Vol. 84, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 30, 1978 Pages 1069-1075

INSULIN REGULATION OF LIPOPROTEIN LIPASE IN CULTURED 3T3-L1 CELLS

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Received September 19,1978

SUMMARY

Lipoprotein lipase activity is produced by the 3T3-L1 cell an established mouse fibroblast line which resembles an adipocyte after reaching a confluent stage of growth. Since insulin has been shown to be an important regulator of lipoprotein lipase in other mammalian systems, a two hour incubation period was utilized to determine if insulin could enhance an acute response of enzyme activity. Over the range of concentrations tested (0.4, 4.0 and 40 ng/ml), insulin increased lipoprotein lipase activity in acetone ether powders of cells (intracellular enzyme) and the activity secreted into the culture medium. A simultaneous decrease in lipoprotein lipase activity releasable with heparin in a subsequent incubation (membrane bound activity) indicates two distinct effects of insulin on the enzyme in this system.

INTRODUCTION

The uptake of chylomicron and very low density lipoprotein triglyceride by adipose tissue is regulated by lipoprotein lipase (1). Following synthesis by the adipocyte, the enzyme is transferred to the capillary endothelium where it is membrane-bound and hydrolyzes triglyceride to glycerol and free fatty acids. Fatty acids are then transported into the fat cell where they are reesterified with glycerol and stored as intracellular triglyceride. Both in man and experimental animals the *in vivo* regulation of lipoprotein lipase by insulin has been previously demonstrated by increases in both plasma postheparin lipolytic activity and enzyme activity in adipose tissue biopsies after feeding or insulin injection (2-6). Increases in lipoprotein lipase have also been demonstrated *in vitro* following incubation of adipose tissue or isolated adipocytes with insulin (6).

Recently, several cell lines obtained from tissue where lipoprotein lipase is synthesized (fat, heart) have been utilized for the measurement and study of lipoprotein lipase in cell culture (7-10). These cells have been shown to

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undergo adipose conversion by the accumulation of triglyceride after growth in high concentrations of insulin (11,12). The 3T3-L1 cell, an established mouse embryo fibroblastic line, is such a cell. We have previously reported the development of lipoprotein lipase in 3T3-L1 cells maintained at a confluent density in the absence of insulin (7). Although growing these cells in high concentrations of insulin (>5 µg/ml) can increase lipoprotein lipase and facilitate the adipose conversion of these cells (13), the physiological responsiveness to insulin cannot be shown under these conditions. Since an acute response of insulin on the enzyme has been demonstrated in the isolated fat cell (6), the responsiveness of lipoprotein lipase to insulin in the 3T3-L1 cell was tested. In addition, location of the subcellular site of insulin action was determined.

Materials and Methods

3T3-L1 cells were obtained from Dr. Howard Green (Massachusetts Institute of Technology), maintained under conditions previously reported (7), and subcloned. Subclone IX, a line previously shown to have the highest lipoprotein lipase activity, was used for these experiments. After reaching a confluent stage of growth, the cell monolayer was trypsinized and cells were plated at 1-2 x 10^5 cells per 60 mm culture dish in Dulbecco-Vogt modified Eagle's medium (Microbiological Associates)(14) supplemented with 10% (v/v) fetal bovine serum (GIBCO) and gentamicin (50 µg/ml, Microbiological Associates). Medium was changed every 2-3 days. After 10-15 days growth, the serum concentration was reduced to 1% (v/v) overnight (12-15 hours) in order to reduce the concentration of insulin or insulin-like growth factors. The cells were then exposed for 2 hours to fresh medium (1% (v/v) fetal bovine serum) containing 0.04, 0.4, 4.0 and 40 ng/ml of porcine insulin (Sigma Chemical Co., purified to electrophoretic homogeneity by G-50 Sephadex and DEAE chromatography courtesy of Dr. C. de Haën). Following the incubation, lipoprotein lipase was measured in the trypsinized cells with modification as previously described (7), and in the culture medium.

Lipoprotein lipase activity present in the culture medium represents the release of enzyme from the cells during the incubation period. The culture medium activity was measured by incubating an aliquot of the culture medium for 45 min at 37° in heparin (200 mg/ml, Upjohn intestinal mucosa), with a triolein substrate emulsified with lecithin in the presence of albumin, serum, and Tris: HCl. The activity present in ammonium chloride extracts of acetone ether powders of cells represents intracellular lipoprotein lipase. The activity releasable from cellular suspensions during a 45 min incubation of the cells with 200 mg/ml heparin indicates residual enzyme on the membrane not secreted into the culture medium during the prior 2 hr incubation with insulin (heparin-releasable activity). Doles' extraction mixture (15) was utilized to terminate all enzyme substrate reactions and free fatty acids were extracted as outlined by Pykalisto *et al.* (16).

