Bimodal effect of insulin on hormone-stimulated lipolysis: relation to intracellular 3′,5′-cyclic adenylic acid and free fatty acid levels

K. S. Desai, K. C. Li, and A. Angel[1]

Department of Medicine, University of Toronto, Canada

Abstract The present study was undertaken to determine the relationship between the antilipolytic and lipolytic effects of insulin on hormone-stimulated lipolysis and the mechanisms of these reactions. The dose-response curve of norepinephrine-stimulated lipolysis in rat adipocytes was not sigmoidal but biphasic in nature. Intracellular free fatty acid levels were linearly related to lipolytic rate and also described a biphasic profile in response to increments in norepinephrine concentration. Intracellular 3′,5′-cyclic AMP levels measured 10 min after addition of increasing concentrations of norepinephrine showed a rise and a plateau followed by a secondary rise. Insulin was antilipolytic at low concentrations of norepinephrine and distinctly lipolytic at high concentrations. The combined antilipolytic and lipolytic effect of insulin is termed the "bimodal" effect of insulin on hormone-stimulated lipolysis. The bimodal effect of insulin correlated positively with changes in peak intracellular 3′,5′-cyclic AMP levels. In the presence of glucose, insulin invariably enhanced lipolysis. It is suggested that the antilipolytic effect of insulin is achieved by both inhibition of adenyl cyclase activity and activation of low-K_m 3′,5′-cyclic AMP phosphodiesterase, the net effect being a low accumulation of 3′,5′-cyclic AMP. On the other hand, the lipolytic effect of insulin probably reflects enhancement of adenyl cyclase activity to an extent that overrides any activation of low-K_m 3′,5′-cyclic AMP phosphodiesterase activity, resulting in an increase in peak adipocyte 3′,5′-cyclic AMP levels.

Supplementary key words adipocytes · antilipolysis · regulation of lipolysis · free fatty acid mobilization

The antilipolytic effect of insulin on hormone-stimulated lipolysis is well established (1–3). This effect of insulin appears to be associated with a decrease in tissue adenyl cyclase activity (4–6) and a decrease in adipose tissue 3′,5′-cyclic AMP levels (7). The inhibition of lipolysis by insulin is not an invariable event because it is not seen when glucose is also present in the incubation medium (2, 8). Indeed, the lipolytic activity of catecholamines is markedly increased rather than decreased with the addition of glucose and insulin (2, 9). One other aspect about the antilipolytic effect of insulin is that it can be overcome by increasing either the dose of lipolytic hormone or the concentration of insulin in the medium (4, 10–12).

A number of studies have appeared in which insulin actually enhanced hormone-stimulated lipolysis in the absence of medium glucose (3, 10, 13–15). Although the inhibition of lipolysis by insulin can be correlated with lowered levels of intracellular 3′,5′-cyclic AMP, the mechanism of the seemingly paradoxical augmentation of hormone-stimulated lipolysis by insulin has not been explained. In an attempt to clarify the mechanism of the stimulatory effect of insulin on norepinephrine-mediated lipolysis, the present study was undertaken. On the basis of preliminary reports (16, 17) and the results reported here, it is proposed that the antilipolytic and lipolytic effect of insulin (termed the "bimodal" effect of insulin) on norepinephrine-stimulated lipolysis is causally related to observed fluctuations in initial rates of cyclic AMP accumulation and/or peak intracellular 3′,5′-cyclic AMP levels.

MATERIALS AND METHODS

Isolated adipocytes were prepared by the method of Rodbell et al. (18), with minor modifications (19), from epididymal fat pads of Wistar rats (160–200 g) raised on Purina chow. Each incubation vial contained 70–100 mg of adipocyte lipid in 2 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% dialyzed bovine serum albumin. The cells were preincubated for 10 min, after which zero-time samples were taken and hormones were added. The in vitro conditions employed were such to ensure sustained intracellular ATP levels during linear rates of lipolysis and nonsaturation of medium albumin by free fatty acids. Glucose was not required to sustain adipocyte ATP and was not added to the incubation medium unless

Abbreviations: 3′,5′-cyclic AMP, 3′,5′-cyclic adenylic acid.

[1] Performed during tenure as a Medical Research Council Scholar.

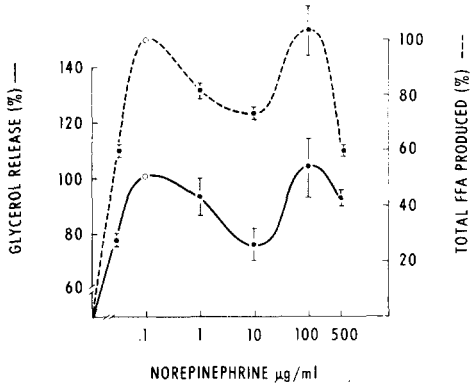

Fig. 1. Effect of norepinephrine (NE) on initial rate of lipolysis. Isolated adipocytes (75–100 mg of lipid) were incubated 30 min with various concentrations of norepinephrine. Cells were separated and medium free fatty acid was measured. Zero-time values were subtracted. Glycerol or free fatty acid release elicited by 0.1 μg/ml norepinephrine was taken as 100% and the other values were normalized. The glycerol and free fatty acid release in response to 0.1 μg/ml norepinephrine was 17.6 ± 1.26 μmoles and 49.2 ± 2.31 μmoles per gram of cell lipid in 30 min, respectively. Each point represents the mean ± SEM (n = 12) of six experiments, each performed in duplicate. The glycerol and free fatty acid output using 10 μg/ml NE was significantly lower than that produced by 0.1 μg/ml NE ($P < 0.01$), and the free fatty acid and glycerol output at 100 μg/ml NE was greater than that produced by 10 μg/ml ($P < 0.01$; statistical analysis by paired difference).

