Insulin-like Effects of Trypsin on Fat Cells

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 microunits 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.

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 Schwartz-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°C. 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.

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).
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 CO2 formed during the next $t_2$ 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, $t_2$ 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.

The rate of glucose utilization by fat cells was estimated from the rate of conversion of $\nu\text{-[U-}^{14}\text{C}]$glucose into $^{14}\text{CO}_2$ and $[^{14}\text{C}]$lipid (10). Since the rates of formation of CO2 and lipid were always parallel to each other, however, only one set of determinations (either of CO2 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...
Effects of trypsin on lipolysis stimulated by dibutyryl cyclic AMP

Aliquots 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, aliquots of cells were incubated with epinephrine as shown.

<table>
<thead>
<tr>
<th>Additives</th>
<th>Glycerol accumulation</th>
<th>Fatty acid accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibutyryl cyclic AMP</td>
<td>Enopenhine</td>
<td>Untreated cells</td>
</tr>
<tr>
<td>mU</td>
<td>µU</td>
<td>nmoles/30 min/10 mg cells</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>460 ± 10</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>551 ± 10</td>
</tr>
<tr>
<td>0</td>
<td>0.55</td>
<td>624 ± 7</td>
</tr>
</tbody>
</table>

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

Fig. 4. Effects of the length of trypsin treatment on the responsiveness of fat cells to epinephrine and insulin. Aliquots of fat cells were exposed to trypsin for either 15 sec or 15 min as shown. The lipolytic activities of treated as well as untreated (intact) cells were determined as usual in the presence of the following: no hormone (open column), 0.55 µU epinephrine (shaded column), or 0.55 µU epinephrine plus 100 microunits per ml of insulin (cross-hatched column), as shown.

The second incubation (t2), reached the maximum, and then decreased gradually (Fig. 1B).

The maximum stimulation of glucose metabolism by trypsin was not greater than that of insulin, and the two effects (at their maxima) were not additive (Fig. 2). The effect of either trypsin or epinephrine plus insulin was greatly inhibited by phlorizin or 3-O-methyl-D-glucose, but not significantly by L-xylene (Fig. 2). Since it has been known that the first two compounds, but not the last one, inhibit the glucose transport system in fat cells, these data indicated that trypsin (like insulin) stimulate glucose metabolism in fat cells presumably by stimulating the glucose transport system in the cell membrane rather than by making

Effects of trypsin on lipolysis induced by epinephrine but not that induced by caffeine. Since it has been known that the first two compounds, but not the last one, inhibit the glucose transport system in fat cells, these data indicated that trypsin (like insulin) stimulate glucose metabolism in fat cells presumably by stimulating the glucose transport system in the cell membrane rather than by making 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 I). 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 were 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.

Fig. 5. Relative antilipolytic effects of insulin and trypsin. Aliquots 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) each cell preparation was supplemented with either 0.55 µU epinephrine (O) 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 rate of lipolysis by epinephrine or caffeine was comparable with that of insulin only at low hormone concentrations (20 to 30 microunits 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 microunits 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 shown) indicated that the effect

*T. Kono and R. J. Ho, unpublished observation.
Table II

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 (cf. "Materials and Methods") of the trypsin preparations modified with DFP and TLCK were 1.0 and 0.7% of the native enzyme.

<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>2.3 ± 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</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>23.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>2.6 ± 0.5</td>
</tr>
</tbody>
</table>

* Nanomoles × (30 min)⁻¹ × (10 mg of cells)⁻¹.

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 treated with trypsin and soybean trypsin inhibitor in the reverse order, and incubated for 2 hours in the same incubation medium. The second group ("recovered") 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.

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-heptanone. 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.⁶

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
FIG. 8. Failure of trypsin to induce insulin-like effects in fat cells that had recovered from a 15-sec treatment. An aliquot of fat cells was treated with trypsin for 15 sec as usual, and the enzyme activity was terminated with soybean trypsin inhibitor. As the control, another aliquot of cells was mixed with trypsin and soybean trypsin inhibitor in the reverse order. Both cell preparations were washed by centrifugation to remove excess soybean trypsin inhibitor, and incubated for 24 hours in fresh buffer for recovery. (Panel A) the rate of glucose metabolism was determined as usual after aliquots of the cell preparations were supplemented with the following: buffer alone (open column), insulin (shaded column), or trypsin followed (15 sec later) by soybean trypsin inhibitor (cross-hatched column). (Panel B) the rate of lipolysis was determined as usual after aliquots of the cell preparations were supplemented with the following: buffer alone (open column), epinephrine (dotted column), insulin and epinephrine (shaded column), or trypsin followed (15 sec later) by soybean trypsin inhibitor and epinephrine (cross-hatched column). The data were corrected for the lipolysis that occurred during the recovery period. The final concentrations of the above additions were: insulin, 1 milliunit per ml; epinephrine, 0.055 μM; trypsin, 1 mg per ml; and soybean trypsin inhibitor, 1 mg per ml. The metabolic activities shown were the units given by 10 mg of cells in 30 min.

Fig. 9. Effects of insulin on the modification of insulin sensitivity of fat cells by trypsin. Fat cells were treated with trypsin for 15 sec either in the presence (○) or absence (△) of insulin (100 milliunits per ml) as shown. As the control, an aliquot of cells was mixed with trypsin and soybean trypsin inhibitor in the reverse order (X). All the cell preparations were then washed extensively under the same conditions. The washed cells were incubated for 2 hours in fresh buffer for recovery. The response of the cells to insulin was tested as usual, but in duplicate.

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.2

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]iodoinsulin (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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Insulin-like Effects of Trypsin on Fat Cells: LOCALIZATION OF THE METABOLIC STEPS AND THE CELLULAR SITE AFFECTED BY THE ENZYME

Tetsuro Kono and Frances W. Barham


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