel to each other, however, only one set of determinations (either of CO$_2$ or of lipid) is presented. The rate of lipolysis (in the absence of glucose) was estimated by determining the accumulation of glycerol and fatty acids in the reaction mixture with the enzymatic method of Wieland (15) and the titrimetric method of Dole and Meinertz (16), respectively. Since the results of these determinations were also parallel to each other, only a set of the data is presented in figures. The accumulation of cyclic AMP in the incubation mixture was determined by an enzymatic method (17) as described by Butcher, Baird, and Sutherland (18). The amount of cells was estimated from the content of malate dehydrogenase in the cell preparation (11); the results are shown in terms of the wet weight of fresh adipose tissue (11). The activity of trypsin was determined spectrophotometrically with N-benzoyl-DL-arginine ethyl ester as the substrate (19).

RESULTS

Effects of Trypsin on Glucose Metabolism—As it can be seen in Fig. 1, glucose metabolism in fat cells was stimulated when fat cells were exposed to trypsin (1 mg per ml) for a short time; the maximum stimulation was observed when the exposure period ($t_1$) was 15 to 30 sec (Fig. 1A). The degree of stimulation was altered, however, when treated cells were incubated further for a second time ($t_2$) after trypsin was inactivated (Fig. 1B). Thus, the stimulation was intensified during the first 20 min of the

* T. Kono, unpublished observation.
EFFECTS OF TRYPSIN ON LIPOLYSIS STIMULATED BY DIBUTYRYL CYCLIC AMP

Alloquots of fat cells were treated with trypsin for 15 sec, and the enzyme reaction was terminated with soybean trypsin inhibitor. The treated and untreated cells were incubated with dibutyryl cyclic AMP at different concentrations as shown. The accumulation of both glycerol and fatty acids are recorded in this table. As controls, alloquots of cells were incubated with epinephrine as shown.

![Graph showing the effects of trypsin on lipolysis](http://www.jbc.org/)

**TABLE 1**

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**Effects of trypsin on lipolysis stimulated by dibutyryl cyclic AMP**

Alloquots of fat cells were treated with trypsin for 15 sec, and the enzyme reaction was terminated with soybean trypsin inhibitor. The treated and untreated cells were incubated with dibutyryl cyclic AMP at different concentrations as shown. The accumulation of both glycerol and fatty acids are recorded in this table. As controls, alloquots of cells were incubated with epinephrine as shown.

![Graph showing the effects of trypsin on lipolysis](http://www.jbc.org/)

**Fig. 5. Relative antilipolytic effects of insulin and trypsin.**

Alloquots of fat cells were either supplemented with different doses of insulin or treated with trypsin for 15 sec as shown at the bottom of the figure. After 15 min of subsequent incubation (t2, see Fig. 1), each cell preparation was supplemented with either 0.55 μM epinephrine (○) or 1 mM caffeine (●). The rate of lipolysis was determined as usual. The experiments with insulin were performed in duplicate, while those with trypsin were carried out in triplicate.

The cells “leaky” to glucose (cf. “Discussion” in Reference 10). Although the data are not shown, trypsin did not produce any significant amount of insulin-like activity in the incubation medium. The latter observation was in agreement with the data obtained by Kuo, Dill, and Holmlund (6) and Kuo (7) with a bacterial proteinase.

**Effects of Trypsin on Lipolysis**—As it can be seen in Fig. 3, the epinephrine-dependent lipolysis in fat cells was inhibited when the cells were exposed to trypsin (1 mg per ml) for 15 sec (t1). However, upon subsequent incubation of trypsin-treated cells for t2 minutes, the inhibitory effect was enhanced during the first 20 min and then decreased gradually.

In contrast, no significant antilipolytic effect of trypsin was observed when dibutyryl cyclic AMP was used as the lipolytic agent (Table 1). These data were similar to those obtained earlier with insulin (20) and suggested that trypsin (like insulin) had little effect on the lipolytic mechanism subsequent to the step activated by cyclic AMP. In addition, neither the antilipolytic effect of trypsin nor that of insulin was evident when trypsin treatment was carried out for 15 min instead of 15 sec (Fig. 4). This observation was consistent with an assumption that trypsin did not inhibit the lipolytic mechanism directly, but initially inhibited the mechanism indirectly by activating the cellular insulin receptor or certain other site, which was destroyed by the enzyme during prolonged incubation.

