Insulin Inhibition of Lipolysis of Human Adipocytes

The Role of Cyclic Adenosine Monophosphate

THOMAS W. BURNS, BOYD E. TERRY, PAUL E. LANGLEY, AND G. ALAN ROBISON

SUMMARY

To gain information on the manner in which insulin suppresses lipolysis in man, isolated adipocytes, prepared from subcutaneous adipose tissue, were incubated with insulin (100 μU/ml) alone and in combination with isoproterenol (10^{-7} M or 10^{-8} M). Cyclic AMP concentration was measured at 60 min; glycerol release, used as an index of lipolysis, was determined at 45 and 75 min. Insulin consistently reduced both basal and stimulated cyclic AMP and glycerol release: the degree of suppression of each was comparable. In subsequent experiments, the ability of insulin to suppress glycerol release stimulated by isoproterenol, theophylline, and dibutyryl cyclic AMP (dbcAMP), respectively, was compared. Insulin substantially reduced the raised levels of cyclic AMP and glycerol release prompted by isoproterenol and theophylline, but it had little effect on increases caused by dbcAMP. These findings support the view that reduction in cyclic AMP is an important component in the regulation of fat mobilization by insulin. DIABETES *28*:957–961, November 1979.

It is generally accepted that one of the principal functions of insulin is to regulate fat mobilization by inhibiting the rate of lipolysis. The massive surge of free fatty acid (FFA) from the periphery seen in diabetic ketoacidosis is attributable to severe insulinopenia. The more modest elevations in serum FFA seen in the fasting state are explicable on the basis of low levels of circulating insulin.[1] In addition, it has been shown that the administration of insulin acutely depresses serum FFA concentrations in man.[2] A number of workers have demonstrated in vitro the ability of insulin to suppress lipolysis in adipose tissue or adipocytes from animals[3] and man.[4]

In spite of the intense interest shown, the question of how insulin acts to suppress lipolysis remains open. As early as 1963, Jungas and Ball suggested that insulin might inhibit lipolysis by reducing the level of cyclic AMP.[5] This possibility was supported by the findings of Butcher et al.[6] using rat epididymal fat pads. Others, such as Jarett et al.[7] and Fain and Rosenberg,[8] have reported finding no change in rat fat cell cyclic AMP under conditions in which insulin inhibited the lipolytic action of catecholamines. Using isolated human cells, we have reported preliminary observations[9] suggesting that insulin causes some decrease in the concentration of cyclic AMP. In the following report, we provide additional evidence that insulin does indeed reduce cyclic AMP and that this action appears to be associated with the reduction in lipolysis in human fat cells.

MATERIALS AND METHODS

Isolated human cells were prepared from subcutaneous adipose tissue samples obtained from volunteer subjects admitted to the Clinical Research Center of University of Missouri Medical Center and from patients undergoing abdominal surgery. Informed consent was obtained. No attempt was made to control the nutritional status of donors. The method of preparing human cells incorporating modifications of Rodbell's procedure[10] have been described.[11] Cells were suspended in Krebs bicarbonate buffer containing 2.5 mM glucose and 4% bovine serum albumin. Flasks containing aliquots of suspended cells were incubated with gentle shaking in an atmosphere of 95% O_2 and 5% CO_2. For determination of cyclic AMP, test substances were incubated in 10 ml of cell suspension. At the end of incubation, the contents of each flask were quick-frozen in liquid nitrogen and were then stored at −70°C for subsequent assay for cyclic AMP. Extracts were prepared essentially as described by Butcher et al.[12] but using a different chromatographic procedure;[13] cyclic AMP was assayed by the radioimmunoassay of Steiner et al.[14] Cyclic AMP was

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expressed as picomoles per gram of triglyceride. To determine glycerol release, which was used as an index of lipolysis, test substances were incubated with 1 ml of cell suspension in each of three to six flasks. After incubation, protein-free filtrates were prepared and frozen for subsequent analysis for glycerol by the enzymatic method of Garland and Randle.[15] Glycerol concentration was expressed as micromoles per gram of triglyceride per unit of time.

