Reduction in adipocyte ATP by lipolytic agents: relation to intracellular free fatty acid accumulation

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ABSTRACT Epinephrine, norepinephrine, ACTH, and dibutyryl 3′,5′-cyclic AMP reduced adipocyte ATP levels during 60 min incubation; glucose displayed a protective effect. The reduction in adipocyte ATP levels could not be attributed solely to: a direct hormone effect, deficiency in metabolic substrate, activation of adenyl cyclase with ATP consumption, loss of adenine nucleotide from the cell or loss of cells during incubation, lipolytic rate per se, or extracellular accumulation of FFA or glycerol.

To determine whether intracellular FFA accumulation was a causative factor, intracellular FFA levels were measured during hormone-stimulated lipolysis. This was accomplished by using sucrose-U-^{14}C as a marker for the extracellular space to correct for contamination of cells by extracellular albumin-bound FFA. These experiments showed that the fall in adipocyte ATP correlated with FFA saturation of medium albumin and progressive accumulation of FFA within the adipocyte. Furthermore, the protective effect of glucose noted above was associated with a marked reduction in intracellular FFA as compared to the extracellular FFA pool.

On the basis of these studies, combined with those in the literature, it is concluded that in vitro effects of lipolytic agents on adipocyte ATP levels are the net result of impaired ATP synthesis (uncoupled oxidative phosphorylation) in the face of normal or augmented ATP consumption.

SUPPLEMENTARY KEY WORDS energy metabolism · metabolic controls · intracellular fatty acid pools · hormone-stimulated lipolysis · epinephrine · norepinephrine · ACTH · dibutyryl 3′,5′-cyclic AMP · adipose tissue DNA · sucrose space

LIPOLYTIC hormones are known to produce a variety of effects in adipose tissue, apart from activating the hydrolysis of triglyceride. Glucose uptake, its conversion to glyceride-glycerol, as well as its oxidation are all increased in adipose tissue exposed to a number of lipolytic hormones (1–4). On the other hand, anabolic reactions such as fatty acid synthesis (1, 2) and protein synthesis (5, 6) are substantially impaired in similarly treated tissues. Under conditions where extracellular fatty acid acceptor is limiting, stimulation of lipolysis is followed by depressed glucose metabolism (3), a fall in oxygen consumption (7), and inhibition of the lipolytic process itself (8). Indeed, continued lipolysis in isolated adipocytes incubated in the absence of glucose results in serious alteration in the structural integrity of the plasma membrane, with leakage of enzymes and other cellular proteins into the bathing medium (9). Many of these effects have been attributed to excessive accumulation of fatty acids within the adipose cell (1–10). This conclusion is supported by the findings that fatty acids added in high concentrations to the incubation medium mimic the effects of lipolytic agents on the uptake and subsequent metabolism of glucose in isolated adipose cells (10) and that lipolytic hormones do not stimulate glucose uptake nor inhibit amino acid transport in glyceride-depleted adipose cell ghosts (11, 12).

There does exist some evidence which indicates that there is an effect of lipolytic hormones on carbohydrate utilization independent of the lipolytic process. Bray and Goodman (13, 14) have shown that catecholamines increase the arabinose space in adipose tissue treated with propranolol to inhibit lipolysis, and they concluded that epinephrine increases glucose oxidation by promoting its entry into the adipocyte. A similar conclusion was

Abbreviations: FFA, free fatty acids.

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reached in studies with isolated adipocytes in which prostaglandin-E_1 blocked epinephrine-stimulated lipolysis without altering augmented glucose oxidation (15).

Recently, reports have appeared showing that catecholamines cause a significant fall in adipose tissue ATP levels (16–19). The implications of this observation are far-reaching, since alterations in the concentration of high-energy nucleotides could affect numerous energy-dependent biosynthetic and transport processes. Furthermore, it suggests that lipolytic hormones could potentially alter the general metabolic integrity of adipose cells incubated in vitro, thereby complicating interpretation of mechanisms underlying the many effects of lipolytic hormones. For this reason it was thought important to study in detail how lipolytic agents lower adipocyte ATP. Part of the data presented here have appeared in preliminary form (19).

MATERIALS AND METHODS

Male Wistar rats weighing 180–220 g and fed ad lib. on Purina Chow were used throughout the study. The animals were killed by a sharp blow to the head, and isolated white adipose cells were obtained by collagenase digestion of epididymal fat as described by Rodbell (4), with minor modifications (20). Cells were suspended in Krebs-Ringer bicarbonate buffer containing 1.27 mM $CaCl_2$ and 5% bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.). In experiments in which fatty acids were added to the incubation medium, albumin concentration was reduced in proportion to the volume of solution added. The buffer albumin had been dialyzed twice against 10 volumes of buffer, and frozen. Stock solutions of medium were thawed and gassed with 95% O_2–5% CO_2 to pH 7.4 before use. The cell–medium mixture was dispensed into 1-oz plastic bottles (Nalgene) so that each flask contained about 200 mg of adipocytes in a final volume of 2 ml. Incubations were carried out in a Dubnoff metabolic shaker oscillating at 90 cycles per min at 37°C with 95% O_2–5% CO_2 gas phase.

Following incubation the contents of each flask were poured into a 15-ml glass centrifuge tube and centrifuged at 300 *g* for 15–30 sec in a clinical bench centrifuge. The infranatant medium was separated from the packed adipose cell "float" by aspiration through a long needle into a syringe.

The adipose cell "float" was denatured by addition of 2 ml of 5% perchloric acid; the mixture was then thoroughly mixed for 30 sec on a Vortex mixer. This mixture was centrifuged for 3–5 min at 300 *g* and the intermediate clear aqueous phase was removed with a needle and syringe for subsequent assay of ATP. To determine adipose cell mass the oil layer was extracted three times with 3 ml of diethyl ether, and an aliquot of the pooled extracts was evaporated and weighed in tared vessels.

