Effects of Prolonged Incubation of Isolated Fat Cells on their Response to Hormones Stimulating Lipolysis and Glucose Metabolism

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1. The metabolism of isolated fat cells from parametrial adipose tissue of starved normal rats was studied during 8 hr. incubation. 2. There was a three- to eight-fold increase in conversion of glucose into carbon dioxide, fatty acids and glycerol during the fourth to eighth hours of incubation in 4% albumin buffer over that seen during the first 4 hr. of incubation. 3. The addition of growth hormone and dexamethasone to fat cells at the start of the incubation period accelerated lipolysis during the first 4 hr. of incubation but no further effect was seen during the fourth to eighth hours of incubation. Addition of growth hormone and dexamethasone to fat cells that had been incubated for 4 hr. did not accelerate lipolysis during the next 4 hr. whether fat cells were incubated with or without glucose. 4. Fat cells incubated for prolonged periods also displayed a reduced sensitivity to the lipolytic action of adrenocorticotrophic hormone. 5. During prolonged incubation there was no damage to the cells as judged by the retention of two soluble cytoplasmic enzymes, lactate dehydrogenase and malate dehydrogenase, within the cells.

Isolated fat cells prepared by incubation of adipose tissue with bacterial collagenase can be separated from other cells, since they float to the top of the incubation medium (Rodbell, 1964). Unlike dispersed cells from the liver, which cease respiration (Laws & Stickland, 1956) and leak many soluble cytoplasmic enzymes such as lactate dehydrogenase and malate dehydrogenase (Takeda, Ichihara, Taniok & Inoue, 1964), adipose cells can metabolize glucose for at least 4 hr. and more glucose is metabolized during the second to fourth hours of incubation than during the first 2 hr. (Fain, Kovacev & Scow, 1965). In addition, adipose cells respond to hormones that affect adipose tissue (Rodbell, 1965).

The present studies were designed to examine the effect of incubating fat cells for periods up to 8 hr. on the metabolism of glucose and their response to hormones.

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MATERIALS AND METHODS

Female Sprague-Dawley rats of the NIH strain (140–160 g.) were fed on a high-fat (40%) diet (Simon, Scow & Chernick, 1961) for 5–7 days before the experiments. The rats were starved overnight and killed by decapitation. Isolated fat cells were prepared by a modification of the procedure of Rodbell (1964); the parametrial adipose tissue was incubated for only 1 hr. in albumin–bicarbonate buffer solution, without glucose, containing bacterial collagenase (Worthington Biochemicals Corp., Freehold, N.J., U.S.A.). A 4% solution of bovine fraction V albumin (lot 22, 312; Armour Laboratories, Kankakee, Ill., U.S.A.) was made up fresh daily in bicarbonate buffer and adjusted to pH 7.4 in an atmosphere of CO₂ + O₂ (5:95). The bicarbonate buffer contained the following: NaCl (127 mm), KCl (2.7 mm), CaCl₂ (1.4 mm), MgCl₂ (0.5 mm), NaHCO₃ (12 mm) and NaH₂PO₄ (4 mm). The albumin–bicarbonate buffer was filtered through a 0.45 µ Millipore filter before use. Fat cells were incubated in 1 oz. polyethylene bottles in a metabolic shaker at 37° in an atmosphere of CO₂ + O₂ (5:95). In those experiments in which glycerol release was measured the plastic bottles were rinsed before use since many lots of the Nalgene bottles were contaminated with glycerol. Incubations were performed in duplicate or

triplicate in each experiment and zero-time samples were taken after 5 min. equilibration.

All statistical comparisons are based on paired comparisons and the significance of the difference was tested by a non-parametric sign test (Siegel, 1956); this test was used since the distribution of some of the data was not normal.

Assay procedures. Samples of the medium were analysed for glycerol by a fluorimetric modification of the procedure of Vaughan (1962) and for free fatty acids by a modification of the procedure of Dole & Meinertz (1960). In some experiments $0.2 \mu\text{C}$ of $\text{D-}[U\text{-}^{14}\text{C}]\text{glucose}$ (New England Nuclear Corp., Boston, Mass., U.S.A.) was added to each bottle, making a final specific activity of $0.04 \mu\text{C}/\mu\text{mole}$. The $^{14}\text{CO}_2$ was determined by the procedure of Fain, Scow & Chernick (1963) and labelled glyceride glycerol and fatty acids by the procedures of Rodbell (1964). The amount of cells added per flask was based on their total fatty acid content.

