Lipolytic Effects of High Concentrations of Insulin on Isolated Fat Cells
Enhancement of the Response to Lipolytic Hormones

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SUMMARY

When isolated rat fat cells were incubated with epinephrine or adrenocorticotropin, release of glycerol and non-esterified fatty acids was greater in the presence of 40 ng. per milliliter of insulin than with 2 ng. per milliliter of insulin. Enhanced lipolysis with the higher dose of insulin occurred in the absence of added carbohydrate, did not involve re-esterification of fatty acids, could not be attributed to the presence of glucagon, and was abolished by incubation with anti-insulin gamma globulin or by prior trypsinization of fat cells. Insulin did not augment lipolysis induced by theophylline or added cyclic 3',5'-adenosine monophosphate (cyclic AMP). In the presence of 5.5 μM epinephrine, the initial rate of lipolysis was decreased by insulin in the amount of 2 ng. per milliliter but not by the higher dose, 40 ng. per milliliter. The ability of fat cells to continue accelerated lipolysis was prolonged by 40 ng. per milliliter of insulin, compared with epinephrine alone. Cycloheximide, and probably puromycin, partially inhibited enhancement of lipolysis by insulin, 40 ng. per milliliter, in the presence of 5.5 μM epinephrine. This effect of cycloheximide first appeared 120 minutes after exposure of cells to the drug. Responses to epinephrine alone and to 2 ng. per milliliter of insulin were not affected by these drugs.

These results imply that insulin can enhance the lipolytic activity of hormonal stimulators of adenylate cyclase by actions which at least in part require protein synthesis. Indirect evidence suggests that cyclic AMP mediates the lipolytic effects of insulin. DIABETES 22:629-36, August, 1973.

When incubated with rat adipose tissue in vitro, insulin ordinarily suppresses lipolysis.1,2 With isolated rat fat cells, inhibition of lipolysis by insulin is dose related over the range of concentrations from 0.04 to about 1 ng. per milliliter.3,4 However, several investigators have observed that higher concentrations of insulin, of the order of 40 ng. per milliliter or greater, may inhibit lipolysis only slightly or not at all.1,5-9 Henceforth we will use the term "lipolytic effect of insulin" to denote any such situation in which lipolysis is greater with a high than with a low concentration of insulin. We now report studies of the mechanisms by which insulin enhances lipolysis.

CHEMICALS

Cyclic 3', 5'-adenosine monophosphate (cyclic AMP), puromycin dihydrochloride, and cycloheximide were obtained from Sigma, epinephrine hydrochloride from Parke-Davis, theophylline from Matheson, Coleman and Bell, adrenocorticotropin (ACTH) from Wilson, twice-crystallized trypsin from Nutritional Biochemicals, and soybean trypsin inhibitor from Worthington. The sources of other reagents were as previously described.10

Crystalline porcine insulin, obtained from Burroughs-Wellcome, was further purified by gel filtration, as described elsewhere.11 We used a fraction (peak "c" of figure 1A in reference 11) with a glucagon content of 3.05 μg. per gram, as determined by radioimmunoassay.12 Anti-insulin gamma globulin (AIG) was prepared...
by anion-exchange chromatography from pooled serum of guinea pigs immunized with Regular insulin (Lilly) in Freund's adjuvant.

METHODS

General. Preparation, incubation, and counting of isolated fat cells were as previously described. Fat cells were always prepared and incubated without added carbohydrate. At the end of each incubation, fat cells were removed by aspiration, and the infranatant medium stored at —35° until assayed. Glycerol was determined by the method of Chernick, and nonesterified fatty acids by the method of Dole and Meinertz.

Trypsinization of fat cells. The method was essentially as described by Kono. Isolated fat cells were prepared as usual, then washed and resuspended in medium containing 20 mg. per milliliter of albumin. Half the cells were then treated for ten minutes with a freshly prepared solution of trypsin in a final concentration of 1 mg. per milliliter at 37°. Trypsinization was stopped by addition of excess soybean trypsin inhibitor, followed immediately by washing five times with ordinary incubation medium. The technic was originally validated (Featherstone and Lavis, unpublished observations) with two types of controls: (1) cells treated as above, but with trypsin and inhibitor in reverse order, and (2) cells treated with plain incubation medium. Since both sets of control cells responded identically to insulin, only the latter type was used in subsequent experiments.