The perchloric acid extracts were neutralized with 10 N KOH and triethanolamine–HCl to pH 7, and ATP, ADP, and AMP were assayed as previously described (21).

Medium glycerol was assayed as described by Garland and Randle (22), and FFA were titrated by the method of Dole and Meinertz (23). Protein was measured according to Lowry, Rosebrough, Farr, and Randall (24), using bovine serum albumin as reference standard. To calculate FFA/albumin molar ratios, the mol wt of albumin was taken as 66,000. DNA was determined according to Burton (25), using salmon sperm DNA as a reference standard.

Measurement of Intracellular FFA

Since separation of cells from medium by centrifugation is incomplete, titration of the adipose cell "float" alone would overestimate intracellular FFA to a variable extent depending on the amount of albumin-bound FFA trapped between adipose cells. To correct for this source of error, the volume of medium trapped in the adipose cell "float" was determined using sucrose-^{14}C as a non-utilizable, nontransportable extracellular marker. Sucrose-^{14}C was added to the starting cell–medium mixture to give a final activity of approximately 2×10^5 cpm/ml. 1 ml of H_2O and 5 ml of isopropyl alcohol–heptane–0.5 N H_2SO_4 40:10:1 (v/v/v) (23) were added to the adipose cell "float" and the mixture was agitated for 30 sec on a Vortex mixer. After further addition of heptane and water (23) the radioactive sucrose originally in the adipose cell "float" partitioned quantitatively into the resultant lower phase (26). 1 ml of the lower phase was taken for assay of radioactivity and 3 ml of the upper phase was washed with acidified H_2O (27) and then titrated to determine FFA. Aliquots of the incubation medium were similarly extracted, titrated, and counted.

The volume of medium entrapped in the adipose cell "float" was calculated by dividing the specific radioactivity of the incubation medium (cpm/ml) by the total radioactivity (cpm) in the lower phase of Dole's extract of the adipose cell "float." Since the FFA content of the adipose cell "float" and incubation medium was determined, the intracellular FFA content could then be calculated. Further details of this method for measuring intracellular FFA levels are the subject of a separate report (26).

Aliquots of the various fluids were counted in 15 ml of Bray's scintillation mixture (28). Radioactivity was assayed at 15°C in a Beckman liquid scintillation spectrometer, model LS-250, equipped with an external standard. Corrections for quenching were not necessary.

Chemicals and Reagents

Norepinephrine hydrochloride, ACTH, and epinephrine bitartrate were purchased from Sigma Chemical Co., St. Louis, Mo.; N_6-O_2 dibutyryl 3′,5′-cyclic AMP and salmon sperm DNA were obtained from Calbiochem, Spring Valley, N.Y. 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., and sucrose-U-^{14}C (SA 10 mCi/mmole) was obtained from Amersham Don Mills, Ontario, Can. Organic solvents were reagent grade. Octanoic, palmitic, and oleic acids, > 99% pure according to the manufacturers' specifications, were obtained from the Hormel Foundation, Austin, Minn., and were converted to their sodium salts (20 mM stock solution) by heating to 70°C–80°C in a water bath with one-third molar excess of 0.1 N NaOH. Fatty soaps were coupled to medium albumin by gentle stirring in a water bath at 45°C. Care was taken to restore the medium pH to 7.4 by gassing with 95% O_2–5% CO_2 prior to addition of adipose cells.

RESULTS

ATP Content of White Fat

The ATP content of epididymal adipose tissue taken from fed rats varied inversely with animal weight (age) (Fig. 1). This is not surprising, since an increase in adipocyte size and lipid content is one of the processes responsible for enlargement of the adipose organ during growth (29, 30). The negative correlation between rat weight and adipose tissue ATP levels was abolished when the results were expressed in terms of DNA rather than tissue weight. This variation in ATP level in relation to tissue weight (lipid) may well account for the variable levels reported by others (17, 18, 21). The constancy of ATP content in relation to tissue DNA (Fig. 1, right) of approximately 400 nmoles of ATP per mg of DNA is in good agreement with the data of Denton, Yorke, and Randle (21) and indicates that the ATP content of white fat is more a function of cellularity than of adiposity. Since enlargement of epididymal fat pads during growth is not only a function of increasing lipid content per cell but also of an increase in cell number (29, 30), the constant ATP/DNA ratios during growth suggest that each of the ATP-containing cell types increases proportionately or, alternatively, that all cell lines (stromal, endothelial, adipose) in the fat pads have identical ATP/DNA ratios.

Since less than one-half of the cells in adipose tissue are lipid-laden adipocytes (4), it is likely that a significant portion of total tissue ATP will be associated with non-adipose cell components. To estimate that portion of tissue ATP in adipocytes proper, the ATP content of different tissue fractions was determined after collagenase digestions. As shown in Table 1, the ATP content of isolated cells was significantly less than that of intact tissue. This is not surprising since the adipocyte isolation procedure removes stromal and vascular elements which contain ATP but very little lipid. It is worth noting that after 60 min of incubation with collagenase the ATP content of freshly digested fragments was identical to that of incubated control tissue, indicating that collagenase treatment does not of itself affect adipocyte ATP levels. Since 17–57% of tissue ATP was stromal or vascular in origin, isolated adipocytes were used in the remainder of the study rather than intact tissue. The ATP content of