Assays of lactate dehydrogenase and malate dehydrogenase were performed on samples of the incubation medium or homogenates prepared by rupture of cells in a Vortex homogenizer. The assay medium for lactate dehydrogenase contained the following: triethylamine hydrochloride (250 mm), adjusted to pH 7.6 with NaOH, NADH (0.03 mm) and pyruvate (10 mm), in a total volume of 1 ml. The assay medium for malate dehydrogenase contained: glycylhydrazine (200 mm), adjusted to pH 9.5 with NaOH, NAD^+ (0.05 mm) and malate (10 mm), in a total volume of 1 ml. In each assay the substrate was added last. Enzyme activity was based on the initial change in the native fluorescence of the nicotinamide nucleotides, measured with an Aminco Fluoromicrophotometer. The wavelength of the incident light was $340 \text{ m}\mu$ and the secondary filter transmitted light above $410 \text{ m}\mu$.

Materials. The hormones used were: bovine growth hormone (NIH-GH-B3) obtained from the Endocrinology Study Section of the National Institutes of Health; Oxycel-purified adrenocorticotrophic hormone (100 units/mg.) purchased from Wilson Laboratories, Chicago 9, Ill., U.S.A.; soluble insulin, Iletin U-40 (Eli Lilly and Co.) purchased locally; and dexamethasone (9α -fluoro- 11β , 17α , 21 -trihydroxy- 16α -methyl-4-pregnene-3,20-dione), obtained from Merck, Sharp & Dohme Research Laboratories, Rahway, N.J., U.S.A. Protein hormones were diluted in albumin buffer.

RESULTS

The conversion of glucose into carbon dioxide was fourfold greater, into fatty acid eightfold greater and into glyceride glycerol 2.5-fold greater by fat cells during the fourth to eighth hours of incubation than during the first 4 hr. (Table 1). The increase in basal glucose metabolism during the second 4 hr. resembled the effect produced by the addition of insulin during the first 4 hr. (Table 1). When insulin was added in the 4–8 hr. period there was an effect which was less than that seen after insulin addition during the 0–4 hr. period. But the sum of the basal increase in glucose metabolism and the insulin effect from 4 to 8 hr. was only

Table 1. *Effects of incubation time and insulin (1 milliu/ml.) on the metabolism of $[U\text{-}^{14}\text{C}]\text{glucose}$ in isolated fat cells*

Fat cells (5 mg./flask) were incubated for 4 or 8 hr. in 2 ml. of medium with $[U\text{-}^{14}\text{C}]\text{glucose}$ (final concn. 2.8 mm). Results are given as means $\pm$ s.e.m. of five experiments and *P* was calculated by the non-parametric sign test (Siegel, 1956).

Time of incubation (hr.)	Insulin + $[^{14}\text{C}]\text{glucose}$ added at (hr.)	Glucose converted ($\mu\text{moles/g. of adipose tissue/4 hr.}$)							
		Carbon dioxide		Fatty acids		Glyceride glycerol		Insulin effect	<i>P</i>
		Control	Insulin	Control	Insulin	Control	Insulin		
4	0	6 $\pm$ 2	41 $\pm$ 23	0.2 $\pm$ 0.04	4.0 $\pm$ 1.6	12 $\pm$ 3	49 $\pm$ 20	37	0.03
8	4	26 $\pm$ 12	34 $\pm$ 17	1.6 $\pm$ 0.07	2.6 $\pm$ 1.0	29 $\pm$ 9	35 $\pm$ 12	6	0.03
Effect due to preincubation on activity 4 to 8 hr.		+20 (<i>P</i> =0.03)		+1.4 (<i>P</i> =0.03)		+17 (<i>P</i> =0.03)			

slightly less than the maximal response obtained with insulin over 0-4 hr. incubation.

