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Cyclic AMP-Dependent Protein Kinase Activity and Lipolysis in Adipose Tissue. Effect of Fasting, Oligomycine and Iodoacetamide

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The release of glycerol into the medium, the concentration of cAMP, and the cAMP-dependent protein-kinase activity were studied in adipocytes and in fat-pads obtained from epididymal adipose tissue of rats under different conditions of feeding.

An increase in the tissue concentration of cAMP and in the protein-kinase activity was observed *in vivo* at 48 and 96 h of fasting. A diminished release of glycerol was found in adipocytes from rats fasted for 48 h, in the absence of glucose, and the maximum concentration of cAMP was inferior to that of fed rats.

Oligomycine and iodoacetamide, in the presence of epinephrine and glucose, produce a diminution in the values of the parameters studied. No significant differences were observed, however, in the responses of tissue obtained from fed and fasting rats to these compounds. The present results confirm previous observations and show the dependence of the lipolytic process on carbohydrate metabolism.

Extensive studies have established the important role that cyclic AMP plays as mediator of the lipolytic effect exercised by hormones on adipose tissue (5), and in the same manner the mechanism through which it exercises its action upon the dissociation of the protein kinase (6). Fasting was characterized by a rise of the plasma cAMP levels in the rat (31) and in man (25). Previous studies have confirmed that the adipose tissue is responsable for part of this increment (20). However, there was a lack of studies to relate these variations *in vivo* of the cAMP levels with the foreseen modifications in the protein kinase activity of the adipose tissue.

We have reported (27) that the fat pads from fasted rats, incubated *in vitro*, have an absolute requirement for glucose so that the epinephrine-stimulated lipolysis can take place. In this context, the rela-

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tionship between energetic metabolism and lipolysis has been widely studied by FASSINA *et al.* (12, 13) and others (8, 17) through the use of inhibitors of glycolysis and oxidative phosphorylation.

The present study was designed to characterize the modifications that fasting can produce in the epinephrine-lipolytic sensitivity of the adipocytes, and the degree of activation *in vivo* of the cyclic AMP-dependent protein kinase in that state. Evidence showed that the inhibitors of glycolysis and oxidative phosphorylization accomplish an apparent dissociation between the cAMP levels and lipolysis, although no significant differences were found between tissues proceeding from fasted and from fed rats.

Materials and Methods

Male Wistar rats weighing 200-250 g were fed on a standard diet or were subjected to different periods of fasting, as indicated. They were anaesthetized systematically with Nembutal. In those experiments in which the levels *in vivo* of cAMP and protein kinase activity were to be determined, the epididymal adipose tissue was rapidly extracted and homogenized in 25 mM phosphate buffer, pH 6.5, 8 mM theophylline, 10 mM EDTA and 0.5 M NaCl (buffer A) as described (6).

The adipocytes were isolated with collagenase according to RODBELL (26). The cells (0.1 ml) were incubated in 0.8 ml Krebs Ringer bicarbonate buffer, pH 7.4, BSA 2 %, with 1 μM epinephrine and 1 mM theophylline in the absence of glucose, in a total volume of 1 ml. The glycerol was determined according to the method of GARLAND *et al.* (15), after adding 10 % TCA to the medium plus adipocytes, centrifuging at 3,000 r.p.m. and eliminating the TCA with H_2O-satured ether. Cyclic AMP was measured in samples deproteinized with 50 % TCA, according to the method of GILMAN (16), modified in the separation of cAMP with charcoal-BSA, as described (31).

In experiments with fat-pads, epididymal adipose tissue was divided into small pieces (20-50 mg) and was randomized so that each flask contained 100-200 mg of tissue. They were incubated in 5 ml KRB-2 % BSA pH 7.4 with 10 μM epinephrine, 5 mM glucose, with or without iodoacetamide and oligomycine. The tissue was extracted from the medium and was rapidly homogenized in cold buffer A. After centrifuging 20,000 $\times$ *g* for 10 min the protein kinase activity was measured immediately in the infranatant according to the method of CORBIN *et al.* (6). The cAMP was determined in the same infranatant without deproteinizing, and the glycerol in the incubation medium, as was described above. Protein concentration was estimated by the method of LOWRY *et al.* (23). The (γ-^{32}P)ATP and (^{3}H)cyclic AMP were supplied by the Radiochemical Center; Histone (type II-A) by Sigma. The protein kinase and its inhibitor were prepared according to the methods of WALSH *et al.* (32) and GILMAN (16), respectively.