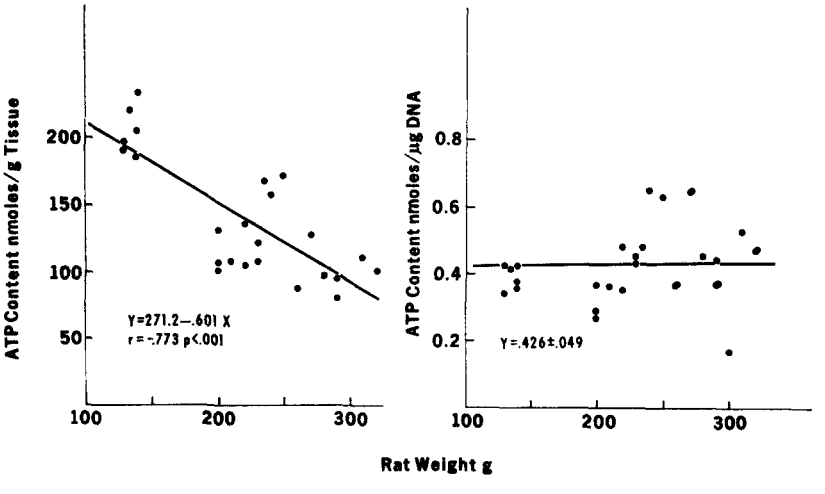

FIG. 1. ATP content of white adipose tissue. For each animal one epididymal fat pad was taken for ATP determinations and the other pad for DNA analysis. ATP was measured in perchloric acid extracts of tissue homogenates, and DNA was determined in the sediment after serial homogenization and centrifugation with 2 × 50 ml of cold acetone and 2 × 50 ml of ethyl ether.

TABLE 1 ATP LEVELS IN ADIPOSE TISSUE COMPONENTS

Expt	Intact Tissue	Partially Digested Fragments	Isolated Cells	Stromal Vascular Sediment	Proportion of Adipose Tissue ATP in Adipocytes†
	nmoles/g lipid				%
1	154 ± 12.3*	176	125	900	83
	n = 4		130		
2	217 ± 12.3	205	93	1300	43
	n = 4		93		
3	277 ± 23.8	280	171	352	64
	n = 8		180		

In each experiment epididymal fat from eight rats (16 pads) was used. Four or eight pads were incubated separately in 2 ml of buffer–5% albumin + 3 mM glucose for 60 min and served as controls. 12 pads were digested with collagenase and separated into component fractions (4). Partially digested fragments were those which did not disrupt completely after agitation with a siliconized glass rod following collagenase treatment. Isolated cells were divided into two batches and analyzed separately. On summing the fractions, 28–35% of tissue ATP was not recovered. This was attributed to the adipocyte isolation procedure, since 20–32% of tissue lipid was also lost.

* Mean ± SEM.

† (ATP content isolated cells/g lipid)/(ATP content intact tissue/g lipid) × 100.

adipocytes obtained from the size range of animals employed was 70–180 nmoles per g of lipid.

Addition of epinephrine to the incubation medium depressed adipose cell ATP significantly (Fig. 2). This occurred at a dose of 0.1 μg/ml, and with further increments in hormone, ATP levels decreased progressively. The fall in cellular ATP was inversely related to glycerol and net FFA production, which were maximal at hormone concentrations exceeding 0.5 μg/ml. Under the conditions employed here, the fall in ATP was between 30 and 70% below control levels after 60 min incubation with 1–10 μg/ml epinephrine or norepinephrine.

Dibutyryl 3′,5′-cyclic AMP, a potent analogue of 3′,5′-cyclic AMP (31), was used in order to bypass endogenous ATP consumption via the adenyl cyclase system and to activate lipase directly (32). A reduction in adipose cell ATP occurred after exposure of cells to dibutyryl 3′,5′-cyclic AMP. However, the pattern of effects differed somewhat from that observed with lipolytic hormones (Fig. 3). Dibutyryl 3′,5′-cyclic AMP was maximally effective within a very narrow dose range (1–2 μmoles/ml), and at a concentration of 2 mM it reduced adipose cell ATP to unmeasurable levels within 1 hr of incubation. This effect of dibutyryl 3′,5′-cyclic AMP on adipose cell ATP was irreversible, indicative of cell death, whereas adipose cells exposed to catecholamines recovered on reincubation in fresh medium (Table 2). Glucose had a salutary effect in restoring adipocyte ATP levels in norepinephrine-pretreated cells.

The fall in ATP with catecholamine treatment was not due to loss of nucleotide from the cell or loss of cells from the system, since reciprocal accumulation of hydrolysis products was shown in nucleotide balance studies (Table 3).

Experiments were next carried out to determine whether the effects of lipolytic hormones on steady-state ATP levels could be the result of lack of metabolic substrate. This explanation must be viewed as an unlikely possibility since addition of pyruvate or oxaloacetate to the incubation system did not prevent the

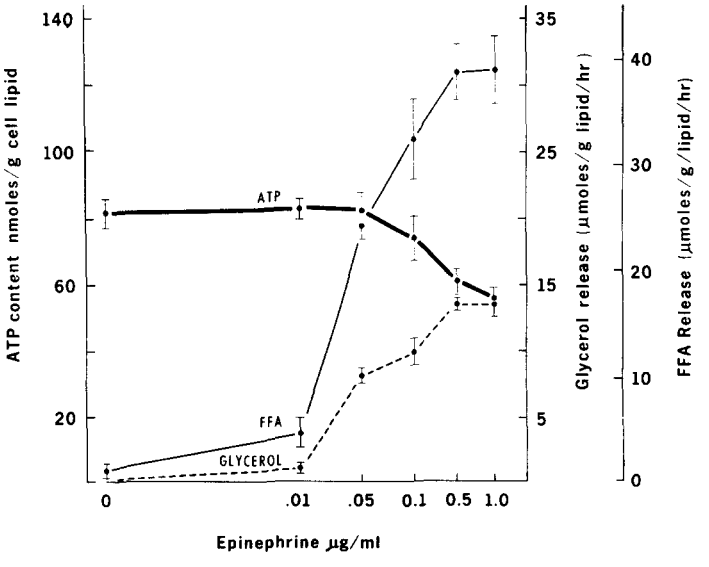

FIG. 2. The effect of epinephrine on adipose cell ATP and lipolysis. Isolated adipocytes were incubated with different amounts of epinephrine for 60 min. Medium and cells were separated by centrifugation and analyzed. Two experiments were combined and each point represents the mean ± SEM of four observations.