Calculations. Each pool of fat cells was prepared from the tissues of from two to four rats. Experimental treatments were performed in triplicate on aliquots from this pool, and the means of the triplicate determinations were taken as single observations for calculations. Therefore, for statistical computations, "n" represented the number of times an experiment was repeated, which was one-third the number of vessels incubated and assayed. The relative effects of different treatments were assessed by paired comparisons, using Student's t distribution. A one-tailed test was employed when the direction of differences was specified in advance by the hypothesis being tested; otherwise a two-tailed test was used.

RESULTS

Release of glycerol and nonesterified fatty acids. In the presence of ACTH or epinephrine (table 1), release of glycerol during two hour incubations was consistently greater with 40 ng. per milliliter than with 2 ng. per milliliter of insulin, whether or not the lower dose of insulin inhibited lipolysis. With 27.8 μM. epinephrine, both concentrations of insulin actually enhanced glycerol release (p < 0.01). In the presence of theophylline, which stimulated glycerol release as strongly as did the lipolytic hormones, both doses of insulin had identical effects. There was no significant augmentation of glycerol release by insulin, 40 ng. per milliliter, in the absence of other lipolytic agents.

The effects of insulin on release of fatty acids were similar to the changes in glycerol production (data not shown). The ratio of fatty acids to glycerol released (table 1) did not differ significantly from 3.0, indicating that re-esterification of fatty acids had been prevented by the omission of carbohydrate from the incubation media. This ratio was not affected by either dose of insulin.

Some incubations were conducted in the absence of divalent cations, a medium which enhances the lipolytic activity of added cyclic AMP. Under these conditions, just as in ordinary medium, lipolysis was greater with 40 ng. per milliliter than with 2 ng. per milliliter of insulin in the presence of epinephrine (table 2). However, the higher dose of insulin had no such effect in the presence of cyclic AMP (table 2). In order to interpret the foregoing results better, we sought to determine whether theophylline, which is thought to inhibit degradation of cyclic AMP, would act differently from a high concentration of insulin. Since the net effect of 40 ng. per milliliter of insulin presumably reflects enhancement superimposed on inhibition of lipolysis, theophylline was tested in the presence of a maximally antilipolytic concentration of insulin. As shown in table 3, in the presence of epinephrine, 0.02 mM. or 0.1 mM. theophylline enhanced lipolysis to about the same degree as did 40 ng. per milliliter of insulin (compare with table 2). However, these doses of theophylline had no synergistic effect in the presence of cyclic AMP (table 3).

Effects of anti-insulin gamma globulin (AIG). Table 4 shows that AIG had no effect on glycerol release in the absence of insulin. AIG, 100 μg. per milliliter, reversed the antilipolytic effect of 2 ng. per milliliter of insulin. With 40 ng. per milliliter of insulin, the lower concentration of AIG reversed the lipolytic effect of insulin, and the higher dose of AIG blocked both the lipolytic and antilipolytic effects of the hormone.

Effects of trypsinization of fat cells. As shown in table 5, controlled trypsinization of fat cells as described under Methods attenuated the inhibition of glycerol release by insulin, 4 ng. per milliliter, and abolished lipolytic enhancement by insulin, 40 or 400 ng. per milliliter. In other experiments (data not shown),
TABLE 1
Effects of insulin on release of glycerol and nonesterified fatty acids