Results

The effect of insulin on acetone ether activity is shown in Fig. 1A. Over the range of insulin concentrations utilized, a significant increase in lipo-

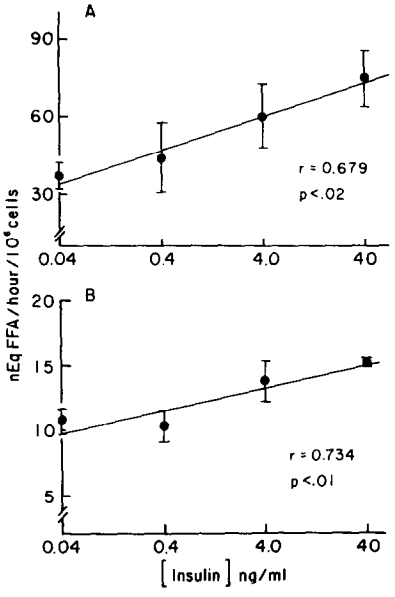

Figure 1: Lipoprotein lipase activity (mean±SE of triplicate measurements) after a 2 hr insulin incubation as a function of insulin concentration in ng/ml. The correlation coefficient of regression (r) and significance level (p) are shown. 1A - acetone ether cellular powder activity; 1B - activity present in the culture medium.

protein lipase activity was seen. Increasing concentrations of insulin also resulted in a release of lipoprotein lipase into the culture medium (Fig. 1B). There was a relationship between insulin stimulated activity in the culture medium and the activity present in acetone ether powders of cells ($r=0.967$, $p<.001$).

The increase in lipoprotein lipase in the culture medium could be simply a reflection of increased intracellular activity with insulin or could be due to enhanced release of enzyme from the cell membrane, a process shown in other systems to be energy dependent (17). If the effect was a reflection of an increase in acetone ether powder activity, lipoprotein lipase releasable from cells during a subsequent incubation with heparin might be expected to be the same or higher. If, however, the release of activity in the culture medium is due to enhanced release from the cell membrane, a decrease in heparin releasable activity should be found.

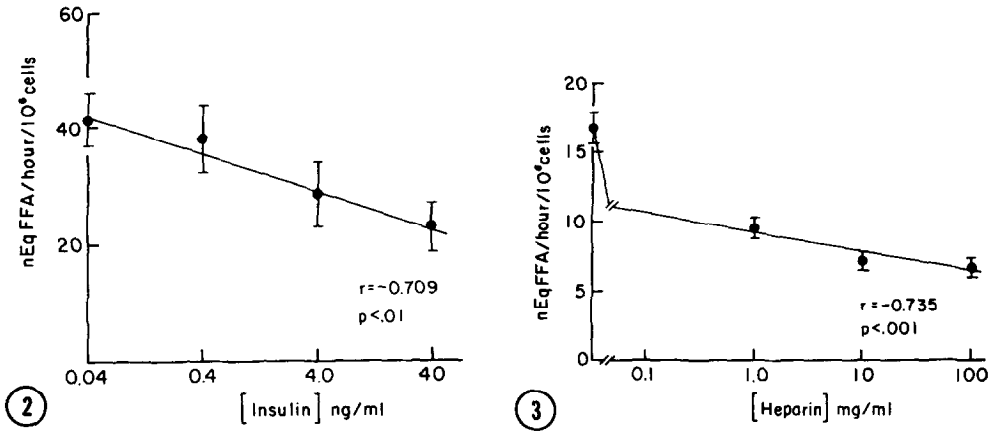

Figure 2: The lipoprotein lipase activity releasable from cellular suspensions during a 45 min incubation with heparin following a prior 2 hr incubation of cells in insulin is plotted on the ordinate. The insulin concentrations utilized are shown on the abscissa. Data points represent the mean±SE of triplicate measurements.

Figure 3: The lipoprotein lipase activity releasable from cellular suspensions during a 45 min incubation with heparin following a prior 2 hr incubation of cells in heparin is plotted on the ordinate with the heparin concentration in mg/ml on the abscissa. The results represent the mean±SE of triplicate measurements.

The lipoprotein lipase releasable with heparin from cellular suspensions decreased with increasing prior insulin concentrations (Fig. 2). This decrease over the range of insulin concentrations utilized during the 2 hr incubation is compatible with a direct effect of insulin on the cell membrane. Cells incubated in 1.0, 10 and 100 mg/ml of heparin for 2 hrs instead of insulin showed a similar decrease in heparin releasable activity (Fig. 3), and results very similar to those shown for insulin (Fig. 2) were obtained. Like insulin, heparin also enhanced a release of lipoprotein lipase into the culture medium, but unlike insulin, heparin had no effect on intracellular activity (data not shown). Similar effects of insulin on lipoprotein lipase were found in 5 experiments (Fig. 4); increases in acetone ether powder and culture medium activity were associated with decreases in activity releasable with heparin from cellular suspensions.