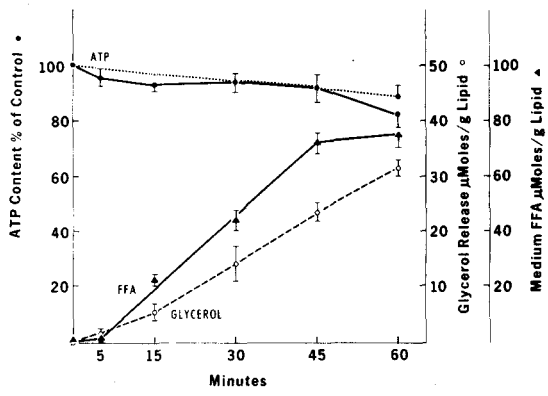

Fig. 2. Time-course study of relationship between lipolysis and adipose cell ATP. Medium and cells were separated by centrifugation and analyzed. Three experiments were combined and each point represents the mean ± SEM of six observations. The ATP levels were normalized, taking the zero-time control value as 100% to simplify presentation. ATP levels in the presence (●——●) and absence (●---●) of norepinephrine, 10 μg/ml.

specifically stated. These criteria of ideal conditions for in vitro lipolysis have been previously proposed (9, 20, 21) and were employed in this study to permit meaningful interpretation of the effects of interventions on initial rates of lipolysis.

At the end of incubation, the contents of each vial was transferred to a 15-ml centrifuge tube and centrifuged at 300 *g* for 15 sec. The medium was separated from the floating material ("cell float") by aspiration through polyethylene tubing (Intramedic PE100) attached to a 20-gauge, 1-inch needle and syringe. Intracellular free fatty acids were determined by first measuring adipose "cell float" free fatty acid content and correcting for extracellular albumin-bound free fatty acid, using [^{14}C]sucrose as an extracellular marker. Further details of this method for measuring intracellular free fatty acids have been reported previously (9, 20). Free fatty acids were titrated by the procedure of Dole and Meinertz (22).

Medium glycerol was measured in deproteinized medium by the method of Garland and Randle (23). Protein measurement was carried out according to Lowry et al. (24), using bovine serum albumin as reference standard. To calculate free fatty acid/albumin molar ratios, the molecular weight of albumin was taken as 66,000. ATP assays were carried out on perchloric acid extracts of adipose cells. The perchloric acid extracts were neutralized with 10 N KOH and triethanolamine–HCl to pH 7, and ATP was assayed as described by Denton, Yorke, and Randle (25).

3′,5′-Cyclic AMP was measured by a modified procedure of the protein binding assay described by Gilman (26). The binding reactions were carried out for 120 min at 0°C in 50 mM sodium acetate buffer, pH 4, in a volume of 100 μl, with 4 μg of binding protein, 15 μg of the protein inhibitor fraction, and 4 pmoles of ^{3}H-labeled 3′,5′-cyclic AMP (14.2 Ci/mmole). At the end of the incubation, the reaction mixture was filtered without prior dilution through a 24-mm cellulose ester Millipore filter, 0.45 μ (type HAWP), and washed with 15 ml of cold 20 mM potassium phosphate buffer, pH 6. Each filter was transferred to a counting vial and digested at room temperature in 1 ml of ethylene glycol monomethyl ether (methyl Cellosolve, Fisher) for 30 min before the addition of a toluene scintillation mixture containing 4 g of 2,5-diphenyloxazole (PPO), 50 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 250 ml of Cellosolve per liter. The counting efficiency was 33%.

Norepinephrine bitartrate and crystalline insulin (lot no. 26B-0700 24 IU/mg) were purchased from Sigma Chemical Co., St. Louis, Mo. Collagenase prepared from *Clostridium histolyticum* was purchased from Worthington Biochemical Corp., Freehold, N.J. Bovine serum albumin fraction V was obtained from Armour Pharmaceutical Co., Chicago, Ill. [U-^{14}C]Sucrose (sp act 10 m Ci/mmole) was obtained from Amersham, Don Mills, Ontario, Canada, and [8-^{3}H]adenosine 3′,5′-cyclic phosphate was purchased from Schwarz/Mann, Orangeburg, N.Y. and used without prior purification. All other reagents were reagent grade.

RESULTS

Biphasic initial rates of lipolysis

The effect of concentration of norepinephrine (NE) on the initial rates of lipolysis is shown in Fig. 1. The initial rates of glycerol and free fatty acid release showed an in-

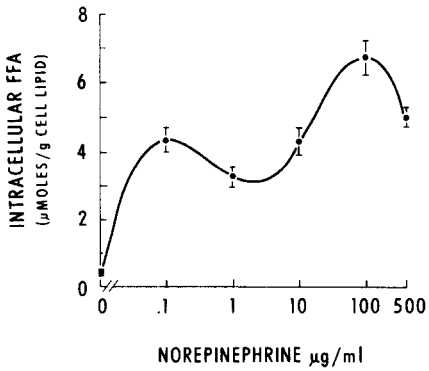

Fig. 3. Effect of norepinephrine on initial rate of intracellular free fatty acid accumulation. Adipocytes from the experiments described in Fig. 1 were extracted, and intracellular free fatty acid levels were determined as described in Materials and Methods. Each point represents the mean ± SEM (n = 12) of six experiments, each performed in duplicate.

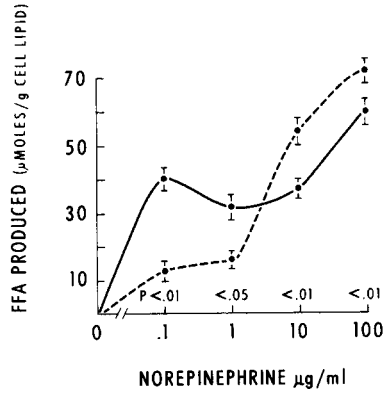

Fig. 4. Effects of insulin on the initial rates of norepinephrine-stimulated lipolysis. Isolated fat cells (75–100 mg/flask) were incubated 30 min in the presence of various concentrations of norepinephrine with (---) or without (——) insulin (100 μU/ml). Medium and cells were separated by centrifugation and analyzed for free fatty acid and glycerol. Zero-time total free fatty acid content was subtracted, and the results represent net free fatty acid release (cells plus medium). Free fatty acid/albumin ratio did not exceed 4. The results of six experiments were combined, and each point represents the mean ± SEM of 12 observations. The antilipolytic effect of insulin at low concentrations of norepinephrine (0.1–1.0 μg/ml) and the lipolytic effect of insulin at high concentrations of norepinephrine (10–100 μg/ml) is termed the bimodal effect of insulin on lipolysis.