<table>
<thead>
<tr>
<th>Lipolytic agent</th>
<th>Lipolytic agent</th>
<th>Insulin</th>
<th>Glycerol release</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a.</td>
<td>2 ng./ml.</td>
<td></td>
</tr>
<tr>
<td>None (7)</td>
<td>None (7)</td>
<td>7.6±1.2</td>
<td>5.0±0.6</td>
<td></td>
</tr>
<tr>
<td>ACTH, /tg./ml.</td>
<td>ACTH, /tg./ml.</td>
<td>0.05 (4)</td>
<td>480±18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 (4)</td>
<td>609±26</td>
<td>N.S.</td>
</tr>
<tr>
<td>(NEFA/glycerol)</td>
<td>(NEFA/glycerol)</td>
<td>(2.9)</td>
<td>221±35</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0 (4)</td>
<td>653±20</td>
<td>N.S.</td>
</tr>
<tr>
<td>Epinephrine, µM.</td>
<td>Epinephrine, µM.</td>
<td>0.55 (5)</td>
<td>477±38</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.5 (5)</td>
<td>616±60</td>
<td>&lt;0.0125</td>
</tr>
<tr>
<td>(NEFA/glycerol)</td>
<td>(NEFA/glycerol)</td>
<td>(3.0)</td>
<td>427±58</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.8 (5)</td>
<td>497±43</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Theophylline, mM.</td>
<td>Theophylline, mM.</td>
<td>0.5 (3)</td>
<td>588±37</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0 (4)</td>
<td>626±43</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0 (4)</td>
<td>696±44</td>
<td>&lt;0.0025</td>
</tr>
<tr>
<td>(NEFA/glycerol)</td>
<td>(NEFA/glycerol)</td>
<td>(3.4)</td>
<td>578±46</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.0 (4)</td>
<td>598±51</td>
<td>&lt;0.0005</td>
</tr>
</tbody>
</table>

Glycerol release

- a. None
- b. 2 ng./ml.
- c. 40 ng./ml.

Significance

- b<a
- c<a
- c>b

N.S.: Not significant

Effects of insulin on release of glycerol and nonesterified fatty acids. Fat cells were incubated for two hours, with additions as indicated in the table. The number of sets of triplicate incubations for each experimental condition is in parentheses, in the column headed "lipolytic agent." In every experiment, net release of glycerol was determined by subtraction of the initial from the final glycerol content of the incubation medium. Results are expressed as nanomoles glycerol/10^6 cells/two hours: mean ± S.E. The significance levels for glycerol release, for differences in the directions shown at the tops of the columns, were obtained by a one-tailed 't' test.17 "N.S." denotes p > 0.05; "." denotes that the hypothesized and observed differences were opposite in direction. For some experimental conditions, net release of nonesterified fatty acids was also determined and the molar ratio of fatty acids to glycerol (NEFA/glycerol) calculated. Mean values for NEFA/glycerol are shown in parentheses, below the glycerol values.

concentrations of insulin up to 1 µg. per milliliter had no detectable lipolytic activity. The lipolytic response to epinephrine, however, remained nearly intact in trypsinized cells. Immunoassay of the media from incubations with the lowest concentration of insulin showed substantial disappearance of insulin during the incubation (table 5). However, identical amounts of insulin remained in the trypsinized-cell and control vessels.

Time course of lipolysis. In the presence of 5.5 µM epinephrine, glycerol release with 40 ng. per milliliter

TABLE 2
Effects of insulin on glycerol release in the absence of divalent cations

<table>
<thead>
<tr>
<th>Lipolytic agent</th>
<th>Lipolytic agent</th>
<th>Insulin</th>
<th>Glycerol release</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>a.</td>
<td>2 ng./ml.</td>
<td></td>
</tr>
<tr>
<td>None (7)</td>
<td>None (7)</td>
<td>4.4±0.5</td>
<td>3.6±0.9</td>
<td>N.S.</td>
</tr>
<tr>
<td>Epinephrine, µM.</td>
<td>Epinephrine, µM.</td>
<td>0.55 (5)</td>
<td>95±57</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.5 (5)</td>
<td>253±80</td>
<td>&lt;0.0025</td>
</tr>
<tr>
<td>Cyclic AMP, mM.</td>
<td>Cyclic AMP, mM.</td>
<td>0.5 (4)</td>
<td>50±11</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 (4)</td>
<td>181±33</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.5 (5)</td>
<td>207±54</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 (6)</td>
<td>282±21</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Glycerol release

- a. None
- b. 2 ng./ml.
- c. 40 ng./ml.