As shown in Fig. 5, the antilipolytic effect of trypsin in the presence of either epinephrine or caffeine was comparable with that of insulin only at low hormone concentrations (20 to 30 micromoles per ml). Although it is not directly related to the present work, it is of interest to note (a) that the inhibitory effect of either insulin at a low concentration (10 micromoles per ml) or trypsin applied for 15 sec was greater with epinephrine as lipolytic agent than with caffeine and (b) that insulin showed a biphasic effect on lipolysis induced by epinephrine but not that induced by caffeine (Fig. 5). Preliminary data (not shown3) indicated that the effect

![Graph showing the effects of trypsin on lipolysis](http://www.jbc.org/)

3 T. Kono and R. J. Ho, unpublished observation.
Effects of modified trypsin preparations on fat cell metabolism

In Experiments 1 through 4, fat cells were incubated for 15 sec with either trypsin or modified trypsin preparations (each, 1 mg per ml) as shown. After 15 sec, the reaction mixture was supplemented with soybean trypsin inhibitor (1 mg per ml). In Experiment 5, cells were mixed with trypsin and soybean trypsin inhibitor (each 1 mg per ml) in the reverse order. The rate of glucose utilization was determined as usual. The proteolytic activities of the trypsin preparations modified with DFP and TLCK were 1.0 and 0.7% of the native enzyme.

In addition, it was shown in Table II (Experiment 5) that no significant insulin-like effects occurred when fat cells were exposed to trypsin preparations that had previously been modified with either diisopropyl phosphofluoridate or 1-chloro-3-tosylamido-7-amino 2-keptonone. It has been known that DFP and TLCK attack preferentially the serine and histidine residues, respectively, that are located at the active center of the enzyme (21, 22).

Effects of Modified Trypsin Preparations on Glucose Metabolism in Fat Cells—The data shown in Table II indicated that no significant insulin-like effects occurred when fat cells were exposed to trypsin preparations that had previously been modified with either diisopropyl phosphofluoridate or 1-chloro-3-tosylamido-7-amino 2-keptonone. It has been known that DFP and TLCK attack preferentially the serine and histidine residues, respectively, that are located at the active center of the enzyme (21, 22). In addition, it was shown in Table II (Experiment 5) that no insulin-like effect occurred and the effect of insulin was not significantly affected when unmodified trypsin was added to a cell suspension that had previously been supplemented with soybean trypsin inhibitor. This observation indicated that soybean trypsin inhibitor was highly effective in terminating the action of trypsin without disturbing fat cell metabolism.

Recovery from Insulin-like Effects of Trypsin—As it can be seen in Fig. 6, reversal of the insulin-like effect on glucose metabolism was not significantly inhibited by cycloheximide. In contrast, recovery of cells from a long (15 min) trypsin treatment was greatly inhibited by the reagent (Fig. 6), as reported previously (11). Similar effects of cycloheximide were observed by measuring the change in lipolytic activity, although the data (not shown) were complicated by the fact that the apparent sensitivity of fat cells to lipolytic agents increases with time.

The cells recovered from the insulin-like effects of trypsin were highly responsive to insulin at 1 milliunit per ml (Figs. 6 and 7A). However, "recovered" cells were not as sensitive to insulin as untreated cells (Fig. 7A). In addition, neither glucose utilization nor lipolysis in "recovered" cells was affected by a second trypsin treatment for 15 sec (Fig. 8), presumably because the cells were not very sensitive to either insulin or trypsin. When fat cells were allowed to recover for 2 hours from a long (15 min) trypsin

Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cells treated with</th>
<th>Glucose utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>Nothing</td>
<td>2.1 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>Trypsin</td>
<td>23.5 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>DFP-trypsin</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>TLCK-trypsin</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>Soybean trypsin-inhibitor</td>
<td>2.6 ± 0.5</td>
</tr>
</tbody>
</table>

* Nanomoles × (30 min)** × (10 mg of cells)**.

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

Fig. 6. A difference in the effects of cycloheximide on the recoveries of fat cells from a brief (15 sec) and a long (15 min) trypsin treatment. All the cells in this experiment were incubated for a total of 210 min either in the presence or absence of 100 μM cycloheximide as shown (± CYCLO). At appropriate intervals, aliquots of cells were treated with trypsin for either 15 sec or 15 min as shown (t1), and the enzyme reaction was terminated with soybean trypsin inhibitor. As controls, aliquots of untreated (INTACT) cells were mixed with trypsin and soybean trypsin inhibitor in the reverse order. Then, after the cells were allowed to recover for the indicated period (t2; either 0 or 2 hours), the rate of glucose metabolism was determined as usual, either in the presence (△) or absence (●) of insulin (1 milliunit per ml). The activity shown was that given by 10 mg of cells in 30 min.