RESULTS

The effect of insulin on cyclic AMP concentration. Isoproterenol, a pure β-adrenergic agonist, is a powerful lipolytic substance when incubated with human cells. To determine optimal concentrations of isoproterenol with which to assess the inhibitory action of insulin, a series of dose-response experiments was done. For these, the concentration of insulin, when present, was held constant (100 μU/ml) and the concentration of isoproterenol was varied from 10^{-9} M to 10^{-5} M. Glycerol release was determined at 4 h and cyclic AMP concentration at 2 h. Results are summarized in Table 1. Even at the highest concentration of isoproterenol used, insulin had a small but definite inhibitory effect on the accumulation of glycerol. In general, however, the inhibitory effect of insulin was more marked at lower concentrations of the catecholamine (10^{-6} M to 10^{-8} M), and concentrations in this range were used in subsequent experiments.

Time course of changes in the concentration of cyclic AMP following addition of insulin. To determine the optimal time to assess the effect of insulin on cyclic AMP, experiments were done in which cyclic AMP was measured at ½, 1, 2, 3, and 4 h after the addition of insulin (100 μU/ml). In these experiments, cells were stimulated with isoproterenol (10^{-7} M), added at time 0. A typical time curve is presented in Figure 1. Maximal inhibition of cyclic AMP appeared to occur at between 1 and 2 h. In these same experiments, glycerol release was also measured; no effect of insulin on lipolysis was noted during the first hour (data not shown).

The effect of insulin on cyclic AMP and glycerol release stimulated by isoproterenol. To more closely define and compare the inhibitory effect of insulin on cyclic AMP and glycerol release, a large number of experiments has been done in which cyclic AMP was measured at 60 min and glycerol release at 45 and 75 min. Because of delay in the effect of insulin on cyclic AMP described above, cells were preincubated with insulin (100 μU/ml) for 1 h before the introduction of isoproterenol (10^{-7} M and 10^{-8} M) at time 0. Sampling times of 45 and 75 min were selected, because the average rate of glycerol release during this interval should approximate the instantaneous rate of lipolysis at 60 min, reflecting the activity of triglyceride lipase at that instant. As discussed by Steinberg,[16] lipase activity is determined, at least in part, by the activity of cyclic AMP–dependent protein kinase. The results are summarized in Table 2. Insulin consistently suppressed cyclic AMP and glycerol release both in the presence and absence of isoproterenol. To further evaluate the relationship between cyclic AMP concentration and glycerol release, the 30-min increment in the latter, i.e., the increase that occurred between 45 and 75 min, was plotted as a function of the concentration of cyclic AMP (Figure 2). Points representing results from flasks containing insulin fell on a curve similar to that defined by points from insulin-free flasks. These findings strongly suggest a causal relationship between the insulin-induced reduction in cyclic AMP and inhibition of glycerol release, and they argue against the concept of an inhibitory effect of insulin on the action of cyclic AMP.

The effect of insulin on dibutyryl cyclic AMP–stimulated lipolysis. The dibutyryl derivative of cyclic AMP, dbcAMP, has been shown to consistently stimulate lipolysis when incubated with human adipocytes. This derivative, or possibly the N^6-monobutyryl product resulting from its hydrolysis, is thought to act primarily by directly stimulating protein kinase,[17] although it may also act by protecting endogenous cyclic AMP from destruction by phosphodiesterase.[18] If insulin suppresses lipolysis primarily by reducing cyclic