Results and Discussion

Cyclic AMP levels and protein kinase activity in adipose tissue. An analysis of the response to fasting of the cAMP concentration and protein kinase activity in the adipose tissue is shown in table I. Epididymal adipose tissue, proceeding from fed rats and rats fasted during 24, 48 and 96 hours, was homogenized rapidly after extraction. The cAMP levels determined in the adipose tissue of rats fasted during 48 and 96 h were significantly higher ($P < 0.05$) than those corresponding to fed rats. These results agree with those previously described in plasma (31), adipose tissue (30) and in liver (29). The increment in cAMP in these conditions can be interpreted as an activation of the tissue adenyl-cyclase in response to

Table I. *Effect of fasting on cyclic AMP levels and protein kinase activity in epididymal adipose tissue.*

Epididymal adipose tissue was extracted from normally fed, and 24, 48 and 96 h fasted rats. It was rapidly homogenized as described in Material and Methods. In bracket is indicated of number of rats in each group. Values are mean ± SEM.

Nutritional state	Intracellular levels of cyclic AMP (pmol/mg protein)	Protein kinase activity (—cAMP/ +cAMP)
Fed (24)	34.3±4.4	0.40±0.01
Fasted-24 h (15)	26.2±2.2	0.34±0.02
Fasted-48 h (16)	50.9±6.3*	0.54±0.03*
Fasted-96 h (18)	65.8±8.6*	0.55±0.04*

* $P < 0.005$ vs. fed rats.

the increased concentrations of glucagon and epinephrine and to a decrease of the insulin levels (30), although detailed studies (29) point out the decrease of the insulin/glucagon molar ratio as the principal factor in explaining the hepatic increment in cAMP during fasting, rather than the separate hormone concentrations.

The protein kinase activity (—cAMP/ +cAMP) follows the same pattern as the cAMP concentration in the feeding conditions studied. Special care was taken in stabilizing the activity ratio of the enzyme, in the crude extract, with 0.5 M NaCl (6). This result confirms *in vivo* the regulation of the protein kinase activity of the adipose tissue previously demonstrated in adipocytes incubated with different hormones (19). These variations in the ratio of the protein kinase activity (0.40 in the controls against 0.55 in the tissue of fasted rats) are not as clear as the ones that are found in studies done *in vitro*, presumably because of the existence of other regulator mechanism of the enzyme activity in the intact animal.

Lipolysis and cAMP concentration in adipocytes. In vitro net formation of cAMP in adipocytes from 96 h fasted rats incubated in the presence of epinephrine and in the absence of glucose is significantly less than that produced in adipocytes from fed rats, during the first five minutes of incubation (fig. 1).

Under these conditions, one can likewise observe a decrease in the cAMP levels, once a maximum peak of synthesis is reached after five minutes of incubation. This decrease has been interpreted as a liberation of a Feed-back Regulator (FR) into the medium, which inhibited the adenyl-cyclase activity (20), and as the result of the liberation of adenosine into the medium, which inhibits both the adenyl-cyclase activity (28) and the hormone-stimulated cAMP accumulation in isolated fat cells (10). After 10 minutes incubation with epinephrine, both levels are equal and are maintained constant for 60 minutes.

In order to interpret this inhibitory ef-

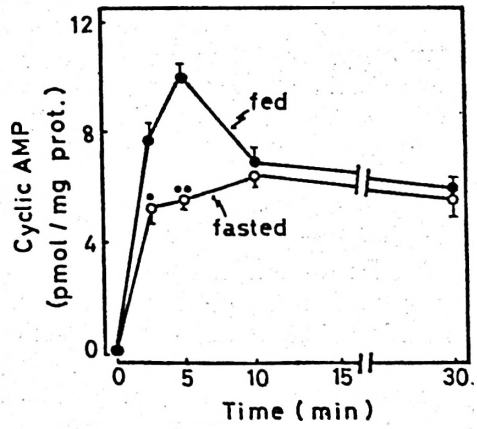

Fig. 1. *Time-course of the accumulation of cyclic AMP in adipocyte form fed (○) and fasted (○) rats.*

Adipocytes were incubated in KRB buffer, pH 7.4, in the presence of 1 μM epinephrine and 1 mM theophylline without glucose. Other experimental conditions are given in Material and Methods. The points represent the mean ± SEM of four experiments. Statistical comparisons versus fed rats are shown by asterisks: * $p < 0.05$, ** $p < 0.001$.