Significance

- b<a
- c<a
- c>b

N.S.: Not significant

Effects of insulin on glycerol release in the absence of divalent cations. Experimental conditions were as indicated in the legend to table 1, except that the fat cells were prepared and incubated in medium from which Ca^2+, Mg^2+, and SO_4^2- had been omitted. Symbols and presentation of results are the same as for table 1.
TABLE 3
Effects of theophylline on glycerol release in the absence of divalent cations

<table>
<thead>
<tr>
<th>Additions</th>
<th>Glycerol release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Epinephrine + theophylline</td>
<td></td>
</tr>
<tr>
<td>1) 5.5 µM. none</td>
<td>125±26 (5)</td>
</tr>
<tr>
<td>2) 5.5 µM. 0.02 mM.</td>
<td>222±29 (5)</td>
</tr>
<tr>
<td>3) 5.5 µM. 0.1 mM.</td>
<td>381±45 (4)</td>
</tr>
<tr>
<td>Significance:</td>
<td></td>
</tr>
<tr>
<td>&quot;p&quot;</td>
<td>2 &gt; 1</td>
</tr>
<tr>
<td></td>
<td>3 &gt; 1</td>
</tr>
<tr>
<td>Cyclic AMP + theophylline</td>
<td></td>
</tr>
<tr>
<td>4) 2 mM. none</td>
<td>241±28 (5)</td>
</tr>
<tr>
<td>5) 2 mM. 0.02 mM.</td>
<td>252±30 (4)</td>
</tr>
<tr>
<td>6) 2 mM. 0.1 mM.</td>
<td>216±27 (3)</td>
</tr>
<tr>
<td>Significance:</td>
<td></td>
</tr>
<tr>
<td>&quot;p&quot;</td>
<td>5 &gt; 4</td>
</tr>
<tr>
<td></td>
<td>6 &gt; 4</td>
</tr>
</tbody>
</table>

Effects of theophylline on glycerol release in the absence of divalent cations. Experimental conditions were as indicated in the legend to table 2. Symbols and presentation of results are the same as for table 1. Significance levels, for differences in the directions indicated in the table, were obtained by a one-tailed "t" test.17

of insulin was greater than with 2 ng. per milliliter at every time examined (figure 1). The initial rate of lipolysis was the same with epinephrine plus insulin, 40 ng. per milliliter, as with epinephrine alone. However, glycerol release was better sustained over the two hour incubation in the presence of insulin.

Effects of inhibitors of protein synthesis. In these experiments, puromycin and cycloheximide were employed in doses sufficient to inhibit protein synthesis by

TABLE 4
Effects of anti-insulin gamma globulin (AIG) on glycerol release

<table>
<thead>
<tr>
<th>Concentration of AIG</th>
<th>Glycerol release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Epinephrine, 5.5 µM.</td>
</tr>
<tr>
<td>100 µg./ml.</td>
<td>413 (385-436)</td>
</tr>
<tr>
<td>400 µg./ml.</td>
<td>413 (408-423)</td>
</tr>
</tbody>
</table>

Effects of anti-insulin gamma globulin (AIG) on glycerol release. Insulin and AIG were allowed to react for twenty minutes at room temperature before addition of fat cells, which were then incubated for one hour. Glycerol release, in nanomoles/10^6 cells/hour, is shown as the mean of one set of triplicate incubations, with the range in parentheses.