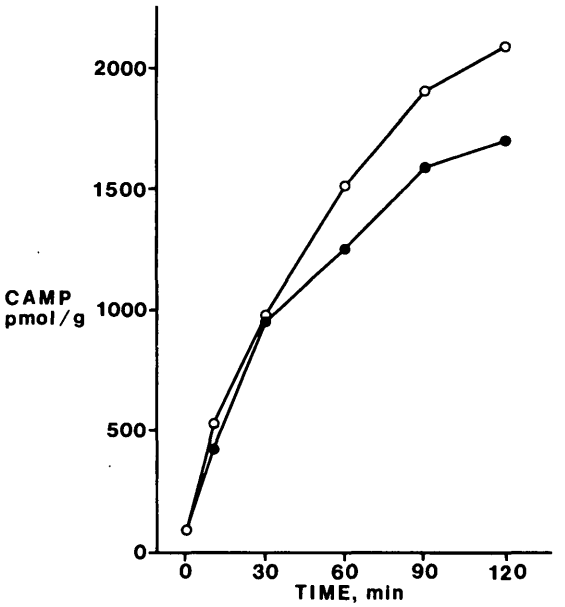

FIGURE 1. The time course of cyclic AMP following the addition of insulin. Human adipocytes were incubated in the presence of isoproterenol (10^{-7} M) alone (open circles) or with isoproterenol plus insulin (100 μU/ml, closed circles). Contents of flasks were frozen at the times indicated for cyclic AMP assay. All flasks contain 4% albumin in Krebs bicarbonate buffer and 20 mg of adipocyte triglyceride per milliliter. The results of a representative experiment are shown.

TABLE 1
Human adipocytes were incubated with varying concentrations of isoproterenol (Is) with and without insulin (Ins), 100 μU/ml; test substances were added at time 0. Glycerol (Gly) is expressed as micromoles released over 4 h/g of lipid; cyclic adenosine monophosphate (cAMP) is expressed as picomoles per gram at 2 h. Results of three experiments are given

Experiment no.	HC 598		HC 599		HC 602	
	Gly	cAMP	Gly	cAMP	Gly	cAMP
Control	2.5	265	1.0	81	1.0	46
Ins	0.8	296	0.7	73	0.9	40
Is 10^{-8} M	2.5	201	13.2	138	4.3	66
Is 10^{-8} M + Ins	1.0	261	5.6	128	1.3	67
Is 10^{-7} M	4.2	688	15.5	318	9.8	241
Is 10^{-7} M + Ins	1.8	440	13.2	281	5.6	154
Is 10^{-6} M	8.6	6550	16.9	615	11.1	2039
Is 10^{-6} M + Ins	6.3	4395	14.3	405	6.7	1576
Is 10^{-5} M	9.0	6702	17.3	1140	11.3	2811
Is 10^{-5} M + Ins	8.8	4660	14.9	452	7.9	2062

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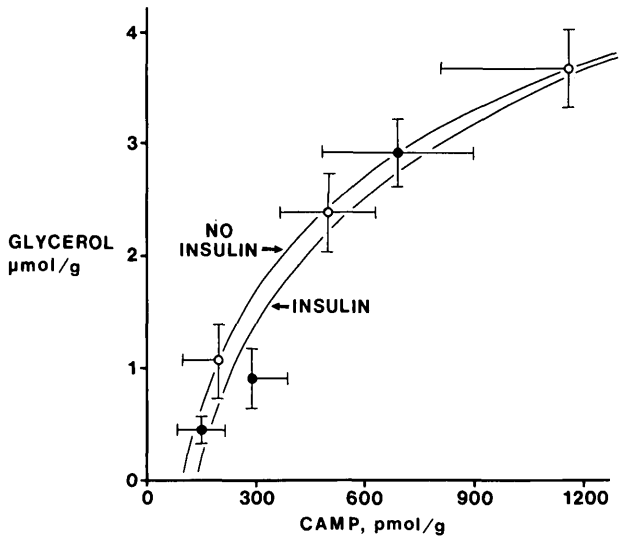
TABLE 2
Human adipocytes were incubated with isoproterenol (Is), 10^{-8} M and 10^{-7} M; half the flasks were preincubated for 1 h with insulin (Ins), 100 μU/ml. Cyclic AMP, expressed as picomoles per gram of lipid, was measured at 60 min. The glycerol released over a 30-min period, from 45 to 75 min, is expressed as micromoles per gram of lipid. Results of nine experiments are given

