The Effect of Glucose, Insulin and Noradrenaline on Lipolysis, and on the Concentrations of Adenosine 3':5'-Cyclic Monophosphate and Adenosine 5'-Triphosphate in Adipose Tissue

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Glycerol release and tissue concentrations of ATP and cyclic AMP were followed during the incubation of adipose tissue with or without glucose, insulin and noradrenaline. Glucose plus insulin or, to a lesser extent, glucose alone increased the accumulation of glycerol during incubations both with and without noradrenaline by slowing the decline in the rate of glycerol release with time. Insulin alone decreased the accumulation by accelerating the fall in glycerol release. In the absence of noradrenaline, ATP and cyclic AMP concentrations were not significantly affected by insulin or glucose. With noradrenaline or noradrenaline plus insulin the ATP concentration gradually fell. With noradrenaline plus glucose the ATP concentration fell rapidly and then stabilized, or, if insulin was also present, returned to the control value. In the presence of noradrenaline, the concentration of cyclic AMP rose during the first 20 min and then fell. Insulin lowered the peak concentration of cyclic AMP, but glucose had no effect either on the peak value or the fall in the concentration of the nucleotide. The increase and fall in the concentration of cyclic AMP with noradrenaline or noradrenaline plus insulin bore similarities to the increase and decline in the lipolytic rate in incubations without glucose. It is proposed that glucose stimulates ATP production by furnishing glycerol 1-phosphate and thus removing free fatty acids, but that it can influence lipolysis by a mechanism which is distinct from any which is mediated by free fatty acids, possibly by inhibiting the inactivation of the lipase.

The accumulation of glycerol during the incubation of adipose tissue with adrenaline is decreased by insulin in the absence of glucose. It is increased, however, by insulin in the presence of glucose, or, to a lesser extent, by glucose alone (Jungas & Ball, 1963). Since glycerol is not readily metabolized by adipose tissue, glycerol release can be taken as a reasonable indication of the lipolytic activity in the tissue (Steinberg & Vaughan, 1965). Thus glucose overcomes the antilipolytic effect of insulin, and can itself enhance lipolysis in adipose tissue. There are now many observations that suggest that the hormonal stimulation of adipose tissue lipase is mediated by cyclic AMP (Klainer *et al.*, 1962; Birnbaumer & Rodbell, 1969; Butcher *et al.*, 1965). The antilipolytic effect of insulin is associated with a fall in the concentration of cyclic AMP in adipose tissue (Butcher *et al.*, 1966). It is not known whether the effects of glucose upon lipolysis can similarly be explained by changes in the intracellular concentration of cyclic AMP. Alternatively glucose could influence lipolysis by altering ATP concentrations, since ATP is required for the stimulation of adipose tissue lipase (Rizack, 1964). The experiments described in the present paper were designed to test these two possibilities. Glycerol release and tissue concentrations of ATP

and cyclic AMP were followed during the incubation of adipose tissue with or without glucose, insulin and noradrenaline. It was found that, under all hormonal conditions, glucose inhibited the fall in the rate of glycerol release which occurred during the incubation, but had no effect on cyclic AMP concentrations in adipose tissue.

Materials and Methods

Rats weighing 160-180 g from an inbred Wistar strain, fed *ad libitum*, were used throughout. Each experiment consisted of eight incubations. For every experiment, one epididymal fat-pad was taken from each of four rats. Each pad was cut into four parts, which were distributed between four flasks containing 5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4 (Umbreit *et al.*, 1964) (with one-half the recommended Ca^{2+}). Each flask received four pieces of tissue (300-400 mg), one from each pad, and one from each region of the pad. The other four pads from the same rats were distributed in an identical manner into four flasks containing 5 ml of the buffer and 6 mM-glucose. This procedure gave pairs of flasks, one with and one without glucose, containing

identical portions of tissue from opposite sides of the rats. Insulin (final concn. 1 munit/ml) and noradrenaline bitartrate (final concn. 5 μ g of base/ml) were added where required. The flasks were gassed with O₂ + CO₂ (95:5), and incubated at 37°C with shaking. Experiments were performed with incubation periods of 10, 20, 40 or 70 min.