The addition of 0.01-1.0 $\mu\text{g.}$ of growth hormone/ml. in the presence of 0.016 $\mu\text{g.}$ of dexamethasone/ml. has been shown to accelerate lipolysis in fat

cells (Fain *et al.* 1965). The lipolytic effect of these hormones is slow in onset since lipolysis was much greater during the second to fourth hours of incubation than during the first 2 hr. of incubation (Fain *et al.* 1965). The results in Table 2 indicate that the addition of growth hormone and dexamethasone at the start of the incubation period stimulates fatty acid release during the first 4 hr. but no further effect was seen when the incubation period was extended to 8 hr. No effect of growth hormone and dexamethasone on either fatty acid or glycerol release could be obtained during the fourth to eighth hours of incubation even when the hormones were added at the beginning of the fourth hour of incubation (Table 3).

To decide whether the increase in basal glucose metabolism was responsible for the failure of action of growth hormone and dexamethasone, the action of these hormones was examined in albumin buffer without glucose. The results are shown in Table 4. The addition of growth hormone and dexamethasone stimulated lipolysis in fat cells incubated without glucose if added at the start of the first 4 hr. incubation period, in confirmation of previous studies (Fain *et al.* 1965). However, if growth hormone and dexamethasone were added at the

Table 2. *Effects of growth hormone and dexamethasone on fatty acid release from incubated fat cells*

Fat cells (20 mg./flask) were incubated for 4 or 8 hr. in 2 ml. of medium containing glucose (2.8 mM) as described in the text. Concentration of additions at zero time: growth hormone, 1.0 $\mu\text{g./ml.}$; dexamethasone, 0.016 $\mu\text{g./ml.}$ Results are given as means $\pm$ S.E.M. of eight experiments. *P* represents probability of difference between means of corresponding pairs calculated by the non-parametric sign test.

Time during which fatty acid release was measured (hr.)	Fatty acid release ($\mu\text{moles/g.}$)		<i>P</i>
	Control	Increase due to hormones	
0-4	66	73 $\pm$ 17	0.004
4-8	32	7 $\pm$ 28	> 0.1

Table 3. *Effects of growth hormone and dexamethasone on lipolysis when added at zero time or after 4 hr. incubation with glucose*

Fat cells (20 mg./flask) were incubated for 4 or 8 hr. in 2 ml. of medium containing glucose (2.8 mM). Growth hormone (1.0 $\mu\text{g./ml.}$) and dexamethasone (0.016 $\mu\text{g./ml.}$) were added at times indicated in the Table. Results are given as means $\pm$ S.E.M. of six experiments and *P* was calculated by the non-parametric sign test.

Time over which fatty acid release was measured (hr.)	Time of addition of hormones (hr.)	Fatty acid release ($\mu\text{moles/g.}$)		<i>P</i>	Glycerol release ($\mu\text{moles/g.}$)		<i>P</i>
		Control	Increase due to hormones		Control	Increase due to hormones	
0-4	0	67	51 $\pm$ 22	< 0.01	35	18 $\pm$ 7	< 0.01
4-8	4	30	-5 $\pm$ 8	> 0.2	40	1 $\pm$ 1	> 0.2

Table 4. *Effects of growth hormone and dexamethasone on fatty acid release from incubated cells in the absence of glucose*

Fat cells (4 mg./flask) were incubated for 4 or 8 hr. in 2 ml. of medium without glucose. Growth hormone (1.0 $\mu\text{g./ml.}$) and dexamethasone (0.016 $\mu\text{g./ml.}$) were added at times indicated in the Table. Results are shown as means $\pm$ S.E.M. of six experiments and *P* was calculated by the non-parametric sign test.

Time over which fatty acid release was measured (hr.)	Time of addition of hormones (hr.)	Fatty acid release in 4 hr. after hormone addition ($\mu\text{moles/g.}$)		<i>P</i>
		Control	Increase due to hormones	
0-4	0	308	160 $\pm$ 80	0.015
0-8	4	101	-34 $\pm$ 67	> 0.6

Table 5. *Effect of adrenocorticotrophic hormone on fatty acid release by fat cells*

Fat cells (3.5 mg./flask) were incubated in 2 ml. of medium containing glucose (2.8 mM). Adrenocorticotrophic hormone was added 1 hr. before the end of the incubation period and the results are shown as the means of seven experiments. Increases due to the hormone are given as means $\pm$ S.E.M. of the paired differences.