	Glycerol Isoproterenol						*cAMP* Isoproterenol					
Exp. no.	Control	0 +Ins	10^{-8}	10^{-8} +Ins	10^{-7}	10^{-7} +Ins	Control	0 +Ins	10^{-8}	10^{-8} +Ins	10^{-7}	10^{-7} +Ins
HC 623	—	—	1.80	0.60	2.50	2.90	—	—	220	30	215	100
HC 624	2.10	1.05	2.45	0.55	3.57	2.75	132	44	164	92	218	132
HC 625	0.03	0.02	4.30	2.80	5.75	4.80	30	18	156	74	274	214
HC 626	2.65	0.65	1.70	0.65	3.10	1.80	52	16	112	36	194	160
HC 627	1.05	0.50	1.85	1.20	3.95	2.65	102	18	306	240	907	670
HC 628	0.45	0.25	2.75	0.40	4.50	3.55	79	412	872	619	2717	1472
HC 646	0.50	0.35	1.05	0.45	2.60	2.40	74	41	670	286	1205	854
HC 647	1.40	0.40	2.25	0.60	3.00	2.30	855	485	1220	835	2445	1745
HC 648	0.35	0.20	3.45	0.80	3.85	3.10	230	145	730	375	2265	930
$\bar{X}$	1.07	0.43	2.40	0.89	3.65	2.93	194	147	494	287	1160	697

AMP, the hormone should have little or no effect on lipolysis stimulated by dbcAMP. To gain information on this point, the effect of insulin on the dose-response curves of dbcAMP, theophylline, and isoproterenol were compared. Theophylline, a potent inhibitor of phosphodiesterase, stimulates lipolysis by allowing the accumulation of endogenous cyclic AMP. The concentrations of the stimulating substances used were as follows: dbcAMP, 10^{-5} to 10^{-3} M; theophylline, 10^{-6} to 10^{-3} M; and isoproterenol, 10^{-9} to 10^{-6} M. In these experiments and those described below, a 4-h incubation was used; cells were not preincubated with insulin. As expected, insulin substantially reduced lipolysis stimulated by theophylline (Figure 3) and (not shown) isoproterenol; in contrast, the effect of insulin on dbcAMP-induced lipolysis was slight.

Since the manner in which dbcAMP stimulates lipolysis could influence the interpretation of these results, additional experiments were done to clarify that issue. To one set of flasks, epinephrine (10^{-5} M), propranolol (3.2×10^{-5} M), and dbcAMP (10^{-5} to 10^{-3} M) were added. To a second set, these same substances plus insulin (100 μU/ml) were added. We have shown that the combination of epinephrine plus propranolol in these concentrations activates α-adrenergic receptors, drastically reduces cyclic AMP formation, and abolishes the stimulatory effect of theophylline. Stimulation of lipolysis by dbcAMP under these conditions would be attributed to an action beyond cyclic AMP formation. dbcAMP did, in fact, stimulate lipolysis, and again there was but a meager reduction of this effect in the presence of insulin (Figure 4). These findings are consistent with the view that reduction in cyclic AMP is a significant component of the mechanism by which insulin suppresses lipolysis, and they argue against the view that insulin interferes with the effect of cyclic AMP on protein kinase.

FIGURE 2. The correlation between intracellular cyclic AMP content and glycerol release. Human adipocytes were incubated in flasks containing 4% albumin in Krebs bicarbonate buffer and 0, 10^{-8}, and 10^{-7} M isoproterenol. Half of the flasks were preincubated for 1 h with insulin, 100 μU/ml. Cyclic AMP, determined at 60 min, is plotted against the glycerol released between 45 and 75 min. Each point is the mean of nine experiments. The horizontal and vertical lines represent the standard errors of the means of cyclic AMP and glycerol values, respectively. The curves were fitted to the points using the regression formula y = a + blog X. The coefficient of correlation r^2 was 0.93 for insulin-containing flasks (closed circles) and 0.99 for flasks without insulin (open circles).