At the end of an incubation period the tissue was quickly removed from the flask and immediately frozen in liquid N₂. The incubation media were kept frozen until analysed. The frozen tissue was ground to a powder in a mortar cooled in liquid N₂ and transferred to a glass homogenizer tube containing 1 ml of ice-cold 5% (w/v) trichloroacetic acid. The tissue powder was allowed to thaw in contact with the acid and was then homogenized manually between the ground-glass faces of the homogenizer tube and a close-fitting glass plunger. Homogenization was continued for ten strokes after the tissue had been disrupted. The homogenate was centrifuged at 0°C, and the clear solution between the precipitated protein and the floating fat layer was removed. A sample of this solution was neutralized and assayed for ATP by the luminescence method of Strehler & Totter (1954) as adapted by Fletcher & Myant (1961). The remainder of the tissue extract was extracted five times with 10 ml of water-saturated diethyl ether. Any residual ether was removed at 70°C, and the solution was stored frozen until assayed for cyclic AMP by the protein-binding method of Gilman (1970). The assay mixture contained 50 mM-sodium acetate-acetic acid buffer, pH 4.0, 1 pmol of cyclic [8-³H]AMP (6.5 Ci/mmol), 20 μ g of a protein kinase inhibitor protein and 3 μ g of protein kinase in a final volume of 200 μ l. The protein kinase preparation bound approximately 0.1 pmol of cyclic AMP/ μ g of protein under these assay conditions. The mixture was incubated at 0°C for 1½ h, diluted with 0.8 ml of ice-cold 20 mM-potassium phosphate buffer, pH 6.0, and filtered through a cellulose ester filter. The filter was immersed in a mixture of 2 ml of ethanol and 10 ml of 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene, and, when clear, was counted in a Beckman LS-250 liquid-scintillation spectrometer. With up to 40 μ l of adipose tissue extract in the assay volume of 200 μ l there was a linear relationship between the amount of extract added and the amount of cyclic AMP detected. At dilutions less than 1:5 (that is, an amount of extract corresponding to more than about 15 mg of adipose tissue in a 200 μ l assay volume) effects of interfering substances were observed. A volume of 20 μ l of tissue extract (300–400 mg of adipose tissue/ml) with a 1:10 dilution in the assay was chosen for the present work. Under these conditions the recovery of known amounts of cyclic AMP was 104 ± 8% (mean ± S.E.M.).

Incubation media were thawed and kept at 100°C for approximately 10 min. When cool, they were

centrifuged, and the clear supernatants assayed for glycerol by the method of Garland & Randle (1962).

Protein kinase and protein kinase inhibitor were prepared from ox muscle by the methods described by Gilman (1970). Other enzymes and phosphoenolpyruvate (tricyclohexammonium salt) were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. Firefly lanterns, pyridine nucleotides, ATP, cyclic AMP and L-arterenol bitartrate were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Pig insulin, which was free from glucagon (lot A62), was supplied by Novo Industries, London E.2, U.K. Cellulose ester filters (0.45 μ m pore size) were obtained from Millipore Corp., Bedford, Mass., U.S.A., and cyclic [8-³H]AMP (ammonium salt) from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Glucose or glucose plus insulin did not significantly increase the amount of glycerol released during the first 10 min of incubation of adipose tissue with noradrenaline (Fig. 1). However, only in the presence of noradrenaline, insulin and glucose was the rate of glycerol release constant throughout the incubation. Under all other conditions the rate of glycerol release declined as the incubation proceeded. This can be seen more clearly in Fig. 2, where the rates of glycerol release, calculated from the curves in Fig. 1, are plotted against time. The rates of glycerol release from adipose tissue in the absence of noradrenaline, which are included in Fig. 2, fell rapidly from the start of the incubation and reached very low values. Noradrenaline, as expected, increased the rate of glycerol release in all cases. Both with and without noradrenaline the decline in the rate of glycerol release with time was decreased by the presence of glucose or glucose plus insulin, but increased by the presence of insulin alone.

The cyclic AMP concentration in adipose tissue in the absence of noradrenaline was not significantly affected by glucose or insulin (Fig. 3). On the addition of noradrenaline the concentration of cyclic AMP increased rapidly and then gradually returned to values similar to those in the control. The highest concentrations of cyclic AMP were observed after 20 min of incubation. At this time cyclic AMP concentrations were all significantly higher ($P < 0.05$, Student's *t* test) in the presence of noradrenaline than in corresponding incubations without noradrenaline. The mean cyclic AMP concentrations at 10 and 20 min with noradrenaline plus insulin were slightly lower than those with noradrenaline alone. Because of the wide variation between experiments the differences

Fig. 1. Time-course of glycerol release from adipose tissue incubated with noradrenaline (□), noradrenaline and insulin (○), noradrenaline and glucose (■) and noradrenaline, insulin and glucose (●)

