Perifused Fat Cells

EFFECT OF LIPOLYTIC AGENTS*

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SUMMARY

In vitro methods currently utilized for the determination of lipolytic responses in adipose tissue have not allowed for the continuous monitoring of rapid successive changes in rates of lipolysis. The perifused fat cell system utilized in this investigation offers a simple, reproducible method by which minute-to-minute changes in the rate of lipolysis of isolated fat cells may be monitored. In this technique, isolated fat cells are placed in a plastic column and perifused with albumin-containing buffer in the absence and presence of various lipolytic agents. Using this technique, we have been able to observe alterations in the rates of glycerol release during the initiation of hormone-induced lipolysis. In addition, we have been able to observe the decline in glycerol release following the removal of the lipolytic hormone.

In the perifused fat cell system, epinephrine, isoproterenol, and norepinephrine elicited a maximal lipolytic response at 10 μM, theophylline at 1 mM, and glucagon at 5 μM. In addition, N6-Oβ-dibutyryl cyclic adenosine 3':5'-mono- phosphate (1 mM), adrenocorticotropic hormone (100 milli-units per ml), and thyroid stimulating hormone (75 milli-units per ml) effectively stimulated lipolysis in the perifused fat cell system, whereas cyclic adenosine 3':5'-monophosphate (1 mM) and serotonin (1 mM) were without effect. Unlike the incubated tissue method, growth hormone (20 μg per ml) was found to be a potent stimulator of lipolysis in this system. The combined administration of theophylline and epinephrine, both at submaximal concentrations, resulted in rates of glycerol release which were significantly greater than the sum of the rates observed during individual administration.

Considerable emphasis in the last decade has been placed on the elucidation of the mechanisms involved in the hormonal activation of lipolysis in adipose tissue. In vitro experiments have been conducted by incubating intact adipose tissue or isolated adipocytes in the presence of various stimulatory agents and measuring the release of fatty acids, or glycerol, or both into the incubation medium. Although utilization of the incubation technique has made it possible to observe hormonally induced changes in lipolysis, it has been difficult to observe the rates of lipolysis during the activation process on a minute-to-minute basis. In addition, it has been impossible to follow the changes in lipolytic rates occurring after the removal or cessation of hormonal stimulus. These limitations have recently been overcome with the development of a perifused fat cell technique (1). Using glycerol release as an index of lipolysis, it is possible to measure the rates of lipolysis during both the activation and inactivation phases of hormone-induced lipolysis. This technique offers the advantage of being able to follow sequentially the release of glycerol by isolated fat cells at time intervals as short as 5 s.

The purpose of the present investigation was to determine the ability of various agents to stimulate lipolysis in the perifused fat cell system. In addition, this study was undertaken to examine the rates of change in glycerol production occurring during the initiation and cessation of lipolysis caused by various stimulatory agents.

EXPERIMENTAL PROCEDURE

Materials

L-Epinephrine, L-norepinephrine, dl-isoproterenol, cyclic AMP, dibutyryl cyclic AMP, bovine serum albumin (Fraction V), 5-hydroxytryptamine (creatinine sulfate complex), porcine ACTH, glycerokinase, and α-glycerophosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, MO. Theophylline was obtained from Merek & Co., Thyropar (TSH) from Armour Pharmaceutical Co., and porcine sonatotropic hormone (GH) from Calbiochem. Crystalline porcine glucagon was kindly supplied by Eli Lilly & Co.

Methods

Preparation of Fat Cells—Isolated fat cells were prepared from the epididymal fat pads of exsanguinated fed Cox Holtzman rats. This investigation was supported by National Institutes of Health Grants AM 15788 and AM 12340 from the United States Public Health Service and in part by National Institutes of Health Fellowships HL 53937 (A. S. K.) and HL 53741 (K. E. L.) from the Heart and Lung Institute.

* The abbreviations used are: cyclic AMP, cyclic adenosine 3':5'-monophosphate; dibutyryl cyclic AMP, N6,0β-dibutyryl cyclic adenosine 3':5'-monophosphate; TSH, thyroid-stimulating hormone; GH, growth hormone; ACTH, adrenocorticotropic hormone.