DISCUSSION

The time course of changes in cyclic AMP during the exposure of human adipocytes to isoproterenol is not as well defined as are the changes in rat fat cells. The pattern of cyclic AMP response with human cells varies somewhat from sample to sample. Even with the same tissue sample, variation can be introduced by factors such as the cell density in test suspensions. With dilute suspensions (e.g., 20 mg triglyceride per milliliter or less), less fatty acid is produced and higher cyclic AMP levels tend to persist. With heavy cell suspensions (e.g., 80–100 mg/ml) large quantities of fatty acids are produced, curtailing cyclic AMP levels. In the latter circumstance, the peak level of nucleotide is seen at 15–30 min; with dilute suspension, the peak level may be delayed. To complicate matters further in human adipocytes, the temporal and quantitative linkage between cyclic AMP and lipolysis has not been closely defined. We do not know with any precision how soon any rise in glycerol release follows a rise in cyclic AMP or the magnitude of increment in glycerol release to expect from a given rise in cyclic

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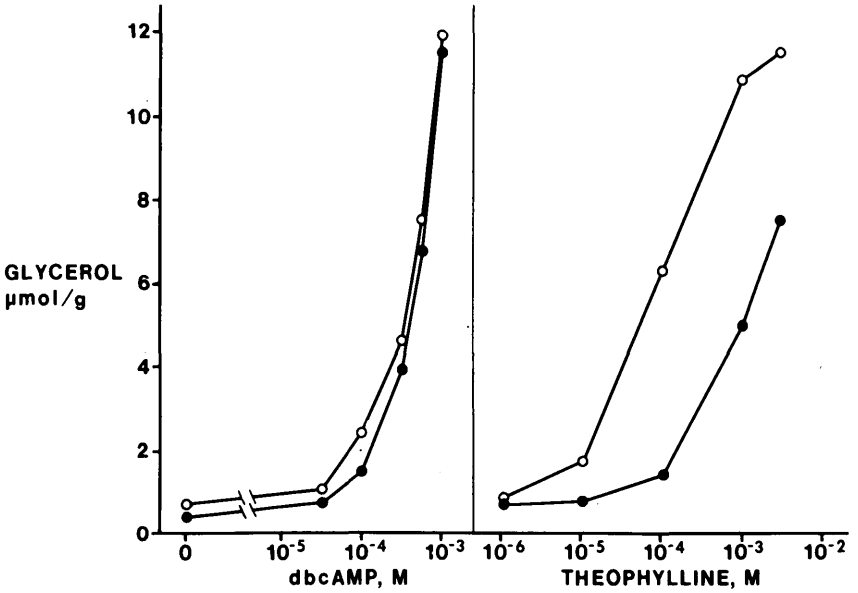
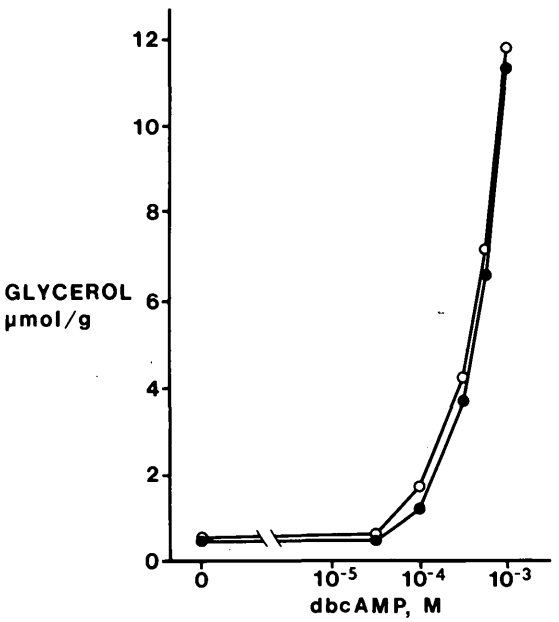


FIGURE 3. The effect of insulin on dbcAMP- and theophylline-stimulated lipolysis. Human adipocytes were incubated for 4 h with varying concentrations of either dbcAMP (left panel) or theophylline (right panel). All flasks contained 4% albumin in Krebs bicarbonate buffer. Flasks containing insulin (100 μU/ml), added at time 0, are represented by closed circles and those without insulin with open circles. The results of a typical experiment are shown.