crease then a decrease followed by a second increase and decrease when fat cells were exposed to increasing concentrations of norepinephrine. This biphasic lipolytic response to catecholamine confirms the observations previously reported by Allen, Hillman, and Ashmore (27). The free fatty acid/glycerol molar ratio at all norepinephrine concentrations was approximately 3. This ratio occurs when glucose is not added to the incubation medium and indicates complete hydrolysis of the triglyceride molecule with negligible reesterification of fatty acid (9, 20, 28).

The reason for the biphasic pattern of lipolysis with increasing concentrations of norepinephrine is not apparent. One possible mechanism for the decrease in lipolytic rate is a reduction in cellular ATP, as it has been shown that catecholamines can cause a significant fall in adipose tissue ATP levels (9, 29). Accordingly, experiments were carried out to determine whether the dip in lipolytic rate seen in the presence of 10 μg/ml norepinephrine was due to changes in the steady state ATP concentration. As seen in Fig. 2, the ATP levels were minimally altered during at least 45 min of incubation. An initial lag in free fatty acid and glycerol output in this experiment confirms our earlier observations (20) and is explained by the fact that during the first few minutes of lipolysis a significant proportion of total free fatty acids produced is retained within the cell and that partial glycerides accumulate within the cell before free glycerol appears in the medium. Glycerol release after 15 min was linear. The free fatty acid output between 5 and 45 min was linear, and the plateau in free fatty acid output after 45 min is attributed to saturation of medium albumin (free fatty acid/albumin = 5.6). The apparent 5% fall in ATP concentration during the first few minutes after the addition of norepinephrine has been observed before (9) and may represent conversion of ATP to 3′,5′-cyclic AMP. Thus, a fall in ATP concentration could not account for the biphasic pattern of lipolysis (Fig. 1). Therefore, attention was next focused on the possible role of intracellular free fatty acids in mediating changes in initial lipolytic rate. This explanation must also be viewed as an unlikely mechanism, as the intracellular free fatty acid concentration also showed a biphasic profile (Fig. 3) similar to that of initial rates of glycerol or free fatty acid production (Fig. 1). This finding would suggest that high intracellular free fatty acid levels are not the basis for the dip and secondary rise (biphasic pattern) in initial rates of lipolysis seen in the presence of norepinephrine.

Effect of insulin on norepinephrine-stimulated lipolysis

Insulin produced complex effects on the biphasic profile of norepinephrine-stimulated lipolysis (Fig. 4). Insulin was antilipolytic at low concentrations of norepinephrine, inhibiting free fatty acid production by almost 75% at 0.1 μg/ml norepinephrine. As the concentration of norepinephrine was increased, the antilipolytic property of insulin was reduced, and at norepinephrine concentrations of 10 and 100 μg/ml, insulin actually increased the initial rate of total free fatty acid release. This bimodal effect of insulin on the biphasic profile of initial rates of norepinephrine-stimulated lipolysis is also seen in analyses of glycerol production (Fig. 5). At low concentrations of norepinephrine, insulin was distinctly antilipolytic. At high concentrations of norepinephrine, insulin produced a 20% increase in glycerol release. The effect of insulin on intracellular free fatty acid levels (Fig. 6) was similar to the effect on total free fatty acid release (Fig. 4) in that it also described a bimodal profile. Thus, insulin displayed a bi-

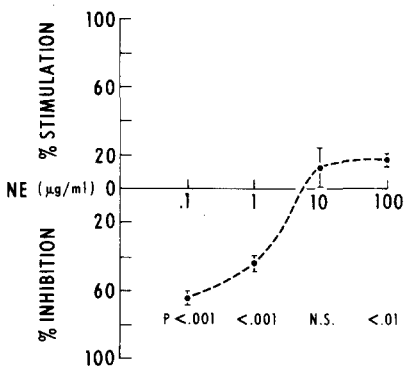

Fig. 5. Effect of insulin on initial rates of norepinephrine-stimulated lipolysis. Glycerol output was measured in media of the experiment described in the legend to Fig. 4. Here glycerol release at each concentration of norepinephrine was subtracted from the values obtained in the presence of insulin and the difference was expressed as percentage inhibition or stimulation.

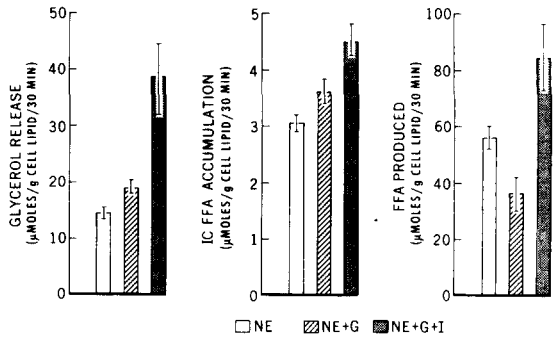

Fig. 7. Effect of glucose (16 mM) and insulin (100 μU/ml) on initial rate of lipolysis and intracellular (IC) free fatty acid levels in norepinephrine-treated (NE, 10 μg/ml) adipocytes. Isolated fat cells were incubated for 30 min. Medium and cells were separated by centrifugation and analyzed. Unstimulated control values were subtracted, and the results reflect net changes in concentrations of lipolytic products. Each bar is the mean ± SEM of eight observations from four experiments. The free fatty acid/glycerol molar ratio for each group was: norepinephrine, 3.2 ± 0.4; norepinephrine + glucose, 2.1 ± 0.3; norepinephrine + glucose + insulin, 2.3 ± 0.4.

modal effect on norepinephrine-induced lipolysis, being antilipolytic at low concentrations of norepinephrine and lipolytic at high concentrations.