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The basal rate of lipolysis, the cells were first perfused with buffer containing different concentrations of bovine serum albumin (BSA) for the time intervals indicated. Glycerol is expressed as nanomoles of glycerol released per min per mg of protein content of the isolated cells.

Fig. 1. Effect of different concentrations of albumin on glycerol release in the perfused fat cell. Two milliliters of packed isolated fat cells were perfused as described under "Experimental Procedure." The cells were perfused sequentially in the presence of 1 μM epinephrine with Krebs-Ringer bicarbonate buffer containing different concentrations of bovine serum albumin (BSA) for the time intervals indicated. Glycerol is expressed as nanomoles of glycerol released per min per mg of protein content of the isolated cells.

RESULTS

Effect of Albumin on Lipolysis—The results obtained when isolated fat cells were perfused with buffer containing different concentrations of albumin in the presence of epinephrine (final concentration 1 μM) are presented in Fig. 1. In order to establish the basal rate of lipolysis, the cells were first perfused with buffer containing 0.5% (w/v) albumin for 10 min before the start of epinephrine infusion. Following the start of infusion of epinephrine, there occurred an increased rate of glycerol release which reached a plateau value in approximately 20 min. When the perfusing buffer was changed to one containing 1.0% (w/v) albumin, there occurred a substantial increase in the lipolytic rate which reached a plateau value in approximately 10 min. Increasing the albumin concentration to 2.0% (w/v) resulted in a small additional increase in glycerol release. In other experiments with epinephrine-stimulated cells, when the perfusing medium was switched from an albumin-containing to an albumin-free buffer, the rate of glycerol release decreased precipitously to near basal levels. The experimental results in the following sections were obtained with buffer containing 1.0% (w/v) albumin.

Epinephrine-stimulated Lipolysis—Rates of glycerol release obtained during the initiation and termination of epinephrine-stimulated lipolysis are recorded in Fig. 2. After a 10 min period of perfusion with drug-free buffer, epinephrine was infused into the cell chamber such that the final concentration was 10 μM. This concentration of epinephrine produced the maximal rate of lipolysis. Following the start of infusion of epinephrine there was a sharp increase in the rate of lipolysis. The lipolytic rate continued to increase until a peak response was attained approximately 20 min after the start of epinephrine administration. Upon cessation of the epinephrine infusion, the rate of lipolysis returned to basal rates within 20 to 25 min.

Theophylline-stimulated Lipolysis—Rates of glycerol release obtained during the initiation and termination of theophylline-induced lipolysis are recorded in Fig. 3. After establishing the basal rate of lipolysis, the fat cells were perfused with buffer containing theophylline at a final concentration of 1 mM. The maximal rate of lipolysis attainable with theophylline was achieved with 1 mM concentration. Following the start of theophylline perfusion, there occurred a sharp increase in the rate of lipolysis. The peak lipolytic response was attained in approximately 20 min after the initiation of theophylline administration. Upon switching to theophylline-free buffer, the rate of glycerol release returned to basal levels within a 10-min period. With this concentration of theophylline (1 mM), the inactivation of lipolysis appeared to proceed at a faster rate than the activation of lipolysis.

Dose Response Curves Obtained with Various Lipolytic Agents—In order to determine whether the lipolytic response was dose-dependent in the perfusion system, studies were undertaken to determine dose-related responses with various known lipolytic agents, the results of which are depicted in Figs. 4 and 5. Fig. 4 represents the alterations in the rate of lipolysis caused by sequential increments in the concentration of theophylline.
From this plot, it would appear that stimulation of lipolysis in plotted for various known lipolytic agents, as shown in Fig. 5. of such reproducible experiments, dose response curves were served in the presence of 0.010 mM theophylline. On the basis concentrations appear to be substantially greater than the rate ob-

maximal rate of lipolysis. Note that the rates of activation observed with these concentrations elicited maximal response at 10 μM, theophylline at 1 mM, and glucagon at 5 μM. The data in Table I indicate the maximal rates of glycerol production that can be attained by these lipolytic agents. The difference in rates of glycerol release (i.e. peak value − basal value) caused by each agent was highly significant when compared to the nonstimulated rates.