Fig. 7. Insulin response of fat cells that had recovered from trypsin treatment for 15 sec (Panel A) or 15 min (Panel B). In the experiments shown in Panels A and B, aliquots of cells were divided into two and three groups, respectively. The first group (intact) was mixed with trypsin and soybean trypsin inhibitor in the reverse order, and incubated for 2 hours in the same incubation medium. The second group ("treated") was treated with trypsin for either 15 sec or 15 min (t1; see Fig. 1), and then incubated for 2 hours for recovery after the inactivation of trypsin. The third group (treated; Panel B only), was incubated with buffer alone for 2 hours, and then treated with trypsin followed by soybean trypsin inhibitor. Aliquots of each cell preparation were then supplemented with the indicated doses of insulin, and the rates of glucose utilization were determined as usual.

Each point in this figure represents the mean value of two observations.
Insulin-like Effects of Trypsin

The decrease in insulin sensitivity by trypsin treatment was not obvious when the treatment was carried out in the presence of insulin, 100 milliunits per ml (Fig. 9). However, as it can be seen in the figure, the basal glucose oxidation of fat cells treated as above did not return to the control level; the reason for this is unknown. In this regard, the cells exposed to insulin alone (100 milliunits per ml) without trypsin treatment did regain a nearly normal rate of basal glucose oxidation (as well as a nearly normal level of sensitivity to the hormone) under the same conditions (the data are not shown). Any protective effect of insulin (such as shown in Fig. 9) was not observed when trypsin treatment was extended to 15 min. This was not surprising, however, since most of the added insulin (100 milliunits per ml) was destroyed by trypsin under these conditions.

DISCUSSION

The data presented in this paper are consistent with an interpretation that trypsin affects fat cell metabolism by regulating the same metabolic reactions that are controlled by insulin. Thus, it was noted that trypsin (like insulin) stimulates glucose metabolism in fat cells by stimulating the glucose transport system in the cell membrane (Fig. 2). This observation is in agreement with the data of previous studies on the effects of trypsin on frog muscle (9) and of a bacterial enzyme on fat cells (6). Previously, it was suggested that insulin and certain proteolytic enzymes may inhibit lipolysis by different mechanisms since, unlike the hormone at a high concentration (1 milliunit per ml), the enzymes did not significantly lower the cellular level of cyclic AMP (8). However, it was noted in the present work that the insulin-like effects of trypsin are comparable with the effects of insulin only at low hormone concentrations (20 to 30 microunits per ml). It is known that the effect of insulin on the level of cyclic AMP in fat cells is maximal when the hormone concentration is 1 milliunit per ml (18) and is small when it is 10 microunits per ml (18).

In agreement with this view, our preliminary data indicated that the effects of dilute insulin and trypsin on the cellular level of cyclic AMP are both small. Naturally, as previously discussed by Kuo and De Renzo (8), this does not exclude the possibility that the concentration of the nucleotide in a certain cellular compartment is greatly affected by either insulin at low concentrations or certain proteolytic enzymes including trypsin. In fact, the present data with dibutyryl cyclic AMP (Table I) are in agreement...
with the theory that trypsin (like insulin [18]) inhibits lipolysis by decreasing the concentration of cyclic AMP.

In addition, the present data are consistent with an assumption that insulin and trypsin interact with the same cellular site. Although it has previously been hypothesized otherwise [12], the bases of the hypothesis were the observations that (a) the antilipolytic effect of trypsin (see Fig. 5) and (b) the effects of trypsin on "recovered" cells (see Fig. 7) were different from those of insulin at a high concentration (1 milliunit per ml). However, no significant differences were observed when the effects of the enzyme were compared with those of insulin at a low concentration (see Figs. 5, 7, and 8). Furthermore, as reported elsewhere, (a) binding of [3H]insulin (as well as native insulin) to fat cells was blocked when the cells were treated with trypsin for 15 sec [23], and (b) the decrease in insulin-binding capacity of fat cells by trypsin was prevented when the cells were exposed to insulin prior to trypsin treatment [23]. The latter observation is in agreement with the present data (Fig. 9), which suggest that insulin prevents the enzymatic modification of the insulin receptor, although the present data (Fig. 9) are inconclusive when considered separately because of a relatively high metabolic activity of "recovered" cells.

Based on the considerations mentioned above, it is suggested as a working hypothesis that trypsin mimics the effects of insulin, which has no proteolytic activity [24], upon binding to the cellular insulin receptor. This hypothesis is compatible with the earlier observation of Kuo et al. that the insulin-like activity of a bacterial proteinase was maintained when the proteolytic activity of the enzyme was abolished by an acid treatment [5]. The binding of trypsin seems to occur at, or in the vicinity of, the serine and histidine residues located at the active center (Table II). It is of interest that histidine is also essential for the hormonal activity of insulin [25-27]. Unlike insulin, however, trypsin seems to modify the cellular insulin receptor making the cells less sensitive than normal to both insulin and trypsin (Figs. 4, 7, 8, and 9). The cause of this change of sensitivity is discussed in the next paper [23].

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