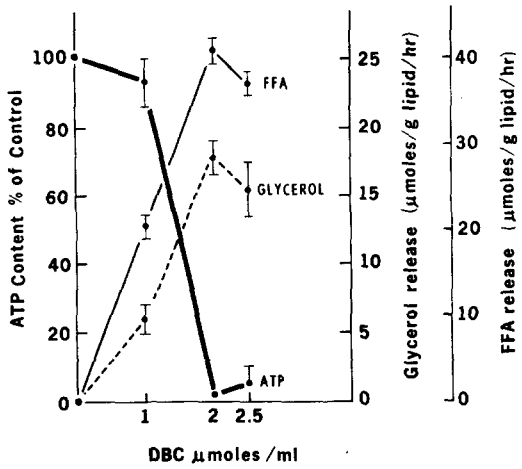

FIG. 3. The effect of dibutyryl 3′,5′-cyclic AMP (*DBC*) on adipose cell ATP levels and lipolysis. Cells were incubated with different concentrations of dibutyryl 3′,5′-cyclic AMP for 60 min and then processed. The results of four experiments were combined and the ATP levels were normalized to simplify presentation. Each point is the mean ± SEM of four observations.

depressive effect of norepinephrine on adipose cell ATP (Table 4).

Glucose addition substantially reversed the depressive effects of norepinephrine (Fig. 4), ACTH (Fig. 5), and dibutyryl 3′,5′-cyclic AMP (Fig. 6) on adipocyte ATP levels. Furthermore, addition of glucose resulted in a greater glycerol release and a reduction in net FFA output, indicating that both augmented lipolysis and enhanced FFA reesterification (Figs. 4, 5, and 6). This effect of glucose in epinephrine-treated tissues has been frequently observed (33, 34), and in relation to the present study it implies that the fall in adipocyte ATP with lipolytic agents is not a direct effect of any lipolytic agent itself. Furthermore, it is not related to lipolytic rate per se nor to the amount of glycerol produced, since addition of glucose resulted in an increased ATP level concomitant with increased lipolysis. Since glucose is the major substrate for α-glycerophosphate production in adipose tissue, and since fatty acid reesterification was enhanced in the glucose-treated group, it seemed likely that the protective effect of glucose was related to the esterification process and that the effects of lipolytic agents on adipose cell ATP were secondary to FFA accumulation.

To explore this possible etiological relationship, the effects of high concentrations of FFA on adipose cell ATP were studied. Long-chain fatty acids were added to adipose cells at concentrations of 4 mM (fatty acid/albumin molar ratios of 6.4) without significant effect on ATP content (Table 5). However, sodium octanoate at a concentration of 8 mM, which far exceeds physiological concentration of FFA, did significantly reduce adipose cell ATP. These results would indicate that the extracellular accumulation of long-chain FFA was not in itself responsible for the effects of lipolytic agents on adipose cell ATP. This conclusion gains further support from experiments in which ATP consumption was deliberately augmented in cells suspended in medium saturated with

TABLE 2 RECOVERY OF ADIPOSE CELL ATP AFTER INCUBATION WITH LIPOLYTIC AGENTS

Group	No. of Observations	Isolated Adipose Cells Incubated with the Following Additions	ATP Content
			% of control
1		None (control)	100
2	2	Dibutyryl 3′,5′-cyclic AMP, 2 mM	2
3	2	As 2, then washed and reincubated 60 min in fresh medium	0
4	2	As 3, plus glucose, 16 mM	0
5	4	Norepinephrine, 1 μg/ml	58 ± 7*
6	4	As 5, then washed and reincubated 60 min in fresh medium	72 ± 3
7	4	As 6, plus glucose, 16 mM	85 ± 6

Adipose cells were incubated with or without lipolytic agents for 60 min in Krebs-Ringer bicarbonate–5% albumin buffer. Groups of cells were assayed for ATP and others were washed three times with fresh media and reincubated for a further 60 min with or without glucose. The results are expressed as % of untreated control, and the data from four experiments were combined for presentation.

* Mean ± SEM.

TABLE 3 ADENINE NUCLEOTIDE BALANCE IN WHITE ADIPOSE CELLS

	ATP	ADP	AMP	Total
	nmoles/g cell lipid			
Control	88 ± 4.4*	20 ± 2.2	13 ± 4.0	122 ± 8.7
Epinephrine, 1.0 μg/ml	65 ± 6.4	37 ± 4.2	34 ± 7.1	136 ± 12.0
P	<0.01	<0.01	<0.05	NS

Isolated adipocytes were incubated with or without epinephrine for 60 min, separated from the medium by centrifugation, and extracted with perchloric acid for assay.

* Mean ± SEM, n = 4.