Effect of glucose on norepinephrine-stimulated lipolysis

Under physiological circumstances of lipolysis, glucose is always present, and it has been implicated in the regulation of triglyceride hydrolysis (30). Therefore, the effects of glucose on the initial rate of norepinephrine-stimulated lipolysis were studied. Fig. 7 shows the effect of glucose and glucose plus insulin on the initial rate of norepinephrine-stimulated lipolysis. It is apparent (left panel) that glucose increased glycerol release, and addition of glucose plus insulin more than doubled the lipolytic rate. Intracellular (IC) free fatty acid levels (Fig. 7, middle panel) were also increased by glucose and glucose plus insulin. Net free fatty acid output into the medium was reduced by the addition of glucose, but with glucose plus insulin, free fatty acid output increased to the highest levels observed. Addition of glucose with or without insulin stimulated free fatty acid reesterification, as evidenced by a fall in free fatty acid/glycerol ratios (see legend to Fig. 7).

Intracellular 3′,5′-cyclic AMP levels

It was of interest to determine the relationship between intracellular 3′,5′-cyclic AMP levels and the biphasic effects of lipolytic hormones. Because it was previously shown (31) that the rise in intracellular 3′,5′-cyclic AMP after hormonal stimulation may be a transient event, a time-course experiment was carried out to determine the pattern of intracellular 3′,5′-cyclic AMP accumulation and lipolysis. Adipocytes were exposed to 0.1 μg/ml norepinephrine, and the intracellular 3′,5′-cyclic AMP was measured at different time intervals. As can be seen in Fig. 8, a rapid rise in 3′,5′-cyclic AMP occurred within

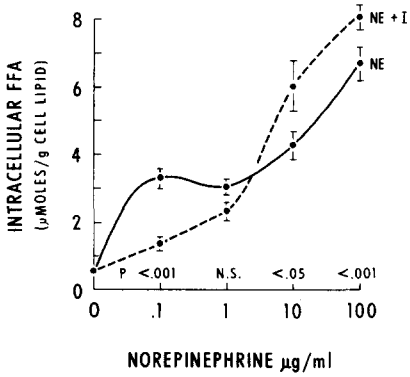

Fig. 6. Effect of insulin on norepinephrine-stimulated intracellular free fatty acid levels. Intracellular free fatty acid levels from the experiment described in the legend to Fig. 4 were measured as described in Materials and Methods and Ref. 9. Norepinephrine (——); norepinephrine + insulin, 100 μU/ml (---).

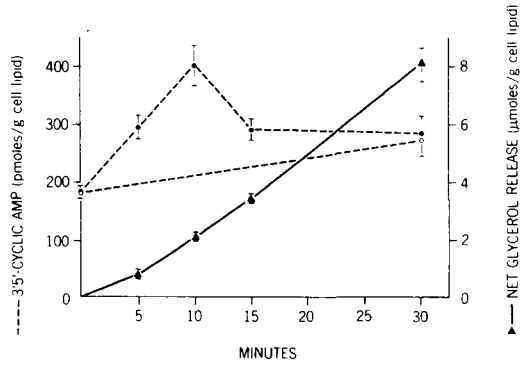

Fig. 8. Effect of norepinephrine (0.1 μg/ml) on adipocyte 3′,5′-cyclic AMP levels. Isolated fat cells (90–115 mg/flask) were incubated in triplicate in the presence (●---●) or absence (○---○) of hormone. Medium and cells were separated by brief centrifugation and analyzed for glycerol and 3′,5′-cyclic AMP, respectively.

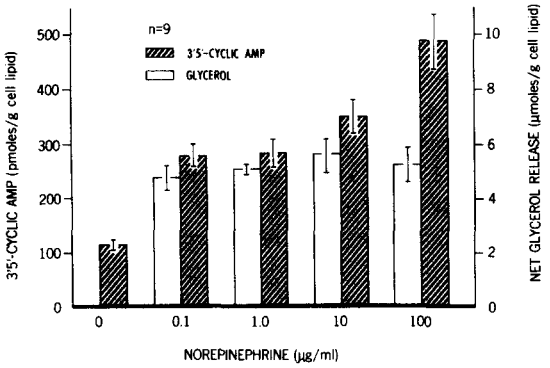

Fig. 9. Effects of norepinephrine on intracellular 3′,5′-cyclic AMP levels. Isolated cells (95–145 mg/flask) were preincubated in 2 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5% bovine serum albumin, at 37°C for 30 min, and hormone was added. After 10 min, cells were separated from medium by brief centrifugation, and the cells were extracted by the addition of 2 ml of cold 5% trichloroacetic acid. Medium glycerol was also determined. The height of each bar represents the mean ± SEM of nine observations from three experiments.

the first 5 min, and a peak level was observed at 10 min. Thereafter, despite the continued presence of norepinephrine and linear lipolysis (glycerol output), the intracellular 3′,5′-cyclic AMP levels fell and by 30 min equaled control values. A slight but insignificant rise in intracellular 3′,5′-cyclic AMP was noted in the control flasks after 30 min of incubation. A lag in glycerol release during the first 10 min of incubation is apparent. This has been noted before (20) and is important because it shows that glycerol output during the first few minutes of incubation may not be a reliable index of lipolytic rate. In subsequent experiments, intracellular 3′,5′-cyclic AMP levels were assayed after 10 min exposure to hormones in order to measure peak levels.