Pieces of adipose tissue were incubated in Krebs-Ringer bicarbonate buffer, pH 7.4 (containing one-half the recommended Ca^{2+}). Noradrenaline ($5\mu\text{g}/\text{ml}$), insulin ($1\text{ munit}/\text{ml}$) and glucose (6mM) were present where indicated. Each experiment consisted of comparable pieces of adipose tissue incubated for the same time under each of the hormonal conditions (details of the distribution of tissue within each experiment are given in the Materials and Methods section). The points represent the mean ($\pm\text{S.E.M.}$) of five, five, four and nine experiments terminated at 10, 20, 40 and 70 min respectively. All values at 70 min were significantly different ($P < 0.05$, Student's t test) from each other.

between the means were not significant. However, the difference between values with and without insulin within each experiment at 10 min or 20 min was statistically significant ($P < 0.05$, paired t test) with a mean of 34% of the control concentration of cyclic AMP. Results with and without glucose at 10 and 20 min, calculated similarly, gave a mean difference of 6%, which was not significant.

The concentration of ATP in adipose tissue was slightly lowered by insulin or glucose, although

these effects were not statistically significant in the present experiments (Fig. 4). Noradrenaline caused a gradual fall in the concentration of ATP in the tissue (value at 70 min less than that at 10 min, $P < 0.01$, Student's t test). Insulin alone did not affect this fall. In the presence of glucose the depletion of ATP on the addition of noradrenaline was rapid. After 10 min of incubation the concentrations of ATP were significantly lower ($P < 0.05$, paired t test) than those in tissues incubated without glucose. Subsequently, however, the ATP concentration in the presence of noradrenaline plus glucose remained constant, and that in the presence of noradrenaline, glucose and insulin slowly rose to within 10% of the control value. It was then significantly higher than those in the other incubations containing noradrenaline ($P < 0.05$, Student's t test).

Discussion

In the present experiments the rate of glycerol release from adipose tissue incubated in the absence of glucose fell as the incubation proceeded. Glucose inhibited this fall under all the hormonal conditions studied, but did not significantly stimulate the rate of glycerol release. The ability of glucose to delay the fall in glycerol release during incubation of adipose tissue with adrenocorticotrophic hormone was noted by Bally *et al.* (1965). Glycerokinase activity in adipose tissue is too low for the results to be explained by the use of glycerol as a source of glycerol 1-phosphate in the absence of glucose (Robinson & Newsholme, 1967). It is apparent that the 'lipolytic' effect which has been attributed to glucose, either in the presence or in the absence of noradrenaline (Jungas & Ball, 1963; Chlouverakis, 1967), is mainly due to an inhibition of the decline in lipolysis with time rather than to a direct stimulation of glyceride breakdown.

In the presence of glucose, noradrenaline causes a rapid depletion of ATP in adipose tissue. This presumably results from the use of ATP for the esterification of fatty acids. Subsequently the ATP concentration remains constant, or, if insulin is also present, rises to its original value, whereas in the absence of glucose it gradually falls throughout the incubation. The increase in oxidation which occurs upon the addition of glucose cannot, even under the most favourable conditions of fully coupled oxidative phosphorylation, produce more ATP than is needed to replace that used for esterification (Ball & Jungas, 1964; Fisher & Ball, 1967). It follows, therefore, that the efficiency of phosphorylation in adipose tissue is depressed in the presence of noradrenaline, but can be improved by the addition of glucose. Free fatty acids can uncouple oxidative phosphorylation in isolated mitochondria (Pressman & Lardy, 1956). If

Fig. 2. Rate of glycerol release from adipose tissue during incubation with and without noradrenaline, insulin and glucose

The rates of glycerol release in the presence of noradrenaline are the slopes (calculated at minute intervals) of the curves given in Fig. 1. Those in the absence of noradrenaline were calculated from similar curves obtained in the same experiments.

the mitochondrial pool of free fatty acids reflects that of the whole cell, then the ability of glucose and glucose plus insulin to increase the synthesis of ATP could be secondary to their ability to lower the free fatty acid concentration in the tissue by stimulating re-esterification (Bally *et al.*, 1960).