AMP. The conditions used in doing the experiments summarized in Table 2 were selected with these limitations in mind. Glycerol released over a 30-min period (45–75 min) was determined and the concentration of cyclic AMP was measured midway in this period (60 min). Not surprisingly, we see a better correlation between cyclic AMP and glycerol in these experiments than in those done to select appropriate doses of isoproterenol (Table 1). In the latter, glycerol release was measured at 4 h and cyclic AMP at 2 h.

Our findings are consistent with some, but apparently not all, studies on the effect of insulin on rat fat cells. For example, Jarett et al.,[7] Fain and Rosenberg,[8] and others found that insulin could inhibit lipolysis in these cells in response to low doses of lipolytic hormones with little or no measurable effect on cyclic AMP. It is quite possible that these apparent, discrepant findings reflect true species differences and that insulin may be capable of inhibiting lipolysis by more than one mechanism, with the effect on cyclic AMP being more important in human adipose tissue than in rat adipocytes. Furthermore, all these effects may be the result of a single primary action of insulin at the receptor level.

FIGURE 4. The effect of insulin on dbcAMP-induced lipolysis in the presence of α-adrenergic receptor stimulation. The experimental conditions used were the same as described for Figure 3, except that all flasks contain epinephrine (10^{-5} M), a mixed agonist, and propranolol (3.2×10^{-5} M), a β blocker. Under these conditions, it is highly likely that dbcAMP is acting beyond cyclic AMP formation. Insulin had little effect on this action (see text).



If insulin does indeed reduce cyclic AMP, as we believe, the mechanism by which this is accomplished is far from understood. Earlier reports[19,20] of the hormone's inhibitory effect on adenylate cyclase have yet to be confirmed. Another possible mechanism is the stimulation of a membrane-bound "low K_m" form of phosphodiesterase.[21–23] However, the finding that insulin has no effect on this enzyme when added directly in broken cell preparations implies the existence of some more primary effect, such as production of an unstable soluble factor. This factor could simultaneously affect phosphodiesterase and, in some cells, other enzymes such as the protein kinase. Support for this latter action can be found in a recent report by Walkenbach et al.,[24] according to which the addition of insulin to rat skeletal muscle leads to reduced sensitivity of the protein kinase to stimulation by cyclic AMP. Walkenbach and his colleagues have proposed that the soluble factor is generated in response to insulin and that this factor then interacts with protein kinase, thereby interfering with the ability of cyclic AMP to bind to it. A similar effect could presumably occur in rat fat cells and might, at times, be more important in these cells than the effect on phosphodiesterase. Conversely, in human fat cells, it would appear that the effect on phosphodiesterase may be more important. In fact, as noted previously in connection with Figures 2 and 3, we could find no evidence for an effect of insulin on the action of cyclic AMP. If the soluble factor postulated by Walkenbach et al. can be identified and characterized, it would be of interest to compare its effects on the enzymes of rat adipocytes with those of human cells.

The net effect of insulin on lipolysis may, of course, be more complex than the foregoing discussion might suggest, since it is inevitably intertwined with effects on other intra-

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cellular processes. For example, it has been shown that FFA have an important negative feedback effect on lipolysis in both rats[25] and man.[26] By promoting esterification of FFA, insulin reduces their intracellular concentration and, thus, indirectly stimulates lipolysis.

Nevertheless, it is likely that one of the effects of insulin in human fat cells is to reduce the intracellular level of cyclic AMP, and it would appear that this is sufficient in humans, if not in rats, to account at least in part for its antilipolytic effect.

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