Experiments were next carried out to determine the relationship between dose of lipolytic hormone and intracellular concentration of 3′,5′-cyclic AMP (Fig. 9). It is apparent that with the lowest concentration of norepinephrine used (0.1 μg/ml), 3′,5′-cyclic AMP levels increased to almost 2.5 times the control values and plateaued despite a 10-fold increase in norepinephrine concentration. At 10 μg/ml norepinephrine, intracellular 3′,5′-cyclic AMP levels increased further, and at 100 μg/ml a second elevation of 3′,5′-cyclic AMP was observed. This biphasic pattern of an initial rapid rise in response to increasing norepinephrine concentrations could well account for the biphasic effect of norepinephrine on initial rate of lipolysis noted in Figs. 1 and 4. It is interesting to note here that, unlike the experiments described in Figs. 1 and 4, there were no differences in net glycerol release in response to increasing concentrations of norepinephrine. This could be attributed to the lag in glycerol release and suggests that it is probably more valid to compare the 10-min 3′,5′-cyclic AMP levels to the 15–20-min glycerol release (i.e., after the linear rate is established) for correlations.

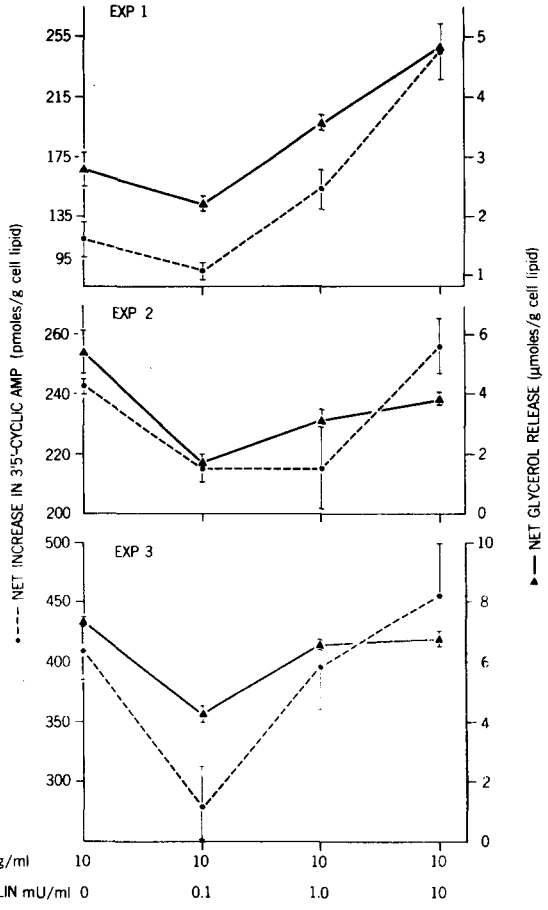

Fig. 10. Bimodal effect of insulin on norepinephrine-stimulated lipolysis. Isolated adipocytes (115–180 mg/flask) were preincubated in triplicate for 30 min prior to addition of hormones in the concentrations shown. After 10 min, cells and medium were separated and analyzed for 3′,5′-cyclic AMP and glycerol, respectively. The data are expressed as increments above nonstimulated control cells, and the results of three separate experiments are shown. Each point is the mean ± SEM of triplicate incubations. The 3′,5′-cyclic AMP levels without hormone addition in experiments 1, 2, and 3 were 115 ± 16.3, 138 ± 61, and 139 ± 7.3 (means ± SEM) pmoles/g of lipid, respectively.

Effect of insulin on intracellular 3′,5′-cyclic AMP levels

The effect of insulin on norepinephrine-stimulated lipolysis was studied in an effort to determine whether the bimodal effect of insulin (Fig. 4) correlated with intracellular 3′,5′-cyclic AMP levels. Three experiments are individually plotted (Fig. 10). In these studies, norepinephrine was constant at 10 μg/ml and the insulin concentration was varied.

In all three experiments, insulin was antilipolytic at 0.1 mU/ml, and suppression of glycerol release was paralleled by a reduction in intracellular 3′,5′-cyclic AMP concentration. In experiment 1, insulin at concentrations of 1 and 10 mU/ml increased the lipolytic effect of 10 μg/ml norepinephrine. This increase in glycerol release was also accompanied by an increase in the intracellular concentration of 3′,5′-cyclic AMP. In experiments 2 and 3, high

concentrations of insulin resulted in a reduction of antilipolytic effect. In these latter two experiments, loss of the antilipolytic effects of insulin was paralleled by increased 3′,5′-cyclic AMP levels.

DISCUSSION

Initial rates of lipolysis in isolated adipocytes display a biphasic pattern for both free fatty acids and glycerol output in response to increasing concentrations of norepinephrine. This profile of a rise and fall followed by a secondary increase in lipolytic rate was first noted by Allen et al. (27, 32) and is presently confirmed (Fig. 1). The first peak and second peak have been arbitrarily designated as Lipolysis I (occurring at a norepinephrine concentration of 3×10^{-8} to 1×10^{-5} M) and Lipolysis II (occurring at a norepinephrine concentration of 1×10^{-5} to 1×10^{-3} M), respectively (27). Although these designations were initially proposed to simplify discussion, they do have a functional basis in that the two areas of the dose-response curve correspond to differences in lipolytic responsiveness with respect to K^+ dependency and β blockade by propranolol (32). The present study has extended the understanding of the mechanism responsible for this profile in that it was shown that the rise and fall in initial rate of lipolysis cannot be attributed to alterations in intracellular ATP concentration, which remained quite constant throughout incubation (Fig. 2), or to intracellular free fatty acid levels, which seemed to reflect fluctuations in lipolytic rate rather than to mediate changes in lipolytic rate. The experimental data reported here do suggest that variations in initial rate of lipolysis are related to changes in 3′,5′-cyclic AMP levels measured shortly after (within 10 min) the addition of lipolytic hormones. It should be noted that intracellular 3′,5′-cyclic AMP levels increased and plateaued at hormone concentrations corresponding to Lipolysis I then increased again at catecholamine concentrations corresponding to Lipolysis II (Fig. 9 and Ref. 27). Thus, it seems reasonable to conclude that the profile of initial lipolytic rates is related to the initial rate of 3′,5′-cyclic AMP accumulation or some function thereof.