fect of fasting on the accumulation of cAMP, one could propose and inhibition of the adenyl-cyclase activity or a stimulation of the phosphodiesterase activity by some of the previously described metabolites, or by a feedback regulation of the products of lipolysis, or both, in cells from fasting rats. BRODIE *et al.* (2) have reported that there was no change in the phosphodiesterase activity in adipocytes membranes prepared after fasting. Although an increase in phosphodiesterase activity has been reported (22) in homogenates of fat cells incubated with insulin it is not likely that this mechanism would be the one responsible for the inhibition of accumulation of cAMP observed during fasting, as a decrease in the levels of insulin in this metabolic state has been established. A more logical supposition based on the rapid mobilization of lipids during fasting, suggests interpretation of this decrease as an inhibitor effect of the FFA on the adenyl-cyclase of the adipocyte (4). During fasting there have been no modifications described in the intracellular concentration of FFA in adipose tissue, nevertheless their concentration in the extravascular extracellular space surrounding the adipocytes could conceivably be much higher (3). Likewise, stability of the inhibitor effect of the FFA throughout different treatments of the adipose tissue has been described (4).

The liberation of glycerol to the medium (index of lipolysis) is also found to be significantly decreased in adipocytes from fasted rats when were incubated with epinephrine and without glucose (figure 2). In normally fed rats, the lipolytic activity of the adipocytes is time-dependent, while in those proceeding from fasted rats, it reaches a maximum after 30 minutes and is maintained for the 60 minutes of incubation. At this time the liberation of glycerol is decreased by 40% with respect to that produced with adipocytes from fed rats.

Our results showing a temporal dissociation between cAMP and glycerol production in fed rats are consistent with previous discoveries in other laboratories (1, 7, 24) although the length of the period during which the cAMP levels are high varies between 5 minutes and greater. This effect has recently been discussed by BIRNBAUN *et al.* (1), who have shown that the transient increment in cAMP is implicated in the initial stimulation of the lipolysis, concluding that the rate of increment of the cAMP is more important than its absolute concentration in determining the degree of activation of the triglyceride lipase.

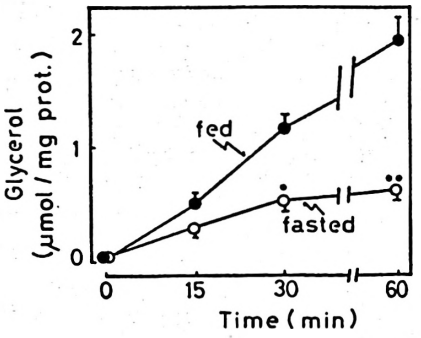

Fig. 2. *Time-course of glycerol release from adipocytes from fed (●) and fasted (○) rats.* Experimental conditions were the same as those of figure 1. Statistical comparisons versus fed rats are shown by asterisks: * $p < 0.01$, ** $p < 0.001$.

In the fasted state an inhibition was observed in both parameters, that is, in accumulation of cAMP and liberation of glycerol in the medium. The rate of inhibition of the lipolytic activity can be related to a lack of stimulation of adenyl cyclase. During prolonged fasting, there is an increased plasma concentration of FFA, which could be responsible for the inhibition observed in the formation of cAMP (fig. 1) and consequently on the glycerol liberation (fig. 2). A decrease in the adenyl-cyclase activity in membranes of adipocytes of rats fed high fat has been described (18). It should be pointed out

that in these experiments with adipocytes there is not any glucose in the incubation medium and this fact could cause a situation similar to that previously described in fat pads where there has also been observed a loss of the lipolytic capacity of epinephrine in tissue from fasting rats. The addition of glucose reverses this situation (27).

In fat pads from fasting rats, accumulation of cAMP and liberation of glycerol in the presence of glucose and epinephrine is greater than that in tissue from fed rats (table II). This could suggest that the FFA accumulate in greater quantity in the tissue of fasting rats, and that the addition of glucose permits their re-esterification with α-glycerophosphate, to produce the absence of inhibition of the adenyl-cyclase system caused by the FFA.

Effect of iodoacetamide and oligomycine on the protein kinase activity, cAMP concentration and lipolysis in fat-pads. The relation between the hormone-stimulated lipolysis and the requirement of ATP, either as the substrate of the adenyl-cyclase or as the phosphorylizing agent of the triglyceride lipase, was studied by various authors (8, 13, 14, 17) using inhibitors of glycolysis (iodoacetate) and of oxidative phosphorylization (oligomycine). FASSINA *et al.* (13, 14) conclude that although the cAMP is the mediator of the hormonal effect, the ATP levels are the rate limiting factor for the intensity and duration of the lipolysis. These and other works have suggested, in regard to the relative importance of the metabolic pathways considered, that the ATP synthesized in glycolysis is fundamental in the synthesis of cAMP, while the ATP produced in oxidative phosphorylation is probably more important in the activation of the lipase (12).