Effect of Dibutyryl Cyclic AMP and Cyclic AMP—Rates of glycerol release obtained when isolated cells were perifused separately with dibutyryl cyclic AMP and cyclic AMP, both at final concentrations of 1 mM have been recorded in Fig. 6. After the start of dibutyryl cyclic AMP infusion, there was a lag period for 6 min after which there was a steady increase in the rate of glycerol release until a plateau value was achieved approximately 35 min after the start of infusion. After the termination of infusion, a short lag period was observed after which the rate of glycerol release slowly approached the basal value. These lag periods occurring after the administration and removal of dibutyryl cyclic AMP have also been seen in other experiments. In contrast to the effect seen with dibutyryl cyclic AMP, cyclic AMP at the concentration employed was without effect in the perifused system. Isolated fat cells which were refractory to exogenous cyclic AMP were stimulated by epinephrine thereby negating the possibility that these cells were refractory to any lipolytic stimulant.

Effect of ACTH, TSH, GH, and Serotonin in Perifused System The data contained in Table II demonstrates that ACTH, TSH, and GH are capable of stimulating lipolysis in the perifused fat cell system. Dose response experiments were not carried out with these agents and, as such, it is not known whether the reported concentrations elicited maximal responses. The pur-
Table I

Effect of epinephrine, isoproterenol, norepinephrine, theophylline, and glucagon on glycerol production in perfused fat cell

Experimental data obtained according to the technique described under "Experimental Procedure" and on the basis of cumulative dose response curves as represented by Fig. 4. The results are presented as mean ± S.E. Basal value refers to the rate of glycerol production just prior to the start of infusion. Peak value refers to the maximal rate of glycerol production attained with the lipolytic agent at the indicated concentration. The statistical significance of the difference caused by each agent was evaluated by means of the Student's t test for paired comparisons.

| Lipolytic agent | Concentration | No. of experiments | Glycerol production | p Value |
|-----------------|---------------|-------------------|---------------------|---------|
| Epinephrine     | 10 µM         | 8                 | 8.3 ± 1.9           | 0.001   |
| Isoproterenol   | 10 µM         | 4                 | 10.1 ± 3.1          | 0.005   |
| Norepinephrine  | 10 µM         | 4                 | 6.1 ± 1.2           | 0.05    |
| Theophylline    | 1000 µM       | 4                 | 9.0 ± 2.2           | 0.01    |
| Glucagon        | 5 µM          | 3                 | 14.1 ± 1.4          | <0.025  |

Table II

Effect of ACTH, TSH, GH, and serotonin on glycerol production in perfused fat cell

Two milliliters of packed isolated cells were perfused as described under "Experimental Procedure." Basal value refers to the rate of glycerol production just prior to the start of a 30-min infusion of the drug. Peak value refers to the rate of glycerol production occurring at the end of the infusion period.

| Compound           | Glycerol production | p Value |
|--------------------|---------------------|---------|
| ACTH               | 100 million units/ml | 6.3     | 77.4 | 71.1 |
| TSH                | 75 million units/ml  | 5.2     | 31.0 | 25.8 |
| GH                 | 20 µg/ml             | 2.0     | 32.4 | 30.4 |
| Serotonin          | 1 µM                 | 7.1     | 8.8  | 1.7  |

**Individual and Combined Administration of Epinephrine and Theophylline at Submaximal Concentrations—Glycerol release by isolated cells when epinephrine and theophylline, both at submaximal concentrations, were infused individually and simultaneously have been recorded in Fig. 7. The infusion of epinephrine (final concentration 0.05 µM) for 20 min resulted in a difference (peak value - basal value) of 16 nmoles of glycerol per min per mg of protein. The infusion of theophylline (final concentration 10 µM) for a 20-min period resulted in a change of 16 nmoles of glycerol per min per mg protein. When epinephrine and theophylline were mixed together, the peak response was not obtained until approximately 30 min after the start of infusion. However, there was a difference of 47 nmoles of glycerol per min per mg of protein after 20 min of infusion. This value was considerably larger than the sum of the differences obtained individually with epinephrine and theophylline over 20-min infusion periods. Note that the difference obtained at any time after the start of the combined infusion was much greater than the sum of the differences of the individual infusions at the same
were readily reproducible with different populations of isolated epinephrine and theophylline. The results of this experiment indicated that maximum rates of lipolysis are observed within 1 hour, there was no change in glycerol release from the control values. Therefore, it would appear that the lipolytic response observed in the perfused fat cell system with this particular GH preparation cannot be observed by conventional incubation methods. It is possible that an inhibitor substance accumulates in the incubated tissue method and this may be washed out in the perfused fat cell system thereby enabling GH to exert its lipolytic action.