If the biphasic effect of catecholamine is mediated through variations in the peak intracellular 3′,5′-cyclic AMP concentration, a number of possible mechanisms must be considered in trying to account for these fluctuations. A rise in 3′,5′-cyclic AMP concentration obviously represents the balance between its synthesis and degradation. With respect to synthesis of 3′,5′-cyclic AMP, activation of adenyl cyclase in fat cells by catecholamines is well established (33, 34). To account for the secondary rise in 3′,5′-cyclic AMP concentration seen with higher doses of hormone (Fig. 9), it is noteworthy that binding of catecholamine to liver plasma membrane displays a biphasic pattern reflecting the binding affinity of low- and high-K_m receptors (35) and that the total number of high-K_m binding sites exceeds that of the low-K_m sites by a factor of 12. If activation of adenyl cyclase is determined by the number of hormone–receptor interactions, and if the binding to the catecholamine receptor of fat cells resembles that of liver, a biphasic lipolytic profile can be explained. This possible mechanism does not preclude other effects consequent to hormone–cell interaction such as enhancement of calcium binding to cell membrane or increased K^+ flux (35, 36).

Alternatively, lipolytic hormones may affect a biphasic lipolytic profile by directly or indirectly altering the rate of 3′,5′-cyclic AMP degradation. The relevance of this mechanism is supported by the fact that prostaglandin E_1 and dibutyryl 3′,5′-cyclic AMP activate phosphodiesterase activity in fibroblasts (37, 38); also, adrenal membrane preparations prepared from rats pretreated with ACTH show enhanced phosphodiesterase activity (39). However, the available data on the effect of lipolytic hormone on phosphodiesterase activity in adipose tissue is conflicting. Attempts to demonstrate fluctuations in phosphodiesterase activity by catecholamines in fat cells (31, 36, 40) have been unsuccessful. On the other hand, Zinman and Hollenberg (41) have recently shown that isoproterenol and dibutyryl 3′,5′-cyclic AMP can increase the V_{max} of the low-K_m 3′,5′-cyclic AMP phosphodiesterase. On the basis of these findings, it is prudent to conclude that the initial lipolytic rate reflects the net effect of activation of both adenylate cyclase and 3′,5′-cyclic AMP phosphodiesterase and that the relative activities of these enzymes depend on the ambient profile and concentration of lipolytic hormone(s).

Insulin exhibited a bimodal effect on norepinephrine-stimulated lipolysis. The finding that the antilipolytic effect of insulin was associated with a reduced initial rate of 3′,5′-cyclic AMP accumulation (Fig. 10) is in harmony with the observations that insulin can prevent the rise in intracellular cyclic AMP levels by inhibiting adenyl cyclase (4–6) and/or by augmenting its hydrolysis through activation of the low-K_m 3′,5′-cyclic AMP phosphodiesterase (42). However, the paradoxical observation of insulin enhancing the lipolytic action of catecholamine (Fig. 4) cannot be explained in the same way. Our finding that the intracellular 3′,5′-cyclic AMP levels paralleled the lipolytic effects of insulin (Fig. 10) suggests a causal relationship. This implies that high concentrations of insulin enhance adenyl cyclase activity or inhibit phosphodiesterase activity or both. Since insulin actually increases the low-K_m phosphodiesterase activity, it is more likely that enhancement of adenyl cyclase is responsible. Recent reports (5, 6) that insulin in high concentrations in the presence of lipolytic hormones can augment adenyl cyclase activity in adipo-

cytes, liver membranes, and adipocyte ghosts support this argument.

It is worth pointing out that although the enhancing effect of insulin on catecholamine-stimulated lipolysis has been observed many times (3, 10, 11, 14, 15, 43, 44), in our hands it is not seen at every experimental attempt. The reason for this variability is not apparent, but variable destruction of insulin receptors by collagenase digestion could be a major contributing factor. It is also worth mentioning that interpretations of total intracellular free fatty acids, ATP, or 3′,5′-cyclic AMP levels have not taken into account the fact that these metabolites are compartmentalized both in a functional sense and among various subcellular organelle systems (19, 21, 34). Whether the fluctuations in initial rates of lipolysis seen with increasing concentrations of norepinephrine or whether the bimodal effect of insulin on hormone-stimulated lipolysis reflects alterations in distribution of free fatty acid, 3′,5′-cyclic AMP, or ATP between pools within the cell is an important consideration but cannot be discerned from the available data.

The initial rate of norepinephrine-induced lipolysis was enhanced by glucose addition (Fig. 7). This effect of glucose has also been observed many times (8, 28, 30, 45) and has been regarded as a protective effect, achieved by promoting fatty acid reesterification and thereby reducing the intracellular free fatty acid concentration, thus preventing inhibition of the lipolytic reaction (2, 30, 45). The results in Fig. 7 show that this is an untenable line of reasoning since intracellular free fatty acid levels are actually increased rather than decreased in the presence of glucose. (This should not be taken to mean that glucose will not protect the cell against the uncoupling effect of free fatty acid accumulation, because it can [8, 46], but the conditions under which that mechanism applies do not correspond to the in vitro conditions employed here [see Refs. 9, 20, and 21 for detailed discussions of optimal conditions for study of in vitro lipolysis].) Insulin in the presence of glucose invariably enhances norepinephrine-stimulated lipolysis (8, 28, 45, 46). Although reesterification of free fatty acid is maximal under these circumstances, intracellular free fatty acid levels are at their highest (Fig. 7) rather than reduced. This finding negates the deinhibition of lipolysis argument, i.e., that the lipolytic effect of glucose in the presence or absence of insulin is related to a reduction in intracellular free fatty acid secondary to enhanced free fatty acid esterification.

The question of physiological or clinical relevance of the lipolytic effect of insulin deserves some comment. Insulin lipoatrophy, which occurs in diabetics at the site of repeated insulin injections, could be the result of enhanced lipolysis in a circumscribed area since the local concentration of insulin at the injection site would certainly correspond to those at which in vitro lipolysis is enhanced (Fig. 10). It is noteworthy that in isolated brown fat cells insulin not only stimulates fatty acid synthesis but also augments preferential mobilization and release of newly synthesized fatty acids (47). Finally, it is of interest to speculate that the increased free fatty acid turnover rate and the increased rate of lipolysis noted in obesity may be causally related to the hyperinsulinemia seen in that state (48, 49). In this regard it is significant that serum free fatty acid levels are not reduced in obese man despite hyperinsulinemia (49, 50, 51). Whether this apparent enhancement of basal lipolysis in obesity represents a lipolytic effect of elevated insulin and glucose levels in vivo, analogous to that observed in vitro, remains to be established.