Effects of epinephrine and insulin on lipolysis by trypsinized fat cells

Hormones added

<table>
<thead>
<tr>
<th>Hormones added</th>
<th>Glycerol release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine, 2.8 µM.</td>
<td>814 (789-828)</td>
</tr>
<tr>
<td>+ insulin, 4 ng./ml.</td>
<td>294 (269-323)</td>
</tr>
<tr>
<td>+ insulin, 40 ng./ml.</td>
<td>428 (376-502)</td>
</tr>
<tr>
<td>+ insulin, 400 ng./ml.</td>
<td>537 (493-585)</td>
</tr>
</tbody>
</table>

Time concentration

<table>
<thead>
<tr>
<th>Time of incubation</th>
<th>Insulin in incubation medium (µg./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start of incubation</td>
<td>4 ng./ml.</td>
</tr>
<tr>
<td>End of incubation</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Effects of insulin on lipolysis by trypsinized fat cells. Trypsinized and control fat cells were prepared as described under Methods. After washing, the cells were distributed in flasks containing hormones as indicated in the table and incubated for two hours. Glycerol release is expressed as mean nanomoles/10^6 cells/two hours, with the range of triplicate incubations in a single experiment shown in parentheses. Radioimmunoassay for insulin was performed on the media from all vessels incubated with a nominal insulin concentration of 4 ng. per milliliter; the results shown are mean values of triplicate incubations and radioimmunoassays.

FIG. 1. Time course of lipolysis. Fat cells were incubated for various times, with additions as indicated in the figure. The points indicate mean glycerol release, in nanomoles/10^6 cells, and the brackets indicate S.E. for three sets of triplicate incubations.
greater than 90 per cent.\textsuperscript{21,22} Table 6 shows the results of two hour incubations. By paired comparisons, neither drug significantly affected stimulation of glycerol release by epinephrine alone, or its suppression by insulin, 2 ng. per milliliter. Mean glycerol release with insulin, 40 ng. per milliliter, was significantly reduced by cycloheximide, although it remained substantially greater than with the low dose of insulin. Puromycin had a similar effect in four of five experiments, which was of borderline statistical significance ($p = 0.084$). Investigation of the time course (table 7) showed that cycloheximide did not reduce enhancement of lipolysis until ninety minutes after epinephrine and 40 ng. per milliliter of insulin were added, when the cells were pretreated with the drug for thirty minutes.

**DISCUSSION**

A lipolytic effect of high concentrations of insulin in vitro has been noted several times since 1963.\textsuperscript{1,5-9} In the cases in which adipose tissue was incubated with glucose, this effect of insulin could be ascribed to acceleration of glucose metabolism, which can promote lipolysis by several mechanisms.\textsuperscript{1,23-27} We have investigated the actions of insulin on lipolysis in the absence of extracellular carbohydrate. Under these conditions, a high concentration of insulin alone did not increase lipolysis. Rather, it permitted a greater response to epinephrine or ACTH than did a smaller amount of insulin. The "lipolytic effect" of insulin therefore really represents synergism with lipolytic hormones. In the presence of a lipolytic hormone, the net effect of insulin on lipolysis is the resultant of its superimposed synergistic and inhibitory actions. Under certain conditions, as with 1 $\mu$g. per milliliter of ACTH or with 5.5 $\mu$M. epinephrine, consideration only of the effects of 40 ng. per milliliter of insulin would erroneously suggest that insulin did not act on lipolysis, when in fact the two opposing actions were balanced.

Might lipolytic activity have been attributable to a contaminant in the preparation of insulin? There was no significant contamination by glucagon: Incubation mixtures with insulin, 40 ng. per milliliter, could have contained no more than 0.12 pg. per milliliter of glucagon, while the threshold for stimulation of lipolysis by glucagon is at least 1,000 pg. per milliliter (Lavis, unpublished results).\textsuperscript{28-30} Contamination with some other activator of adenylate cyclase seems unlikely in light of our results with theophylline, because lipolysis induced by activation of adenylate cyclase typically is enhanced by methylxanthines.\textsuperscript{31}