The incubation of adipose tissue with cyclic AMP, which has been implicated as an intracellular mediator of hormone-stimulated lipolysis, has generally resulted in a lack of lipolytic response. In epididymal fat pads, exogenous cyclic AMP has been reported to inhibit lipolysis (9) and counteract the lipolytic effect of epinephrine (10). However, lipolysis in fat cells and fat pad fragments can be stimulated by cyclic AMP when incubated in a simple phosphate-0.9% NaCl medium (11). In the perfused fat cell system, cyclic AMP was found to have no effect on the basal rate of lipolysis. In contrast, dibutyryl cyclic AMP, an effective lipolytic agent in incubated fat pads and isolated fat cells (12), substantially stimulated lipolysis in the perfused fat cell system. The greater efficacy of dibutyryl cyclic AMP has been attributed to its resistance to degradation by phosphodiesterase and its greater cell permeability (13). Referring to the results contained in Fig. 6, there was a 6-min lag period between the administration of dibutyryl cyclic AMP and the initial increase in the rate of lipolysis. It may be speculated that during this period, dibutyryl cyclic AMP was permeating the cell membrane and thereby increasing its intracellular concentration to effective stimulatory levels.

Theophylline has been reported to act synergistically with catecholamines in the in vitro stimulation of lipolysis (14–16) and elevation of cyclic AMP levels (17) in adipose tissue. In the present study, the rate of glycerol release observed in the presence of theophylline and epinephrine at submaximal concentrations was greater than the sum of the rates of glycerol release observed during the administration of theophylline or epinephrine alone. This greater than additive effect was also observed when the initial rates of activation of lipolysis were compared. Similar to results obtained in the incubation method (14, 15), the rate of lipolysis obtained with a maximal concentration of theophylline in the perfused system could not be further increased by epinephrine. Thus, again, results obtained in the perfused fat cell system parallel results obtained in the tissue incubation technique.

The unparalleled feature of the perfused fat cell system resides in the ability to measure sequentially lipolytic rates over short intervals of time. An important point to be made at this time is that the lipolytic rates observed during the activation and inactivation phases of hormone-induced lipolysis in this system appear not to be dependent upon drug equilibration and washout from the cell chamber, as evidenced by previous observations with labeled epinephrine (1). Conflicting reports exist in the literature concerning the rate at which catecholamines activate the lipolytic process. Angel et al. (18) and Kuo and De Renzo (19) have published data indicating a 5- to 10-min delay before norepinephrine stimulates lipolysis in isolated fat cells while Manginelli et al. (20) have indicated that maximum rates of lipolysis are observed within 1
min of addition of epinephrine. Our data with the perifused fat cell system indicate that epinephrine produces an immediate stimulation of lipolysis but 10 to 15 min are required before maximum rates are obtained. This is in agreement with the work of Scow (21) using perfused rat adipose tissue which showed that fatty acid release was greater in the second 10-min period than in the first 10 min after addition of epinephrine.

With the perifused fat cell system it is possible not only to determine the maximal rate of lipolysis attainable with various lipolytic agents, but also to examine the rate of activation of lipolysis during the initiation process. More importantly, prior to the development of this system, methods were not available for observing the decline in rates of lipolysis during the inactivation process. Thus, the development of the perifused fat cell system should prove to be useful in elucidating the biochemical events occurring during the initiation, continuance, and cessation of hormone-induced lipolysis.

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