The authors are grateful to Misses Winifreda Asico and Judy Halmagyi for expert technical assistance. We wish to acknowledge Dr. Mitchell Halperin for helpful discussions. Our thanks go also to Mrs. Cathryn West for preparation of manuscripts. The work was supported by the Medical Research Council of Canada and the Ontario Heart Foundation. Dr. K. S. Desai was supported by Fellowship grants from the Department of Medicine, Toronto General Hospital, and K. C. Li was the recipient of a Faculty of Medicine summer scholarship.

Manuscript received 11 September 1972 and in revised form 4 April 1973; accepted 6 July 1973.

REFERENCES

1. Perry, W. F., and H. F. Bowen. 1962. Factors affecting the in vitro production of non-esterified fatty acid from adipose tissue. *Can. J. Biochem. Physiol.* **40:** 749–755.
2. Jungas, R. L., and E. G. Ball. 1963. Studies on the metabolism of adipose tissue. XII. The effects of insulin and epinephrine on free fatty acid and glycerol production in the presence and absence of glucose. *Biochemistry.* **2:** 383–388.
3. Fain, J. N., V. P. Kovacev, and R. O. Scow. 1966. Antilipolytic effect of insulin in isolated fat cells of the rat. *Endocrinology.* **78:** 773–778.
4. Jungas, R. L. 1966. Role of cyclic-3′,5′-AMP in the response of adipose tissue to insulin. *Proc. Nat. Acad. Sci. USA.* **56:** 757–763.
5. Hepp, K. D., and R. Renner. 1972. Insulin action on the adenyl cyclase system: antagonism to activation by lipolytic hormones. *FEBS Lett.* **20:** 191–194.
6. Illiano, G., and P. Cuatrecasas. 1972. Modulation of adenylate cyclase activity in liver and fat cell membranes by insulin. *Science.* **175:** 906–908.
7. Butcher, R. W., C. E. Baird, and E. W. Sutherland. 1968. Effects of lipolytic and antilipolytic substances on adenosine 3′,5′-monophosphate levels in isolated fat cells. *J. Biol. Chem.* **243:** 1705–1712.
8. Hall, C. L., and E. G. Ball. 1970. Factors affecting lipolytic rates in rat adipose tissue. *Biochim. Biophys. Acta.* **210:** 209–220.
9. Angel, A., K. S. Desai, and M. L. Halperin. 1971. Reduction in adipocyte ATP by lipolytic agents: relation to intracellular free fatty acid accumulation. *J. Lipid Res.* **12:** 203–213.