The question of contamination was also approached by an attempt to inhibit responses to insulin, by treatment of fat cells with trypsin. Under the conditions we used, such treatment reduces high-affinity binding of insulin to plasma membranes\textsuperscript{16,34-35} and blocks stimulation of glucose metabolism and inhibition of lipolysis by the hormone.\textsuperscript{16,34,35} Trypsinization inhibits insulin action fairly specifically, since the fat cells retain their characteristic responses to epinephrine, ACTH, oxytocin, growth hormone and glucocorticoids, and various non-hormonal stimuli.\textsuperscript{16,34,35} Although trypsin does render fat cells unresponsive to the lipolytic effects of glucagon,\textsuperscript{16,36} as noted above our preparation of insulin was essentially free of glucagon. Immunoassay of the

\begin{table}[h]
\centering
\caption{Effects of inhibitors of protein synthesis on glycerol release}
\begin{tabular}{|c|c|c|c|c|}
\hline
Additions & Glycerol release & Significance & \multicolumn{2}{c|}{Significance} \\
 & & & $<p$ & \\
\hline
 & a. None & b. 2 ng./ml. & c. 40 ng./ml. & \\
1. Epinephrine, 5.5 $\mu$M. & 519±67 & 355±55 & 625±124 & $<0.005$ & c<a \\
2. Epinephrine, 5.5 $\mu$M. + puromycin, 0.1 mM. & 542±84 & 335±41 & 537±89 & $<0.005$ & N.S. \\
3. Epinephrine, 5.5 $\mu$M. + cycloheximide, 0.01 mM. & 497±68 & 327±38 & 498±81 & $<0.01$ & — \\
\hline
Significance: & $<p$ & N.S. & N.S. & $=0.08$ \\
$3 \neq 1$ & N.S. & N.S. & $<0.05$ \\
\hline
\end{tabular}
\end{table}

Effects of inhibitors of protein synthesis on glycerol release. Fat cells were incubated for two hours, with additions as noted in the table. Glycerol release is expressed as nanomoles/10^6 cells/two hours; mean ± S.E. for five sets of triplicate incubations. The significance levels for effects of insulin, in the directions shown at the tops of the three columns at the right, were obtained by a one-tailed $t$ test.\textsuperscript{37} The significance levels for effects of the inhibitors, shown in the two lowest rows, were obtained by the two-tailed $t$ test.\textsuperscript{37} Symbols are the same as for table 1.

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incubation media demonstrated that the reduced responses of tryptinized cells to insulin could not be attributed to degradation of the hormone by residual trypsin. Parenthetically, most of the rather extensive insulin-removing activity in these incubations was contributed by the bovine serum albumin in the media; this activity varies among different lots of albumin (unpublished observations). Therefore, the observed failure of trypsinized fat cells to respond to insulin, 40 ng. per milliliter, is consistent with the hypothesis that lipolytic activity is intrinsic to the hormone.

Further evidence for specificity was provided by the results with AIG. However, the AIG, which was obtained by immunization with commercial insulin, might have contained antibodies to a contaminant. Ultimately, experiments with synthetic insulin will be required to prove that lipolytic activity inheres in insulin molecules. For now, all the indirect evidence is consistent with this postulate, and there are no indications to the contrary.

Our studies of the mechanism of the lipolytic effect of insulin permit rejection of two possibilities. First, in the absence of added carbohydrate insulin did not influence re-esterification of fatty acids. Second, since insulin had no lipolytic activity in the presence of theophylline, it probably did not directly stimulate adenylate cyclase.

In our experiments, the response to insulin depended not merely on conditions of stimulated lipolysis, as had previously been suggested, but on the nature of the lipolytic stimulus. These results may be interpreted in the context of the concepts that lipolytic hormones augment the synthesis, and methylxanthines inhibit the degradation of intracellular cyclic AMP, which in turn activates a triglyceride lipase. If these theories be true, then actions exerted at or subsequent to the step(s) by which cyclic AMP activates the lipase should have identical effects in the presence of equally lipolytic concentrations of epinephrine, ACTH, or theophylline. Since this was not the case for 40 ng. per milliliter of insulin, its action presumably is exerted elsewhere. A postulated lipolytic action of insulin mediated by increased concentrations of cyclic AMP would be consistent with the above theories and with our results.