At first sight it appears that the effect of glucose on lipolysis might also be explained by its stimulation of esterification. The decrease in the fall in lipolytic rate which is brought about by glucose or glucose plus insulin is certainly related qualitatively to a decrease in the concentration of free fatty acids in adipose tissue (Hall & Ball, 1970). Moreover, the presence of a high concentration of free fatty acids in some tissue compartment can lead to a depression of lipolysis (Bally *et al.*, 1965; Rodbell, 1965; Angel *et al.*, 1971; Cushman & Rizack, 1970). However, it is not known

whether free fatty acids can inhibit the lipase directly. Also, Hall & Ball (1970) have pointed out that the changes in fatty acid concentrations are not quantitatively similar to the changes in lipolysis, and that the extra lipolytic activity elicited by glucose and insulin in the presence of dibutyryl-cyclic AMP or theophylline is not accompanied by any alteration in free fatty acid concentration in adipose tissue. In the present experiments there is a clear similarity with noradrenaline and noradrenaline plus insulin between the increase and subsequent fall in the cyclic AMP concentration and the stimulation and subsequent decline in the lipolytic rate. It is possible, therefore, that the gradual decline of the lipolytic rate in adipose tissue in the absence of glucose is merely the result of the fall in the concentration of cyclic AMP and is not related to the accumulation of

Fig. 3. Cyclic AMP concentrations in adipose tissue during incubation with and without noradrenaline, insulin and glucose

See the legend to Fig. 1 for details. In each experiment the results were expressed as a percentage of the value obtained in the incubation with no additions. The points represent the mean ($\pm$ S.E.M.) of eight, nine, four and eight experiments terminated at 10, 20, 40 and 70 min respectively. Incubations were performed (a) with no additions (Δ), with glucose ($\blacktriangle$), with noradrenaline ($\square$), with noradrenaline and glucose ($\blacksquare$); and (b) with insulin (∇), with insulin and glucose ($\blacktriangledown$), with noradrenaline and insulin ($\circ$) and with noradrenaline, insulin and glucose ($\bullet$). The mean concentration of cyclic AMP in tissue with no additions was 132 ± 10 pmol/g wet wt.

Fig. 4. ATP concentrations in adipose tissue during incubation with and without noradrenaline, insulin and glucose

See the legend to Fig. 1 for incubation details and the legend to Fig. 3 for explanation of symbols. In each experiment the results were expressed as a percentage of the value obtained in the incubation with no additions. The points represent the mean ($\pm$ S.E.M.) of four, four, three and five experiments terminated at 10, 20, 40 and 70 min respectively. The mean ($\pm$ S.E.M.) concentration of ATP in tissue with no additions was 109 ± 5 nmol/g wet wt.

free fatty acids in the tissue. Ignorance of the details of compartmentation and the physical state of fatty acids in adipose tissue prevents any definite conclusions being drawn from these results, but they suggest

that glucose might influence lipolysis by a mechanism that is distinct from any that is mediated by free fatty acids.

Glucose does not retard the fall in lipolytic rate in

adipose tissue by maintaining the concentration of cyclic AMP and thus the stimulation of the activity of the lipase. It had no effect on either the peak concentrations of cyclic AMP after the addition of nor-adrenaline or the subsequent fall. These results differ from those of Ho (1970), who found that the concentration of cyclic AMP in isolated fat-cells was some 30% higher in the presence of glucose than in its absence. The experimental conditions used by Ho (1970), however, were different from those employed here. Manganiello *et al.* (1971) found no effect of glucose on cyclic AMP concentrations in isolated fat-cells incubated with adrenaline, but in their experiments glucose also had no effect on glycerol release.

The stimulation of the activity of the hormone-sensitive lipase in adipose tissue involves the activation of protein kinase by cyclic AMP and the subsequent phosphorylation of the lipase to its active form (Huttunen & Steinberg, 1971). There is also evidence that the tissue contains a lipase phosphatase which can inactivate the enzyme (Huttunen & Steinberg, 1971). The present results do not indicate that the activity of protein kinase or the activation reaction itself are affected by glucose, although this possibility cannot be eliminated. They do, however, support the hypothesis that the inactivation of the lipase by the lipase phosphatase is inhibited in the presence of glucose and is further inhibited if glucose uptake and metabolism is increased by insulin. Such an action of glucose could be independent of any release of inhibition of the active lipase by removal of fatty acids. It has recently been suggested that the increase in lipolysis in adipose tissue from starved rats is due to an inhibition of the lipase-inactivating system (Schwartz & Jungas, 1971).

There is little evidence at present to indicate how glucose could affect either the activation or the inactivation of the lipase. Lipolysis in adipose tissue homogenates is depressed by metal ions (Corbin *et al.*, 1970; Gorin & Shafrir, 1967; Wade *et al.*, 1970) and ATP plus Mg^{2+} ions (Corbin *et al.*, 1970; Wade *et al.*, 1970) and is stimulated by the removal of metal ions (Mosinger & Vaughan, 1967) or by fructose 1,6-diphosphate (Chlouverakis, 1968). It is probable, therefore, that any effect of glucose upon the lipolytic activity of adipose tissue involves a complicated interplay between ATP, metal ions and, perhaps, free fatty acids.

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