Incubation time (hr.)...	Fatty acid release (μ moles/g.)			
	4		8	
	Hormone (μ g./ml.)	Mean	Increase due to hormone	Mean
0	135		425	
0.002	370	+ 235 $\pm$ 85	335	- 90 $\pm$ 65
0.01	840	+ 705 $\pm$ 175	615	+ 190 $\pm$ 140
0.1	1400	+ 1265 $\pm$ 200	1175	+ 750 $\pm$ 120

Table 6. *Release of lactate dehydrogenase and malate dehydrogenase from isolated fat cells (5 mg./flask) into incubation medium*

Enzyme activity is expressed as μ moles of NADH oxidized or NAD⁺ reduced/min./ml. of medium or homogenate. Results are given as means $\pm$ S.E.M. Experimental details are given in the text.

Duration of incubation (hr.)	No. of experiments	Lactate dehydrogenase			Malate dehydrogenase		
		Activity in medium	Activity in cell homogenate	Release (%)	Activity in medium	Activity in cell homogenate	Release (%)
0	4	0.1 $\pm$ 0.04	1.2 $\pm$ 0.05	9	0.2 $\pm$ 0.01	3.6 $\pm$ 0.05	5
4	6	0.1 $\pm$ 0.01	1.2 $\pm$ 0.02	8	0.3 $\pm$ 0.04	3.1 $\pm$ 0.1	10
8	7	0.1 $\pm$ 0.01	1.0 $\pm$ 0.2	10	0.2 $\pm$ 0.01	3.0 $\pm$ 0.1	9

start of the second 4 hr. of incubation they had no effect on lipolysis.

The effect of prolonged incubation on the sensitivity of fat cells to another lipolytic hormone, adrenocorticotrophic hormone, was examined. Table 5 records the response of isolated fat cells to three concentrations of adrenocorticotrophic hormone added at the third and seventh hours of incubation. In cells that had been incubated for 7 hr., as opposed to those that had been incubated for 3 hr., there was no lipolytic effect of 0.001 or 0.01 μ g. of adrenocorticotrophic hormone/ml. If the concentration of the hormone was increased to 0.1 μ g./ml. a lipolytic effect was apparent in cells incubated for 7 hr. before its addition.

The possibility that there was leakage of soluble enzymes out of the fat cell as has been described for isolated liver cells (Takeda *et al.* 1964) was examined. The presence of two soluble cytoplasmic enzymes, lactate dehydrogenase and malate dehydrogenase, was measured in the medium and cells. After 8 hr. incubation there was no fall in the lactate-dehydrogenase content of the cells and less than a 20% decrease in malate dehydrogenase. No appreciable increases in amounts of these

enzymes were detected in the incubation medium over the 8 hr. period of incubation (Table 6).

DISCUSSION

The evidence from this study indicates that there is a marked increase in glucose metabolism by isolated fat cells during the fourth to eighth hours of incubation and a reduced sensitivity to lipolytic hormones. Similar effects on glucose metabolism can be obtained by the addition of insulin at the start of a 4 hr. incubation period. Fain, Kovacev & Scow (1966) have noted that in the presence of insulin there is no lipolytic effect of growth hormone and dexamethasone whether cells are incubated with or without glucose and that the effect of small but not of large concentrations of adrenocorticotrophic hormone is blocked by insulin. The similarity in the effects of prolonged incubation and of insulin suggests that insulin might be responsible for the rise in glucose metabolism during prolonged incubation. Alp & Recant (1964) found that preparations of human plasma albumin stimulated glucose metabolism by adipose tissue during a 2 hr. incubation period. However, if

there is insulin in the albumin it is difficult to understand why the effect of insulin is not manifest during the first 4 hr. of incubation.

Herrera, Phillips & Renold (1965) have noted that even after incubation of adipose tissue from starved rats for longer periods than those used in the present experiments there was no marked increase in glucose metabolism. But there was an increase in glucose metabolism with time if the tissue was incubated with pyruvate, insulin and glucose, or serum. In the present experiments the increase in glucose metabolism of adipose cells on prolonged incubation occurred in the absence of added serum, insulin, or pyruvate.

The increase in glucose metabolism during prolonged incubation could conceivably be due to spontaneous changes in the plasma membrane of the fat cells which allow increased entry of glucose and also decrease net lipolysis. If a membrane change of this sort occurs the evidence suggests it is not due to non-specific damage during incubation as has been found for incubated liver cells (Takeda *et al.* 1964), since there is no leakage of soluble cytoplasmic enzymes from the cells into the incubation medium.

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