TABLE 4 EFFECT OF NOREPINEPHRINE ON ADIPOSE CELL ATP CONTENT

Additions	Relative ATP Content
None	100
NE (1 μg/ml)	60 ± 6*
NE (1 μg/ml) + pyruvate (10 μmoles/ml)	71 ± 7
NE (1 μg/ml) + oxaloacetate (10 μmoles/ml)	53 ± 5

Adipocytes (200 mg/flask) were incubated 60 min in 2 ml of buffer–albumin solution with various additions. NE, norepinephrine. Pyruvate and oxaloacetate were added as Na salts. To facilitate presentation of data the ATP content of control cells is designated 100 and the results are expressed in relative terms (% of control). The results of four experiments, each in duplicate, were combined.

* Mean ± SEM, n = 8.

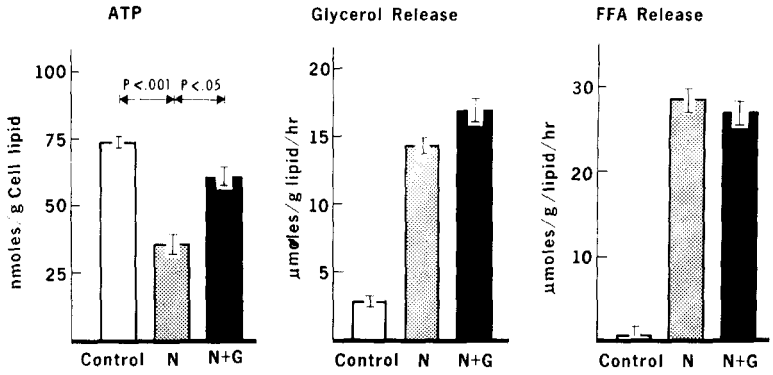

FIG. 4. Protective effect of glucose against reduction in adipocyte ATP by norepinephrine (*N*). Isolated adipose cells were incubated 60 min with or without N (1 µg/ml). Glucose (*G*), 16 µmoles/ml, added to flasks containing N prevented the fall in adipocyte ATP. The results of two experiments were combined, and each bar and vertical bracket represents the mean ± SEM of eight observations.

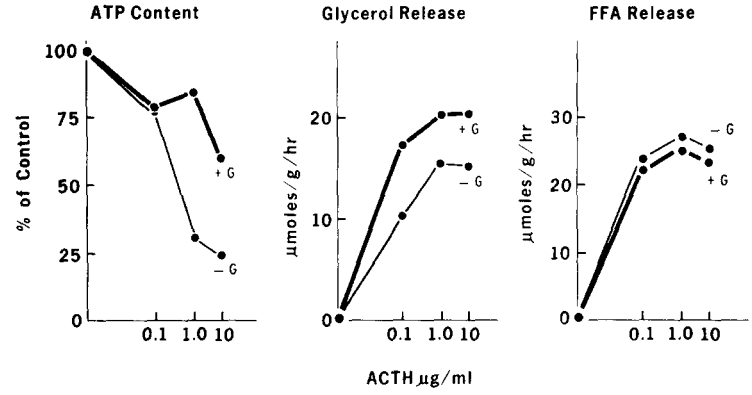

FIG. 5. Reduction in adipocyte ATP by ACTH and the protective effect of glucose. Isolated adipocytes were incubated 60 min with different amounts of ACTH in the presence (+*G*) or absence (−*G*) of glucose, 16 µmoles/ml. Each point is the average of duplicate observations.

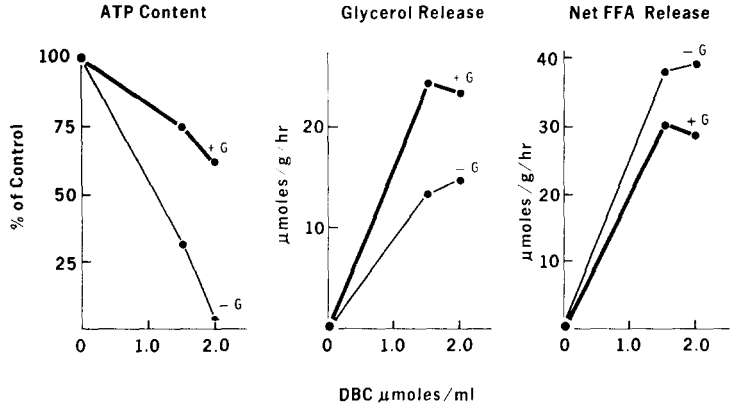

FIG. 6. Reduction in adipocyte ATP by dibutyryl 3′,5′-cyclic AMP (*DBC*) and the protective effect of glucose (*G*), 16 µmoles/ml. Each point is the average of duplicate observations.

FFA. In this experiment (Fig. 7) ATP levels were not reduced under conditions of enhanced fatty acid esterification where ATP turnover during the first 30 min of incubation was approximately 500 times the steady-state concentration (calculated by dividing ATP required for esterification by cell ATP level at zero time; see legend to Fig. 7).

This shows that augmented ATP consumption together with a high concentration of extracellular FFA does not alter steady-state ATP levels in adipocytes

TABLE 5 EFFECT OF ADDED FATTY ACID ON ATP LEVELS IN ADIPOSE CELLS

Expt	Additions	Concentration	FFA/Albumin Molar Ratio	Relative ATP Content
		mM		*% of control*
	None (control)	—	—	100
1	Na palmitate	2.9	4.3	103
2	Na oleate	4.0	6.4	106
3	Na octanoate	6.0	8.7	92
		8.0	11.7	84
		16.0	23.4	68

The sodium salts of the various fatty acids were added to Krebs-Ringer bicarbonate–albumin buffer and the mixture was gassed again to ensure a constant pH of 7.4. Adipocyte ATP levels were determined and the results are expressed relative to the unsupplemented control. Three separate experiments were carried out and each value is the mean of triplicate observations. Each flask contained 250 mg of cells in 2 ml of Krebs-Ringer bicarbonate buffer–5% albumin medium. Incubation 60 min.

because ATP synthesis can keep pace. The data suggest that the reduction in ATP level consequent to lipolytic stimulation cannot be simply the result of enhanced ATP utilization.