In support of this interpretation is the report that a high concentration of insulin enhances activation by catecholamines of fat cell adenylate cyclase, suggesting that increased formation of cyclic AMP could mediate the lipolytic effects of insulin. Whether 40 ng. per milliliter of insulin might also inhibit degradation of cyclic AMP remains moot. Other investigators have found the cyclic AMP-phosphodiesterase activity of fat cells to be activated equally by 800 μU. per milliliter or 100 μU. per milliliter of insulin. However, the reported experiments were performed in the absence of lipolytic hormones, a condition under which the higher dose of insulin would have had no lipolytic effect. No one has yet reported measurements of low-Km phosphodiesterase activity of amounts of cyclic AMP in fat cells, under conditions favorable to enhancement of lipolysis by insulin.

In our experiments, insulin did not consistently enhance the lipolytic activity of exogenous cyclic AMP, but neither did amounts of theophylline which augmented epinephrine-stimulated lipolysis to the same degree as did insulin. Therefore, a possible theophylline-like action of insulin could not be either confirmed or excluded. These results do not imply that theophylline...
cannot potentiate lipolysis induced by cyclic AMP, for higher concentrations of theophylline did so (unpublished results). Rather, we infer that incubation with cyclic AMP, contrary to expectation, is a poor way to detect small degrees of theophylline-like action. It is doubtful that experiments with added cyclic AMP can help to elucidate mechanisms of lipolysis.

Kinetic studies showed that a high concentration of insulin, in the presence of epinephrine, enhanced lipolysis in two ways: (1) by immediate acceleration of the rate of lipolysis, in comparison with the smaller dose of insulin, and (2) by improvement in the ability of cells to sustain that rate for more than one hour. The second effect of insulin is not simply a matter of improved cellular viability or protection against the deleterious effects of accumulated fatty acids, because such actions should have been apparent in the presence of theophylline. The relationship of cyclic AMP to these dual effects is unknown. When fat cells are exposed to lipolytic hormones, the concentration of intracellular cyclic AMP ordinarily rises rapidly and then declines. Further investigations of the mechanisms governing these changes may indicate possible sites of regulation by insulin.

Cycloheximide, and probably puromycin, reduced but did not abolish enhancement of lipolysis by 40 ng per milliliter of insulin. The effect of cycloheximide did not appear until ninety minutes after insulin and epinephrine were added, which was 120 minutes after the drug was added. These drugs did not inhibit lipolysis or insulin action nonspecifically, since neither the response to epinephrine alone nor the antilipolytic effect of 2 ng per milliliter of insulin was affected. From these results we infer that synthesis of new protein is required for lipolysis to be maximally sustained in response to insulin. By analogy, the delayed increase in lipolysis induced by growth hormone and glucocorticoids is also inhibited by cycloheximide or puromycin. However, these hormones elicit no immediate lipolytic response, implying a mechanism different from that of insulin.

Appreciation of the lipolytic activity of insulin helps explain our previous finding that 1 mM. cysteine together with crystalline insulin, 1,000 μU per milliliter, inhibited epinephrine-induced lipolysis less strongly than did the insulin alone. In this range of concentrations of crystalline insulin, lipolysis increases with increasing dose (Lavis, unpublished results). Therefore, the added cysteine behaved as would an additional amount of insulin, a result consistent with the other insulin-like actions of the thiol.

The concentration of insulin required for exhibition of lipolytic activity is clearly supraphysiologic. However, when insulin is injected subcutaneously, its concentration at the site of administration would be in the lipolytic range. Conceivably the local lipoatrophy which occurs in some patients taking insulin could be related to the lipolytic effect of the hormone.

To recapitulate, a high concentration of insulin can enhance lipolysis induced by hormonal activators of adenylate cyclase. New protein synthesis and, presumably, cyclic AMP mediate this effect, which consists of at least two kinetically distinguishable actions. The physiologic significance of these phenomena remains to be established.

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