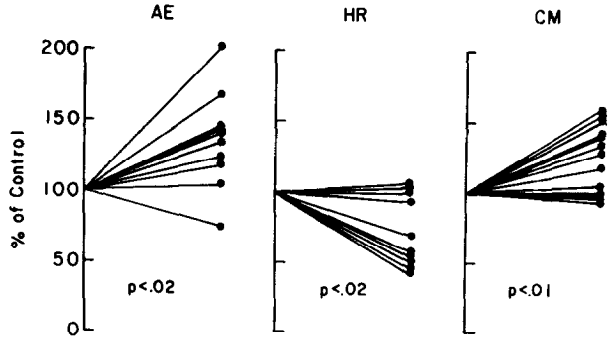

Figure 4: The effect of 0.4, 4.0 or 40 ng/ml insulin on lipoprotein lipase activity in five experiments. The data is represented by percentage of control activity (0.04 ng/ml) and is shown for acetone ether cellular powders (AE), activity releasable with heparin from cellular suspensions (HR) and that present in the culture medium (CM) following a 2 hr incubation in insulin. Discrimination between different insulin concentrations is not shown. Each point represents the mean of triplicate measurements. The paired t test was used to compare the enzyme activity alterations from the insulin treated group with the non-insulin treated control group (0% of control).

It is noteworthy that these cells frequently undergo change in tissue culture. For example, in some cultures of 3T3-L1 cells, the exponential phase of growth is prolonged, the cells multilayer and lipoprotein lipase can no longer be measured. If contact inhibition occurs above 1.5×10^6 cells/60 mm dish, an insulin effect on lipoprotein lipase is frequently absent. Both of the effects are more likely to occur as in vitro passage numbers increase from the original subclone IX. Finally, if cells are allowed to remain confluent for more than several weeks, the cell layer will peel off the culture dish and be lost. Many factors, therefore, appear to be important in determining whether these cells are in an optimal experimental condition for examination of lipoprotein lipase.

Discussion

In work with intact rat epididymal fat pads and isolated adipocytes, Garfinkel et al. have previously worked out a model for the secretion of lipoprotein lipase from adipocytes (18,19). By gel chromatography they have been able to distinguish 2 forms of the enzyme. As originally postulated, the in-

tracellular, or "b" fraction, corresponds to the activity present in acetone ether powders of cells. The extracellular, or "a" fraction, is membrane-bound and releasable by heparin. A transformation of the intracellular and the extracellular form appears to occur in conjunction with secretion of the enzyme from the fat cell. The same group has subsequently shown that following insulin treatment in vivo (1 u/kg), extracts of intact rat adipose tissue acetone ether powders separated by gel chromatography showed similar increases in both "a" and "b" fractions (6). However, in adipocytes isolated from contralateral fat pads from the same insulin-treated rats, an increase in secreted enzyme activity was observed nearly 4 hrs before an increase in acetone ether powder activity could be measured. Thus, in isolated adipocytes insulin increased lipoprotein lipase activity predominantly outside the adipocyte, while in intact adipose tissue an increase in both "a" and "b" fractions was seen.

In 3T3-L1 cells Wise and Green have shown the ability of heparin to release lipase activity into the culture medium (13). They have also shown that cycloheximide is effective in decreasing intracellular enzyme activity in the presence of 5 μg/ml insulin. In their studies, the contribution of de novo protein synthesis to the release of membrane-bound activity into the culture medium was not determined.

Insulin, therefore, appears to have two effects on lipoprotein lipase. The first is the primary demonstrated effect on enzyme synthesis; the second, a local membrane effect on release of enzyme. The latter effect may be mediated through an action of insulin upon calcium, which has been shown to be important for lipoprotein release (20) and is influenced by binding of insulin to its receptor (21), or upon microtubule function, which has been demonstrated to play an important role in many secretory processes (22).

Acknowledgments

Presented in part at the Annual Meeting of the American Federation for Clinical Research, San Francisco, May 1, 1978.

Supported by NIH grants AM 02456, AM 15312, and HL 18687, a grant from the

American Diabetes Association, Washington Affiliate, Inc., and a research fellowship award from the Juvenile Diabetes Foundation.

We thank Ms. Martha Kimura for her excellent technical assistance and Ms. Sharon Kemp for preparing this manuscript.

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