Since extracellular FFA did not seem to be responsible for the fall in ATP levels, attention was focused on the intracellular compartment, and experiments were performed in which FFA accumulation was intentionally restricted to the cell by incubating adipocytes in medium saturated with fatty acid. On addition of lipolytic hormone to this preparation, ATP levels fell to a greater extent than cells suspended in unsupplemented medium (Table 6), and the fall in ATP occurred at a lipolytic

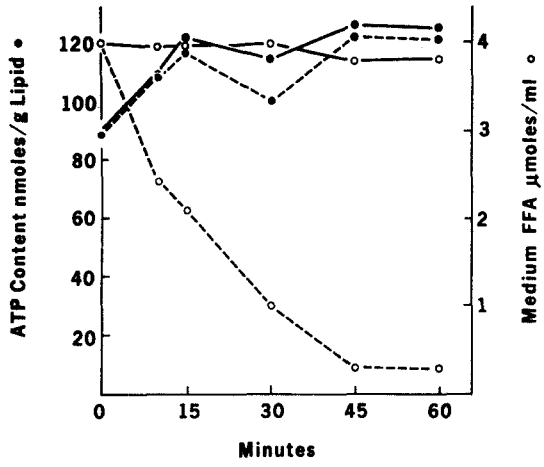

FIG. 7. Effect of increased ATP consumption on adipocyte ATP levels. Isolated cells were incubated in medium saturated with sodium oleate (4 mM, FFA/albumin molar ratio 7.4:1) with (-----) or without (———) glucose (16 mM) plus insulin (100 µU/ml). Each flask contained 235 ± 15 mg of cells in 2 ml of medium. In the presence of glucose and insulin net FFA esterification was 24 µmoles/g cells/30 min. This amount of esterification requires a minimum of 48 µmoles of ATP. ●, ATP; ○, medium FFA.

TABLE 6 EFFECT OF NOREPINEPHRINE ON ATP LEVELS OF ADIPOSE CELLS INCUBATED IN MEDIUM SATURATED WITH FFA

Expt	Addition	ATP	Medium FFA	Glycerol Release
		nmoles/g of lipid	*µmoles/g of lipid*	
1	None	95 ± 5	0.9 ± 0.6	0.7 ± 0.5
	NE (1 µg/ml)	86 ± 8	33.7 ± 1.5	14.9 ± 0.6
	NE (10 µg/ml)	59 ± 3	32.7 ± 2.1	16.4 ± 0.7
	NE (1 µg/ml) + oleate*	50 ± 3	31.7 ± 1.0	2.5 ± 0.6
2	Oleate	94 ± 2	33.0 ± 1.0	1.3 ± 0.4
	Oleate + NE (10 µg/ml)	33 ± 6	33.3 ± 1.5	6.2 ± 0.2
	Oleate + NE (10 µg/ml) + glucose (16 µmoles/ml) + insulin (1 mU/ml)	48 ± 4	28.6 ± 2.2	15.5 ± 0.9
3	NE (1 µg/ml)	89 ± 4	20.2 ± 0.9	9.2 ± 0.6
	NE (1 µg/ml) + oleate	71 ± 5	28.4 ± 1.6	3.7 ± 0.6
	NE (10 µg/ml)	81 ± 4	23.3 ± 1.6	11.4 ± 0.7
	NE (10 µg/ml) + oleate	50 ± 5	30.5 ± 0.6	6.0 ± 0.7

Isolated adipocytes were incubated in 2 ml of buffer–albumin for 60 min with various additions. Each flask contained 190–210 mg of adipose cells, and the initial FFA/albumin molar ratios where sodium oleate was added were 4.7, 5.9, and 4.7 in Experiments 1, 2, and 3, respectively. Each value is the mean ± SEM of triplicate incubations.

* 4 µmoles/ml.

level (glycerol output) far below that ordinarily seen (compare with Figs. 2–6). Thus, despite an apparent blunting of lipolytic response, restriction of FFA accumulation to the cell resulted in a profound drop in steady-state ATP.

To ascertain the possible role of intracellular FFA in mediating the changes in adipocyte ATP levels, a technique was developed to measure intracellular FFA (see Methods); the results of these studies are shown in Figs. 8–10. In Fig. 8 the relationship between glycerol and FFA output on the one hand, and adipocyte ATP on the other, is shown. While glycerol release (lipolysis) was nearly linear throughout the incubation, FFA output began to plateau at 15 min, and after 30 min of incubation, net output of FFA had all but ceased at a medium concentration of 4–5 mM. Calculation of FFA/albumin molar ratios showed that the plateau in medium FFA occurred at a molar ratio of 5–6, which corresponds to saturation of Sites I and II of the albumin molecule (35, 36). Since reesterification of FFA does not occur to any significant extent in the absence of added glucose (8, 26), the fall in FFA output cannot be attributed to synthesis of glyceride and suggests that FFA must be retained within the adipose cells. This deduction was confirmed by direct analysis of intracellular FFA. In Fig. 9 de-