The present results (table II) confirm this hypothesis in fat pads incubated with glucose, whether they be from fed or fas-

Table II. *Effect of iodoacetamide and oligomycine on cyclic AMP levels, protein kinase activity and glycerol release in fat-pads from fed and 48 h fasted rats.*
Epididymal fat-pads were incubated in KBR buffer pH 7.4 in the presence of 1 μM epinephrine and 5 mM glucose, with or without 1 mM iodoacetamide or 40 μg/ml oligomycine. General procedure is given in Material and Methods. In bracket is indicated the percent of the inhibition respect to their controls (glucose + epinephrine). Values are mean ± SEM, for 5 experiments. Statistical comparisons versus glucose + epinephrine groups are shown by asterisks: * $p < 0.05$; ** $p < 0.025$; *** $p < 0.0005$.

Additions to the medium	cAMP (pmol/mg prot.)	Prot. kinase (—cAMP/+cAMP)	Glycerol (nmol/mg prot.)
Fed			
glucose + epinephrine	175.2 ± 13.2 (100)	0.65 ± 0.03 (100)	41.0 ± 2.1 (100)
glucose + epinephrine + iodoacetamide	56.2 ± 5.3* (32)	0.52 ± 0.02*** (80)	34.27 ± 2.4** (84)
glucose + epinephrine + oligomycine	84.1 ± 6.7* (50)	0.54 ± 0.02*** (83)	22.87 ± 2.1* (45)
Fasted			
glucose + epinephrine	218.0 ± 8.9 (100)	0.69 ± 0.01 (100)	106.7 ± 6.1 (100)
glucose + epinephrine + iodoacetamide	126.0 ± 10.5* (58)	0.48 ± 0.03* (70)	81.32 ± 5.0** (77)
glucose + epinephrine + oligomycine	185.0 ± 8.6** (85)	0.62 ± 0.02 (91)	62.33 ± 3.5* (58)

ted rats. In both metabolic states the accumulation of cAMP in the presence of iodoacetamide is found to be greatly decreased. The liberation of glycerol, however, is only slightly decreased in identical conditions. A different situation occurs with oligomycine, that is, a greater production of cAMP corresponding to a greater inhibition of glycerol liberation. These observations could be related to the principal requirement for ATP, proceeding from oxidative phosphorylation, for the stimulation of the lipase, as was previously described (12, 21). It has also been observed that the state of fasting does not modify the pattern of cAMP formation, protein kinase activity of the liberation of glycerol in the presence of these inhibitors, with respect to that observed in the tissue of fed rats.

The protein kinase activity determined in same situations is not well co-ordinated with cAMP levels: the same ratio of the protein kinase activity is found in different cAMP levels. The interpretation of this fact is not clear, although it would be related to the existence of different intracellular pools of cAMP (9).

In summary, the results presented confirm previous observations (12, 13) of the different uses of ATP proceeding from the glycolysis of oxidative phosphorylation and make evident the dependence of the lipolytic process on carbohydrate metabolism. Direct conclusions cannot be drawn, however, about the degree of participation of each pathway considered in the lipolysis.

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Resumen

Se estudia la liberación de glicerol al medio, la concentración de AMPc y la actividad de la proteína quinasa dependiente del AMPc y la actividad de la proteína quinasa dependiente del AMPc en adipocitos y en trozos de tejido adiposo epididimal obtenidos en diversas condiciones de alimentación.

A 48 y 96 h de ayuno se observa *in vivo* un aumento en la concentración de AMPc y de la actividad proteína quinasa. En adipocitos obtenidos de ratas en ayuno de 48 h se encuentra, en ausencia de glucosa, una liberación disminuida de glicerol, y la concentración máxima de AMPc es menor que la encontrada en ratas alimentadas.

La oligomicina y la iodoacetamida, en presencia de epinefrina y glucosa, producen una disminución de los parámetros estudiados. Sin embargo, no se observan diferencias significativas en la respuesta a estos compuestos del tejido obtenido en ratas alimentadas y en ayunas. Los resultados confirman previas observaciones y demuestran la dependencia del proceso lipolítico del metabolismo de los carbohidratos.

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