10. Chlouverakis, C. 1967. Factors affecting the inhibitory action of insulin on lipolysis in a glucose-free medium *Endocrinology.* **81:** 521–526.
11. Goodman, H. M. 1969. The effects of insulin on lipolysis evoked by cyclic AMP and its dibutyryl analog. *Proc. Soc. Exp. Biol. Med.* **130:** 97–100.
12. Hepp, K. D., L. A. Menahan, O. Wieland, and R. H. Williams. 1969. Studies on the action of insulin in isolated adipose tissue cells. II. 3′,5′-Nucleotide phosphodiesterase and antilipolysis. *Biochim. Biophys. Acta.* **184:** 554–565.
13. Kovacev, V. P., and R. O. Scow. 1966. Effect of hormones on fatty acid release by rat adipose tissue in vivo. *Amer. J. Physiol.* **210:** 1199–1208.
14. Goodridge, A. G. 1968. Lipolysis in vitro in adipose tissue from embryonic and growing chicks. *Amer. J. Physiol.* **214:** 902–907.
15. Hales, C. N., T. M. Chalmers, M. C. Perry, and D. R. Wade. 1968. Investigations of the hormonal control of lipolysis. *In* Protein and Polypeptide Hormones. M. Margoulies, editor. Excerpta Medica Foundation, Amsterdam. 432–443.
16. Desai, K. S., and A. Angel. 1971. Effects of insulin and glucose on initial rates of lipolysis and intracellular FFA levels in isolated adipocytes. *Proc. Can. Fed. Biol. Soc.* **14:** 92. (Abstr.)
17. Desai, K. S., and A. Angel. 1971. Lipolytic effects of insulin. *Clin. Res.* **19:** 789.
18. Rodbell, M., A. B. Jones, G. E. Chiappe de Cingolani, and L. Birnbaumer. 1968. The actions of insulin and catabolic hormones on the plasma membrane of fat cells. *Recent Progr. Hormone Res.* **24:** 215–254.
19. Angel, A. 1970. Studies on the compartmentation of lipid in adipose cells. I: Subcellular distribution, composition, and transport of newly synthesized lipid: liposomes. *J. Lipid Res.* **11:** 420–432.
20. Angel, A., K. S. Desai, and M. L. Halperin. 1971. Intracellular accumulation of free fatty acids in isolated white adipose cells. *J. Lipid Res.* **12:** 104–111.
21. Angel, A., K. S. Desai, and M. L. Halperin. 1971. Free fatty acid and ATP levels in adipocytes during lipolysis. *Metabolism.* **20:** 87–99.
22. Dole, V. P., and H. Meinertz. 1960. Microdetermination of long-chain fatty acids in plasma and tissues. *J. Biol. Chem.* **235:** 2595–2599.
23. Garland, P. B., and P. J. Randle. 1962. A rapid enzymatic assay for glycerol. *Nature.* **196:** 987–988.
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
25. Denton, R. M., R. E. Yorke, and P. J. Randle, 1966. Measurement of concentrations of metabolites in adipose tissue and effects of insulin, alloxan-diabetes and adrenalin. *Biochem. J.* **100:** 407–419.
26. Gilman, A. G. 1970. A protein binding assay for adenosine 3′:5′-cyclic monophosphate. *Proc. Nat. Acad. Sci. USA.* **67:** 305–312.
27. Allen, D. O., C. C. Hillman, and J. Ashmore. 1969. Studies on a biphasic lipolytic response to catecholamines in isolated fat cells. *Biochem. Pharmacol.* **18:** 2233–2240.
28. Rodbell, M. 1965. Modulation of lipolysis in adipose tissue by fatty acid concentration in fat cell. *Ann. N.Y. Acad. Sci.* **131:** 302–314.
29. Hepp, D., D. R. Challoner, and R. H. Williams. 1968. Studies on the action of insulin in isolated adipose tissue cells. I. Stimulation of incorporation of 32P-labeled inorganic phosphate into mononucleotides in the absence of glucose. *J. Biol. Chem.* **243:** 4020–4026.
30. Bally, P. R., H. Kappeler, E. R. Froesch, and A. Labhart. 1965. Effect of glucose on spontaneous limitation of lipolysis in isolated adipose tissue: a potential regulatory mechanism. *Ann. N.Y. Acad. Sci.* **131:** 143–156.
31. Manganiello, V. C., F. Murad, and M. Vaughan. 1971. Effects of lipolytic and antilipolytic agents on cyclic 3′,5′-adenosine monophosphate in fat cells. *J. Biol. Chem.* **246:** 2195–2202.
32. Allen, D. O., and P. J. MacLaren. 1970. Effect of potassium ion, theophylline and propranolol on the biphasic lipolytic response to some catecholamines. *Biochem. Pharmacol.* **19:** 2569–2578.
33. Butcher, R. W., R. J. Ho, H. C. Meng, and E. W. Sutherland. 1965. Adenosine 3′,5′-monophosphate in biological materials. II. The measurement of adenosine 3′,5′-monophosphate in tissues and the role of the cyclic nucleotide in the lipolytic response of fat to epinephrine. *J. Biol. Chem.* **240:** 4515–4523.
34. Kuo, J. F., and E. C. De Renzo. 1969. A comparison of the effects of lipolytic and antilipolytic agents on adenosine 3′,5′-monophosphate levels in adipose cells as determined by prior labeling with adenine-8-^{14}C. *J. Biol. Chem.* **244:** 2252–2260.
35. Shlatz, L., and G. V. Marinetti. 1972. Hormone–calcium interactions with the plasma membrane of rat liver cells. *Science.* **176:** 175–177.
36. Perry, M. C., and C. N. Hales. 1970. Factors affecting the permeability of isolated fat-cells from the rat to [^{42}K] potassium and [^{36}Cl] chloride ions. *Biochem. J.* **117:** 615–621.
37. Manganiello, V., and M. Vaughan. 1972. Prostaglandin E_1 effects on adenosine 3′:5′-cyclic monophosphate concentration and phosphodiesterase activity in fibroblasts. *Proc. Nat. Acad. Sci. USA.* **69:** 269–273.
38. D'Armiento, M., G. S. Johnson, and I. Pastan. 1972. Regulation of adenosine 3′:5′-cyclic monophosphate phosphodiesterase activity in fibroblasts by intracellular concentrations of cyclic adenosine monophosphate. *Proc. Nat. Acad. Sci. USA.* **69:** 459–462.
39. Ng, S. S., and Y. C. Kong. 1971. The regulatory role of phosphodiesterase in adenosine cyclic 3′,5′-monophosphate mediated ACTH action. *Experientia.* **27:** 1257–1258.
40. Allen, D. O., and J. B. Clark. 1971. Effect of various antilipolytic compounds on adenyl cyclase and phosphodiesterase activity in isolated fat cells. *Advan. Enzyme Regul.* **9:** 99–112.
41. Zinman, B., and C. H. Hollenberg. 1972. Effect of insulin and catecholamines on the low K_m 3′,5′-cyclic nucleotide phosphodiesterase activity of rat adipocytes. Proceedings of the Fourth International Congress of Endocrinology. 157. (Abstr.)
42. Loten, E. G., and J. G. T. Sneyd. 1970. An effect of insulin on adipose-tissue adenosine 3′:5′-cyclic monophosphate phosphodiesterase. *Biochem. J.* **120:** 187–193.
43. Kono, T. 1972. A paradoxical effect of insulin on lipolysis in isolated rat epididymal fat cells. *Federation Proc.* **31:** 243Abs.
44. Solomon, S. S., J. S. Brush, and A. E. Kitabchi. 1970. Antilipolytic activity of insulin and proinsulin on ACTH

and cyclic nucleotide-induced lipolysis in the isolated adipose cell of rat. *Biochim. Biophys. Acta.* **218:** 167–169.

45. Chlouverakis, C. 1967. The action of glucose on lipolysis. *Metabolism.* **16:** 469–472.
46. Ho, R. J., R. England, and H. C. Meng. 1970. Effect of glucose on lipolysis and energy metabolism in fat cells. *Life Sci.* **9(1):** 137–150.
47. Angel, A. 1969. Brown adipose cells: spontaneous mobilization of endogenously synthesized lipid. *Science.* **163:** 288–290.
48. Smith, U. 1970. Effect of glucose and insulin on lipolysis rates in human fat cells of different sizes. *FEBS Lett.* **11:** 8–10.
49. Nestel, P. J., H. M. Whyte. 1968. Plasma free fatty acid and triglyceride turnover in obesity. *Metabolism.* **17:** 1122–1128.
50. Knittle, J. L., and F. Ginsberg-Fellner. 1972. Effect of weight reduction on in vitro adipose tissue lipolysis and cellularity in obese adolescents and adults. *Diabetes.* **21:** 754–761.
51. Yalow, R. S., S. M. Glick, J. Roth, and S. A. Berson. 1965. Plasma insulin and growth hormone levels in obesity and diabetes. *Ann. N.Y. Acad. Sci.* **131:** 357–373.