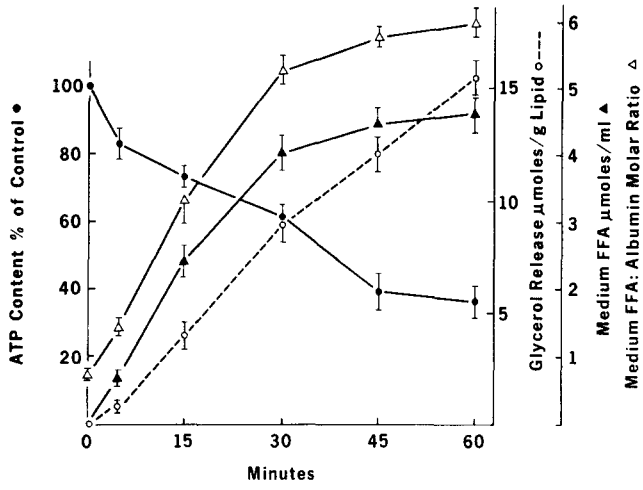

FIG. 8. Effect of norepinephrine on adipose cell ATP levels in relation to release of products of lipolysis. Isolated cells (200–225 mg/flask) were preincubated 10 min before zero-time samples were taken and norepinephrine (10 μg/ml) was added. Zero-time glycerol and FFA concentrations in the medium were subtracted from timed determinations. FFA/albumin molar ratios were not adjusted. The results of three experiments were combined and each point represents the mean ± SEM of six observations.

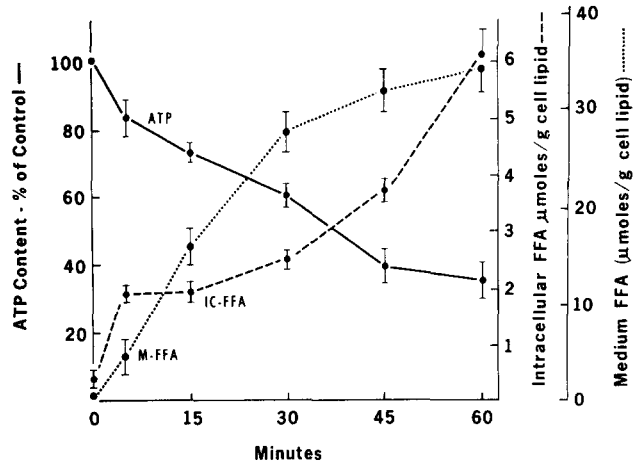

FIG. 9. Relation between intracellular (*IC*) and medium (*M*) FFA accumulation and adipocyte ATP levels during lipolysis. Isolated adipocytes (195–215 mg/flask) were preincubated 10 min before addition of norepinephrine (10 μg/ml) and analysis of zero-time samples. Medium and cells were separated by centrifugation and FFA was determined by titration. The amount of medium contaminating the adipose cell "float" was determined by the amount of extracellular marker (sucrose-U-^{14}C) retained (see Methods for details). The ATP levels were taken from the experiments described in Fig. 8. Three experiments were combined and each point is the mean ± SEM of six observations.

tailed information on the pattern of intra- and extracellular FFA accumulation is given. Accumulation of FFA in the intracellular compartment occurred throughout incubation and followed a definite and reproducible pattern. The basal adipocyte FFA content was 0.36 μmole/g and within 5 min exposure to norepinephrine a 5-fold increase occurred. The FFA level then plateaued for about 10 min, after which it steadily and progressively rose, reaching 6.1 μmoles/g at 60 min. This latter rise in intracellular FFA corresponds in time to the plateau in net FFA release and saturation of medium albumin and with the progressive decline in adipocyte ATP level (Fig. 8). It is significant that at the end of incubation intracellular FFA accounted for 12% of the total free acids produced.

These results strongly support the conclusion that the effects of lipolytic agents on ATP levels are secondary to intracellular accumulation of FFA or their derivatives. If this is so, one might anticipate that the protective effect of glucose noted earlier (Figs. 4–6) might be related to the reduction in intracellular FFA. Indeed, this was found in the experiment (Fig. 10) in which addition of glucose to norepinephrine-treated cells, while increasing both lipolysis and reesterification, prevented accumulation of FFA within the adipose cell.

DISCUSSION

The present report constitutes a systematic exploration of a number of mechanisms potentially responsible for the depressive effect of lipolytic agents on adipocyte ATP levels. The results of this study indicate that the fall in adipocyte ATP content cannot be attributed to a direct effect of the hormone itself, a deficiency in metabolic substrate, activation of the adenyl cyclase system with consumption of ATP by its conversion to 3′,5′-cyclic AMP, loss of adenine nucleotide from the cell, or accumulation of extracellular FFA or glycerol. The weight of evidence from experiments herein described and those reported by others (16, 18) suggests that the progressive fall in ATP is causally related to intracellular accumulation of free fatty acids. The strength of this conclusion is substantially enhanced by the direct demonstration of intracellular FFA accumulation coincident with a reduction in adipocyte ATP levels.

It would seem that intracellular FFA levels rapidly increase following catecholamine stimulation and that the pattern of FFA accumulation follows a definite profile. A rapid increase in intracellular FFA occurred within 5 min exposure to lipolytic hormone and plateaued at 2 μmoles/g of cell lipid (approximately 5 times the basal level). After 15 min incubation another increase in intracellular FFA concentration was observed. This second increment in adipocyte FFA developed more gradually than the first and correlated with a progressive decline in FFA release into the incubation medium and with a decline in cellular ATP. The arrest of medium FFA accumulation at this time was due to saturation of albumin binding sites and this undoubtedly accounts for the reciprocal rise in intracellular FFA. Surprisingly, the intracellular FFA pool reached levels of 6–9 μmoles/g

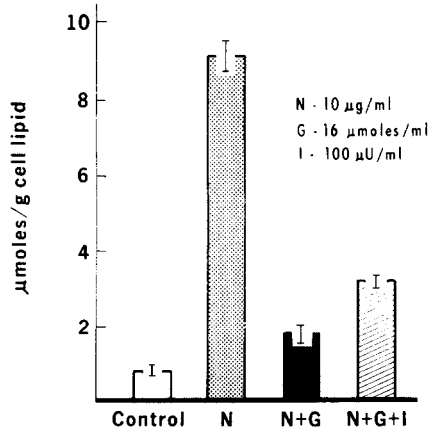

FIG. 10. The effect of glucose (*G*) (16 μmoles/ml) and insulin (*I*) (100 μU/ml) on intracellular FFA levels in norepinephrine (*N*) (10 μg/ml)-treated adipocytes. Control = no additions. Isolated cells were incubated 60 min and analyzed. Glucose alone and glucose plus insulin prevented intracellular accumulation of FFA. Each bar is the mean ± SEM of triplicate observations. The glycerol output (μmoles/g cell lipid) for each treatment was: control, 0.52 ± 0.01; N, 15.50 ± 0.31; N + G, 19.60 ± 0.55; and N + G + I, 23.60 ± 1.00. The net FFA release (μmoles/g cell lipid) was: control, 0.77 ± 0.20; N, 30.06 ± 1.00; N + G, 14.19 ± 0.30; and N + G + I, 21.56 ± 1.30.

cell lipid (Figs. 9 and 10), accounting for up to 15% of the total FFA produced after 1 hr of incubation.

It is likely that the initial plateau in FFA at 2 μmoles/g of cells reflects the balance between the affinity of intracellular fatty acid acceptors on organelle membranes (8, 26, 37) and that of medium albumin. Further, it has been shown that under conditions of maximal and linear lipolysis where saturation of medium albumin is not approached, intracellular FFA levels remain at 2–3 μmoles/g cell lipid (26). Once the binding capacity of cytoplasmic particles was reached, a further increment in FFA would result in a disporportionately large increase in unbound FFA, the form most likely to be responsible for the fall in adipocyte ATP. This is precisely what occurs as medium albumin becomes saturated and output of FFA is impeded with intracellular retention and accumulation of fatty acid (Fig. 9).

Since steady-state ATP levels are the net result of both synthesis and degradation, a fall in ATP concentration may be caused by depressed synthesis or enhanced hydrolysis, or both. While the present study does not in any way measure the effect of lipolytic hormones on ATP turnover, certain evidence from the literature and from the present work suggests that the fall in ATP is secondary to a combination of altered synthesis and augmented utilization. It is well known that long-chain FFA uncouple oxidative phosphorylation in isolated mitochondrial preparations (38, 39). That this occurs in intact adipose tissue has been claimed for interscapular brown fat cells stimulated by catecholamines (40). The studies of Hepp, Challoner, and Williams (16) provide good suggestive evidence that this occurs in white fat since ^{32}P incorporation into adipocyte ATP is depressed by catecholamine treatment at a time when oxygen consumption is augmented by 30%. Since adipocytes were able to maintain ATP levels at a time when ATP turnover was increased many hundredfold (Fig. 7), and since in the presence of lipolytic hormones glucose addition is associated with an increased Q_{O_2} (33) yet prevents the fall in adipocyte ATP (Figs. 4–6), and because ATP conversion to 3′,5′-cyclic AMP through adenyl cyclase activation occurs during hormone-stimulated lipolysis (41), it is concluded that the fall in ATP due to FFA accumulation is secondary to suppressed synthesis in the face of normal or augmented ATP consumption.

Many effects of lipolytic agents on adipocyte metabolism have been attributed to intracellular FFA accumulation. If the "primary" effect of lipolytic hormones is activation of glyceride hydrolysis, and subsequent effects of fatty acid accumulation such as the fall in ATP levels are termed "secondary," any metabolic deviation attributable to the fall in ATP levels should be termed a "tertiary" effect of the lipolytic agent. In this regard it is possible that depressed fatty acid synthesis (2), decreased amino acid transport and protein synthesis (5, 42), and decreased K^+ transport (43, 44) as well as inhibition of lipolysis itself (8) in adipose tissue preparations exposed to lipolytic hormones represent such tertiary effects. It follows that strict attention must be paid to conditions of incubation in studies concerning the effects of lipolytic agents or other energy-requiring biosynthetic or transport processes, since meaningful interpretation of results may be impossible unless intracellular FFA and ATP levels are known. This is especially important where dibutyryl 3′,5′-cyclic AMP is used (45, 46) since concentrations of 2 mM produced an irreversible depression of ATP levels under conditions employed here (Table 2 and Fig. 3).

The protection afforded by glucose against the ATP-lowering effect of lipolytic agents did not occur with alternate substrates such as pyruvate and oxaloacetate. This is attributable to the fact that glycerophosphate production from glucose is far greater than that potentially available from pyruvate (47) and suggests that the protective effect of glucose is related to contraction of a critical FFA pool by esterification. In this respect it is of interest that white adipocyte mitochondria are characterized by a very high affinity for FFA (37) and, in addition, serve as a template for esterification of fatty acids to α-glycerophosphate (20, 48). The strategic proximity of fatty acid binding and esterification on the mitochondrial membrane might well constitute an important mechanism geared to protect the cell from untoward effects of excessive accumulation of fatty acids or its derivatives. Furthermore, white adipose cell mitochondria, unlike

those of liver or muscle, do not readily oxidize α-glycerophosphate (49), an advantageous arrangement where esterification is to be promoted (50). Brown cell mitochondria oxidize α-glycerophosphate more readily than do white cell mitochondria (51), and this fact might help explain the difference between the two tissues with respect to their susceptibility to uncoupling effects of intracellular FFA (52, 53). Thus, both types of adipose cells possess intrinsic systems associated with mitochondrial membranes which either protect the cell from untoward effects of fatty acid accumulation or perhaps, at a more subtle level, serve to modulate the desired physiological response to fatty acid accumulation.

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